Estudi del paper de les proteïnes JNK en el desenvolupament de trastorns metabòlics i cognitius

Study on the role of the JNK proteins in the development of metabolic and cognitive disruptions

Oriol Busquets Figueras

UNIVERSITAT DE BARCELONA
Facultat de Farmàcia
i Ciències de l'Alimentació

TESI DOCTORAL

Estudi del paper de les proteïnes JNK en el desenvolupament de trastorns metabòlics i cognitius

Study on the role of the JNK proteins in the development of metabolic and cognitive disruptions

ORIOL BUSQUETS FIGUERAS

RECERCA, DESENVOLUPAMENT I CONTROL DE MEDICAMENTS

DIRECTORS :

DR. ANTONI CAMINS ESPUNY I DR. JAUME FOLCH LÓPEZ

TUTOR :

DR. ANTONI CAMINS ESPUNY

2019

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

PROGRAMA DE DOCTORAT
RECERCA, DESENVOLUPAMENT I CONTROL DE MEDICAMENTS

Estudi del paper de les proteïnes JNK en el desenvolupament de trastorns metabòlics i cognitius

Study on the role of the JNK proteins in the development of metabolic and cognitive disruptions

Memòria presentada per **Oriol Busquets Figueras** per a optar al títol de doctor per la Universitat de Barcelona

Dr. Antoni Camins Espuny (Director / Tutor)

Dr. Jaume Folch López (Director)

Oriol Busquets Figueras (Doctorand)

Aquesta tesi doctoral ha estat realitzada al Departament de Farmacologia, Química Terapèutica i Toxicologia (Facultat de Farmàcia i Ciències de l'Alimentació) dintre del grup de recerca "Grup de Recerca en envelliment i neurodegeneració (GREN)", amb reconeixement de la Generalitat de Catalunya com a Grup Consolidat (2018 SGR 625). El grup també pertany a la fundació CIBERNed (Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas) i és part de l'Institut de Neurociències de la Universitat de Barcelona (Grup de Recerca en Neurofarmacologia: Prevenció de l'envelliment).

Durant la tesi doctoral el doctorant ha gaudit de diverses beques, ajuts i premis:

- Premi a la millor presentació oral al "II PhD Workshop" de l'Institut de Neurociències. Desembre 2017. Facultat de Biologia. Universitat de Barcelona. Barcelona.
- Ajut per a l'assistència al "European Meeting of Neuroscience – 3rd Edition". Maig 2018. Institut des Neurosciences – Centre de Recherche Inserm. Grenoble, França.
- Ajut per a l'assistència a Congressos Internacionals de l'Institut de Neurociències. Juliol 2018. FENS (Forum of European Neuroscience). Berlin, Alemanya.
- Borsa de viatge per a activitats relacionades amb la recerca de la Facultat de Farmàcia de la Universitat de Barcelona. Juliol 2018. FENS (Forum of European Neuroscience). Berlin, Alemanya.

- Premi a la millor presentació oral al "5th MetNet International Annual Meeting. Targeting Metabolism to Develop New Therapies" de la Societat Catalana de Biologia (Secció de Senyalització Cel·lular i Metabolisme). Maig 2019. Institut d'Estudis Catalans. Barcelona.
- Ajut per a la realització d'estades a l'estranger per a l'any 2019-2020 dirigida a investigadors de l'Institut de Neurociències (NEUROESTADA 19/20). Juny-Agost 2019. Brown University. Providence; Rhode Island; Estats Units.

La present tesi doctoral s'ha dut a terme gràcies al finançament rebut de la fundació CIBERNed (CB06/05/2004 i PI2016/01; Proyectos Colaborativos CIBERNED 2016. Alterations in glycolipidic metabolism and the development of Alzheimer's disease dementia) i la concessió del projecte d'investigació del Ministeri d'Economia i Competitivitat (SAF2017-84283-R).

Publicacions derivades de la present tesi doctoral

Auladell C, de Lemos L, Verdaguer E, Ettcheto M, **Busquets O**, Lazarowski A, Beas-Zarate C, Olloquequi J, Folch J and Camins A. Role of JNK isoforms in the kainic acid experimental model of epilepsy and neurodegeneration. Front Biosci. 2017; 22:795–814.

Busquets O, Ettcheto M, Pallàs M, Beas-Zarate C, Verdaguer E, Auladell C, Folch J and Camins A. Long-term exposition to a high fat diet favours the appearance of β-amyloid depositions in the brain of C57BL/6J mice. A potential model of sporadic Alzheimer's disease. Mech Ageing Dev. 2017; 162:38–45.

Patraca I, Martínez N, **Busquets O**, Martí A, Pedrós I, Beas-Zarate C, Marin M, Ettcheto M, Sureda F, Auladell C, Camins A and Folch J. Anti-inflammatory role of Leptin in glial cells through p38 MAPK pathway inhibition. Pharmacol Reports. 2017;69(3):409–18.

Ettcheto M, Sánchez-López E, Pons L, **Busquets O**, Olloquequi J, Beas-Zarate C, Pallàs M, García ML, Auladell C, Folch J and Camins A. Dexibuprofen prevents neurodegeneration and cognitive decline in APPswe/PS1dE9 through multiple signalling pathways. Redox Biol. 2017; 13:345–52.

Folch J, **Busquets O**, Ettcheto M, Sánchez-López E, Pallàs M, Beas-Zarate C, Marin M, Casadesús G, Olloquequi J, Auladell C and Camins A. Experimental Models for Aging and their Potential for Novel Drug Discovery. Curr Neuropharmacol. 2017;16(10):1466–83.

Ettcheto M, Abad S, Petrov D, Pedrós I, **Busquets O**, Sánchez-López E, Casadesús G, Beas-Zarate C, Carro E, Auladell C, Olloquequi J, Pallàs M, Folch J and Camins A. Early Preclinical Changes in Hippocampal CREB-Binding Protein Expression in a Mouse Model of Familial Alzheimer's Disease. Mol Neurobiol. 2018;55(6):4885–95.

Busquets O, Ettcheto M, Verdaguer E, Castro-Torres RD, Auladell C, Beas-Zarate C, Folch J and Camins A. JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid. Neuropharmacology. 2018; 131:440–52.

Folch J, **Busquets O**, Ettcheto M, Sánchez-López E, Castro-Torres RD, Verdaguer E, García ML, Olloquequi J, Casadesús G, Beas-Zarate C, Pelegrí C, Vilaplana J, Auladell C and Camins C. Memantine for the treatment of dementia: A review on its current and future applications. J Alzheimer's Dis. 2018;62(3):1223–40.

Folch J, Ettcheto M, **Busquets O**, Sánchez-López E, Castro-Torres RD, Verdaguer E, Manzine PR, Rabieipoor S, García ML, Olloquequi J, Beas-Zarate C, Auladell C and Camins A. The implication of the brain insulin receptor in late onset Alzheimer's disease dementia. Pharmaceuticals. 2018;11(1):1–16.

Ettcheto M, Sánchez-López E, Gómez-Mínguez Y, Cabrera H, **Busquets O**, Beas-Zarate C, García ML, Carro E, Casadesús G, Vázquez-Carrera M, Folch J and Camins A. Peripheral and Central Effects of Memantine in a Mixed Preclinical Mice Model of Obesity and Familial Alzheimer's Disease. Mol Neurobiol. 2018;55(9):7327–39.

Jara-Moreno D, Castro-Torres RD, Ettcheto M, Auladell C, Kogan MJ, Folch J, Verdaguer E, Cano A, **Busquets O**, Delporte C and Camins A. The Ethyl Acetate Extract of Leaves of Ugni molinae Turcz. Improves Neuropathological Hallmarks of Alzheimer's Disease in Female APPswe/PS1dE9 Mice Fed with a High Fat Diet. J Alzheimer's Dis. 2018;66(3):1175–91.

Camins A, Ettcheto M, **Busquets O**, Manzine PR, Castro-Torres RD, Beas-Zarate C, Verdaguer E, Sureda F, Bulló M, Olloquequi J, Auladell C and Folch J. Triple GLP-1/GIP/glucagon receptor agonists, a potential novel treatment strategy in Alzheimer's disease. Expert Opin Investig Drugs. 2018;28(1): 93-97.

Castro-Torres RD, Landa J, Rabaza M, **Busquets O**, Olloquequi J, Ettcheto M, Beas-Zarate C, Folch J, Camins A, Auladell C and Verdaguer E. JNK Isoforms Are Involved in the Control of Adult Hippocampal Neurogenesis in Mice. Both in Physiological Conditions and in an Experimental Model of Temporal Lobe Epilepsy. Mol Neurobiol. 2019.

Busquets O, Eritja À, López BM, Ettcheto M, Manzine PR, Castro-Torres RD, Verdaguer E Olloquequi J, Vázquez-Carrera M, Auladell C, Folch J and Camins A. Role of brain c-Jun N-terminal Kinase 2 in the control of the insulin receptor and its relationship with cognitive performance in a high-fat diet preclinical model. J Neurochem. 2019;149(2):161–310.

Manzine PR, Ettcheto M, Cano A, **Busquets O**, Marcello E, Pelucchi S, Di M, Endres K, Olloquequi J, Camins A and Regina M. ADAM10 in Alzheimer' s disease: Pharmacological modulation by natural

compounds and its role as a peripheral marker. Biomed Pharmacother. 2019;113.

Ettcheto M, Cano A, **Busquets O**, Manzine PR, Sánchez-López E, Castro-Torres RD, Beas-Zarate C, Verdaguer E, García ML, Olloquequi J, Auladell C, Folch J and Camins A. A metabolic perspective of late onset Alzheimer's disease. Pharmacol Res. 2019;145.

Ettcheto M, **Busquets O**, Camins A. Potential preventive disease-modifying pharmacological strategies to delay late onset Alzheimer' s disease. Neural Reg Res. 2019;14(10):1721–5.

Folch J, Olloquequi J, Ettcheto M, **Busquets O**, Sánchez-López E, Cano A, Espinosa-Jiménez T, García ML, Beas-Zarate C, Casadesús G, Bulló M, Auladell C and Camins A. The involvement of peripheral and brain insulin resistance in late onset Alzheimer's dementia. Front Aging Neurosci. 2019; 11(236): 1-16.

Comunicacions en congressos

Orals

Pharmacological inhibition of JNK1 by Licochalcone A leads to neuroprotection. **Busquets O**, Ettcheto M, Verdaguer E, Castro-Torres RD, Auladell C, Beas-Zarate C, Folch J, Camins A. II PhD Workshop of the Institute of Neurosciences (Universitat de Barcelona). Facultat de Biologia. Barcelona (Spain). December 2017.

Estudi del paper de la dieta en l'aparició de processos neurodegeneratius en models preclínics. **Busquets O**. Seminaris de Recerca de la Facultat de Farmàcia i Ciències de la Alimentació de la Universitat de Barcelona. Barcelona (Spain). May 2018.

Role of the JNK2 in the control of the activity of the Insulin Receptor and its consequences on cognitive performance. **Busquets O**, Ettcheto M, Manzine PR, Rabieipoor S, Verdaguer E, Castro-Torres RD, Auladell C, Folch J, Camins A. European Meeting of Neuroscience for PhD students – 3rd Edition. Grenoble Institut Neurosciences – Centre de Recherche Inserm. Grenoble (France). May 2018.

c-JUN N-terminal Kinase 1: A key to modulate brain metabolism. **Busquets O**, Ettcheto M, Verdaguer E, Auladell C, Folch J, Camins A. 5th MetNet International Annual Meeting. Targeting Metabolism to Develop New Therapies. Societat Catalana de Biologia (Secció de Senyalització Cel·lular i Metabolisme). Institut d'Estudis Catalans. Barcelona (Spain). May 2019.

Combinatory therapeutic strategies for Alzheimer's disease: Current and future options. Presentation delivered by Dr. Antoni Camins; Oriol Busquets was a co-author. XXVIII Foro Nacional de Investigación en Salud. Ciudad y Puerto de Acapulco Guerrero. Mexico. October 2019.

Cinasa c-Jun N-terminal 1: Un element clau per a la protecció davant de dèficits cognitius derivats de trastorns metabòlics. **Busquets O**, Espinosa-Jiménez T, Ettcheto M, Verdaguer E, Auladell C, Folch J, Camins A. XII Jornades de Recerca de la Facultat de Farmàcia i Ciències de l'Alimentació. Barcelona (Spain).

Pòsters

Licochalcone A, an inhibitor of JNK1, a drug to prevent cytotoxic and neuroinflammatory damage in a temporal lobular epilepsy mica model. **Busquets O**, Ettcheto M, Verdaguer E, Auladell C, Pallàs M, Folch J, Camins A. X Simposi Neurobiologia. Institut d'Estudis Catalans. Barcelona (Spain). May 2016.

Memantine improves metabolic alterations in APP/PS1 mice model of familial Alzheimer's disease fed with high fat diet. Ettcheto M, Sánchez-López E, **Busquets O**, Pallàs M, García ML, Folch J, Camins A. IV Congreso Internacional de Investigación e Innovación en Enfermedades Neurodegenerativas (CiiiEN). Alicante (Spain). September 2016.

Strategies for Alzheimer's disease prevention: pegylated biodegradable dexibuprofen nanospheres administration to APPswe/PS1dE9. Ettcheto M, Sánchez-López E, **Busquets O**, García ML, Pallàs M, Folch J, Camins A. 2nd FALAN (Federation of Latin

American and Caribbean Neuroscience Societies) Congress. Buenos Aires (Argentina). October 2016

Licochalcone A, an inhibitor of JNK1, a drug to prevent cytotoxic and neuroinflammatory damage in a temporal lobular epilepsy mica model. **Busquets O**, Ettcheto M, Verdaguer E, Auladell C, Pallàs M, Folch J and Camins A. PhD Workshop of the Institute of Neurosciences (Universitat de Barcelona). Facultat de Psicologia. Barcelona (Spain). December 2016.

The use of Licochalcone A as a pre-emptive pharmacological inhibition of JNK1, leads to neuronal protection against excitotoxic insults derived of kainic acid. **Busquets O**, Ettcheto M, Verdaguer E, Auladell C, Folch J, Camins A. 37th Sociedad Española de Farmacologia National Meeting with guest society British Pharmacological Society. Barcelona (Spain). June 2017.

JNK1 inhibition by Licochalcone A leads to neuronal protection. **Busquets O**, Ettcheto M, Verdaguer E, Castro-Torres RD, Auladell C, Beas-Zarate C, Camins A, Folch J. Alzheimer's Global Summit. Lisbon (Portugal). September 2017.

Dexibuprofen prevents cognitive and metabolic parameters associated with Alzheimer's disease in APPswe/PS1dE9 mice. Ettcheto M, Sánchez-López E, Gorriz S, **Busquets O**, Auladell C, Folch J, Camins A. II PhD Workshop of the Institute of Neurosciences (Universitat de Barcelona). Facultat de Biologia. Barcelona (Spain). December 2017.

Downregulation of the insulin receptor pathway leads to a decline in cognitive performance in a model of sporadic Alzheimer's disease. **Busquets O**, López BM; Ettcheto M, Manzine PR, Rabieipoor S, Verdaguer E, Castro-Torres RD, Auladell C, Folch J, Camins A. 11th FENS Forum of Neuroscience. Berlin (Germany). July 2018.

Effects of dexibuprofen in APPswe/PS1dE9 mice fed with a high fat diet in BACE1 and ADAM10 protein levels. Manzine PR, Ettcheto M, Sánchez-López E, Gorriz S, **Busquets O**, Auladell C, Folch J, Cominetti M, Camins A. 11th FENS Forum of Neuroscience. Berlin (Germany). July 2018.

Targeting JNK1: a key to enhance insulin receptor signalling. **Busquets O**, Eritja A, Ettcheto M, Verdaguer E, Auladell C, Folch J, Camins A. III PhD Workshop of the Institute of Neurosciences (Universitat de Barcelona). Facultat de Medicina i Ciències de la Salut. Barcelona (Spain). October 2018.

"*Science is magic that works*"

Kurt Vonnegut

Agraïments

Primer de tot, dono les gràcies al Dr. Antoni Camins i el Dr. Jaume Folch, per haver-me permès dur a terme la meva tesi doctoral al laboratori de la Facultat de Farmàcia i Ciències de l'Alimentació (UB), i donar-me l'oportunitat per a exercir com a professor associat a la Facultat de Medicina i Ciències de la Salut (URV). També, dono les gràcies a la Dra. Carme Auladell, per obrir-me les portes del departament de Biologia Cel·lular de la Facultat de Biologia (UB) i sempre acollir-me amb els braços oberts. Aquests anys han estat plens d'experiències de tota mena, i sé que ho recordaré sempre.

En segon lloc, agraeixo de tot cor l'ajuda i amistat de tots aquells que he conegut dintre el món de la recerca. Especialment a la Miren, que va ser la meva guia des del primer dia, ella em va ensenyar i preparar fins que, de mica en mica, vaig començar a treballar pel meu compte. Tot i això, mai vaig estar sol, en cada nou disseny, en cada nou problema o entrebanc hi va haver altres companys que em van donar un cop de mà. M'agradaria destacar l'Ester, en qui vaig confiar des del primer moment i que sempre ha sigut crítica i sincera. D'ella he après mil i una coses. També, dono les gràcies a l'Emma, que ha tingut paciència infinita davant els meus dubtes i preguntes i, que sempre m'ha tractat amb tota la cura i estima del que disposava. Finalment, en Raúl, sempre malhumorat i sardònic, però seriós i responsable a la vegada. Amb ell he après i rigut en parts iguals.

Però això no és tot, qui més hi havia al meu voltant? Qui m'ha ajudat tant a treballar com a relaxar-me? Doncs una llista molt llarga de persones. En aquesta llista hi trobem tota la resta d'investigadors del

laboratori, tècnics o la Mar, la nostra administrativa, així com els treballadors de l'estabulari. Tots han aportat el seu granet de sorra. Per últim, també vull mencionar els estudiants que han passat per el nostre grup de recerca, els hagi ensenyat jo directament o no, tots han sigut diferents i especials a la seva manera.

A nivell més personal, hi ha tres grups de persones dels quals no em puc oblidar. Ells no han estat amb mi al laboratori, però han hagut d'aguantar-me, donar-me suport i cuidar-me.

En el primer grup trobem els amics. La Sofia, la Laia, el Marc i l'Andrea, amics propers d'ençà que vam començar a estudiar a la universitat. També hi trobem l'Aina, qui m'ha acompanyat des de l'adolescència i, l'Aaron, així com amb els companys de pis. A tot ells els agraeixo totes les hores i experiències que hem compartit. Tots ells són especials, m'han escoltat i animat. Amb ells he passat moltes hores, en grup o en solitari, i amb cadascun tinc una relació diferent, però totes igual de bones.

En el segon grup trobem la família. La meva mare, decidida i forta, i a la vegada tossuda i difícil. El meu pare, tranquil i intel·ligent, sempre amb un nou llibre a la mà. Els dos sempre m'han animat a superar-me i a tirar endavant. També hi trobem la meva germana, amb qui a vegades sembla que funcionem millor en la distància però que en el fons ens entem. En aquest grup també hi ha la meva àvia materna, sempre patint. Sempre llesta per a explicar-te una nova idea o repetir alguna anècdota. Amb ella comparteixo un humor molt especial amb el que, en més d'una ocasió, hem compartit estones molt divertides. A més a més, els avis paterns sempre m'han donat suport i

ànims per a tirar endavant i perseguir els meus objectius. Així com els tiets, cosins i, com no podia faltar, els animalons que tenim per casa, que sempre estan contents de veure't i preparats per una nova aventura. A tots ells me'ls estimo de tot cor.

L'últim grup està compost només per una persona. Algú especial i important per a mi, que ara és lluny però sempre porto molt a prop i que només el futur sap que ens espera.

Index

INDEX

ACRONYMS

AD	Alzheimer's disease
AED	Antiepileptic Drug
AKT	Protein Kinase B
AMPA	Amino-3-hydroxy-5-Methyl-4-isoxazolepropionic Acid
ARC	Activity-Regulated Cytoskeleton-associated protein
ATF4	Activating Transcription Factor 4
ATF6	Activating Transcription Factor 6
ATP	Adenosine Triphosphate
BAX	B-Cell Lymphoma 2-like protein 4
BCL-2	B-Cell Lymphoma 2
BCL-XL	B-Cell Lymphoma extra-large
BDNF	Brain-Derived Neurotrophic Factor
BIM	B-Cell Lymphoma 2-like protein 11
BIP	Immunoglobulin heavy chain-Binding Protein
BMI	Body Mass Index
CA	Cornu Ammonis
CHOP	C/EBP Homologous Protein
CREB	cAMP Response Element-Binding
DG	Dentate Gyrus
DIABLO	Direct IAP-Binding protein with Low PI
DNA	Deoxyribonucleic Acid
EIF2α	Eukaryotic Initiation Factor 2 alpha
ER	Endoplasmic Reticulum
ERK	Extracellular signal-Related Kinase

GABA	γ-aminobutyric acid
GLP1	Glucagon-Like Peptide 1
GLUT	Glucose Transporter
GPX	Glutathione Peroxidase
GRP78	Glucose-Regulated Protein 78
GSH	L-γ-Glutamyl-L-cysteinylglycine
GSK3β	Glycogen Synthase Kinase 3 β
HFD	High Fat Diet
IGF1R	Insulin-like growth factor 1 receptor
IL1β	Interleukin 1β
IR	Insulin Receptor
IRE1	Inositol-Requiring kinase/Endoribonuclease 1
IRS	Insulin Receptor Substrate
JIP	JNK-Interacting scaffold Protein
JNK	c-JUN N-terminal Kinase
KA	Kainic Acid
LIC-A	Licochalcone A
MAPK	Mitogen Activated Protein Kinase
MKK	Mitogen Activated Protein Kinase Kinase
NFκB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NMDA	N-Methyl-D-Aspartate
NORT	Novel Object Recognition Test
NOS	Nitric Oxide Synthase
OXPHOS	Oxidative Phosphorylation
PERK	Protein Kinase activated by double-stranded RNA (PKR)-like ER Kinase

PGC1α	Peroxisome Proliferator-Activated Receptor gamma (PPARγ) Coactivator 1-alpha
PI3K	Phosphoinositol-3-Kinase
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
PRX	Peroxiredoxin
PTP1B	Protein Tyrosine Phosphatase 1B
ROS	Reactive Oxygen Species
SAPK	Stress-Activated Protein Kinase
SE	Status Epilepticus
SOCS	Suppressor Of Cytokine Signalling
SMAC	Second Mitochondria-derived Activator of Caspases
SOD	Superoxide Dismutase
T2DM	Type 2 Diabetes Mellitus
T3D	Type 3 Diabetes
TLE	Temporal Lobe Epilepsy
TLR4	Toll-Like Receptor 4
TNFα	Tumour Necrosis Factor alpha
TRAF2	Tumour Necrosis Factor Receptor-Associated Factor 2
UPR	Unfolded Protein Response
WHO	World Health Organization
XBP1	X-box Binding Protein 1

ABSTRACT

Many past reports on the c-JUN N-terminal Kinases (JNKs) did not take into account the existing differences in the activity of each of the isoforms. And so, therapeutic proposals that regulated the JNKs unspecifically encountered setbacks of import. The aim of the present thesis was to contribute to current understanding of the role of individual JNK isoforms in the development of pathology and, to appraise any therapeutic interest derived of their modulation for temporal lobe epilepsy and the metabolic-cognitive syndrome.

Reported results demonstrated that the knock-out JNK1 had neuroprotective effects against excitotoxic damage derived of the administration of kainic acid, a model of temporal lobe epilepsy. Thus, Licochalcone A (LIC-A), a JNK1 inhibitor, was tested for its potential as a therapeutic agent. Results confirmed that when animals were pre-treated with LIC-A they were protected from the effects of kainic acid, as demonstrated by the absence of degenerating cells and sclerotic tissue, as well as lower neuroinflammatory responses in astrocytes and microglia.

Additionally, the metabolic consequences of a chronic feeding of a fat-enriched diet (High fat diet; HFD) were also assessed. Data demonstrated that HFD caused the appearance of peripheral and central insulin resistance as a result of mitochondrial and endoplasmic stress, dysregulation of autophagy and other alterations. In the end, it led to the appearance of cognitive impairments. Parallelly, the effects of the ablation of JNK2 were evaluated and, it was determined that it favoured the appearance of these same alterations, especially when combined with HFD. On the contrary, the knockout of JNK1 protected against the

metabolic consequences of a chronic feeding with HFD, showing improved sensibility to insulin, reduced body weight and more efficient mitochondrial activity. Moreover, these animals were protected against the appearance of metabolic-derived cognitive dysfunctions.

RESUM

Molts estudis previs sobre el paper de les cinases c-JUN N-terminal (JNK) no tenien en compte les diferències existents en l'activitat de cadascuna de les isoformes. Això va provocar que les propostes terapèutiques que regulaven les JNK de manera inespecífica es trobessin amb problemes importants. L'objectiu de la present tesi doctoral era ampliar els coneixements que es tenen actualment sobre el paper individual de les isoformes de les JNK i, avaluar qualsevol interès terapèutic que pugui derivar de la seva modulació per a l'epilèpsia del lòbul temporal i afectacions cognitives derivades del metabolisme.

Estudis previs van demostrar que la inactivació genètica de la JNK1 tenia efectes neuroprotectors davant el dany citotòxic derivat de l'administració d'àcid kainic, un model d'epilèpsia del lòbul temporal. Per tant, es va posar a prova el potencial terapèutic de la Licochalcona A (LIC-A), un inhibidor de la JNK1. Els resultats van confirmar que quan els animals eren pretractats amb LIC-A, aquests quedaven protegits dels efectes de l'àcid kainic, tal i com ho demostrava l'absència de cèl·lules en degeneració ni de teixit escleròtic, així com una menor resposta neuroinflamatòria en astròcits i micròglia.

A més a més, es van estudiar les conseqüències metabòliques d'una alimentació crònica amb una dieta rica en greixos (HFD). Els resultats demostraven que la dieta provocava l'aparició de resistència la insulina a escala central i perifèrica com a resultat d'estrès en les mitocòndries i el reticle endoplasmàtic, desregulacions de l'autofàgia, entre altres. Al final, això portava a l'aparició d'afectacions cognitives. Paral·lelament, es van avaluar els efectes de la inactivació genètica de la JNK2 i, es va determinar

que afavoria l'aparició d'aquestes mateixes alteracions, especialment quan es combinava amb HFD. Per contra, la inactivació de JNK1 protegia de les conseqüències metabòliques d'una ingesta crònica de HFD, afavorint una major sensibilitat a la insulina, un menor pes corporal i una activitat mitocondrial més eficient. A més a més, aquests animals estaven protegits davant l'aparició de dèficits cognitius derivats d'alteracions metabòliques.

RESUMEN

Muchos estudios previos sobre el papel de las quinasas c-JUN N-terminal (JNK) no tenían en cuenta las diferencias existentes en la actividad de cada una de las isoformas. Esto provocó que las propuestas terapéuticas que regulaban las JNK de forma inespecífica se encontraran con problemas importantes. El objetivo de la presente tesis doctoral era ampliar los conocimientos que se tienen actualmente sobre el papel individual de las isoformas de las JNK y, evaluar cualquier interés terapéutico que pueda derivar de su modulación para la epilepsia del lóbulo temporal y afectaciones cognitivas derivadas del metabolismo.

Estudios previos demostraron que la inactivación genética de la JNK1 tenía efectos neuroprotectores ante el daño citotóxico derivado de la administración de ácido kaínico, un modelo de epilepsia del lóbulo temporal. Por tanto, se puso a prueba el potencial terapéutico de la Licochalcona A (LIC-A), un inhibidor de la JNK1. Los resultados confirmaron que cuando los animales eran pretratados con LIC-A, estos quedaban protegidos de los efectos del ácido kaínico, tal y como lo demostraba la ausencia de células en degeneración ni de tejido esclerótico, así como una menor respuesta neuroinflamatoria en astrocitos y microglía.

Además, se estudiaron las consecuencias metabólicas de una alimentación crónica con una dieta rica en grasas (HFD). Los resultados demostraban que la dieta provocaba la aparición de resistencia a la insulina a escala central y periférica como resultado de estrés en las mitocondrias y el retículo endoplasmático, desregulaciones de la autofagia, entre otros. Al final, esto llevaba a la aparición de afectaciones

cognitivas. Paralelamente, se evaluaron los efectos de la inactivación genética de la JNK2 y, se determinó que favorecía la aparición de estas mismas alteraciones, especialmente cuando se combinaba con HFD. Por lo contrario, la inactivación de JNK1 protegía ante las consecuencias metabólicas de una ingesta crónica de HFD, favoreciendo una mayor sensibilidad a la insulina, un menor peso corporal y una actividad mitocondrial más eficiente. Además, estos animales quedaban protegidos ante la aparición de déficits cognitivos derivados de alteraciones metabólicas.

Introduction

INTRODUCTION

Modern societies are characterized by technological innovations that have transformed human life in fundamental ways. In developed countries, people live longer and healthier. Now, it is possible to cure diseases that were once considered death sentences. Still, there are significant woes that need to be addressed.

An increase in stress and anxiety, unbalanced and unhealthy diets, dominating sedentary lifestyles and exposure to pollution and radiation are just a few of these struggles, and they have paramount impact on human health. Understanding how these factors influence the development of complex diseases is crucial in order to develop safe, state-of-the-art treatments.

This thesis discusses some molecular mechanisms responsible for the regulation of healthy physiology, and how these same systems bring about the development of disease. Specifically, it focuses on the study of the c-JUN N-terminal Kinases (JNKs) and their ability to control a wide range of cellular mechanisms in physiological and pathological conditions, as well as their potential as therapeutic targets in the context of neurodegenerative processes.

MITOGEN ACTIVATED PROTEIN KINASES

The maintenance of homeostasis is key to cellular physiology. It is possible thanks to highly specialized biochemical mechanisms that respond to intra- and extracellular stimuli and induce alterations and regulatory modifications.

The mitogen-activated protein kinases (MAPKs) are a superfamily of intracellular, ancient and highly conserved serine (Ser)/threonine (Thr) kinases that regulate multiple cellular functions by converting stimuli into responses. They can be found in most eukaryotic cells and are responsible for the control of cell proliferation, differentiation, migration, senescence and apoptosis among other processes (*Cargnello M and Roux PP, 2011*). Substrates for these kinases are located in the cytoplasm, mitochondria, Golgi apparatus, endoplasmic reticulum (ER) and the nucleus. The signals transmitted via the various cascades modulate the activity of a large number of transcription factors and suppressors, as well as chromatin remodelling proteins (*Plotnikov A et al., 2011*).

This family of kinases is composed of four main cascades that propagate signals through sequential phosphorylation with the aid of scaffold proteins. These regulators have the ultimate role of inducing modifications in gene expression but are also implicated with proteins in the plasma membrane and the cytosol, thereby regulating additional cellular mechanisms. These proteins are the extracellular signal-related kinases (ERK1/2), ERK5, p38 and the JNKs (**Figure 1**) (*Sabapathy K, 2012*; *Sun Y et al., 2015*).

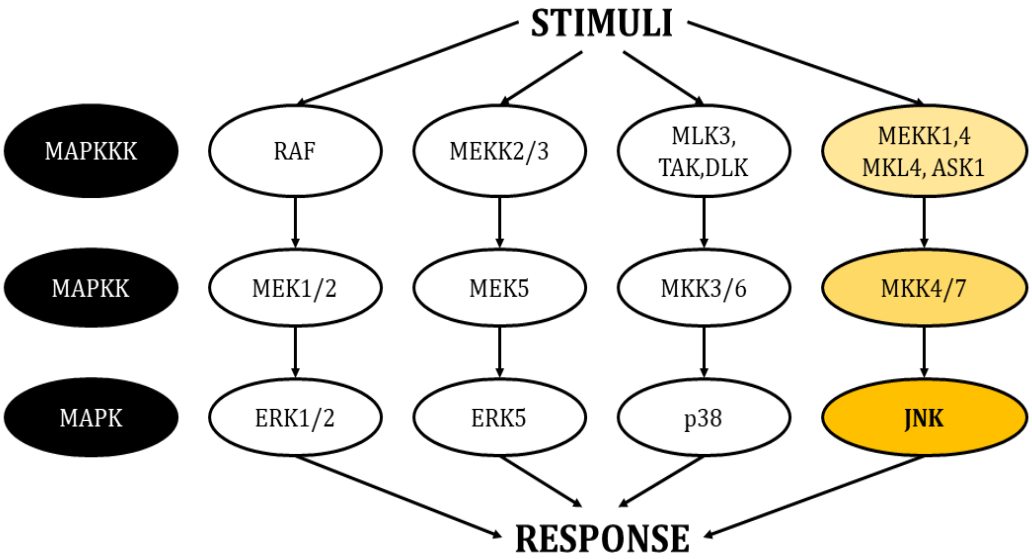


Figure 1. Schematic of the cascades in the MAPK pathway. Each pathway responds to different stimuli and creates an appropriate response (modified from *Cargnello M and Roux PP, 2011*).

Each of the MAPK cascades responds to different sets of stimuli, prompting the appropriate responses according to the environment surrounding the cell. In general:

- ERK1/2 are activated by growth factors, insulin, ligands linked to the activity of G-protein coupled receptors, cytokines, osmotic pressure and microtubule disorganization (*Pinto DJ et al., 2005*).
- ERK5 becomes stimulated by growth factors, oxidative stress and hyperosmolarity (*Wang X et al., 2006*).
- p38 isoforms have severe responses to environmental agents like oxidative stress and UV radiation, as well as to inflammatory cytokines (*Cuadrado A and Nebreda AR, 2010*).

- The JNKs, also known as stress-activated protein kinases (SAPK), are triggered by multiple stimuli, including heat shock, ionizing radiation, oxidative stress, deoxyribonucleic acid (DNA)-damaging agents, cytokines, ultraviolet irradiation, protein inhibitors, G protein coupled receptors, as well as deprivation and presence of growth factors… (*Davis RJ, 2000; Cui J et al., 2007*).

C-JUN N-TERMINAL KINASES

The JNKs are stress-response elements that become activated through dual phosphorylation in Thr and tyrosine (Tyr) residues by the Mitogen Activated Protein Kinase Kinase 4 (MKK4) and 7 (MKK7) with the JNK-interacting scaffold protein (JIP) (*Yasuda J et al., 1999*).

Over the years, three isoforms have been described for the JNKs: JNK1, JNK2 and JNK3. These variants share 85% of identity and are coded by three genes: (*Mapk8 (Jnk1)*, *Mapk9 (Jnk2)* and *Mapk10 (Jnk3)*). These can generate up to 10 spliced protein products, with molecular masses of 46 or 55 KDa. Isoforms JNK1 and JNK2 can be found almost ubiquitously but, JNK3 is only expressed in the brain, testes and cardiac myocytes (*Cui J et al., 2007; Coffey ET, 2014*). During embryonic development, isoforms JNK1 and JNK2 are the first to be expressed at E7, while JNK3 starts its expression at E11 (*Borsello T, 2012; Yarza R et al., 2016*). Studies using knockout animals have determined that mice deficient in any of the three genes are viable without apparent morphological abnormalities in recent developmental stages. Also, it has been described that lack of expression of both Mapk8 (JNK1) and Mapk9 (JNK2) is non-viable in

mice. Triple JNK knockouts have been tested *in vitro* and cause neuronal hypertrophy and reduction in the number of dendrites. However, an increase in lifespan is also observed due to a reduction of the capacity to activate apoptosis (*Borsello T, 2012*).

In vitro studies have shown that JNK activation can be transient, often leading to cellular survival signals, or sustained, leading to cell death. In most cases, when activated, these kinases relocalize from the cytoplasm into the nucleus where they phosphorylate the cJUN transcription factor in the Ser63/73 residues (*Sabapathy K, 2012*).

Additional substrates for the JNKs include p53, activating transcription factor 2, heat shock factor 1 c-Myc and JUNB, among others. Continuous discoveries, the description of new mechanisms and the widespread effects of these kinases point to the possibility that there are still many other substrates that are yet to be identified (*Cui J et al., 2007*; *Cargnello M and Roux PP, 2011*).

Role of the JNKs in the development of disease

Many reports describe how, in pathological states, JNK activity is largely upregulated, indicating that these kinases play a significant role as originators and aggravators of pathological features (*Sabapathy K, 2012*).

When cells are exposed to a stress, the initial response of the JNKs is to activate survival mechanisms, but if the alteration prolongs in time or is too intense, the JNKs will promote cellular death (*Green DR and Llambi F, 2015*). Specifically, sustained activation favours the

activation of apoptotic and necrotic cellular mechanisms, coupled with the upregulated expression of cytotoxic ligands, like tumour necrosis factor α (TNFα) or interleukin 1 β (IL1β), along with increased concentration of reactive oxygen species (ROS) (*Ventura JJ et al., 2006; Madhumita D et al., 2009; Koch P et al., 2015*). Also, the JNKs regulate the intrinsic apoptotic pathway through mitochondria by modulating the activity of the members of the Bcl2 family; activating pro-apoptotic agents and inhibiting anti-apoptotic ones (*Tournier C et al., 2000*).

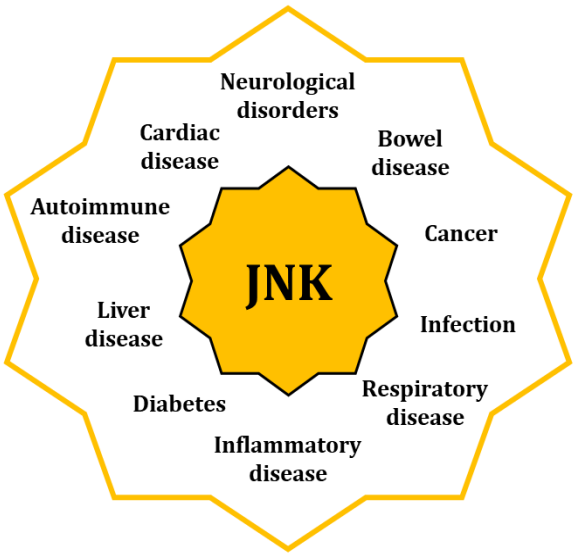


Figure 2. Increased activity of the JNKs is associated with pathologies of different nature through their regulatory control of multiple cell mechanisms.

Pathologies linked to the activity of the JNKs can be found in almost all systems and apparati - neurological, coronary, hepatobiliary, respiratory, autoimmune, inflammatory, as well as cancer, diabetic alterations and obesity. In many cases, these conditions are associated

with mitochondrial and ER dysregulations, as well as with the disruption of other cellular mechanisms like autophagy or cell cycle progression (**Figure 2**) (*Cui J et al., 2007; Sabapathy K, 2012*).

JNKs in subcellular compartments

Mitochondria

Mitochondria are double-membraned organelles critical for the regulation of many cellular processes (*Waldbaum S and Patel M, 2010*). Their primary function lies in the production of intermediaries in the Krebs's Cycle and the maintenance of chemiosmotic balances between the inner and intermembrane spaces, allowing for the synthesis of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) (*Picard M et al., 2011*).

The mitochondrial electron transport chain pumps protons (H^+) into the intermembrane space efficiently. Despite this, some electrons leak into the inner matrix, becoming a source for the generation of ROS molecules. These particles are usually defined as partially-reduced metabolites of oxygen possessing higher reactivity that molecular oxygen (O_2) (*Thannickal V and Fanburg B, 2000*). There is growing evidence that indicates that a majority of the superoxide radicals (O_2^-) are generated by the OXPHOS complexes I and III (*Muller F et al., 2004*). Research suggests that about 1-4% of the O_2 that is used in cellular respiration is converted into O_2^-, which may be later transformed into hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) or peroxynitrite ($ONOO^-$) (Boveris A, 1984; *Andreyev A et al., 2005*).

Physiologically-speaking, the concentration of ROS molecules follows a pattern of transient fluctuations that have regulatory functions, requiring very tight control (*Martindale JL and Holbrook NJ, 2002*). Organisms have evolved multiple methods to counteract the production and accumulation of ROS. These include the use of non-enzymatic molecules like glutathione, vitamins A, C, E and flavonoids, as well as enzymatic ROS-degrading agents like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and peroxiredoxin (PRX). Additional antioxidant protection can be achieved through the activity of L-γ-glutamyl-L-cysteinylglycine (GSH)-linked antioxidant enzymes (*Ott M et al., 2007*).

Unfortunately, the mechanisms responsible for the degradation of ROS are not always enough to counteract their production, leading to an increase in their concentration and accumulation, and the subsequent damage to most macromolecules. This situation is known as "oxidative stress" (*Martindale JL and Holbrook NJ, 2002*). Examples of the consequences include: damage to DNA, direct oxidation and inactivation of iron-sulphur (Fe-S) proteins, increased lipid peroxidation, as well as alteration of vital mitochondrial functions, inner membrane barrier properties and potential (*Ott M et al., 2007*).

Oxidant injury elicits a wide spectrum of responses ranging from growth arrest to cell death through the release of pro-apoptotic proteins from mitochondrial intermembrane spaces. It has been described that the JNKs would play major role in this mechanism (*Martindale JL and Holbrook NJ, 2002; Ott M et al., 2007*).

The JNKs signal for cell death through the activation of pro-apoptotic proteins like the B-cell lymphoma 2-like protein 4 (BAX) or B-cell lymphoma 2-like protein 11 (BIM). It may also inhibit the anti-apoptotic protein B-cell lymphoma 2 (BCL-2) and B-cell lymphoma extra-large (BCL-XL). In the end, its actions will affect mitochondrial membrane integrity and cause the release of cytochrome c, Second Mitochondria-derived Activator of Caspases (SMAC) and Direct IAP-Binding protein with Low PI (DIABLO) proteins (**Figure 3**) (*Hanawa N et al., 2008; Chambers JW and LoGrasso PV, 2011*).

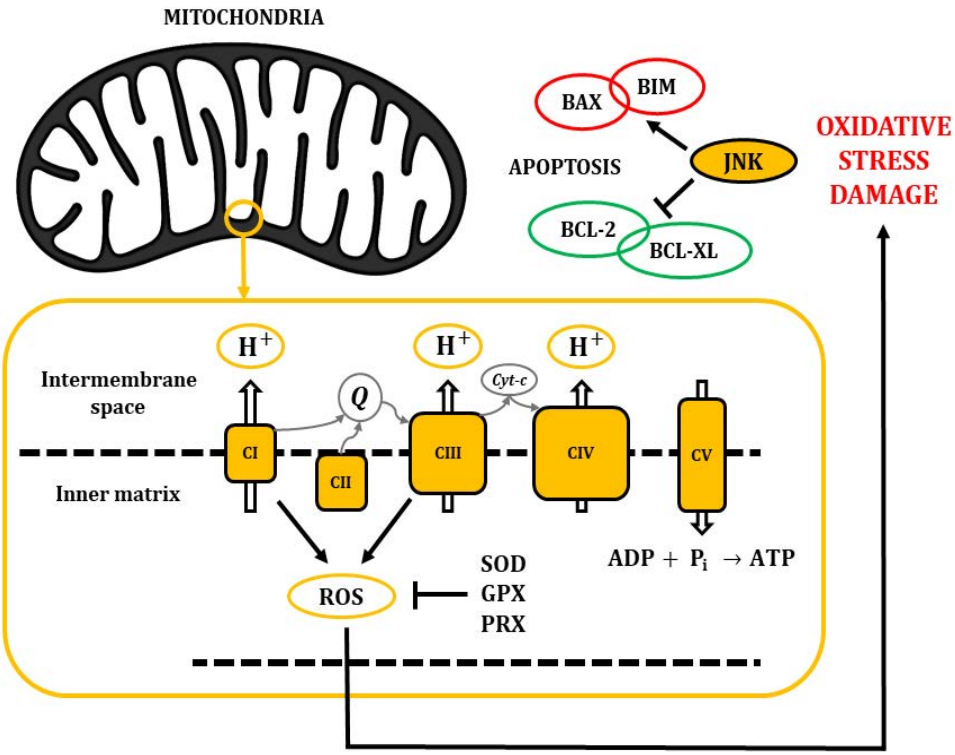


Figure 3. The electron transport chain is the pathway that pumps H^+ from the cytosol into the intermembrane space of the mitochondria, creating a gradient that returns into the inner matrix through the ATP synthase (CV), allowing for the synthesis of ATP. Accumulation of ROS leads to oxidative stress and damage to macromolecules, inducing pro-apoptotic activity through the activation of the JNKs.

Endoplasmic reticulum

The ER is a dynamic organelle found in all eukaryotic cells. It is made up of membranous structures that are both interconnected and branched to form tubules, vesicles and cisternae (*Schöntal A, 2012*). ER lumen provides a subcellular oxidative compartment that is important for the formation of disulphide bonds and proper protein folding (*Xu C et al., 2005; Schöntal A, 2012*). It also participates in the synthesis of fatty acids and phospholipids, assembly of lipid bilayers, carbohydrate metabolism, calcium homeostasis... (*Schöntal A, 2012*).

Proper ER functionality depends on the maintenance of homeostatic conditions. This state can be significantly affected by a diverse range of elements, like oxygen pressure, glucose, temperature, acidosis, calcium and energy levels to name a few (*Schöntal A, 2012*).

ER lumen is rich in calcium-dependent chaperones such as the master regulator immunoglobulin heavy chain-binding protein (BIP; also known as glucose-regulated protein 78 (GRP78)) (*Haas I and Wabl, 1983*). In physiological conditions, part of the BIP molecules are bound to specific transmembrane proteins in their N-terminal region, inhibiting their activity and maintaining them in an inactive state. These proteins are the protein kinase activated by double-stranded RNA (PKR)-like ER kinase (PERK), the inositol-requiring kinase/endoribonuclease 1 (IRE1), and activating transcription factor 6 (ATF6) (*Xu C et al., 2005*).

When ER balances are disturbed, protein folding is negatively affected, causing an accumulation of unfolded and misfolded proteins. This situation favours the release of BIP in order to respond to the

increased need for protein folding enzymes, and the posterior triggering of the unfolded protein response (UPR) through the activation of the three signalling pathways (**Figure 4**) (*Kaneto H et al., 2005; Malhotra J and Kaufman R, 2007; Ron D and Walter P, 2007*).

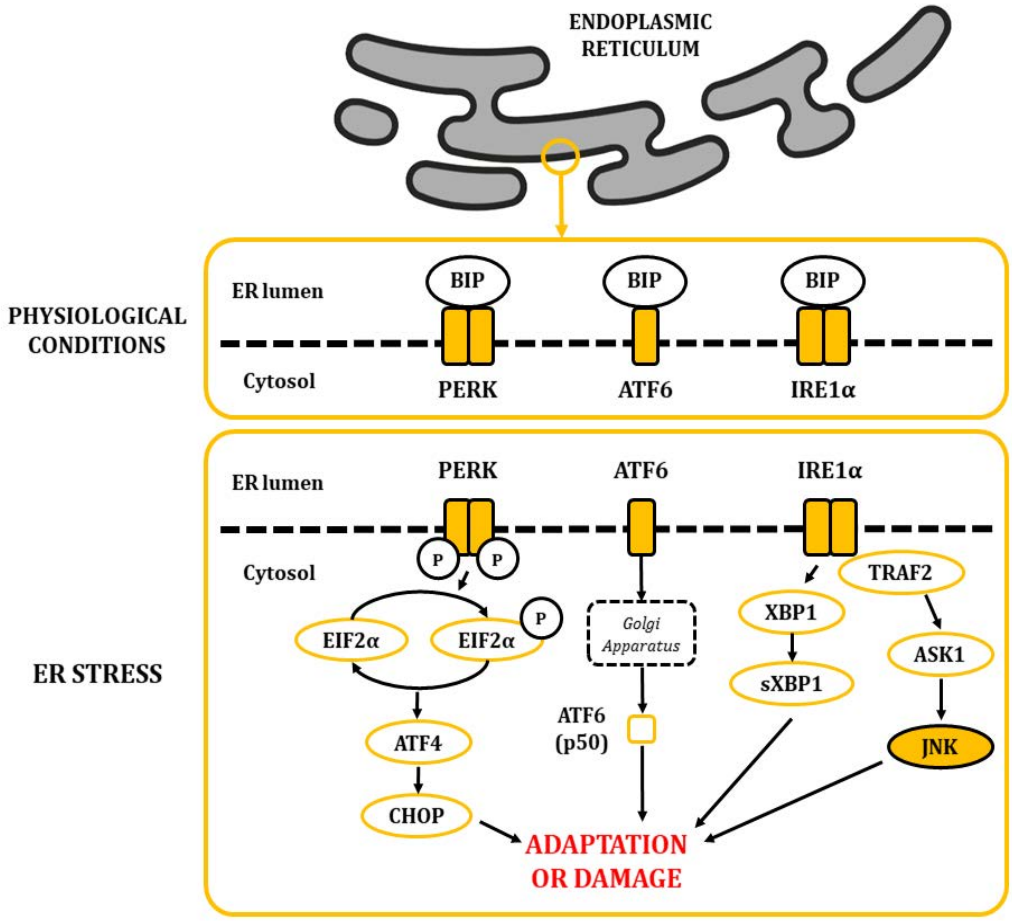


Figure 4. Alteration of ER homeostasis favours the accumulation of unfolded and misfolded proteins in the lumen of the ER. This situation leads to the dissociation of BIP from the PERK, ATF6 and IRE1α proteins, allowing for their activation and posterior signalling of the UPR.

The first pathway. When the PERK pathway is activated, PERK subunits homodimerize and autophosphorylate. Later, they phosphorylate the eukaryotic initiation factor 2 alpha (EIF2α) in serine residues, causing a decrease in global protein synthesis. This step is expected to reduce protein influx into the ER, ameliorating cytotoxic damage caused by accumulated proteins (*Harding H et al., 2000*). Furthermore, it increases the translation of the activating transcription factor 4 (ATF4) into the nucleus, and the stimulation of genes related to the recovery of homeostasis and/or the adaptation to the environment (*Fels D and Koumenis C, 2006*).

The second pathway. After releasing BIP from ATF6, it translocates to the Golgi apparatus where it becomes proteolytically cleaved, releasing a p50 fragment from ATF6. This part migrates into the nucleus where it stimulates the expression of genes linked to the resistance to ER stress damage (*Szegezdi E et al., 2006; Lin J et al., 2008; Kaneko M et al., 2017*).

The third pathway. IRE1α has two functions after becoming homodimerized and autophosphorylated. For its first function, IRE1α cleaves mRNA molecules of X-box binding protein 1 (XBP1). The spliced products are transcription factors that control the expression of genes that stimulate ER-associated protein degradation (*Ron D and Hubbard S, 2008*). As a second function, this protein activates a cascade through TNF receptor-associated factor 2 (TRAF2) that results in the activation of the JNKs (*Urano F et al., 2000*).

When stresses are mild or only last for a short-term, activation of this mechanism allows for necessary adaptation to the environment

or neutralisation of the stress. However, if they are too severe or long-lasting, they favour the activation of pro-apoptotic molecules that will trigger cell death to dispense of dysfunctional cells (*Xu C et al., 2005*; *Schöntal A, 2012*). For example, the C/EBP homologous protein (CHOP) becomes highly expressed through the activation of the PERK pathway through ATF4 and is a key initiator of pro-apoptotic mechanisms. CHOP stimulates the expression of BIM, BID and BAX, which in turn promote the repression of anti-apoptotic BCL-2. This mechanism is highly coordinated and parallel to the activity of the JNKs (Nishitoh H, 2012). Signalling of the IRE1α pathway also promotes cell death by activating caspases, a large family of cysteine proteases that either relay or act as the actual effectors of apoptosis (*Lin J et al., 2008)*.

Additional consequences of JNK activation likely go further, causing for other harmful physiological alterations. As an example, when ER stress occurs and the JNKs are activated through the IRE1α pathway, BCL-2 activity is suppressed, disrupting its binding to beclin 1, an essential autophagy regulator (*Hotamisligil GS and Davis RJ, 2016)*.

JNKs in the brain

It has been described that the JNKs have higher activity in the brain than in any other tissue, suggesting they may be key players in the regulation of protein function and gene expression in the central nervous system (*Coffey ET and Courtney MJ, 1997; Xu X et al., 1997; Borsello T, 2012*). Each of the three JNK genes show the highest

expression in the neocortex, followed by the hippocampus, thalamus and midbrain (*Coffey ET, 2014*).

Using *in situ* hybridation in mice, it was determined that the JNK isoforms are distributed differently within adult brain tissue. The JNK1 isoform is found in the somatic space, axons and dendrites in the cortical layers III and IV. It shows significantly high expression in the cytosol and dendrites of thalamic neurons. The JNK2 isoform is found in the same areas but its limited in its localization, found only in the cytosol and nucleus. The JNK3 isoform is found at the highest concentration in layers III and V (pyramidal neuron layers) in the cortex and in the nuclei of Purkinje cells. Additionally, isoforms 1 and 3 are the most predominant in the hippocampus, with JNK1 expression being at its highest in the *cornu ammonis* 3 (CA3), CA4 and hilus of the dentate gyrus (DG). JNK3 is mostly localized to the somas of hippocampal pyramidal neurons (*Lee J et al., 1999*). On a cellular level, the JNKs are primarily distributed in the cytoplasm but, as it has been previously mentioned, they can be found in other cellular compartments. In neurons, the JNKs have been localized in peripheral structures like dendritic spines and synapses, being relevant in their maintenance and dynamics (*Mao L and Wang J, 2016*).

The JNKs play roles in development, cellular proliferation, differentiation and morphogenesis, as well as neuronal pathfinding and synaptic maintenance. It is also thought that the JNKs regulate neuronal migration by phosphorylating cytoskeletal proteins. Furthermore, they are involved in neuronal death through apoptotic mechanisms after cellular insults, suggesting that they play important roles in neurodegenerative pathologies and in the physiology of

neuropsychiatric disorders (*Cui J et al., 2007, Weston C and Davis R, 2007; Coffey ET, 2014*).

The JNKs are also involved in the development of neuroinflammatory responses, a feature described as highly important in most degenerative disorders. They are expressed in microglia, astrocytes and oligodendrocytes, which are cells that contribute to this mechanism (*Haeusgen W et al., 2009; Kaminska B et al., 2009*). In initial stages, an inflammatory response causes for the activation of microglial cells and posterior release of cytokines which will favour the reactivity of the rest of the tissue through the upregulation of factors like cJUN and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (*Borsello T, 2012*).

DISEASES LINKED TO THE ACTIVITY OF THE JNKs

In this thesis, two different diseases have been studied due to the relevance of the JNKs in their neuropathophysiology: temporal lobe epilepsy (TLE) and the metabolic-cognitive syndrome.

TEMPORAL LOBE EPILEPSY

Epilepsy is a chronic, noncommunicable, neurological disease of the brain that affects people of all ages and is characterized by a predisposition for recurrent seizures (*World Health Organization (WHO); Vezzani A et al., 2011*). Epidemiological data indicates that it contributes 1-2% of the global burden of disease with around 50 million people affected by epilepsy worldwide (80% of them live in developing countries) (*WHO; Prilopko L, 2005; Vezzani A et al., 2011; Steinhäuser C et al., 2016*).

Seizures are brief episodes of involuntary movement that may be partial or full bodied. They are produced as a result of abnormal transient electrical discharges by firing neurons in different brain areas (epileptogenic focuses), and a posterior spreading across the cortex through the recruiting of other neuronal populations (*WHO; Pinto D et al., 2005*). TLE is the most common form of human epilepsy (40% of all cases). Its development involves three stages (**Figure 5**) (L*évesque M et al.,2016; Nirwan N et al., 2018)*:

1. *Initial insult:* leads to the appearance *of status epilepticus* (SE). Insults include situations like severe encephalitis, febrile illness,

alcohol or drug abuse, exposure to chemical agents..., which can be combined with other factors like genetic predisposition and developmental dysfunctions (*Lévesque M and Avoli M, 2016*).

SE has been defined as a period of tonic-clonic seizure activity that may or may not lead to a lack of consciousness, lasts at least 30 min and is accompanied by an increase in intracranial pressure, release of cytokines, chemokines, lipid mediators and other molecules, and a decrease in cerebral blood flow. This medical condition is life-threatening and needs to be treated rapidly or it may lead to permanent neuronal damage and synaptic reorganization (*Cherian A and Sanjeev T, 2009; Lévesque M et al., 2016; Seinfeld S et al., 2016*).

2. *Latent period (epileptogenesis)*: time between the initial insult and the first clinical manifestation of spontaneous seizures. This period ranges anywhere from a few days to many years in humans. During this period, there is no epileptic activity but, it is assumed that alterations in neuronal and glial circuitry are occurring. For example, restructuration of pre/post-synaptic terminals, changes in ion channels, dysfunction of homeostatic mechanisms, as well as strong and persistent inflammatory states in astrocytes and microglial cells in the microenvironment of the neuronal tissue that can last for over 3 to 4 months (*Lévesque M et al., 2016; Hiragi T et al., 2018; Rana A and Musto A, 2018*). Additional alterations associated to the latent period include aberrant neurogenesis in the subgranular zone of the DG, coupled with ectopic positioning of new cells in the hilus (*Zhong Q et al., 2016*; *Hiragi T et al., 2018*).

3. *Chronic period*: There is an occurrence of spontaneous complex focal seizures due to a state of neuronal hyperexcitability and hypersynchrony. They may show clustering profiles in which they occur many times a day over multiple consecutive days (*Lévesque M and Avoli M, 2016*).

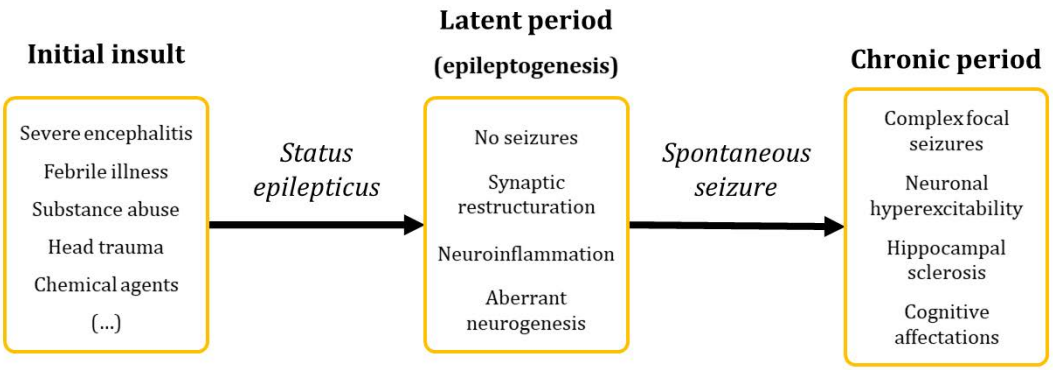


Figure 5. Time course of the different stages leading to the development of TLE. Initial insult → Latent period → Chronic period.

Neuroanatomical examinations of the brains of patients with TLE have shown that they typically display sclerosis in the CA1, CA3 and hilus of the DG of the hippocampus, coupled with granule cell dispersion and sprouting of aberrant mossy fibres in the molecular layer of the DG (*Lévesque M and Avoli M, 2016*). TLE will also cause cognitive sequelae on memory, attention, language, praxis, executive function, judgment, insight and problem solving (*Zhao F et al., 2014; Tramoni-Negre E et al., 2017*).

Current standardized treatments for epilepsy include a wide group of molecules with multiple mechanisms of action (antiepileptic drugs;

AEDs). But, in about 30% of patients affected by this illness, these strategies are ineffective and the pathology becomes pharmacoresistant or refractory. It has been hypothesized that this outcome is the consequence of a lack of understanding of all the mechanisms involved in the pathology. After all, AEDs only target the symptoms of epilepsy, not the underlying pathology. In this situation, surgical resection of the affected tissue is the only alternative but, in some occasions, it is impracticable or it only reduces the occurrence of seizures as a result of an insufficient resection or a lack of clear identification of their focus (*Vezzani A et al., 2011; Lévesque M et al., 2016; Lévesque M and Avoli M, 2016; Nirwan N et al., 2018; Rana A and Musto A, 2018*).

Preclinical models

In the last four decades, researchers have developed multiple models that allow to reproduce some of the electroencephalographic, behavioural and neuropathological features of TLE (*Lévesque M et al., 2016; Nirwan N et al., 2018*). These protocols induce an initial brain injury that favours the development of an SE-like state and a posterior latent period that will eventually result in the appearance of spontaneous seizures (*Lévesque M et al., 2016*). These models allow for the study of both the pathophysiology of the disease and the efficacy and safety profile of potential anti-epileptic molecules, just like their mechanism of action (*Nirwan N et al., 2018*). Some examples of these models include:

- *Kainic acid (KA)* (first described by *Ben-Ari et al., 1979*): cyclic analogue of L-glutamate that behaves as an agonist of amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and ionotropic KA receptors. It was first extracted and isolated from a red alga usually found in tropical and subtropical waters (*Digenea simplex*). The hippocampus and amygdala are often the sites of origin of seizures, which later propagate to the neocortex. It produces the neuronal degeneration typically found in humans with TLE. The chronic phase reproduces approximately two weeks after the initial administration (*Nadler JV, 1979; Cavalheiro E et al., 1982; Nirwan N et al., 2018*).

KA receptors are expressed in the amygdala, entorhinal cortex, basal ganglia, cerebellum and hippocampus. Here, they are mostly located in the CA1 and CA3 regions, making them highly susceptible to the excitotoxic damage induced by KA (*Vincent P and Mulle C, 2009; Lévesque M and Avoli M, 2016*).

- *Pilocarpine* (first described by *Turski et al., 1983*): agonist of cholinergic muscarinic and N-Methyl-D-aspartate (NMDA) receptors. The epileptogenic focus is limited to the hippocampus, posterior propagation affects the amygdala and the neocortex. Just like in KA, spontaneous seizures appear around 2 weeks after injection (Hamilton S et al., 1997; *Lévesque M et al., 2016*).

This model has been combined with a pre-treatment of lithium, causing a reduction in seizure threshold by increasing the permeability of the blood-brain barrier, requiring a much lower pilocarpine dosage in order to induce SE (*Marchi N et al., 2007*).

These models are the most used and valid for the reproduction of human TLE. Yet, none of them can reproduce all the features of the pathology in humans. It is necessary to mention that effects vary between species and strains. There is high variability in mortality rates and posterior neuronal damage. Other elements that play a significant role in the response include age, supplier, colony, feeding, handling... (*Holmes G and Thompson J, 1988; Langer M et al., 2011*).

Seizure symptoms developed by the systemic administration of these substances can be scored in order to evaluate the severity of the response, and to be able to quantify the possible differences obtained by the experimental conditions. *Stage 0* signifies no response. *Stage 1* denotes chewing or facial twitches. *Stage 2* is chewing and head nodding or wet dog shakes while *stage 3* is unilateral forelimb clonus. *Stage 4* is bilateral forelimb clonus and rearing, and finally. *stage 5* is bilateral forelimb clonus, rearing and falling (Racine R et al, 1972).

Other models to induce TLE-like states include:

- *Electric kindling*: repetitive electrical stimulation protocols can induce the appearance of a chronic hyperexcitable state that can precipitate the occurrence of spontaneous seizures. It is a highly malleable method that allows for targeting of specific regions and allows for easy manipulation of experimental parameters. Controversially, this model does not cause visible lesions (no hippocampal sclerosis), leading to doubts on its validity (Mcintyre D and Leech C, 1969).
- *Organophosphate pesticides*: these molecules mirror the characteristics of pilocarpine-induced SE through the

hyperstimulation of cholinergic receptors. Neurodegenerative damage is usually observed in the CA1, CA3 and DG areas of the hippocampus (*De Aranjo FM et al., 2010*).

- *Flurothyl* (2,2,2-trifluoroethyl ether): it is volatile and fast acting stimulant that induces SE in rodents. Effects are variable depending on the age of the subject (*Nirwan N et al., 2018*).
- *Cobalt-homocysteine*: homocysteine affects the synthesis of NMDA and γ-aminobutyric acid (GABA), while cobalt causes pathological lesions that impairs amino acid metabolism. The exact pathological mechanisms in this preclinical model are not completely understood to date (*Nirwan N et al., 2018*).
- *Orphenadrine*: induces SE through the blockade of muscarinic and histaminic receptors (*Rejdak K et al., 2011*).
- *Thiocolchicoside*: causes SE by blocking $GABA_A$ receptors and activating strychnine-sensitive glycine receptors. Additionally, it has muscle relaxant properties (antiinflammatory and analgesic effects) (*Giavina Bianchi P et al., 2009*).
- *Quinolinic acid*: amino acid metabolite of tryptophan that behaves as endogenous excitatory molecules of glutamatergic signalling pathways (*Schwarcz R et al., 1984*).

Pathophysiology

On a molecular level, TLE is characterized by profound alterations in cellular homeostasis. As it has been previously mentioned, one of the consequences of seizure activity is the development of sclerotic tissue, derived by induction of cellular death in the neural tissue,

coupled with inflammatory and immunological reactions (*Liu G et al., 2011; Chatzikonstantinou A, 2014*). The appearance of gliosis, characterized by proliferation and hypertrophy of glial cells, increases tissue damage and promotes the secretion of cytokines and chemokines (IL1, TNFα, IL6, IL10, interferon α and β, etc.). Crosstalk between neurons and glial cells in this situation reduces seizure threshold in neurons (*Puttachary S et al., 2015*).

Seizures cause for activation of the apoptotic extrinsic pathway, via the activation of response elements like the TNF receptor 1. Furthermore, the intrinsic pathway is activated as a result of high levels of calcium in the cytoplasmic compartment. This accumulation also has negative consequences in a diverse array of organelles, including the mitochondria and ER, resulting in the increased production and release of molecules like ROS and cytochrome c, and the upregulation of caspase activity. The activation of IRE1α in the ER, as well as the release of ROS in the mitochondria also favours the maintenance of a constant activated state in the JNKs (*Henshall DC, 2007; Waldbaum S and Patel M, 2010; Liu G et al., 2011; Puttachary S et al., 2015; Tai T et al., 2017*).

The use of preclinical models for TLE has allowed to describe the roles of the different JNK isoforms. Specifically, combining TLE models with isoform-specific JNK knockouts, has led to the discovery that when JNK1 and JNK3 are absent, mice become highly resistant to the damage resulting from KA (*Yang DD et al., 1997; Schauwecker P, 2000; Coffey ET, 2014; de Lemos L et al., 2018; Castro-Torres R et al., 2019*). Furthermore, it has been demonstrated that their absence reduces the occurrence and severity of seizures, as well as glial reactivity and the

expression of proinflammatory genes (*Tai T et al., 2017; de Lemos L et al., 2018*). JNK absence also prevents the alteration of neurogenic cell subpopulations after KA insults (*Castro-Torres R et al., 2019*).

THE METABOLIC-COGNITIVE SYNDROME

Insulin resistance, or the loss of sensibility of cellular insulin receptors (IR) to the action of insulin, causes impairments in glucose uptake both in central and peripheral regions. This condition, of supposed multifactorial origin, results in an increase in hepatic glucose production, favouring states of hyperglycaemia and/or compensatory hyperinsulinemia. Many pathologies like diabetes, cardiovascular disease, fatty liver disease, impaired lung function, mild cognitive impairment and Alzheimer's disease (AD) have been linked with this condition (*Duque-Guimarães D and Ozanne S, 2013; Dodd GT and Tiganis T, 2017; McCracken E et al., 2018; Onyango A, 2018; Zafar U et al., 2018*).

Insulin Signalling

Insulin is a pancreatic polypeptide hormone produced by β cells in response to nutritional stimuli. It is responsible for the control of glucose concentrations in blood by stimulating their uptake into cells through its interaction with the IR (*Lee J and Pitch P, 1994; Boucher J et al., 2014*). All tissues depend on this mechanism for the maintenance of their metabolism, especially muscle, adipose tissue, liver and the brain. It also plays a role in the control of development, cell growth and division among other physiological functions (*Lee J and Pitch P, 1994; Haeusler R et al., 2018*).

The IR is a tetrameric tyrosine kinase protein made up of two extracellular α subunits and two transmembrane β subunits joined by

disulphide bonds. In their resting state, α subunits behave as inhibitors of the tyrosine kinase activity of the β subunits. When insulin binds to the IR, this inhibition is relieved due to the induction of conformational changes, allowing for the autophosphorylation of the IR in tyrosine residues (*Lee J and Pitch P, 1994; Boucher J et al., 2014; Saltiel A, 2015; Haeusler R et al., 2018*).

Following this, insulin receptor substrate (IRS) molecules are recruited and, when activated through tyrosine phosphorylation, they will be the ones responsible for signal transduction within the cell (*Boucher J et al., 2014; Copps K and White M, 2012*). One of the pathways that respond to the activation of IRS is the phosphoinositol-3-kinase (PI3K)/Protein Kinase B (AKT) pathway. This axis controls and regulates the function of many cellular mechanisms. One of them is the recruitment of glucose transporters (GLUT) from intracellular storage vesicles to the cell surface. GLUT are glucose carriers that translocate glucose molecules from the exterior to the cellular cytoplasm (*Lee J and Pitch P, 1994; Haeusler R et al., 2018*). Other examples of functions of the PI3K/AKT pathway include the activation of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and the cAMP response element-binding (CREB) or, the inhibitory control of glycogen synthase kinase 3 β (GSK3β) and pro-apoptotic molecules BAX, BAD and caspase 9 (*Yamaguchi H and Wang H, 2001; Gardai S et al., 2004; Saltiel A, 2015; Haeusler R et al., 2018*). Furthermore, AKT activity has been linked with the regulation and control of calcium influx in both mitochondria and ER membranes (**Figure 6**) (Phillips M and Voeltz G, 2016).

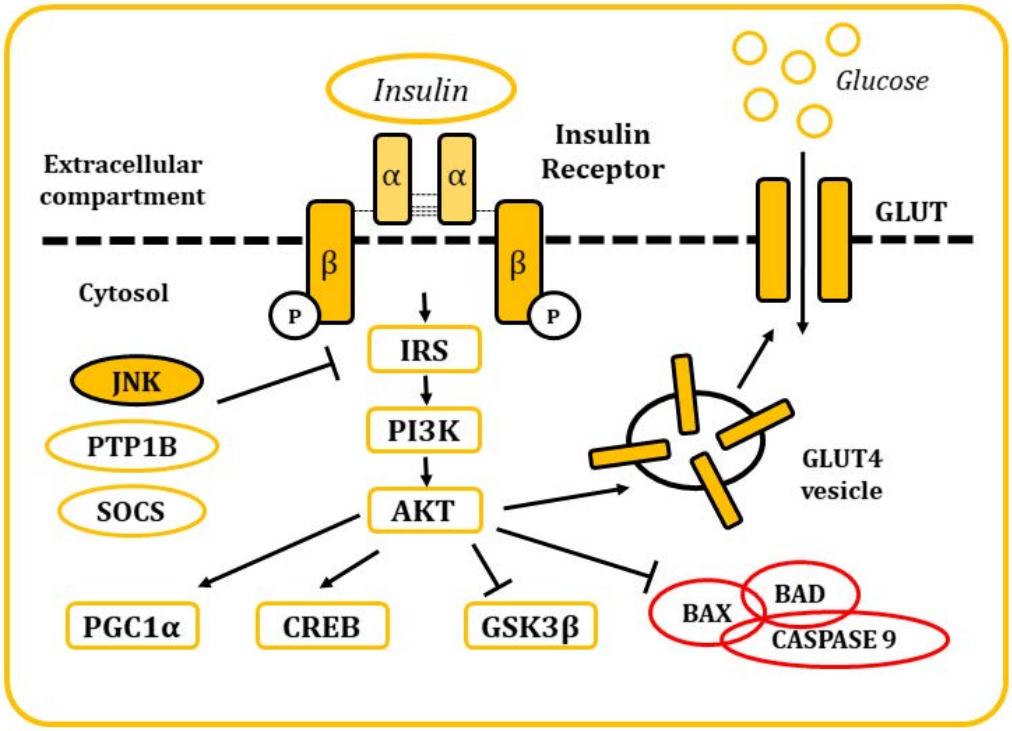


Figure 6. Representation of the cellular response after insulin stimulation. Insulin binds to the IR and causes its activation. The posterior signalling cascade stimulates the uptake of glucose through the GLUT transporters. Also, this signalling pathway is responsible for the regulation of multiple cellular functions and response mechanisms.

There are many regulatory mechanisms associated with the signalling of the IR, allowing for a tight control on the maintenance of homeostasis. Yet, these mechanisms can also become dysregulated and thus cause pathological insulin resistance states. In situations where insulin concentrations increase (hyperinsulinemia), the receptor becomes down-regulated, going so far as to become internalized into vesicles and, in some occasions, going through

lysosomal degradation (*Haeusler R et al., 2018*). IR signalling is also inhibited by inflammatory cytokines, which in turn will activate stimuli response elements like IKB kinase β or the JNKs. These kinases have been described to phosphorylate the IRS molecules in serine residues, causing for the development of states of decrease in insulin sensitivity (insulin resistance) and metabolic dysfunction (*Hirosumi J et al., 2002; Osborn O and Olefsky J, 2012; Boucher J et al., 2014*). Other elements responsible for the inhibitory control of insulin signalling include molecules like the protein tyrosine phosphatase 1B (PTP1B), responsible for the dephosphorylation of both the IR and IRS proteins. Also, the suppressor of cytokine signalling (SOCS) proteins reduce IR signalling by either occupying the phoshotyrosine activity site on the IR or recruiting ubiquitin ligases that will induce proteasomal degradation of IRS (*Emanuelli B et al., 2000; Haeusler R et al., 2018*).

All these mechanisms that can induce states of insulin resistance have also been described in other conditions like obesity (visceral adiposity), systemic hypertension, hypercoagulation and atherogenic dislipidemia. Experts have determined that when at least three of these alterations occur, there is a state known as metabolic syndrome. Worldwide, this condition is responsible for substantial morbidity and premature mortality (*McCracken E et al., 2018*). Epidemiological data in the United States has determined that over 30% of the adult population are affected by these conditions and, that percentages increase to over 43% when measuring adults over 60 years of age (*O'Neill S et al., 2015*).

Regarding conditions like overweight and obesity, they are one of the main risk factors for the development of metabolic syndrome,

becoming, in many cases, the source or a coadjuvant for the appearance of all or some of the other previously mentioned alterations (*Després JP and Lemieux I, 2006; Engin A, 2017*). The WHO has defined overweight and obesity as abnormal or excessive fat accumulations that impair health. It is considered that an individual suffers from overweight when their body mass index (BMI) value is greater than or equal to 25 kg/m^2; and obesity when the BMI is greater than or equal to 30 kg/m^2 (*WHO*).

Epidemiological data from 2016 determined that 39% of the adult world population was overweight and 13% was obese, showing a clear upward tendency on the incidence of these conditions. Their appearance is highly entitled to an environmental factor, associated to the consumption of unbalanced, unhealthy and calorie-rich foods, as well as to the lack of physical exercise typical of sedentary lifestyles. But, in some cases, there is also a genetic factor that causes alterations in the physiological function of molecules like leptin, leptin receptor or proopiomelanocortin (*WHO; Yaswen L et al, 1999; Pelloux V et al, 2002*). Importantly, in the past it was believed that metabolic alterations were the source for the appearance of only peripheral diseases but now, it is clear that the central nervous system is also highly affected (*Dmitry P et al., 2015; Zhigang L et al., 2015; Kullmann S et al., 2016; Cardoso S et al., 2019*).

Insulin action in the brain

The brain is a tissue highly sensitive to the effects of insulin due to its high distribution of IRs. The highest concentration of these

receptors can be found in the hypothalamus, olfactory areas, limbic regions, neocortex, basal ganglia, cerebellum, choroid plexus and hippocampus (*Hill JM et al., 1986; Kullmann S et al., 2016; Bilotta F et al., 2017; Gralle M, 2017*). Insulin activity controls glucose metabolism and the mobilization of GLUT4 transporters in neurons and glial cells. Also, it regulates multiple metabolic pathways in peripheral tissues through the regulation of hypothalamic activity. Furthermore, this hormone modifies synaptic plasticity, regulates neurogenesis and neurite outgrowth, has neuroprotective effects, as well as a role in memory, cognitive modulation and oligodendrocyte proliferation, differentiation and myelination, just like control of glial functionality (**Figure 7**) (*Gralle M, 2017; Al Haj Ahmad RM and Al-Domi HA,* 2018).

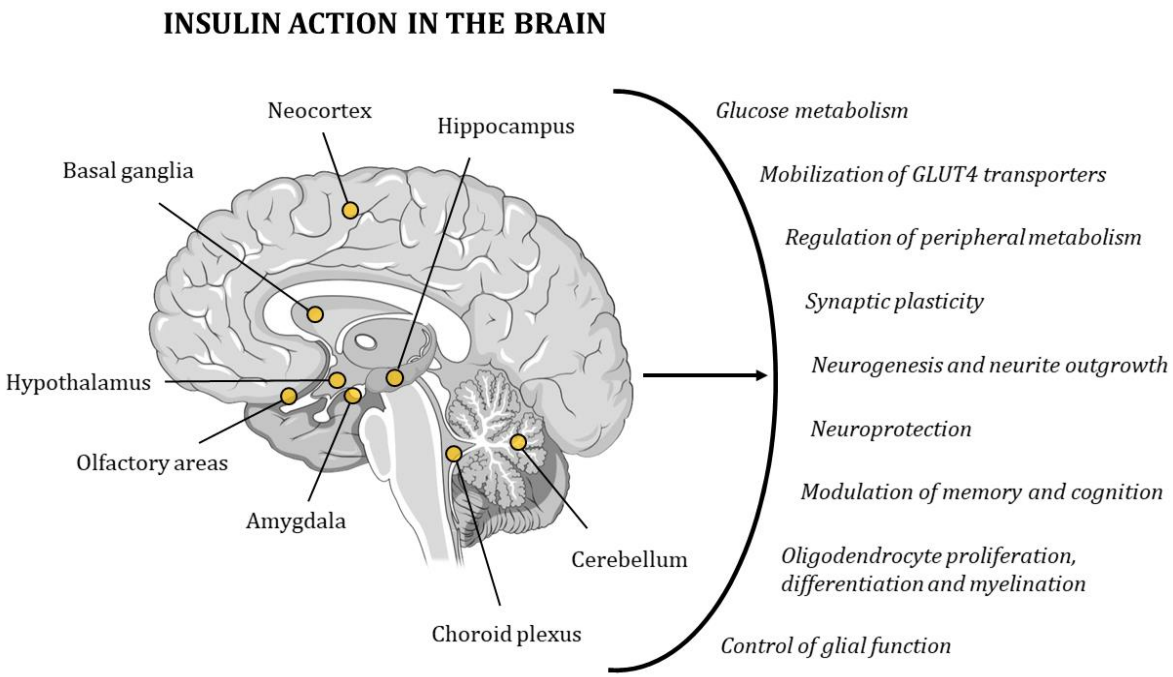


Figure 7. Insulin has a very strong effect on the functionality of the brain. The areas with the highest abundance of IRs have been labelled in this figure.

Metabolism and cognitive affectations

Over the years, multiple studies have demonstrated a relationship between the appearance of cognitive deficits and phenomena like insulin resistance. Dr. Siegfried Hoyer was the first to mention this relationship. On his studies on the neuropathology of AD, he observed that a desensitization of the IRs might be a cause for the development of neurodegenerative hallmarks classically linked to dementia (*Henneberg N and Hoyer S, 1995*). Later, the Rotterdam study revealed an increased risk of dementia in diabetic patients, stablishing a clear relationship between hyperglycaemia and hyperinsulinemia states and the development of pathologies like AD (*Ott A et al., 1999*). Additionally, the analysis of human samples also yielded clear data demonstrating that AD patients show defective insulin signalling and response to this hormone just like altered levels and/or altered activation of components of the insulin signalling pathway (*de Felice F et al. 2014*). In 2005, Dr. Suzanne M de la Monte coined the term type 3 diabetes (T3D) to define as state of brain insulin resistance plus insulin deficiency that, in some occasions can overlaps with Type 2 Diabetes Mellitus (T2DM) (*Steen E et al., 2005; de la Monte SM and Wands JR, 2008*). In the last decade, many other researchers have backed up this hypothesis of the metabolic-cognitive syndrome with new discoveries and ground-breaking research projects (*Kroner Z, 2009; Ma L et al., 2015; Kullmann S et al., 2016; Mittal K et al., 2016*).

Metabolic-related cognitive dysfunction develops through different stages, being the two initial ones potentially reversible with the appropriate strategy (*Koekkoek, PS et al., 2015*):

1. *Cognitive decrements* occur when slow and subtle changes in cognitive performance appear, giving rise to slight complaints but with no effect on daily life activities.
2. M*ild cognitive impairment* characterizes for cognitive complaints and impaired performance in cognitive testing. In this stage daily life activities remain unaffected but, slight complex instrumental functions may be minimally impaired. It usually occurs in individuals between the ages of 60-65.
3. *Dementia* is the last stage. This state presents cognitive affectations that run in parallel to behavioural changes and impaired performance on cognitive testing. Functional deficits are severe. In most cases it occurs in patients over 65 years of age.

Given the metabolic component of these cognitive dysregulations, strategies for the treatment of diabetic and obesity situations might be able to prevent and alleviate them. It has been described that maintaining a healthy diet and active lifestyle is a potential method to improve insulin sensitivity on both peripheral and central tissue through the reversal of metabolic defects caused by conditions like obesity (*Krentz A, 1996; Kullmann S et al., 2016*). Importantly, the capacity of the body to regulate food intake and the control of energy reserves is highly determined in the very early life and thus, these balanced and healthy practices must be stablished shortly after birth (nutritional programming) (*Symonds, ME et al., 2009*).

The use of insulin or insulin analogues has been described as another possible therapeutic approach. In small scale clinical, an intranasal treatment has afforded some degree of memory improvement and protection against further cognitive deterioration

(*Reger M et al 2006, 2008*). Problematically, some studies have described that, over time, this strategy may promote an insensitization of the IRs. Finally, the use of antidiabetic drugs like metformin, peroxisome proliferator-activated receptor gamma (PPARγ) agonists, sulfonylureas, glucagon-like peptide 1 (GLP1) agonists has also been proposed (*Kullmann S et al., 2016*).

Preclinical models

The preclinical study of insulin resistance in the periphery and in central tissues can be accomplished using models that include environmental agents like diets and chemical agents, as well as genetic models to modulate the expression of specific genes linked to the activity of insulin. These models have been used in the past to dissect the cellular mechanisms that relate to this alteration, just like for the test of new therapeutic strategies. These models show similar metabolic alterations, including hyperglycaemia, hyperinsulinemia, glucose intolerance, hyperleptinemia and insulin resistance both in peripheral and central areas. Some examples include:

- Diet:
    - *High fat diets* (HFD; Western diets): typically composed of higher proportions of fat, alone or in combination with glucose/fructose (*Biessels GJ and Reagan LP, 2015*). These models typically induce obesity combined with increased levels in blood of free fatty acids, which favour the formation of other lipidic biomolecules like ceramides (*Yang ZH et al., 2012; Sah S et al., 2016*).

- *Cafeteria Diets*: these models share high similarity with HFD models but, they differ in the fact that they are much more palatable to the animals, increasing food ingestion by animals through voluntary hyperphagia. Typical foods served to the animals include bacon, sausages cookies, soft drinks, condensed milk… (*Sah S et al., 2016*)
- *Fructose/Glucose/Sucrose-enriched drink/diet* (*Yang ZH et al., 2012; Sah S et al., 2016; Sangüesa G et al., 2018*).

- Genetic models:
  - *Knockouts of the IR gene* have allowed to understand its cellular role. Animals of this model show slight growth retardation, as well as severe cases of hyperinsulinemia and pancreatic β-cell degranulation. Mice death usually occurs shortly afterwards due to diabetic ketoacidosis. Multiple knockout conditional models have also been produced to circumvent any problems linked to the negative effects of lack of IR during embryotic and early life development. These models also allow the design of tissue-specific knock-out (*Nandi A et al., 2004*).

    The use of lentiviral particles with antisense sequences for the downregulation of the expression of the IR gene is another model that has been used to evaluate the role of IRs in the hypothalamus (*Grillo CA et al., 2007*)
  - Some other knockout models include the genes: *insulin-like growth factor 1 receptor (IGF1R), IRS, AKT, GLUT4,* brain-derived neurotrophic factor *(BDNF)*… (*Nandi A et al., 2004; Sah S et al., 2016*), just like mutations in the *leptin* (mice

ob/ob) and *leptin receptor gene* (mice db/db and Zucker rats) (*Biessels GJ and Reagan LP, 2015*).

- Chemical agent:
  - *Streptozotocin* is an antimicrobial and chemotherapeutic agent. It is a cytotoxic agent for β-cells that is administered to animals to induce diabetic states (*Sah S et al., 2016; Delikkaya B et al., 2019*). This model can be also combined with HFDs to induced further metabolic dysregulations and a much more severe response.

When tested for cognitive capabilities, these animal models show impaired performance, especially when tested for complex tasks, indicating that affectations are mostly located in prefrontal and hippocampal areas (*Biessels GJ and Reagan LP, 2015*).

Pathophysiology

One of the most striking elements that occurs in this pathologic scenario is that after a prolonged peripheral hyperinsulinemia state, there is a decrease in the number of IRs in the blood brain barrier, reducing the transport of insulin into the brain and causing a decrease in insulin action (*Schwartz M et al., 1990*). This condition also characterizes for a situation of mitochondrial dysfunction and ROS overproduction, as well as an increased activation of the UPR in the ER due to the activation of stress responses (*Cui J et al., 2007; Ozcan L et al., 2009; Schöntal A, 2012; Boucher J et al., 2014; de Felice F et al. 2014; Biessels GJ and Reagan LP, 2015; Hotamisligil GS and Davis RJ, 2016; Kaneko M et al., 2017; Onyango A, 2018; Zafar U et al., 2018*).

Furthermore, a chronic inflammatory state in astrocytes and microglia is developed favouring tissue damage and degeneration through the release of cytokines like TNFα, IL1β and IL-6, which later interact with receptors such as the toll-like receptor 4 (TLR4) (*de Felice F et al. 2014; Hotamisligil GS and Davis RJ, 2016; Kullmann S et al., 2016; Kaneko M et al., 2017*). This situation is usually aggravated in situations of obesity in which there is an excessive accumulation of fat in the perivisceral areas and the waist. Additionally, in this situation there is also an increase in plasma of other lipidic molecules like sphingolipids and ceramides, which have been associated with insulin resistance (*Özcan U et al., 2004; Zafar U et al., 2018*)

What is more, all these dysregulations and the subsequent cellular stress, increase the activation of stress-response elements like the JNKs which, in turn will phosphorylate other molecules like the IRSs in serine residues. Thus, favouring further impairment on the signalling of insulin, as well as the release, activation and overproduction of proapoptotic molecules (*Kaneto H et al., 2005; Sabapathy K, 2012; Schöntal A, 2012; Biessels GJ and Reagan LP, 2015; Hotamisligil GS and Davis RJ, 2016; Onyango A, 2018; Zafar U et al., 2018*). Additional alterations associated to an overactivation of the JNKs in a situation of insulin resistance include: negative activation of the hypothalamus-pituitary-thyroid axis and activation of PTP1B and SOCS3 molecules (*Hotamisligil GS and Davis RJ, 2016*)

As a result, dysregulations in metabolic activity have adverse effects on brain function and structure, inducing brain atrophy through loss of grey matter, reduced integrity of white fibre tracts, white matter hyperintensity, infarcts and microbleeds. Alterations

can either be global or region specific with a particularly detrimental effect in the medial temporal lobe, which includes the hippocampus. Other alterations include loss of neurons, axodendritic pruning, reduced synaptic plasticity and integrity, as well as decreased neurogenesis, cell proliferation and reduction in the number of dendritic spines. In the end, all affectations will lead to impairments in learning, memory and other cognitive functions (*Biessels GJ and Reagan LP, 2015; Kullmann S et al., 2016*).

Published preclinical research has demonstrated that modulation of the activity of the JNKs through a pharmacological approach or, genetic modulation, can have both beneficial and detrimental effects on insulin sensitivity. It depends on which of the JNK isoforms is being modulated and also, in which tissue the modification is occurring:

- Whole body absence of JNK1 in knockout models has been shown to increase sensitivity to insulin and reduce body weight, even when animals are chronically exposed to HFD (*Cui J et al., 2007; Grivennikov S et al., 2007; Solinas G and Karin M, 2010; Sabapathy K, 2012; Koch P et al., 2015*). Furthermore, these animals show reduced oxidative damage and inflammatory responses, just like lower anxiety levels and increased neurogenesis (*de Lemos L et al., 2017; Mohammad H et al., 2018*). Importantly, when conditional knockouts for the JNK1 were produced in neurons, animals showed superior sensitivity to insulin than in the previous model, as well as protection against the damage associated with chronic HFD feeding (*Belgardt BF et al., 2010; Sabio G and Davis RJ, 2010*).

- Lack of JNK2 in the whole body is the model that has been the least studied both in the periphery and the brain, there being controversy between its actual effect on the control of energy metabolism. There is data on its importance in the maintenance of proper ER activity (*Raciti M, 2012*).
- Finally, knockout of JNK3 in a situation of no metabolic stress has been shown to have no detrimental effects. Yet, when animals are fed HFD, it induces severe hyperphagia due to leptin dysregulation, causing the development of a severe obesity phenotype (*Vernia S et al., 2016*).

THE JNKs AS POTENTIAL THERAPEUTIC TARGETS

Considering how the JNKs seem to have roles in the development of many pathologic situations, multiple pharmacological options have been proposed with the objective of modulating the JNK pathway (**Figure 8**) (*Cui J et al., 2007; Bogoyevitch M and Arthur P, 2008*).

Initial research led to the synthesis of the first generation of chemical anthrapyrazole ATP competitive inhibitors like SP600125 and CEP1347. These small molecules were tested both *in vitro* and *in vivo* and showed successful results in the prevention of JNK-dependent apoptosis mechanisms, reduction of cytokine production and enhancement in the recovery of situations like ischemia-reperfusion damage (*Sabapathy K, 2012; Coffey ET, 2014*). Other studies demonstrated neuroprotective effects in models of stroke and spinal cord injury (*Guan Q et al., 2006; Yoshimura K et al., 2011*).

The problem with these compounds was that they showed poor bioavailability in the brain and had to be administered via intraventricular injections or infusions to achieve effective doses (*Graczyk P, 2013*). Moreover, they showed significant cellular toxicity, as well as lack of specificity. Some of them have shown interaction with over 70 different molecules (*Coffey ET, 2014*). Moreover, these compounds inhibited the phosphorylation of all substrates of these kinases, causing in some models, lack of effectivity as a treatment or, aggravation of the pathology at hand. For example, treatment with SP600125, 7 days after transient focal cerebral ischemia in mice causes for an increase in infarct volume, along with an inhibition of vascular remodelling (*Bogoyevitch M and Arthur P, 2008; Murata Y et*

al., 2012). Second generation ATP-competitive inhibitors like CC-401, AS601245 and Compound A showed lower toxicity but remained highly target unspecific making them low in therapeutic value (*Sabapathy K, 2012*).

Posterior research allowed for the synthesis of ATP non-competitive inhibitors based on the function and structure of scaffold proteins like JIP. These compounds targeted the substrate-docking site. The D-JNKI-1 inhibitor peptide demonstrated to be effective in multiple variety of pathological situations, allowing for a reduction in the production of amyloid β or showing neuroprotective mechanisms in a model of myocardial ischemic injury. Assays seemed to indicate that these molecules presented higher target specificity (*Bogoyevitch M and Arthur P, 2008*). Even more, it was described D-JNKI-1 had high JNK3 inhibitory capacity in mitochondria (*Zhao Y et al., 2012*).

Increased specificity and the favourable results for these molecules in a wide spectrum of pathologies, encouraged companies into developing clinical trials using some of the previously mentioned compounds. The trials were oriented towards determining their toxicity in humans and potential therapeutic applications in Phase 2 and 3 trials. In most cases, studies were discontinued due to their lack of efficacy or therapeutic capacity when tested in human models (*Bogoyevitch M and Arthur P, 2008*)

The main problem is that these inhibitors cannot directly modulate JNK activity targeting only the affected tissue. Also, they inhibit all JNK isoforms which, may cause for a net balance of no therapeutic benefit or even, in some cases, a worsening of the pathological state. The use

of genetic experimental models in mice has shown that the JNK isoforms have, in some occasions, overlapping and redundant functions but also that, in many others, each of them have specific roles that vary in a cell-type and signal-dependent manner. For example, JNK1 and JNK2 show opposite effects on the turnover and activity over cJUN (*Sabapathy K et al., 2004*). Furthermore, a preclinical model of joint damage in mice demonstrated that JNK1 deficiency caused a reduction of inflammatory cell infiltration while lack of JNK2 showed no benefit (*Guma M et al., 2011*).

Problematically, the first initial attempts at designing isoform specific inhibitors were not successful. One of the problems they encountered was their very low capacity to go through the blood brain barrier, reducing very significantly their bioavailability in the brain. Next, the new compounds inhibited cytochrome P450, an essential cellular component required for lipid, hormone and drug metabolism, making them non-viable due to the very high possibility for the appearance of negative side effects (*Kamenecka T et al., 2010*).

The design and synthesis of isoform-specific inhibitors is highly complicated. Within the ATP binding pocket, the different JNK isoforms can only be differentiated in two positions in the amino acidic sequence when comparing between JNK1 and 2 but, when comparing 1 and 3 or 2 and 3 there is only one point of variation (*Koch P et al., 2015*). Nonetheless, several natural molecules have been described as isoform specific inhibitors.

Compounds like latifolians A and B are benzylisoquinolones extracted from the *Gnetum latifolium* plant (found in Papua New Guinean) and have been described as inhibitors of the JNK3 isoform (*Rochfort, SJ, 2005*).

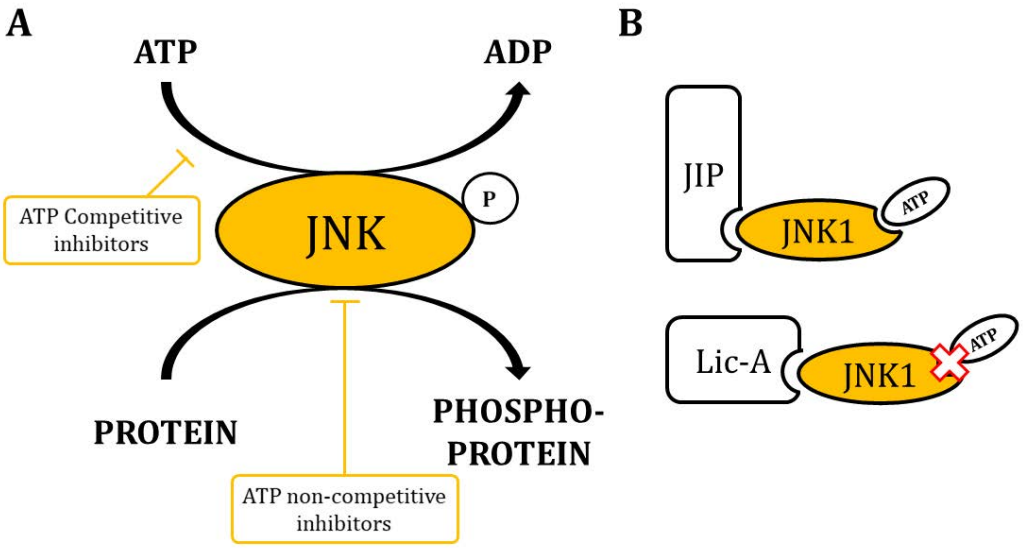


Figure 8. A) Diagram of the mechanism of action of ATP competitive and non-competitive inhibitors. B) Lic-A competes with JIP for the binding with JNK1 and causes for changes in the conformation of the ATP binding cleft.

Licochalcone A (LIC-A), is a chalcone phenolic component found in the roots of liquorice and has been described as a specific inhibitor of the JNK1 (*Glycyrrhiza inflata*). Like many traditional herbal medicines and foods, it exhibits anti-inflammatory and antioxidative effects. Mechanistically, LIC-A competes with JIP for the binding with JNK1, and causes distortions in the conformation of the ATP binding cleft, reducing its activity (**Figure 8B**). Importantly, like in most drugs an excessive daily dosage of 50 g can lead to negative effects through a significant rise in blood pressure. In preclinical studies, a dosage of

10-20 mg/kg has proven to have beneficial effects and to be well tolerated by mice. (*Yao K et al., 2014; Liou CJ et al., 2019*).

In conclusion, the scientific community has a lot of knowledge on the JNKs and their role in pathological situations yet, it seems that it still is not enough. This thesis aimed to understand further their role in the pathophysiological mechanisms involved in the development of TLE and the metabolic-cognitive syndrome.

Hypothesis & Objectives

HYPOTHESIS & OBJECTIVES

The JNKs are stress-response elements that have been associated with the development of diseases in many tissues, including the brain. Yet, there are still many unknowns on the role of each of the isoforms. This thesis aims to understand better the role of the JNK isoforms in the development of two different pathologies:

- The use of KA, a model for the study of TLE, has allowed to understand the protective effect of the knockout of JNK1 and JNK3 against cytotoxicity (*Yang DD et al., 1997; de Lemos et al., 2018*). Yet, to this date no specific inhibitors for isoform JNK1 have been tested for their potential as pharmacological therapeutic strategies.

  Objective:

  1. Evaluate the preventive neuroprotective effect of a single dose of LIC-A (JNK1 inhibitor) prior to an administration of KA, as a model of TLE, on young adult *wild-type* mice (2 months of age).

- Obesity, as a metabolic condition, is believed to affect the brain, favouring the disruption of homeostasis and the appearance of hallmarks typical of neurodegenerative pathologies like AD. Furthermore, the appearance of insulin resistance has been linked with the development of cognitive deficits (T3D hypothesis) (*de la Monte and Wands JR, 2008*). Importantly, previous data has established that the knockout of different JNK isoforms have variable effects on the development of obesity and insulin

sensitivity (*Hirosumi J et al., 2002; Sabio G and Davis RJ, 2010; Solinas G and Karin M, 2010*).

Objectives:

1. Determine the consequences of a long-term chronic exposition to HFD (16 months) in the appearance of AD-related hallmarks in *wild-type* C57BL/6J mice.
2. Evaluate the effects of the knockout of JNK1 and JNK2, combined with a chronic exposition to HFD (9 months), on the development of obesity, insulin resistance and cognitive impairments in mice.

Results

Publication I

JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid.

Busquets O, Ettcheto M, Verdaguer E, Castro-Torres RD, Auladell C, Beas-Zarate C, Folch J and Camins A.

Neuropharmacology. 2018; 131:440–52.

Neuropharmacology 131 (2018) 440–452

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid

Oriol Busquets [a, b, c, d], Miren Ettcheto [a, b, c, d], Ester Verdaguer [c, d, e], Ruben D. Castro-Torres [b, e, f], Carme Auladell [c, d, e], Carlos Beas-Zarate [f], Jaume Folch [a, c, **], Antoni Camins [b, c, d, *]

[a] Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Tarragona, Spain
[b] Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain
[c] Biomedical Research Networking Centre in Neurodegenerative Diseases (CIBERNED), Madrid, Spain
[d] Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain
[e] Departament de Biologia Cellular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain
[f] Departamento de Biología Celular y Molecular, C.U.C.B.A., Universidad de Guadalajara y División de Neurociencias, Sierra Mojada 800, Col. Independencia, Guadalajara, Jalisco 44340, Mexico

ARTICLE INFO

Article history:
Received 9 June 2017
Received in revised form
13 September 2017
Accepted 23 October 2017
Available online 28 October 2017

Keywords:
Kainic acid
Epilepsy
Neurodegeneration
Licochalcone A
Hippocampus

ABSTRACT

The mitogen-activated protein kinase family (MAPK) is an important group of enzymes involved in cellular responses to diverse external stimuli. One of the members of this family is the c-Jun-N-terminal kinase (JNK). The activation of the JNK pathway has been largely associated with the pathogenesis that occurs in epilepsy and neurodegeneration. Kainic acid (KA) administration in rodents is an experimental approach that induces status epilepticus (SE) and replicates many of the phenomenological features of human temporal lobe epilepsy (TLE).

Recent studies in our group have evidenced that the absence of the JNK1 gene has neuroprotective effects against the damage induced by KA, as it occurs with the absence of JNK3. The aim of the present study was to analyse whether the pharmacological inhibition of JNK1 by Licochalcone A (Lic-A) had similar effects and if it may be considered as a new molecule for the treatment of SE. In order to achieve this objective, animals were pre-treated with Lic-A and posteriorly administered with KA as a model for TLE. In addition, a comparative study with KA was performed between *wild type* pre-treated with Lic-A and single knock-out transgenic mice for the *Jnk1* $^{-/-}$ gene.

Our results showed that JNK1 inhibition by Lic-A, previous to KA administration, caused a reduction in the convulsive pattern. Furthermore, it reduced phosphorylation levels of the JNK, as well as its activity. In addition, Lic-A prevented hippocampal neuronal degeneration, increased pro-survival anti-apoptotic mechanisms, reduced pro-apoptotic biomarkers, decreased cellular stress and neuroinflammatory processes. Thus, our results suggest that inhibition of the JNK1 by Lic-A has neuroprotective effects and that; it could be a new potential approach for the treatment of SE and neurodegeneration.

1. Introduction

Temporal lobe epilepsy (TLE) is the most common form of human epilepsy (Lévesque and Avoli, 2013). It is characterized by the occurrence of unpredictable and recurrent focal seizures (Luckewold et al., 2015) Currently, although there are effective treatments for this pathology, there is the problem of drug-resistant epilepsy in certain patients, a condition defined by the *International League against Epilepsy*, as the persistence of epileptic

* Corresponding author. Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain.
** Corresponding author. Biomedical Research Networking Centre in Neurodegenerative Diseases (CIBERNED), Madrid, Spain.
E-mail address: camins@ub.edu (A. Camins).

https://doi.org/10.1016/j.neuropharm.2017.10.030
0028-3908/

seizures despite using at least two treatments with appropriate antiepileptic applications. In this case, the only therapeutic alternative in such patients is the surgical resection of the brain's epileptic tissue. For this reason, the development of new therapeutic approaches is necessary.

Although there are no preclinical models that reproduce all the features of TLE, some experimental models have been used over the past decades due to their high capacity of reproduction of human epilepsy (Jefferys et al., 2016). One of these well-known, thoroughly described models is the administration of kainic acid (KA, 2-carboxy- 4-isopropenylpyrrolidin-3-ylacetic acid). This model, which was originally reported by Ben-Ari (Ben-Ari et al., 1979), features TLE allowing for a deep study of this pathology.

The neurotoxin KA was first isolated in the red alga *Digenea simplex* which is found in tropical and subtropical waters (Fernández-Espejo, 1996). KA is an analogue of glutamate, thus, local or systemic administration of KA in rodents leads to a pattern of repetitive limbic seizures that lasts for several hours and is followed, after a variable latency period, by the chronic phase, which is characterized by the occurrence of spontaneous limbic seizures (Hammer et al., 2008). These seizures cause extensive brain damage, often associated with aberrant axonal reorganization, an increase in reactivity of the glia (increased proliferation and hypertrophy of astrocytes and microglia), as well as dysregulation of cellular homeostasis (Mechanisms and Initiation, 2002). The c-Jun N-terminal kinases (JNKs), also called stress-activated protein kinases (SAPK), belong to the family of mitogen-activated protein kinases (MAPK). These enzymes are activated by KA and are involved in stress responses and cell death (Derijard et al., 1994; Kallunki et al., 1994, 1996; Kyriakis et al., 1994; Auladell et al., 2017). Moreover, JNKs are also involved in the processes of neuronal cell loss in cerebral ischemia-induced neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Mechanisms and Initiation, 2002; Lerma, 2013). There are different JNK isoforms, encoded by three different genes, *Jnk1*, *Jnk2*, and *Jnk3*. In mammals, JNK1 (MAPK8) and JNK2 (MAPK9) proteins are expressed ubiquitously, whereas JNK3 (MAPK10) is mostly expressed in the brain and, to a lesser extent, in the heart and testes (Yuan et al., 2003; Brecht et al., 2005).

Several synthetic compounds have shown to be able to inhibit this pathway. Among them, the best evaluated molecules at a preclinical level are SP600125 and AS601245, both unspecific inhibitors of all JNK isoforms (Bennett et al., 2001; Carboni et al., 2008). However, it is necessary to develop specific inhibitors for each isoform to avoid possible side effects derived of the different roles that the three isoforms play within the tissues. The JNK3 isoform is the one that has been classically related on the development of neurotoxic responses after KA administration and, several groups, have reported interesting results using a competitive inhibitor of the JNK3, the D-JNK-1. Specifically, this inhibitor has effect on the pool of this protein found in the mitochondria (Borsello et al., 2003; Repici et al., 2007; Spigolon et al., 2010; Zhao et al., 2012). In addition, our group recently demonstrated that the JNK1 would also have high importance in the regulation of brain damage induced by KA (de Lemos et al., 2017). One promising pharmacological inhibitors of the JNK1 belongs to the group of chalcones, which are found in many natural products (fruits, vegetables, spices, tea …). The roots of liquorice (Glycyrrhiza inflata) have various pharmacological properties, including anti-inflammatory, anti-oxidative and anti-carcinogenic activities (Shibata, 2000; Funakoshi-Tago et al., 2010). One of these chalcones, Licochalcone-A (Lic-A), has been reported to target JNK1 and JNK2 but only has inhibitory effect on the activity of the JNK1 (Yao et al., 2014).

Since the JNK1 is involved in neuronal death induced by KA, and Lic-A selectively inhibits JNK1, it was hypothesized that a pre-treatment with Lic-A, previous to KA, would reduce the consequences of epileptic seizures compared to mice only treated with KA. The results revealed a decrease in the severity of seizures and neuronal damage, along with reductions in apoptotic biomarkers and neuroinflammation. Moreover, in order to confirm that the neuroprotective effect of Lic-A against KA is, in fact, a consequence of the inhibition of the JNK1, several biomarkers that are usually altered by KA were studied versus mice pre-treated with Lic-A before KA injection, and single knock-out transgenic mice for the *Jnk1*$^{-/-}$gene also treated with KA. The analyses revealed similar effects in both experimental models. So, for the first time, it has been demonstrated that in vivo pharmacological inhibition of JNK1 by Lic-A has neuroprotective effects in mice. Therefore, this compound may constitute a new potential drug that should be evaluated in additional experimental models for the treatment of epilepsy and also other neurodegenerative pathologies.

2. Materials and methods

2.1. Animals

2.1.1. Evaluation of the potential application of Lic-A as a neuroprotectant

C57BL6/J *wild-type* mice of two months of age were used in this study (n = 15 for each experimental group). Four experimental groups were established; animal distribution can be seen in Table 1.

2.1.2. Comparative evaluation of the effects of Lic-A inhibition and *Jnk1* −/− transgenesis against KA insults

Two months old C57BL6/J *wild-type* and *Jnk1* $^{-/-}$ single knock-out transgenic mice were used in this study (n = 4 for each experimental group). The generation and characterization of the *Jnk1*$^{-/-}$ single knockout mouse has been previously described (Dong et al., 1998). Animal distribution for this study can be seen in Table 1.

Throughout all the experiments, mice were housed in a temperature and humidity controlled environment, with regular 12-h light/dark cycle, food and water were available *ad libitum*. The experiments were conducted in accordance with the Council of Europe Directive (2010)/63. The procedure was registered at the *Department of Agricultura, Ramaderia i Pesca* of the *Generalitat de Catalunya*. Ref. Number order 8852.

2.2. Pre-treatment, treatment and sample extraction

The different administrations established in Table 1 were all done intraperitoneally as follows: Saline volume administration was standardized at 10 μl/g in order to keep a similar blood volume affectation on all animals. KA was administered in a 30 mg/kg dosage (Sigma-Aldrich, K-2389, USA)(Junyent et al., 2009) and Lic-A at 15 mg/kg (Calbiochem®, 435800-50MG, Denmark).

In both studies (Study 1 and 2) the animals were pre-treated 3-h before KA treatment with either saline or Lic-A. 3, 24 or 72-h after KA administration, the animals were sacrificed in the appropriate method. In study 2 the samples were limited to the 3-h period.

2.3. Evaluation of seizure-related behaviour

After the exposure to KA, the evaluation of seizures was performed according to Morrison et al., (1996). Briefly, highest stage of seizures was evaluated for 2-h after injection. Each seizure score was determined depending on the overall seizure-related behaviour for each 5-min period. Each seizure score was multiplied by the number of 5-min periods in which the animal received that score.

Table 1
Schematic of animal distribution, experimental groups description, pre-treatments, treatments and sacrifices timing. A. Animal distribution for initial evaluation of the effects of Lic-A as a neuroprotectant. B. Animal distribution for the comparative assays of effect of KA between the JNK1 inhibition by Lic-A and the single knock-out transgenic for *Jnk1* −/−.

		Pre-treatment		Lic-A	Treatment		Sacrifice
		Saline	Lic-A		Saline	KA	
Experimental groups(n = 15 for each)	Saline	x		3-h	x		3, 24, 72-h
	KA	x				x	
	Lic-A		x		x		
	Lic-A + KA		x			x	

		Pre-treatment		Elapse	Treatment	Sacrifice
		Saline	Lic-A		KA	
Experimental groups(n = 4 for each)	KA	x		3-h	x	3-h
	Lic-A + KA		x		x	
	Jnk1−/− + KA	x			x	

These numbers were added together for a raw score, and the final seizure ratings were derived from these raw scores. Raw scores less than 50 were assigned a rating of 1, those between 50 and 79 received a rating of 2, and those 80 and above received a rating of 3. Animals exhibiting no seizure activity received a rating of 0.

2.4. Immunoblot analysis

Protein extraction for western blot analysis was performed in fresh tissue samples obtained from animals sacrificed through neck dislocation. Previous to the actual protein extraction, the samples were pulverized with liquid nitrogen. Posteriorly, samples were homogenized on a lysis buffer (Tris HCl 1M pH 7.4, NaCl 5M, EDTA 0.5M pH 8, Triton, distilled H_2O), a protease inhibitor (Complete Mini, EDTA-free; Protease inhibitor cocktail tablets, 11836170001, Roche Diagnostics GmbH, Germany) and a phosphatase inhibitor (Phosphatase inhibitor cocktail 3, P0044, Sigma-Aldrich, USA). After a 30-min incubation at 4 °C, the samples were centrifuged at 14,000 rpm for 10-min at 4 °C and the supernatant was recovered and frozen at −80 °C until use.

After protein extraction, protein concentration was evaluated through a Pierce™ BCA Protein Assay Kit (#23225; Thermo Scientific, USA). Sequentially, 10 μg per sample were denatured at 95 °C for 5-min in a sample buffer (0.5M Tris HCl, pH 6.8, 10% glycerol, 2%(w/v) SDS, 5%(v/v) 2-mercaptoethanol, 0.05% bromophenol blue). Electrophoresis was performed on acrylamide gels at 100 V and they were transferred to polyvinylidene difluoride sheets (Immobilon®-P Transfer Membrane; IPVH00010; Merk Millipore Ltd., USA) (200 mA). Then, membranes were blocked for 1-h with 5% non-fat milk dissolved in TBS-T buffer (0.5 mM Tris; NaCl, Tween® 20 (P1379, Sigma-Aldrich, USA), pH 7.5), washed with TBS-T 3 times for 5-min and incubated with the appropriate primary antibody (Table 2) overnight shaking at 4 °C. Subsequently, blots were washed thoroughly in TBS-T buffer and incubated at room temperature for 1-h with the appropriate secondary antibody (Table 2). Ultimately, blots were exposed to a chemoluminescence detection agent (Pierce® ECL Western Blotting Substrate; #32106; Thermo Scientific, USA). Protein levels were determined using Image Lab version 5.2.1 (Bio-Rad Laboratories). Measurements were expressed as arbitrary units and all results were normalized to the corresponding GAPDH used as a loading control.

2.5. Kinase activity assay

The kinase activity assay was performed after an immunoprecipitation of the P-JNK enzyme from a protein extraction sample (200 μg were used per sample). To minimize the detection of cross-reactive proteins a pre-cleaning procedure was performed: protein samples were incubated with 50 μl Protein A/G PLUS -Agarose (sc-2003, Santa Cruz Biotechnology, USA) for 1-h at 4 °C with gentle shaking in a rotor. This step was followed by a centrifugation at 3500*g* for 5-min to remove the supernatants to clean tubes. Next, samples were incubated with 3 μl of antibody plus 30 μl of Protein A/G PLUS-Agarose overnight at 4 °C with gentle shaking. On the next day, samples were centrifuged at 3500*g* for 5-min and the supernatant was discarded. Several cleaning steps were performed afterwards with STEN buffer (0.5M NaCl, 50 mM Tris, pH 7.6, 2 mM EDTA, 0.2% NP-40) before the samples were deemed ready for the activity assay.

This procedure was performed following the SAPK/JNK Kinase Assay Kit instructions (Nonradioactive) (#9810, Cell Signaling, USA).

2.6. Immunofluorescence

Mice used for immunofluorescence studies were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused with 4% paraformaldehyde (PFA) diluted in 0.1M phosphate buffer (PB). Brains were removed and stored in the same solution overnight at 4 °C and 24-h later, they were cryoprotected in 30% sucrose-PFA-PB solution. Coronal sections of 20 μm of thickness were obtained by a cryostat (Leica Microsystems).

On the first day, free-floating sections were washed three times with 0.1 mol/L PBS pH 7.35 and after, five times with PBS-T (PBS 0.1 M, 0.2% Triton X-100). Then, they were incubated in a blocking solution containing 10% fetal bovine serum (FBS), 1% Triton X-100 and PBS 0.1M + 0.2% gelatin for 2-h at room temperature. After that, slices were washed with PBS-T five times for 5-min each and incubated with the primary antibody overnight (Table 3). On the second day, brain slices were washed with PBS-T 5 times for 5 min and incubated with the appropriate secondary antibody for 2-h at room temperature (Table 3). Later, sections were co-stained with 0.1 μg/ml Hoechst 33258 (861405; Sigma-Aldrich, St Louis, MO, USA) for 15-min in the dark at room temperature and washed with PBS 0.1M. Finally, slides were mounted using Fluoromount G (#19984-25; Electron Microscopy Sciences, USA). Image acquisition was performed with an epifluorescence microscope (Olympus BX61 Laboratory Microscope, Melville, NY-Olympus America Inc.).

2.7. Fluoro-Jade B staining

Neurodegeneration was assessed using Fluoro-Jade B staining (AG310, Millipore, USA) (Schmueda, 2000). Slides were rinsed with

Table 2
Primary and secondary antibodies for western blotting.

Primary antibody	Reference	Company
Anti-cJUN N-Terminal Kinase 1 (phosphor Thr183); P-JNK1	ab47337	Abcam
Anti-cJUN (E254)	ab32137	
Anti-cJUN (phosphor Ser 73); P-cJUN	ab30620	
Anti-Neuronal nitric oxide synthase; nNOS	ab1376	
Anti-ADAM10	ab39177	
Anti-BCL-2 like protein 11; BIM	AB17003	Millipore
Anti-α-Spectrin	MAB1622	
Anti-Glyceraldehyde-3-phosphate dehydrogenase (6C5), GAPDH	MAB374	
Anti-cJUN N-Terminal Kinase; JNK	9252	Cell Signaling Technology
Anti-cJUN N-Terminal Kinase (phosphor Thr183/Tyr185) (G9); P-JNK	9255	
Anti-cJUN N-Terminal Kinase 1 (2C6); JNK1	3708	
Anti-AKT	9272	
Anti-AKT (phosphor Ser473) (D9E); P-AKT	4060	
Anti-cAMP response element binding (48H2); CREB	9197	
Anti-cAMP response element binding (phospho Ser133); P-CREB	9198	
Anti-B-cell lymphoma 2 (50E3); BCL-2	2870	
Anti-Inducible nitric oxide synthase; iNOS	2982	
Anti-Peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PGC1Cα	sc-13067	Santa Cruz Biotechnology
Anti-Tumor necrosis factor alpha; TNFα	sc-133192	
Anti-BCL-2 like protein 4 (B9); BAX	sc-7480	
Secondary antibody	**Reference**	**Company**
Goat Anti-Mouse IgG (Horseradish peroxidase conjugate) - Pierce™ Antibody	31430	Thermo Scientific
Goat Anti-Rabbit IgG (Horseradish peroxidase conjugate) - Pierce™ Antibody	31460	

Table 3
Primary and secondary antibodies for immunofluorescence.

Primary antibody	Reference	Company
Anti-Glial Fibrillary Acidic Protein; GFAP	Z0334	Dako
Anti-Ionized calcium-binding adapter molecule 1; IBA1	019–19741	Wako
Anti-NESTIN	MAB353	Millipore
Anti-Neuronal Nuclear Antigen (A60); NeuN	MAB377	
Anti-B-cell lymphoma 2 (N-19); BCL-2	2870	Cell Signaling Technology
Anti-BCL-2 like protein 4; BAX	sc-7480	Santa Cruz Biotechnology
Secondary antibody	**Reference**	**Company**
AlexaFluor 488 Donkey Anti-Rabbit	A21206	Life Technology
AlexaFluor 594 Goat Anti-Rabbit	A11012	
AlexaFluor 488 Donkey Anti-Mouse	A21202	
AlexaFluor 594 Goat Anti-Mouse	A11005	

phosphate buffer solution (PBS), followed by two washes in distilled water. Afterwards, slides were immersed in 0.6 g/L potassium permanganate (KMnO4) for 15-min in the dark. Then, after two washes in distilled water, the slides were transferred to the staining solution containing 0.1 mL/L acetic acid and 0.004 mL/L of the fluorophore Fluoro-Jade B for 30 min in the dark. Slides were rinsed in distilled water, and then submerged directly into xylene and mounted in DPX medium (06522, Sigma-Aldrich, USA). Slides were analysed using an epifluorescence microscopy (Olympus BX61 Laboratory Microscope, Melville, NY-Olympus America Inc.).

2.8. Evaluation of labelled cells

In order to obtain results on cellular numbers, sections corresponding to the hippocampal areas between Bregma −1.34 to −2.46 mm, in accordance with the Atlas reported by Franklin and Paxinos (2012), were used to determine the number of positively-labelled cells in 40 mm^2 areas of each section (4–6 animals/genotype and age, 4–8 sections/animal), in both the CA3 and CA1. The density of counted cells was expressed as number of cells per square millimetre of tissue examined, using a conversion factor ($3{,}5 \cdot 10^4$) calculated for 100X magnification. Counts used to determine numerical densities of cells were made with a 25-point eyepiece morphometric grid (NE35, Electron Microscopy Sciences, Hatfield, PA) attached to the ocular of an optical microscope (Optical Microscope Olympus BX61) (Olloquequi et al., 2010).

2.9. Statistical analysis

All results were represented as MEAN ± SD. Statistical analysis was performed trough different methods:

- Evaluation of the potential application of Lic-A as a neuroprotectant: The saline experimental group was compared against KA and Lic-A groups through one-way ANOVA ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Tuckey post-test was performed. The KA group was compared against the Lic-A group through *t*-test analysis ($ p < 0.05, $$ p < 0.01, $$$ p < 0.001).
- Comparative evaluation of the effects of Lic-A inhibition and Jnk1 −/− transgenesis against KA insults: The KA experimental group was compared against the Lic-A + KA and *Jnk1* $^{-/-}$ + KA groups through %-way ANOVA ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Tuckey post-test was performed.

All analyses and graph representations were performed in the program Graph Pad Prism for Windows version 6.01; Graph Pad Software, Inc.

3. Results

3.1. Evaluation of the potential application of Lic-A as a neuroprotectant

3.1.1. Lic-A attenuates the induction of seizure-related behaviours induced by KA

The pre-treatments with Lic-A caused a reduction of seizures compared to mice treated only with KA (Fig. 1). Specifically, animals in the KA experimental group showed a rating value of 2.46 ± 0.16, while those pre-treated with Lic-A had a rating of 1.83 ± 0.17

(reduction on a 25.6%) These evaluations were performed as its described in the corresponding subsection of the *Materials and Methods*.

3.1.2. Lic-A reduces JNK phosphorylation levels and activity

In previous studies, it has been demonstrated that KA administration on rodents induces the activation of MAPK (de Lemos et al., 2010; Ettcheto et al., 2015). Accordingly, we examined in the hippocampus of all our experimental groups, the phosphorylation levels of JNK at various time points after KA administration (3, 24 and 72-h).

The results obtained in Western blot revealed a significant increase of JNK phosphorylation levels (P-JNK) in the hippocampal tissues at 3-h after KA injection. Contrarily, the animals pre-treated with Lic-A before KA showed a phosphorylation decrease compared with the KA experimental group (69.7% of decrease). Moreover, Lic-A prevented the increase of JNK phosphorylation even against the control group (69.2% of decrease). The time course response of animals treated with Lic-A and Lic-A + KA would also suggest a reversible inhibition of the JNK seeing how, over time, it restores back to the response levels of the saline group (Fig. 2A). Because it has been reported that JNK1 could be and specific target of Lic-A, we evaluated the effects of KA on this specific isoform 3-h after the exposition to KA. The phosphorylation values of P-JNK1 showed high correlation with the ones observed with the total P-JNK protein (Fig. 2B).

To corroborate these observations, the levels of total and phosphorylated forms of cJUN were evaluated after 3-h of treatment. cJUN is one of the targets phosphorylated downstream of JNK1 (Sabapathy et al., 2004). In fact, exposure to KA increased significantly the levels of total and phosphorylated forms of cJUN. By contrast, the exposure to Lic-A reduced this stress-associated response in the P-cJUN, both Lic-A and Lic-A + KA experimental groups (61.9% and 28.4% of decrease respectively of saline and KA groups) (Fig. 2B).

Further demonstration of JNK inhibition by Lic-A was evaluated through the assay of SAPK/JNK activity (Fig. 2C). Results showed a significant increase in the kinase activity in mice exposed to KA compared to control, while it was significantly reduced in mice pre-treated with Lic-A (42.3% of reduction).

3.1.3. Lic-A protects against neurodegeneration and induces cell survival responses

Neurodegeneration is an indicator of brain damage, clearly detected in the CA1 and CA3 hippocampal areas 24-h past the KA treatment. Fluoro-Jade B stain was used as a method of detection of neurodegenerating neurons. Only the animals in the KA experimental group showed a typical pattern of high density of degenerating cells (Schmueda, 2000); those that had been pre-treated with Lic-A had no labelling (Fig. 3). Concurrently, an evaluation on the number of surviving neurons was performed. Using the NeuN antigen in an immunofluorescence assay as an indicator of alive mature neurons, it was detected how there was a significant reduction on them in the KA experimental group versus all others (Fig. 4).

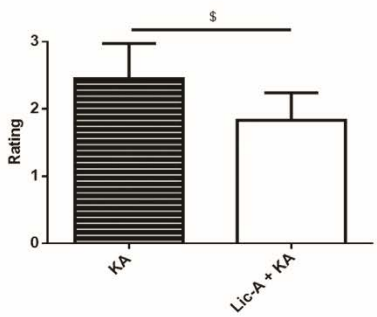


Fig. 1. Evaluation of seizure-related behaviours. The quantification of the responses was performed according to the one described by Morrison et al. (1996). $ $p < 0.05$ KA vs Lic-A + KA.

3.1.4. Lic-A reduces neural proliferation responses after KA damage

When studying the damage caused by KA in the hippocampus in mice, the subsequent proliferation of neural cells was also evaluated. The granule cell layer of the hippocampus is preserved after the exposure to KA. After the damage, it leads to the development of neurogenesis mechanisms in the subgranular zone (SGZ). To test if there were changes in this process, with Lic-A pre-treatment before KA, an immunofluorescence assay using an antibody against NESTIN, as a marker for stem cells, was performed. The results obtained evidenced a significant reduction of nestin immunopositive cells in mice pre-treated with Lic-A before KA compared to mice only treated with KA (Fig. 5).

3.1.5. Lic-A decreases the need for induction of survival pathways after KA treatment

The molecular pathways involved in the response against cell cytotoxicity, caused by the exposure to KA, include the survival signaling pathway. The main effector under the regulation of the JNKs is the AKT protein, which is modulated by phosphorylation on the Ser473 residue. Thus, 3-h after the exposure to KA there was a significant increase in the phosphorylated status of AKT (Fig. 6). Lic-A pre-treatment caused for a significant reduction of this phosphorylated status of the AKT (45.6% of reduction). Correspondingly, the cAMP responsive element-binding protein (CREB), that is found downstream of AKT in the survival pathway, showed the same pattern levels of phosphorylation as the previously described protein (41% of reduction in the Lic-A + KA experimental group). Further analysis of AKT and CREB 24-h after the exposure to KA revealed no significant variations of the protein status in any experimental group.

3.1.6. Lic-A promotes anti-apoptotic responses and reduces pro-apoptotic biomarkers

In order to evaluate further the data obtained in the previous section, other biomarkers associated with the development of apoptotic mechanisms were analysed. The study of biomarkers through western blot was limited to the time period 3-h after the administration of KA in order to evaluate the early response signals. As it was expected, KA promoted the increase of protein levels of B-cell lymphoma 2 (BCL-2), BCL-2-like protein 4 (BAX), BCL-2 like protein 11 (BIM) and the products of α-spectrin (aS): cleaved α-spectrin by calpain (CaSBC) and cleaved α-spectrin by caspase 3 (CaSBC3) (Fig. 7). Also, Lic-A pre-treatments reduced significantly the levels of these same proteins; interestingly BCL-2 was increased further (increase of BCL-2 by 14%, decrease of BAX (31%), BIM (48.9%), CaSBC (38.7%) and CaSBC3 (49.1%)).

Next, immunofluorescence assays against BCL-2 and BAX on the tissue samples extracted 24-h after the administration of KA were performed. The results were very similar to those discussed for the western blot. BAX had increased its levels in the KA experimental group and Lic-A had reduced its response (Fig. 8). In addition, BCL-2 had also increased its levels with KA but, Lic-A had promoted further its levels (Fig. 9).

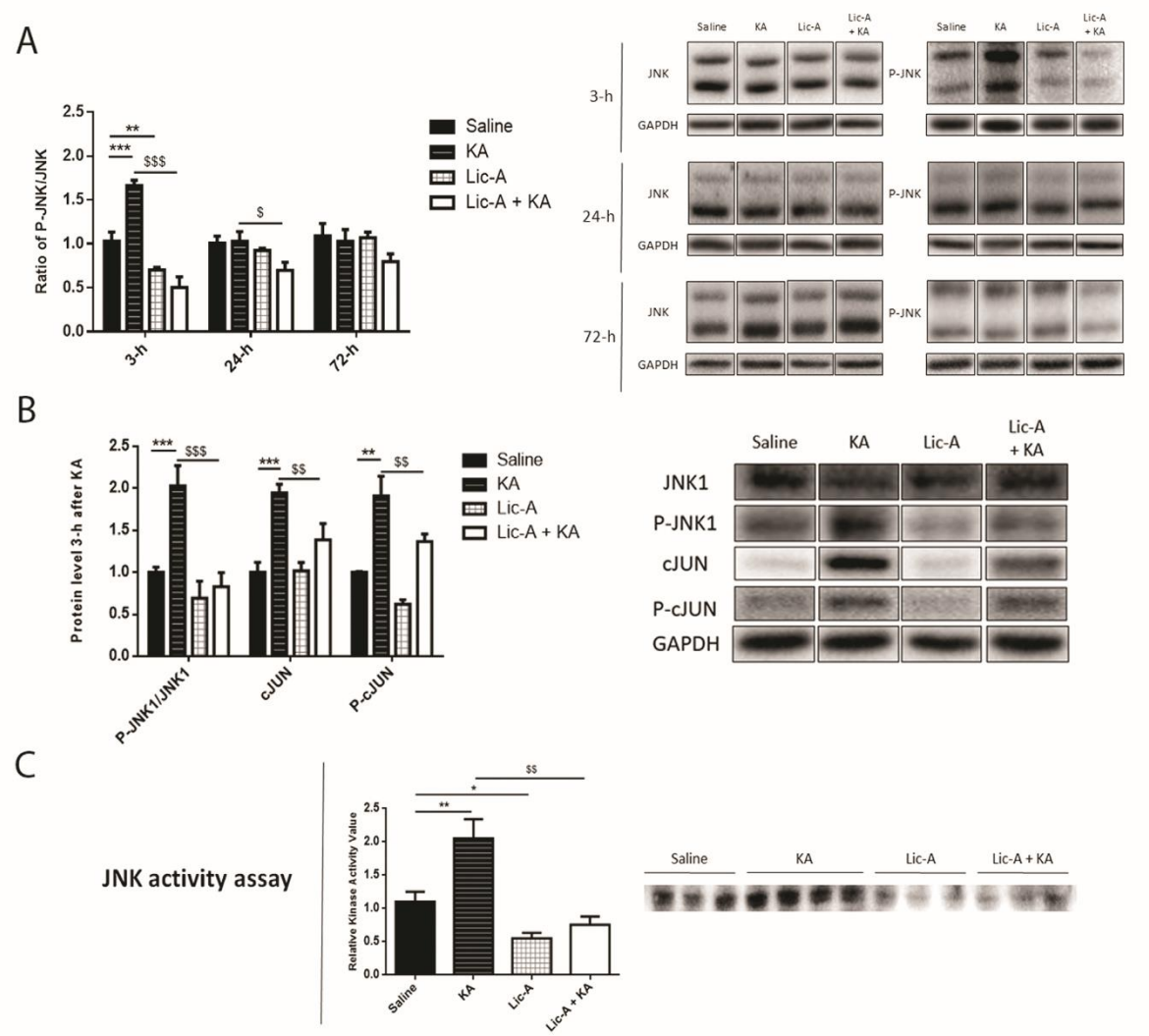


Fig. 2. A. Detection of protein levels for JNK and P-JNK 3, 24 and 72-h after KA administration. B. Western blot analysis for JNK1, P-JNK1, cJUN and P-cJUN at 3-h after KA administration. C. Results of the SAPK/JNK kinase activity assay on protein samples extracted a 3-h treatment of KA. Results were represented as MEAN ± SD. One-way ANOVA: *p < 0.05 Saline vs KA and Lic-A, **p < 0.01 Saline vs KA and Lic-A and ***p < 0.001 Saline vs KA and Lic-A. Student t-test: $ p < 0.05 KA vs Lic-A + KA, $$ P < 0.01 KA vs Lic-A + KA and $$$ p < 0.001 KA vs Lic-A + KA.

3.1.7. Lic-A decreases oxidative stress and neuroinflammatory responses

Oxidative stress is one of the key factors that causes damage derived of KA. Several studies by western blot with protein samples of animals exposed to KA for 3-h were performed. Significant increases in the levels of the peroxisome-proliferator-activated receptor-gamma-coactivator-1-alpha (PGC1α) were detected, a mitochondrial protein associated with the development of stress responses. In addition, significant increases in pro-inflammatory biomarkers: tumor necrosis factor α (TNFα), induced nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) were detected. All these biomarkers that found themselves increased due to the stress and damage caused by KA, were significantly reduced when the animals had undergone a pre-treatment with Lic-A (9.5, 33.5, 38.49% and 25.8% of decrease respectively). Interestingly, Lic-A also seems to decrease the presence of proADAM10 and ADAM10 (40.31% and 24.8% of decrease respectively), a metalloprotease associated with the activation of soluble TNFα (Fig. 10).

Given the oxidative stress results, several immunofluorescence assays to evaluate its consequences on the long term were performed. Immunolabeling of the glial fibrillary acidic protein (GFAP) 72-h after the administration of KA (Fig. 11), and the ionized calcium-binding adapter molecule 1 (IBA1) 24-h after the administration of KA (Fig. 12), in the DG of the hippocampus, revealed how the Lic-A pre-treatment had also caused for a significant reduction of the reactivity of these two cellular types, being highly reactive for the KA experimental group.

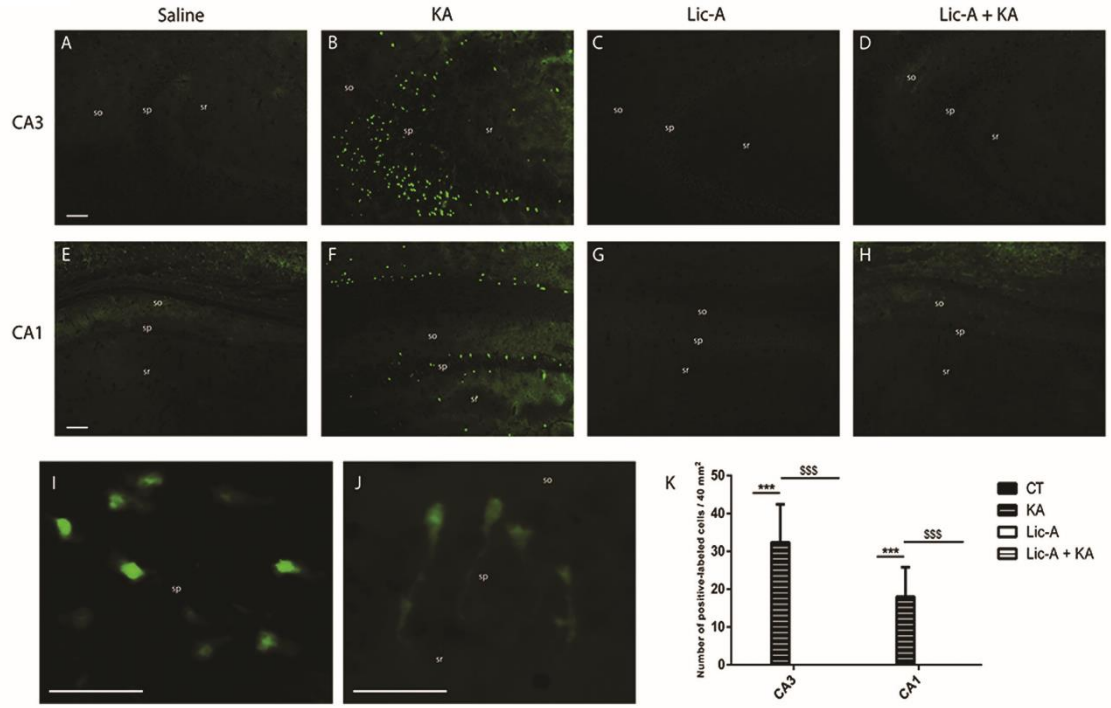


Fig. 3. Fluoro-Jade B staining (green) of mouse brains for the detection of degenerating neurons in the CA3 (Images A-D) and CA1 (Images E-H). Images I and J show magnifications of both CA3 and CA1 images for the KA experimental group. Image K presents a graphical representation of the results of quantification of the number of Fluoro-Jade B positively-labelled cells/40 mm^2. Results were represented as MEAN ± SD. ***p < 0.001 Saline vs KA and Lic-A and $$$ p < 0.001 KA vs Lic-A + KA. Samples were obtained 24-h after the appropriate pre-treatment and treatment. All images have a scale bar of 100 μm. Abbreviations: so = stratum oriens, sp = stratum pyramidal and sr = stratum radiatum. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Comparative evaluation of the effects of Lic-A inhibition and Jnk1 −/− transgenesis against KA insults

To confirm that the neuroprotective action of Lic-A is mediated through the inhibition of JNK1, the levels of several proteins (P-JNK/JNK, P-AKT/AKT, BCL-2, BAX and aS and its cleaved products CaSBC and CaSBC3) that had already been analysed previously in the 3-h after KA time period were evaluated. The results evidenced that Lic-A caused a reduction of these proteins as occurred in the Jnk1$^{-/-}$ mice (Fig. 13).

4. Discussion

The data obtained in the present work demonstrates the neuroprotective effect of in vivo pharmacological selective inhibition of JNK1 by Lic-A, specifically in a KA-induced TLE mice model. Furthermore, the specificity of this molecule was studied further comparing the effects of Lic-A versus single knock-out transgenic mice for the *Jnk1* gene.

First of all, it was evidenced how a pre-treatment with Lic-A before KA was able to reduce seizure-related behaviours. This was obtained through an evaluation of the convulsive response of animals after KA administration. Since Lic-A is a specific inhibitor of JNK1, as it has been demonstrated in vitro models (Yao et al., 2014), the results obtained point out to the fact that JNK1 has a role on diminishing seizures, but the pathway through which this occurs is still unknown. Considering the basis of convulsions, associated with high calcium levels in the cytoplasmic environment, it could be possible that the JNKs have some control over the contractibility of muscular cells or the activity of calcium-dependent enzymes. Tai et al., 2017 has reported similar data referring to the anticonvulsive effect of the inhibition of the JNKs. But, they do not report the mechanism through which this would occur.

TLE has been associated many times with high values of activating phosphorylation of the JNKs in mice, rats and humans (Jeon et al., 2000; Sabapathy, 2012), which eventually leads to neuronal death (Harper and Lograsso, 2001; Verdaguer et al., 2003). In our results, this data was confirmed through a time course, treating animals for 3, 24 and 72 h with KA. It was observed how KA caused a significant increase in JNK phosphorylation at 3 h, as it was reported before in others studies from our group (Auladell et al., 2017). Lic-A reduced the phosphorylated state of the JNKs. Specifically, significant reductions in the phosphorylation levels of total JNK as well as JNK1 after being treated with KA for 3-h were observed. The inhibition of the JNK1 phosphorylated status, supports the data reported by Yao et al. (2014) demonstrating the specificity of Lic-A. Additionally, the results of the Lic-A experimental group points out to a mechanism of reversible inhibition when observing the time course treatment. Furthermore, the analysis of cJUN, a JNK downstream early transcription factor, added more evidences to the importance of the JNK1 in KA induced neuronal death. The role of JNK1 in this process was also supported when neuronal degeneration was evaluated by Fluoro-Jade B stain 24 h after KA treatment. The animals that had undergone a pre-treatment with Lic-A showed no neurodegenerating neurons in the main areas of the hippocampus typically affected by KA (Nadler

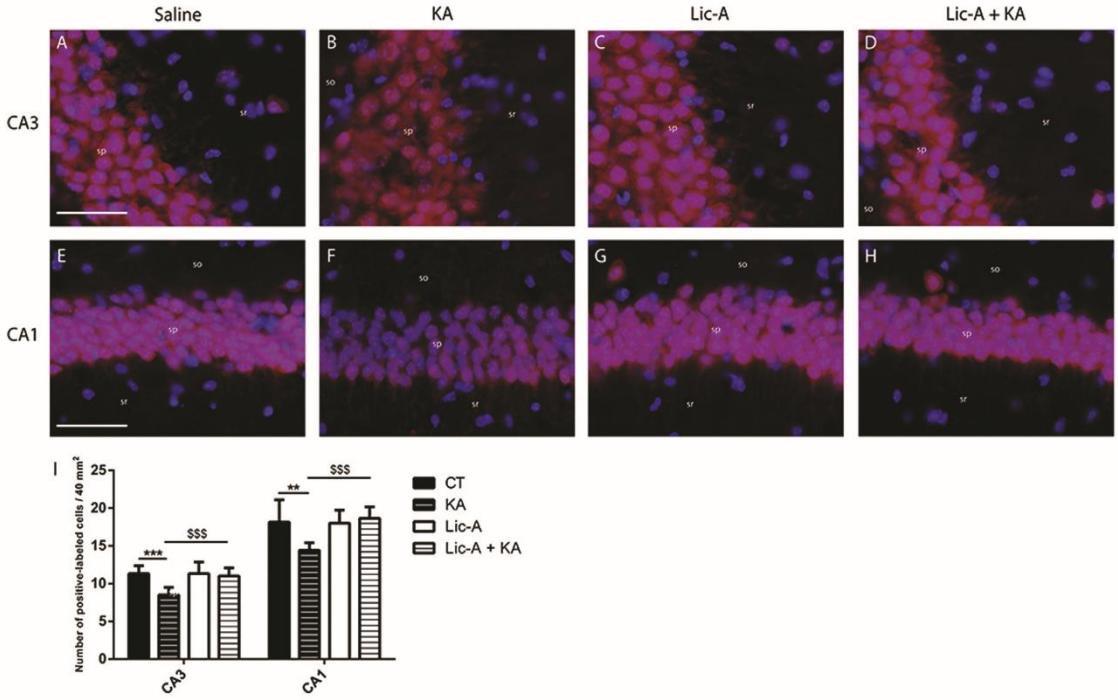


Fig. 4. Identification and quantification of the number of surviving neurons 24 h after KA insult in the CA3 (Images A-D) and CA1 (Images E-H). Detection by NeuN immunofluorescence. Image I is a graphical representation of the results of quantification of the number NeuN positively-labelled cells/40 mm^2. Results were represented as MEAN ± SD. **p < 0,01 Saline vs KA and Lic-A, ***p < 0.001 Saline vs KA and Lic-A and $$$ p < 0.001 KA vs Lic-A + KA. All images have a scale bar of 100 μm. Abbreviations: so = stratum oriens, sp = stratum pyramidal and sr = stratum radiatum.

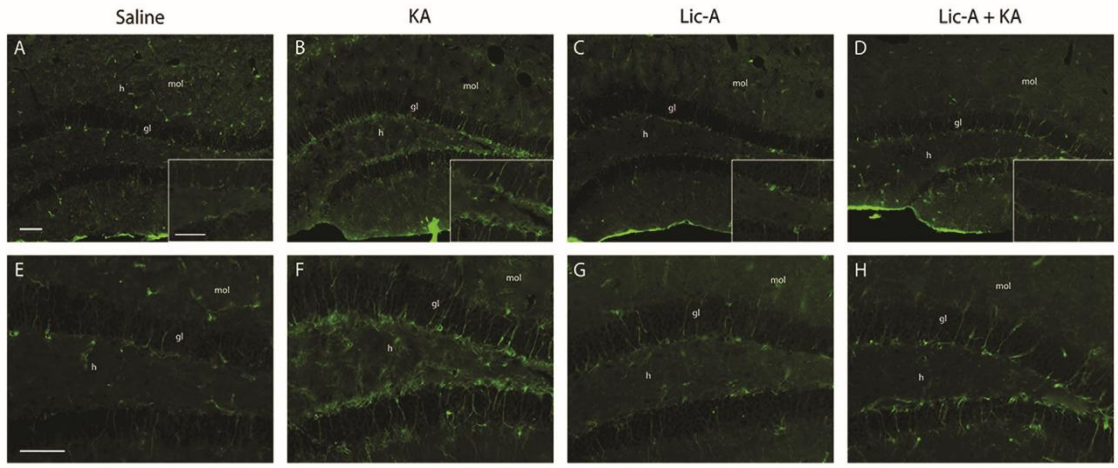


Fig. 5. Immunofluorescence labelling of NESTIN in the DG of the hippocampus (green) on samples after 24-h of appropriate treatment. All images have a scale bar of 100 μm. Abbreviations: h = hilus, gl = granular layer and mol = molecular layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and Cuthbertson, 1980; Nadler et al., 1980; French et al., 1982). A posterior analysis, in the same time period, on the number of survival neurons through the detection of the NeuN protein, a typical biomarker of mature neurons, increased the amount of evidence on the neuroprotectant effect of Lic-A through its inhibition of the JNK1. It must be noted, that our group has already published data demonstrating how the deletion of the *Jnk1* gene has neuroprotective effects in models of KA (de Lemos et al., 2017).

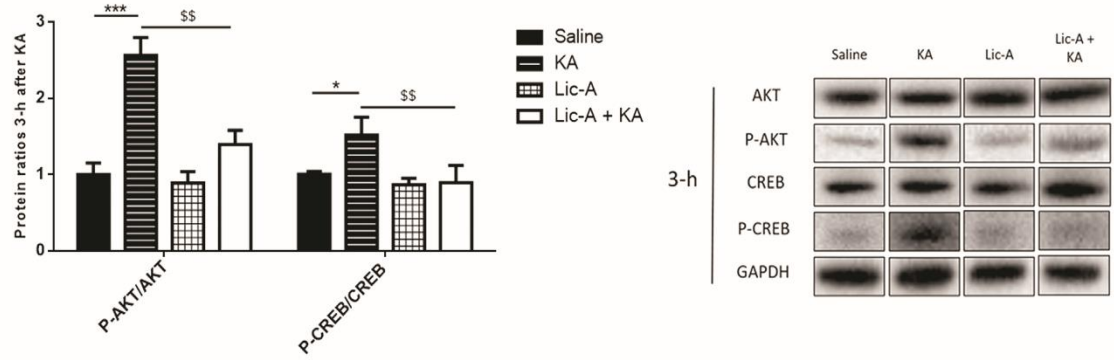


Fig. 6. Analysis of the phosphorylation levels of AKT and CREB survival proteins through western blot 3-h after KA-induced damage. Results were represented as MEAN ± SD. *p < 0.05 Saline vs KA and Lic-A, ***p < 0.001 Saline vs KA and Lic-A and $$ p < 0.01 KA vs Lic-A + KA.

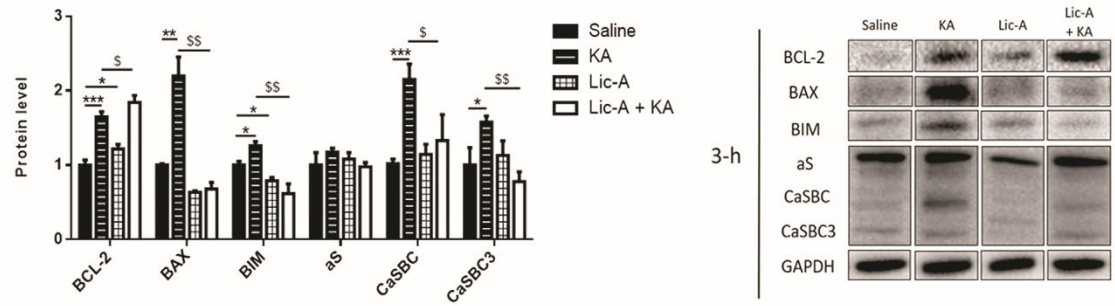


Fig. 7. Western blot quantification of apoptotic cascade biomarkers: BCL-2, BAX, BIM, aS, CaSBC and CaSBC3. Analysis 3-h after KA administration. Results were represented as MEAN ± SD. *p < 0.05 Saline vs KA and Lic-A, **p < 0.01 Saline vs KA and Lic-A, ***p < 0.001 Saline vs KA and Lic-A, $ p < 0.05 KA vs Lic-A + KA and $$ p < 0.01 KA vs Lic-A + KA.

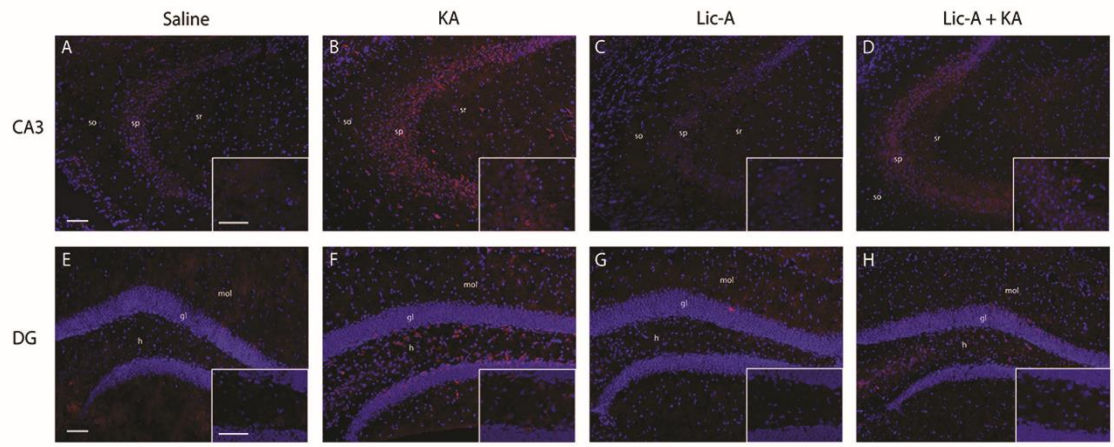


Fig. 8. BAX immunofluorescence detection in the CA3 (Images A-D) and DG (Images E-H) regions of the hippocampus 24-h after exposure to KA (red). Nucleus of cells have been stained in blue using Hoechst. All images have a scale bar of 100 μm. Abbreviations: so = stratum oriens, sp = stratum pyramidal, sr = stratum radiatum, h = hilus, gl = granular layer and mol = molecular layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Moreover, following KA treatment, there is increase in the proliferation on neural cells. This situation becomes a harmful event and induces alterations in the network circuits due to the formation of aberrant synaptic contacts (Parent and Murphy, 2008; Jessberger et al., 2007). This abnormal network is the cause for the development of spontaneous seizures in the so called chronic epilepsy (Mathern et al., 1993). The reduction of nestin immunopositive cells observed in mice pre-treated with Lic-A before KA, in

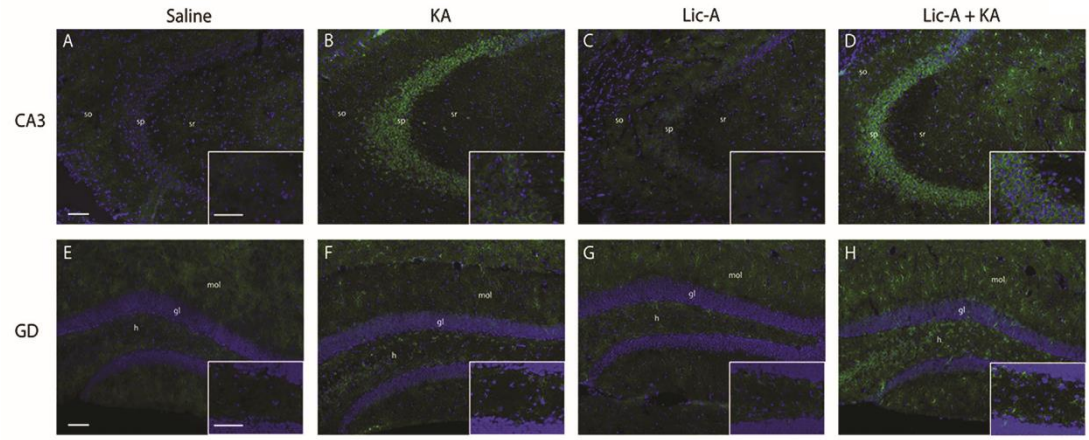


Fig. 9. Immunofluorescence against BCL-2 detection in the CA3 (Images A-D) and DG (Images E-H) regions of the hippocampus 24-h after exposure to KA (green). Nucleus of cells have been stained in blue using Hoechst. All images have a scale bar of 100 μm. Abbreviations: so = stratum oriens, sp = stratum pyramidal, sr = stratum radiatum, h = hilus, gl = granular layer and mol = molecular layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

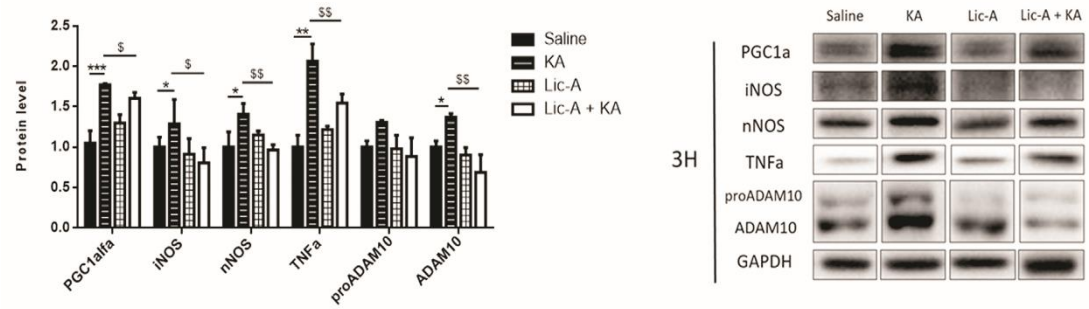


Fig. 10. Evaluation of PGC1α, iNOS, nNOS, TNFα, proADAM10 and ADAM10 protein levels through western blot with samples extracted 3-h after KA administration. Results were represented as MEAN ± SD. *p < 0.05 Saline vs KA and Lic-A, **p < 0.01 Saline vs KA and Lic-A, ***p < 0.001 Saline vs KA and Lic-A, $ p < 0.05 KA vs Lic-A + KA and $$ p < 0.01 KA vs Lic-A + KA.

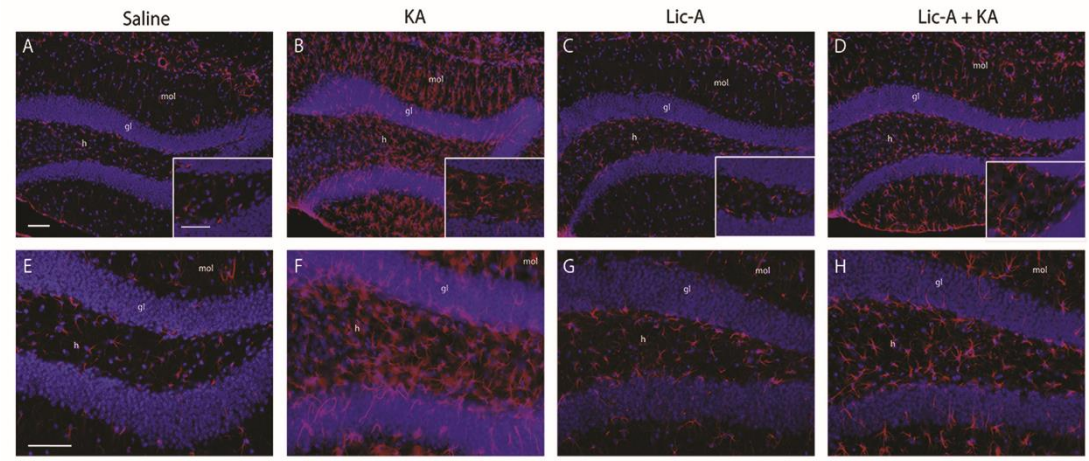


Fig. 11. GFAP immunodetection with fluorescence in the DG of the hippocampus (red). Assay performed on samples 72-h after KA treatment. Nucleus of cells have been stained in blue using Hoechst. All images have a scale bar of 100 μm. Abbreviations: h = hilus, gl = granular layer and mol = molecular layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

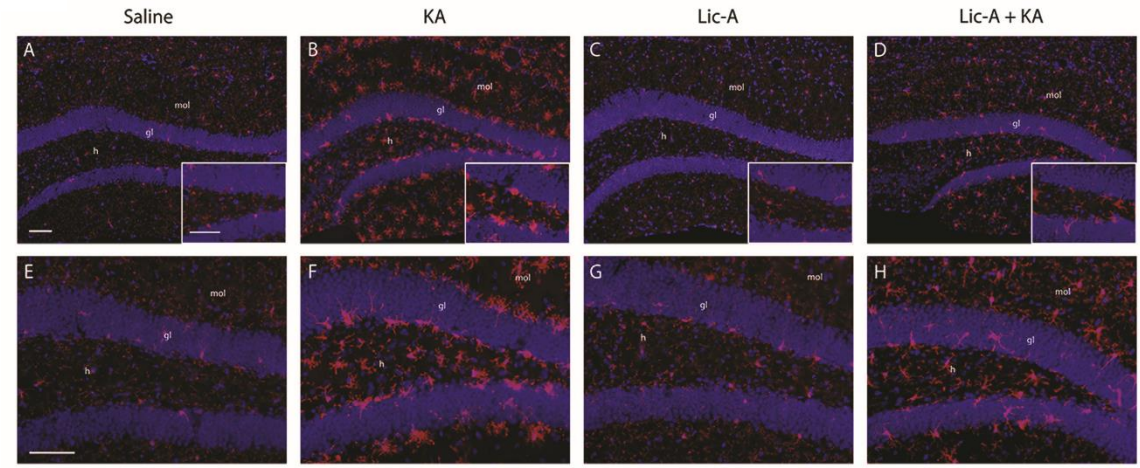


Fig. 12. Detection of IBA1 through immunofluorescence in the DG of the hippocampus (red). Assay performed on samples 24-h after KA-induced damage. Nucleus of cells have been stained in blue using Hoechst. All images have a scale bar of 100 μm. Abbreviations: h = hilus, gl = granular layer and mol = molecular layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

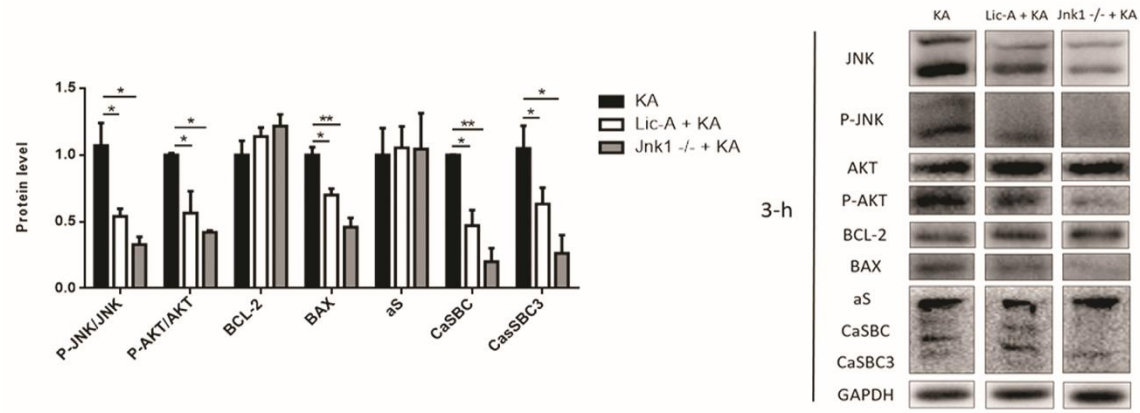


Fig. 13. Comparative evaluation of the effects of Lic-A inhibition and *Jnk1* −/− transgenesis against KA insults. Western blot analysis of protein levels of P-JNK/JNK, P-AKT/AKT, BCL-2, BAX, aS, CaSBC and CaSBC3 3-hafter KA administration. Results were represented as MEAN ± SD. *p < 0.05 and **p < 0.01.

comparison to mice only treated with KA, suggested there was a lower chance for the formation of aberrant synaptic contacts and therefore a reduction of spontaneous seizures though a chronic status.

After the exposition to KA, many molecular pathways involved in the degeneration of neuronal cells are activated like cell survival responses. One of these is the phosphoinositide 3-kinase (PI3K)/AKT pathway (Dunleavy et al., 2013). The activation of this pathway, specifically, through the phosphorylation of AKT in the Ser473 residue, allows for the initiation of survival responses. This phosphorylation was highly increased in those animals that had been treated with KA for 3-h, Lic-A reduced significantly this phosphorylation. This data together with the rest obtained in this study, backs up our hypothesis that JNK1 inhibition is able to reduce the stress and damage that the cells suffer and, consequently, there is a lesser need for the activation of survival mechanisms. These results correlate perfectly with the data already obtained of less convulsive responses and a decrease in neuronal degeneration in the group of animals pre-treated with Lic-A. Moreover, CREB, a transcription factor downstream of AKT, which has been reported to modulate synaptic plasticity (Benito and Barco, 2010) showed similar patterns of response and, as a result, the data adds up on the conclusion that Lic-A protects neurons and prevents the formation of defective synaptic contacts (Parent and Murphy, 2008; Jessberger et al., 2007) reinforcing the role of JNK1 in KA-induced neuronal damage.

It has already been described that the JNKs have a role in the regulation of apoptosis (Liou et al., 2003), in the present paper it was demonstrated how the inhibition of JNK1 diminishes apoptotic responses caused by KA. Several apoptotic signals were analysed 3-h after treatment. KA effects and the subsequent activation of the JNKs lead to a promotion of pro-apoptotic biomarkers like BAX, responsible for the activation of BIM which inhibits BCL-2, an anti-apoptotic protein. Also, it has been demonstrated that JNK activation is able to inhibit BCL-2 further (Dhanasekaran and Reddy, 2008). The experiments performed in mice pre-treated with Lic-A

before KA administration, showed lower activation of this pathway, decreasing the presence of BAX and BIM and promoting highly the presence of BCL-2, thus modifying the balance between apoptotic and anti-apoptotic proteins. Interestingly, in the experimental group of animals only with Lic-A, there was already an increase in anti-apoptotic protein BCL-2, corroborating the efficiency of Lic-A and its inhibition of JNK1 in preventing the activation of the apoptotic pathway. Moreover, this data is supported by the analysis on Lic-A pre-treated animals on the generation of products of aS, obtained by the activation of pro-apoptotic enzymes caspase 3 and calpain. These results are very interesting since Lic-A has also been reported to be a pro-apoptotic molecule in carcinogenic cell cultures (Park et al., 2015), thus arising hypothesis on its possible applications.

Moreover, linked to cellular stress, mitochondrial function alteration was evaluated, a reduction of PGC1α was observed. PGC1α is a transcriptional coactivator that regulates mitochondrial biology and is an important mediator against reactive oxygen species (ROS) (St-Pierre et al., 2006). In our analysis, 3-h after the administration of KA, the levels of PGC1α had increased significantly, being reduced by Lic-A. KA administration causes excitotoxicity that is responsible for the seizures described in the model of TLE and a posterior inflammatory process as it has been demonstrated detecting appearance of gliosis by IBA1 and GFAP reactive immunofluorescence in the hippocampus of animals treated with KA. Early inflammatory targets were also detected 3-h after KA administration, specifically iNOS, nNOS and TNFα protein levels. The results showed similar pattern of response increasing their levels 3-h after KA administration, and as it was expected, Lic-A was able to reduce the levels of both proteins. The metalloproteinases TACE (ADAM17) and ADAM10 are the primary enzymes that catalyse the release of membrane anchored proteins from the cell surface. Then, they would be responsible for the cleavage of TNFα from its transmembrane to its soluble form. The soluble form of TNFα is the one that binds much more efficiently with the TNFαR1 receptor, related to inflammatory and apoptotic pathways (Zheng et al., 2004). While ADAM17 has been the main protein described as the one responsible for this release, the focus of this study was on the levels of ADAM10 since it is more specific of neuronal tissues. Also, it is found in myelinic structures and has a role in development and growth of the neuronal network, making it an interesting target for the study of the well-being of the neuronal tissue (Huovila et al., 2005). Surprisingly, Lic-A also reduced the protein levels of ADAM10. Nonetheless, it is important to note that the anti-inflammatory property of Lic-A could come through the inhibition of JNK1 and also from its properties as a chalcone.

In order to corroborate further that JNK1 has important role in excitotoxicity and that Lic-A could be a potential pharmacological drug for the treatment of diseases where JNK1 is involved, a study comparing the specific inhibition of the JNK1 by Lic-A against single knock-out transgenic mice for the Jnk1$^{-/-}$ was performed. Both groups showed high correlation between them, the levels of P-JNK/JNK were reduced, as well as P-AKT/AKT ratio and other proteins related to apoptosis such as BAX and aS. The only difference encountered between the two JNK1 inhibition methods was in the fact that transgenic mice always had a much more significant response, this would be due to the complete ablation of the gene compared to the reversible and partial inhibition obtained by the drug.

In conclusion, our results suggest that the inhibition of JNK1 can prevent neuronal degeneration in a mice experimental model of TLE, and that Lic-A, a specific inhibitor of JNK1, is a potential drug with neuroprotectant applications for epilepsy.

Conflict of interest

All authors don't have any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations. All authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Science and Innovation PI2016/01, CB06/05/0024 (CIBERNED) and the European Regional Development Founds. Research team from UB and URV belongs to 2014SGR-525 from Generalitat de Catalunya. CBZ is supported by grants from CONACyT México (No. 0177594) and RDCT from Postdoctoral fellowship CONACYT No. 298337 and the Doctoral Program in Sciences in Molecular Biology in Medicine, LGAC Molecular Bases of Chronic Diseases-Degenerative and its Applications (000091, PNPC, CONACyT). The authors are grateful to Roxanne Rowles for the English revision of the manuscript.

References

Auladell, C., de Lemos, L., Verdaguer, E., Ettcheto, M., Busquets, O., Lazarowski, A., Beas-Zarate, C., Olloquequi, J., Folch, J., C.A, 2017. Role of JNK isoforms in the kainic acid experimental model of epilepsy and neurodegeneration. Front. Biosci. 795–814.

Ben-Ari, Y., Lagowska, J., Tremblay, E., La Salle, G.L.G., 1979. A new model of focal status epilepticus: intra-amygdaloid application of kainic acid elicits repetitive secondarily generalized convulsive seizures. Brain Res. 163, 176–179.

Benito, E., Barco, A., 2010. CREB's control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. Trends Neurosci. 33, 230–240.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., Anderson, D.W., 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc. Natl. Acad. Sci. 98, 13681–13686.

Borsello, T., Clarke, P.G.H., Hirt, L., Vercelli, A., Repici, M., Schorderet, D.F., Bogousslavsky, J., Bonny, C., 2003. A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. Nat. Med. 9, 1180–1186.

Brecht, S., Kirchhof, R., Chromik, A., Willesen, M., Nicolaus, T., Raivich, G., Wessig, J., Waetzig, V., Goetz, M., Claussen, M., Pearse, D., Kuan, C.Y., Vaudano, E., Behrens, A., Wagner, E., Flavell, R.A., Davis, R.J., H.T, 2005. Specific pathophysiological functions of JNK isoforms in the brain. Eur. J. Neurosci. 363–377.

Carboni, S., Boschert, U., Gaillard, P., Gotteland, J.-P., Gillon, J.-Y., Vitte, P.-A., 2008. AS601245, a c-Jun NH 2 -terminal kinase (JNK) inhibitor, reduces axon/dendrite damage and cognitive deficits after global cerebral ischaemia in gerbils. Br. J. Pharmacol. 153, 157–163.

Dong, Chen, Yang, Derek D., Wysk, Mark, Alan Whitmarsh, J., Roger Davis, J., R.A.F., 1998. Defective T cell differentiation in the absence of Jnk1. Science 5396, 2092–2095.

de Lemos, L., Junyent, F., Verdaguer, E., Folch, J., Romero, R., Pallàs, M., Ferrer, I., Auladell, C., C.A, 2010. Differences in activation of ERK1/2 and p38 kinase in Jnk3 null mice following KA treatment. J. Neurochem. 5, 1315–1322.

de Lemos, L., Junyent, F., Camins, A., Castro-Torres, R.D., Folch, J., Olloquequi, J., Beas-Zarate, C., Verdaguer, E., Auladell, C., 2017 Jun 29. Neuroprotective effects of the absence of JNK1 or JNK3 isoforms on kainic acid-induced temporal lobe epilepsy-like symptoms. Mol. Neurobiol. https://doi.org/10.1007/s12035-017-0669-1 [Epub ahead of print].

Derijard, B., Hibi, H., Wu, I.H., Barrett, T., Su, B., Deng, T., K.M., D.R, 1994. JNK1: a protein kinase stimulated by UV light and Ha-ras that binds and phosphorylates the c-Jun activation domain. Cell 1025–1037.

Dhanasekaran, D.N., Reddy, E.P., 2008. JNK signaling in apoptosis. Oncogene 27, 6245–6251.

Dunleavy, M., Provenzano, G., Henshall, D.C., Bozzi, Y., 2013. Kainic acid-induced seizures modulate Akt (SER473) phosphorylation in the hippocampus of dopamine D2 receptor knockout mice. J. Mol. Neurosci. 49, 202–210.

Ettcheto, M., Junyent, F., de Lemos, L., Pallas, M., Folch, J., Beas-Zarate, C., Verdaguer, E., Gómez-Sintes, R., Lucas, J.J., Auladell, C., C.A, 2015. Mice lacking functional fas death receptors are protected from kainic acid-induced apoptosis in the Hippocampus. Mol. Neurobiol. 1, 120–129.

Fernández-Espejo, E., 1996. Basic neurobiology of hippocampal formation. Rev. Neurol. 24, 779–784.

Franklin, Paxinos, 25 Oct 2012. The Mouse Brain in Stereotaxic Coordinates, fourth ed., ISBN 9780123910578.

French, E.D., Aldinio, C., Schwarcz, R., 1982. Intrahippocampal kainic acid, seizures and local neuronal degeneration: relationships assessed in unanesthetized rats.

Neuroscience 7, 2525–2536.
Funakoshi-Tago, M., Nakamura, K., Tsuruya, R., Hatanaka, M., Mashino, T., Sonoda, Y., K.T, 2010. The fixed structure of Licochalcone A by alpha, beta-unsaturated ketone is necessary for anti-inflammatory activity through the inhibition of NF-kappaB activation. Int. Immunopharmacol. 5, 562–571.
Hammer, J., Alvestad, S., Osen, K.K., Skare, Ø., Sonnewald, U., Ottersen, O.P., 2008. Expression of glutamine synthetase and glutamate dehydrogenase in the latent phase and chronic phase in the kainate model of temporal lobe epilepsy. Glia 56, 856–868.
Harper, S.J., Lograsso, P., 2001. Signalling for survival and death in neurones: the role of stress-activated kinases, JNK and p38. Cell. Signal 13, 299–310.
Huovila, A.P.J., Turner, A.J., Pelto-Huikko, M., Kärkkäinen, I., Ortiz, R.M., 2005. Shedding light on ADAM metalloproteinases. Trends biochem. 30 (7), 413–422.
Jefferys, J., Steinhäuser, C., Bedner, P., 2016 Feb 15. Chemically-induced TLE models: topical application. J. Neurosci. Meth. 260, 53–61.
Jeon, S.H., Kim, Y.S., Bae, C.-D., Park, J.-B., 2000. Activation of JNK and p38 in rat hippocampus after kainic acid induced seizure. Exp. Mol. Med. 32, 227–230.
Jessberger, S., Zhao, C., Toni, N., Clemenson, G.D., Li, Y., Gage, F.H., 2007. Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling. J. Neurosci. 27, 9400–9407.
Junyent, F., Utrera, J., Romero, R., Pallàs, M., Camins, A., Duque, D., A.C, 2009. Prevention of epilepsy by taurine treatments in mice experimental model. J. Neurosci. Res. 87, 1500–1508.
Kallunki, T., Su, B., Tsigelny, I., Sluss, H.K., Dérijard, B., Moore, G., Davis, R., Karin, M., 1994 Dec 15. JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Genes Dev. 8 (24), 2996–3007.
Kallunki, T., Deng, T., H.M., K.M, 1996. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 929–939.
Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Ruble, E.A., Ahrnad, M.F., A.J., W.J, 1994. The stress activated protein kinase subfamily of c-Jun kinases. Nature 156–216.
Lerma, J.M.J., 2013. Kainate receptors in health and disease. Neuron 292–311.
Lévesque, M., Avoli, M., 2013. The kainic acid model of temporal lobe epilepsy. Neurosci. Biobehav. Rev. 37, 2887–2899.
Liou, A.K.F., Clark, R.S., Henshall, D.C., Yin, X.M., Chen, J., 2003. To die or not to die foyr neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. Prog. Neurobiol. 69, 103–142.
Lucke-wold, B.P., Nguyen, L., Turner, R.C., Logsdon, A.F., Chen, Y., Smith, K.E., Huber, J.D., Matsumoto, R., Rosen, C.L., Tucker, E.S., Richter, E., 2015. Traumatic Brain Injury and Epilepsy: Underlying Mechanisms Leading to Seizure, vol. 33, pp. 13–23.
Mathern, G.W., Cifuentes, F., Leite, J.P., Pretorius, J.K., Babb, T.L., 1993. Hippocampal EEG excitability and chronic spontaneous seizures are associated with aberrant synaptic reorganization in the rat intrahippocampal kainate model. Electroencephalogr. Clin. Neurophysiol. 87, 326–339.
Mechanisms, B., Initiation, F.S., 2002. Basic mechanisms underlying s izures and epilepsy. Pathophysiology of Seizures: An Alteration in the Normal Balance of Inhibition and Excitation Pathophysiology 9–11.
Morrison, R.S., Schwartzkroin, P.A., Jurgen, H., Donehower, A., 1996. Loss of the P53 Tumor Suppressor Cell Death Gene Protects Neurons, vol. 76, pp. 1337–1345.
Nadler, J.V., Perry, B.W., Gentry, C., Cotman, C.W., 1980. Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. J. Comp. Neurol. 192, 333–359.
Nadler, J.V., Cuthbertson, G.J., 1980. Kainic Acid Neurotoxicity toward Hippocampal Formation: Dependence on Specific Excitatory Pathways, vol. 195, pp. 47–56.
Olloquequi, J., Ferrer, J., Montes, J.F., Rodríguez, E., Montero, M.A., García-Valero, J., 2010. Differential lymphocyte infiltration in small airways and lung parenchyma in COPD patients. Respir. Med. 104, 1310–1318.
Parent, J.M., Murphy, G.G., 2008. Mechanisms and functional significance of aberrant seizure-induced hippocampal neurogenesis. Epilepsy 49, 19–25.
Park, M.R., Kim, S.G., Cho, I.A., Oh, D., Kang, K.R., Lee, S.Y., Moon, S.M., Cho, S.S., Yoon, G., Kim, C.S., Oh, J.S., You, J.S., Kim, D.K., Seo, Y.S., Im, H.J., Im, J.S., Park, M.R.I, Kim, S.G.2, Cho, I.A.2, Oh, D.2, Kang, K.R., Lee, S.Y., Moon, S.M., Cho, S.S., Yoon, G., Kim, C.S., Oh, J.S., You, J.S., Kim, D.K., K.J, 2015. Licochalcone-A induces intrinsic and extrinsic apoptosis via ERK1/2 and p38 phosphorylation-mediated TRAIL expression in head and neck squamous carcinoma FaDu cells. Food Chem. Toxicol. 34–43.
Repici, M., Centeno, C., Tomasi, S., Forloni, G., Bonny, C., Vercelli, A., Borsello, T., 2007. Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation. Neuroscience 150, 40–49.
Sabapathy, K., 2012. Role of the JNK Pathway in Human Diseases, 1st Ed, Progress in Molecular Biology and Translational Science. Elsevier Inc.
Sabapathy, K., Hochedlinger, K., Nam, S.Y., Bauer, A., Karin, M., Wagner, E.F., 2004. Distinct roles for JNK1 and JNK2 in regulating JNK activity and c-Jun-dependent cell proliferation. Mol. Cell 15, 713–725.
Schmueda, L.H.K., 2000. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res. 874, 123–130.
Shibata, S., 2000. A drug over the millennia: pharmacognosy, chemistry, and pharmacology of licorice. Yakugaku Zasshi 120, 849–862.
Spigolon, G., Veronesi, C., Bonny, C., Vercelli, A., 2010. c-Jun N-terminal kinase signalling pathway in excitotoxic cell death following kainic acid-induced status epilepticus. Eur. J. Neurosci. 31, 1261–1272.
St-Pierre, J., Drori, S., Uldry, M., Silvaggi, J.M., Rhee, J., Jäger, S., Handschin, C., Zheng, K., Lin, J., Yang, W., Simon, D.K., Bachoo, R., Spiegelman, B.M., 2006. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell 127, 397–408.
Tai, T.Y., Warner, L.N., Jones, T.D., Jung, S., Concepcion, F.A., Skyrud, D.W., Fender, J., Liu, Y., Williams, A.D., Neumaier, J.F., D'Ambrosio, R., Poolos, N.P., 2017. Anti-epileptic action of c-Jun N-terminal kinase (JNK) inhibition in an animal model of temporal lobe epilepsy. Neuroscience 349, 35–47.
Verdaguer, E., Jordà, E., Stranges, A., Canudas, A.M., Jiménez, A., Sureda, F.X., P.M., C.A, 2003. Inhibition of CDKs: a strategy for preventing kainic acid-induced apoptosis in neurons. Ann. N. Y. Acad. Sci. 1010, 671–674.
Yao, K., Chen, H., Lee, M.H., Li, H., Ma, W., Peng, C., Song, N.R., Lee, K.W., Bode, A.M., Dang, Z., Dong, Z., 2014. Licochalcone A, a natural inhibitor of c-Jun N-terminal kinase 1. Cancer Prev. Res. 7, 139–149.
Yuan, C.Y., Whitmarsh, A.J., Yang, D.D., Liao, G., Schloemer, A.J., Dong, C., Bao, J., Banasiak, K.J., Haddad, G.G., Flavell, R.A., Davis, R.J., R.P, 2003. A critical role of neural-specific JNK3 for ischemic apoptosis. Proc. Natl. Acad. Sci. 15184–15189.
Zhao, Y., Spigolon, G., Bonny, C., Culman, J., Vercelli, A., Herdegen, T., 2012. The JNK inhibitor D-JNKI-1 blocks apoptotic JNK signaling in brain mitochondria. Mol. Cell. Neurosci. 49, 300–310.
Zheng, Y., Saftig, P., Hartmann, D., C.B, 2004. Evaluation of the con- tribution of different ADAMs to tumor necrosis factor(TNF) shedding and ofthe function of the TNF ectodomain in ensuring selective stimulated shedding by the TNF convertase (TACE/ADAM17). J. Biol. Chem. 42898–42906.

Publication II

Long-term exposition to a high fat diet favours the appearance of β-amyloid depositions in the brain of C57BL/6J mice.

A potential model of Alzheimer's disease.

Busquets O, Ettcheto M, Pallàs M, Beas-Zarate C, Verdaguer E, Auladell C, Folch J and Camins A.

Mech Ageing Dev. 2017; 162:38–45.

Mechanisms of Ageing and Development 162 (2017) 38–45

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

Long-term exposition to a high fat diet favors the appearance of β-amyloid depositions in the brain of C57BL/6J mice. A potential model of sporadic Alzheimer's disease

Oriol Busquets[a,b,d], Miren Ettcheto[b,d], Mercè Pallàs[b,d,e], Carlos Beas-Zarate[f], Ester Verdaguer[c,d,e], Carme Auladell[c,d,e], Jaume Folch[a,d,**], Antoni Camins[b,d,e,*]

[a] Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Tarragona, Spain
[b] Departament de Farmacologia, Toxicologia i Química Terapeurica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain
[c] Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain
[d] Biomedical Research Networking Center in Neurodegenerative Diseases (CIBERNED), Madrid, Spain
[e] Insitutut de Neurociències, Universitat de Barcelona, Barcelona, Spain
[f] Laboratorio de Regeneración y Desarrollo Neural, Instituto de Neurobiología, Departamento de Biología Celular y Molecular, CUCBA, Guadalajara, Mexico

ARTICLE INFO

Article history:
Received 2 June 2016
Received in revised form
26 September 2016
Accepted 11 November 2016
Available online 15 November 2016

Abbreviations:
Aβ, β-amyloid
AD, Alzheimer's disease
AMPK, AMP-activated protein kinase
ANS, autonomic nervous system
BACE1, beta-site APP-cleaving enzyme 1
BBB, brain blood barrier
CSF, cerebrospinal fluid
DIO, diet-induced obesity
HCD, high calorie diet
HFD, high-fat diet
IGF-1, insulin-like growth factor-1
LOAD, late-onset Alzheimer's disease
MAPK, mitogen-activated protein kinase
PTP1β, protein tyrosine phosphatase 1β
T2DM, type 2 diabetes mellitus

ABSTRACT

Aims: The sporadic and late-onset form of Alzheimer's disease (AD) constitutes the most common form of dementia. This non-familiar form could be a consequence of metabolic syndrome, characterized by obesity and the development of a brain-specific insulin resistance known as type III diabetes. This work demonstrates the development of a significant AD-like neuropathology due to these metabolic alterations.
Methods: C57BL/6J mice strain were divided into two groups, one fed with a diet rich in palmitic acid (high-fat diet, HFD) since their weaning until 16 months of age, and another group used as a control with a regular diet. The analyses were carried out in the dentate gyrus area of the hippocampus using a Thioflavin-S stain and immunofluorescence assays.
Results: The most significant finding of the present research was that HFD induced the deposition of the βA peptide. Moreover, the diet also caused alterations in different cell processes, such as increased inflammatory reactions that lead to a decrease in the neuronal precursor cells. In addition, the results show that there were also dysregulations in normal autophagy and apoptosis, mechanisms related to βA formation.
Conclusions: The present findings confirm that HFD favors the formation of βA depositions in the brain, a key feature of AD, supporting the metabolic hypothesis of sporadic AD.

1. Introduction

Diseases like type-II diabetes mellitus (T2DM), atherosclerotic cardiovascular disease and metabolic syndrome are derived from population's life expectancy continuous growth and the worsening of people's life habits. These diseases together with other degenerative conditions have a metabolic origin and are associated with central/upper body fat accumulation, hypertension, dyslipidemia and hyperglycemia (McGill, 2014). The development of obesity and metabolic syndrome is related to an excessive consumption of red meats, refined sugars, high fat foods and refined grains that contain

* Corresponding author at: Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Tarragona, Spain.
** Corresponding author at: Corresponding autor Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia, Centros de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED). Universitat de Barcelona. Barcelona, Avinguda/Joan XXIII, Spain.
E-mail address: camins@ub.edu (A. Camins).

http://dx.doi.org/10.1016/j.mad.2016.11.002
0047-6374/

high concentrations of saturated and trans-fatty acids (Freeman et al., 2014; McGill, 2014). Metabolic syndrome is one of the most complex and heterogeneous diseases and affects many organs like liver, kidney, gut, pancreas and brain (Hristova, 2013).

Metabolic derangements resulting of obesity cause inflammation, insulin resistance, endoplasmic reticulum stress and impairment of cognitive functions (De Felice and Lourenco, 2015); going so far as being related in epidemiological studies to Alzheimer's disease (AD) (de la Monte and Wands, 2008; Julien et al., 2010; De Felice and Ferreira, 2014; Grillo et al., 2015).

Given recent published findings that provide evidence that HFD causes obesity, insulin resistance and aggravates several AD markers, we chose this experimental approach as our method to study the mechanisms that lead to AD progression (Nuzzo et al., 2015). Previous results from our group in both C57BL/6J and APP/PS1 mice indicate that continuous feeding with HFD, starting at the time of weaning, is sufficient to induce a metabolic syndrome and appears to have direct effects on brain insulin regulation and mitochondrial function. Moreover, through the Morris Water Maze Test (MWM) and the Novel Object Recognition Test (NOR), a significant cognitive decline was evidenced in those animals (Giacco, 2011; Petrov et al., 2015).

There is growing evidence to believe that obesity as it occurs in aging, promotes low-grade systemic inflammation, including the brain (Tucsek et al., 2014; Tang et al., 2015; Pistell et al., 2010). Macrophages, microvascular endothelial cells and adipocytes release a wide range of inflammatory mediators into the bloodstream, such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin (IL) −6 and −8 (Nousen et al., 2013). This systemic inflammation, could also affect most cells ability to progress in the cell cycle and, in turn, their ability to generate new ones. The positive or negative effects of pro- and anti-inflammatory cytokines still need for further understanding but is an important point to be assessed (Borsini et al., 2015; Singh et al., 2012). The dentate gyrus (DG), a structural and functional part of the hippocampus involved in the formation of memory, is one of the brain areas that shows clear neurogenesis ability, allowing for the formation of new neurons, that later migrate into other areas of the tissue. As it is later glimpsed in this research, this neurogenesis system is indeed negatively affected by the neuroinflammation derived of the development of a metabolic syndrome.

Another cellular mechanism that would be affected by the metabolic syndrome is autophagy (Russell et al. 2014; Lipinski et al., 2010). This cellular process allows for the recycling of cellular components to sustain the viability of cells when they have been outstripped of their exogenous nutrient supply and it allows for the renewal of cellular components. Also, a link exists between insulin resistance and autophagy (Yoshizaki, 2012). As it has been previously reported in several papers through the study of these mechanisms in multiple mice models, the appearance of insulin resistance causes the suppression of autophagy, although the mechanism through which it occurs is yet to be clear. In this line, p53 is a protein identified to be involved in the autophagy program. Autophagy and p53 have a negative feedback loop in which p53 induces autophagy, which then limits p53 activation (Kruiswijk et al., 2015). The B-cell lymphoma 2 (BCL-2) also regulates autophagy and is part of an interplay in which p53 inhibits BCL-2 through its phosphorylation and, in turn, BCL-2 inhibits several proteins downstream of the activation of p53, like the p53 upregulated modulator of apoptosis (PUMA), the neuro-oncological ventral antigen (NOVA), the BCL-2-like protein 11 known as BIM and the BCL-2-like protein 4 known as BAX (Maiuri et al., 2010). Moreover, BCL-2 inhibits the BECLIN1, a protein that participates in the formation of the autophagosome (Park et al., 2009; Lorin et al., 2010).

Table 1
Description of caloric content of the CT diet versus HFD diet.

	CT	HFD
	Kcal%	Kcal%
Protein	24.0	16.4
Carbohydrate	58.0	38.6
Fat	18.0	45.0
Total	100.0	100.0
Ingredients		Kcal
Casein, 30 Mesh		912
Maltodextrin 10		680
Corn Starch		1424
Soybean Oil		225
Coconut Oil		2277
Vitamin Mix V10001		40
Total		5558

Since our group has already published data on several alterations due to short-term feeding of HFD (Petrov et al., 2015). In the present study, we tried to discern the negative consequences of a long-term feeding on a HFD and its implications in the alteration of multiple cell processes. The results obtained through histochemical stains and immunofluorescence techniques supported the link between metabolic alterations and the appearance of βA deposit together with glial reactivity. The analyses of different proteins related to apoptosis and to autophagy suggested that these alterations would be the result of impairment in cellular process such as apoptosis and autophagy activity. Moreover, the negative impact of the metabolic syndrome would also have consequences on the progression of neurogenesis mechanisms.

2. Materials and methods

2.1. Animals

16-months old *wild-type* C57BL/6J mice were used in this study. They were separated in two study groups: those fed with regular control diet (CT) and those fed with a HFD. Animals were maintained under standard animal housing conditions with a 12-h dark–light cycle with free access to food and water. Animal procedures were conducted according to ethical guidelines (European Communities Council Directive 2010/63/EU) and approved by the local ethical committee (UB). Every effort was made to minimize animal suffering and to reduce the number of animals used.

2.2. Diet

HFD was purchased from Research Diets, Inc. (Product D08061110). It is made out of hydrogenated coconut oil. The contents of this diet are shown in Table 1. Both CT and HFD were fed to the animals from their weaning until they were sacrificed at 16 months of age.

2.3. Antibodies

The primary and secondary antibodies used in this study have been listed in Tables 2 and 3.

2.4. Immunofluorescence

Mice used for immunofluorescence studies were anesthetized by intraperitoneal injection of ketamine (d = 100 mg/kg) and xylazine (d = 10 mg/kg) and perfused with 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate buffer (PB). Brains were removed and stored in the same solution overnight at 4 °C and 24 h later, they were cryoprotected in 30% sucrose-PFA-PB solution. Coronal

List of primary antibodies used in the immunofluorescence procedure.

Primary Antibody	Reference	Company	Antigen	Source	Concentration
Anti-GFAP	ab7260	Abcam	Glial Fibrillary Acidic Protein	Rabbit	1:1000
Anti-Iba1	019–19741	Wako	Microglia	Rabbit	1:500
Anti-LC3 A/B	ab128025	Abcam	Microtubule-associated protein 1A/1B-light chain 3	Rabbit	1:200
Anti-Nestin	MAB353	Chemicon	Nestin	Mouse	1:200
Anti-ULK1	Ab128859	Abcam	ULK1	Rabbit	1:200
Bcl-2 (N-19)	sc-492	Santa Cruz	B-cell lymphoma 2	Rabbit	1:500
p53 (C-11)	sc-55476	Santa Cruz	p53	Mouse	1:200
β-catenin	sc-1496	Santa Cruz	β-catenin	Goat	1:200
12F4	SIG-39142	BioLegend	β-Amyloid 1–42 peptide	Mouse	1:1000

Table 3
List of secondary antibodies used in the immunofluorescence procedure.

Secondary Antibody	Reference	Company	Antigen	Source	Concentration
AlexaFluor 594	A11005	Life Technology	Mouse IgG	Goat	1:200
AlexaFluor 594	A11012	Life Technology	Rabbit IgG	Goat	1:200
AlexaFluor 488	A11055	Life Technology	Goat IgG	Donkey	1:200
AlexaFluor 488	A21202	Life Technology	Mouse IgG	Donkey	1:200
AlexaFluor 488	A21206	Life Technology	Rabbit IgG	Donkey	1:200

sections of 20 μm of thickness were obtained by a cryostat (Leica Microsystems).

On the first day, free-floating sections were washed three times with 0.1 mol/L PBS pH 7.35 and after five times with PBS-T (PBS 0.1 M, 0.2% Triton X-100). Then, they were incubated in a blocking solution containing 10% fetal bovine serum (FBS), 1% Triton X-100 and PBS 0.1 M + 0.2% gelatin for 2 h at room temperature. After that, slices were washed with PBST (PBS 0.1 M, 0.5% Triton X-100) five times for 5 min each and incubated with the primary antibody overnight. On the second day, brain slices were washed with PBS-T (PBS 0.1 M, 0.5% Triton X-100) 5 times for 5 min and incubated with the appropriate secondary antibody for 2 h at room temperature. Later, sections were co-stained with 0.1 μg/ml Hoechst 33258 (Sigma-Aldrich, St Louis, MO, USA) for 15 min in the dark at room temperature and washed with PBS 0.1 M. Finally, the slides were mounted using Fluoromount G (EMS) image acquisition was performed with an epifluorescence microscope fluorescence filter (BX61 Laboratory Microscope, Melville, NY-Olympus America Inc.).

2.5. Thioflavin-S staining

The stain solution was made of Thioflavin-S diluted with PBS 0.1 mol/L on a 0.0033% concentration. Brain coronal sections were incubated for 8 min in darkness at room temperature.

2.6. Quantification of results

All images and quantifications shown in this paper were obtained from coronal sections from Bregma 1.34–to Bregma 2.46 mm in the mouse brain. NESTIN and ULK1 positive cells quantification was obtained solely from the DG of at least 3 animals from each experimental group. For plaque quantification, similar and comparable histological areas were selected, focusing on having the hippocampus and the whole cortical area positioned adjacently.

In order to quantify the differential fluorescence relative intensity in the images, ImageJ software was used. Relative intensity quantification numbers were obtained under the following formula: CTCF (Corrected Total Cell Fluorescence) = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

2.7. Statistical analysis

Statistical analysis was performed with unpaired *t*-test. Data are presented as means ± SEM, and differences are considered significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

3. Results

3.1. βA peptide accumulation and increased glial reactivity

The presence of βA peptide depositions is a hallmark of AD. Their presence was evaluated through the Thioflavin-S stain, used for the detection of fibrillar aggregates, and using the 12F4 antibody to detect βA diffuse plaques. In both detection methods there was a significant appearance of deposits of the βA peptide in the HFD experimental group versus what occurred in controls (Fig. 1).

Two antibodies were used to analyze the glial response in the DG of the brain between those animals fed with CT and HFD. Astrogliosis, a feature observed in different brain pathologies, was identified using an antibody against glial fibrillary acidic protein (GFAP), the main constituent of the intermediate filament system of adult astrocytes, (Pekny et al., 2014). Also, with an antibody against ionized calcium-binding adapter molecule 1 (Iba1), the morphology of microglial cells was studied. (Deininger, 2002). An augment in astrocyte and microglial response was detected.

3.2. Reduction in neural precursors neurogenesis

Using the anti-NESTIN antibody as a protein marker of neural stem cells, we visualized a decline of these cells in the subgranular zone of the DG (SGZ) of the hippocampus in mice fed with a HFD (Fig. 4).

3.3. Reduction in the activation of apoptotic mechanisms

A reduction in the apoptotic processes was observed using an immunofluorescence against p53 and BCL-2. Thus, by an anti-p53 antibody, a protein associated with tumor suppression, DNA damage and induction of apoptosis, we detected a reduction in the p53 transcription factor of the cytoplasm of different hippocampal neurons in HFD animals in comparison with CT mice (Fig. 5). The immunofluorescence against BCL-2, which has a role in promoting cellular survival and inhibiting actions of pro-apoptotic proteins,

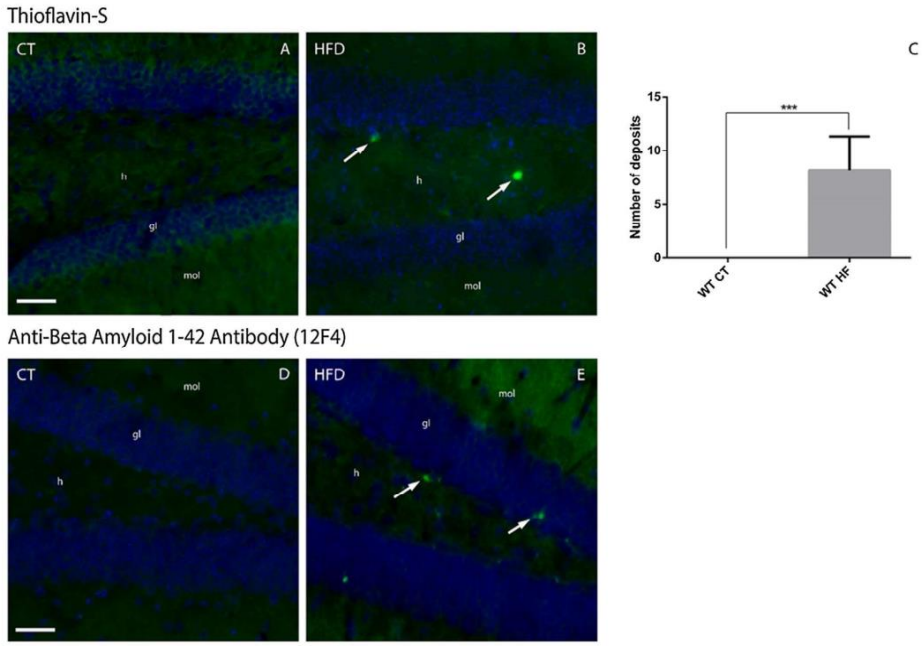


Fig. 1. Stain with Thioflavin-S and 12F4 antibody for the detection of βA fibrillar aggregates and diffuse plaques respectively in coronal hippocampal sections obtained from C57BL/6J 16-months old animals. Images A and B correspond to CT and HFD animals with a Thioflavin-S stain; Detection with 12F4 is shown in images C and D. Comparison between both experimental groups shows presence of several deposits in the DG of the hippocampus in both detection methods. Tissue is also stained with Hoechst (blue). Arrows indicate the presence of the fibrillar aggregates or diffuse plaques. Graphic C shows statistical analysis of the quantification of βA depositions in both the hippocampus and cortex of CT and HFD experimental groups. Statistical analysis was obtained through unpaired Student's *t*-test with p-value < 0.001. Scale bar represents 200 μm. Abbreviations: mol: molecular layer, gl: granular layer, h: hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

revealed an increase of BCL-2 immunopositive cells in HFD mice versus CT, mainly noticed in the dentate gyrus of the hippocampus (Fig. 6). This observed data was supported with the analysis of fluorescence intensity.

3.4. Autophagy impairment

To analyze if the autophagic activity was altered by HFD mice, we studied the hippocampal distribution pattern of different proteins, such as ULK1 (Serine/threonine-protein kinase 1), LC3 (microtubule-associated proteins light chain 3) and β-catenin. No significant differences in ULK1 immunopositive cells were detected between the experimental animal groups (Fig. 7). However, the levels of LC3 and β-catenin complexes were reduced in different areas of the hippocampus, such as DG (hilus, molecular and granular layers) and in the *cornu ammonis 1* (CA1) field, specifically in the *stratum lacunosum-moleculare* (slm) and *stratum radiatum* (sr). Quantification of relative fluorescence intensity values reaffirmed the results with a significant decrease on the fluorimetric response on the WT HF experimental group (p value < 0.001) (Fig. 8).

4. Discussion

The intent of this experimental work was to support the hypothesis on the link between the development of a metabolic syndrome associated with insulin resistance with sporadic AD. Interestingly, we focused on the role of and neuroinflammatory cellular processes with the reduction in neurogenesis along with the disruption of normal autophagic mechanisms that lead to the appearance of βA depositions in the brain. Overall, this distorted situation that had been reached after a long-life exposure to negative environmental exposure (long-term feeding with a HFD) would promote the development of neuropathological disorders (De Felice, 2013; Heni et al., 2015).

In order to carry through the present study, we used mice fed with a HFD. The long term feeding of this diet lead to increases in body weight, peripheral hyperglycemia, hyperinsulinemia and insulin resistance together with a mitochondrial dysfunction (Khalyfa et al., 2013; Takalo et al., 2014; Petrov et al., 2015). As members of our group had already described it, the feeding of this diet caused an increase on the concentration of soluble βA in the brain along with the development of memory deficits, supporting the association between metabolic disorders and AD. (Petrov et al., 2015). In addition, it had also been detected an increase in glial reactivity combined with an escalation in BACE-1 (β-site amyloid precursor protein cleaving enzyme 1) levels, a protein related with the cleavage of the APP peptide. All these data supported an induction of the amyloidogenic pathway in HFD mice as occur with familial AD (Glass et al., 2010; Lee et al., 2008; Patil et al., 2006; Nuzzo et al., 2015). Our results provide evidence that alterations in specific cellular processes induce the appearance of βA deposits.

One of the cellular mechanism that trigger this situation is the inflammatory response that occurs in the neuronal tissue of the brain (Figs. 2 and 3), as a consequence of the alterations in intracellular signaling molecules. Among them, it has been reported that metabolic disorders affect the activity of c-Jun N-Terminal kinase (JNK), protein Kinase R (PKR) and the inhibitor of nuclear factor

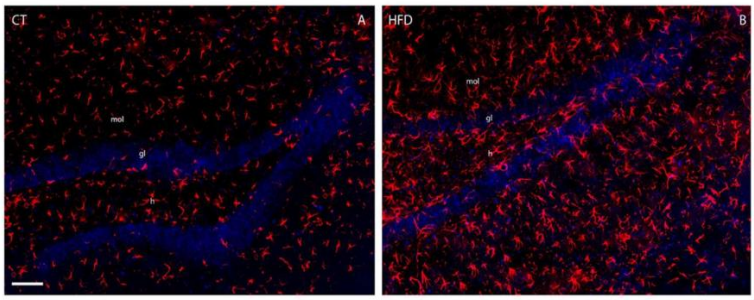


Fig. 2. Immunofluorescence against GFAP in coronal hippocampal sections obtained from C57BL/6J 16-months old animals. Images A and B correspond to CT and HFD diets respectively. Comparison between A and B reveal that astrocytes (red) of HFD fed animals display higher reactivity (bigger size and more ramified profiles). Tissue is also stained with Hoechst (blue). Scale bar represents 100 μm. Abbreviations: mol: molecular layer, gl: granular layer, h: hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

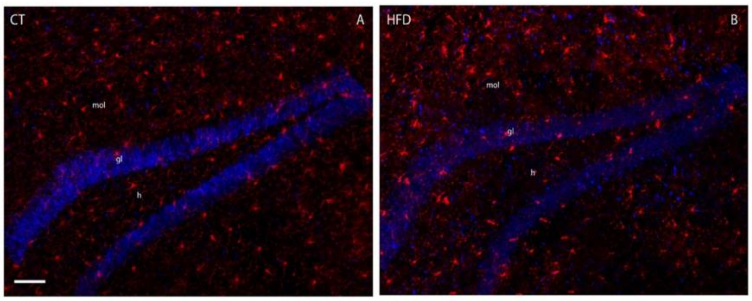


Fig. 3. Microglial reactivity observed using an anti-Iba1 antibody (red) in the DG of the hippocampus after a 16-month feeding of a CT and HFD. Images A and B correspond to CT and HFD diets respectively. Comparison between experimental groups shows an increase in the microglial response in the HFD fed animals versus CT. Tissue is also stained with Hoechst (blue). Scale bar represents 100 μm. Abbreviations: mol: molecular layer, gl: granular layer, h: hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

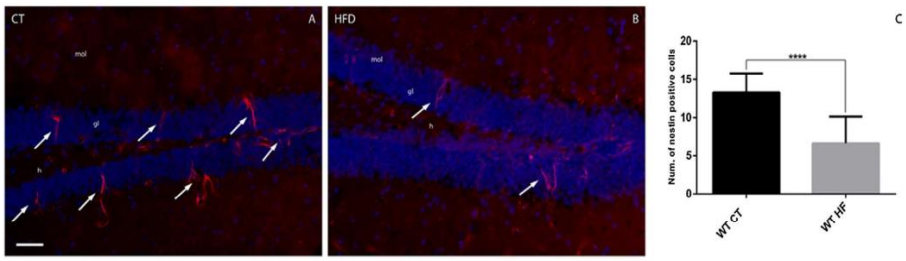


Fig. 4. Detection of Nestin positive cells counterstained with Hoechst in the DG of the hippocampus from CT and HFD fed animals. Images A and B correspond to CT and HFD diets respectively. Comparison between A and B reveal that in the HFD-fed experimental group there would be a decrease in the fluorescence response both from NPCs in the granular layer (red). Nestin positive cells are indicated in the images with arrows. Graphic C shows a quantification of nestin positive cells in the DG of multiple animals per group in similar and comparable Bregma areas. Scale bar represents 200 μm. Abbreviations: mol: molecular layer, gl: granular layer, h: hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

kappa −B Kinase (IKK). All these enzymes would initiate intracellular cascades that lead to the release of inflammatory mediators into the bloodstream (Nousen et al., 2013). This situation ends with the disruption of proper blood-brain barrier function making the brain tissue more sensible to outer metabolic alterations (Takeda et al., 2013).

Park (2010) found that an increase in neuroinflammation affects the progression of neural progenitors within the brain. According to these results, we found a decline in the nestin immunopositive cells in the SGZ, a protein that has a role in the survival and self-renewal of neural stem cells. Consequently, it is suggested that HFD can cause a reduction in the neuronal renovation that affects brain's plasticity and cognitive performance, parameters all decreased in the neuropathology of AD (Singh et al., 2012).

The down-regulation in HFD-fed animals of LC3 that could form a complex with β-catenin promoting its degradation and enhancing autophagy, pointed out to an alteration in the autophagic system (Jia et al., 2014). The β-catenin blockade prevents the degradation of p62 and cellular components targeted to the autophagosome, thereby preventing completion of autophagy. This impairment of autophagy was supported with the reduction found in cytoplasmic p53 and an up-regulation of BCL-2 protein,

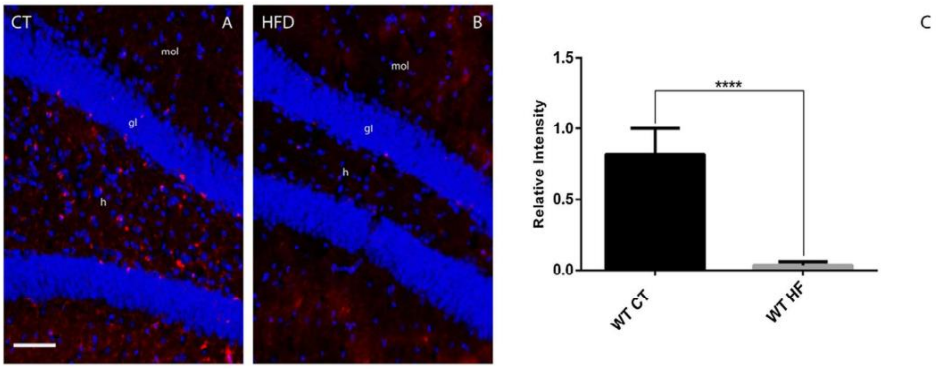


Fig. 5. Immunofluorescence against p53 in coronal hippocampal sections obtained from C57BL/6J 16-months old animals. Images A and B correspond to CT and HFD diets respectively. The results reveal a fluorescence response in the cytoplasm of the cells in the CT groups that has nearly disappeared in the HFD fed animals. Tissue is also stained with Hoechst (blue). Graphic C shows statistical analysis of difference in relative fluorescence intensity between CT and HFD experimental groups. Statistical analysis was obtained through unpaired Student's *t*-test with p-value < 0.0001. Scale bar represents 200 μm. Abbreviations: mol: molecular layer, gl: granular layer, h: hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

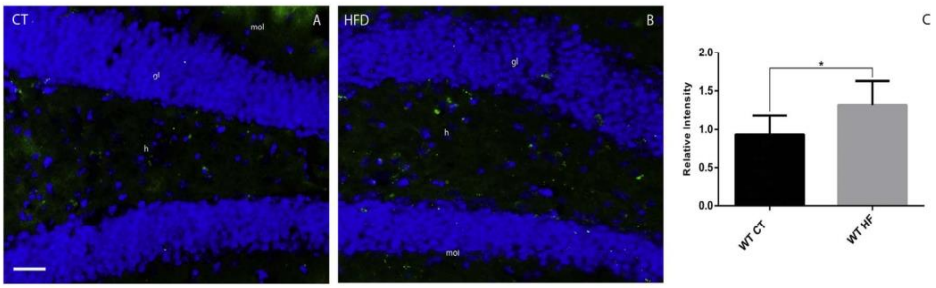


Fig. 6. BCL-2 detection in coronal hippocampal sections obtained from C57BL/6J 16-months old animals. Images A and B correspond to CT and HFD diets respectively. Comparison between A and B reveal that HFD-fed mice have much higher relative fluorescence intensity against BCL-2 protein (green). Tissue is also stained with Hoechst (blue). Graphic C shows statistical analysis of difference in relative fluorescence intensity between CT and HFD experimental groups. Statistical analysis was obtained through unpaired Student's *t*-test with p-value < 0.0001. Scale bar represents 200 μm. Abbreviations: mol: molecular layer, gl: granular layer, h: hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

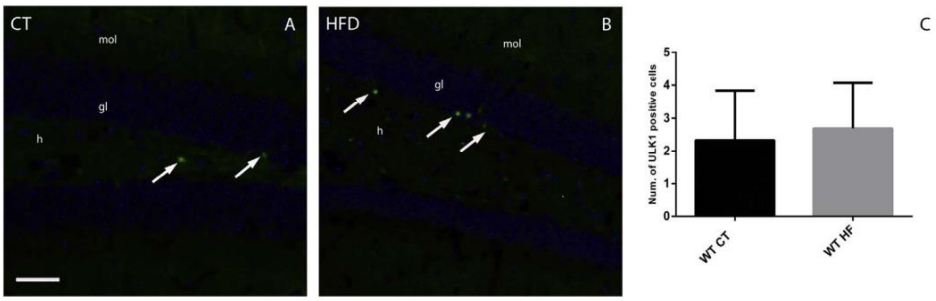


Fig. 7. ULK1 positive cells were detected in coronal hippocampal sections obtained from C57BL/6J 16-months old animals. Images A and B correspond to CT and HFD diets respectively. Comparison between A and B show higher relative fluorescence intensity in the HFD experimental group (green). Tissue is also stained with Hoechst (blue). Arrows indicate the presence of ULK1 positive cells in the tissue. Graphic C shows statistical analysis shows no significant differences between CT and HFD experimental groups. Statistical analysis was obtained through unpaired Student's *t*-test with p-value < 0.0001. Scale bar represents 200 μm. Abbreviations: mol: molecular layer, gl: granular layer, h: hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecules intervene as regulators in several steps on the formation of the autophagosome (Roussy et al., 2008). This process is important for an adequate regulation of protein homeostasis in neurons. It degrades damaged or unwanted components and recycles those destined for use in energy production and other biosynthetic reactions. Therefore, we can suggest that the increase of βA depositions in the cortex and hippocampus of the elder mice could be the result of a dysfunction of autophagy (Takechi et al., 2010a, 2010b). Thus, we theorized that autophagy is involved in the aging of the brain and in age-related neurodegenerative disorders.

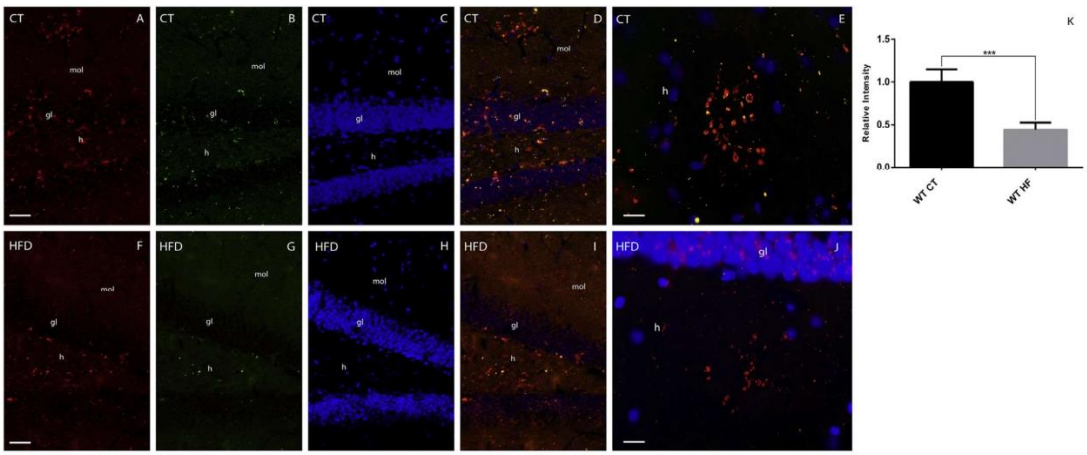


Fig. 8. Representative LC3 and β-catenin detection in coronal hippocampal sections obtained from C57BL/6J 16-months old animals. Images A–E and F–J correspond to CT and HFD diets respectively. From left to right we see in the first column (A and F) immunohistochemistry against LC3 (red), next (B and G) immunohistochemistry against β-catenin (green), next (C and H) Hoechst stain (blue) and lastly, merge of all three colors (D and I, E and F). Scale bar for images A–D and F–I represents 200 μm. Scale bar for E and J represents 20 μm. There is a reduction in the relative fluorescence response in the HFD animals, in both LC3 and β-catenin, in contrast with CT. Abbreviations: mol: molecular layer, gl: granular layer, h: hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To summarize, the observed βA peptide deposition in hippocampus of 16-months old wild-type C57BL/6J mice should not be considered as a spurious observation but, as a signal of how many other systems are distorted, since it is accompanied with significant alterations of cell normal processes, such as increases in neuroinflammation, reduced neural proliferation and decline of autophagy.

Conflict of interest

All authors don't have any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations. All authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data.

Acknowledgement

OB, MP, AC, ME, EV, and JF belong to 2014SGR 525 from Generalitat de Catalunya.

References

Borsini, A., Zunszain, P.A., Thuret, S., Pariante, C.M., 2015. The role Please check article title and journal in Ref. Kruiswijk et al. (2015).of inflammatory cytokines as key modulators of neurogenesis. Trends Neurosci., http://dx.doi.org/10.1016/j.tins.2014.12.006.

De Felice, F.G., 2013. Alzheimer's disease and insulin resistance: translating basic science into clinical applications. J. Clin. Invest. 123 (2), 531–539.

De Felice, F.G., Ferreira, S.T., 2014. Inflammation, defective insulin signaling, and mitochondrial dysfunction as common molecular denominators connecting type 2 diabetes to Alzheimer disease. Diabetes 63 (7), 2262–2272.

De Felice, F.G., Lourenco, M.V., 2015. Brain metabolic stress and neuroinflammation at the basis of cognitive impairment in Alzheimer's disease. Front. Aging Neurosci. 7, 1–8, http://dx.doi.org/10.3389/fnagi.2015.00094.

de la Monte, S.M., Wands, J.R., 2008. Alzheimer's disease is type 3 diabetes-evidence reviewed. J Diabetes Sci Technol 2, 1101–1113, http://dx.doi.org/10.1177/193229680800200619.

Freeman, L.R., Haley-Zitlin, V., Rosenberger, D.S., Granholm, A.-C., 2014. Damaging effects of a high-fat diet to the brain and cognition: a review of proposed mechanisms. Nutr. Neurosci. 17, 241–251, http://dx.doi.org/10.1179/1476830513Y.0000000092.

Giacco, F., 2011. Oxidative stress and diabetic complications. Circ. Res. 107, 1058–1070, http://dx.doi.org/10.1161/CIRCRESAHA.110.223545.Oxidative.

Glass, C.K., Saijo, K., Winner, B., Marchetto, M.C., Gage, F.H., 2010. Mechanisms underlying inflammation in neurodegeneration. Cell 140, 918–934, http://dx.doi.org/10.1016/j.cell.2010.02.016.

Grillo, C.A., Piroli, G.G., Lawrence, R.C., Wrighten, S.A., Green, A.J., Wilson, S.P., Sakai, R.R., Kelly, S.J., Wilson, M.A., Mott, D.D., Reagan, L.P., 2015. Hippocampal Insulin Resistance Impairs Spatial Learning and Synaptic Plasticity. Diabetes 64 (11), 3927–3936.

Heni, M., Kullmann, S., Preissl, H., Fritsche, A., Häring, H.-U., 2015. Impaired insulin action in the human brain: causes and metabolic consequences. Nat. Rev. Endocrinol. 11, 701–711, http://dx.doi.org/10.1038/nrendo.2015.173.

Hristova, M.G., 2013. Metabolic syndrome – from the neurotrophic hypothesis to a theory. Med. Hypotheses 81, 627–634, http://dx.doi.org/10.1016/j.mehy.2013.07.018.

Jia, Z., Wang, J., Wang, W., Tian, Y., XiangWei, W., Chen, P., Ma, K., Zhou, C., 2014. Autophagy eliminates cytoplasmic??-catenin and NICD to promote the cardiac differentiation of P19CL6 cells. Cell. Signal. 26, 2299–2305, http://dx.doi.org/10.1016/j.cellsig.2014.07.028.

Julien, C., Tremblay, C., Phivilay, A., Berthiaume, L., Émond, V., Julien, P., Calon, F., 2010. High-fat diet aggravates amyloid-beta and tau pathologies in the 3xTg-AD mouse model. Neurobiol. Aging 31, 1516–1531, http://dx.doi.org/10.1016/j.neurobiolaging.2008.08.022.

Khalyfa, a, Carreras, a, Hakim, F., Cunningham, J.M., Wang, Y., Gozal, D., 2013. Effects of late gestational high-fat diet on body weight, metabolic regulation and adipokine expression in offspring. Int. J. Obes. (Lond). 37, 1481–1489, http://dx.doi.org/10.1038/ijo.2013.12.

Kruiswijk, F., Labuschagne, C.F., Vousden, K.H., 2015. p 53 in survival, death and metabolic health: a lifeguard with a licence to kill. Nat. Rev. Mol. Cell Biol. 16, http://dx.doi.org/10.1038/nrm4007.

Lee, Y.-H., Tharp, W.G., Maple, R.L., Nair, S., Permana, P., a. Pratley, R.E., 2008. Amyloid precursor protein expression is upregulated in adipocytes in obesity. Obesity (Silver Spring). 16, 1493–1500, http://dx.doi.org/10.1038/oby.2008.267.

Lipinski, M.M., Zheng, B., Lu, T., Yan, Z., Py, B.F., Ng, A., Xavier, R.J., Li, C., Yankner, B.A., Scherzer, C.R., Yuan, J., 2010. Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. Proc. Natl. Acad. Sci. 107, 14164–14169, http://dx.doi.org/10.1073/pnas.1009485107.

Lorin, S., Pierron, G., Ryan, K.M., Codogno, P., Djavaheri-Mergny, M., 2010. Evidence for the interplay between JNK and p53-DRAM signalling pathways in the regulation of autophagy. Autophagy 6, 153–154, http://dx.doi.org/10.4161/auto.6.1.10537.

Maiuri, M.C., Galluzzi, L., Morselli, E., Kepp, O., Malik, S.A., Kroemer, G., 2010. Autophagy regulation by p53. Curr. Opin. Cell Biol. 22, 181–185, http://dx.doi.org/10.1016/j.ceb.2009.12.001.

McGill, A.-T., 2014. Causes of metabolic syndrome and obesity-related co-morbidities Part 1: A composite unifying theory review of human-specific co-adaptations to brain energy consumption. Arch. public Heal. Arch. belges santeî publique 72, 30, http://dx.doi.org/10.1186/2049-3258-72-30.

Nousen, E.K., Franco, G.G., Sullivan, E.L., 2013. Unraveling the mechaisms responsible for the comorbidity between metabolic syndrome and mental health disorders. Neuroendocrinology 98, 254–266, http://dx.doi.org/10.1159/000355632.Unraveling.

Nuzzo, D., Picone, P., Baldassano, S., Caruana, L., Messina, E., Marino, A., Cappello, F., Mulè, F., Di, M., 2015. Insulin resistance as common molecular denominator linking obesity to alzheimer's disease. Curr. Alzheimer Res., 1–13.

Park, K.J., Lee, S.H., Lee, C.H., Jang, J.Y., Chung, J., Kwon, M.H., Kim, Y.S., 2009. Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death. Biochem. Biophys. Res. Commun. 382, 726–729, http://dx.doi.org/10.1016/j.bbrc.2009.03.095.

Patil, S., Sheng, L., Masserang, A., Chan, C., 2006. Palmitic acid-treated astrocytes induce BACE1 upregulation and accumulation of C-terminal fragment of APP in primary cortical neurons. Neurosci. Lett. 406, 55–59, http://dx.doi.org/10.1016/j.neulet.2006.07.015.

Pekny, M., Wilhelmsson, U., Pekna, M., 2014. The dual role of astrocyte activation and reactive gliosis. Neurosci. Lett. 565, 30–38, http://dx.doi.org/10.1016/j.neulet.2013.12.071.

Petrov, D., Pedrós, I., Artiach, G., Sureda, F.X., Barroso, E., Pallàs, M., Casadesús, G., Beas-zarate, C., Carro, E., Ferrer, I., Vazquez-carrera, M., Folch, J., Camins, A., 2015. High-fat diet-induced deregulation of hippocampal insulin signaling and mitochondrial homeostasis deficiencies contribute to Alzheimer disease pathology in rodents. BBA – Mol. Basis Dis., http://dx.doi.org/10.1016/j.bbadis.2015.05.004.

Pistell, P.J., Morrison, C.D., Gupta, S., Knight, A.G., Keller, J.N., Ingram, D.K., Bruce-Keller, A.J., 2010. Cognitive impairment following high fat diet consumption is associated with brain inflammation. J. Neuroimmunol. 219, 25–32, http://dx.doi.org/10.1016/j.jneuroim.2009.11.010.

Roussy, I.G., Roussy, I.G., Paris, U., Paris, U., Paris, S., Paris, S., Federico, N., Federico, N., Biotecnologiche, S., Biotecnologiche, S., Sperimentale, F., Sperimentale, F., Institute, D.T., Institute, D.T., Vergata, T., Vergata, T., 2008. A dual role of p53 in the control of autophagy 1. Autophagy 8627, 810–814, 6486[pii].

Russell, R.C., Tian, Y., Yuan, H., Park, H.W., Chang, Y., Kim, H., Neufeld, T.P., Dillin, A., Guan, K., 2014. ULK1 induces autophagy by phosphorylating Beclin-1 and activating Vps34 lipid kinase. Nat. Cell Biol. 15, 741–750, http://dx.doi.org/10.1038/ncb2757.ULK1.

Singh, R.B., Gupta, S., Dherange, P., De Meester, F., Wilczynska, A., Alam, S.E., Pella, D., Wilson, D.W., 2012. Metabolic syndrome: a brain disease. Can. J. Physiol. Pharmacol. 90, 1171–1183, http://dx.doi.org/10.1139/y2012-122.

Takalo, M., Haapasalo, A., Martiskainen, H., Kurkinen, K.M.A., Koivisto, H., Miettinen, P., Khandelwal, V.K.M., Kemppainen, S., Kaminska, D., Mäkinen, P., Leinonen, V., Pihlajamäki, J., Soininen, H., Laakso, M., Tanila, H., Hiltunen, M., 2014. High-fat diet increases tau expression in the brain of T2DM and AD mice independently of peripheral metabolic status. J. Nutr. Biochem. 25, 634–641, http://dx.doi.org/10.1016/j.jnutbio.2014.02.003.

Takechi, R., Galloway, S., Pallebage-Gamarallage, M.M.S., Lam, V., Mamo, J.C.L., 2010a. Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk. Prog. Lipid Res. 49, 159–170, http://dx.doi.org/10.1016/j.plipres.2009.10.004.

Takechi, R., Galloway, S., Pallebage-Gamarallage, M.M.S., Wellington, C.L., Johnsen, R.D., Dhaliwal, S.S., Mamo, J.C.L., 2010b. Differential effects of dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-beta. Br. J. Nutr. 103, 652–662, http://dx.doi.org/10.1017/S0007114509992194.

Takeda, S., Sato, N., Ikimura, K., Nishino, H., Rakugi, H., Morishita, R., 2013. Increased blood-brain barrier vulnerability to systemic inflammation in an Alzheimer disease mouse model. Neurobiol. Aging 34, 2064–2070, http://dx.doi.org/10.1016/j.neurobiolaging.2013.02.010.

Tang, Y., Purkayastha, S., Cai, D., 2015. Hypothalamic microinflammation: a common basis of metabolic syndrome and aging. Trends Neurosci. 38, 36–44, http://dx.doi.org/10.1016/j.tins.2014.10.002.

Tucsek, Z., Toth, P., Sosnowska, D., Gautam, T., Mitschelen, M., Koller, A., Szalai, G., Sonntag, W.E., Ungvari, Z., Csiszar, A., 2014. Obesity in aging exacerbates blood-brain barrier disruption, neuroinflammation, and oxidative stress in the mouse hippocampus: effects on expression of genes involved in beta-amyloid generation and Alzheimer's disease. J. Gerontol. – Ser. A Biol. Sci. Med. Sci. 69, 1212–1226, http://dx.doi.org/10.1093/gerona/glt177.

Yoshizaki, T., 2012. Autophagy in insulin resistance. Anti-Aging Med. 9, 180–184.

Publication III

Role of brain c-Jun N-terminal Kinase 2 in the control of the insulin receptor and its relationship with cognitive performance in a high-fat diet preclinical model.

Busquets O, Eritja À, López BM, Ettcheto M, Manzine PR, Castro-Torres RD, Verdaguer E, Olloquequi J, Vázquez-Carrera M, Auladell C, Folch J and Camins A.

J Neurochem. 2019;149(2):161–310.

Journal of Neurochemistry JNC

JOURNAL OF NEUROCHEMISTRY | 2019 | 149 | 255–268 doi: 10.1111/jnc.14682

ORIGINAL ARTICLE

Role of brain c-Jun N-terminal kinase 2 in the control of the insulin receptor and its relationship with cognitive performance in a high-fat diet pre-clinical model

Oriol Busquets*†‡§, Àuria Eritja*, Blanca M. López*, Miren Ettcheto*†‡§, Patricia R. Manzine*¶, Rubén D. Castro-Torres***††, Ester Verdaguer‡§**, Jordi Olloquequi‡‡, Manuel Vázquez-Carrera*§§¶¶, Carme Auladell‡§**, Jaume Folch†‡ and Antoni Camins*‡§

*Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain

†Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain

‡Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain

§Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain

¶Department of Gerontology, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil

**Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

††Departamento de Biología Celular y Molecular, C.U.C.B.A., Universidad de Guadalajara y División de Neurociencias, Guadalajara, Jalisco, Mexico

‡‡Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile

§§Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Barcelona, Spain

¶¶Institut de Recerca Sant Joan de Déu (IR-SJD), Barcelona, Spain

Received September 26, 2018; revised manuscript received December 20, 2018; accepted February 1, 2019.

Address correspondence and reprint requests to Antoni Camins, Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain. Av. Joan XXIII 27/31, E-08028 Barcelona, Spain. E-mail: camins@ub.edu

Abbreviations used: AD, Alzheimer's disease; AKT, protein kinase B; APP, amyloid precursor protein; ATF3, activation transcription factor 3; ATF4, activation transcription factor 4; BIP, 78-KDa glucose-regulated protein; Cd86, cluster of differentiation 86; CHOP, C/EBP homologous protein; CREB, cAMP-response element binding; CT, control diet; DI, discrimination ratio; EIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; GFAP, glial fibrillary acidic protein; GSK3β, glycogen synthase kinase 3β; GTT, glucose tolerance test; HFD, high-fat diet; IBA1, ionized calcium-binding adapter molecule 1; IF, immunofluorescence; IR, insulin receptor; ITT, insulin tolerance test; *Jnk2*$^{-/-}$, JNK2 knock-out animals; Cd206, cluster of differentiation 206; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; NFκB, nuclear factor kappa light chain of activated b cells; NORT, novel object recognition test; P-AKT, phospho-protein kinase B; P-ATF2, phospho-activation transcription factor 2; P-CREB, phospho-cAMP response element binding; P-EIF2α, phospho-eukaryotic initiation factor 2α; PERK, protein kinase R-like endoplasmic reticulum kinases; PFA, paraformaldehyde; P-GSK3β, phospho-glycogen synthase kinase 3β; P-IR, phospho-insulin receptor; P-PERK, phospho-protein kinase R-like endoplasmic reticulum kinases; PSD95, post-synaptic density protein 95; PTP1B, protein tyrosine phosphatase 1b; RRID, research resource identifiers; RT-PCR, real-time–polymerase chain reaction; T2DM, type 2 diabetes mellitus; T3D, type 3 diabetes; WT, wild type; XBP1, X-box protein 1.

Abstract

Insulin resistance has negative consequences on the physiological functioning of the nervous system. The appearance of type 3 diabetes in the brain leads to the development of the sporadic form of Alzheimer's disease. The c-Jun N-terminal kinases (JNK), a subfamily of the Mitogen Activated Protein Kinases, are enzymes composed by three different isoforms with differential modulatory activity against the insulin receptor (IR) and its substrate. This research focused on understanding the regulatory role of JNK2 on the IR, as well as study the effect of a high-fat diet (HFD) in the brain. Our observations determined how JNK2 ablation did not induce compensatory responses in the expression of the other isoforms but led to an increase in JNKs total activity. HFD-fed animals also showed an increased activity profile of the JNKs. These animals also displayed endoplasmic reticulum stress and up-regulation of the protein tyrosine phosphatase 1B (PTP1B) and the suppressor of cytokine signalling 3 protein. Consequently, a reduction in insulin sensitivity was detected and it is correlated with a decrease on the signalling of the IR. Moreover, cognitive impairment was observed in all groups but only wild-type genotype animals fed with HFD showed neuroinflammatory responses. In conclusion, HFD and JNK2 absence cause alterations in normal cognitive activity by altering the signalling of the IR. These affectations are related to the appearance of endoplasmic reticulum stress and an increase in the levels of inhibitory proteins like PTP1B and suppressor of cytokine signalling 3 protein.

Keywords: ER stress, high-fat diet, JNK, metabolism, neuroinflammation, PTP1B.

J. Neurochem. (2019) **149**, 255–268.

Cover Image for this issue: doi: 10.1111/jnc.14502.

In the last few decades, the prevalence of obesity has maintained a growing trend all over the world (Abarca-Gómez *et al.* 2017). The dramatic increase in the number of individuals suffering this condition is the consequence of an easy availability to calorie-dense foods, sedentary lifestyles and a combination of genetic predisposition and learnt behaviour. In addition, inappropriate dietary upbringing and unhealthy childcare of infants is causing for early-on appearance of obesity which, over the years, will have further complications as they age. Multiple data have demonstrated that obesity constitutes an important risk factor for the appearance of a number of chronic conditions including hypertension, heart disease, stroke, certain cancers and other pathologies like type 2 diabetes mellitus (T2DM) (Bray 2004).

T2DM is associated with alterations in glucose homeostasis as a result of insulin resistance (Kahn 1978). The insulin receptor (IR) is a transmembrane receptor that is found ubiquitously in peripheral and central tissues and has major roles in cellular function (De Meyts 2016). Several years ago, the Rotterdam Study reported evidence on a relationship between T2DM and the risk of developing dementia (Ott *et al.* 1999). Specifically, pre-clinical research data have revealed associations between IR activity and neuroplasticity, as well as modulation of learning and memory processes (Cheng *et al.* 2010; Liu *et al.* 2015). This link has been studied by different research groups and the concept of a brain-specific insulin resistance has been hypothesized (Henneberg and Hoyer 1995; de la Monte 2012). This condition has been labelled as type 3 diabetes and it is believed to be the progression towards the sporadic form of Alzheimer's disease (AD) (de la Monte and Wands 2008; Mittal *et al.* 2016).

High-fat diet (HFD) pre-clinical models have been established as an approach to reproduce the consequences of obesity on the organism while affecting both peripheral and central systems (Kothari *et al.* 2017). It has been reported that HFD reduces the activity of the IR, increases endoplasmic reticulum (ER) stress as well as up-regulates neuroinflammatory responses and impairs cognitive function leading to the development of AD-like pathologies (Correia *et al.* 2012; De Felice and Ferreira 2014). Published evidence from our research group and others have demonstrated how HFD leads to increases in gene expression and protein levels of the amyloid precursor protein and its product both on its soluble and insoluble deposited states (Nuzzo *et al.* 2015; Ettcheto *et al.* 2016; Busquets *et al.* 2017).

The c-Jun N-terminal kinases (JNK), a subfamily of the MAPK, are modulators of many cellular pathways (Johnson and Nakamura 2007; Solinas and Becattini, 2017). These proteins are expressed from three genes (*Mapk8*, *Mapk9* and *Mapk10*) that turn up into ten variant protein products, which are grouped within three different isoforms (JNK1, JNK2 and JNK3). Interestingly, these isoforms have been described to be heterogeneously distributed throughout the body and tissues, as well as characterized for their different functions (Haeusgen *et al.* 2009; Pal *et al.* 2016). Several studies have reported their presence in the brain (Carboni *et al.* 1998; Brecht *et al.* 2005).

The JNKs have been described to be activated in obesity situations, promoting the inhibition of the IR and favouring inflammatory responses (Hirosumi *et al.* 2002). Interestingly though, it seems that the isoforms have divergent effects. When studying different isoform-specific knock-out mice it was seen that *Jnk1*$^{-/-}$ mice were leaner than the control and, when they were fed with HFD, the ablation protected them

from developing impaired glucose tolerance and insulin resistance (Hirosumi *et al.* 2002). *Jnk2*$^{-/-}$ animals are the least studied models and thus very little information can be provided. Finally, *Jnk3*$^{-/-}$ mice showed severe increases in body weight when fed with HFD. Body weight values were well over those observed in wild-type (WT) mouse (Vernia *et al.* 2016).

In addition to these data, the JNKs are also regulators of cellular homeostasis and controllers of intracellular stresses from the ER, which is also affected by exposition to a HFD. Lastly, these kinases control some essential elements for proper cognitive performance like the cytoskeleton (Sánchez *et al.* 2000; Bjorkblom 2005), playing important roles in the definition of dendritic and neurite architecture and morphogenesis. They also play specific roles in synaptic plasticity and neurogenesis in the brain (Zdrojewska and Coffey 2014).

Consequently, the main purpose of this study was to evaluate the role of the JNK2 isoform and HFD on the development of altered conditions in the brain. We aimed to understand better how it affects IR signalling and ER stress while observing its consequences on cognitive performance and neuroinflammation.

Materials and methods

Animals and diet

Male C57BL/6J WT (research resource identifiers: MGI:5657312) and knock-out transgenic mice for the *Mapk9* gene that codifies for the JNK2 protein (*Jnk2* $^{-/-}$) were used. Transgenic animals were obtained and characterized following the method described by Dong *et al.* (1998). In all cases, animals were obtained from established breeding couples in the animal facility (Animal facility from the Pharmacy and Food Sciences Faculty from the University of Barcelona; approval number C-0032). Study was not pre-registered.

Right after the weaning and throughout their growth, animals were fed control (CT) diet or palmitic acid-enriched HFD (45% fat content, Research Diets, Inc.; product D12451; Table S1). Animals were allocated by using consecutive litters. Specifically, each litter was fully assigned to an experimental group until enough animals had been obtained for the study: WT CT [n = 15; after excluding two animals during the novel object recognition test (NORT) (see later)], WT HFD (n = 12; after excluding four animals during the NORT), *Jnk2* $^{-/-}$ CT (n = 15; after excluding three animals during the NORT) and *Jnk2* $^{-/-}$ HFD (n = 17; after excluding one animal during the NORT). The number of mice per group differed because in each litter there was a different numbers of animals born and only males were used for the study. Litters were born one after the other in the different breeding pairs of WT and *Jnk2*$^{-/-}$ animal genetic lines. Animals were number 1–69 and identified by using ear markings. Animals were housed in boxes of no less than two animals per cage and no more than four. No previous sample size calculations were performed. Although unlikely, it cannot be discarded that littermate pooling might create differences in the general data sets owing to the purity of the homozygous background of the breeding pairs. Animals were grown until 9 months of age and underwent monthly weight controls.

The animals were kept under stable conditions of temperature and humidity, 12 h light/dark cycles and food and water *ad libitum* (Animal facility from the Pharmacy and Food Sciences Faculty from the University of Barcelona). Animals were treated at all times under the ethic directions defined by the European Committee (European Communities Council Directive 2010/63/EU) and the manipulation protocols were previously approved by the ethic committee from the University of Barcelona. It was made sure that animal numbers, their stress and pain were kept under a necessary minimum following the appropriate animal manipulation ethic methodologies. An experimental design flowchart has been depicted in Fig. 1.

Glucose tolerance test – insulin tolerance test

Previous to the beginning of the test, animals were fasted for 6 h. In both tests, animals were injected in the intraperitoneal cavity. In glucose tolerance test (GTT) mice were administered a glucose dosage of 1 g/kg, whereas in insulin tolerance test (ITT), insulin was administered at a dosage of 0.75 IU/kg. Next, blood samples were analysed from the tail vein in consecutive time periods. For GTT, samples were extracted at 5, 15, 30, 60, 120 and 180 min after the administration of glucose, in ITT the measurements were made 15, 30, 45, 60 and 90 min after insulin injection. Animals were continuously observed and monitored and, in those cases in which blood glucose concentrations dropped under a concentration of 20 mg/dL, animals were administered a glucose dosage of 1 g/kg and kept under observation until glucose blood levels stabilized and normal behaviour was observed.

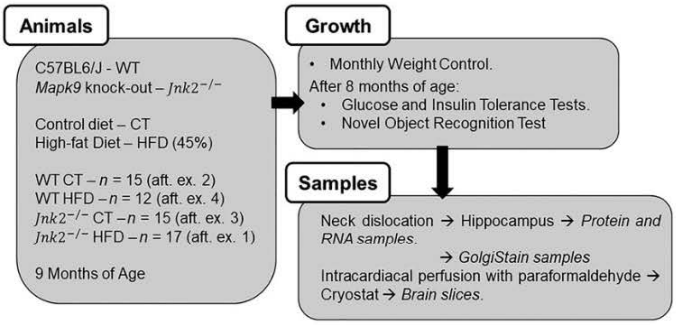


Fig. 1 Flowchart schematic describing the experimental design of the study. n = number of animals. aft. ex. = after excluding during the NORT.

Novel object recognition test (NORT)

In the NORT test, animals were evaluated in a room with an open field box (50 × 50 × 20 cm) surrounded by black curtains and constant controlled illumination. The animals were placed in the open field area 3 consecutive days for 10 min in order to habituate them to the space. The amount of time spent in the central area of the field was registered. In the 4th day, two identical objects were placed in the open field space. Exploration time for each of the objects was evaluated over a total of 10 min. Animals that showed significant preference for one of the objects over the other were excluded from posterior analysis. Significant preference was defined after statistical comparison of observation time for each object. A total of 10 animals were excluded from all experimental groups. In the 5th day, one of the objects was replaced for a new one and the same observation and data acquiring were made.

All spaces and objects were properly cleaned and prepared between animals in order to eliminate odour cues. All objects were chosen randomly for the test in order to eliminate the variability associated with colours and shapes preference. Simple randomization, the objects were blindly chosen from a box and assigned for each test. All data were obtained from recordings using the program Smart 3.0 (Panlab, Barcelona, Spain). Quantifications of exploration time were determined through a ratio: Discrimination ratio = (time spent exploring the new object − time spent exploring the known object)/total exploration time.

Golgi stain

Golgi stain procedures were done following the direction of the kit purchased from FD Neurotechnologies, Inc. (FD Rapid GolgiStain™ Kit; Cat #PK401, Columbia, MD, USA). Images were obtained from a BX61 Laboratory Microscope (Olympus America Inc., Melville, NY, USA).

Dendritic spine numbers were quantified by selecting granular neurons in the dentate gyrus (DG) of the hippocampus. Measurement was done at least 50 µm from the soma along consecutive 10 µm fragments on secondary branches starting 10 µm after branching from the primary dendrite. Spine density was calculated by dividing the number of spines per segment and was expressed as the number of spines per 10 µm of dendrite. Three animals were chosen from each experimental group by taking animals from different litters. It allowed to correct for any biases developed caused by factors like pregnancy conditions or number of siblings as it has been described to affect cognitive development. From each animal at least five neurons were checked. Neurons were chosen from those that showed clear staining. No neurons were included in the analyses if their dendritic arborization crossed paths with those nearby. Spine density value for each neuron was the result of the mean of five consecutive measurements in the same secondary branch. Quantifications were performed by a blinded experimenter.

Immunoblot analysis

Fresh brains were extracted right after killing (neck dislocation). Hippocampus area was dissected and kept frozen at −80°C until use. Samples were cryohomogenized using liquid nitrogen. Proteins were extracted using a lysis buffer (Tris HCl 1 M pH 7.4, NaCl 5 M, EDTA 0.5M pH 8, Triton, distilled H_2O) containing a protease (Complete Mini, EDTA-free; Protease Inhibitor cocktail tablets, 11836170001; Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitor cocktail (Phosphatase Inhibitor Cocktail 3, P0044; Sigma-Aldrich, St. Louis, MO, USA). Samples concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Waltham, MA, USA) and 10 µg was used for each assay.

Immunoblot procedure was already described in Busquets *et al.* (2018);. In brief, for electrophoresis acrylamide gels of 10–12% concentration were used and run at 100 V. Transference was performed through standard wet transference at constant 200 mA for 100 min or semi-dry at 2.5 A for 20 min. Primary and Secondary antibodies are described in Table S2.

Results were obtained from chemoluminescence detection using the Pierce® enhanced chemiluminescence Western Blotting Substrate (#32106; Thermo Scientific), a Bio-Rad Universal Hood II Molecular Imager and the Image Lab v5.2.1 software (Bio-Rad Laboratories, Hercules, CA, USA). Measurements were expressed in arbitrary units and all results were normalized with the corresponding loading control (glyceraldehyde-3-phosphate dehydrogenase; GAPDH).

Kinase activity assay

The P-JNK enzyme was immunoprecipitated from hippocampal protein samples. Assay preparation and procedure were previously described in Busquets *et al.* (2018).

Immunofluorescence

Animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After making sure the animals were in the no-pain sleep phase they were intracardiacally perfused with 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate buffer. After perfusion, brains were removed and stored in PFA overnight at 4°C. The next day the solution was changed into PFA + 30% sucrose. Coronal sections of 20 µm were obtained by a cryostat (Leica Microsystems, Wetzlar, Germany), kept in a cryoprotectant solution and −20°C in the freezer.

Immunofluorescence (IF) protocol has already been described in previous publications (Busquets *et al.* 2017, 2018). Information regarding the antibodies used in IFs can be found in Table S3.

Image acquisition was performed with an epifluorescence microscope (Olympus BX61 Laboratory Microscope; Olympus America Inc.). Fluorescence intensity was quantified as Corrected Total Cell Fluorescence Value as described in Busquets *et al.* (2017).

Real-time–polymerase chain reaction

For RNA extraction, samples were added TRIsure™ (BIO-38033; Bio line GmbH, London, UK), homogenized using a vortex and left to rest for several minutes. They were centrifuged for 5 min at 12 000 *g* and 4°C. Supernatant was transferred into new tubes and chloroform was added. After another centrifuge, phases were separated and the superior layer was collected into a new tube. Later, isopropanol was added and samples were left to rest in ice. After at least 10 min, they were centrifuged again for 10 min at 14 000 *g* and 4°C. Next, 70% ethanol was added and samples were centrifuged for 5 min at 7500 *g* and 4°C. Lastly, supernatant was removed, pellet was left to dry and it was dissolved in DEPC H_2O. RNA extraction products were kept at −80°C until use. RNA concentration and integrity were assessed using a NanoDrop (Thermo Scientific).

For reverse transcription, 2 µg of RNA was used following the protocols described by the High Capacity cDNA Reverse Transcription Kit (4368813; Applied Biosystems, Foster City, CA,

USA). cDNA samples were used in equivalent quantities and each was analysed in triplicate for each gene. Primer sequences can be found in Table S4. SyBr Green reagent was used coupled with ROX (Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X); K0253; Thermo Scientific) on a Step One Plus RT-PCR system (Life Technologies, Grand Island, NY, USA). RT-PCR cycle parameters are as follows: stage 1: 95°C 10 min; stage 2: 95°C 10 s, 60°C 30 s and 70°C 1 min (40 cycles; melting curve: 95°C 15 s, 60°C 60 s and 95°C 15 s).

All results were normalized with *Gapdh* as housekeeping gene. The WT CT experimental group was used as the calibrator to evaluate gene expression variations. Measurements were calculated through the double-delta Ct method and expressed in arbitrary units.

Xbp1 splicing detection

The qualitative evaluation of the spliced state of X-box protein 1 (XBP1) was examined by gel electrophoresis after a PCR amplification (Forward Primer: TGAGAACCAGGAGTTAAGAACAC; Reverse Primer: TTCTGGGTAGACCTCTGGGAGTTCC). The methodology has been previously described by Guo *et al.* (2007). PCR cycle parameters: stage 1: 94°C 2 min; stage 2: 94°C 1 min, 62°C 1 min and 72°C 1 min (34 cycles) and stage 3: 72°C 5 min.

Statistical analysis

Data were presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. All four experimental groups were compared through two-way ANOVA. Tukey's was performed ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Researchers were blinded to treatment when data sets were analysed. All analyses and graph representations were performed in the program Graph Pad Prism for Windows version 6.01; GraphPad Software Inc., San Diego, CA, USA. Data were previously checked for normality (D'Agostino–Pearson normality test) and outliers (Grubb's test). In all figures, only relevant significant comparisons were shown: WT CT versus WT HFD, WT CT versus *Jnk2*$^{-/-}$ CT, WT CT versus Jnk2$^{-/-}$ HFD, *Jnk2*$^{-/-}$ CT versus *Jnk2*$^{-/-}$ HFD and WT HFD versus *Jnk2*$^{-/-}$ HFD.

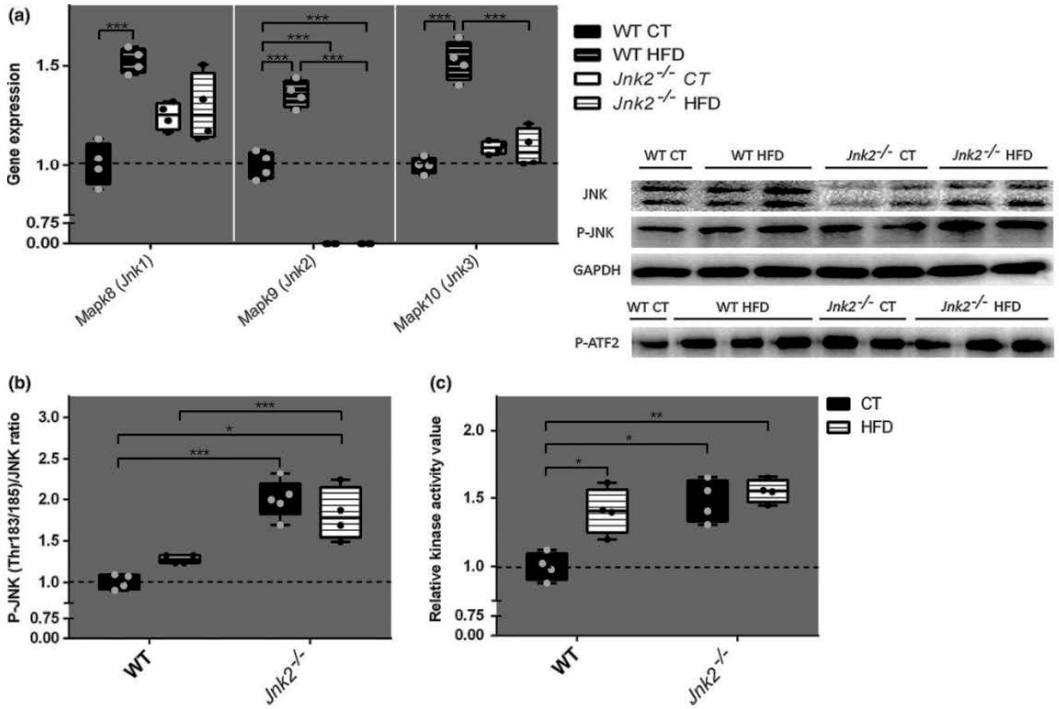


Fig. 2 Analysing the c-Jun N-terminal kinases (JNKs). (a) RT-PCR quantification for the *Mapk8*, *Mapk9* and *Mapk10* genes expression. Multiple comparison: *Mapk8* [*** WT control diet (CT) vs. WT high-fat diet (HFD)], *Mapk9* (*** WT CT vs. WT HFD, *** WT CT vs. *Jnk2*$^{-/-}$ CT and *** WT HFD vs. *Jnk2*$^{-/-}$ HFD) and *Mapk10* (** WT CT vs. WT HFD and ** WT HFD vs. *Jnk2*$^{-/-}$ HFD). (b) Immunoblot evaluation of hippocampal total and phosphorylated JNK ratio (Thr183/185). Multiple comparison: *** WT CT versus *Jnk2*$^{-/-}$ CT, *** WT CT versus *Jnk2*$^{-/-}$ HFD and * WT HFD versus *Jnk2*$^{-/-}$ HFD. (c) Quantification of relative kinase activity value for the P-ATF2 (Thr71) protein. Multiple comparison: * WT CT versus WT HFD, * WT CT versus *Jnk2*$^{-/-}$ CT and ** WT CT versus *Jnk2*$^{-/-}$ HFD. $n \geq 4$ (n = number of animals). Data were presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Results

Mapk9 knock-down and HFD feeding increase activity rates of JNK in the hippocampus

Gene expression for *Mapk8*, *Mapk9* and *Mapk10* was quantified through RT-PCR (Fig. 2a). WT HFD experimental group showed significant up-regulation on the expression of all genes when compared to WT CT ($p < 0.001$ for *Mapk8* and *Mapk9*; $p < 0.01$ for *Mapk10*). *Jnk2*$^{-/-}$ experimental groups had no expression of the *Mapk9* gene ($p < 0.001$).

Evaluation of the ratio between phosphorylated (Thr183/185) and total JNK protein levels determined significant increased values: $p < 0.001$ WT CT versus *Jnk2*$^{-/-}$ CT, $p < 0.001$ WT CT versus *Jnk2*$^{-/-}$ HFD and $p < 0.05$ WT HFD versus *Jnk2*$^{-/-}$ HFD (Fig. 2b). Similar results were observed when performing an activity assay for this same enzyme (P-JNK Thr183/185): $p < 0.05$ WT CT versus WT HFD, $p < 0.05$ WT CT versus *Jnk2*$^{-/-}$ CT and $p < 0.01$ WT CT versus *Jnk2*$^{-/-}$ HFD (Fig. 2c).

Alteration in physiological metabolic parameters

Several measurements were performed on the animals during their growth (Fig. 3). First, body weight was controlled monthly (Fig. 3a). HFD groups showed higher body weight values at 9 months of age ($p < 0.001$ WT CT vs. WT HFD and $p < 0.05$ *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD). Also, JNK2 ablation led to increased body weight: $p < 0.05$ WT CT versus *Jnk2*$^{-/-}$ CT and $p < 0.001$ WT CT versus *Jnk2*$^{-/-}$ HFD. Additional data were gathered with the GTT and ITT tests. Time-related variation in blood glucose concentration was determined by comparing area under the curve values between experimental groups extrapolated from the curves of response throughout the tests. In the GTT, all experimental groups showed significant increases when compared against the control: WT HFD ($p < 0.05$), *Jnk2*$^{-/-}$ CT ($p < 0.05$) and

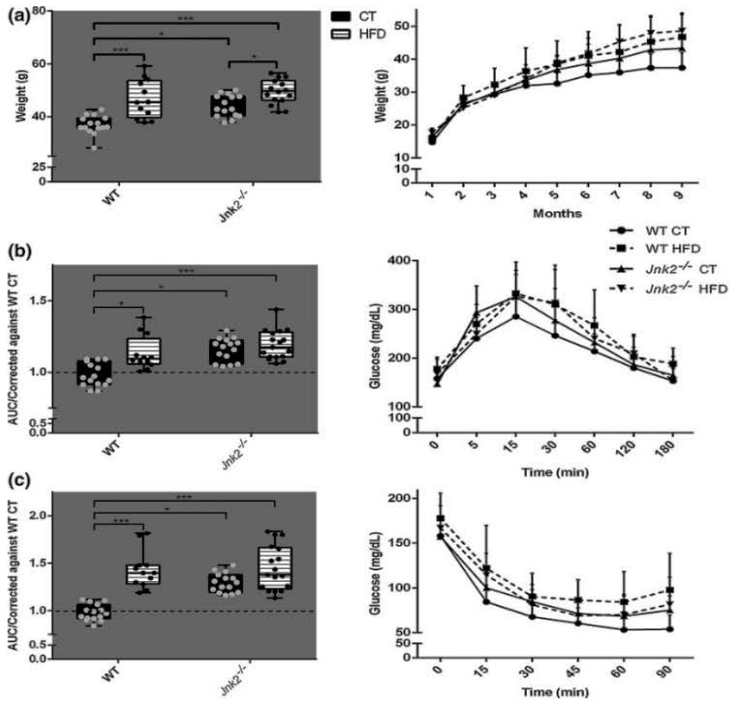


Fig. 3 Evaluating metabolic parameters. (a) Analysis and representation of the increase in animal body weight in the 9-month period of the study. Multiple comparison: *** WT control diet (CT) versus WT high-fat diet (HFD), * WT CT versus *Jnk2*$^{-/-}$ CT, *** WT CT versus *Jnk2*$^{-/-}$ HFD and * *Jnk2*$^{-/-}$ CT versus *Jnk2*$^{-/-}$ HFD. (b) Extrapolation of GTT results by determination of area under the curve values and posterior normalization to control group. Multiple comparison: * WT CT versus WT HFD, * WT CT versus *Jnk2*$^{-/-}$ CT and *** WT CT versus *Jnk2*$^{-/-}$ HFD. (c) Extrapolation of insulin tolerance test results by determination of area under the curve values and posterior normalization to control group. Multiple comparison: *** WT CT versus WT HFD, * WT CT versus *Jnk2*$^{-/-}$ CT and *** WT CT versus *Jnk2*$^{-/-}$ HFD. $n \geq 12$ (n = number of animals). Data were presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

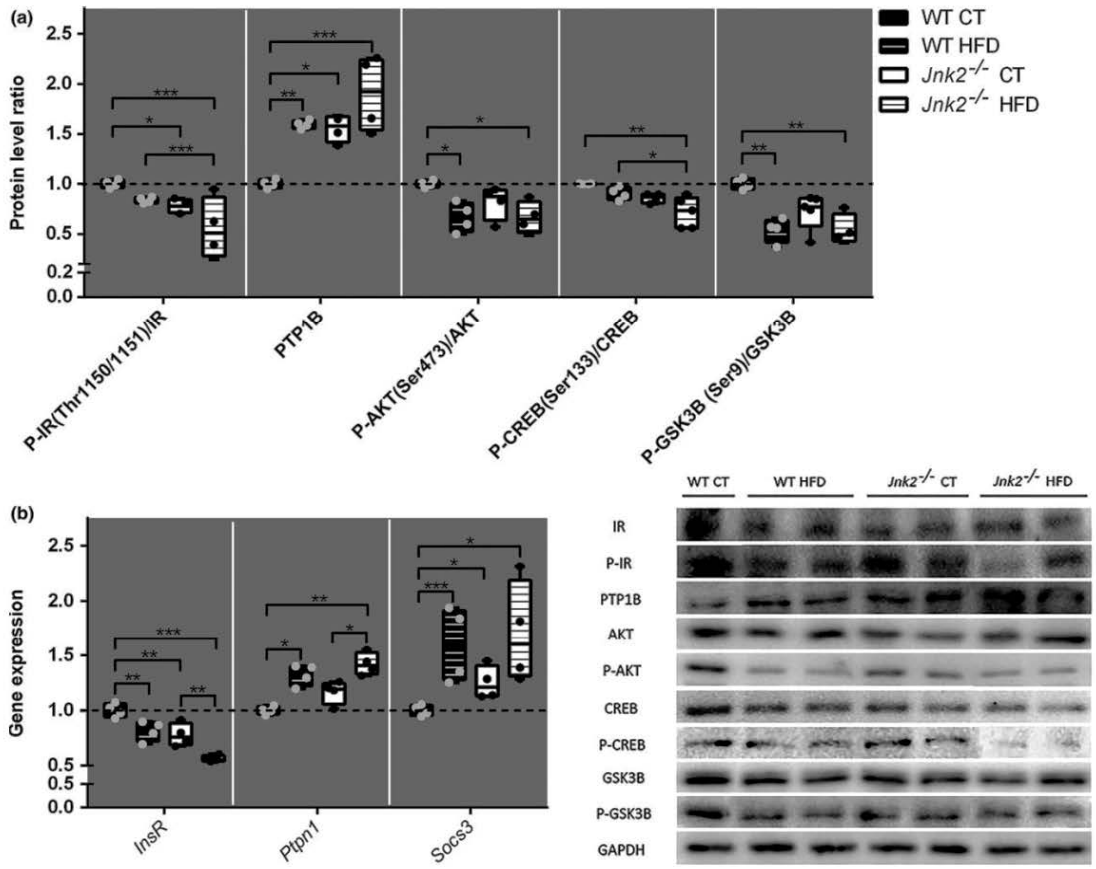


Fig. 4 Study of the IR signalling pathway. (a) Immunoblot detection of insulin receptor (IR) and related signalling proteins: P-IR (Thr1150/1151)/IR ratio [* WT control diet (CT) vs. *Jnk2*$^{-/-}$ CT, *** WT CT vs. *Jnk2*$^{-/-}$ high-fat diet (HFD) and *** *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD)], PTP1B (** WT CT vs. WT HFD, * WT CT vs. *Jnk2*$^{-/-}$ CT and *** WT CT vs. *Jnk2*$^{-/-}$ HFD), phospho-protein kinase B (P-AKT) (Ser473)/protein kinase B (AKT) ratio (* WT CT vs. WT HFD and * WT CT vs. *Jnk2*$^{-/-}$ HFD), phospho-cAMP response element binding (P-CREB) (Ser133)/CREB ratio (** WT CT vs. *Jnk2*$^{-/-}$ HFD and * WT HFD vs. *Jnk2*$^{-/-}$ HFD), P-GSK3B (Ser9)/GSK3B ratio (** WT CT vs. WT HFD and ** WT CT vs. *Jnk2*$^{-/-}$ HFD). (b) Quantification of gene expression variations: *InsR* (** WT CT vs. WT HFD, ** WT CT vs. *Jnk2*$^{-/-}$ CT, *** WT CT vs. *Jnk2*$^{-/-}$ HFD and ** *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD), *Ptpn1* (* WT CT vs. WT HFD, ** WT CT vs. *Jnk2*$^{-/-}$ HFD and * *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD) and *Socs3* (*** WT CT vs. WT HFD, * WT CT vs. *Jnk2*$^{-/-}$ CT and * WT CT vs. *Jnk2*$^{-/-}$ HFD). $n \geq 4$ (n = number of animals). Data were presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (*$p < 0.05$).

Jnk2$^{-/-}$ HFD ($p < 0.001$) (Fig. 3b). Similarly, ITT assays showed similar responses ($p < 0.001$ WT CT vs. WT HFD, $p < 0.05$ WT CT vs. *Jnk2*$^{-/-}$ CT and $p < 0.001$ WT CT vs. *Jnk2*$^{-/-}$ HFD) (Fig. 3c).

Next, hippocampal proteins of the IR signalling pathway were detected. The calculation of the ratio between total and phosphorylated protein determined significant reductions as a result of the lack of JNK2, as well as for the effects of HFD ($p < 0.05$ WT CT vs. *Jnk2*$^{-/-}$ CT). Additional response elements to the signalling of the IR were examined. In all cases there was a reduction on their phosphorylated ratios: phospho-protein kinase B (Ser473)/protein kinase B ($p < 0.05$ WT CT vs. WT HFD and WT CT vs. *Jnk2*$^{-/-}$ HFD), phospho-cAMP response element binding (Ser133)/cAMP response element binding ($p < 0.01$ WT CT vs. WT HFD and $p < 0.05$ WT HFD vs. *Jnk2*$^{-/-}$ HFD) and P-GSK3B (Ser9)/GSK3B ($p < 0.01$ WT CT vs. WT HFD and WT CT vs. *Jnk2*$^{-/-}$ HFD) (Fig. 4a). Also, protein tyrosine

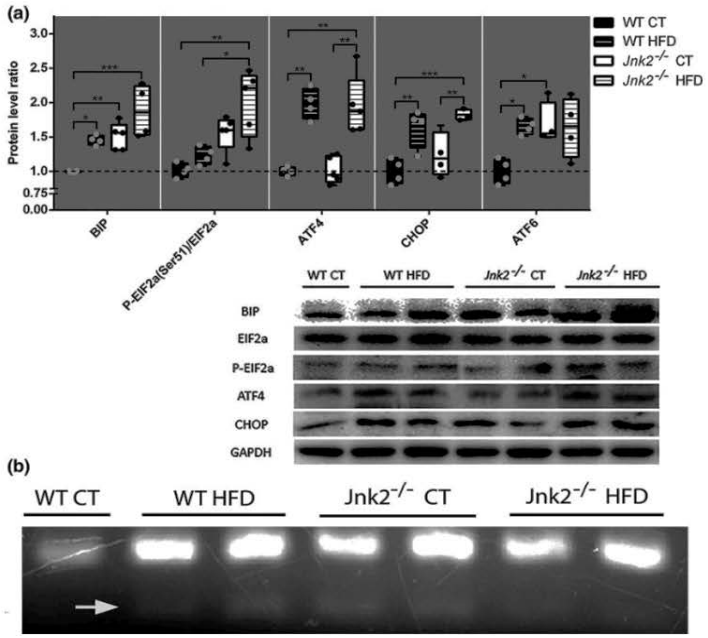


Fig. 5 Endoplasmic reticulum (ER) stress status evaluation. (a) Detection of ER stress-related proteins: 78-KDa glucose-regulated protein [* WT control diet (CT) vs. WT high-fat diet (HFD), ** WT CT vs. *Jnk2*$^{-/-}$ CT and *** WT CT vs. *Jnk2*$^{-/-}$ HFD], P-EIF2a (Ser51)/EIF2a ratio (** WT CT vs. *Jnk2*$^{-/-}$ HFD and * WT HFD vs. *Jnk2*$^{-/-}$ HFD), ATF4 (** WT CT vs. WT HFD, ** WT CT vs. *Jnk2*$^{-/-}$ HFD and ** *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD), C/EBP homologous protein (CHOP) (** WT CT vs. WT HFD, *** WT CT vs. *Jnk2*$^{-/-}$ HFD and ** *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD) and ATF6 (* WT CT vs. WT HFD and * WT CT vs. *Jnk2*$^{-/-}$ HFD). (b) Qualitative evaluation of ER stress through XBP1 mRNA splicing. In WT HFD, *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ HFD experimental groups, the presence of a smaller band in the lower part of the gel would indicate the presence of a spliced version of XBP1 mRNA, one of the mechanisms of signalling of stress in the ER. $n \geq 4$ (n = number of animals). Data were presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (*$p < 0.05$, **$p < 0.01$).

phosphatase 1b (PTP1B) presented noticeable increases in its protein levels in all experimental groups versus the control ($p < 0.01$ WT CT vs. WT HFD, $p < 0.05$ WT CT vs. *Jnk2*$^{-/-}$ CT and $p < 0.001$ WT CT vs. *Jnk2*$^{-/-}$ HFD).

Finally, quantification of gene expression variations was performed. *InsR* showed decreased values in all experimental groups versus the control ($p < 0.01$ WT CT vs. WT HFD, WT CT vs. *Jnk2*$^{-/-}$ CT and WT HFD and *Jnk2*$^{-/-}$ HFD; $p < 0.01$ WT CT vs. *Jnk2*$^{-/-}$ HFD) while *Ptpn1* and *Socs3* showed increases (Fig. 4b): *Ptpn1* ($p < 0.05$ WT CT vs. WT HFD and *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD; $p < 0.01$ WT CT vs. *Jnk2*$^{-/-}$ HFD) and *Socs3* ($p < 0.001$ WT CT vs. WT HFD, $p < 0.05$ WT CT vs. *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ HFD).

Evaluation of HFD-induced ER stress

Owing to the importance of proper ER function in the memory process, levels of several proteins of the ER were assessed (Fig. 5a): 78-KDa glucose-regulated protein ($p < 0.05$ WT CT vs. WT HFD, $p < 0.01$ WT CT vs. *Jnk2*$^{-/-}$ CT and $p < 0.001$ WT CT vs. *Jnk2*$^{-/-}$ HFD), P-EIF2a (Ser51)/EIF2a ($p < 0.01$ WT CT vs. *Jnk2*$^{-/-}$ HFD and $p < 0.05$ WT HFD vs. *Jnk2*$^{-/-}$ HFD), activation transcription factor 4 (ATF4) ($p < 0.01$ WT CT vs. WT HFD, *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD and WT CT vs. *Jnk2*$^{-/-}$ HFD), C/EBP homologous protein ($p < 0.01$ WT CT vs. WT HFD and *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD; $p < 0.001$ WT CT vs. *Jnk2*$^{-/-}$ HFD) and ATF6 ($p < 0.05$ WT CT vs. WT HFD and *Jnk2*$^{-/-}$ CT).

Complementarily, a qualitative evaluation of the splicing of the XBP1 mRNA showed how all the experimental groups but the WT CT showed a spliced product of lower molecular weight (in the gel it can be observed as a faint band at the lower part of the image (arrow) (Fig. 5b).

Determination of cognitive decline through NORT, quantification of dendritic spines and involved biomarkers

When the animals reached the 8 months of age, they were tested for memory and learning capabilities through the

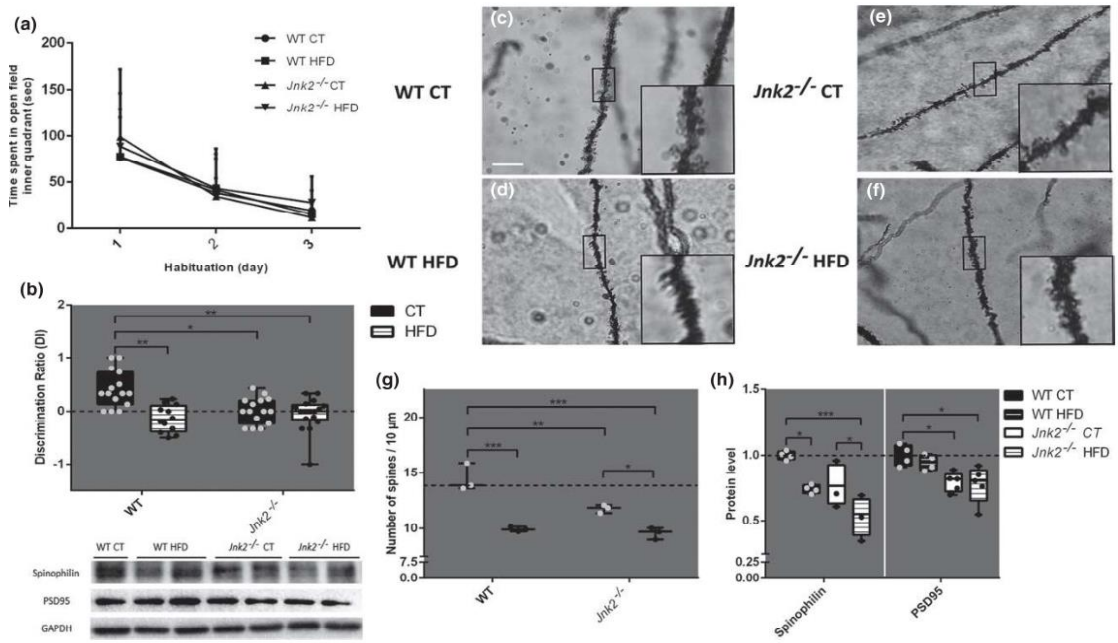


Fig. 6 Behavioural assessment of cognitive performance and related biomarkers. (a) Novel object recognition test (NORT) open field habituation. Time spent in inner quadrant (seconds; sec). (b) Results for the quantification of the discrimination ratio (DI). Multiple comparison: ** WT control diet (CT) versus WT high-fat diet (HFD), * WT CT versus *Jnk2*$^{-/-}$ CT and ** WT CT versus *Jnk2*$^{-/-}$ HFD. $n \geq 12$ (n = number of animals). (c–f) Optical microscope images of brain GolgiStain. Scale bar: 10 μm. (g) Quantification of dendritic spines for each 10 μm. Multiple comparison: *** WT CT versus WT HFD, ** WT CT versus *Jnk2*$^{-/-}$ CT, *** WT CT versus *Jnk2*$^{-/-}$ HFD and * *Jnk2*$^{-/-}$ CT versus *Jnk2*$^{-/-}$ HFD. $n = 3$ (n = number of animals). From each animal at least five neurons were checked. Spine density value for each neuron was the result of the mean of five consecutive measurements in the same secondary branch. (h) Immunoblot detection of Spinophilin (* WT CT vs. WT HFD, *** WT CT vs. *Jnk2*$^{-/-}$ HFD and *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD) and PSD95 (* WT CT vs. *Jnk2*$^{-/-}$ CT and * WT CT vs. *Jnk2*$^{-/-}$ HFD). $n \geq 4$ (n = number of animals). Data were presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (*$p < 0.05$, **$p < 0.01$).

NORT (Fig. 6). No differences were observed between groups in the open field exploration time in the habituation period. Results showed reductions in the discrimination ratio of all groups when compared to WT CT (Fig. 6b): $p < 0.05$ for WT CT versus *Jnk2*$^{-/-}$ CT and $p < 0.01$ for WT CT versus WT HFD and *Jnk2*$^{-/-}$ HFD.

Some samples were used for Golgi Stain in order to determine the state and number of dendritic spines of pyramidal neurons in the DG of the hippocampus (Fig. 6c–f). Experimental groups that had been exposed to HFD or lacked JNK2 presented smaller and shorter dendritic spines (qualitative evaluation). Quantification of dendritic spines number determined significant differences in all experimental groups when compared with the WT CT: $p < 0.001$ WT CT versus WT HFD and *Jnk2*$^{-/-}$ HFD, $p < 0.01$ WT CT versus *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ CT versus *Jnk2*$^{-/-}$ HFD (Fig. 6g).

Protein levels for spinophilin, a protein highly present in dendritic spines, and the post-synaptic density protein 95 (PSD95) were determined through immunoblot (Fig. 6h). Spinophilin showed significant reductions between WT CT and WT HFD ($p < 0.05$), WT CT and *Jnk2*$^{-/-}$ HFD ($p < 0.001$) and *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ HFD ($p < 0.05$). Also, statistical analysis for PSD95 showed significant decrease between WT CT and both *Jnk2*$^{-/-}$ transgenic groups ($p < 0.05$).

Analysis of changes in neuroinflammatory-related cells and other biomarkers

Visualization of astroglia and microglia was performed through the detection of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (IBA1) proteins. Representative images of all four experimental groups can be found in Fig. 7(a–d) for GFAP and E-H for

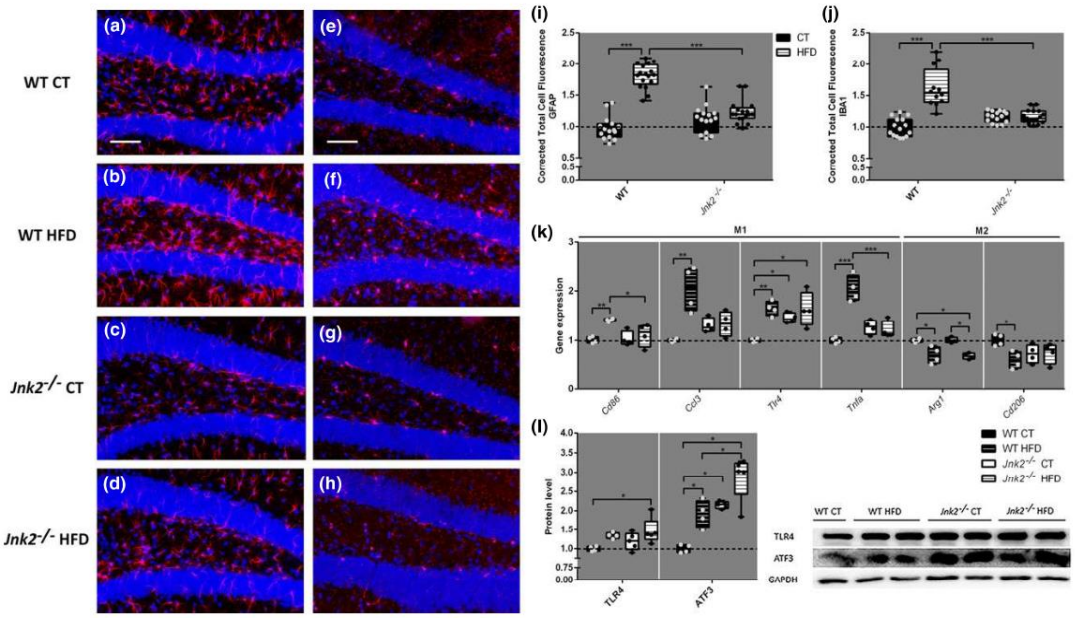


Fig. 7 Evaluation of inflammatory responses. Representative images for the detection of astrocytes (a–d) and microglia (e–h) in the DG of the hippocampus. All samples are co-stained with Hoechst for the detection of cellular nucleus (blue). Scale bar: 200 μm. Quantification of relative fluorescence was calculated as corrected total cell fluorescence for each experimental group: GFAP [i; *** WT control diet (CT) vs. WT high-fat diet (HFD) and *** WT HFD vs. *Jnk2*$^{-/-}$ HFD] and IBA1 (j; ** WT CT vs. WT HFD and *** WT HFD vs. *Jnk2*$^{-/-}$ HFD). $n \geq 15$ (n = number of biological replicates). (k) Quantification of expression variation for multiple microglia profile characterization-related genes: cluster of differentiation 86 (** WT CT vs. WT HFD and * WT CT vs. *Jnk2*$^{-/-}$ HFD), *Ccl3* (** WT CT vs. WT HFD), *Tlr4* (** WT CT vs. WT HFD, * WT CT vs. *Jnk2*$^{-/-}$ CT and * WT CT vs. *Jnk2*$^{-/-}$ HFD), *Tnfa* (*** WT CT vs. WT HFD and *** WT HFD vs. *Jnk2*$^{-/-}$ HFD), *Arg1* (* WT CT vs. WT HFD, * WT CT vs. *Jnk2*$^{-/-}$ HFD and *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD) and cluster of differentiation 206 (*Cd206*) (* WT CT vs. WT HFD). (l) Semi-quantification of protein levels for TLR4 (* WT CT vs. Jnk2$^{-/-}$ HFD) and ATF3 (* WT CT vs. WT HFD, * WT CT vs. *Jnk2*$^{-/-}$ CT, * WT CT vs. *Jnk2*$^{-/-}$ HFD and * WT HFD vs. *Jnk2*$^{-/-}$ HFD). $n \geq 4$ (n = number of animals). Data were presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (*$p < 0.05$).

IBA1. Quantification of the CTCF determined significant increases in the relative intensity fluorescence value in the WT HFD experimental group for both experiments: GFAP (Fig. 7i; $p < 0.001$ WT CT vs. WT HFD and WT HFD vs. *Jnk2*$^{-/-}$ HFD) and IBA1 (Fig. 7j; $p < 0.01$ WT CT vs. WT HFD and $p < 0.001$ WT HFD vs. *Jnk2*$^{-/-}$ HFD).

Further gene expression variation analyses allowed for the characterization of the state of M1 (pro-inflammatory) and M2 (immunosuppressant) microglia states (Fig. 7k): cluster of differentiation 86 ($p < 0.01$ WT CT vs. WT HFD and $p < 0.05$ WT HFD vs. *Jnk2*$^{-/-}$ HFD), *Ccl3* ($p < 0.01$ WT CT vs. WT HFD), *Tlr4* ($p < 0.01$ WT CT vs. WT HFD and $p < 0.05$ WT CT vs. *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ HFD), *Tnfa* ($p < 0.001$ WT CT vs. WT HFD and WT HFD vs. *Jnk2*$^{-/-}$ HFD), *Arg1* ($p < 0.05$ WT CT vs. WT HFD, WT CT vs. *Jnk2*$^{-/-}$ HFD and *Jnk2*$^{-/-}$ CT vs. *Jnk2*$^{-/-}$ HFD) and cluster of differentiation 206 ($p < 0.05$ WT CT vs. WT HFD).

Complementarily, inflammation response-related proteins were examined: TLR4 ($p < 0.05$ WT CT vs. *Jnk2*$^{-/-}$ HFD) and ATF3 ($p < 0.05$ WT CT vs. WT HFD, *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ HFD; $p < 0.05$ WT HFD vs. *Jnk2*$^{-/-}$ HFD) (Fig. 7l).

Discussion

Previous publications have described how obesity increases the activity of the JNKs (Ip and Davis 1998; Hirosumi *et al.* 2002). This increase causes pro-inflammatory and pro-apoptotic responses, as well as reductions in insulin sensitivity (Nguyen *et al.* 2005; Solinas *et al.* 2006). However, in most cases, this activity is evaluated as a whole and not in an isoform-specific manner. Also, their role in the hippocampus has never been studied attentively. Considering their prominent control on synaptic plasticity (Sánchez *et al.* 2000;

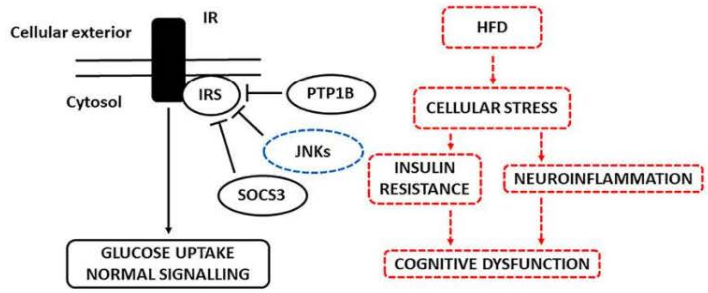


Fig. 8 Final schematic for the mechanisms involved in this study.

Zdrojewska and Coffey 2014), our research group considers them key targets for the treatment of neurodegenerative pathologies in the brain. In this study, their regulation of the IR and its consequences on cognition were evaluated in a model of JNK2 ablation and HFD feeding.

Many authors have confirmed the presence of all JNK isoforms in the brain, including in the hippocampus (Carboni *et al.* 1998; Brecht *et al.* 2005; Coffey 2014). Reinecke *et al.* (2013) reported that the lack of one of the isoforms caused for compensatory changes in the expression of the others. However, no differences were observed in the *Jnk2*$^{-/-}$ experimental groups when compared to the control. Interestingly, when evaluating the ratio between the phosphorylated state of P-JNK and total protein, a significant increase was detected. Similar results were observed when running a kinase activity assay. Thus, compensatory mechanisms seem to exist between the isoforms; independent of gene expression but linked to their activity.

Published data have described the effects of specific knockouts of JNK1 and JNK3 on the control of body weight (Hirosumi *et al.* 2002; Vernia *et al.* 2016) but there is controversy on the effect of JNK2. In our case, analysis of body weight values detected increases in those animals that had been knocked out for the *Mapk9* gene. Also, long-term feeding of HFD caused body weight gain. The increased activity of the JNKs in all experimental groups might be the reason for this variation; favouring IR inhibition and higher formation of adipose tissue reserves (Morton *et al.* 2006). This hypothesis would be supported by our results demonstrating a reduced sensitivity to insulin through the GTT and ITT tests. Furthermore, alterations on IR functioning would have consequences on cognitive performance. Grillo and coworkers reported how when administering lentiviral virus that contained antisense sequences for the brain IR, cognitive capabilities were affected (Grillo *et al.* 2015). Analysis of the PTP1B, Suppressor of Cytokine Signalling 3 protein (SOCS3) and other IR signalling downstream proteins would back up these conclusions.

PTP1B has been postulated to be a possible target for the treatment of pathologies like T2DM (Ganou *et al.* 2018) as it is a direct inhibitor of the signalling of the IR through its phosphatase activity (Vieira *et al.* 2017). Higher levels of PTP1B would result in higher blood glucose concentration, increased food ingestion and body weight. Similarly, research on SOCS3 has shown similar effects. (Mori *et al.* 2004; Torisu *et al.* 2007). Furthermore, it is also believed that these proteins regulate other pathways like ER homeostasis, synaptic plasticity and neuroinflammation (Panzhinskiy *et al.* 2013). Finally, the JNKs are inductors of these two proteins as it has been demonstrated in models of HFD-induced obesity (Szegezdi *et al.* 2006; MohammadTaghvaei *et al.* 2012) or by using JNK inhibitors like in the research by Gao and colleagues (Gao *et al.* 2017).

In our results, all experimental groups showed increased ER stress when compared against the control group. These data were in accordance with that reported by Raciti and colleagues (Raciti *et al.* 2012), which described how JNK2 activation was required in order to maintain pro-survival mechanisms in the cell. Increased ER stress would also favour the activation of the JNKs by increasing the activation of ATF6 and the posterior splicing of the XBP1 mRNA (Salvadó *et al.* 2014). Moreover, multiple research groups have linked alterations of the ER and the appearance of alterations in synaptic plasticity and cognitive dysfunction (Özcan *et al.* 2003; Liang *et al.* 2015). It has also been demonstrated how phosphorylation of the protein EIF2α has profound effects on synaptic function and memory impairment in several pre-clinical models of neurological diseases (Ma *et al.* 2014; Trinh and Klann 2014; Lourenco *et al.* 2015).

Hence, the evidence suggests that all these mechanisms would be the cause for the subsequent cognitive decline. In our study, we evaluated the cognitive response of the animals using the NORT behavioural test. The results showed clear impairment in the animals for memory formation as a result of the HFD and the transgenesis of the *Mapk9* gene. In addition, the use of a GolgiStain in brain slices and the immunoblot of synaptic proteins like Spinophilin and PSD95 demonstrated significant decline in their values versus the control. Some researchers have linked these alterations to the reduction of proteins like the brain-derived neurotrophic factor (Molteni *et al.* 2002;

Kanoski *et al.* 2008). These results would support the evidence on the relationship between the development of insulin resistance and the appearance of reduction in cognitive performance.

Finally, inflammation has been reported to have a close relationship with the appearance of cognitive dysfunctions (Bocarsly *et al.* 2015; de Felice and Lourenco 2015). As it has been previously described, HFD caused an induction of reactive astrocytes and microglia (Busquets *et al.* 2017) but animals that have had their JNK2 ablated showed no changes in the cellular reactivity neither when being fed CT or HFD diet. Multiple biomarkers of the M1 and M2 microglia profiles were evaluated, as well as proteins like TLR4 and ATF3. The detection of increased gene expression for *Ccl3*, *Arg1*, cluster of differentiation 86 and *Tnfa* in the WT HFD experimental group would indicate an increased presence of M1 (pro-inflammatory) microglia (Zhang *et al.* 2017), but in those animals that lacked JNK2 no such increase appeared. Possibly, the lack of pro-inflammatory response in these animals would be derived on the direct control of the JNKs on the activation of glial cells or as a result of other compensatory mechanisms. Increased levels of the anti-inflammatory ATF3 could be one of the reasons for the observed results (Gilchrist *et al.* 2006; Hunt *et al.* 2012).

In conclusion, the importance of the JNK2 isoform in the proper cellular function of the brain tissue has been demonstrated, just like the negative effects of the HFD. Also, the relationship between metabolic alterations like insulin resistance, ER stress and neuroinflammation for proper cognitive performance has been confirmed; specially by biomarkers like PTP1B and SOCS3 proteins on the modulation of the IR. Further study of the isoforms and the effects of insulin-related biomarkers will favour the design of new drugs for the treatment of AD-like sporadic neurodegenerative pathologies (Fig. 8).

Acknowledgments and conflict of interest disclosure

OB, AC, ME, CA, EV and JF belong to 2014SGR 525 from Generalitat de Catalunya. Also, this study was partly supported by funds from the Spanish Ministerio de Economía y Competitividad (SAF2017-84283-R to AC, SAF2015-64146-R to MVC), the Generalitat de Catalunya (2014SGR-525 to AC and 2014SGR-13 to MVC) and Spanish Ministry of Science and Innovation (PI2016/01). CIBER de Enfermedades Neurodegenerativas (CIBERNED) (Grant CB06/05/2004 to AC) and CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM) (Grant CB07/08/0003 to MVC; Instituto de Salud Carlos III Project). RDCT is supported by Postdoctoral fellowship CONACYT No. 298337 and the Doctoral Program in Sciences in Molecular Biology in Medicine, LGAC Molecular Bases of Chronic Diseases-Degenerative and its Applications (000091, PNPC, CONACyT). PRM is supported by grants 2015/26084-1 and 2017/13224-5, São Paulo Research Foundation (FAPESP) – Brazil. No authors have any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations. All authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data.

All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Description of nutritional content for control (CT) and high-fat diet (HFD).

Table S2. List of primary and secondary antibodies used for immunoblot analysis.

Table S3. List of primary and secondary antibodies used for immunofluorescence (IF) assays.

Table S4. Sequence specification for primers used in real-time–polymerase chain reaction (RT-PCR).

References

Abarca-Gómez L., Abdeen Z. A., Hamid Z. A. *et al.* (2017) Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. *Lancet* **390**, 2627–2642.

Bjorkblom B. (2005) Constitutively active cytoplasmic c-Jun N-terminal kinase 1 is a dominant regulator of dendritic architecture: role of microtubule-associated protein 2 as an effector. *J. Neurosci.* **25**, 6350–6361.

Bocarsly M. E., Fasolino M., Kane G. A., LaMarca E. A., Kirschen G. W., Karatsoreos I. N., McEwen B. S. and Gould E. (2015) Obesity diminishes synaptic markers, alters microglial morphology, and impairs cognitive function. *Proc. Natl Acad. Sci.* **2**, 201511593.

Bray G. A. (2004) Medical consequences of obesity. *J. Clin. Endocrinol. Metab.* **89**, 2583–2589.

Brecht S., Kirchhof R., Chromik A. *et al.* (2005) Specific pathophysiological functions of JNK isoforms in the brain. *Eur. J. Neurosci.* **21**, 363–377.

Busquets O., Ettcheto M., Pallàs M., Beas-Zarate C., Verdaguer E., Auladell C., Folch J. and Camins A. (2017) Long-term exposition to a high fat diet favors the appearance of β-amyloid depositions in the brain of C57BL/6J mice. A potential model of sporadic Alzheimer's disease. *Mech. Ageing Dev.* **162**, 38–45.

Busquets O., Ettcheto M., Verdaguer E., Castro-Torres R. D., Auladell C., Beas-Zarate C., Folch J. and Camins A. (2018) JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid. *Neuropharmacology* **131**, 440–452.

Carboni L., Carletti R., Tacconi S., Corti C. and Ferraguti F. (1998) Differential expression of SAPK isoforms in the rat brain. An in situ hybridisation study in the adult rat brain and during post-natal development. *Mol. Brain Res.* **60**, 57–68.

Cheng A., Hou Y. and Mattson M. P. (2010) Mitochondria and neuroplasticity. *ASN Neuro* **2**, e00045.

Coffey E. T. (2014) Nuclear and cytosolic JNK signalling in neurons. *Nat. Rev. Neurosci.* **15**, 285–299.

Correia S. C., Santos R. X., Carvalho C., Cardoso S., Candeias E., Santos M. S., Oliveira C. R. and Moreira P. I. (2012) Insulin signalling, glucose metabolism and mitochondria: major players in Alzheimer's disease and diabetes interrelation. *Brain Res.* **1441**, 64–78.

De Felice F. G. and Ferreira S. T. (2014) Inflammation, defective insulin signalling, and mitochondrial dysfunction as common molecular denominators connecting type 2 diabetes to Alzheimer disease. *Diabetes* **63**, 2262–2272.

De Meyts P. (2016). The insulin receptor and its signal transduction network. Endotext.

Dong C., Yang D. D., Wysk M., Whitmarsh A. J., Roger J., Davis R. J. and Flavell R. A. (1998) Defective T cell differentiation in the absence of Jnk1. *Science* **5396**, 2092–2095.

Ettcheto M., Petrov D., Pedros I., Alva N., Carbonell T., Beas-Zarate C., Pallas M., Auladell C., Folch J. and Camins A. (2016) Evaluation of neuropathological effects of a high-fat diet in a presymptomatic Alzheimer's disease stage in APP/PS1 mice. *J. Alzheimers Dis.* **54**, 233–251.

de Felice F. G. and Lourenco M. V. (2015) Brain metabolic stress and neuroinflammation at the basis of cognitive impairment in Alzheimer's disease. *Front. Aging Neurosci.* **7**, 1–8.

Ganou C. A., Eleftheriou P. T., Theodosis-Nobelos P., Fesatidou M., Geronikaki A. A., Lialiaris T. and Rekka E. A. (2018) Docking analysis targeted to the whole enzyme: an application to the prediction of inhibition of PTP1B by thiomorpholine and thiazolyl derivatives$. *SAR QSAR Environ. Res.* **29**, 133–149.

Gao S., Howard S. and Lograsso P. V. (2017) Pharmacological inhibition of c-Jun N-terminal kinase reduces food intake and sensitizes Leptin's anorectic signaling actions. *Sci. Rep.* **7**, 1–12.

Gilchrist M., Thorsson V., Li B., Rust A. G., Korb M., Kennedy K., Hai T., Bolouri H. and Aderem A. (2006) Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* **441**, 173–178.

Grillo C. A., Piroli G. G., Lawrence R. C. *et al.* (2015) Hippocampal insulin resistance impairs spatial learning and synaptic plasticity. *Diabetes* **64**, 3927–3936.

Guo W., Wong S., Xie W., Lei T. and Luo Z. (2007) Palmitate modulates intracellular signalling, induces endoplasmic reticulum stress, and causes apoptosis in mouse 3T3-L1 and rat primary preadipocytes. *Am. J. Physiol. Endocrinol. Metab.* **293**, 576–586.

Haeusgen W., Boehm R., Zhao Y., Herdegen T. and Waetzig V. (2009) Specific activities of individual c-Jun N-terminal kinases in the brain. *Neuroscience* **161**, 951–959.

Henneberg N. and Hoyer S. (1995) Desensitization of the neuronal insulin receptor: a new approach in the etiopathogenesis of late-onset sporadic dementia of the Alzheimer type (SDAT)? *Arch. Gerontol. Geriatr.* **21**, 63–74.

Hirosumi J., Tuncman G., Chang L., Görgün C. Z., Uysal K. T., Maeda K., Karin M. and Hotamisligil G. S. (2002) A central role for JNK in obesity and insulin resistance. *Nature* **420**, 333–336.

Hunt D., Raivich G. and Anderson P. N. (2012) Activating transcription factor 3 and the nervous system. *Front. Mol. Neurosci.* **5**, 1–17.

Ip Y. T. and Davis R. J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK) - from inflammation to development. *Curr. Opin. Cell Biol.* **10**, 205–219.

Johnson G. L. and Nakamura K. (2007) The c-jun kinase/stress-activated pathway: regulation, function and role in human disease. *Biochim. Biophys. Acta* **1773**, 1341–1348.

Kahn C. R. (1978) Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. *Metabolism* **27**, 1893–1902.

Kanoski S. E., Meisel R. L., Mullins A. J. and Davidson T. L. (2008) The effect of energy-rich diets on discrimination reversal learning and on BDNF in the hippocampus and prefrontal cortex of the rat. *Behav. Brain Res.* **182**, 57–66.

Kothari V., Luo Y., Tornabene T., O'Neill A. M., Greene M. W., Geetha T. and Babu J. R. (2017) High fat diet induces brain insulin resistance and cognitive impairment in mice. *Biochim. Biophys. Acta* **1863**, 499–508.

Liang L., Chen J., Zhan L., Lu X., Sun X., Sui H., Zheng L., Xiang H. and Zhang F. (2015) Endoplasmic reticulum stress impairs insulin receptor signalling in the brains of obese rats. *PLoS ONE* **10**, 1–14.

Liu Z., Patil I. Y., Jiang T., Sancheti H., Walsh J. P., Stiles B. L., Yin F. and Cadenas E. (2015) High-fat diet induces hepatic insulin resistance and impairment of synaptic plasticity. *PLoS ONE* **10**, 1–16.

Lourenco M. V., Ferreira S. T. and De Felice F. G. (2015) Neuronal stress signalling and eIF2α phosphorylation as molecular links between Alzheimer's disease and diabetes. *Prog. Neurobiol.* **129**, 37–57.

Ma T., Trinh M. A., Wexler A. J., Bourbon C., Gatti E., Pierre P., Cavener D. R. and Klann E. (2014) Suppression of eIF2α kinases alleviates AD-related synaptic plasticity and spatial memory deficits. *Nat. Neurosci.* **16**, 1299–1305.

Mittal K., Mani R. J. and Katare D. P. (2016) Type 3 diabetes: cross talk between differentially regulated proteins of type 2 diabetes mellitus and Alzheimer's disease. *Sci. Rep.* **6**, 1–8.

MohammadTaghvaei N., Taheripak G., Taghikhani M. and Meshkani R. (2012) Palmitate-induced PTP1B expression is mediated by ceramide-JNK and nuclear factor κB (NF-κB) activation. *Cell. Signal.* **24**, 1964–1970.

Molteni R., Barnard R. J., Ying Z., Roberts C. K. and Gómez-Pinilla F. (2002) A high-fat, refined sugar diet reduces hippocampal brain-derived neurotrophic factor, neuronal plasticity, and learning. *Neuroscience* **112**, 803–814.

de la Monte S. M. and Wands J. R. (2008) Alzheimer's disease is type 3 diabetes-evidence reviewed. *J. Diabetes Sci. Technol.* **2**, 1101–1113.

de la Monte S. (2012) Brain insulin resistance and deficiency as therapeutic targets in Alzheimer' s disease. *Curr. Alzheimer Res.* **9**, 35–66.

Mori H., Hanada R., Hanada T., Aki D., Mashima R., Nishinakamura H., Torisu T., Chien K. R., Yasukawa H. and Yoshimura A. (2004) Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. *Nat. Med.* **10**, 739–743.

Morton G. J., Cummings D. E., Baskin D. G., Barsh G. S. and Schwartz M. W. (2006) Central nervous system control of food intake and body weight. *Nature* **443**, 289–295.

Nguyen M. T. A., Satoh H., Favelyukis S., Babendure J. L., Imamura T., Sbodio J. I., Zalevsky J., Dahiyat B. I., Chi N.-W. and Olefsky J. M. (2005) JNK and tumor necrosis factor-α mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. *J. Biol. Chem.* **280**, 35361–35371.

Nuzzo D., Picone P., Baldassano S., Caruana L., Messina E., Gammazza A., Cappello F., Mulè F. and Carlo M. (2015) Insulin resistance as common molecular denominator linking obesity to Alzheimer's disease. *Curr. Alzheimer Res.* **12**, 723–735.

Ott A., Stolk R. P., van Harskamp F., Pols H. A. P., Hofman A. and Breteler M. M. B. (1999) Diabetes mellitus and the risk of dementia: the Rotterdam Study. *Neurology* **53**, 1937–1942.

Özcan U., Cao Q., Yilmaz E., Lee A.-H., Iwakoshi N. N., Özdelen E., Tuncman G., Görgun C., Glimcher L. H. and Hotamisligil G. S. (2003) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* **299**, 1033–1036.

Pal M., Febbraio M. A. and Lancaster G. I. (2016) The roles of c-Jun NH2-terminal kinases (JNKs) in obesity and insulin resistance. *J. Physiol.* **594**, 267–279.

Panzhinskiy E., Ren J. and Nair S. (2013) Protein tyrosine phosphatase 1B and insulin resistance: role of endoplasmic reticulum stress/reactive oxygen species/nuclear factor kappa B axis. *PLoS ONE* **8**, e77228.

Raciti M., Lotti L. V., Valia S., Pulcinelli F. M. and Di Renzo L. (2012) JNK2 is activated during ER stress and promotes cell survival. *Cell Death Dis.* **3**, e429.

Reinecke K., Herdegen T., Eminel S., Aldenhoff J. B. and Schiffelholz T. (2013) Knockout of c-Jun N-terminal kinases 1, 2 or 3 isoforms induces behavioural changes. *Behav. Brain Res.* **245**, 88–95.

Salvadó L., Barroso E., Gómez-Foix A. M., Palomer X., Michalik L., Wahli W. and Vázquez-Carrera M. (2014) Pparβ/δ prevents endoplasmic reticulum stress-associated inflammation and insulin resistance in skeletal muscle cells through an ampk-dependent mechanism. *Diabetologia* **57**, 2126–2135.

Sánchez C., Díaz-Nido J. and Avila J. (2000) Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. *Prog. Neurobiol.* **61**, 133–168.

Solinas G. and Becattini B. (2017) JNK at the crossroad of obesity, insulin resistance, and cell stress response. *Mol. Metab.* **6**, 174–184.

Solinas G., Naugler W., Galimi F., Lee M.-S. and Karin M. (2006) Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. *Proc. Natl Acad. Sci.* **103**, 16454–16459.

Szegezdi E., Logue S. E., Gorman A. M. and Samali A. (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* **7**, 880–885.

Torisu T., Sato N., Yoshiga D., Kobayashi T., Yoshioka T., Mori H., Iida M. and Yoshimura A. (2007) The dual function of hepatic SOCS3 in insulin resistance in vivo. *Genes Cells* **2**, 143–154.

Trinh M. A. and Klann E. (2014) Translational control by eIF2a kinases in long-lasting synaptic plasticity and long-term memory. *Neurobiol. Learn. Mem.* **105**, 93–99.

Vernia S., Morel C., Madara J. C. *et al.* (2016) Excitatory transmission onto AgRP neurons is regulated by cjun NH2-terminal kinase 3 in response to metabolic stress. *Elife* **5**, 1–18.

Vieira M. N. N., Lyra e Silva N. M., Ferreira S. T. and De Felice F. G. (2017) Protein tyrosine phosphatase 1B (PTP1B): a potential target for Alzheimer's therapy? *Front. Aging Neurosci.* **9**, 1–9.

Zdrojewska J. and Coffey E. T. (2014) The impact of JNK on neuronal migration. *Adv. Exp. Med. Biol.* **800**, 37–57.

Zhang F., Zhong R., Li S., Fu Z., Cheng C., Cai H. and Le W. (2017) Acute hypoxia induced an imbalanced M1/M2 activation of microglia through NF-κB signalling in Alzheimer's disease mice and wild-type littermates. *Front. Aging Neurosci.* **9**, 1–12.

Publication IV

c-Jun N-terminal Kinase 1 ablation protects against metabolic-induced hippocampal cognitive impairments.

Busquets O, Ettcheto M, Eritja À, Espinosa-Jiménez T, Verdaguer E, Olloquequi J, Beas-Zarate C, Castro-Torres RD, Auladell C, Bulló M, Folch J and Camins A.

Under review in Journal of Molecular Medicine

(Submitted - 27th August 2019).

c-Jun N-terminal Kinase 1 ablation protects against metabolic-induced hippocampal cognitive impairments

Oriol Busquets [1,2,3,4], Miren Ettcheto [1,2,3,4], Àuria Eritja [1], Triana Espinosa-Jiménez [1,3,4], , Ester Verdaguer [3,4,5], Olloquequi J [6], Beas-Zarate C [7], Castro-Torres RD [7], Carme Auladell [3,4,5], Mònica Bulló [2], Jaume Folch [2,3], Antoni Camins [1,3,4*].

[1] Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain.

[2] Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain.

[3] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain.

[4] Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain.

[5] Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain.

[6] Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile.

[7] Departamento de Biología Celular y Molecular, C.U.B.C.B.A., Universidad de Guadalajara y División de Neurociencias, Sierra Mojada 800, Col. Independencia, Guadalajara, Jalisco, Mexico.

* Correspondence and reprint requests to Antoni Camins PhD, Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain. Av. Joan XXIII 27/31, 08028. Tel: +34 93 4024531, Fax: +34 934035982, Mail: camins@ub.edu

Abstract

The development of metabolic alterations like insulin resistance has been associated with dysfunctions in mitochondrial oxidative capacity, induction of neuroinflammatory responses, and the appearance of cognitive impairments in the brain. The c-Jun N-terminal Kinase 1 (JNK1) is a potential key modulator of these mechanisms.

The current study identifies a protective effect of whole body JNK1 knock-out in the presence of a high-fat diet (HFD). Specifically, the data suggest that mice missing JNK1 show increased insulin sensitivity and mitochondrial activity, as well as reduced body weight, and astrocyte and microglial reactivity. Finally, these animals are also protected against HFD-induced cognitive impairments as assessed through NORT, the observation of dendritic spines, and the levels of BDNF or other proteins like spinophilin and ARC. Thus, modulation of JNK1 activity seems like a promising approach for the design of therapies aimed at treating metabolic-induced cognitive impairments.

Introduction

Mild cognitive impairment is a human syndrome characterized by the appearance of small cognitive complaints that do not affect to the performance of simple tasks (1). It usually occurs in patients over 65 years old, can aggravate over time, and eventually lead to the appearance of a dementia after 1 year (2). Longitudinal studies in humans have observed how its progression is linked to dysregulations in the metabolism of glucose, a characteristic of diabetic alterations (2).

The nervous system depends on the metabolism of glucose to maintain its physiological activity (3). In preclinical studies, alterations in the oxidation of this carbohydrate have been linked with a decrease in lifespan and the appearance of slow-building affectations that cause neurodegeneration (4). It has been described that the insulin receptor (IR) and its signalling pathway play a role in the modulation of these mechanisms. Also, the IR has been studied for its importance in the development of diabetic complications and proper functionality of cognition-related areas like the hippocampus and the prefrontal cortex (5). On this note, Grillo and colleagues showed how silencing of the gene for the IR in the hippocampus caused major spatial learning impairments when using antisense sequences in lentivirus (6). These results correlate with previously reported studies in which sporadic forms of Alzheimer's disease have been associated with the desensitization of the IR (7,8), a paradigm that has later been labelled as Type 3 Diabetes (T3D) (9,10). Thus, investigating mechanisms for the modulation of this pathway may prove relevant to identify new approaches to treat these afflictions.

The c-Jun N-terminal Kinases (JNK) are very active stress-response elements that participate in the control of many cellular mechanisms (11). Short term activation is believed to be necessary for survival and the maintenance of physiological functions, while long term activation has been described to cause the appearance of cellular stress and the induction of pathophysiological mechanisms. For example, it has been reported that there is high JNK activity in the hypothalamus during obesity (12,13). In 2010, Sabio and colleagues reported that isoform 1 (JNK1) is a highly active JNK isoform (14). High JNK1 activity has been linked with the appearance of reactive stress responses, conditions like obesity, and pathologies like diabetes or anxiety and neurodegenerative disorders (15,16). On a molecular level, JNK1 is activated by cytokines, mitochondrial and endoplasmic reticulum stress, and hyperlipidaemia among many other stimuli, all of which are hallmarks of metabolic pathologies (4). Furthermore, JNK1 regulates inhibitory serine phosphorylation of the IR substrate (IRS) proteins, which impairs insulin signalling. Yet, it has been observed that blocking of the Ser307 residue, a point of JNK1 activity, does not avoid insulin resistance but rather causes for a further increase, indicating the existence of multiple parallel and redundant mechanisms through which JNK1 promotes metabolic alterations when it is activated (20,21).

In order to study these mechanisms, whole body knock-out animals for JNK1 (*Jnk1*$^{-/-}$) have been previously used by several authors. It has been reported that JNK1 is necessary for the accumulation of visceral fat and, thus, its absence is protective against obesity, enhances sensitivity to insulin, and induces antiinflammatory effects in models

of obesity induced with a high fat diet (HFD)(16,19). Studies revealed that these animals showed metabolic protection against the effects of HFD for over 40 weeks and maintained a high level of tolerance and protection against oxidative damage in the adipose and hepatic tissues (20). Controversially, Becattini and co-workers reported that this genetic modification, while beneficial for the control of peripheral metabolic alterations, caused for mild oxidative damage in the skin of mice at the age of 11 and 20 months when exposed to a HFD, yet effects were lower than in previously studied models like *Drosophila* or *Caenorhabditis elegans* (20). Finally, researchers like Mohammad H and colleagues described how *Jnk1*$^{-/-}$ animals show lower anxiety levels and increased neurogenesis (21). Similar results on the differences of neurogenic activity of the JNK1 transgenic animals have been reported by our research group (22). Complementarily, tissue-specific knock-outs of the JNK1 in the adipose tissue, muscle and liver have also been described. In all cases, animals showed amelioration of metabolic alterations, but effects were not as significant as those produced by the whole body knock-out (23–25). Exceptionally, conditional neuron JNK1 knock-out mice showed dramatic sensitivity to insulin in the brain and the periphery, reduced inflammatory responses and complete protection against HFD in the hypothalamus (26). Authors described very high energy expenditure rates, caused by the increased production of triiodothyronine (T3) and thyroid stimulating hormone (TSH) hormones (26,27).

Consequently, the present research is intended to validate the hypothesis of a molecular regulatory factor linking metabolic dysregulations and cognitive loss, to demonstrate the role of JNK1

isoform in these mechanisms, and suggest a putative target for future pharmacological strategies.

Research Design and Methods

Animals and Diet

Male C57BL/6J wild-type (WT) and *Mpak8* monotransgenic knock-out animals were used for this study. The *Mapk8* gene codifies for the protein JNK1 (*Jnk1*$^{-/-}$). Transgenic animals were obtained and characterized following the method described by Dong and colleagues(28). All animals used in this study were obtained from established breeding couples in the animal facility (Pharmacy and Food Sciences Faculty; University of Barcelona; Approval number C-0032).

The animals were fed with either control (CT) or palmitic acid-enriched diets (High-Fat Diets; HFD; 45% fat content. Research Diets, Inc.; product D12451) from their weaning until they were euthanized. Thus, four experimental groups were established: WT CT, WT HFD, *Jnk1*$^{-/-}$ CT and *Jnk1*$^{-/-}$ HFD. Animals were randomly assigned into each experimental group in a non-blinded manner and 12-15 animals were used per group. They were grown until 9 months of age and underwent monthly weight controls. Environmental conditions of temperature and humidity were kept stable. Also, animals were kept under a 12h light/dark cycle and had food and water available at all times (Pharmacy and Food Sciences Faculty; University of Barcelona). During all procedures the European Committee bioethics directives

were followed (European Communities Council Directive 2010/63/EU) and all protocols were previously approved by the ethic committee from the University of Barcelona. In all cases, it was made sure that animal numbers, stress, and pain were kept under a necessary minimum.

Glucose Tolerance Test (GTT) – Insulin Tolerance Test (ITT)

Tests were conducted as previously described (29). In short, animals were fasted 6 hours previous to the tests and posteriorly injected with either glucose (1 g/kg) or insulin (0.75 ui/kg) in the intraperitoneal cavity. Peripheral glucose concentrations were calculated using a glucometer (Accu-Check, Roche) at different time points right before (basal) and after the administration: GTT (5, 15, 30, 60, 120 and 180 min) and ITT (15, 30, 45, 60 and 90 min). Animals were monitored throughout the test and, if any of the subjects dropped below 20 mg/dl in the ITT, they were administered a dose of glucose (1 g/kg). Blood glucose levels and behaviour where checked regularly until they were stable.

Novel Object Recognition Test (NORT)

Experimental procedure was adapted from a publication by Bevins RA and Besheer J(30). The week previous to the test, animals were handled for a few minutes every day in order to reduce manipulation stress. To reduce environmental cues, tests were conducted in an open field box (50x50x20cm) surrounded by black curtains. Initial testing consisted of three days in which the animals were introduced to the open field box for 10 minutes and were allowed to explore it freely (habituation period). Motor activity and stress for each animal

was evaluated by the quantification of the speed, total distance and time spent in the inner quadrant. On the following day, two identical objects were introduced into the open field and the exploration time for each object was quantified. Animals that showed significant preference for one of the objects over the other were excluded. The next day, one of the objects was substituted by a new one and exploration time was quantified again.

During the experimental procedure all spaces and objects were properly cleaned previous to the introduction of the animals in order to eliminate odour cues. Objects were randomized by blindly choosing from a box in each session so as to reduce any possible preference effects caused by colour or shape. All recordings and data were obtained using the program Smart 3.0 (Panlab). Motor activity and stress data was presented as a curve in which the mean and standard deviation were presented. Area under the curve was extrapolated for posterior statistical analysis. Discrimination ratio (DI) was calculated using the formula: DI = (Time spent exploring the new object – Time spent exploring the known object)/Total exploration time.

Western Blot

Protein detection was performed from protein extracts of hippocampal tissue of mice euthanized by neck dislocation. Protein extraction and posterior western blot assays were performed as previously described (29). References for the antibodies used for these assays have been described in **Supplementary Material 1**. Detections were performed through chemoluminescence using Pierce® ECL Western Blotting Substrate (#32106, Thermo Scientific,

USA), a Bio-Rad Universal Hood II Molecular Imager and the Image Lab v5.2.1 software (Bio-Rad Laboratories). Measurements were expressed in arbitrary units and all results were normalised with the corresponding loading control (Glyceraldehyde-3-phosphate dehydrogenase; GAPDH).

Immunofluorescence

Prior to perfusion with 4% paraformaldehyde, animals were anesthetized through an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Posterior brain fixation, sectioning and labelling through immunofluorescence have been previously described(29).

Antibodies used for IF have been included in **Supplementary Material 1**. Images were acquired from an epifluorescence microscope (Olympus BX61 Laboratory Microscope, Melville, NY-Olympus America Inc.). Number of SOD1-positive cells was quantified in the hilus of the *dentate gyrus* of the hippocampus.

Real Time – Polymerase Chain Reaction (RT-PCR)

Gene expression was quantified after hippocampal RNA extraction. Posteriorly, RNA samples were retrotranscribed into cDNA and used for RT-PCR (29). Specific protocol details are described in a previous publication from our research group. Primer sequences for the RT-PCR are in **Supplementary Material 2**.

Citrate Synthase Activity Colorimetric Assay Kit

Activity was detected from tissue homogenates as described in the protocol by BioVision, Inc. (Citrate Synthase Activity Colorimetric

Assay Kit, K318) and corrected for sample-protein content through a Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Waltham, MA, USA).

Golgi Stain

Protocol was followed as described by the manufacturer ((FD Rapid GolgiStain™ Kit; Cat #PK401; FD Neurotechnologies, Inc.) and images were obtained from a BX61 Laboratory Microscope (Melville NY-Olympus America Inc.).

Dendritic spines were quantified as previously described(29). Briefly, granular neurons from the *dentate gyrus* of the hippocampus were chosen and measurements were performed at least 50 μm from the soma. At minimum, 5 consecutive 10 μm sections were collected and 5 neurons were quantified per animal. Neurons were chosen from those that showed clear staining. No neurons were included in the analyses if their dendritic arborisation crossed paths with those nearby. Four animals per experimental group were used.

Statistical analysis

Results were presented as interleaved boxes and whiskers with each of the obtained values being represented. The box represents the median and the 25^{th} to 75^{th} percentiles in the extremes. Maximum and minimum values were represented as whiskers. All experimental groups were tested through two-way ANOVA and Tukey's. All analyses and graph representations were performed in the program Graph Pad Prism for Windows version 6.01; Graph Pad Software, Inc. Experimenters were blinded to data sets when calculating for statistical differences. Only relevant significant values were

represented with their corresponding p-value. In all figures and graph representations the f value and degrees of freedom for ANOVA was included.

Results

Transgenic JNK1 knock-out mice show lower body weight, higher insulin sensitivity and no negative insulin related or inflammatory alterations after long-term HFD feeding.

Evaluation of periphery parameters demonstrated how lack of JNK1 reduced animal body weight and increased responsiveness to insulin when evaluated in the ITT. WT HFD animals showed significant increases in body weight, as well as, increased blood glucose concentrations both in the GTT and ITT. HFD caused for mild increases in weight and glucose concentrations in the *Jnk1*$^{-/-}$ HFD experimental group but values were similar to those of WT CT animals (**Figure 1**).

On a molecular level, the IR/AKT signalling axis showed a significant increase of the activating phosphorylation rates in the *Jnk1*$^{-/-}$s when compared with WTs. GSK3β auto-phosphorylated inhibitory Ser9 residue showed similar tendencies. Additionally, IDE protein levels were significantly reduced in the WT HFD experimental group versus the WT CT. Finally, protein levels for the PTP1B were strongly increased in the WT HFD animals while *Jnk1*$^{-/-}$s experimental groups showed non-significant, slightly lower values than WT CT (*p=0.1143 and p=0.0816* respectively) (**Figure 2**).

Analysis of the profiles of both astrocytes and microglia revealed that cells were more reactive in the WT HFD when compared against the control. Reactiveness was evaluated regarding the size, colour intensity, number and ramification rates of the detected cells. In the *Jnk1*$^{-/-}$ experimental groups, a reduction in these same characteristics was observed even below control levels (**Figure 3**).

Lack of JNK1 increases mitochondrial oxidative phosphorylation, antioxidant enzymes and protects against HFD-induced dysregulations.

Detection of OXPHOS complexes showed an overall tendency towards higher protein levels in the *Jnk1*$^{-/-}$ animals, especially when comparing against the WT HFD mice, which present significant reductions on CI and CII versus WT CT. Exceptionally, CII was strongly affected by HFD even in the *Jnk1*$^{-/-}$ HFD experimental group. Importantly, CIII is highly upregulated in both *Jnk1*$^{-/-}$ experimental groups. Similar upward tendencies were observed in PGC1α and PPARγ, as well as in antioxidant enzymes SOD1 and GPX1 (**Figure 4A**). Evaluation of gene expression rates for *Pgc1α*, *Pparγ*, *Sod1,* and *Gpx1* showed significant upregulation in the *Jnk1*$^{-/-}$ animals (**Figure 4B**). Additionally, quantification of the number of SOD-positive cells in the hilus region of the *dentate gyrus* of the hippocampus showed the same trends (**Figure 5A-B**). Finally, 4-HNE level were significantly increased in WT HFD and reduced in *Jnk1*$^{-/-}$s (**Figure 4**) and the activity of citrate synthase was mildly higher in the *Jnk1*$^{-/-}$ experimental groups (**Figure. 5C**).

Absence of JNK1 increases motor activity and protects against cognitive impairment by maintaining dendritic spines and synapse-related proteins even after chronic exposure to HFD.

During the habituation period, three parameters related to motor activity were quantified: time spent in the open field inner quadrant, total distance, and mean speed. In all three measures, *Jnk1*$^{-/-}$ animals showed higher values than their controls (**Figure 6A-C**). Assessment of long-term memory consolidation through the NORT determined that only WT HFD animals had reductions in the discrimination ratio index (**Figure 6D**).

Detection of the protein levels of BDNF demonstrated a significant increase in the *Jnk1*$^{-/-}$ mice. Synapse-related proteins ARC, neurexin 2 and neuroligin 3 showed tendencies towards an increase in animals lacking JNK1 (**Figure 6E**). Additionally, significant reductions were observed for the WT HFD experimental group versus WT CT in synaptophysin, neurexin 2 and neuroligin 3 protein levels while *Jnk1*$^{-/-}$ HFD showed no diet-induced effects (**Figure 6E**). Finally, alterations on neurexin 2 and neuroligin 3 were confirmed in an immunofluorescence detection in the *cornu ammonis 3* region of the hippocampus (**Figure 7**).

Moreover, spinophilin, P-Pyk2, and DBN1, found in dendritic arborisations and spines, showed similar upward tendencies in *Jnk1*$^{-/-}$s (**Figure 6E**), which were correlated with the values in the PAK1/LIMK1 axis (**Figure 8A**). Similarly, quantification on the number of dendritic spines showed clear reductions in the WT HFD experimental group while *Jnk1*$^{-/-}$s showed no differences against WT

CT (**Figure 8B**). Furthermore, WT HFD spines showed smaller and shorter profiles.

Discussion

The results from the present investigation demonstrate, for the first time, the role of JNK1 in the context of the appearance of cognitive deficits and metabolic alterations (31)..

The appearance of hyperglycaemia and loss of insulin sensitivity in the periphery and central tissues has been previously reported in obesity models with HFD, both by us and by other research groups (29, 35). It is believed that this situation derives of the development of mitochondrial and endoplasmic reticulum stress which, in turn increases the activity of the JNKs, kinases responsible for the inhibitory phosphorylation of the IRS1. Moreover, other molecules like PTP1B and SOCS3, which are phosphatases of the tyrosine activation points, also become upregulated (36–38). These mechanisms were confirmed in a previous publication from our group in which the effects of the removal of JNK2 was studied (29). Additionally, other alterations like the increased production of ceramides, favour the appearance of metabolic dysfunctions, impairments of neuronal plasticity, reduced myelin maintenance, glial and neuronal cell death, neurodegeneration, and cognitive affectations (39).

Alternatively, modulating the activity of the JNK1 seems like a promising approach to reverse this situation. As it has been

summarized in the introduction, the whole body removal of JNK1 or the neuron-specific silencing of this kinase causes mice to become significantly more sensitive to insulin even when exposed to a HFD for a long term (24,26). In the brain, when analysing hippocampal extracts, significant increases in the phosphorylation rates of the IR, AKT and GSK3β proteins were observed when comparing against the control, thus indicating higher activation of the IR axis. So, higher activity of the insulin signalling pathway after the modulation of JNK1 would favour the maintenance of glucose homeostasis and increase cell survival and activity, allowing for the protection of brain function against metabolic diseases.

When evaluating the state of the mitochondria, a reduction in oxidative capacity was observed in WT HFD mice. This conclusion was drawn from the detection of a decrease in the levels of the OXPHOS complexes, levels and expression of antioxidant enzymes, and PGC1α and PPARγ agents. Similar observations in other tissues have already been reported by other researchers (40). Impairments in PGC1α have been associated with the appearance of cognitive affectations through the down-regulation of BDNF (41). Furthermore, evidence suggests that PPARγ has protective roles through the regulation of SOD activity (42) and, consequently, its alteration might participate in mitochondrial dysregulation. Importantly, animals that lacked JNK1 and where exposed to CT diet showed increased mitochondrial function, even when detecting the activity of citrate synthase enzyme, a component of the Krebs Cycle. So, higher mitochondrial activity will account for increased energy expenditure. This conclusion, together with the fact that JNK1 is involved with anxiety behaviours and other

mechanisms like the maintenance of reservoirs in the adipose tissue, and the observed increased motor activity of the *Jnk1*$^{-/-}$, could account for the reduction in the total body weight of these animals. *Jnk1*$^{-/-}$ HFD animals showed high resilience to the negative effects of HFD in the mitochondria, thus indicating the potential protective capabilities of this kinase.

As a result of metabolic affectations there is also increased release of molecules like cytokines (TNFα, IL6, etc.) and eicosanoids (prostaglandins, leukotrienes, etc.) by adipocytes (43,44), which induce inflammatory responses. In the brain, astrocytes and microglia respond strongly to those molecules (45). The appearance of chronic inflammatory reactions in the brain leads to degeneration due to the induction of apoptotic mechanisms in neurons and other neural types (46,47). It has been described that HFD, as a method to cause environmentally-induced obesity in preclinical models, induces increased cellular reactivity (29,32). In our study, these same observations were made and, most significantly, it was clear that *Jnk1*$^{-/-}$ animals presented qualitatively lower reactivity even against the control group. This result is in accordance with the relevance that JNK1 has in the activation of this cellular type (48).

In the end, the metabolic alterations caused by the HFD lead to the appearance of cognitive impairments (34). Our results have shown clear affectations in the capacity of animals to generate long-term memory as assessed by the NORT. Furthermore, the analysis of proteins like synaptophysin and neurexin related to the establishment of synaptic connections supported these assumptions, together with the reduction in the number and size of dendritic spines

as assessed by the Golgi Stain. Similar tendencies towards a reduction in the levels of other molecules like BDNF or Drebrin were observed, but statistical analysis deem them non-significant. Nonetheless, reported bibliography has already described the negative effects of dysregulation of metabolism in cognitive function (3). Yet, once again, *Jnk1*$^{-/-}$ experimental animals showed evidence of the protective and beneficial effects of the modulation of the activity of this kinase. These experimental groups had normal cognitive capacity in the NORT and, in some cases, showed higher levels for proteins related to the maintenance of synapses and dendritic spine density. Especially relevant results were observed in the high upregulation of BDNF, the maintenance of neurexin and neuroligin proteins, as observed both through immunoblot and immunofluorescence, and the increase activation of PAK1 and LIMK1 proteins, responsible of the inhibition of Cofilin, a known destabilising element of cytoskeletal microfilaments of the structure of dendritic spines (49).

In conclusion, metabolic dysregulations and posterior cognitive impairments seem to be prevented when modulating the activity of JNK1. So, it is of interest to consider this target for the design of future strategies to treat these pathologies, taking into account that the use of a partial, pharmacological approach will most likely avoid the reported skin oxidative damage in the whole body knock-out animals. Additionally, the use of a molecule derived from natural products like Licochalcone A, a compound already tested by our research group in a model of temporal lobe epilepsy(50), will prove to cause less secondary side effects than those typically caused by the chronic use of synthetic drugs.

Acknowledgments

The research group is partly supported by funds from the Spanish Ministerio de Economía y Competitividad (SAF2017-84283-R to AC), the Generalitat de Catalunya (2014SGR-525 to AC) (2017 SGR 625 to CA) and CIBER de Enfermedades Neurodegenerativas (CIBERNED) (Grant CB06/05/2004 to AC).

OB was the lead scientist of this research he worked on the experimental design, procedures and the manuscript. AE and TEJ contributed on the experiments. ME took part in experimental design and experimental trouble solving. EV and CA provided the first animals to create the colony, helped with the experimental design and corrected the manuscript. JF and AC provided with ideas and helped write and correct the manuscript.

References

1. Gauthier S, Reisberg B, Zaudig M, Petersen RC, Ritchie K, Broich K, et al. Mild cognitive impairment. Lancet. 2006;367(9518):1262–70.
2. Drzezga A, Lautenschlager N, Siebner H, Riemenschneider M, Willoch F, Minoshima S, et al. Cerebral metabolic changes accompanying conversion of mild cognitive impairment into alzheimer's disease: A PET follow-up study. Eur J Nucl Med Mol Imaging. 2003;30(8):1104–13.
3. Mergenthaler P, Lindauer U, Dienel GA, Meisel A. Sugar for the brain: the role of glucose in physiological and pathological brain function. Trends Neurosci. 2013;36(10):587–97.
4. Belgardt BF, Mauer J, Brüning JC. Novel roles for JNK1 in metabolism. Aging (Albany NY). 2010;2(9):621–6.
5. De Felice FG, Lourenco M V. Brain metabolic stress and neuroinflammation at the basis of cognitive impairment in

Alzheimer's disease. Front Aging Neurosci. 2015;7(May):1–8.

6. Grillo CA, Piroli GG, Lawrence RC, Wrighten SA, Green AJ, Wilson SP, et al. Hippocampal insulin resistance impairs spatial learning and synaptic plasticity. Diabetes. 2015;64(11):3927–36.

7. Henneberg N, Hoyer S. Desensitization of the neuronal insulin receptor: a new approach in the etiopathogenesis of late-onset sporadic dementia of the Alzheimer type (SDAT)? Arch Gerontol Geriatr. 1995;21(1):63–74.

8. Hoyer S, Henneberg N, Knapp S, Lannert H, Martin E. Brain glucose metabolism is controlled by amplification and desensitization of the neuronal insulin receptor. Ann N Y Acad Sci. 1996;17(777):374–9.

9. de la Monte SM, Wands JR. Alzheimer's disease is type 3 diabetes-evidence reviewed. J Diabetes Sci Technol. 2008;2(6):1101–13.

10. Steen E, Terry BM, J. Rivera E, Cannon JL, Neely TR, Tavares R, et al. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease – is this type 3 diabetes? J Alzheimer's Dis. 2005;7:63–80.

11. Sabapathy K. Role of the JNK Pathway in human diseases. 1st ed. Vol. 106, Progress in Molecular Biology and Translational Science. Elsevier Inc.; 2012. 145–169 p.

12. Araujo EP, De Souza CT, Bordin S, Zollner RL, Saad MJA, Velloso LA, et al. Consumption of a Fat-Rich Diet Activates a Proinflammatory Response and Induces Insulin Resistance in the Hypothalamus. Endocrinology. 2005;146(10):4192–9.

13. Prada PO, Zecchin HG, Gasparetti AL, Torsoni MA, Ueno M, Hirata AE, et al. Western diet modulates insulin signaling, c-jun N-terminal kinase activity, and insulin receptor substrate-1 ser307 phosphorylation in a tissue-specific fashion. Endocrinology. 2005;146(3):1576–87.

14. Sabio G, Davis RJ. CJun NH2-terminal kinase 1 (JNK1): Roles in metabolic regulation of insulin resistance. Trends Biochem Sci. 2010;35(9):490–6.

15. Mohammad H, Marchisella F, Ortega-Martinez S, Hollos P, Eerola K, Komulainen E, et al. JNK1 controls adult hippocampal

neurogenesis and imposes cell-autonomous control of anxiety behaviour from the neurogenic niche. Mol Psychiatry. 2018;23(2):362–74.

16. Grivennikov S, Vilcu C, Naugler W, Wynshaw-Boris A, Solinas G, Luo J-L, et al. JNK1 in Hematopoietically Derived Cells Contributes to Diet-Induced Inflammation and Insulin Resistance without Affecting Obesity. Cell Metab. 2007;6(5):386–97.

17. Copps KD, Hancer NJ, Opare-Ado L, Qiu W, Walsch C, White MF. Irs1 Serine 307 Promotes Insulin Sensitivity in Mice Kyle. Cell Metab. 2010;11(1):84–92.

18. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-Mediated Inhibition of Insulin Receptor Tyrosine Kinase Activity in TNF-α- and Obesity-Induced Insulin Resistance. Science (80-). 1996;271(5249):665–70.

19. Solinas G, Karin M. JNK1 and IKKβ: molecular links between obesity and metabolic dysfunction. FASEB J. 2010;24(8):2596–611.

20. Becattini B, Zani F, Breasson L, Sardi C, D'Agostino VG, Choo MK, et al. JNK1 ablation in mice confers long-term metabolic protection from diet-induced obesity at the cost of moderate skin oxidative damage. FASEB J. 2016;30(9):3124–32.

21. Mohammad H, Marchisella F, Ortega-Martinez S, Hollos P, Eerola K, Komulainen E, et al. JNK1 controls adult hippocampal neurogenesis and imposes cell-autonomous control of anxiety behaviour from the neurogenic niche. Mol Psychiatry. 2018;23(2):362–74.

22. de Lemos L, Junyent F, Camins A, Castro-Torres RD, Folch J, Olloquequi J, Beas-Zarate C, Verdaguer E AC. Neuroprotective effects of the absence of JNK1 or JNK3 isoforms on kainic acid-induced temporal lobe epilepsy-like symptoms. Mol Neurobiol. 2017;

23. Sabio G, Cavanagh-Kyros J, Jin Ko H, Young Jung D, Gray S, Jun JY, et al. Prevention of Steatosis by Hepatic JNK1. Cell Metab. 2009;10(6):491–8.

24. Sabio G, Kennedy NJ, Cavanagh-Kyros J, Jung DY, Ko HJ, Ong H, et al. Role of Muscle c-Jun NH2-Terminal Kinase 1 in Obesity-

Induced Insulin Resistance. Mol Cell Biol. 2010;30(1):106–15.

25. Sabio G, Das M, Mora A, Zhang Z, Jun JY, Hwi JK, et al. A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. Science (80-). 2008;322(5907):1539–43.

26. Belgardt BF, Mauer J, Wunderlich FT, Ernst MB, Pal M, Spohn G, et al. Hypothalamic and pituitary c-Jun N-terminal kinase 1 signaling coordinately regulates glucose metabolism. Proc Natl Acad Sci. 2010;107(13):6028–33.

27. Sabio G, Cavanagh-Kyros J, Barrett T, Jung DY, Ko HJ, Ong H, et al. Role of the hypothalamic-pituitary-thyroid axis in metabolic regulation by JNK1. Genes Dev. 2010;24(3):256–64.

28. Chen Dong, Derek D. Yang, Mark Wysk, Alan J. Whitmarsh, Roger J. Davis RAF. Defective T Cell Differentiation in the Absence of Jnk1. Science. 1998;5396(282):2092–5.

29. Busquets O, Eritja À, López BM, Ettcheto M, Manzine PR, Castro-Torres RD, et al. Role of brain c-Jun N-terminal Kinase 2 in the control of the insulin receptor and its relationship with cognitive performance in a high-fat diet preclinical model. J Neurochem. 2019;149(2):161–310.

30. Bevins R a, Besheer J. Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study "recognition memory". Nat Protoc. 2006;1(3):1306–11.

31. E. González-Reyes R, Aliev G, Avila-Rodrigues M, E. Barreto G. Alterations in Glucose Metabolism on Cognition: A Possible Link Between Diabetes and Dementia. Curr Pharm Des. 2016;22(7):812–8.

32. Busquets O, Ettcheto M, Pallàs M, Beas-Zarate C, Verdaguer E, Auladell C, et al. Long-term exposition to a high fat diet favors the appearance of β-amyloid depositions in the brain of C57BL/6J mice. A potential model of sporadic Alzheimer's disease. Mech Ageing Dev. 2017;162:38–45.

33. Cordner Z a., Tamashiro KLK. Effects of high-fat diet exposure on learning & memory. Physiol Behav. 2015;152:363–71.

34. Freeman LR, Haley-Zitlin V, Rosenberger DS, Granholm A-C. Damaging effects of a high-fat diet to the brain and cognition: a

review of proposed mechanisms. Nutr Neurosci. 2014;17(6):241–51.

35. Yang ZH, Miyahara H, Takeo J, Katayama M. Diet high in fat and sucrose induces rapid onset of obesity-related metabolic syndrome partly through rapid response of genes involved in lipogenesis, insulin signalling and inflammation in mice. Diabetol Metab Syndr. 2012;4(1):1–10.

36. Morrison CD, Huypens P, Stewart LK, Gettys TW. Implications of crosstalk between leptin and insulin signaling during the development of diet-induced obesity. Biochim Biophys Acta - Mol Basis Dis. 2009;1792(5):409–16.

37. Thon M, Hosoi T, Ozawa K. Possible integrative actions of leptin and insulin signaling in the hypothalamus targeting energy homeostasis. Front Endocrinol (Lausanne). 2016;7(OCT):1–7.

38. Bastard J-P, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw. 2006;17(1):4–12.

39. De La Monte SM, Tong M, Nguyen V, Setshedi M, Longato L, Wands JR. Ceramide-mediated insulin resistance and impairment of cognitive-motor functions. J Alzheimer's Dis. 2010;21(3):967–84.

40. García-Ruiz I, Solís-Muñoz P, Fernández-Moreira D, Grau M, Colina F, Muñoz-Yagüe T, et al. High-fat diet decreases activity of the oxidative phosphorylation complexes and causes nonalcoholic steatohepatitis in mice. Dis Model Mech. 2014;7(11):1287–96.

41. Jodeiri Farshbaf M, Ghaedi K, Megraw TL, Curtiss J, Shirani Faradonbeh M, Vaziri P, et al. Does PGC1α/FNDC5/BDNF Elicit the Beneficial Effects of Exercise on Neurodegenerative Disorders? NeuroMolecular Med. 2016;18(1):1–15.

42. Garcia-Fuentes E, Murri M, Garrido-Sanchez L, Garcia-Serrano S, García-Almeida JM, Moreno-Santos I, et al. PPARγ expression after a high-fat meal is associated with plasma superoxide dismutase activity in morbidly obese persons. Obesity. 2010;18(5):952–8.

43. Hardwick JP, Eckman K, Lee YK, Abdelmegeed MA, Esterle A, Chilian WM, et al. Eicosanoids in Metabolic Syndrome. Vol. 66, Advances in Pharmacology. 2013. 157–266 p.

44. Wisse BE. The inflammatory syndrome: The role of adipose tissue cytokines in metabolic disorders linked to obesity. J Am Soc Nephrol. 2004;15(11):2792–800.

45. Norden DM, Trojanowski PJ, Villanueva E, Navarro E, Godbout JP. Sequential Activation of Microglia and Astrocyte Cytokine Expression Precedes Increased Iba-1 or GFAP Immunoreactivity following Systemic Immune Challenge. Glia. 2016;64(2):300–16.

46. Chen WW, Zhang X, Huang WJ. Role of neuroinflammation in neurodegenerative diseases (Review). Mol Med Rep. 2016;13(4):3391–6.

47. Amor S, Peferoen LAN, Vogel DYS, Breur M, van der Valk P, Baker D, et al. Inflammation in neurodegenerative diseases - an update. Immunology. 2014;142(2):151–66.

48. Solinas G, Becattini B. JNK at the crossroad of obesity, insulin resistance, and cell stress response. Mol Metab. 2017;6(2):174–84.

49. Shaw AE, Bamburg JR. Peptide regulation of cofilin activity in the CNS: A novel therapeutic approach for treatment of multiple neurological disorders. Vol. 175, Pharmacology and Therapeutics. 2017. p. 17–27.

50. Busquets O, Ettcheto M, Verdaguer E, Castro-Torres RD, Auladell C, Beas-Zarate C, et al. JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid. Neuropharmacology. 2018;131:440–52.

Figure Legends

Figure 1. Periphery metabolic parameters: (A) Weight: WT CT = 13, WT HFD = 12, *Jnk1*$^{-/-}$ CT = 13, *Jnk1*$^{-/-}$ HFD = 14; (B) GTT: WT CT = 8, WT HFD = 11, *Jnk1*$^{-/-}$ CT = 11, *Jnk1*$^{-/-}$ HFD = 10; (C) ITT: WT CT = 9, WT HFD = 11, *Jnk1*$^{-/-}$ CT = 11, *Jnk1*$^{-/-}$ HFD = 10. [x]/Time progression curves are presented as the mean and standard deviation. Results are presented as interleaved boxes and whiskers. The box represents the

median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Statistical analysis: two-way ANOVA and Tukey's (** $p < 0.01$ and *** $p < 0.001$).

Figure 2. Evaluation of biomarkers associated with the cellular signalling of insulin through the detection of protein levels. Results are presented as scatter plots representing individual values. MEAN ± SD. n = 4. Statistical analysis: two-way ANOVA and Tukey's (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Figure 3. Analysis on the reactive profiles of astrocytes (GFAP; green; first column) and microglia (IBA1; green; second column) in the *dentate gyrus* of the hippocampus. Representative images were presented. Scale bar: 200 µm. Abbreviations: mol – molecular layer, gl – granular layer, h – hilus.

Figure 4. Detection of mitochondrial oxidative phosphorylation complexes and antioxidant enzymes through (A) protein levels and (B) gene expression. Results are presented as scatter plots representing individual values. MEAN ± SD. n = 4. Statistical analysis: two-way ANOVA ($ $p < 0.05$ and $$ $p < 0.01$) and Tukey's (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Figure 5. (A) Representative images of labelling against SOD1 (red) in the *dentate gyrus* of the hippocampus. Cellular nuclei were stained with Hoechst (blue). Scale bar: 1. 200 µm and 2. 100 µm. Abbreviations: mol – molecular layer, gl – granular layer, h – hilus. (B) Quantification of the number of SOD1-positive cells/10 mm^2. n = 6. (C) Results of the citrate synthase activity assay. n = 6. Results are

presented as scatter plots representing individual values. MEAN ± SD. Statistical analysis: two-way ANOVA and Tukey's (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Figure 6. Behavioural assessment through NORT in an open field. (A) Time spent in open field inner quadrant (sec), (B) Total distance (cm; x1000), (C) Mean speed (cm/sec) and (D) Discrimination ratio The first three measurements (A, B and C) were quantified during the habituation period. WT CT = 12, WT HFD = 11, *Jnk1*$^{-/-}$ CT = 11, *Jnk1*$^{-/-}$ HFD = 11; [x]/Time progression curves are presented as the mean and standard deviation. Results are presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. (E) Protein level detection against: BDNF, SPINOPHILIN, P-PYK2(Thr402)/PYK2, DBN1, SYNAPTOPHYSIN, ARC, NEUREXIN 2 and NEUROLIGIN 3. Results are presented as scatter plots representing individual values. MEAN ± SD. Statistical analysis: two-way ANOVA and Tukey's (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Figure 7. Distribution of Neurexin 2 (Red, presynaptic protein) and Neuroligin 3 (Green, postsynaptic protein) in the *cornu ammonis 3* area of the hippocampus. Cellular nuclei are stained with Hoechst (Blue). Scale bar: 200 μm. Abbreviation: so – *stratum oriens*, sp – pyramidal layer, slu – *stratum lucidum* and *stratum radiatum*.

Figure 8. (A) Determination of the levels for the PAK1 and LIMK protein axis. (B) Representative images and quantification of the number of dendritic spines for each of the experimental groups. Scale

bar: 5 µm. Results are presented as scatter plots representing individual values. MEAN ± SD. n = 4. Statistical analysis: two-way ANOVA ($$ $p < 0.01$) and Tukey's (* $p < 0.05$ and *** $p < 0.001$).

FIGURE 1

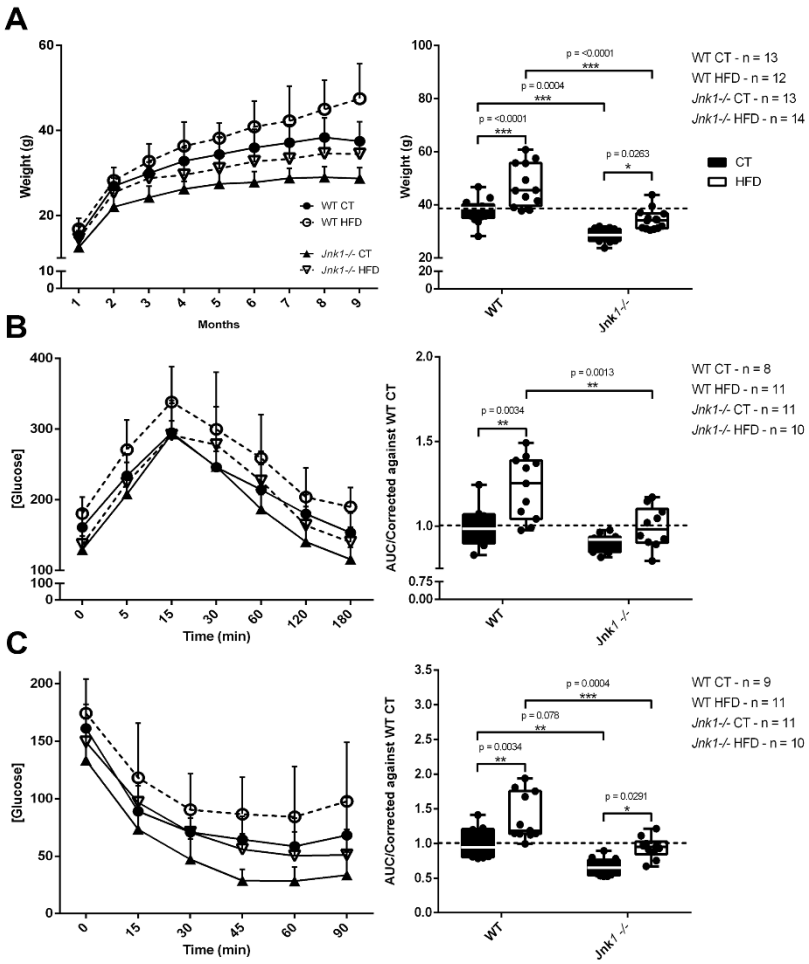


FIGURE 2

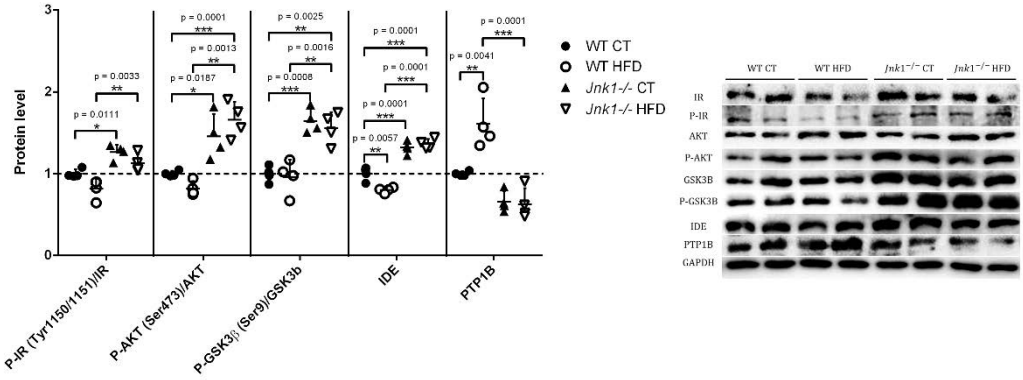


FIGURE 3

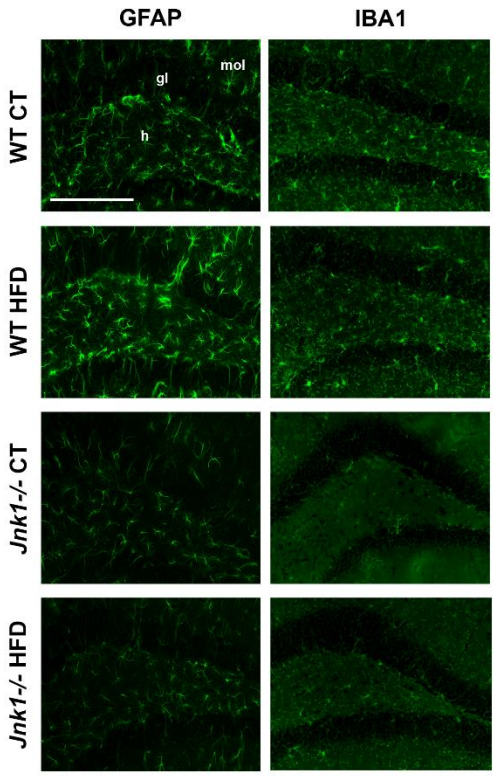


FIGURE 4

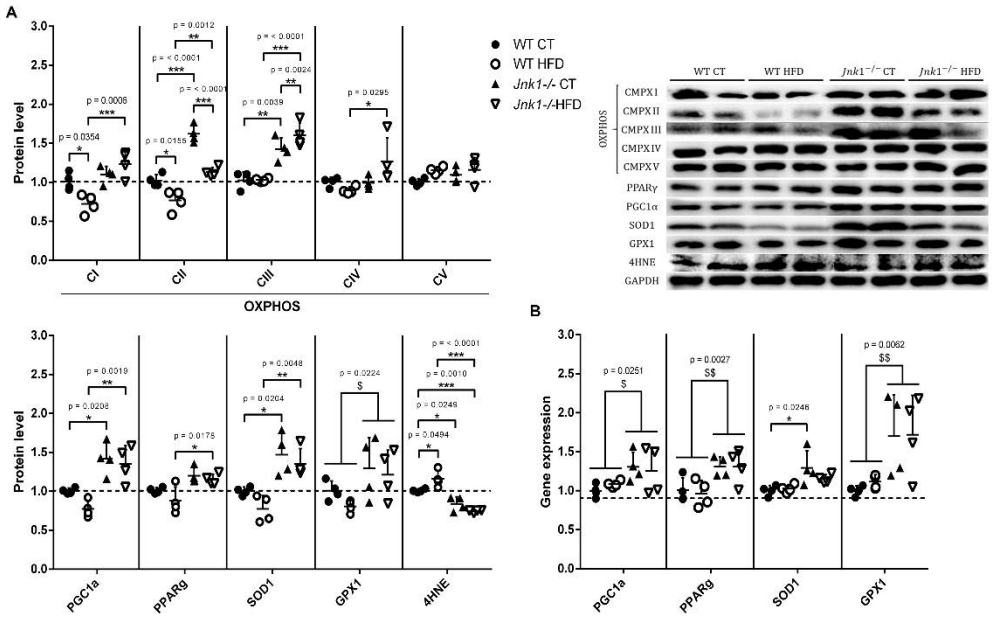


FIGURE 5

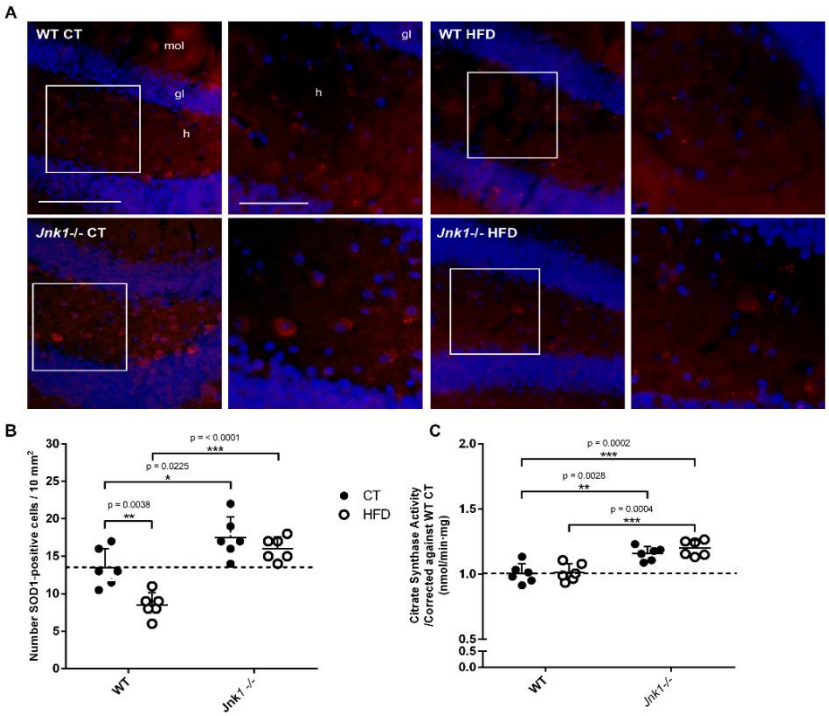


FIGURE 6

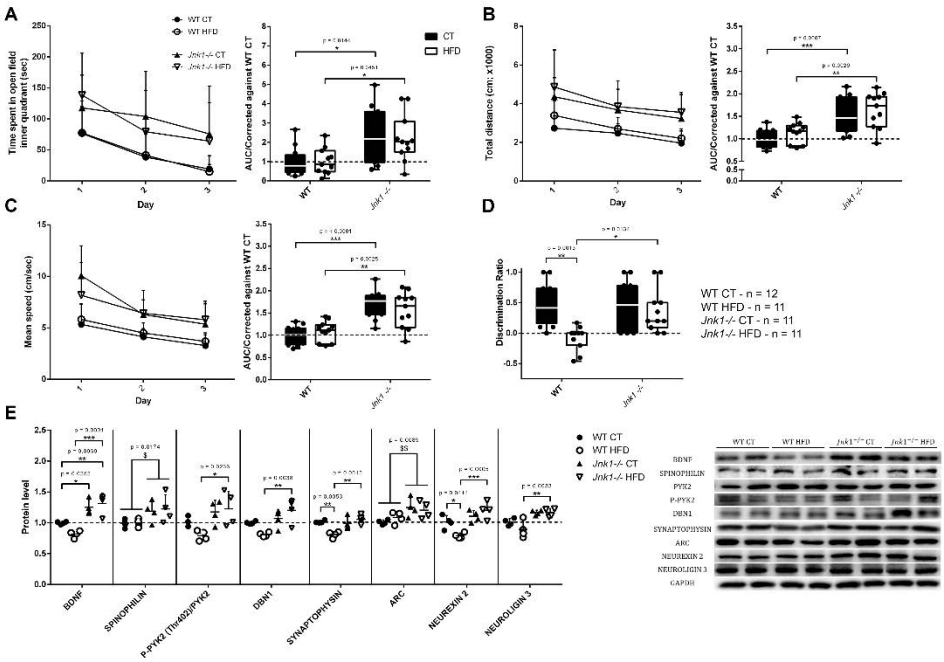


FIGURE 7

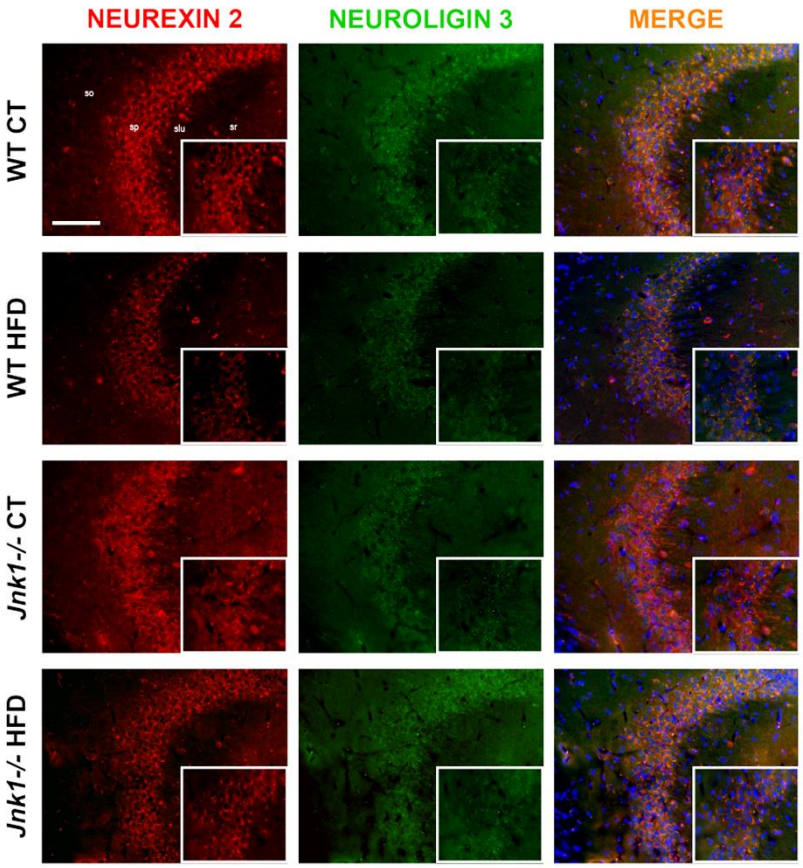
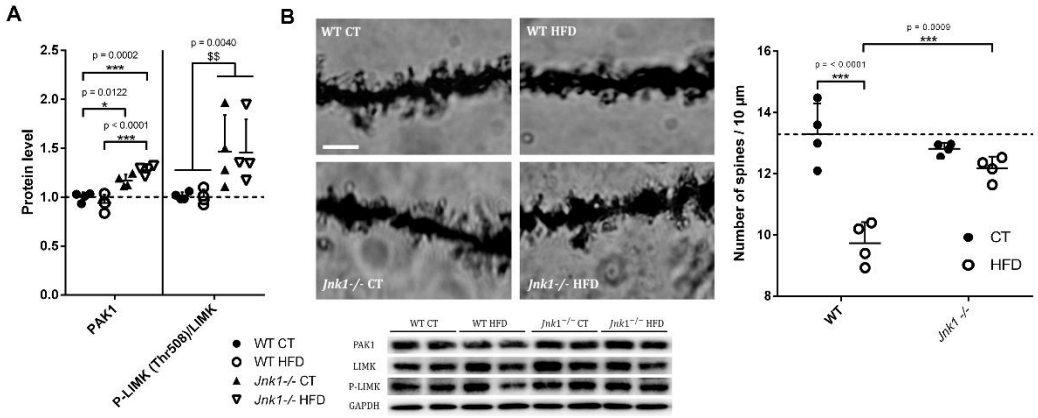


FIGURE 8



Supplementary Material 1. List of antibodies used in the study.

Primary Antibody	Company	Reference
4-Hydroxinonenal (4-HNE)	Abcam	ab46545
Arg 3.1; Activity-regulated cytoskeleton-associated protein (ARC)		ab23382
Insulin Degrading Enzyme (IDE)		ab32216
Neurexin 2		ab34245
Neuroligin 3		ab186307
Oxidative phosphorylation complexes (OXPHOS)		ab110413
Peroxisome proliferator-activated receptor gamma coactivator 1- alpha (PGC1α)		ab54481
Spinophilin		ab18561
Glycogen Synthase Kinase 3β (27C10) (GSK3β)	Cell Signalling	9315
Glycogen Synthase Kinase 3β phospho Ser9 (P-GSK3β)		9336
LIM kinase 1 (LIMK1)		3842
LIM kinase 1 phospho Thr508 (P-LIMK1)		3841
p21-activated kinase 1 (PAK1)		2602
Peroxisome proliferator-activated receptor gamma (PPARγ)		2430
Protein Kinase B (AKT)		9272
Protein Kinase B phospho Ser473 (P-AKT)		4060
Protein Tyrosine Kinase 2 (PYK2)		3292

Protein Tyrosine Kinase 2 phospho Tyr402 (P-PYK2)		3291
Synaptophysin		M0776
Protein Tyrosine Phosphatase 1B (PTP1B)		ABS40
Developmentally-regulated brain protein; Drebrin 1 (DBN1)	Dako	ABN207
Glutathione Peroxidase 1 (GPX1)	Millipore	NBP1-33620
Superoxide Dismutase 1 (SOD1)		NBP2-24915
Brain-derived Neurotrophic Factor (5H8) (BDNF)	Novus Biologicals	sc-65514
Insulin Receptor (CT-3) (IR)		sc-57342
Insulin Receptor (10C3) phospho Thr1150/1151 (P-IR)	Santa Cruz Biotechnology	sc-81500

Secondary Antibody	Company	Reference
Alexa Fluor 488 Donkey Anti-Rabbit	Life Technology	A21206
Alexa Fluor 594 Goat Anti-Rabbit		A11012
Alexa Fluor 594 Goat Anti-Mouse		A11005
Goat Anti-Rabbit IgG (Horseradish peroxidase conjugate) - Pierce™ Antibody	Thermo Scientific	31460
Goat Anti-Mouse IgG (Horseradish peroxidase conjugate) - Pierce™ Antibody		31430

Supplementary Material 2. List and sequences for the RT-PCR primers.

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
Gpx1	CACAGTCCACCGTGTATGCC	GTGTCCGAACTGATTGCACG
Pgc1α	TCTCAGTAAGGGGCTGGTTG	TTCCGATTGGTCGCTACACC
Pparγ	GCTGTTATGGGTGAAACTCTGG	ATAGGCAGTGCATCAGCGAA
Sod1	GGAACCATCCACTTCGAGCA	CCCATGCTGGCCTTCAGTTA

Discussion

DISCUSSION

The aim of this thesis was to understand further the role of each the JNK isoforms in pathological states, as well as evaluate their potential as therapeutic targets. Specifically, two different diseases were reproduced in murine models: TLE and the metabolic-cognitive syndrome. In both cases, previous data from our research group and others set the foundations for the design of each of the projects.

In the past, research had already stablished the response of the JNK3 isoform in the KA preclinical model of TLE. Specifically, the team of Yang and colleagues described that the knockout of JNK3 was neuroprotective against excitotoxic damage (*Yang DD et al., 1997*). Importantly, in 2018, the team of Dr. Carme Auladell (Department of Cell Biology, Physiology and Immunology, Biology Faculty; University of Barcelona) demonstrated for the first time that the lack of JNK1 had similar effects (*de Lemos L et al., 2018*). Given the need for continuous research on new and effective therapies for TLE, LIC-A (JNK1 inhibitor) was tested for its potential as a therapeutic and damage-preventive molecule.

Publication I: *JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid.*

In this study, the injection of KA produced severe episodes of seizures on animals (*Ben-Ari et al., 1979*), due to a significant activation of the neuronal tissue in the hippocampus, focal point for the appearance of a hyperexcitable state in the KA model (*Nadler JV, 1979; Cavalheiro E et al., 1982; Nirwan N et al., 2018*).

This situation led to an acute and sustained activation of the JNKs as a result of increased levels of stress molecules like PGC1α, TNFα and others like nitric oxide synthase (NOS). Also, there was a dysregulation of AKT and CREB, elements necessary for the maintenance of cell survival stimuli (*Zhao F et al., 2014; Tramoni-Negre E et al., 2017*). Furthermore, there was cell degeneration as a result of the activation of proapoptotic mechanisms, which caused the appearance of sclerotic areas in the granular layer of the hippocampus (*Chatzikonstantinou A, 2014*). Moreover, there was a disruption of normal neurogenic activity, coupled with severe inflammatory responses from both astrocytes and microglia (*Dudek EF, 2002*). These alterations favoured the formation of aberrant synaptic contacts, critical hallmarks towards the development of the chronic state of the pathology (*Mathern GW et al., 1993*).

Prominently, animals that had been pre-treated with a single dose of LIC-A showed significant resilience to excitotoxic damage. Specifically, initial evaluations determined that this experimental group showed lower severity of seizures, an effect that had already been described by another research group when treating animals with SP600125 (*Tai TY et al., 2017*). The exact mechanisms responsible for this outcome are yet to be determined but, our research group hypothesized that the inhibition of JNK1 caused a

reduction in the intensity of seizures through a decrease in muscle responsiveness and a downregulation of the activity of enzymes responsible for the control of calcium homeostasis.

Additionally, in our experiments the LIC-A pre-treatment favoured complete protection against neuronal degeneration. In fact, no alterations were observed in the granular layers of the CA of the hippocampus. Furthermore, a reduction in the intensity of damage-derived neurogenic activity was observed, thus reducing the probability for the appearance of a chronic TLE state (*Mathern GW et al., 1993*). This situation also correlated with a reduction of proapoptotic molecules like BAX, BIM and cleaved products of α-spectrin and, importantly, with a significant upregulation of the anti-apoptotic protein BCL-2.

Finally, results also pointed to a significant reduction in the reactive state of both astrocytes and microglia, a consequence of the inhibition of JNK1, kinase responsible for the activation of these cells (*Solinas G and Becattini B, 2017*).

Regarding the studies on the metabolic-cognitive syndrome, previous data by the team of Dr. Antoni Camins (Department of Pharmacology, Toxicology and Therapeutic Chemistry, Pharmacy and Food Sciences Faculty; University of Barcelona) and Dr. Jaume Folch (Department of Biochemistry and Biotechnology, Medicine and Health Sciences Faculty; University Rovira i Virgili), suggested that

dysregulations in normal metabolism have a significant role in the aetiology and development of brain cognitive affectations and neurodegenerative processes. Specifically, studies with the APPswe/PD1dE9 familial AD mice model demonstrated that a chronic feeding with HFD worsened and accelerated the pathological outcome (*Ettcheto M et al, 2016 and 2018*).

That is why this thesis aimed to evaluate the consequences of metabolic dysregulations induced by HFD in the brain of *wild-type* mice. Also, it focused on determining the outcome of a genetic ablation of isoforms JNK1 and JNK2.

Publication II: *Long-term exposition to a high fat diet favours the appearance of β-amyloid depositions in the brain of C57BL/6J mice. A potential model of sporadic Alzheimer's disease.*

Metabolic dysregulations induced by obesity have been proven to affect negatively the brain (*Dmitry P et al., 2015; Zhigang L et al., 2015; Kullmann S et al., 2016; Cardoso S et al., 2019*). In *Publication II*, the aim was to pinpoint some of the mechanisms involved in this alteration, and demonstrate the similarity of these to some of the features described as part of the pathophysiology of AD.

The first result that was presented was the detection of β-amyloid accumulations in the brain of *wild-type* mice that had been chronically fed with HFD (16 months). Similar observations had been previously reported by another research group, which also detected alterations in the normal rates of phosphorylation of the TAU protein, just like

reductions in the abundance of the IR in the hippocampus (*Nuzzo D et al., 2015*). The appearance of this last alteration together with such classical hallmarks of the pathophysiology of AD was in agreement by the ideas postulated in the metabolic hypothesis of this disease. Nonetheless, this data was also an indicator of the dysregulated state of other cellular mechanisms.

Correspondingly, it was observed that the chronic feeding with HFD caused an increase in the reactive state of both astrocytes and microglia, which prompted a neuroinflammatory response. As it has been previously mentioned, the development of a reactive state in these cells causes the release of cytokines and other substances, affecting the surrounding tissue and promoting its dysregulation and eventual degeneration (*de Felice F et al. 2014; Hotamisligil GS and Davis RJ, 2016; Kullmann S et al., 2016; Kaneko M et al., 2017*). In the present research, one of the observed consequences of this state was a reduction in the number of progenitor neuronal cells (nestin-positive cells) found in the DG of the hippocampus.

Another alteration observed in this study was a reduction in the abundance of proteins linked to autophagy, a mechanism necessary for the maintenance of normal physiological activity through the recycling and degradation of damaging or long-lived biomolecules (*Ravanan P et al., 2017*). A dysregulation of this cellular process might explain the increased concentration of soluble β-amyloid found in the brain of animals chronically fed with HFD (*Wakabayashi T et al., 2019*) and also, for its eventual precipitation and accumulation in the form of senile plaques as observed in this study.

Publication III: *Role of brain c-Jun N-terminal Kinase 2 in the control of the insulin receptor and its relationship with cognitive performance in a high-fat diet preclinical model.*

The development of insulin resistance has been associated with the appearance of cognitive dysfunction (*Henneberg N and Hoyer S, 1995; Ott A et al., 1999; de Felice F et al. 2014; Steen E et al., 2005; de la Monte SM and Wands JR, 2008*). It is believed that this alteration derives of a downregulation of both peripheral and brain metabolism and, that the JNKs have a role in the regulation of this processes (*Hotamisligil GS and Davis RJ, 2016*). Importantly, each of the isoforms responds differently, controlling separate systems and pathways (*Cui J et al., 2007; Grivennikov S et al., 2007; Solinas G and Karin M, 2010; Sabapathy K, 2012; Koch P et al., 2015; Vernia S et al., 2016*). Thus, in *Publication III* the aim was to determine the effects of whole-body ablation of JNK2, coupled with a chronic feeding with HFD (9 months) on hippocampal insulin sensitivity and cognitive performance.

The first assay in this publication focused on determining the state of activation of the JNKs. It was observed that a chronic feeding of HFD, and the subsequent obesity and metabolic syndrome conditions, caused significant activation of these kinases (*Ip YT and Davis RJ, 1998; Hirosumi J et al., 2002*). Furthermore, it was observed that lack of JNK2 also caused an increase of total JNK activity, even when the animals were not exposed to HFD, indicating to the existence of some kind of regulatory mechanism between the isoforms.

Moreover, animals that had been exposed to the HFD, lacked JNK2 or had both variables together, showed reduced tolerance to glucose and sensibility to insulin action. It was determined that this situation was derived, in part, from an increased inhibitory activity of the JNKs, coupled with an increased abundance of molecules like PTP1B and SOCS3, inhibitors of the signalling capacity of the IR and its substrates (*Emanuelli B et al., 2000; Haeusler R et al., 2018*). Also, these responses were upregulated by an activation of the UPR in the endoplasmic reticulum and an increased inflammatory state (*Panzhinskiy E et al., 2013; Hotamisligil GS and Davis RJ, 2016*). Surprisingly, animals that had been knocked out for the expression of JNK2 showed no increased astrocyte or microglial reactivity when compared to the control, even when fed HFD. This result would be in accordance with published data that demonstrates that the JNKs are required for the polarization of the M1 microglia subtype (proinflammatory) (*Hotamisligil GS and Davis RJ, 2016*). There is also evidence on increased polarization towards the M2 subtype (antiinflammatory) when the JNKs are inhibited but, in our results, this response was not reproduced. There is a possibility that this response derives only of the regulation of another isoform.

Finally, behavioural tests determined that all experimental groups showed cognitive affectations during the novel object recognition test (NORT) when compared against the control. This correlated with a reduction in the number of dendritic spines found in neuronal dendrite arborisations and with the decrease on the levels of proteins like spinophilin and PSD95.

Publication IV: *c-Jun N-terminal Kinase 1 ablation protects against metabolic-induced hippocampal cognitive impairments.*

Following up from the results presented in *Publication III*, in Publication *IV* we analysed the effects of the knockout of JNK1 in the same experimental model of HFD (7 months).

In accordance to published data, animals that lacked JNK1 showed lower body weight, normal tolerance to glucose and increased sensitivity to insulin in the periphery, as well as protection against the effects of HFD (*Cui J et al., 2007; Grivennikov S et al., 2007; Solinas G and Karin M, 2010; Sabapathy K, 2012; Koch P et al., 2015*). Furthermore, when analysing protein extracts from the hippocampus of these animals, it was clear that the knockout of JNK1 caused for an increased activation of the insulin signalling pathway in this tissue. These results were backed up with the observation of a significant decrease in the reactive state of astrocytes and microglia in the dentate gyrus of the hippocampus, in accordance with previous publications (*Solinas G and Becattini B, 2017*).

Moreover, when analysing the state of mitochondria, it was confirmed that HFD reduced the oxidative capacity of this organelle and, that the diet favoured a decrease in the levels of antioxidant enzymes, thus favouring an increased production and accumulation of ROS (*García-Ruiz I et al., 2014*). In animals lacking JNK1, it was observed how there was an increased abundance of mitochondrial molecules (OXPHOS complexes and antioxidant enzymes), pointing to a high and efficient activity of this organelle. Also, a decrease in the levels of 4-hidroxinonenal (4-HNE) pointed to a reduction on the

accumulation of peroxidation products derived of lipid oxidation, elements highly damaging for cells (*Pillon NJ et al., 2012*).

Next, data regarding the behavioural and cognitive state of animals was analysed. Results from the open field test and NORT led to the conclusion that the ablation of JNK1 significantly reduced anxiety levels (*Mohammad H et al., 2018*), and increased motor activity, as well as protected animals from the development of cognitive affectations derived of a chronic feeding with HFD. This conclusion was backed up by the analysis of levels of multiple proteins linked to the maintenance of synaptic plasticity and cognitive function like BDNF, spinophilin or the Activity-Regulated Cytoskeleton-associated protein (ARC), as well as by the evaluation of the state and number of dendritic spines and the detection of the abundance of proteins like neuroligin and neurexin in the CA3 from the hippocampus. These results were also confirmed by an increase in the levels of enzymes responsible of the inhibition of the activity of cofilin, a known destabilizing element of microtubules in synaptic structures (*Shaw AE et al., 2017*). Finally, previous research has determined that reactive microglial cells are sensible to phagocytise synaptic structures (*Hao S et al., 2016*). The previously mentioned result on the reduced reactivity of these cells would also be evidence of the amelioration obtained through the ablation of JNK1.

With all these results in mind, it is our belief that the JNKs play a significant role in the development of neurodegenerative pathology. Also, that the inhibition of JNK1 seems to be a very promising therapeutic strategy. That is why we propose that a treatment with LIC-A would be an effective strategy for TLE and cases of metabolic-

cognitive syndrome (**Figure 9**). In fact, published data by Liou and colleagues already hints to the truth behind this idea. Their research demonstrates that a treatment with LIC-A is able to ameliorate conditions like obesity and hepatic non-alcoholic fatty liver disease in a model of HFD (*Liou, C et al., 2019*). Furthermore, the use of LIC-A would bypass other side effects derived of a complete inhibition of JNK1 activity, like isoform activity compensation, dysregulated physiological mechanisms or the appearance of skin oxidative stress as reported by Becattini B (*Becattini B et al., 2016*).

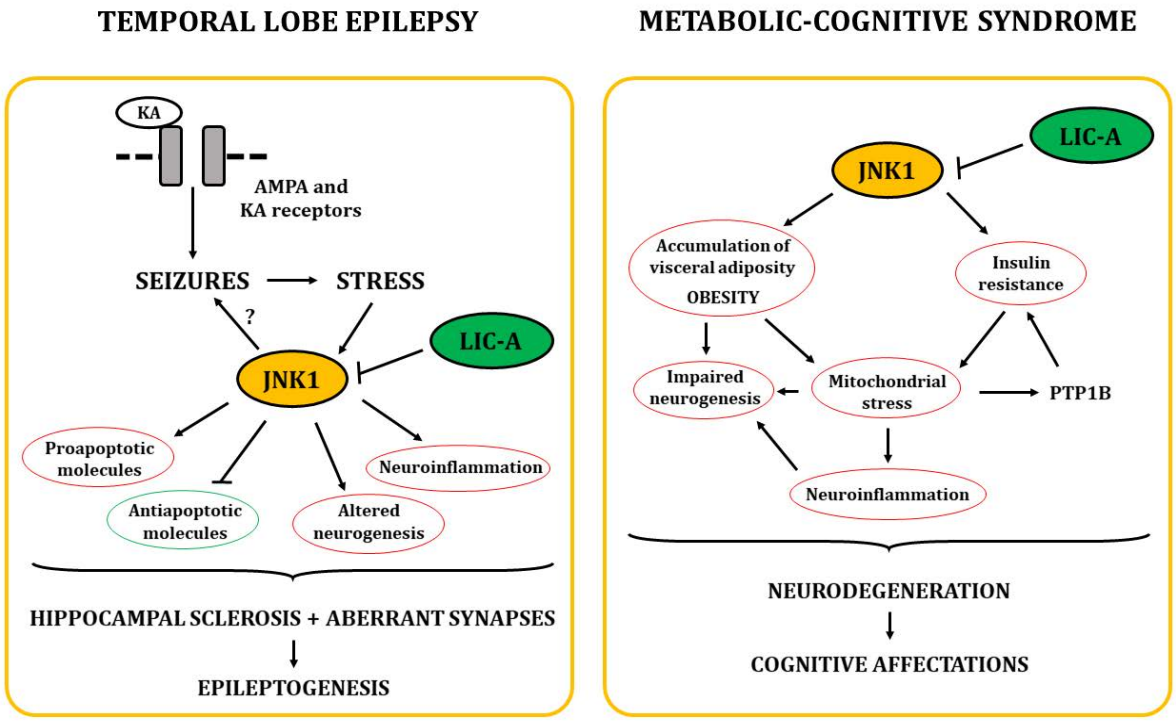


Figure 9. Final chart describing the pathways regulated by JNK1 and their involvement in the development of TLE and the metabolic-cognitive syndrome. Also, the mechanism of action of LIC-A is represented.

Finally, the use of encapsulated nanoparticles of LIC-A could also be an interesting improvement to this therapeutic proposal (higher bioavailability, targeted treatment, lower hepatic first pass effect and toxicity…), as it has been demonstrated in our group in previously reported research (*Sánchez-López E et al., 2016, 2017 and 2018; Cano A et al., 2018 and 2019*).

Conclusions

CONCLUSIONS

The main conclusions derived of the present doctoral thesis are:

1. The inhibition of JNK1 by LIC-A is an effective preventive neuroprotective strategy against the effects of KA. The observed beneficial effects were:
    - Lower seizure severity.
    - Absence of any degenerating cells or sclerotic areas in the hippocampus after the KA insult. Also, lower levels of pro-apoptotic proteins and higher anti-apoptotic.
    - Reduced insult-response neurogenesis.
    - Decreased reactivity in astrocytes and microglia.

Overall, the results point to an improved prognosis of the pathology and a lower chance for the appearance of a chronic epileptic state.

---

2. Chronic feeding with HFD has severe negative consequences on normal physiology. The studies have revealed that it causes:
    - Increased fat accumulation and release of stress molecules, as well as mitochondrial and endoplasmic reticulum stress and higher reactivity in astrocytes and microglia.
    - Higher production of β-amyloid, leading to its eventual accumulation in the form of senile plaques.
    - Appearance of phenomena of glucose intolerance and insulin resistance as a result of increased activation of the JNKs and other inhibitory molecules like PTP1B and SOCS3.

- Decreased neurogenic activity, coupled with reduced autophagy and anti-apoptotic proteins.
- Reduced cognitive performance in the NORT.

In the end, there is enough evidence to conclude that all these alterations favour the appearance of cognitive affectations derived of significant dysregulations in normal metabolism and physiology.

3. The knockout of JNK2 is detrimental for the maintenance of proper metabolic and cognitive activity. The consequences were:
   - Lower tolerance for glucose and appearance of insulin resistance situations both in the periphery and central area.
   - Increased endoplasmic reticulum stress.
   - Reduced cognitive performance and abundance of proteins like spinophilin and PSD95, as well as a decrease in dendritic spines in neurons of the DG of the hippocampus.

These results are a demonstration that the JNK isoforms have, in some occasions, different roles and behaviours in the cell. This conclusion is an evidence of the importance of targeting single isoforms for therapeutic approaches in order to avoid negative outcomes derived of an unspecific modulation.

4. The inactivation of JNK1 favours the maintenance of proper metabolic and cognitive activity, and confers resistance against alterations derived of chronic HFD feeding. There was:
   - Lower body weight, normal glucose tolerance and increased sensitivity to insulin.
   - Reduced cellular reactivity in astrocytes and microglia.

- Enhanced mitochondrial activity.
- Decreased anxiety, higher motor activity and preserved cognitive function even when chronically exposed to HFD. There was also higher abundance of certain proteins linked to proper cognitive function like BDNF and ARC.

This data points to the significant role of JNK1 in the development of pathology and how, its negative modulation can become an effective therapeutic approach.

Bibliography

BIBLIOGRAPHY

A

Al Haj Ahmad RM, Al-Domi HA. Thinking about brain insulin resistance. Diabetes Metab Syndr Clin Res Rev. 2018;12(6):1091–4.

Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial Metabolism of Reactive Oxygen Species. Biochem. 2005;70(2):200–14.

Auladell C, de Lemos L, Verdaguer E, Ettcheto M, Busquets O, Lazarowski A, et al. Role of JNK isoforms in the kainic acid experimental model of epilepsy and neurodegeneration. PubMed Commons. Front Biosci. 2017; 22:795–814.

B

Becattini B, Zani F, Breasson L, Sardi C, D'Agostino VG, Choo MK, et al. JNK1 ablation in mice confers long-term metabolic protection from diet-induced obesity at the cost of moderate skin oxidative damage. FASEB J. 2016;30(9):3124–32.

Belgardt BF, Mauer J, Wunderlich FT, Ernst MB, Pal M, Spohn G, et al. Hypothalamic and pituitary c-Jun N-terminal kinase 1 signalling coordinately regulates glucose metabolism. Proc Natl Acad Sci. 2010;107(13):6028–33.

Ben-Ari Y, Lagowska J, Tremblay E, Le Gal G, Salle LA. A new model of focal status epilepticus: intra-amygdaloid application of kainic acid elicits repetitive secondarily generalized convulsive seizures. Brain Research. 1979; 163(1):176-179.

Biessels GJ, Reagan LP. Hippocampal insulin resistance and cognitive dysfunction. Nat Rev Neurosci. 2015;16(11):660–71.

Bilotta F, Lauretta MP, Tewari A, Haque M, Hara N, Uchino H, et al. Insulin and the Brain: A Sweet Relationship with Intensive Care. J Intensive Care Med. 2017;32(1):48–58.

Bogoyevitch MA, Arthur PG. Inhibitors of c-Jun N-terminal kinases-JuNK no more? Biochimica et Biophysica Acta - Proteins and Proteomics. 2008; 1784 (1):76–93.

Borsello T. The JNK signalling transduction pathway in the brain. Front Biosci. 2012; E4(6):2110–20.

Boucher J, Kleinridders A, Ronald Kahn C. Insulin receptor signalling in normal and insulin-resistant states. Cold Spring Harb Perspect Biol. 2014;6(1).

Boveris A. Determination of the Production of Superoxide Radicals and Hydrogen Peroxide in Mitochondria. Methods Enzymol. 1984;105(C):429–35.

C

Cano A, Ettcheto M, Chang JH, Barroso E, Espina M, Kühne BA, et al. Dual-drug loaded nanoparticles of Epigallocatechin-3-gallate (EGCG)/Ascorbic acid enhance therapeutic efficacy of EGCG in a APPswe/PS1dE9 Alzheimer's disease mice model. J Control Release. 2019; 301:62–75.

Cano A, Ettcheto M, Espina M, Auladell C, Calpena AC, Folch J, et al. Epigallocatechin-3-gallate loaded PEGylated-PLGA nanoparticles: A new anti-seizure strategy for temporal lobe epilepsy. Nanomedicine Nanotechnology, Biol Med. 2018;14(4):1073–85.

Cardoso S, Moreira PI. Diabesity and brain disturbances: A metabolic perspective. Mol Aspects Med. 2019; 66;71-79.

Cargnello M, Roux PP. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. Microbiol Mol Biol Rev. 2011;75(1):50–83.

Castro-Torres RD, Landa J, Rabaza M, Busquets O, Olloquequi J, Ettcheto M, et al. JNK Isoforms Are Involved in the Control of Adult Hippocampal Neurogenesis in Mice, Both in Physiological Conditions and in an Experimental Model of Temporal Lobe Epilepsy. Mol Neurobiol. 2019; 56 (8): 5856-5865.

Cavalheiro EA, Riche DA, Le Gal La Salle G. Long-term effects of intrahippocampal kainic acid injection in rats: A method for inducing spontaneous recurrent seizures. Electroencephalogr Clin Neurophysiol. 1982;53(6):581–9.

Chambers JW, LoGrasso P V. Mitochondrial c-Jun N-terminal Kinase (JNK) signalling initiates physiological changes resulting in amplification of reactive oxygen species generation. J Biol Chem. 2011;286(18):16052–62.

Chatzikonstantinou A. Epilepsy and the hippocampus. Hippocampus Clin Neurosci. 2014; 34:121–42.

Cherian A, Sanjeev T. Status epilepticus. Ann Indian Acad Neurol. 2009;12(3):140–53.

Coffey ET. Nuclear and cytosolic JNK signalling in neurons. Nat Rev Neurosci. 2014;15(5):285–99.

Coffey ET, Courtney MJ. Regulation of SAPKs in CNS neurons. Biochem Soc Trans. 1997;25(4):568.

Copps KD, White MF. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia. 2012; 55(10):2565–82.

Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. Biochem J. 2010;429(3):403–17.

Cui J, Zhang M, Zhang YQ, Xu ZH. JNK pathway: Diseases and therapeutic potential. Acta Pharmacol Sin. 2007;28(5):601–8.

D

Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell. 2000;103(2):239–52.

De Araujo Furtado M, Lumley LA, Robison C, Tong LC, Lichtenstein S, Yourick DL. Spontaneous recurrent seizures after status epilepticus induced by soman in Sprague-Dawley rats. Epilepsia. 2010;51(8):1503–10.

De Felice FG, Lourenco M V., Ferreira ST. How does brain insulin resistance develop in Alzheimer's disease? Alzheimer's Dement. 2014;10(1 SUPPL.): S26–32.

de la Monte SM, Wands JR. Alzheimer's disease is type 3 diabetes-evidence reviewed. J Diabetes Sci Technol. 2008;2(6):1101–13.

de Lemos L, Junyent F, Camins A, Castro-Torres RD, Folch J, Olloquequi J, et al. Neuroprotective Effects of the Absence of JNK1 or JNK3 Isoforms on Kainic Acid-Induced Temporal Lobe Epilepsy-Like Symptoms. Mol Neurobiol. 2018;55(5):4437–52.

Delikkaya B, Moriel N, Tong M, Gallucci G, de la Monte SM. Altered expression of insulin-degrading enzyme and regulator of calcineurin in the rat intracerebral streptozotocin model and human apolipoprotein E-ε4–associated Alzheimer's disease. Alzheimer's Dement Diagnosis, Assess Dis Monit. 2019; 11:392–404.

Després J-P, Lemieux I. Abdominal obesity and metabolic syndrome. Nature. 2006; 444:1–7.

Dhanasekaran DN, Reddy EP. JNK signalling in apoptosis. Oncogene. 2008;27(48):6245–51.

Dodd GT, Tiganis T. Insulin action in the brain: Roles in energy and glucose homeostasis. J Neuroendocrinol. 2017;29(10):1–14.

Dudek EF. Role of glial cells in seizures and epilepsy: intracellular calcium oscillation and spatial buffering. Epilepsy Curr. 2002;2(4):137–9.

Duque-Guimarães DE, Ozanne SE. Nutritional programming of insulin resistance: Causes and consequences. Trends in Endocrinology and Metabolism. 2013; 24(10):525-535.

E

Emanuelli B, Peraldi P, Filloux C, Sawka-Verhelle D, Hilton D, Van Obberghen E. SOCS-3 is an insulin-induced negative regulator of insulin signalling. J Biol Chem. 2000;275(21):15985–91.

Engin A. Obesity and Lipotoxicity: The Definition and Prevalence of Obesity and Metabolic Syndrome. Adv Exp Med Biol. 2017;960: 1-17.

Ettcheto M, Sánchez-López E, Gómez-Mínguez Y, Cabrera H, Busquets O, Beas-Zarate C, et al. Peripheral and Central Effects of Memantine in a Mixed Preclinical Mice Model of Obesity and Familial Alzheimer's Disease. Mol Neurobiol. 2018;55(9):7327–39.

Ettcheto M, Petrov D, Pedros I, Alva N, Carbonell T, Beas-Zarate C, et al. Evaluation of neuropathological effects of a high-fat diet in a presymptomatic Alzheimer's disease stage in APP/PS1 mice. J Alzheimer's Dis. 2016;54(1):233–51.

F

Federico A, Cardaioli E, Da Pozzo P, Formichi P, Gallus GN, Radi E. Mitochondria, oxidative stress and neurodegeneration. J Neurol Sci. 2012;322(1–2):254–62.

Fels DR, Koumenis C. The PERK/eIF2α/ATF4 module of the UPR in hypoxia resistance and tumour growth. Cancer Biology and Therapy. 2006; 5(7):723–728.

G

García-Ruiz I, Solís-Muñoz P, Fernández-Moreira D, Grau M, Colina F, Muñoz-Yagüe T, et al. High-fat diet decreases activity of the oxidative phosphorylation complexes and causes non-alcoholic steatohepatitis in mice. Dis Model Mech. 2014;7(11):1287–96.

Gardai SJ, Hildeman DA, Frankel SK, Whitlock BB, Frasch SC, Borregaard N, et al. Phosphorylation of Bax ser184 by AKT regulates its activity and apoptosis in neutrophils. J Biol Chem. 2004 May 14;279(20):21085–95.

Giavina-Bianchi P, Giavina-Bianchi M, Tanno LK, Ensina LFC, Motta AA, Kalil J. Epileptic seizure after treatment with thiocolchicoside. Ther Clin Risk Manag. 2009;5(1):635–7.

Graczyk P. JNK inhibitors as anti-inflammatory and neuroprotective agents. Futur Med Chem. 2013;5(5):539–51.

Gralle M. The neuronal insulin receptor in its environment. Vol. 140, Journal of Neurochemistry. Blackwell Publishing Ltd; 2017. p. 359–67.

Green DR, Llambi F. Cell death signalling. Cold Spring Harb Perspect Biol. 2015;7(12):1–24.

Grillo CA, Tamashiro KL, Piroli GG, Melhorn S, Gass JT, Newsom RJ, et al. Lentivirus-mediated downregulation of hypothalamic insulin receptor expression. Physiol Behav. 2007;92(4):691–701.

Grivennikov S, Vilcu C, Naugler W, Wynshaw-Boris A, Solinas G, Luo J-L, et al. JNK1 in Hematopoietically Derived Cells Contributes to Diet-Induced Inflammation and Insulin Resistance without Affecting Obesity. Cell Metab. 2007;6(5):386–97.

Guan QH, Pei DS, Liu XM, Wang XT, Xu T Le, Zhang GY. Neuroprotection against ischemic brain injury by SP600125 via suppressing the extrinsic and intrinsic pathways of apoptosis. Brain Res. 2006;1092(1):36–46.

Guma M, Ronacher LM, Firestein GS, Karin M, Corr M. JNK-1 deficiency limits macrophage-mediated antigen-induced arthritis. Arthritis Rheum. 2011;63(6):1603–12.

H

Haas I, Wabl M. Immunoglobulin heavy chain binding protein. Nature. 1983;306(5941):387–9.

Haeusgen W, Boehm R, Zhao Y, Herdegen T, Waetzig V. Specific activities of individual c-Jun N-terminal kinases in the brain. Neuroscience. 2009;161(4):951–9.

Haeusler RA, McGraw TE, Accili D. Metabolic Signalling: Biochemical and cellular properties of insulin receptor signalling. Vol. 19, Nature Reviews Molecular Cell Biology. Nature Publishing Group; 2018; 19(1):31–44.

Hamilton SE, Loose MD, Qi M, Levey AI, Hille B, Mcknight GS, et al. Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc Natl Acad Sci USA. 1997;94(24):13311–6.

Hanawa N, Shinohara M, Saberi B, Gaarde WA, Han D, Kaplowitz N. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. J Biol Chem. 2008;283(20):13565–77.

Hao S, Dey A, Yu X, Stranahan AM. Dietary obesity reversibly induces synaptic stripping by microglia and impairs hippocampal plasticity. Brain Behav Immun. 2016; 51:230–9.

Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell. 2000;5(5):897–904.

Henneberg N, Hoyer S. Desensitization of the neuronal insulin receptor: a new approach in the etiopathogenesis of late-onset sporadic dementia of the Alzheimer type (SDAT)? Arch Gerontol Geriatr. 1995;21(1):63–74.

Henshall D. Apoptosis signaling pathways in seizure-induced neuronal death and epilepsy. Biochem Soc Trans. 2007;35(2):421–3.

Hill JM, Lesniak MA, Pert CB, Roth J. Autoradiographic localization of insulin receptors in rat brain: Prominence in olfactory and limbic areas. Neuroscience. 1986;17(4):1127–38.

Hiragi T, Ikegaya Y, Koyama R. Microglia after Seizures and in Epilepsy. Cells. 2018;7(4):26.

Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, et al. A central role for JNK in obesity and insulin resistance. Nature. 2002;420(6913):333–6.

Holmes G, Thompson J. Effects of kainic acid on seizure susceptibility in the developing brain. Brain Res. 1988;467(1):51–9.

Hotamisligil GS, Davis RJ. Cell signaling and stress responses. Cold Spring Harb Perspect Biol. 2016;8(10):1–20.

I

Ip YT, Davis RJ. Signal transduction by the c-Jun N-terminal kinase (JNK) - from inflammation to development. Curr Opin Cell Biol. 1998; 10:205–19.

K

Kamenecka T, Jiang R, Song X, Duckett D, Chen W, Ling YY, et al. Synthesis, biological evaluation, X-ray structure, and pharmacokinetics of aminopyrimidine c-jun-N-terminal kinase (JNK) inhibitors. J Med Chem. 2010;53(1):419–31.

Kaminska B, Gozdz A, Zawadzka M, Ellert-Miklaszewska A, Lipko M. MAPK signal transduction underlying brain inflammation and gliosis as therapeutic target. Anatomical Record. 2009; 292(12):1902–13.

Kaneko M, Imaizumi K, Saito A, Kanemoto S, Asada R, Matsuhisa K, et al. ER Stress and Disease: Toward Prevention and Treatment. Biol Pharm Bull Pharm Bull. 2017;40(9):1337–43.

Kaneto H, Matsuoka TA, Nakatani Y, Kawamori D, Miyatsuka T, Matsuhisa M, et al. Oxidative stress, ER stress, and the JNK pathway in type 2 diabetes. J Mol Med. 2005;83(6):429–39.

Koch P, Gehringer M, Laufer SA. Inhibitors of c-Jun N-terminal kinases: An update. J Med Chem. 2015;58(1):72–95.

Koekkoek PS, Kappelle LJ, van den Berg E, Rutten GEHM, Biessels GJ. Cognitive function in patients with diabetes mellitus: Guidance for daily care. Lancet Neurol. 2015;14(3):329–40.

Krentz A j. Insulin resistance. BMJ. 1996;313(7069):1385.

Kroner Z. The relationship between Alzheimer's disease and diabetes: Type 3 diabetes? Altern Med Rev. 2009;14(4):373–9.

Kullmann S, Heni M, Hallschmid M, Fritsche A, Preissl H, Häring HU. Brain insulin resistance at the crossroads of metabolic and cognitive disorders in humans. Physiol Rev. 2016;96(4):1169–209.

L

Langer M, Brandt C, Löscher W. Marked strain and substrain differences in induction of status epilepticus and subsequent development of neurodegeneration, epilepsy, and behavioural alterations in rats. Strain and substrain differences in an epilepsy model in rats. Epilepsy Res. 2011;96(3):207–24.

Lee JK, Park J, Lee YD, Lee SH, Han PL. Distinct localization of SAPK isoforms in neurons of adult mouse brain implies multiple signalling modes of SAPK pathway. Mol Brain Res. 1999;70(1):116–24.

Lee J, Pilch PF. The insulin receptor: structure, function, and signalling. Am J Physiol Cell Physiol. 1994; 266:319–34.

Lévesque M, Avoli M. The kainic acid model of temporal lobe epilepsy. Neurosci Biobehav Rev. 2016; 37:2887–99.

Lévesque M, Avoli M, Bernard C. Animal models of temporal lobe epilepsy following systemic chemoconvulsant administration. J Neurosci Methods. 2016; 260:45–52.

Liang L, Chen J, Zhan L, Lu X, Sun X, Sui H, et al. Endoplasmic reticulum stress impairs insulin receptor signalling in the brains of obese rats. PLoS One. 2015;10(5):1–14.

Lin JH, Walter P, Yen TSB. Endoplasmic Reticulum Stress in Disease Pathogenesis. Annu Rev Pathol Mech Dis. 2008; 3:399-425.

Liou C-J, Lee Y-K, Ting N-C, Chen Y-L, Shen S-C, Wu S-J, et al. Protective Effects of Licochalcone A Ameliorates Obesity and Non-Alcoholic Fatty Liver Disease Via Promotion of the Sirt-1/AMPK Pathway in Mice Fed a High-Fat Diet. Cells. 2019;8(5):447.

Liu G, Guo H, Guo C, Zhao S, Gong D, Zhao Y. Involvement of IRE1α signalling in the hippocampus in patients with mesial temporal lobe epilepsy. Brain Res Bull. 2011;84(1):94–102.

Liu Z, Patil IY, Jiang T, Sancheti H, Walsh JP, Stiles BL, et al. High-fat diet induces hepatic insulin resistance and impairment of synaptic plasticity. PLoS One. 2015;10(5):1–16.

M

Ma L, Wang J, Li Y. Insulin resistance and cognitive dysfunction. Clin Chim Acta. 2015; 444:18–23.

Madhumita D, Sabio G, Jiang F, Rincón M, Flavell RA, Davis RJ. Induction of hepatitis by JNK-mediated expression of TNFα. Cell. 2009;136(2):249–60.

Malhotra JD, Kaufman RJ. The endoplasmic reticulum and the unfolded protein response. Vol. 18, Seminars in Cell and Developmental Biology. Elsevier Ltd; 2007;18(6):716–31.

Mao LM, Wang JQ. Synaptically Localized Mitogen-Activated Protein Kinases: Local Substrates and Regulation. Vol. 53, Molecular Neurobiology. Humana Press Inc.; 2016;53(9):6309–6315.

Marchi N, Oby E, Batra A, Uva L, De Curtis M, Hernandez N, et al. In vivo and in vitro effects of pilocarpine: Relevance to ictogenesis. Epilepsia. 2007;48(10):1934–46.

Martindale JL, Holbrook NJ. Cellular response to oxidative stress: Signalling for suicide and survival. J Cell Physiol. 2002;192(1):1–15.

Mathern GW, Cifuentes F, Leite JP, Pretorius JK, Babb TL. Hippocampal EEG excitability and chronic spontaneous seizures are associated with aberrant synaptic reorganization in the rat intrahippocampal kainate model. Electroencephalogr Clin Neurophysiol. 1993;87(5):326–39.

McCracken E, Monaghan M, Sreenivasan S. Pathophysiology of the metabolic syndrome. Clin Dermatol. 2018;36(1):14–20.

Mcintyre DC, Leech CK. A Permanent Change in Brain Function Resulting from Daily Electrical Stimulation. Exp Neurol. 1969; 25:295–330.

Mendelson KG, Contois L-R, Tevosian SG, Davis RJ, Paulson KE. Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. Proc Natl Acad Sci. 1996;93(23):12908–13.

Mittal K, Mani RJ, Katare DP. Type 3 Diabetes: Cross Talk between Differentially Regulated Proteins of Type 2 Diabetes Mellitus and Alzheimer's Disease. Sci Rep. 2016; 6:1–8.

Mohammad H, Marchisella F, Ortega-Martinez S, Hollos P, Eerola K, Komulainen E, et al. JNK1 controls adult hippocampal neurogenesis and imposes cell-autonomous control of anxiety behaviour from the neurogenic niche. Mol Psychiatry. 2018;23(2):362–74.

Muller FL, Liu Y, Van Remmen H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. J Biol Chem. 2004;279(47):49064–73.

Murata Y, Fujiwara N, Seo JH, Yan F, Liu X, Terasaki Y, et al. Delayed Inhibition of c-Jun N-Terminal Kinase Worsens Outcomes after Focal Cerebral Ischemia. J Neurosci. 2012;32(24):8112–5.

N

Nadler JV. Kainic acid: neurophysiological and neurotoxic actions. Life Sci. 1979;24(4):289–99.

Nandi A, Kitamura Y, Kahn CR, Accili D. Mouse Models of Insulin Resistance. Physiol Rev. 2004;84(2):623–47.

Nirwan N, Vyas P, Vohora D. Animal models of status epilepticus and temporal lobe epilepsy: A narrative review. Rev Neurosci. 2018;29(7):757–70.

Nishitoh H. CHOP is a multifunctional transcription factor in the ER stress response. Journal of Biochemistry. 2012;151(3):217–219.

Nuzzo D, Picone P, Baldassano S, Caruana L, Messina E, Gammazza A, et al. Insulin Resistance as Common Molecular Denominator Linking Obesity to Alzheimer's Disease. Curr Alzheimer. 2015;12(8):723–35.

O

O'Neill S, O'Driscoll L. Metabolic syndrome: A closer look at the growing epidemic and its associated pathologies. Obes Rev. 2015;16(1):1–12.

Onyango AN. Cellular Stresses and Stress Responses in the Pathogenesis of Insulin Resistance. Vol. 2018, Oxidative Medicine and Cellular Longevity. Hindawi Limited; 2018; 4321714.

Osborn O, Olefsky JM. The cellular and signalling networks linking the immune system and metabolism in disease. Nat Med. 2012; 18:363–374.

Ott A, Stolk RP, van Harskamp F, Pols HAP, Hofman A, Breteler MMB. Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology. 1999;53(9):1937–1937.

Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. Apoptosis. 2007;12(5):913–22.

Ozcan L, Ergin AS, Lu A, Chung J, Sarkar S, Nie D, et al. Endoplasmic reticulum stress plays a central role in development of leptin resistance. Cell Metab. 2009;9(1):35–51.

Özcan, U., Cao, Q., Yilmaz, E., Lee, A.H., Iwakoshi, N.N., Özdelen, E., Tuncman, G., Görgün, C., Glimcher, L.H. and Hotamisligil GS, Ozcan U, Cao Q, Yilmaz E, Lee A-H, Iwakoshi NN, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science. 2004;306(5695):457–61.

P

Panzhinskiy E, Ren J, Nair S. Protein Tyrosine Phosphatase 1B and Insulin Resistance: Role of Endoplasmic Reticulum Stress/Reactive Oxygen Species/Nuclear Factor Kappa B Axis. PLoS One. 2013;8(10).

Pelloux V, Lahlou N, Cabrol S, Bougnères P, Dina C, Froguel P, et al. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature. 2002;392(6674):398–401.

Petrov D, Pedrós I, Artiach G, Sureda FX, Barroso E, Pallàs M, et al. High-fat diet-induced deregulation of hippocampal insulin signalling and mitochondrial homeostasis deficiencies contribute to Alzheimer disease pathology in rodents. Biochim Biophys Acta - Mol Basis Dis. 2015;1852(9):1687–99.

Phillips MJ, Voeltz GK. Structure and function of ER membrane contact sites with other organelles. Nat Rev Mol Cell Biol. 2016; 17:69–82.

Picard M, Taivassalo T, Gouspillou G, Hepple RT. Mitochondria: Isolation, structure and function. J Physiol. 2011;589(18):4413–21.

Pillon NJ, Croze ML, Vella RE, Soulère L, Lagarde M, Soulage CO. The lipid peroxidation by-product 4-hydroxy-2-nonenal (4-HNE) induces

insulin resistance in skeletal muscle through both carbonyl and oxidative stress. Endocrinology. 2012;153(5):2099–111.

Pinto DJ, Patrick SL, Huang WC, Connors BW. Initiation, propagation, and termination of epileptiform activity in rodent neocortex in vitro involve distinct mechanisms. J Neurosci. 2005;25(36):8131–40.

Plotnikov A, Zehorai E, Procaccia S, Seger R. The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. Biochim Biophys Acta - Mol Cell Res. 2011; 1813(9):1619–33.

Prilopko L (WHO). Epilepsy Care in the World. Epilepsy Health Atlas. 2005.

Puttachary S, Sharma S, Stark S, Thippeswamy T. Seizure-induced oxidative stress in temporal lobe epilepsy. BioMed Research International. Hindawi Limited; 2015; 745613.

R

Racine RJ. Modification of seizure activity by electrical stimulation: II. Motor seizure. Electroencephalogr Clin Neurophysiol. 1972; 31:281–94.

Raciti M, Lotti L V., Valia S, Pulcinelli FM, Di Renzo L. JNK2 is activated during ER stress and promotes cell survival. Cell Death Dis. 2012;3(11): e429-10.

Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. Oncogene. 2007;26(22):3100–12.

Rana A, Musto AE. The role of inflammation in the development of epilepsy. Vol. 15, Journal of Neuroinflammation. BioMed Central Ltd.; 2018; 15(1):144.

Ravanan P, Srikumar IF, Talwar P. Autophagy: The spotlight for cellular stress responses. Life Sci. 2017;188:53–67.

Reger MA, Watson GS, Frey WH, Baker LD, Cholerton B, Keeling ML, et al. Effects of intranasal insulin on cognition in memory-impaired older adults: Modulation by APOE genotype. Neurobiol Aging. 2006;27(3):451–8.

Reger MA, Watson GS, Green PS, Baker LD, Chorleton B, Fishel MA, et al. Intranasal insulin administration dose-dependently modulates verbal memory and plasma β-amyloid in memory-impaired older adults. J Alzheimers Dis. 2008;13(3):323–31.

Rejdak K, Nieoczym D, Czuczwar M, Kiś J, Wlaź P, Turski WA. Orphenadrine induces secondarily generalized convulsive status epilepticus in rats. Brain Res Bull. 2011;84(6):389–93.

Rochfort S, Towerzey L, Carroll A, King G, Michael A, Pierens G, et al. Latifolians A and B, novel JNK3 kinase inhibitors from the Papua New Guinean plant Gnetum latifolium. J Nat Prod. 2005;68(7):1080–2.

Ron D, Hubbard SR. How IRE1 Reacts to ER Stress. Cell. 2008; 132 (1):24–6.

Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007; 8:519–29.

S

Sabapathy K. Role of the JNK Pathway in human diseases. Progress in Molecular Biology and Translational Science. 2012;106:145–169.

Sabapathy K, Hochedlinger K, Nam SY, Bauer A, Karin M, Wagner EF. Distinct roles for JNK1 and JNK2 in regulating JNK activity and c-Jun-dependent cell proliferation. Mol Cell. 2004;15(5):713–25.

Sabio G, Davis RJ. CJun NH2-terminal kinase 1 (JNK1): Roles in metabolic regulation of insulin resistance. Trends Biochem Sci. 2010;35(9):490–6.

Sah SP, Singh B, Choudhary S, Kumar A. Animal models of insulin resistance: A review. Pharmacol Reports. 2016;68(6):1165–77.

Saltiel AR. Insulin Signalling in the Control of Glucose and Lipid Homeostasis. Metab Control. 2015; 233:51-71.

Sánchez-López E, Egea MA, Cano A, Espina M, Calpena AC, Ettcheto M, et al. PEGylated PLGA nanospheres optimized by design of experiments for ocular administration of dexibuprofen-in vitro, ex vivo and in vivo characterization. Colloids Surfaces B Biointerfaces. 2016; 145:241–50.

Sánchez-López E, Ettcheto M, Egea MA, Espina M, Calpena AC, Folch J, et al. New potential strategies for Alzheimer's disease prevention: pegylated biodegradable dexibuprofen nanospheres administration to APPswe/PS1dE9. Nanomedicine Nanotechnology, Biol Med. 2017;13(3):1171–82.

Sánchez-López E, Ettcheto M, Egea MA, Espina M, Cano A, Calpena AC, et al. Memantine loaded PLGA PEGylated nanoparticles for Alzheimer's disease: In vitro and in vivo characterization. J Nanobiotechnology. 2018;16(1):1–16.

Sangüesa G, Roglans N, Montañés JC, Baena M, Velázquez AM, Sánchez RM, et al. Chronic Liquid Fructose, but not Glucose, Supplementation Selectively Induces Visceral Adipose Tissue Leptin Resistance and Hypertrophy in Female Sprague-Dawley Rats. Mol Nutr Food Res. 2018;62(22):1–18.

Schauwecker PE. Seizure-induced neuronal death is associated with induction of c-Jun N-terminal kinase and is dependent on genetic background. Brain Res. 2000;884(1–2):116–28.

Schönthal AH. Endoplasmic Reticulum Stress: Its Role in Disease and Novel Prospects for Therapy. Scientifica (Cairo). 2012; 2012:1–26.

Schwarcz R, Brush GS, Foster AC, French ED. Seizure activity and lesions after intrahippocampal quinolinic acid injection. Exp Neurol. 1984;84(1):1–17.

Schwartz MW, Figlewicz DF, Kahn SE, Baskin DG, Greenwood MRC, Porte D. Insulin binding to brain capillaries is reduced in genetically obese, hyperinsulinemic Zucker rats. Peptides. 1990;11(3):467–72.

Shaw AE, Bamburg JR. Peptide regulation of cofilin activity in the CNS: A novel therapeutic approach for treatment of multiple neurological disorders. Pharmacology and Therapeutics. 2017; 175:17–27.

Solinas G, Becattini B. JNK at the crossroad of obesity, insulin resistance, and cell stress response. Mol Metab. 2017;6(2):174–84.

Solinas G, Karin M. JNK1 and IKKβ: molecular links between obesity and metabolic dysfunction. FASEB J. 2010;24(8):2596–611.

Steen E, Terry BM, J. Rivera E, Cannon JL, Neely TR, Tavares R, et al. Impaired insulin and insulin-like growth factor expression and signalling mechanisms in Alzheimer's disease – is this type 3 diabetes? J Alzheimer's Dis. 2005; 7:63–80.

Steinhäuser C, Grunnet M, Carmignoto G. Crucial role of astrocytes in temporal lobe epilepsy. Neuroscience. 2016; 323:157–69.

St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. J Biol Chem. 2002;277(47):44784–90.

Sun Y, Liu WZ, Liu T, Feng X, Yang N, Zhou HF. Signalling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J Recept Signal Transduct. 2015; 35(6):600–4.

Symonds ME, Sebert SP, Hyatt MA, Budge H. Nutritional programming of the metabolic syndrome. Nat Rev Endocrinol. 2009;5(11):604–10.

Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep. 2006;7(9):880–5.

T

Tai TY, Warner LN, Jones TD, Jung S, Concepcion FA, Skyrud DW, et al. Antiepileptic action of c-Jun N-terminal kinase (JNK) inhibition in an animal model of temporal lobe epilepsy. Neurosci. 2017; 349:35–47.

Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signalling. Am J Physiol Lung Cell Mol Physiol. 2000;279(6):1005–28.

Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, et al. Requirement of JNK for Stress- Induced Activation of the Cytochrome c–Mediated Death Pathway. Science. 2000; 288:870–4.

Tramoni-Negre E, Lambert I, Bartolomei F, Felician O. Long-term memory deficits in temporal lobe epilepsy. Revue Neurologique. 2017; 173(7-8): 490–7.

Turski WA, Schwarz M, Czuczwar SJ, Kleinrok Z, Turski L, Cavalheiro EA. Limbic seizures produced by pilocarpine in rats: Behavioural,

electroencephalographic and neuropathological study. Behav Brain Res. 1983;9(3):315–35.

U

Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, et al. Coupling of Stress in the ER to Activation of JNK protein kinases by Protein Kinase IRE1. Science. 2000;287(5453):664–6.

V

Ventura JJ, Hübner A, Zhang C, Flavell RA, Shokat KM, Davis RJ. Chemical genetic analysis of the time course of signal transduction by JNK. Mol Cell. 2006;21(5):701–10.

Vernia S, Morel C, Madara JC, Cavanagh-Kyros J, Barrett T, Chase K, et al. Excitatory transmission onto AgRP neurons is regulated by cjun NH2-terminal kinase 3 in response to metabolic stress. Elife. 2016; 5:1–18.

Vezzani A, French J, Bartfai T, Baram TZ. The role of inflammation in epilepsy. Nature Reviews Neurology. 2011; 7 (1):31–40.

Vincent P, Mulle C. Kainate receptors in epilepsy and excitotoxicity. Neuroscience. 2009; 158(1):309–23.

W

Wakabayashi T, Yamaguchi K, Matsui K, Sano T, Kubota T, Hashimoto T, et al. Differential effects of diet- and genetically-induced brain insulin resistance on amyloid pathology in a mouse model of Alzheimer's disease. Mol Neurodegener. 2019;14(1):1–18.

Waldbaum S, Patel M. Mitochondria, oxidative stress, and temporal lobe epilepsy. Epilepsy Research. 2010; 8(1):23–45.

Wang X, Finegan KG, Robinson AC, Knowles L, Khosravi-Far R, Hinchliffe KA, et al. Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress. Cell Death Differ. 2006;13(12):2099–108.

Welty FK, Alfaddagh A, Elajami TK. Targeting inflammation in metabolic syndrome. Translational Research. Mosby Inc.; 2016; 167(1):257–80.

Weston CR, Davis RJ. The JNK signal transduction pathway. Current Opinion in Cell Biology. 2007; 19(2):142–9.

X

Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: Cell life and death decisions. Journal of Clinical Investigation. 2005; 115(10): 2656–64.

Xu X, Raber J, Yang D, Su B, Mucke L. Dynamic regulation of c-Jun N-terminal kinase activity in mouse brain by environmental stimuli. Proc Natl Acad Sci. 1997; 94:12655–60.

Y

Yamaguchi H, Wang HG. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. Oncogene. 2001;20(53):7779–86.

Yang DD, Kuan CY, Whitmarsh AJ, Rincón M, Zheng TS, Davis RJ, et al. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature. 1997;389(6653):865–70.

Yang ZH, Miyahara H, Takeo J, Katayama M. Diet high in fat and sucrose induces rapid onset of obesity-related metabolic syndrome partly through rapid response of genes involved in lipogenesis, insulin signalling and inflammation in mice. Diabetol Metab Syndr. 2012;4(1):1–10.

Yao K, Chen H, Lee MH, Li H, Ma W, Peng C, et al. Licochalcone A, a natural inhibitor of c-Jun N-terminal kinase 1. Cancer Prev Res. 2014;7(1):139–49.

Yarza R, Vela S, Solas M, Ramirez MJ. c-Jun N-terminal kinase (JNK) signalling as a therapeutic target for Alzheimer's disease. Frontiers in Pharmacology. 2016; 6:321.

Yasuda J, Whitmarsh AJ, Cavanagh J, Sharma M, Davis RJ. The JIP Group of Mitogen-Activated Protein Kinase Scaffold Proteins. Mol Cell Biol. 1999;19(10):7245–54.

Yaswen L, Diehl N, Brennan MB, Ute Hochgeschwender. Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nat Med. 1999;5(9):3–7.

Yoshimura K, Ueno M, Lee S, Nakamura Y, Sato A, Yoshimura K, et al. C-Jun N-terminal kinase induces axonal degeneration and limits motor recovery after spinal cord injury in mice. Neurosci Res. 2011;71(3):266–77.

Z

Zafar U, Khaliq S, Ahmad HU, Manzoor S, Lone KP. Metabolic syndrome: an update on diagnostic criteria, pathogenesis, and genetic links. Hormones. 2018;17(3):299–313.

Zhao F, Kang H, You L, Rastogi P, Venkatesh D, Chandra M. Neuropsychological deficits in temporal lobe epilepsy: A comprehensive review. Ann Indian Acad Neurol. 2014;17(4):374–82.

Zhao Y, Spigolon G, Bonny C, Culman J, Vercelli A, Herdegen T. The JNK inhibitor D-JNKI-1 blocks apoptotic JNK signaling in brain mitochondria. Mol Cell Neurosci. 2012;49(3):300–10.

Zhong Q, Ren BX, Tang FR. Neurogenesis in the Hippocampus of Patients with Temporal Lobe Epilepsy. Curr Neurol Neurosci Rep. 2016;16(2):1–9.

Annexes

Annex I

Role of JNK isoforms in the kainic acid experimental model of epilepsy and neurodegeneration.

Auladell C, de Lemos L, Verdaguer E, Ettcheto M, Busquets O, Lazarowski A, Beas-Zarate C, Olloquequi J, Folch J and Camins A.

Front Biosci. 2017; 22:795–814.

[Frontiers in Bioscience, Landmark, 22, 795-814, January 1, 2017]

Role of JNK isoforms in the kainic acid experimental model of epilepsy and neurodegeneration

Carme Auladell[1,4,10], Luisa de Lemos[2], Ester Verdaguer[1,4,10], Miren Ettcheto[3,4], Oriol Busquets[3,4], Alberto Lazarowski[6], Carlos Beas-Zarate[7,8], Jordi Olloquequi[9], Jaume Folch[3,5], Antoni Camins[3,4,10]

[1]Departament de Biologia Cellular, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain, [2]Instituto Gulbenkian de Ciencia, Rua Quinta Grande 6,2780-156 Oeiras, Portugal, [3]Unitat de Farmacologia i Farmacognosia, Facultat de Farmacia, Universitat de Barcelona, Barcelona, Spain, [4]Biomedical Research Networking Center in Neurodegenerative Diseases (CIBERNED), Madrid, Spain, [5]Unitat de Bioquimica i Biotecnologia, Facultat de Medicina i Ciencies de la Salut, Universitat Rovira i Virgili, Reus, Tarragona, Spain, [6]Instituto de Investigaciones en Fisiopatologia y Bioquimica Clinica (INFIBIOC), Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina, [7]Departamento de Biología Celular y Molecular, C.U.C.B.A., Universidad de Guadalajara and Division de Neurociencias, Sierra Mojada 800, Col. Independencia, Guadalajara, Jalisco 44340, Mexico, [8]Centro de Investigacion Biomédica de Occidente (CIBO), Instituto Mexicano del Seguro Social (IMSS), Jalisco 44340, Mexico, [9]Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile, [10]Institute of Neurosciences, University of Barcelona, Spain

TABLE OF CONTENTS

1. ABSTRACT

Chemoconvulsants that induce status epilepticus in rodents have been widely used over the past decades due to their capacity to reproduce with high similarity neuropathological and electroencephalographic features observed in patients with temporal lobe epilepsy (TLE). Kainic acid is one of the most used chemoconvulsants in experimental models. KA administration mainly induces neuronal loss in the hippocampus. We focused the present review inthe c-Jun N-terminal kinase-signaling pathway (JNK), since it has been shown to play a key role in the process of neuronal death following KA activation. Among the three isoforms of JNK (JNK1, JNK2, JNK3), JNK3 is widely localized in the majority of areas of the hippocampus, whereas JNK1 levels are located exclusively in the CA3 and CA4 areas and in dentate gyrus. Disruption of the gene encoding JNK3 in mice renders neuroprotection to KA, since these animals showed a reduction in seizure activity and a diminution in hippocampal neuronal apoptosis. In light of this, JNK3 could be a promising subcellular target for future therapeutic interventions in epilepsy.

2. INTRODUCTION

According to the latest publication of the ILAE (International League Against Epilepsy), epilepsy is defined as a brain disorder characterized by an enduring predisposition to generate seizures and a hyper-synchronous firing of neurons, that leads to neurobiological, cognitive and social consequences (1). The gradual process by which the brain develops epilepsy is known as epileptogenesis and it is divided into three stages: the acute event (the triggering insult or initial seizure), a latent period (clinically silent) and spontaneous

seizures. In humans, the latent period can last for months or years (2-5). Therefore, epileptogenesis is a dynamic process by which the brain becomes epileptic and begins to generate spontaneous recurrent seizures (5).

There are several causes of epilepsy, such as tumors, trauma, metabolic dysfunction, infection and vascular disease, among others (5). However, some forms of epilepsy can occur due to a genetic predisposition (6). Few genes related to this neurological disorder have been identified. One group of these genes are related to ion channels, transporters and receptors, while other genes correlate with synaptic inhibition mediated by GABA neurotransmitter (6).

The signs and symptoms of seizures depend on the type, being the convulsive one the most common (60%) (4-6). These seizures derive in a systematic metabolic derangement, including hypoxia, hypotension and hypoglycaemia, leading to a reduction in high-energy phosphatases in the brain and thus potentially causing devastating effects in brain tissue. Systemic complications, such as cardiac arrhythmias, pulmonary edema, hyperthermia and muscle breakdown can also occur (3). The convulsive status epilepticus (SE) is clinically defined when the patient suffers a prolonged electrical and clinical convulsive seizure activity longer than five minutes, or more than one seizure within a five-minute period, without returning to the normal state between them. It is a neurological emergency, associated with a high mortality rate, particularly if treatment is delayed.

Two-thirds of convulsive seizures are focal (or partial) and one third is general. Focal convulsive seizures are circumscribed in a specifically brain area and are originated by abnormal electrical activity in a small group of neurons that have the ability to enhance excitability (epileptiform activity) to neighboring regions (5). The symptoms depend on the location of the focus. The intense firing of neurons may be the result of different factors such as altered cellular properties or altered synaptic connections, caused by a local scar, blood clot or tumor, frequently accompanied by unusual behaviours or consciousness alteration (7-10). Unlike the focal seizure, a primary generalized seizure disrupts normal activity in both cerebral hemispheres simultaneously. They are a 40% of non-convulsive seizures, depending on whether the seizures are associated with tonic (the person's muscles initially stiffen and they lose consciousness) or clonic movements (the individual's muscles begin to spasm and jerk).

Temporal lobe epilepsy (TLE) is the most common form of human epilepsy with focal seizures, covering 40% of all cases of this neurological disorder. A common subtype is the mesial temporal lobe epilepsy (MTLE), characterized by the presence of seizures originated in limbic areas of the mesial temporal lobe, particularly in the hippocampus, amygdala, and in the parahippocampal gyrus and its connections (11-15). This type of epilepsy is associated with an "early first insult", such as febrile seizures, a prolonged focal seizure, infection of the central nervous system (CNS), or head trauma, among others, most often occurring in the first five years of life (2-12). Patients frequently suffer from cognitive impairment, especially related to memory, and behaviour disturbances.

Its pathophysiological substrate is usually hippocampal sclerosis, the most common epileptogenic lesion found in patients with this type of epilepsy (MTLE-HS). Familial forms of MTLE-HS have been recognized, but no causal gene or linkage has been identified so far. This subtype of epilepsy is the most common cause of surgical and refractory epilepsy in adulthood (11,12).

In most cases, it takes years for any noticeable damages to appear in MTLE-HS, but it is still very important to treat the disease early and as effectively as possible, since it is often refractory to the drug treatment. Nonetheless, MTLE-HS can be abolished in most patients by surgical treatment.

In reference to the cellular basis of the epilepsy, the current axiom in epileptic research states that whereas a fine balance between excitatory (depolarizing) and inhibitory (hyperpolarizing) signals characterize normal brain function, a change in this balance leads to seizures. This imbalance is achieved by either increased excitation, reduced inhibition (i.e. des-inhibition) or both. These results in an abnormal neuronal discharge associated with a large release of the excitatory neurotransmitter glutamate, which is ultimately responsible for seizures. One of the goals of both basic research and therapeutic applications, during recent years, has been to counter-balance the sustained excitation by developing drugs that would trigger repolarization (i.e. impairment of GABAergic inhibition in epilepsy). Several lines of evidence support this approach: i) GABA is the main inhibitory neurotransmitter in the neuronal structures involved in MTLE; ii) enhancers of GABAergic inhibition (such as benzodiazepines or barbiturates) are commonly used as antiepileptic drugs; and iii) discharges can be detected following the pharmacological blockade of GABA receptors.

3. CURRENT TREATMENT OF EPILEPSY

The currently used antiepileptic drugs are heterogeneous, since they have different mechanisms of action, even though they are not completely well understood (13-16). One group interacts mainly through the regulation of sodium channels (blockade). This is the case of phenytoin, carbamazepine, oxcarbamazepine, valproate, lamotrigine, topiramate, lacosamide and also zonisamide (13,14). *In vitro* pharmacological studies evidenced that zonisamide and lacosamide enhance slow inactivation of voltage dependent sodium channels

and reduce calcium entry through voltage dependent calcium channels, thereby stabilizing hyperexcitable neuronal membranes (14-16).

Another group of drugs used for the treatment of epilepsy activates the gamma-amino-butyric acid ($GABA_A$) receptor. This leads to an increase in the intracellular Cl^- influx (ion concentration), which induces hyperpolarization of the membrane and inhibition of action potentials, hence rendering the neuron unresponsive for a period. This group includes benzodiazepines, barbiturates, topiramate, felbamate, tiagabine □a potent and selective GABA reuptake inhibitor in neuronal and glial cells□ and gabapentin, which inhibits the release of monoamine neurotransmitters and increases GABA turnover in several brain areas (13-15). Another group of antiepileptic drugs includes pregabalin, gabapentin, topiramate and lamotrigine, which block high voltage calcium channels.

Retigabine is a different antiepileptic drug from all those currently approved. It acts primarily as a neuronal potassium channel opener, stabilizing the resting membrane potential and controlling neuronal electrical excitability. Thus, retigabine prevents the onset of discharge of epileptogenic potential action (14,16).

Felbamate blocks the NMDA receptor, a specific type of ionotropic glutamate receptor that under local membrane depolarization becomes permeable to Ca^{++}. The influx of Ca^{++} further depolarizes the neuron and contributes to Ca^{++}-mediated neuronal injury under conditions of excessive neuronal activation (16).

Perampanel is a selective non-competitive antagonist of ionotropic AMPA-type (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, a subtype of non-NMDA glutamatergic receptor) glutamate receptors in postsynaptic neurons. Although the exact mechanism by which it exerts its antiepileptic effect in humans is not yet fully elucidated, its administration is associated with a decreased frequency of seizures (13,16).

Interestingly, topiramate has more than one mechanism of action. Thus, in addition to blockade of Na^+ channels, increases $GABA_A$ receptor activation and blocks the AMPA receptors (13,14). This drug was fisrt used as an anticonvulsant and now is used to treat epilepsy in children and adults. It is also used for the treatment of Lennox-Gastaut-Syndrome, a severe form of epilepsy that shows seizure activity before the 4 years of age.

4. EXPERIMENTAL ANIMAL MODELS USED TO STUDY EPILEPSY

The understanding of epileptic disorders has derived from appropriate animal models that have allowed the study of this disease (17).

The status epilepticus defined as 5 minutes or more of continuous seizures can be induced by systemic or intracerebral administration of different convulsive agents, such as kainic acid (KA) (2-carboxy-4-isopropenylpyrrolidin-3-ylacetic acid), a potent neuroexcitatory amino acid that binds and activates receptors for glutamate or pilocarpine, which acts through the activation of the M1 muscarinic receptor (17-27). Prolonged seizure activity cause permanent neuronal damage and synaptic reorganization; these conditions are often associated to the development of "chronic" epilepsy (17-19).

The procedures for KA and pilocarpine injection are well established in rodents (18-33). However, the effect of these chemoconvulsants differs among animal strains (17,23). After the acute status epilepticus is induced, there is a period of free crisis □known as the latent phase□ which can last for weeks. Finally, the chronic phase, characterized by spontaneous and recurrent seizures (17-23), emerges. The anatomical defects induced are very similar to those developed in humans with MTLE-HS. Therefore, these experimental models allow us to evaluate the pathological changes that occur in the chronic phase, but also to assess those ones appearing in the early stages and during epileptogenesis. In a recent publication (17), a comparison between the mechanisms of action of the two abovementioned pro-convulsants was performed, showingthat pilocarpine provokes a rapid status epilepticus when compared to KA. Moreover, pilocarpine has a higher mortality than KA in rodents (17,23). Nonetheless, experimental studies suggest that both models are very useful in assessing anticonvulsant drug effectivity. Anti-epileptic drugs that are already on the market, such as phenobarbital or carbamazepine, show an anti-convulsant effect in animal models treated with both neurotoxins (22). Another interesting point is that both compounds favour the neuronal loss, being pilocarpine a more quickly neuronal loss inducer, whichs explains its more lethal effects in rodents (17). There areother experimental models that induce seizures and are also useful to evaluate the efficacy of anticonvulsants, such as pentylenetetrazole (PTZ) which cause convulsions and the repeated subconvulsive electrical stimulations (kindling) in high doses (22).

The systemic administration of KA could be intraperitoneal, intravenous or subcutaneous. On the other hand, KA can be administered locally or directly in the brain (intrahippocampal, intraamygdala or intracortical) with the advantage that the brain injury effects are more localized and provides a lesser animal mortality (17-23). The main advantage of intraperitoneal injection as compared to intracranial administration, is probably the simplicity and feasibility of the procedure. In all cases, KA triggers a pattern of repeated seizures for several hours, followed by a latency period and a subsequent spontaneous occurrence of seizures (20-25).

A single systematic injection (30 mg/kg in mice) of a convulsive dose of KA results in a limbic (SE), inducing features similar to those observed in MTLE-HS adult human epilepsy, although followed by high mortality rates (17-23). Recently, Umpierre *et al*. (2016) evidenced that a systematic low dose of KA produces SE without acute degree of mortality. Interestingly, the caused pathology had the same features observed when common doses were applied, including hippocampal neurodegeneration and astrogliosis (27-33).

5. THE HIPPOCAMPUS, THE FIRST KA TARGET

The brain region most affected by seizures, both in animal models and humans, is the hippocampus. It is a small region of the brain that forms part of the limbic system and it is involved in processes such as memory and learning (34,35). Particularly, the hippocampus seems to play a major role in declarative memory, the type of memory involving concepts or events that can be purposely recalled (36). It also participates in the detection of novel stimuli, the conduct of search and in stress response. Therefore, it is critical in habituation processes like awareness and classical conditioning (37). Together with the amygdala, the thalamus, the hypothalamus, the septal area, the olfactory cortex and other CNS structures, the hippocampus is part of the limbic system (37,38).

In reference to the hippocampal architecture in rodents, the hippocampus is a complex alocortical cylindrical structure composed of different subfields. One is the dentate gyrus, a separate structure that is a tightly packed layer of small granule cells wrapped around the end of the hippocampus proper (37-39). Next, there is the *Cornu Ammonis* area (CA) considered the "hippocampus proper". This CA area is subdivided in CA4, CA3, CA2 and CA1. The CA4 area underlies the dentate gyrus and is also called the hilus or hilar region. It contains mossy cells, thus it is not filled with densely packed pyramidal cells as occurs in the other CA areas. CA4 is followed by CA3, then there is a very small zone called CA2 and after there is the CA (13, 41). Next to the CA1 there is the subiculum, followed by a pair of ill-defined areas, the presubiculum and parasubiculum. Finally, there is the enthorhinal cortex. The CA subfield, the dentate gyrus and the subiculum configurate the "hippocampal formation" (38-41).

One crucial question is whether the hippocampal-parahippocampal network dysregulation and memory deficits observed in epilepsy are state or trait characteristics of the disorder. The parahippocampal gyrus, a grey matter cortical region of the brain that surrounds the hippocampus, transmits its signals through the enthorinal cortex to the dentate gyrus via granule cell fibers, known collectively as the perforant path (39-41). The mossy fibres, which project from the granular neurons of dentate gyrus and establish synapses with the proximal dendrites of CA3 pyramidal neurons, play a crucial role during the status epilepticus, as demonstrated by the formation of novel aberrant mossy fibres on the hyppocampal neurons, as well as by an increase in the density of KA receptors and, therefore, to the reduction of seizure threshold (39-41). CA3 neurons then fires to CA1 neurons via Schaffer collaterals, which synapse in the subiculum, the main output of the hippocampus. It also receives input from the entorhinal cortical layer III pyramidal neurons. Collectively, the dentate gyrus, CA1 and CA3 areas of the hippocampus compose the trisynaptic loop.

6. MECHANISMS OF NEURODEGENERATION INDUCED BY KA

The neurotoxin KA was first isolated in the red alga, *Digenea simplex*, which is found in tropical and subtropical waters (41). KA is a non-degradable analogue of glutamate, and a potent neurotoxin that acts through glutamate receptors present in the mossy fibres in the hippocampus. Excessive stimulation of glutamate receptors induces excitotoxicity, which is the main cause of cell death in CNS diseases (42-44).

There are two groups of glutamate receptors: ionotropic (iGluR) and metabotropic receptors (mGluR) (44-49). The first group (iGluR) regulates the passage of ions through the neuronal membrane. When glutamate binds to iGluRs, conformational changes resulting in the opening of the channel occur, which increase the flow of Ca^{++} and/or Na^{+} into the cytosol, and K^{+} into the extracellular environment. iGluRs include three main type of receptors, the NMDAR (N-Methyl-D-aspartate receptor), the AMPAR (Amino Methylphosphoric Acid Receptor) and KARs (Kainic Acid Receptors) (45-49). AMPAR and KARR are also known as non-NMDA receptors, since they are not sensitive to selective antagonists of NMDAR, have lower affinity for glutamate and their activation kinetics is much faster than those of NMDAR (23,38). The KARs family is divided into two subfamilies, the GluR5-7 and KAR1-2 (4,5). These receptors are expressed in the amygdala, entorhinal cortex, basal ganglia, cerebellum, cortex and hippocampus, where high levels of KAR1 and KAR2 have been detected (45-51). It has been shown that KAR are located in both presynaptical and postsynaptic areas, being KAR1 predominant in CA3 pyramidal neurons, while KAR2 is detected in CA3 and CA1. This distribution pattern makes the hippocampal region the most susceptible to KA excitotoxicity (45-48). Several studies suggest that KA epileptogenic effect on pyramidal neurons of CA3 is caused by activation of their KARs, preferentially expressed in synaptic mossy fibers of the stratum lucidum region (slm), to promote the release of glutamate in the synaptic cleft (19,25). Thus, this is the area where seizures begin

Role of JNK isoforms epilepsy and neurodegeneration

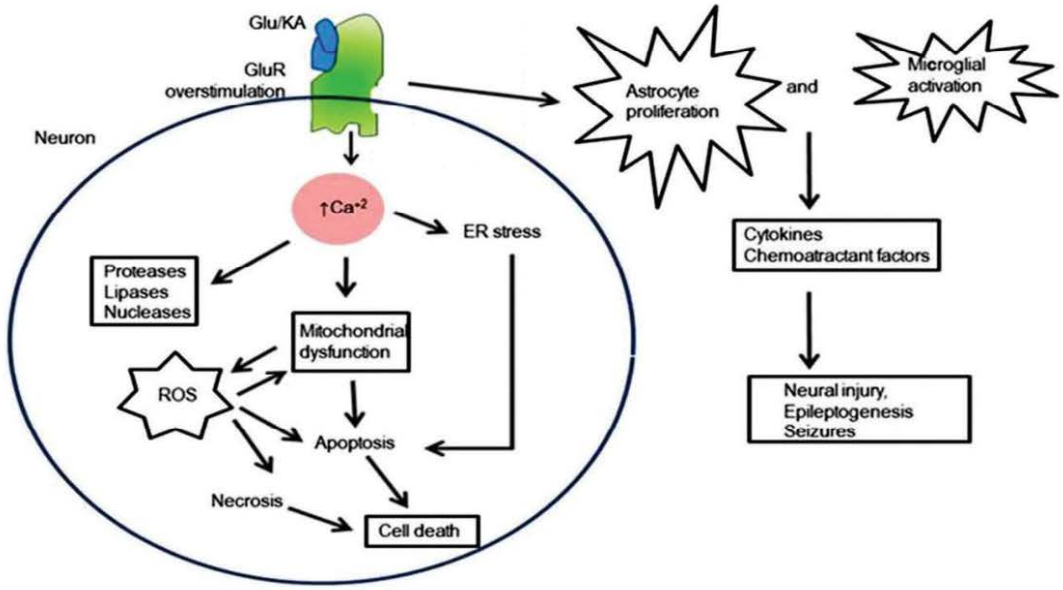


Figure 1. Kainic acid treatment induce excitability with a high increase in calcium levels an inflammation trough the activation of microglia and astrocyte.

and develop (50). Also GluR5 receptors, which are expressed in GABAergic interneurons of the CA1, as well as GluR6 receptors expressed predominantly in CA3, are involved in the excitatory action of KA (49-60). The second group (mGluR), modifies their interaction with other cytosolic proteins upon binding of glutamate. This leads to the activation of specific intracellular signaling cascades (46-49).

The excitation produced upon binding of KA to ionotropic glutamate receptors induces many cellular events, including massive influx of Ca^{2+}, leading to depolarization of the cell membrane (56-61). These conditions favour the production of ROS, which, in turn, induce structural and functional changes in proteins and DNA damage and consequently cell death. In addition, high levels of intracellular calcium trigger the release of nitric oxide synthetase (NOS), which modulates the glutamatergic transmission and can induce changes in mitochondrial function, generating more free radicals (61-65). Finally, there is also a glial activation related to an inflammatory response and neuronal death (23-25,50,54). All these events contribute to neuronal damage, and consequently to cell death (Figure 1).

During seizures, death receptors TNFR1 (tumour necrosis factor receptor 1) and FasR (Fas receptor) are activated, leading to the extrinsic pathway of apoptosis (63-66). Moreover, calcium entry also activates the intrinsic pathway of apoptosis. Hence, the altered mitochondrial function, triggers the mobility of apoptosis regulator Bax (bcl-2-like protein 4) to mitochondrial outer membrane, the cytochrome c release and subsequent activation of cysteine proteases proteins, such as caspase 9. Finally, this activates caspase 3, the main executor of the apoptotic processes (65-72).

Both *in vivo* and *in vitro* studies demonstrated that KA induces microglia activation (Figure 1), releasing high levels of proinflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-12 (IL-12), which together with Interleukin 18 (IL-18), are involved in upregulating Interferon-gamma production (IFNγ) (72-78). Moreover, the administration of KA induces the expression of high levels of other cytokines, like the tumor necrosis factor (TNFa, which could have both neuroprotective and neurotoxic properties, in contrast to the role of other proinflammatory cytokines (73-78).

Interestingly, one of the earliest changes occurring in conditions of KA-induced neuronal hyperexcitability, is an immediate gene overexpression response. Thus, an increase in the expression of mRNAs encoding heat shock proteins such as *hsp27*, *hsp70* and *hsp72* is induced (78-80). *In vivo* studies have shown that, while high expression of *hsp72* can be harmful to cells, overexpression of *hsp27* and *hsp70* have a protective function against excitotoxic damage, although it fails to rescue cells from excitotoxic death (67,77-80). It has also been detected an overexpression of *fos*, *jun* and *Erg1* genes in different hippocampal regions (79).

Role of JNK isoforms epilepsy and neurodegeneration

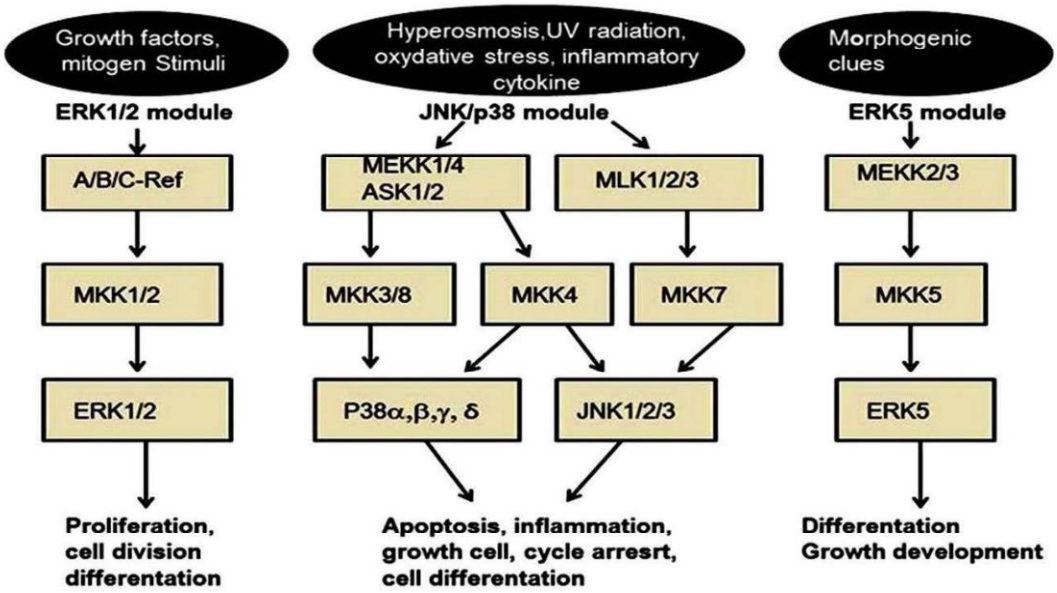


Figure 2. Simplified overview of mammalian MAPK cascade.

The proto-oncogene *jun* and *fos* encode proteins that form the complex AP1 (activator protein 1), regulating the expression of other genes (77,78). Likewise, following KA administration, there is an increase in neuropeptides, like somatostatin (SOM) and neuropeptide Y (NPY), among others (79).

Therefore, the KA experimental model provides a chance to understand the mechanisms of neuronal death. Considering that this process is present in neurodegenerative diseases, such as AD, PD or amyotrophic lateral sclerosis (ALS), the use of KA experimental model could be an important tool in epilepsy knowledge but in biomedical research in general(49-54). Furthermore, activation of glutamate receptors by KA induces the activation of Mitogen Activated Protein Kinases (MAPKs). In this review, we will focus on the c-Jun N-terminal kinase pathway (JNKs), which is clearly involved in the process of apoptotic neuronal death (22,27,28,30).

7. MITOGEN ACTIVATED PROTEIN KINASES (MAPKS)

MAPKs are a family of cytosolic protein kinases that regulate cellular processes such as cellular proliferation, differentiation and apoptosis (81-87). They are activated in response to various extracellular signals or stimuli, such as hormones, growth factors, cytokines or several stresses (83-86). There is a high correlation between the type of stimulus that activates the MAPK pathway and the specific role they develop.

MAPKs pathway is regulated through three sequential phosphorylation kinases (Figure 2). Thus, the first step in this pathway of signal transduction is the phosphorylation of threonine (Thr) and tyrosine (Tyr) residues that occurs in conserved motifs Thr-X-Tyr, located in the kinase subdomain VIII of the four MAPKs (85). This phosphorylation is gradual and starts in the membrane receptors associated with other G protein kinases or adapters, which induce the activation of MAPKKK or MAP3K (MAPK Kinase Kinase Kinase). These, in turn, phosphorylate the MAPKK or MAP2K (MAPK Kinase Kinase) that finally generates the physiological response by the activation of this biochemical pathway (Figure 2) (84-87). This activation system is highly conserved in eukaryotes. Besides, the activation of MAPK pathways through phosphorylation is regulated by the engagement and interaction with regulatory molecules called anchor proteins (Scaffold Proteins) (83-90).

Currently, four groups of MAPKs that regulate different cellular processes have been described: ERK1/2 -Extracellular Signal Regulated Kinase 1/2- with an important role in proliferation and differentiation, JNK1-3 -c-Jun N-terminal kinase 1-3-, also called SAPKs -Stress-Activated Protein Kinases-, p38MAPKα, β, γ, δ, which are activated by cellular stressand ERK5, which is activated by stress and mitogenic signals (83-95). Each one of these MAPKs has its specific MAP2Ks and

Role of JNK isoforms epilepsy and neurodegeneration

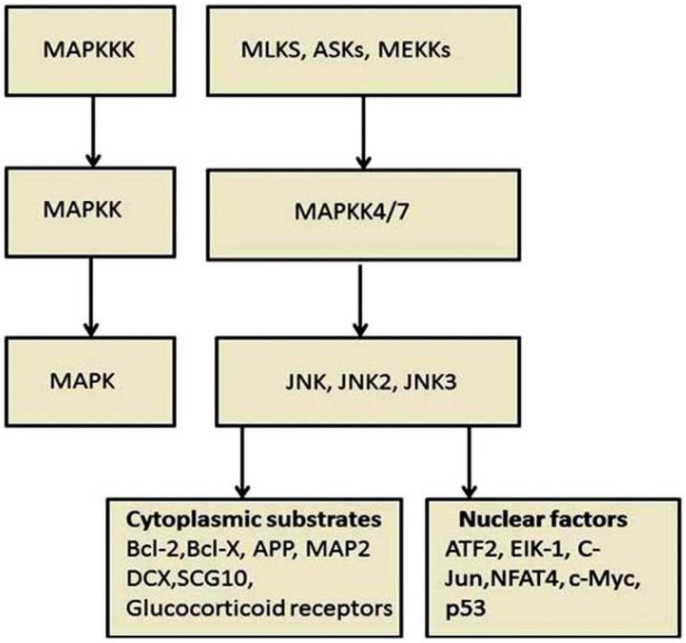


Figure 3. Simplified representation of JNK pathway.

MAP3Ks that, in turn, regulate different cellular processes (Figure 2).

7.1. The c-Jun N-terminal kinase signaling pathway (JNK)

The c-Jun N-terminal Kinase (JNK) is a member of MAPK signaling proteins (95-97).It was initially identified and purified in the liver of rodents exposed to cycloheximide by Kyriakis *et al*., (1994) as an active protein (96). JNK proteins are sensitive to stress and apoptosis-related receptors, and are activated by specific ligands of GPCRs and RTKs receptors (96-99). Many extracellular stimuli activate JNK, such as the cytokines TNF-α and IL-1β. Other cellular stress conditions that activate JNK include ultraviolet light, heat shock, osmotic shock, growth factor withdrawal and a number of toxins including hydrogen peroxide, anisomycin and cyclohexamide (96). Kinases that activate JNK include MKK-4 and -7, and upstream of these kinases there are, in turn, a number of kinases including TAK (Tat-associated kinase), MLK-3 (Mixed Lineage Protein-3), MEKK-1 and -4, and ASK-1 (Apoptosis Signal-Regulating Kinase). Specifically, MEKK1-4 and ASK1-2 are mostly induced by environmental stresses and activate MKK4, while MKK7 activated by cytokines is phosphorylated by MLK1/2/3 (96-100). Both MKK4 and MKK7 activate JNK by dual phosphorylation in the TPY (Thr-Pro-Tyr) motif. The cooperation of the two kinases is required for full activation of JNK (Figure 3) (97-101).

Many substrates, both in the cytoplasm and nucleus, are phosphorylated by JNK. Nuclear substrates include AP-1 from Jun family (c-Jun, junB, JunD), ATF-2 (Activating Transcription Factor 2), ELK-1 protein, NFAT (nuclear factor of activated T cells), c-Myc oncogene, the tumor suppressor p53 (Figure 3). Cytoplasmic substrate include Bcl-2 (B-cell lymphoma 2), Bcl-xL (B-cell lymphoma-extra-large), glucocorticoid receptors, membrane proteins such as APP (Amyloid Precursor Protein), MAP2 (Microtubule-Associated Protein 2), DCX (Doublecortin) and SCG10, a microtubule destabilizing factor (Figure 3) (85,86,94). MARCKSL1 (MARCKS-like protein 1) is another substrate phosphorylated by JNK, which is widely expressed in nervous tissue and has a key role in neural tube formation. The phosphorylation on C-terminal residues (S120, T148, and T183) enables MARCKSL1 to bundle and stabilize F-actin, increase filopodium numbers and dynamics, and retard migration in neurons (86,94). The neurofilament heavy chain is also phosphorylated by JNK, implicating this kinase in neurite outgrowth and regeneration. Moreover, different studies evidence that JNK directly targets chromatin modifiers, driving histone phosphorylation and acetylation. Thus, JNKs can exert an influence on

gene expression not only in stress responses but also during neuronal differentiation (98-103).

However, the first identified and better described JNK substrate is c-Jun, which increases its stability and its transcriptional activity when phosphorylated at residues of Ser63 and Ser73 (8,6,89,94). The c-Jun protein can form homodimers or heterodimers with other proteins, such as transcription factor ATF2, or components of the c-Fos family. This latter heterodimerization is followed by the AP-1 complex formation, which activates the transcription of various genes involved in processes related to proliferation, differentiation, apoptosis or immune response (50). High expression of *c-jun* and high levels of protein precede periods of cell death, such as those that occur during embryonic development, in trauma situations, in cerebral ischemia and after convulsions (80,95-100). A similar induction of this gene was detected in neurodegenerative diseases such as AD, in ALS and PD (43).

There are different JNK isoforms, encoded by three different genes, *jnk1*, *jnk2*, and *jnk3*. Specifically, ten isoforms of JNK having a homology of 85% have been identified from alternative splicing of the gene products: JNK1α1, JNK1α1, JNK1α2, JNK1α2, JNK2α1, JNK2α1, JNK2α2, JNK2α2, JNK3α1 and JNK3α2 (102-104). These isoforms have a molecular weight ranging from 46 to 54 kDa. In many cases, the *jnk1* gene generates a 46 kDa protein product, while the *jnk2* gene produces a protein of 54 kDa and *jnk3* gene produces a variant of 46 and 54 kDa (103).

In mammals JNK1 (MAPK8) and JNK2 (MAPK9) proteins are expressed ubiquitously, whereas JNK3 (MAPK10) is preferentially expressed in the brain and, to a lesser extent, in the heart and testes (104-106).Studies by *in situ* hybridization in rodents suggested that *jnk3* is widely expressed in neocortex, hippocampus, thalamus, and midbrain followed by *jnk1* and *jnk2 (*104-107). In rodents, JNK1 protein levels are high during brain development, in contrast to JNK2 and JNK3; however, they decrease in postnatal stages, maintaining high levels in the olfactory bulb of adults. Specifically, in the hippocampus, the JNK3 is widely dispersed in the majority of hippocampal areas, whereas JNK1 is found exclusively in the CA3 and CA4 areas, as well as in dentate gyrus (103-106). The subcellular distribution of JNK isoforms differs, thus JNK1 is located largely in the cytosol, whereas JNK3 is in the nucleus. Regarding JNK2, it is distributed both in the cytosol and in nucleus.

There are differences among the isoforms in reference to the activation of transcription factors. Thus, some studies suggest a greater ability of JNK2 in phosphorylating c-Jun substrate as compared to nuclear JNK1 (104-106). JNK1 isoform is more involved in the phosphorylation of cytosolic substrates as MAP2 and MAP1B (Microtubule-Associated Protein 1B), thus highlighting the importance of JNK1 in controlling the activity of microtubules and axonal elongation. In addition, MAP2 phosphorylation plays an important role in defining cell architecture (85). However, JNK1 could participate in other functions, such as controlling synaptic plasticity, through the transcription factors ATF-2 and c-Jun (85,96). Moreover, differential effects in chromatin modification exist between JNKs. Thus, JNK2 and JNK3 specifically phosphorylate serine 10 of histone H3 *in vitro*, an event that is associated with the relaxation of chromatin and active gene transcription (99).

7.2. Functional interactions between JNK pathway and JIP proteins

Cells ensure specificity and efficiency of a signalling cascade through the interaction of a variety of anchoring proteins that, despite the lack of enzyme activity, are added to various components of the same signalling pathway forming functional modules (107-110).

The JNK signalling pathway interacts with specific anchoring proteins, known as JIPs (c-Jun–N-terminal-kinase-interacting-proteins), forming a JNK signalling functional module. JIPs facilitate the activation of JNK in addition to different components of the MAPK cascade, as MKK7 and some MAP3K as MLK3 or DLK, forming a multi-enzyme complex.(109,110) JIP family consists of several members, JIP1, JIP2, JIP3 and JIP4 (8, 90).

JIP1 has been the best characterized. The gene encoding this protein, *jip1*, is expressed ubiquitously (111-113). JIP1 can interact with several MAP3K proteins (MEKK3, MLK3 and DLK), but only with one MAP2K (MKK7). Furthermore, JIP1 interacts with JNK isoforms, having a higher affinity for JNK1 isoform (109-113). Mice carrying a targeted deletion of the JNK-binding domain of JIP-1 have been found to be less vulnerable to KA-induced cell death in the hippocampus (113). It has been described that other kinases can phosphorylate JIP1 and change its affinity to the MAPKs (114). Specifically, JIP1 can bind to AKT promoting its activation and suppressing JNK signaling in neurons through the inhibition of the interaction between JNK and JIP1. However, exposure of these cells to an excitotoxic stress promotes the AKT release from JIP1, thereby increasing the interaction between JNK and JIP1, and triggering JNK activation through JIP1-JNK module (112-116).

JIP2 and JIP3 protein are expressed in neurons and neuroendocrine cells (113). JIP2 interacts specifically with MKK7 (109-115). JIP3 interacts with MEKK1, ASK1 and MLK3, and also with MAP2Ks, MKK4 and MKK7. However, it has been shown that JIP3 has a higher affinity for the JNK3 isoform than either JNK1 or JNK2 (112-114). A recent study reported an important role of JIP3 in seizures because the underexpression of JIP3 results in an anticonvulsive effect after KA administration (117,118).

The JIP4 protein is the most recently identified member of the JIP family (119). It interacts with MEKK3, MKK4 and MAPKs (JNK and p38a) (117-120).

7.3. Involvement of JNK in neurodegenerative mechanisms

Activation of the JNK pathway in the nervous system has been associated with neuronal cell death processes, either occurring naturally during embryonic development or arising in different brain pathologies (30). It has been proposed that the duration of JNK activation can determine the type of response. Thus, sustained JNK activation is related to apoptosis, whereas transient activation is related with cell survival and proliferation processes (121-128).

The two major pathways that initiate the apoptotic process are classified into the extrinsic pathway, activated by death receptors, and the intrinsic pathway, initiated by mitochondrial events. JNK appears to have a central role in both pathways, since it activates apoptotic signalling through upregulation of proapoptotic genes, and it is able to directly modulate mitochondrial activity through phosphorylation of both anti- and pro-apoptotic proteins (121,123).

Some authors support that upon activation of JNK, it translocates to the mitochondria, inducing the release of cytochrome c, which, in combination with Apaf-1, activates caspase-9 (122). However, the specific mechanism by which JNK mediates the release of cytochrome c is not clearly established. It has also been shown that JNK induces breakage of Bid (a pro-apoptotic member of the Bcl-2 family), or the phosphorylation of Bax (member of the Bcl-2 gene family) and Bad (Bcl-2-associated death promoter) (122).

In addition to the above described mechanisms, JNK can regulate the stability and transcriptional activity of p53, a key protein that mediates the regulation of the proapoptotic genes, apoptosis induced by DNA damage and oxidative stress (122-126).This indicates that JNK may cause both proapoptotic and antiapoptotic responses, even in the same cell type.

In particular, the JNK kinases play an important role in cerebral ischemia-induced excitotoxicity, epilepsy and in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) (33,48,123,125).

It is well-known that AD is primarily characterized by the formation of neurofibrillary tangles consisting of tau protein hyperphosphorylation, and by the presence of β-amyloid (βA) plaques formed from the abnormal proteolysis of APP peptide (122-128). Recent studies suggest that the JNK pathway may play an important role in neuronal death and synaptic loss associated with AD (129-134). Furthermore, it has been described in neuronal cell cultures that βA-induced neurotoxicity is mediated through an increase in JNK activity, leading to neuronal death (130). Stimulation with βA and APP proteolysis requires a subsequent activation of c-Jun, caspase-8 and the expression of FasL (131). Similarly, it has been demonstrated that phosphorylation of JNK is related with hyperphosphorylation of Tau in neurodegeneration (129-132).

Likewise, it has been reported that the JNK signalling pathway probably playsa crucial role in PD (134-140). The main cause of this neurodegenerative disease is an increase of oxidative stress that causes the selective death of dopaminergic neurons of the substantia nigra (135-141). The degeneration of the nigrostriatal pathway produces clinical alterations such as akinesia, tremor, rigidity and postural disorders. Different studies showed that JNK is activated both in cultured of dopaminergic neurons subject to neurotoxicity by MPP^+ and following administration of MPTP (1-methyl-4-phenyl-1, 2,3,6 tetrahydropyridine) to nigrostriatal level (30).

It has also been demonstrated that there is JNK activation in experimental models of HD (30). This pathology is characterized by the selective loss of striatal neurons, mainly caused by a mutation in the *htt* gene (huntingtin) (30). *In vitro* studies, both in hippocampal neurons and striatal neurons treated with the neurotoxin 3-nitropropionic acid (3NP), evidenced that activation of JNK and c-Jun is associated with neuronal degeneration (30). However, there are currently no studies that describe the inhibitory effect of the JNK pathway in animal models of the disease (132-133,142).

The neuronal loss induced in a KA epileptic model could be mediated by the JNK activation and nuclear translocation induced by c-Jun phosphorylation, hence allowing the formation of the AP-1 complex which, in turn, induces transcription of proapoptotic factors. Different findings indicate that JNK-AP1 complex increases the expression of proapoptotic genes such as *tnf*, *fasl* and *bak* (Bcl-2 homologous antagonist/killer) (121-124).

The differential role of JNK isoforms in neurodegeneration and the specific mechanisms that regulate each isoform are little known. Analysis of genetically modified animals, as is the case of knockout mice for specific isoforms, provides the first evidence to understand the role of each isoform (142-145).

7.4. Specific isoform knock-out models of the JNK pathway

In the last decade, the use of murine genetic models has provided a major advance in the study of differential functions of each isoform of JNK

Role of JNK isoforms epilepsy and neurodegeneration

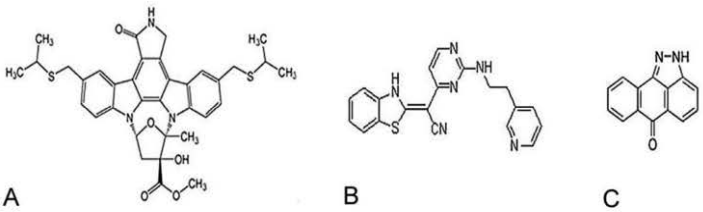


Figure 4. Molecular structure of JNK inhibitors: A. CEP-1347; B. AS601245; C. SP600125.

in vivo (143-146). Specific deletions of *jnk1*, *jnk2* and *jnk3* genes produce viable animals with normal development (143-147). The double knockout *jnk1/jnk2* is lethal, since the neural tube cannot be properly closed due to a deficiency in apoptosis, a crucial event in development process. However, the double knockout *jnk2/jnk3* and *jnk1/jnk3* are viable, thus demonstrating that both isoforms, JNK1 and JNK2, are essential in the regulation of apoptosis during embryonic brain development (141-150).

Some authors have described compensatory mechanisms in the expression of JNK isoforms in mice deficient in one of them. Thus, it has been shown that there is an increase in JNK1 levels in brain of $jnk2^{-/-}$ mice and an increase in JNK2 levels in $jnk3^{-/-}$ mice (104). Different studies with $jnk1^{-/-}$ and $jnk2^{-/-}$ mice showed that JNK1 has a central role in obesity and insulin resistance, while JNK2 could be involved in diabetes and in resistance to the neurotoxin MPTP (148,150). Furthermore, as mentioned above, both isoforms are involved in the regulation of brain development (148).

Interestingly, mice deficient in *jnk3* gene are less susceptible to excitotoxic stimuli, and have a reduced neuronal death in specific areas of the hippocampus after the administration of the neurotoxin KA. In these mice, a significant reduction of phosphorylation of c-Jun and of AP-1 activity was detected (144,145). Also, $jnk3^{-/-}$ mice exhibit reduced neuronal death in ischemic processes as well as in an experimental model of Parkinson's disease, established with the use of MPTP neurotoxin (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-hydroxydopamine (150). Several *in vitro* studies show that cortical and hippocampal neurons deficient in JNK3 are resistant to βA toxicity and to oxygen and glucose deprivation (129-132). In addition, *in vivo* studies show that JNK3-deficient mice exhibit neuroprotection and decrease of seizures after KA administration, as well as resistance to apoptosis induced by hypoxia-ischemia (143,151).

It has been established that knockout mouse model for *c-jun* in which this gene is replaced with the mutant gene *junAA,* is viable. *JunAA* differs from the *wild-type* at serine 63 and 73, which are replaced by alanine. This knockout shows neuroprotection in different areas of the hippocampus after KA injections, suggesting that c-Jun protein can be the key substrate for JNK3 in the KA model (151). Nonetheless, the function of the individual JNK isoforms in the regulation of apoptotic pathways remains elusive.

Further knowledge of JNK physiological functions would be obtained with the deletion of JNK activators MKK4 and MKK7. However, mice deficient for MKK4 or MKK7 and double knockout *mkk4/mkk7* are unviable due to defects in hepatocyte proliferation (151). Moreover, since we have seen that the response of KA injections is different in neuronal and glial cells, it would be useful to study what happens when JNK pathway activity is specifically silenced in neurons or glial cells in the KA experimental model. This will be achieved by using conditional knockouts for *mkk4 and mkk7* genes using the Cre-loxP-System under specific neural promoters (GFAP and CAMKII).

Our findings will enable us to find therapeutic targets that can be used to silence JNK activity and therefore control the evolution of epileptogensis disease.

7.5. Inhibition of JNK pathway

Pharmacological inhibition of JNK is a well-known strategy for the protection of neuronal death. Several molecules have shown to be able to inhibit this pathway. Among them, the most important are CEP-1347, a specific inhibitor of MLK family; SP600125 and AS601245, both selective inhibitors of JNK activity, and D-JNKI a permeable peptide inhibitor of JNK that prevents phosphorylation of c-Jun (Figure 4) (135-141). The inhibitor CEP-1347 acts on MLKs kinases that activate the JNK pathway by competing for the ATP binding site (136). This compound, although it has low selectivity for JNK, shows neuroprotective effects in experimental mice models of PD, treated with MPP^+ (1-methyl-4-phenylpyridinium) in nigrostriatal neurons and prevents the toxicity of βA in neurons.(135). Although CEP-1347 is safe and well tolerated in patients with PD, is not effective in the treatment of this neurodegenerative disease. Further studies are needed to elucidate there mechanism of action (135,136).

SP600125 is a reversible inhibitor that competes for the ATP binding site with high selectivity for the three isoforms of JNK. The neuroprotective effect of SP600125 has been demonstrated in experimental models of Parkinson's disease, in ischemic processes and neurotoxic mechanisms of Alzheimer's disease (137,148). However, high doses of the drug may inhibit other kinases such as MKK3, MKK4, MKK6, and MKK7 (3,5,81).

The AS601245 inhibitor has been developed with a similar mechanism of action as SP600125, having a higher affinity toward the JNK3 isoform that JNK1 and JNK2 (138,139). It has been shown that AS601245 prevents neuronal death in cerebral ischemia models, and it also has an anti-inflammatory effect in models of rheumatoid arthritis (138,139). However, this drug does not provide sufficient efficiency to develop clinical trials intended for use in neurodegenerative diseases.

Moreover, a different strategy to inhibit the JNK pathway is the use of peptides that recognize the substrate binding domain or regulatory proteins thereof, such as JIPs. In this line, the D-JNKI peptide has been developed. It does not inhibit the enzymatic activity of JNK, but blocks the binding to its substrates (140). This peptide prevents phosphorylation of c-Jun, and thus exerts a neuroprotective effect in different models of excitotoxicity *in vitro*; it also protects from apoptotic cell death in experimental models of ischemia (140).

7.6. JNK3 as possible therapeutic target in neurodegenerative diseases

The specific JNK3 expression in brain makes it possible to consider this isoform as a potential therapeutic target for neurodegenerative diseases. As described above, *jnk3*$^{-/-}$ mice are viable and show protection against various apoptotic stimuli such as βA toxicity, oxygen and glucose deprivation, MPTP neurotoxin and KA, used as a model of mesial MTLE-HS. This data demonstrates the important role that JNK3 plays in neuronal death and explain why there is now currently interest in studying the involvement of this specific isoform in the development of neurological diseases (149-151). However, the high homology between JNK isoforms hinders the development of antibodies and selective inhibitors for each isoform. Thus, it is still unexplained why JNK3 plays a stronger role than the other two isoforms against brain damage, and whether it is the only JNK isoform with a neuroprotective role (105,106,130).

8. CONCLUSION

The KA experimental model shares similarities with the MTLE-HS epilepsy diseases, regarding the initial brain assaults, behavioral abnormalities associated to seizures and neuropathology. The use of this experimental animal model is a valuable tool to understand the mechanisms underlying epileptogenesis, specifically to study the potential pathways involved in the process of neuronal loss that occur in neurodegenerative processes. It is important to note that this model induces extensive neuronal damage in hippocampal structures, mainly a loss of pyramidal cells in the CA1 and CA3 regions. Considering that JNK pathway is activated in neuronal death, we focused on the analyses of JNK protein in mice KA experimental model, using different knock-outs for JNK (*jnk1*$^{-/-}$, *jnk2*$^{-/-}$ *and jnk3*$^{-/-}$). Previous data supported that *jnk3*$^{-/-}$ mice were protected from excitotoxicity induced with KA, and showed a decrease in seizures caused by this substance. Given this assumption, future studies should be performed treating this *jnk3*$^{-/-}$ knockout mice with KA. This will allow the evaluation of the potential genes involved in neuronal cell death/protection and will provide more tools to design effective drugs for neuronal protection. In addition, we suggest that JNK pathway inhibitors may constitute new adjuvant drugs for preventing seizure-induced neuronal death and may be used as potential therapeutic strategy for epileptogenesis.

9. ACKNOWLEDGEMENTS

CA, AC, ME, EV, and JF belong to 2014SGR 525 from Generalitat de Catalunya.

10. REFERENCES

1. R.S. Fisher, W. Van Emde Boas, W. Blume, C. Elger, P. Genton, Lee: Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE) *Epilepsia* 46, 470-472 (2005) DOI: 10.1111/j.0013-9580.2005.66104.x
2. M. Barker-Haliski, H.S. White: Glutamatergic Mechanisms Associated with Seizures and Epilepsy. *Cold Spring Harb Perspect Med.* 5, a022863. (2015) DOI: 10.1101/cshperspect.a022863
3. J.A. Gorter, E.A. van Vliet, E. Aronica: Status epilepticus, blood-brain barrier disruption, inflammation, and epileptogenesis. *Epilepsy Behav*. 49, 13-16 (2015) DOI: 10.1016/j.yebeh.2015.04.047
4. Pitkänen, K. Lukasiuk, F.E. Dudek, K.I. Staley. Epileptogenesis. *Cold Spring Harb Perspect Sep* 18;5(10) pii: a022822. DOI: 10.1101/cshperspect.a022822
5. K. Staley: Molecular mechanisms of epilepsy.

Nat Neurosci. 18, 367-372 (2015)
DOI: 10.1038/nn.3947

6. J. Noebels: Pathway-driven discovery of epilepsy genes. *Nat Neurosci.* 18, 344-350 (2015)
DOI: 10.1038/nn.3933
7. J.T. Paz, J.R. Huguenard: Microcircuits and their interactions in epilepsy: is the focus out of focus? *Nat Neurosci.* 18, 351-359(2015).
DOI: 10.1038/nn.3950
8. A.Y. Reid, R.J. Staba: Limbic networks: clinical perspective. *Int Rev Neurobiol.* 114, 89-120 (2014)
DOI: 10.1016/B978-0-12-418693-4.00005-4
9. K.S. Wilcox, A. Vezzani: Does brain inflammation mediate pathological outcomes in epilepsy? *Adv Exp Med Biol.* 813, 169-183 (2014)
DOI: 10.1007/978-94-017-8914-1_14
10. C.R. Houser: Do structural changes in GABA neurons give rise to the epileptic state? *Adv Exp Med Biol*. 813, 151-160 (2014)
DOI: 10.1007/978-94-017-8914-1_12
11. F. Cendes, A.C: Sakamoto, R. Spreafico, W. Bingaman, A.J. Becker: Epilepsies associated with hippocampal sclerosis. *Acta Neuropathol.* 128, 21-37 (2014)
DOI: 10.1007/s00401-014-1292-0
12. Chatzikonstantinou: Epilepsy and the hippocampus. *Front Neurol Neurosci.* 34, 121-142 (2014)
DOI: 10.1159/000356435
13. E. Trinka, J. Höfle, M. Leitinger, F. Brigo: Pharmacotherapy for Status *Epilepticus. Drugs.* 75, 1499-1521 (2015)
DOI: 10.1007/s40265-015-0454-2
14. X. Wang, J. Jin, R. Chen: Combination drug therapy for the treatment of status epilepticus. *Expert Rev Neurother*. 15, 639-654 (2015)
DOI: 10.1586/14737175.2015.1045881
15. K. Bates: Epilepsy: current evidence-based paradigms for diagnosis and treatment. *Prim Care.* 42, 217-232 (2015)
DOI: 10.1016/j.pop.2015.01.006
16. M. Biale, S.I. Johannessen, R.H. Levy, E. Perucca, T. Tomson, H.S. White: Progress report on new antiepileptic drugs: A summary of the Twelfth Eilat Conference (EILAT XII) *Epilepsy Res.* 111, 85-141 (2015)
DOI: 10.1016/j.eplepsyres.2015.01.001
17. M. Lévesque, M. Avoli, C. Bernard: Animal models of temporal lobe epilepsy following systemic chemoconvulsant administration. *J Neurosci Methods.* 260, 45-52 (2016)
DOI: 10.1016/j.jneumeth.2015.03.009
18. Y. Ben-Ari, J. Lagowska: Epileptogenic action of intra-amygdaloid injection of kainic acid. *C R Acad Sci Hebd Seances Acad Sci D.* 287, 813-816 (1978)
19. B.P. Grone, S.C. Baraban: Animal models in epilepsy research: legacies and new directions. *Nat Neurosci.* 18, 339-43 (2015)
DOI: 10.1038/nn.3934
20. J.V. Nadle: Kainic acid as a tool for the study of temporal lobe epilepsy. *Life Sci* 29, 2031-2042 (1981)
DOI: 10.1016/0024-3205(81)90659-7
21. J.E. Cavazos, S.M. Jones, D.J. Cross: Sprouting and synaptic reorganization in the subiculum and CA1 region of the hippocampus in acute and chronic models of partial-onset epilepsy. *Neuroscience* 126, 677-688 (2004)
DOI: 10.1016/j.neuroscience.2004.04.014
22. L. Kandratavicius, P.A. Balista, C. Lopes-Aguiar, R.N. Ruggiero, E.H. Umeoka, N. Garcia-Cairasco, L.S. Bueno-Junior, J.P. Leite: Animal models of epilepsy: use and limitations. *Neuropsychiatr Dis Treat.* 10, 1693-705 (2014)
DOI: 10.2147/NDT.S50371
23. M. Lévesque, M. Avoli: The kainic acid model of temporal lobe epilepsy. *Neurosci Biobehav Rev*. 37 (2013) 2887-2899.
DOI: 10.1016/j.neubiorev.2013.10.011
24. Z. Chen, R.S. Duan, H.C. Quezada, E. Mix, I. Nennesmo, A. Adem, B. Winblad, J. Zhu: Increased microglial activation and astrogliosis after intranasal administration of kainic acid in C57BL/6 mice. *J Neurol* 62, 207-218 (2005)
DOI: 10.1002/neu.20099
25. Y. Ben-Ari. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neurosci* 14, 375-403 (1985)
DOI: 10.1016/0306-4522(85)90299-4
26. Y. Ben-Ari, M. Gho: Long-lasting modification of the synaptic properties of rat CA3 hippocampal neurones induced by kainic

acid. *J Physiol.* 404, 365-384 (1988)
DOI: 10.1113/jphysiol.1988.sp017294

27. Kondratyev, K. Gale: Latency to onset of status epilepticus determines molecular mechanisms of seizure-induced cell death. *Brain Res Mol Brain Res.* 121, 86-94 (2004)
DOI: 10.1016/j.molbrainres.2003.11.005

28. T.P. Sutula, J. Hagen, A. Pitkänen. Do epileptic seizures damage the brain? *Curr Opin Neurol*. 16, 189-95 (2003)
DOI: 10.1097/00019052-200304000-00012

29. A.D. Umpierre, I.V. Bennett, L.D. Nebeker, T.G. Newell, B.B. Tian, K.E. Thomson, H.S. White, J.A. White, K.S. Wilcox. Repeated low-dose kainate administration in C57BL/6J mice produces temporal lobe epilepsy pathology but infrequent spontaneous seizures. *Exp Neurol.* 279, 116-126 (2016)
DOI: 10.1016/j.expneurol.2016.02.014

30. Pitkänen, T.P. Sutula: Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. *Lancet Neurol.* 1, 173-81 (2002)
DOI: 10.1016/S1474-4422(02)00073-X

31. T. Borsello, G. Forloni: JNK signalling: a possible target to prevent neurodegeneration. *Curr Pharm Des.* 13, 1875-1886 (2007)
DOI: 10.2174/138161207780858384

32. T. Bartsch, P. Wulff: The hippocampus in aging and disease: From plasticity to vulnerability. *Neuroscience.* 19, 1-16 (2015)
DOI: 10.1016/j.neuroscience.2015.07.084

33. F.E. Dudek: Role of Glial Cells in Seizures and Epilepsy: Intracellular Calcium Oscillations sn Spatial Buffering. *Epilepsy Curr.* 2, 137-139 (2002)
DOI: 10.1046/j.1535-7597.2002.t01 -1-00052.x

34. Y.R. Chen, T.H. Tan: The c-Jun N-terminal kinase pathway and apoptotic signaling. *Int J Oncol* 16, 651-662 (2000)
DOI: 10.3892/ijo.16.4.651

35. B.A. Strange, M.P. Witter, E.S. Lein, E.I. Moser: Functional organization of the hippocampal longitudinal axis. *Nat Rev Neurosci.* 15 655-669 (2014)
DOI: 10.1038/nrn3785

36. T. Bartsch, P. Wulff: The hippocampus in aging and disease: From plasticity to vulnerability. *Neuroscience*. 309, 1-16 (2015)
DOI: 10.1016/j.neuroscience.2015.07.084

37. L. Tatu, F. Vuillier: Structure and vascularization of the human hippocampus. *Front Neurol Neurosci*. 34, 18-25 (2014)
DOI: 10.1159/000356440

38. J.J. Chrobak, A. Lörincz, G. Buzsáki: Physiological patterns in the hippocampo-entorhinal cortex system. *Hippocampus*. 10, 457-465 (2000)
DOI:10.1002/1098-1063(2000)10:4<457:AID-HIPO12>3.0.CO;2-Z

39. S.C. Shalin, R. Egli, S.G. Birnbaum, T.L. Roth, J.M. Levenson, J.D., Sweatt: Signal transduction mechanisms in memory disorders. *Prog Brain Res.* 157, 25-41 (2006)
DOI: 10.1016/S0079-6123(06)57003-7

40. P. Duarte-Guterman, S. Yagi,C. Chow, L.A. Galea: Hippocampal learning, memory, and neurogenesis: Effects of sex and estrogens across the lifespan in adults. *Horm Behav.* 74, 37-52 (2015)
DOI: 10.1016/j.yhbeh.2015.05.024

41. E. Fernández-Espejo: Basic neurobiology of hippocampal formation. *Rev Neurol.* 24, 779-784 (1996)

42. S. Murakami, T. Takemoto, Z. Shimizu: The effective principle of Diginea simplex Aq. I. Separation of the effective fractions by liquid chromatography. *J Pharm Soc Japan* 73, 1026 (1953)

43. S. Bahn, B. Volk, W. Wisden: Kainate receptor gene expression in the developing rat brain. *J Neurosci.* 14, 5525-5547 (1994)

44. X.T. Jin, Y. Smith: Localization and functions of kainate receptors in the basal ganglia. *Adv Exp Med Biol.* 717, 27-37 (2011).
DOI: 10.1007/978-1-4419-9557-5_3

45. V. Crépel, C. Mulle: Physiopathology of kainate receptors in epilepsy. *Curr Opin Pharmacol.* 20, 83-88 (2015)
DOI: 10.1016/j.coph.2014.11.012

46. M. Carta, S. Fièvre, A. Gorlewicz, C. Mulle: Kainate receptors in the hippocampus. *Eur J Neurosci.* 39, 1835-1844 (2014)
DOI: 10.1111/ejn.12590

47. E. Szczurowska, P. Mareš. NMDA and AMPA receptors: development and status epilepticus. *Physiol Res*. 62 Suppl 1:S21-38 (2013)

48. J. Lerma, J.M. Marques: Kainate receptors in health and disease. *Neuron.* 80, 292-311 (2013)

DOI: 10.1016/j.neuron.2013.09.045

49. T.S. Sihra, G. Flores, A. Rodríguez-Moreno: Kainate receptors: multiple roles in neuronal plasticity. *Neuroscientist.* 20, 29-43(2014). DOI: 10.1177/1073858413478196
50. X.Y. Zheng, H.L. Zhang, Q. Luo, J. Zhu: Kainic acid-induced neurodegenerative model: potentials and limitations. *J Biomed Biotechnol.* 457079 (2011) DOI: 10.1155/2011/457079
51. F. Zheng,K.D. Phelan: The role of canonical transient receptor potential channels in seizure and excitotoxicity. *Cells.* 3, 288-303 (2014) DOI: 10.3390/cells3020288
52. J. Gonzalez, J.C. Jurado-Coronel, M.F. Ávila, A. Sabogal, F. Capani, G.E. Barreto. NMDARs in neurological diseases: a potential therapeutic target. *Int J Neurosci.* 125, 315-327 (2015) DOI: 10.3109/00207454.2014.940941
53. V. Vyklicky, M. Korinek, T. Smejkalova, A. Balik, B. Krausova, M. Kaniakova, K. Lichnerova, J. Cerny, J. Krusek, I. Dittert, M. Horak, L. Vyklicky: Structure, function, and pharmacology of NMDA receptor channels. *Physiol Res.* 63. Suppl 1:S191-203 (2014)
54. Y. Ben-Ari, R. Cossart: Kainate, a double agent that generates seizures: two decades of progress. *Trends Neurosci*. 23, 580-587(2000) DOI: 10.1016/S0166-2236(00)01659-3
55. E.B. Bloss,R.G. Hunter. Hippocampal kainate receptors. *Vitam Horm.* 82, 167-184 (2010) DOI: 10.1016/S0083-6729(10)82009-6
56. Lin, B. Dibling: The true face of JNK activation in apoptosis. *Aging Cell* 1, 112-116 (2002) DOI: 10.1046/j.1474-9728.2002.00014.x
57. T. Herdegen, P. Skene. M. Bahr. The c-Jun transcription factor-bipotential mediator of neuronal death, survival and regeneration. *Trends Neurosci.* 20. 227-231 (1997) DOI: 10.1016/S0166-2236(96)01000-4
58. Q. Wang, S. Yu, A. Simonyi, G. Y. Sun, A.Y. Sun. Kainic acid-mediated excitotoxicity as a model for neurodegeneration. *Mol Neurobiol.* 31, 3-16 (2005) DOI: 10.1385/MN:31:1-3:003
59. E.J. Shin, J.H. Jeong, Y.H. Chung, W.K. Kim, K,H. Ko, J.H. Bach, J.S. Hong, Y. Yoneda, H.C. Kim: Role of oxidative stress in epileptic seizures. *Neurochem Int.* 59, 122-137 (2011) DOI: 10.1016/j.neuint.2011.03.025
60. R.J. Reiter, L.C. Manchester, D.X. Tan: Neurotoxins: free radical mechanisms and melatonin protection. *Curr Neuropharmacol.* 8 194-210 (2010) DOI: 10.2174/157015910792246236
61. Lau, M. Tymianski: Glutamate receptors, neurotoxicity and neurodegeneration. *Pflugers Arch.* 460, 525-542 (2010) DOI: 10.1007/s00424-010-0809-1
62. S.S. Oja, P. Saransaari: Modulation of taurine release by glutamate receptors and nitric oxide. *Prog Neurobiol.* 62, 407-425 (2000) DOI: 10.1016/S0301-0082(00)00005-8
63. M. Gonzalez-Zulueta, L.M. Ensz, G. Mukhina, R.M. Lebovitz, R.M. Zwacka, J.F. Engelhardt, L.W. Oberley, V.L.Dawson, T.W. Dawson: Manganese superoxide dismutase protects nNOS neurons from NMDA and nitric oxide-mediated neurotoxicity. *J Neurosci.* 18, 2040-2055 (1998)
64. D.C. Henshall, T. Araki, C.K. Schindler, S. Shinoda, J.Q. Lan, R.P. Simon: Expression of death-associated protein kinase and recruitment to the tumor necrosis factor signaling pathway following brief seizures. *J Neurochem.* 86, 1260-1270 (2003) DOI: 10.1046/j.1471-4159.2003.01934.x
65. S. Shinoda, S.L. Skradski, T. Araki, C.K. Schindler, R. Meller, J.Q. Lan, W. Taki, R.P. Simon, D.C. Henshall: Formation of a tumour necrosis factor receptor 1 molecular scaffolding complex and activation of apoptosis signal-regulating kinase 1 during seizure-induced neuronal death. *Eur J Neurosci.* 17, 2065-2076 (2003) DOI: 10.1046/j.1460-9568.2003.02655.x
66. D.A. Flusberg, P.K. Sorger: Surviving apoptosis: life-death signaling in single cells. *Trends Cell Biol.* 25, 446-458 (2015) DOI: 10.1016/j.tcb.2015.03.003
67. D.B. Wang, C. Kinoshita, Y. Kinoshita, R.S. Morrison: p53 and mitochondrial function in neurons. *Biochim Biophys Acta.* 1842, 1186-1197 (2014) DOI: 10.1016/j.bbadis.2013.12.015
68. Almeida. Genetic determinants of neuronal vulnerability to apoptosis. *Cell Mol Life Sci.* 70, 71-88 (2013). DOI: 10.1007/s00018-012-1029-y

69. J.L. Franklin. Redox regulation of the intrinsic pathway in neuronal apoptosis. *Antioxid Redox Signal*. 14, 1437-1448 (2011). DOI: 10.1089/ars.2010.3596

70. L. Galluzzi, K. Blomgren, G. Kroemer. Mitochondrial membrane permeabilization in neuronal injury. *Nat Rev Neurosci.* 10, 481-494 (2009) DOI: 10.1038/nrn2665

71. Kondratyev, K. Gale: Latency to onset of status epilepticus determines molecular mechanisms of seizure-induced cell death. *Brain Res Mol Brain Res.* 5, 86-94 (2004) DOI: 10.1016/j.molbrainres.2003.11.005

72. N. Donovan, E.B. Becker, Y. Konishi Y, A. Bonni: JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. *J Biol Chem.* 277, 40944-40949 (2002) DOI: 10.1074/jbc.M206113200

73. X.M. Zhang, J. Zhu: Kainic Acid-induced neurotoxicity: targeting glial responses and glia-derived cytokines. *Curr Neuropharmacol.* 9, 388-398 (2011) DOI: 10.2174/157015911795596540

74. B.A. Miller, F. Sun, R.N. Christensen, A.R. Ferguson, J.C. Bresnahan, M.S. Beattie: A sublethal dose of TNFalpha potentiates kainate-induced excitotoxicity in optic nerve oligodendrocytes. *Neurochem Res.* 30, 867-875 (2005) DOI: 10.1007/s11064-005-6880-x

75. M. Oprica, C. Eriksson, M. Schultzberg: Inflammatory mechanisms associated with brain damage induced by kainic acid with special reference to the interleukin-1 system. *J Cell Mol Med*. 7, 127-140 (2003) DOI: 10.1111/j.1582-4934.2003.tb00211.x

76. A.A. Farooqui, W. Yi Ong, X.R. Lu, B. Halliwell, L.A. Horrocks: Neurochemical consequences of kainate-induced toxicity in brain: involvement of arachidonic acid release and prevention of toxicity by phospholipase A(2) inhibitors. *Brain Res Brain Res Rev.* 38, 61-78 (2001) DOI: 10.1016/S0169-328X(01)00214-5

77. M. Oprica, C. Eriksson, M. Schultzberg: Inflammatory mechanisms associated with brain damage induced by kainic acid with special reference to the interleukin-1 system. *J Cell Mol Med.* 7, 127-140 (2003) DOI: 10.1111/j.1582-4934.2003.tb00211.x

78. Sanz, A. Estrada, I. Ferrer, A.M. Planas: Differential cellular distribution and dynamics of HSP70, cyclooxygenase-2, and c-Fos in the rat brain after transient focal ischemia or kainic acid. *Neuroscience.* 80, 221-232 (1997) DOI: 10.1016/S0306-4522(97)00089-4

79. A.M. Planas, M.A. Soriano, I. Ferrer, E. Rodríguez Farré: Kainic acid-induced heat shock protein-70, mRNA and protein expression is inhibited by MK-801 in certain rat brain regions. *Eur J Neurosci*. 7, 293-304 (1995) DOI: 10.1111/j.1460-9568.1995.tb01065.x

80. T. Borsello, P.G. Clarke, L. Hirt, A. Vercelli, M. Repici, D.F. Schorderet, J. Bogousslavsky, C. Bonny: A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat Med.* 9, 1180-1186 (2003) DOI: 10.1038/nm911

81. Anthony, A.A. Oliva, C.M. Atkins, L. Copenagle, G.A. Banker: Activated c-Jun NTerminal Kinase Is Required for Axon Formation. *J Neurosci.* 26, 9462-9470 (2006) DOI: 10.1523/JNEUROSCI.2625-06.2006

82. R.S. Sloviter, L. Ali-Akbarian, K.D. Horvath, K.A. Menkens: Substance P receptor expression by inhibitory interneurons of the rat hippocampus: enhanced detection using improved immunocytochemical methods for the preservation and colocalization of GABA and other neuronal markers. *J Comp Neurol.* 12, 283-305 (2001) DOI: 10.1002/1096-9861 (20010212) 430:3 <283: AID-CNE1031>3.0.CO;2-V DOI: 10.1002/1096-9861 (20010212) 430:3 <283:AID-CNE1031>3.3.CO;2-M

83. M. Aouadi, B. Binetruy, L. Caron, Y.L. Marchand-Brustel, F. Bost: Role of MAPKs in development and differentiation: lessons from knockout mice. *Biochimica.* 88, 1091-1098 (2006) DOI: 10.1016/j.biochi.2006.06.003

84. J. Avruch: MAPKinase pathways: the first twenty years. *Biochim Biophys Acta.* 1773, 1150- 1160 (2007) DOI: 10.1016/j.bbamcr.2006.11.006

85. L. Chang, M. Karin: Mammalian MAP kinase signalling cascades. *Nature.* 410, 37-40 (2001)

DOI: 10.1038/35065000
DOI: 10.1038/35068583

86. J.M. Kyriakis, J. Avruch: Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.* 81: 807-869 (2001)

87. L. de Lemos, F. Junyent, E. Verdaguer, J. Folch, R. Romero, M. Pallàs, I. Ferrer, C. Auladell, A. Camins: Differences in activation of ERK1/2 and p38 kinase in Jnk3 null mice following KA treatment. *J Neurochem.* 114, 1315-1322 (2010)
DOI: 10.1111/j.1471-4159.2010.06853.x

88. R.M. Biondi, R.A. Nebred: Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *Biochem J.* 372, 1-13 (2003)
DOI: 10.1042/bj20021641

89. D.N. Dhanasekaran, K. Kashef, C.M. Lee, H. Xu, E.P. Reddy: Scaffold proteins of MAP-kinase modules. *Oncogene*. 26, 3185-3202 (2007)
DOI: 10.1038/sj.onc.1210411

90. T.G. Boulton, M.H. Cobb: Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul.* 2, 357-371 (1991)
DOI: 10.1091/mbc.2.5.357

91. T.G. Boulton, S.H. Nye, D.J. Robbins, N.Y. Ip, E. Radziejewska, S.D. Morgenbesser, R.A. DePinho, N. Panayotatos, M.H. Cobb, G.D. Yancopoulos: ERK's: a family of proteinserine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell.* 65, 663-675 (1991)
DOI: 10.1016/0092-8674(91)90098-J

92. Cuenda, S. Rousseau: p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta.* 1773, 1358-1375 (2007)
DOI: 10.1016/j.bbamcr.2007.03.010

93. Y. Keshet, R. Seger: The MAP Kinase Signaling Cascades: A system of hundreds of components regulates a diverse array of physiological functions. *Methods in Molecular Biology*. 661, 3-38 (2010)
DOI: 10.1007/978-1-60761-795-2_1

94. T.H. Chao, M. Hayashi, R.I. Tapping, Y. Kato, J.D. Lee: MEKK3 directly regulates MEK5 activity as part of the big mitogen- activated protein kinase 1 (BMK1) signaling pathway. *J Biol Chem.* 274 36035-36038 (1999)
DOI: 10.1074/jbc.274.51.36035

95. J.M. Kyriakis, P. Banerjee, E. Nikolakaki, T. Dai, E.A. Rubie, M.F. Ahmad, J. Avruch, J.R. Woodgett: The stress-activated protein kinase subfamily of c-Jun kinases. *Nature.* 369, 156-160 (1994)
DOI: 10.1038/369156a0

96. R.J. Davis Signal transduction by the JNK group of MAP kinases. *Cell.* 2, 239-252 (2000)
DOI: 10.1016/S0092-8674(00)00116-1

97. M.A. Bogoyevitch, B. Kobe: Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. *Microbiol Mol Biol Rev.* 70, 1061-1095 (2006)
DOI: 10.1128/MMBR.00025-06

98. K.A. Gallo, G.L. Johnson: Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nat Rev Mol Cell Biol*. 3, 663-672 (2002)
DOI: 10.1038/nrm906

99. E.T. Coffey: Nuclear and cytosolic JNK signaling in neurons. *Nat Rev Neurosci.* 15, 285-299 (2014)
DOI: 10.1038/nrn3729

100. E.T. Coffey, V. Hongisto, M. Dickens, R.J. Davis, M.J. Courtney: Dual roles for c-Jun N-terminal kinase in developmental and stress responses in cerebellar granule neurons. *J Neurosci.* 20, 7602-7613 (2000)

101. H. Kishimoto, K. Nakagawa, T. Watanabe, D. Kitagawa, H. Momose, J. Seo, G. Nishitai, N. Shimizu, S. Ohata, S. Tanemura, S. Asaka, T. Goto, H. Fukushi, H. Yoshida, A. Suzuki, T. Sasaki, T. Wada, J.M. Penninger, H. Nishina, T. Katada: Different properties of SEK1 and MKK7 in dual phosphorylation of stress-induced activated protein kinase SAPK/JNK in embryonic stem cells. *J Biol Chem*. 278, 16595-16601 (2003)
DOI: 10.1074/jbc.M213182200

102. K.G. Finegan, C. Tournier: The mitogen-activated protein kinase kinase 4 has a prooncogenic role in skin cancer. *Cancer Res.* 70, 5797-5806 (2010)
DOI: 10.1158/0008-5472.CAN-09-3669

103. S. Gupta, T. Barrett, A.J. Whitmarsh, J. Cavanagh, H.K. Sluss, B. Derijard, R.J. Davis: RJ. Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J.* 15, 2760-2770 (1996)

104. S. Brecht, R. Kirchhof, A. Chromik, M.

Willesen, T. Nicolaus, G. Raivich, J. Wessig, V. Waetzig, M. Goetz, M. Claussen, D. Pearse, C.Y. Kuan, E. Vaudano, A. Behrens, E. Wagner, R.A. Flavell, R.J. Davis, T. Herdegen: Specific pathophysiological functions of JNK isoforms in the brain. *Eur Journal Neurosci.* 21, 363-377 (2005)
DOI: 10.1111/j.1460-9568.2005.03857.x

105. C.Y. Kuan, Whitmarsh AJ, Yang DD, Liao G, Schloemer AJ, Dong C, Bao J, Banasiak KJ, Haddad GG, Flavell RA, Davis RJ, Rakic P. A critical role of neural-specific JNK3 for ischemic apoptosis. *Proc Natl Acad Sci USA*. 100, 15184-15189 (2003)
DOI: 10.1073/pnas.2336254100

106. L. Carboni, R. Carletti, S. Tacconi, C. Corti, F. Ferraguti: Differential expression of SAPK isoforms in the rat brain. An in hybridisation study in the adult rat brain and during post-natal development. *Brain Res Mol Brain Res*. 60, 57-68 (1998)
DOI: 10.1016/S0169-328X(98)00166-1

107. L. Resnick, M. Fennell. Targeting JNK3 for the treatment of neurodegenerative disorders. *Drug Discov Today.* 9, 932-939 (2004)
DOI: 10.1016/S1359-6446(04)03251-9

108. N. Kelkar, S. Gupta, M. Dickens, R.J. Davis: Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. *Mol Cell Biol.* 20, 1030-1043 (2000)
DOI: 10.1128/MCB.20.3.1030-1043.2000

109. A.H. Kim, T. Sasaki, M.V. Chao: JNK-interacting protein 1 promotes Akt1 activation. *J Biol Chem.* 278, 29830-29836 (2003)
DOI: 10.1074/jbc.M305349200

110. J. Yasuda, A.J. Whitmarsh, J. Cavanagh, M. Sharma, R.J. Davis: The JIP group of mitogen-activated protein kinase scaffold proteins. *Mol Cell Biol.* 19, 7245-7254 (1999)
DOI: 10.1128/MCB.19.10.7245

111. Ikeda, K. Hasegawa, M. Masaki, T. Moriguchi, E, Nishida, Y. Kozutsumi, S. Oka, T. Kawasaki: Mixed lineage kinase LZK forms a functional signaling complex with JIP-1, a scaffold protein of the c-Jun NH(2)-terminal kinase pathway. *J Biochem.* 130, 773-781 (2001)
DOI: 10.1093/oxfordjournals.jbchem.a003048

112. A.J. Whitmarsh: The JIP family of MAPK scaffold proteins. *Biochem Soc Trans.* 34, 828-832 (2006)
DOI: 10.1042/BST0340828

113. A.J. Whitmarsh, C.Y. Kuan, N.J. Kennedy, N. Kelkar, T.F. Haydar, J.P. Mordes, M. Appel, A.A. Rossini, S.N. Jones, R.A. Flavell, P. Rakic, R.J. Davis: Requirement of the JIP1 scaffold protein for stress-induced JNK activation. *Genes Dev.* 15, 2421-2432 (2001)
DOI: 10.1101/gad.922801

114. D. Nihalani, H.N. Wong, L.B. Holzman: Recruitment of JNK to JIP1 and JNK-dependent JIP1 phosphorylation regulates JNK module dynamics and activation. *J Biol Chem.* 278, 28694-28702 (2003)
DOI: 10.1074/jbc.M304212200

115. S. Negri, A. Oberson, M. Steinmann, C. Sauser, P. Nicod, G. Waeber, D.F. Schorderet, C. Bonny: cDNA cloning and mapping of a novel islet-brain/JNK-interacting protein. *Genomics.* 64, 324-330 (2000)
DOI: 10.1006/geno.2000.6129

116. Z. Wang, Y. Chen, Y. Lü, X. Chen, L. Cheng, X. Mi, X. Xu, W. Deng, Y. Zhang, N. Wang, J. Li, Y. Li, X. Wang; Effects of JIP3 on epileptic seizures: Evidence from temporal lobe epilepsy patients, kainic-induced acute seizures and pentylenetetrazole-induced kindled seizures. *Neuroscience.* 300, 314-324 (2015)
DOI: 10.1016/j.neuroscience.2015.05.008

117. Matsuura H, Nishitoh H, Takeda K, Matsuzawa A, Amagasa T, Ito M, Yoshioka K, Ichijo H. Phosphorylation-dependent scaffolding role of JSAP1/JIP3 in the ASK1-JNK signaling pathway. A new mode of regulation of the MAP kinase cascade. *J Biol Chem.* 277 (2002) 40703-40709.
DOI: 10.1074/jbc.M202004200

118. N. Kelkar, C.L. Standen, R.J. Davis: Role of the JIP4 scaffold protein in the regulation of mitogen-activated protein kinase signaling pathways. *Mol Cell Biol.* 25, 2733-2743 (2005)
DOI: 10.1128/MCB.25.7.2733-2743.2005

119. B.K. Sun, J.H. Kim, H.N. Nguyen, S. Oh, S.Y. Kim, S. Choi, H.J. Choi, Y.J. Lee, J.J. Song. MEKK1/MEKK4 are responsible for TRAIL-induced JNK/p38 phosphorylation. *Oncol Rep.* 25, 537-544 (2011)

120. J. Liu, A. Lin. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res.* 15 36-42 (2005)
DOI: 10.1038/sj.cr.7290262

121. 121, G. Verma, M. Datta: The critical role of JNK in the ER-mitochondrial crosstalk during apoptotic cell death. *J Cell Physiol.* 227, 1791-1795 (2012)
DOI: 10.1002/jcp.22903

122. Camins, F.X. Sureda, F.Junyent, E. Verdaguer, J. Folch, C. Beas-Zarate, M. Pallas: An overview of investigational antiapoptotic drugs with potential application for the treatment of neurodegenerative disorders. *Expert Opin Investig Drugs.* 19, 587-604 (2010)
DOI: 10.1517/13543781003781898

123. Ferrer, R. Blanco, M. Carmona: Differential expression of active, phosphorylation dependent MAP kinases, MAPK/ERK, SAPK/JNK and p38, and specific transcription factor substrates following quinolinic acid excitotoxicity in the rat. *Mol. Brain Res.* 94, 48-58 (2001)
DOI: 10.1016/S0169-328X(01)00198-X

124. Ferrer, T. Gomez-Isla, B. Puig, M. Freixes, E. Ribé, E. Dalfó, J. Avila: Current advances on different kinases involved in tau phosphorylation and implications in Alzheimer's disease and taupathies. *Curr Alzheimer Res.* 2, 3-18 (2005)
DOI: 10.2174/1567205052772713

125. N.V. Oleinik, N.I. Krupenko, S.A. Krupenko: Cooperation between JNK1 and JNK2 in activation of p53 apoptotic pathway. *Oncogene.* 26, 7222-7230 (2007)
DOI: 10.1038/sj.onc.1210526

126. Y.R. Chen, T.H. Tan: The c-Jun N-terminal kinase pathway and apoptotic signaling (review) Int J Oncol. 16, 651-662 (2000)
DOI: 10.1016/S0039-9140(00)00551-8

127. G. Verma, M. Datta: The critical role of JNK in the ER-mitochondrial crosstalk during apoptotic cell death. *J Cell Physiol.* 227, 1791-1795 (2012)
DOI: 10.1002/jcp.22903

128. D. Bozyczko-Coyne, T.M. O'Kane, Z.L. Wu, P. Dobrzanski, S. Murthy, J.L.: Vaught, Scott RW CEP-1347/KT-7515, an inhibitor of SAPK/JNK pathway activation, promotes survival and blocks multiple events associated with Abeta-induced cortical neuron apoptosis. *J Neurochem*. 77, 849-863 (2001)
DOI: 10.1046/j.1471-4159.2001.00294.x

129. S. Gourmaud, C. Paquet, J. Dumurgier, C. Pace, C. Bouras, F. Gray, J.L. Laplanche, E.F. Meurs, F. Mouton-Liger,J. Hugon: Increased levels of cerebrospinal fluid JNK3 associated with amyloid pathology: links to cognitive decline. *J Psychiatry Neurosci*. 40, 151-61 (2015)
DOI: 10.1503/jpn.140062

130. S.O. Yoon, D.J. Park, J.C. Ryu, H.G. Ozer, C. Tep, Y.J. Shin, T.H. Lim, L. Pastorino, A.J. Kunwar, J.C. Walton, A.H. Nagahara, K.P. Lu, R.J. Nelson, M.H. Tuszynski, K. Huang: JNK3 perpetuates metabolic stress induced by Aβ peptides. *Neuron.* 75. 824-837 (2012)
DOI: 10.1016/j.neuron.2012.06.024

131. W.T. Kimberly, J.B. Zheng, T. Town, R.A. Flavell, D.J. Selkoe. Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. *J Neurosci.* 25, 5533-5543 (2005)
DOI: 10.1523/JNEUROSCI.4883-04.2005

132. S. Carboni, B. Antonsson, P. Gaillard, J.P. Gotteland, J.Y. Gillon, P.A. Vitte: Control of death receptor and mitochondrial-dependent apoptosis by c-Jun N-terminal kinase in hippocampal CA1 neurones following global transient ischaemia. *J Neurochem.* 92, 1054-1060 (2005)
DOI: 10.1111/j.1471-4159.2004.02925.x

133. Q.H. Guan, D.S. Pei, Q.G. Zhang, Z.B. Hao, T.L. Xu, G.Y. Zhang: The neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via nuclear and non-nuclear pathways. *Brain Res.* 1035, 51-59 (2005)
DOI: 10.1016/j.brainres.2004.11.050

134. M.S. Saporito, B.A. Thomas, R.W. Scott. MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo. *J Neurochem.* 75, 1200-1208 (2000)
DOI: 10.1046/j.1471-4159.2000.0751200.x

135. Parkinson Study Group PRECEPT Investigators. Mixed lineage kinase inhibitor CEP-1347 fails to delay disability in early Parkinson disease. *Neurology.* 69, 1480-1490 (2007)
DOI: 10.1212/01.wnl.0000277648.63931.c0

136. W. Wang, L. Shi, Y. Xie, C. Ma,W. Li, X. Su, S. Huang, R. Chen, Z. Zhu, Z. Mao, Y. Han, M. Li: SP600125, a new JNK inhibitor, protects

dopaminergic neurons in the MPTP model of Parkinson's disease. *Neurosci Res.* 48, 195-202 (2004)
DOI: 10.1016/j.neures.2003.10.012

137. S. Carboni, A. Hiver, C. Szyndralewiez, P. Gaillard, J.P. Gotteland, P.A. Vitte: AS601245 (1,3-benzothiazol-2-yl (2-[[2-(3-pyridinyl) ethyl] amino]-4 pyrimidinyl) acetonitrile): a c-Jun NH2-terminal protein kinase inhibitor with neuroprotective properties. *J Pharmacol Exp Ther*. 310, 25-32 (2004)
DOI: 10.1124/jpet.103.064246

138. S. Carboni, U. Boschert, P. Gaillard, J. P. Gotteland, J.Y. Gillon, P.A. Vitte: AS601245, a c-Jun NH2-terminal kinase (JNK) inhibitor, reduces axon/dendrite damage and cognitive deficits after global cerebral ischaemia in gerbils. *Br J Pharmacol.* 153 157-163(2008)
DOI: 10.1038/sj.bjp.0707574

139. C. Bonny, A. Oberson, S. Negri, C. Sauser, D.F. Schorderet: Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes.* 50, 77-82 (2001)
DOI: 10.2337/diabetes.50.1.77

140. M. Repici, C. Centeno, S. Tomasi, G. Forloni, C. Bonny, A. Vercelli, T. Borsello: Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation. *Neuroscience.* 150, 40-49 (2007).
DOI: 10.1016/j.neuroscience.2007.08.021

141. Behrens, M. Sibilia, E.F. Wagner: Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat Genet.* 21, 326-329 (1999).
DOI: 10.1038/6854

142. Dunn, C. Wiltshire, A. MacLaren, D.A. Gillespie. Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. *Cell Signal*. 14, 585-593 (2002)
DOI: 10.1016/S0898-6568(01)00275-3

143. F. Junyent, L. de Lemos, E. Verdaguer, J. Folch, I. Ferrer, D. Ortu-o-Sahagún, C. Beas-Zárate, R. Romero, M. Pallàs, C. Auladell, A. Camins:.Gene expression profile in JNK3 null mice: a novel specific activation of the PI3K/AKT pathway. *J Neurochem.* 117 244-252 (2011)
DOI: 10.1111/j.1471-4159.2011.07195.x

144. F. Junyent, L. de Lemos, E. Verdaguer, M. Pallàs, J. Folch, C. Beas-Zárate, A. Camins, C. Auladell: Lack of Jun-N-terminal kinase 3 (JNK3) does not protect against neurodegeneration induced by 3-nitropropionic acid. *Neuropathol Appl Neurobiol.* 38, 311-321 (2012)
DOI: 10.1111/j.1365-2990.2011.01214.x

145. D. Petrov, M. Luque, I. Pedrós, M. Ettcheto, S. Abad, M. Pallàs, E. Verdaguer, C. Auladell, J. Folch, A. Camins: Evaluation of the Role of JNK1 in the Hippocampus in an Experimental Model of Familial Alzheimer's Disease. *Mol Neurobiol*. Nov 12 (2015)
DOI: 10.1007/s12035-015-9522-6

146. W.T. Kimberly, J.B. Zheng, T. Town, R.A. Flavell, D.J. Selkoe. Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. *J Neurosci*. 25, 5533-5543 (2005)
DOI: 10.1523/JNEUROSCI.4883-04.2005

147. P. Gaillard, I. Jeanclaude-Etter, V. Ardissone, S. Arkinstall, Y. Cambet, M. Camps, C. Chabert, D. Church, R. Cirillo, D. Gretener, S. Halazy, A. Nichols, C. Szyndralewiez, P.A. Vitte, J.P. Gotteland: Design and synthesis of the first generation of novel potent, selective, and in vivo active (benzothiazol-2-yl) acetonitrile inhibitors of the c-Jun N-terminal kinase. *J Med Chem.* 48 4596-4607 (2005)
DOI: 10.1021/jm0310986

148. S.R. Brucknerm, S.P. Tammariello, C.Y. Kuan, R.A. Flavell, R. Rakic, S. Estus: JNK3 contributes to c-Jun activation and apoptosis but not oxidative stress in nerve growth factor deprived sympathetic neurons. *J Neurochem.* 78, 298-303 (2001)
DOI: 10.1046/j.1471-4159.2001.00400.x

149. C.Y. Kuan, D.D. Yang, D.R. Samanta Roy, R.J. Davis, P. Rakic, R.A. Flavell: The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron.* 22, 667-676 (1999)
DOI: 10.1016/S0896-6273(00)80727-8

150. S. Hunot, M.Vila, P. Teismann, R.J. Davis, E.C. Hirsch, S. Przedborski, P. Rakic P, R.A. Flavell: JNK mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease. *Proc Natl Acad Sci USA.* 101, 665-670 (2004)

DOI: 10.1073/pnas.0307453101

151. C.Y. Kuan, R.E. Burke: Targeting the JNK signaling pathway for stroke and Parkinson's diseases therapy. *Curr Drug Targ CNS Neurol Disord.* 4, 63-67 (2005)
DOI: 10.2174/1568007053005145

Key Words: Kainic Acid, HippocampuS, c-Jun N-Terminal Kinase Signaling Pathway, Apoptosis, Neuroprotection, Review

Send correspondence to: Antoni Camins, Unitat de Farmacologia I Farmacognosia, Facultat de Farmacia, Centros de Investigacion Biomedica en Red de Enfermedades Neurodegenerativas (CIBERNED), Universitat de Barcelona, Barcelona, Avda/Joan XXIII, Spain, Tel: 34 93 4024531, Fax: 34 934035982, E-mail: camins@ub.edu

Annex II

The implication of the brain insulin receptor in late onset Alzheimer's disease dementia.

Folch J, Ettcheto M, Busquets O, Sánchez-López E, Castro-Torres RD, Verdaguer E, Manzine PR, Rabieipoor S, García ML, Olloquequi J, Beas-Zarate C, Auladell C and Camins A.

Pharmaceuticals. 2018;11(1):1–16.

pharmaceuticals

MDPI

Review

The Implication of the Brain Insulin Receptor in Late Onset Alzheimer's Disease Dementia

Jaume Folch [1,2], Miren Ettcheto [1,2,3,4], Oriol Busquets [1,2,3,4], Elena Sánchez-López [2,5,6], Rubén D. Castro-Torres [2,3,4,7,8], Ester Verdaguer [2,4,7], Patricia R. Manzine [3,9], Saghar Rabiei Poor [3], María Luisa García [5,6], Jordi Olloquequi [10], Carlos Beas-Zarate [8], Carme Auladell [2,4,7] and Antoni Camins [2,3,4,*]

1 Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, 43201 Reus, Spain; jaume.folch@urv.cat (J.F.); emiren@gmail.com (M.E.); oriolbusquets@gmail.com (O.B.)
2 Biomedical Research Networking Centre in Neurodegenerative Diseases (CIBERNED), 28031 Madrid, Spain; esanchezlopez@ub.edu (E.S.-L.); rubendario@gmail.com (R.D.C.-T.); everdaguer@ub.edu (E.V.); cauladell@ub.edu (C.A.)
3 Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Av. Joan XXIII 27/31, E-08028 Barcelona, Spain; patricia_manzine@yahoo.com.br (P.R.M.); shagar.rabii@gmail.com (S.R.P.)
4 Institut de Neurociències, Universitat de Barcelona, E-08028 Barcelona, Spain
5 Unitat de Farmàcia, Tecnologia Farmacèutica i Fisico-química, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, E-08028 Barcelona, Spain; rdcm@ub.edu
6 Institute of Nanoscience and Nanotechnology (IN2UB), University of Barcelona, Barcelona E-08028, Spain
7 Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, E-08028 Barcelona, Spain
8 Laboratorio de Regeneración y Desarrollo Neural, Instituto de Neurobiología, Departamento de Biología Celular y Molecular, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Zapopan 44600, Mexico; carlosbeas55@gmail.com
9 Department of Gerontology, Federal University of São Carlos (UFSCar), São Carlos 13565-905, Brazil
10 Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca 3460000, Chile; jolloquequi@gmail.com
* Correspondence: camins@ub.edu; Tel.: +34-93-4024-531; Fax: +34-93-4035-982

Received: 29 December 2017; Accepted: 25 January 2018; Published: 29 January 2018

Abstract: Alzheimer's disease (AD) is progressive neurodegenerative disorder characterized by brain accumulation of the amyloid β peptide (Aβ), which form senile plaques, neurofibrillary tangles (NFT) and, eventually, neurodegeneration and cognitive impairment. Interestingly, epidemiological studies have described a relationship between type 2 diabetes mellitus (T2DM) and this pathology, being one of the risk factors for the development of AD pathogenesis. Information as it is, it would point out that, impairment in insulin signalling and glucose metabolism, in central as well as peripheral systems, would be one of the reasons for the cognitive decline. Brain insulin resistance, also known as Type 3 diabetes, leads to the increase of Aβ production and TAU phosphorylation, mitochondrial dysfunction, oxidative stress, protein misfolding, and cognitive impairment, which are all hallmarks of AD. Moreover, given the complexity of interlocking mechanisms found in late onset AD (LOAD) pathogenesis, more data is being obtained. Recent evidence showed that Aβ42 generated in the brain would impact negatively on the hypothalamus, accelerating the "peripheral" symptomatology of AD. In this situation, Aβ42 production would induce hypothalamic dysfunction that would favour peripheral hyperglycaemia due to down regulation of the liver insulin receptor. The objective of this review is to discuss the existing evidence supporting the concept that brain insulin resistance and altered glucose metabolism play an important role in pathogenesis of LOAD. Furthermore, we discuss AD treatment approaches targeting insulin signalling using anti-diabetic drugs and mTOR inhibitors.

Keywords: Alzheimer's; insulin resistance; amyloid; TAU; cognition; insulin receptor; type 2 diabetes

Pharmaceuticals **2018**, *11*, 11

1. Introduction

Prevention is a key factor when trying to reduce the impact of age-related diseases like cardiovascular alterations, cancer and dementias. These pathologies do not only create substantial personal and family burdens but, unsustainable increases in the public health economic costs in developed populations. The most common form of dementia is Alzheimer's disease (AD) [1–7]. The number of patients diagnosed with AD is rapidly increasing worldwide and becoming a common cause of death in aging populations [4–7]. Moreover, no effective treatments have been established yet to prevent or delay the progression of AD [8,9].

AD has been classified into two groups, depending on its onset: the first classification is familial AD, related to genetic alterations of the amyloid beta precursor protein (AβPP) and preselinins (PS1) [1–5]. This subgroup represents approximately about 3% of the diseased patients [1,4]. The other classification group is the late onset form (LOAD), also known as sporadic. It accounts for the remaining 97% of diagnoses [1,4]. Historically, the neuropathological characteristics of AD were described through the amyloidogenic hypothesis by Selkoe (1992) [9,10]. They were: cognitive loss, abnormal accumulations of Aβ and hyperphosphorylation of TAU protein in areas of the cerebral cortex and hippocampus [6–10]. Initially, the Aβ peptide is generated from the catabolism of AβPP, a plasmatic membrane protein with a single domain found in different cellular types, including neurons, astrocytes and oligodendrocytes [10,11]. This protein is cleaved by α-, β-, and γ-secretase enzymes, as well as, a complex of proteins containing presenilin 1 (PS1) [4,10,11]. In neuropathological situations, AβPP is metabolized predominantly by the amyloidogenic pathway in which the Aβ cleaving enzyme 1 (BACE 1; β-secretase) breaks AβPP by the N-terminal end while γ-secretases cleave the C-terminal end, obtaining Aβ40 and Aβ42 fragments that remain in the extracellular space [4,10].

Since the presence of amyloid plaques has been repeatedly demonstrated not to be strictly correlated with AD symptoms, considerable research has focused on understanding its actual role. Now, one of the working hypotheses is that Aβ42 accumulates in the form of several soluble species, oligomers and protofibres, which contain potentially high toxic properties [11]. As the concentration of these molecules increases, they continue to aggregate into insoluble fibres that are the main constituents of Aβ plaques. Nevertheless, some authors suggest that the cognitive impairment correlates best with alterations on the TAU protein than the Aβ plaques, suggesting a predominant role for TAU in the pathogenesis of AD [4,9–11]. Therefore, in the field of AD research, there are multiple approaches to consider when trying to understand the origin of this disease.

Another hallmark that has been associated as a risk factor for LOAD is the ε4 allele of the apolipoprotein E (APOE) gene [12–16]. This information was demonstrated when studying how the response to intranasal insulin differed between carriers of different apolipoprotein ε4 alleles [14]. APOE genotype influences peripheral glucose and insulin metabolism. Also, as it occurs in other diseases, gender has an influence in the affection and incidence of pathology. APOE positive carrier women have higher risk than men to develop LOAD and, respond less favourably to insulin related therapies [13–16]. Importantly, the molecular basis of this association has remained elusive for decades. Yet, recent findings determined that APOE4 interacts with insulin receptors (IR), impairing its trafficking between endosomes and the plasmatic membranes, by trapping them and favouring the development of impaired insulin signalling [16]. Furthermore, the association between T2DM and LOAD amyloid pathology is specific among carriers of the apolipoprotein E (APOE) ε4 gene allele, compared to the common ε3 allele and the protective ε2 allele [16]. All these new data reinforce the metabolic hypothesis that T2DM appears as a key factor involved in LOAD [17–20].

Currently, only symptomatic therapies are available and their effects are modest (acetylcholinesterase inhibitors and NMDA antagonists) [8]. Memantine (MEM), a low-affinity voltage-dependent uncompetitive antagonist of NMDA receptors (NMDAR), is currently being prescribed for the treatment of AD, jointly with acetylcholinesterase inhibitors such as galantamine, donepezil, and rivastigmine [20–22]. Since it is a low-affinity antagonist, it blocks the NMDAR but it is rapidly displaced from it, avoiding prolonged receptor blockade and the associated negative side effects on

Pharmaceuticals **2018**, *11*, 11

learning and memory that have been observed in high affinity NMDAR antagonists. Unfortunately, and despite its high prevalence and mortality, there are no effective disease-modifying therapies at present.

For the last 25 years, the main focus of research has been on senile plaques, considering them the main source of the symptoms of AD. Consequently, therapeutic approaches have focused on this biomarker. Recent studies at preclinical level and in LOAD patients show that an antibody (aducanumab) penetrates in the brain and reduces soluble and insoluble Aβ42 in a dose-dependent manner [23]. In patients with mild LOAD, a year of treatment by administering monthly intravenous infusions of aducanumab, reduces cerebral Aβ and patients show cognitive improvements. These results suggest that the amyloidogenic hypothesis may contribute to the development of LOAD exacerbating its consequences, possibly along with other factors such as metabolic alterations, glia activation, mitochondrial alteration and oxidative stress, among others [23]. Thus, the classical definition of AD that attributes the main role to plaques and tangles as the main responsible source of the neuropathophysiology should be modified.

Accordingly, the main objective of this review is to summarize the information regarding metabolic alterations and the appearance of LOAD, especially associated with type II diabetes mellitus (T2DM). Finally, the pathways associated with the IR signalling and its inhibition will be presented. Our aim is to evaluate the possible application of drugs involved in the regulation/modulation of brain IR in LOAD treatment, in order to improve cognitive performance and deter the development of AD.

2. The Hippocampal Insulin Receptor Is a Key Target in Physiological Cognitive Processes and Neurodegeneration

In 1985, previous to the establishment of the amyloidogenic hypothesis as the paradigm for the study of AD, Hoyer, proposed the concept of central insulin resistance and dysfunctional insulin signalling in LOAD (Table 1) [24–28]. Insulin resistance is defined as a situation in the human organism in which it does not respond sufficiently to the physiological levels of insulin [29–39]. It is involved in the onset of the metabolic syndrome. Yet, even though this idea had already been theorized, these conclusions did not begin to be established until the publication of the Rotterdam study, a clinical report that described the connection between T2DM and LOAD, revealing that those patients that had been diagnosed with diabetes had higher risk of dementia [33,34]. Subsequent clinical and epidemiological studies have confirmed this potential association demonstrating that the alteration of metabolic parameters, such as hyperglycaemia and hyperinsulinemia, correlates positively with the development of pathology related to LOAD, mainly with the cognitive loss [35–41].

Table 1. Examples of IR signalling pathway alterations in the brain in late onset Alzheimer's disease.

Reference	Physiological Alterations	Pathological Effects
Biessels and Reagan, 2015 [36]	Down regulation in neurogenesis were associated with reductions in dendritic spine density in CA1 pyramidal neurons.	Learning and memory loss.
Hoyer, S., 2004 [26]	Decline in ATP levels (mitochondrial alteration). PKB activity inhibition GSK3 activity increase.	Amount in TAU phosphorylation. Oxidative stress increases
De Felice, F.G., 2014 [29]	Neuroinflammation and TNFα increase associated with neuronal ER stress and JNK activation	Brain IR down regulation and synaptic alteration.
Grillo, C.A., 2015 [42]	Hippocampal-specific insulin resistance using a lentiviral vector expressing an IR antisense sequence	Down regulation of GluN2B and GluA1 phosphorylation at synapses. Memory failure independent of peripheral metabolic alterations.
Hoyer, S., 1994 [28]	Insulin modulates levels of acetylcholine and norepinephrine neurotransmitters,	Cognition loss

Table 1. *Cont.*

Reference	Physiological Alterations	Pathological Effects
Frolich, L.D., 1999 [25]	Formation and deposition of advanced glycation end products (AGEs)	Up-regulate APP via oxidative stress and Aβ production enhancement
De Felice and Ferreira, 2014 [30]	mTOR dysregulation	Learning and memory deficits, cell cycle reentry
Craft, S. 2012 [6]	Insulin resistance increases vascular dysfunction	Vascular dementia
Craft, S. 2005 [43]	Insulin resistance inhibits IDE activity	Aβ levels Increase

Under physiological conditions when insulin binds to the IR, a cascade regulates key downstream serine/threonine kinases such as, protein kinases B (AKT/PKB), mechanistic target of rapamycin (mTOR), and extracellular signal-regulated kinases (ERK), that eventually phosphorylate serine/threonine residues of the insulin receptor substrates (IRS), inhibiting insulin signalling in a negative feedback regulation (Figure 1) [35–41,44–46]. In neurons, the phosphoinositide 3-kinase (PI3K), AKT, glycogen synthase kinase 3β (GSK3β), BCL-2 agonist of cell death (BAD), fork-head box (FOX), mTOR and the mitogen activated protein kinase (MAPK) pathways are critical for cell survival signalling and are regulated by the activity of the IR [43,47,48]. Therefore, alteration of the physiological activity on these pathways might be the source of alteration in normal neuronal performance, supporting the hypothesis that brain insulin resistance could promote LOAD, precisely by inhibition of these pathways [39,41,45].

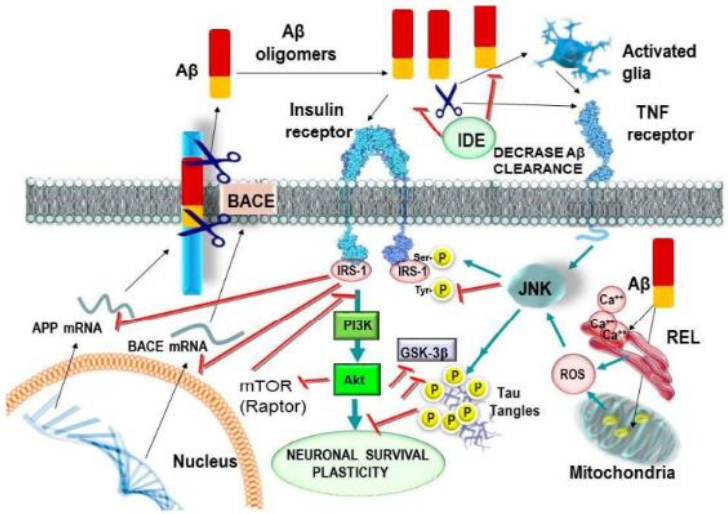


Figure 1. Consequences of insulin and Aβ interactions on reduced neuronal IR signalling. In type 2 diabetes, there can be decreased or increased levels of insulin in brain, along with IR desensitization. Soluble Aβ oligomers block IR. Increased Aβ levels will compete for insulin degrading enzyme (IDE) against cerebral insulin. Reduced IR signalling results in downstream negative effects on PI3K activity and proteins like PKB/AKT. Consequences of this include: reduced glucose metabolism and increased oxidative stress which modulate APP and JNK activity. Moreover, reduced GSK3β phosphorylation leads to up-regulation in TAU phosphorylation and Aβ formation. Likewise, Aβ promotes the activation of microglia increasing the levels of cytokines, mainly TNFα that activates JNK that subsequently inhibits the brain IR. On the other hand, Aβ can alter the endoplasmic reticulum and the mitochondria, generating free oxygen radicals that modulate APP and JNK.

The metabolic hypothesis associated with the appearance of LOAD is based on the fact that cerebral IRs are widely distributed in the brain, existing in higher densities in the olfactory bulb,

Pharmaceuticals **2018**, *11*, 11

hypothalamus, cerebral cortex and hippocampus [28,49]. Frölich and colleagues reported a significant reduced level of CNS IRs in LOAD patients [24–28]. Research from the group led by de la Monte, demonstrated significant decreases in insulin and insulin growth factor (IGF-I) receptor levels in LOAD frontal cortex, hippocampus, and hypothalamus of AD patients [45,50]. In addition, the same research group correlated the decrease in gene expression and protein levels of insulin, IGF1 receptors and other downstream molecules, with impaired acetylcholine production and cognitive performance in LOAD brains. Another recent study strengthened this hypothesis by demonstrating significant alterations in mRNA expression profiles of genes related to insulin signalling in the cortex and hippocampus [51]. Intriguingly, the highest differences in mRNA expression were detected in the hippocampal region of the brain, the main area associated with the cognitive process [51].

A recently published work by the group of Butterfield, reported new information about the complexity of LOAD [44]. In essence, they suggested that human and preclinical studies have provided convincing evidence that in the brains of many LOAD patients and rodents there is a decrease in energy metabolism and, in particular, a decrease in glucose utilization. As a consequence, that LOAD will represent a metabolic disease in which brain glucose utilization and energy production are altered is gaining attention [24–28,39]. Additional data by Grillo and co-workers reported in a very interesting study, that insulin resistance in the hippocampus would prevent the correct structuring of memory, which would be directly related to cognitive loss [42,52]. The administration of a lentiviral vector expressing an antisense sequence of the brain IRs to rats caused for cognitive loss. Using this experimental approach, the authors were able to decrease the number of IR in the hippocampus without affecting peripheral glucose homeostasis, thus generating a specific rat model of altered brain insulin signalling in the hippocampus [42]. This study demonstrated that insulin resistance in the hippocampus might induce a neuroplasticity deficit, including deficits in spatial learning and memory. In addition, the hippocampal levels of the phosphorylated GluN2B and GluA1 subunits were reduced, providing a possible molecular evidence on how the deficit in synaptic transmission occurs when there are alterations on the insulin signalling in the hippocampus [52].

Concurrently, the de Felice research group has demonstrated that Aβ oligomers bind to IR, causing for their removal from the neuronal surface membrane, causing its cellular internalization and, thereby providing an evidence for brain insulin resistance in LOAD [19,29]. In addition, some authors have reported that insulin prevents detrimental effects of Aβ oligomers on the inactivation of brain IR [53]. Furthermore, insulin promotes the release of intracellular synaptic Aβ, and regulates expression of insulin-degrading enzyme (IDE), a protease involved in clearance of Aβ (Table 1) [17].

A possible conclusion from the outcome of these studies might suggest that the presence of Aβ peptide may not be the only factor necessary for cognitive loss. However, Aβ paired with a process of obesity and hence, peripheral and central insulin resistance, may exacerbate the onset of LOAD and worsen cognitive loss. Then, LOAD should be considered globally as a brain expression of a metabolic disease of the whole organism, and correspondingly should not focus only on events that occur in the brain [32].

3. Molecular Bases of Insulin Receptor Modulation

Insulin is a peptide hormone of 5.8 KDa that is synthesized and secreted by the pancreatic β cells. Once released to the blood vessels, insulin is transported to the brain through the blood brain barrier (BBB) and binds to their cognate receptors [24,25]. The IR is a glycoprotein consisting of an extracellular α subunit (135 KDa) that inhibits the activity of the β-transmembrane subunit (95 KDa) [26–28]. The IRs belongs to the tyrosine kinase receptor superfamily. When insulin binds to the α subunit, it dimerizes to form the α2β2 complex in the cell membrane, it leads to autophosphorylation of the beta subunit on Tyr1158, Tyr1162, and Tyr1163, which constitutes the first step in IR activation.

It has been shown that the activation of the IR tyrosine kinase, leads to the recruitment and phosphorylation of several substrates, including IRS1-4, the adapter protein SHC, growth factor receptor-bound protein-2 (Grb-2 or GAB1), dedicator of cytokinesis (DOCK1), casitas B-lineage

lymphoma (CBL) and an interacting protein called APS which are associated proteins, all of which provide specific binding sites for the recruitment of other proteins of the signalling cascade [49]. These phospho-tyrosine residues bind to IRS 1 and 2 in order to initiate several signalling pathways, including the PI3K-AKT pathway [54,55]. PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). This conversion favours the activation of the PKB/AKT through the 3-phosphoinositide dependent kinase (PDK). PIP3 recruits AKT to the plasma membrane, where it becomes phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1), which regulates the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane in the hippocampus.

In the brain, IR activation promotes neuronal survival through the phosphorylation of the FOXO transcription factor, favouring its way out of the nucleus of the cell [55–58]. FOXO is a transcription factor involved in the expression of pro-apoptotic mediators, thus contributing to the process of cell death. All this processes that are regulated by the signalling of the IR, result in deleterious effects on synaptic function and cognitive impairment. The activation of IR tyrosine kinase also results in the activation of the RAS/MAPKs pathway. The stress activated protein kinases (SAPK) or MAPK, include extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), p38 and the c-Jun-N-terminal kinases (JNKs) [56]. The JNK family is made up by 3 genes that codify for 10 different products classified into 3 different isoforms: JNK1 (*Mapk8*), JNK2 (*Mapk9*) and JNK3 (*Mapk10*) and while JNK1 and JNK2 are ubiquitously expressed, JNK3 expression is principally restricted to regions of the brain, heart and testis. When activated, the JNK1 phosphorylates IRS-1 in the serine residues (IRS-1pSer) [29]. This alteration blocks the signalling of the insulin pathway and favours peripheral resistance to this hormone. Based on this activation sequence, JNK1 appears as a key protein to investigate novel therapeutic targets that prevent the development T2DM [54,56,58].

4. Relationship between Insulin Receptor Activation and TAU Phosphorylation

Recent studies have suggested a potential link between impaired insulin signalling and pathogenic alterations of TAU. As we stated before, the activation of IR, IRS1 and 2 initiates several signalling pathways, including the conversion of PIP2 into PIP3, which favours the activation of PKB/AKT and, consequently, leads to the translocation of GLUT4 to the plasma membrane [56,59–62]. Concurrently, AKT signalling affects other diverse cellular responses, like neuronal survival and TAU phosphorylation. Other important targets such as the GSK3β are also regulated by AKT, inhibiting its activation by phosphorylation. Insulin resistance reinforces the activation of GSK3β leading to increased phosphorylation levels of TAU protein and, the subsequent formation of neurofibrillary tangles, one of the hallmarks of AD neuropathology [59–62]. Schubert and co-workers investigate the biochemical processes associated with neurodegeneration in a brain specific IR knockout (NIRKO) mice model [63]. They reported that NIRKO mice presented decreased AKT activity thus, having the previously mentioned increases in GSK3β activation and TAU hyperphosphorylation at specific sites associated with LOAD. This data, along with other being produced in the same line on the study of metabolic alterations, confirm how altered insulin signalling in the brain leads to the appearance of classical hallmarks of LOAD and, demonstrates, how neuronal insulin resistance predisposes for the appearance of pathologies. Freude and co-workers reported that brain insulin receptor specifically modulates TAU phosphorylation at Ser202, a key site which predisposes for tangle formation after peripheral insulin injection in mice [64]. The effects of injected insulin on TAU were abolished in the NIRKO mice.

Studies performed in *post-mortem* brains from patients with tauopathies including AD, Pick's disease, corticobasal degeneration, and progressive supranuclear palsy, showed increases in phosphorylated IRS1 levels which, as we have already mentioned, is a specific marker of insulin resistance. Interestingly, two independent research groups published their research on alterations in brain insulin receptors and downstream pathway in LOAD. Liu and colleagues reported that the insulin-signalling pathway is decreased in LOAD brain and demonstrated that alteration in insulin

Pharmaceuticals **2018**, *11*, 11

signaling may contribute to LOAD through the hyperphosphorylation of TAU [59]. In addition, authors suggest that brain insulin resistance is also correlated with calpain activation, a protease involved in cyclin-dependent kinase (CDK5) activation, a kinase involved in the phosphorylation of TAU [35]. Similar results were reported by other authors:

- Talbot and co-workers reported that LOAD patients show impaired brain insulin-signaling transduction with reduced tyrosine kinase activity of the IR [35]. IR and its receptor analogous IGF1R form heterodimers (IR/IGF1R) that modulate the selectivity and affinity for insulin and IGF1 in the activation of signaling molecules [65].
- Yarchoan and co-workers reported an increase in serine phosphorylation of IRS1 (inactivation), the phosphorylation of IRS1 on multiple serine residues can inhibit IRS1 activity, leading to insulin resistance in the hippocampus in LOAD and other tauopathies [54].

Finally, recent data reported that insulin accumulates intraneuronally together with hyperphosphorylated TAU in LOAD and several other tauopathies suggesting that hyperphosphorylated TAU-bearing neurons is a causative factor involved in the brain insulin resistance observed in LOAD (Table 1) [66].

5. Role of the Glucose Transporter 4 in Cognition

Glucose transporter 4 (GLUT4) is found in peripheral tissues like the skeletal muscle, heart, and adipose tissue [67–69]. Its role in the physiology of the cell is mainly the transport from the extracellular space into the citosol for its metabolism. Thus, in response to the activation of the IR signaling cascade by insulin, GLUT4 is translocated to the plasma membrane to facilitate glucose entry into the cell. Moreover, GLUT4 is found in brain regions such as the cerebellum, and especially the hippocampus. At the hippocampal level, the cognitive improvement effects related to insulin may occur via upregulation in GLUT4-mediated glucose uptake [68]. Thus hippocampal GLUT4 overexpression could be a target to improve the cognitive process in AD. This could be the case of quercetin which improves cognitive dysfunction mediated by chronic unpredicted stress, through upregulation of GLUT4 expression in the hippocampus [69].

6. Effects of Aβ Oligomers on Brain Insulin and Peripheral Metabolic Tissues

Recent hypothesis suggests that since diabetes increases both Aβ production and TAU phosphorylation, both T2DM and Aβ may cooperate to induce neurodegeneration in LOAD [70,71]. It has been pointed out that soluble Aβ peptide oligomers would act as synaptotoxins [10–12]. Moreover, since Aβ and insulin are both amyloidogenic peptides sharing a common sequence recognition motif, it is possible that both molecules are able to bind to the IR. Given this assumption, Aβ may also potentiate insulin resistance through antagonistic effects, blocking the downstream pathway and facilitating the phosphorylation of GSK3β. Thus, the aging process associated with insulin resistance, jointly with Aβ production and hyperphosphorylation of TAU, can have a synergic effect leading to neuronal dysfunction.

In addition to the effects of Aβ oligomers on TAU phosphorylation, a recent study using a mice model of AD, reported peripheral metabolic changes in plasma and liver extracts [72]. Also, Zhang and co-workers demonstrated in APPswe/PS1E9 mice that the Aβ42 peptide induces hepatic insulin resistance in vivo through the activation of the Janus Kinase 2 (JAK2), suggesting that inhibition of Aβ42 peptide production in the brain may be a novel strategy for the treatment of insulin resistance and therefore T2DM [73–75].

As, an overview, we can state that LOAD has a multifactorial component and should be addressed as a disease affecting the whole organism [70,71]. Moreover, preclinical studies in rodents have established that the oligomers of Aβ administered directly to hippocampal neurons induce synaptic loss and neuronal dysfunction, which eventually leads to memory loss [11]. Likewise, intracerebroventricular (icv) administration of Aβ oligomers causes behavioral changes and AD-like

pathology in primates, providing an excellent model for investigating AD-related mechanisms [72]. Furthermore, Clarke and co-workers reported that intracerebral injected Aβ causes peripheral glucose intolerance and insulin resistance, as well as, inflammatory processes in the hypothalamus and adipose tissue, along with alterations of GLUT-4 insulin-induced cell membrane translocation in skeletal muscle [76].

Accordingly, Aβ peptides generated in the brain reach the hypothalamus and alter the body's energy balance, favouring the apparition of a T2DM. In this line, Arietta-Cruz et al. demonstrated an increase in plasma glucose levels when injecting β25-35 into the hypothalamus of rat as a consequence of enhanced hepatic glucose production [77,78].

In addition, generated brain Aβ could accumulate in peripheral tissues such as the pancreas and skeletal muscle contributing to the negative effect on peripheral glucose metabolism [79]. Thus, when trying to explain this complicated bidirectional process between LOAD and T2DM, recent reported data suggests the existence of something called Factor X, a molecule or pathway that would be the bridge between Aβ as responsible of LOAD and T2DM. In addition, authors suggest that characterization of Factor X will be important in order to the development of a potential therapeutic target for LOAD prevention [70].

7. Is BACE1 a Potential Bridge between Aβ and T2DM?

BACE1 is involved in LOAD as the enzyme responsible for the rate-limiting step in Aβ production through the cleaving of the amyloid precursor protein (APP). It has been demonstrated that monomers of Aβ1-42 augment BACE1 gene transcription activation through the MAPK8/JNK1-MAPK9/JNK2 signalling pathway and by interfering with its lysosomal degradation leading to an amyloid vicious cycle [80–82].

Interestingly, recent data demonstrated at the preclinical level that neuronal expression of human BACE1 causes systemic diabetic complications via the induction of hypothalamic impairment, insulin resistance, hepatic deficits and global glucose alterations [79,80]. Using the PLB4 mouse it was demonstrated that the risk of diabetes when BACE1 is overexpressed in neurons increases, providing for a complex mechanistic interaction between T2DM and LOAD. Human (h) BACE1 neurogenic knockout has recently been shown to induce Aβ accumulation, promotes brain inflammation and generates LOAD-like phenotypes in mice in the absence of expression of mutant APP, suggesting that BACE1 represents a molecular risk factor for AD related to the aging process [83].

Plucińskaí and colleagues showed that the overexpression in neurons of the amyloidogenic enzyme, BACE1, is sufficient to increase the risk of developing T2DM [80]. Therefore, this study demonstrates that neuronal BACE1 causes metabolic dysregulation throughout the body along with brain inflammation and cognitive impairment related to the process of amyloidosis. The PLB4 mouse presents a diabetic profile, thus demonstrating that neuronal BACE1 is in part responsible for the appearance of these peripheral metabolic alterations [84].

Therefore, even though the hypothesis states that diabetic complications promote the onset and or progression of AD, the reverse scenario may also apply. This is also in agreement with the potential hypothesis that hyperglycaemia can also originate in the brain and affect the rest of the body (Figure 2). Meakin and colleagues demonstrated that knockout mice for BACE1$^{-/-}$ are thin, resistant to obesity induced by high fat diet and show an increase in insulin sensitivity in peripheral tissues with a regulation of improved glucose metabolism throughout the body [85]. These results outline a novel aspect of BACE1 function in the regulation of metabolic homeostasis and, provide a possible connection between T2DM and AD [85].

Pharmaceuticals **2018**, *11*, 11

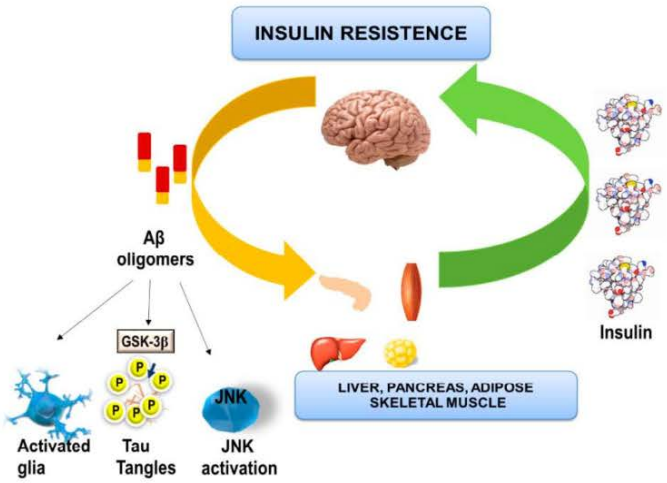


Figure 2. Aβ acting on the hypothalamus can dysregulate energy homeostasis in the human organism through a neuroinflammatory process. Furthermore, in the hippocampus, activation of glial reactivity could increase cytokine levels (such as TNF-α), activating c-Jun N-terminal Kinase and inducing IR resistance and TAU phosphorylation. Likewise, brain generated Aβ could accumulate in peripheral tissues such as the pancreas and skeletal muscle, favouring the appearance of T2DM.

8. Potential Pharmacological Approaches for Late Onset Alzheimer's Disease Treatment Related with the Regulation of Insulin Metabolism

For all of the above, development of LOAD would pivot on the loss of IR functionality, oxidative stress and loss of control of protein homeostasis [86–90]. In order to modulate these mechanisms, different pharmacological approaches are proposed which may act in a combined and, potentially, synergistic manner. On the one hand, it may be appropriate to combine the use of antidiabetic drugs such as pioglitazone, intranasal insulin, NMDAR antagonists such as memantine and inhibitors of mTOR activity such as rapamycin and its derivatives (rapalogs) [22,44,91–102]. In all cases, they are drugs that have been validated in different Phase II (pioglitazone) and III (rapamycin) clinical trials. Since in no case did these molecules improve the evolution of patients with LOAD (in the different Phase III studies), this allows for the possibility of studying their possible synergies when administered in combination.

8.1. Antidiabetic Drugs. Modulators of Proliferation of Activated Gamma Peroxisome Receptor. Pioglitazone

Pioglitazone is an orally active antidiabetic drug in the family of thioazolidinediones, also called "insulin sensitizers" [93,94]. Pioglitazone is a potent and selective receptor agonist for the proliferation of activated gamma peroxisomes receptor (PPARγ). These receptors regulate the transcription of a number of genes that respond to insulin [93]. PPARγs are found in most tissues in which insulin exerts its action: adipose tissue, skeletal muscle and liver. Activation of these receptors regulates the transcription of genes involved in the control of glucose production, transport and its utilization. In relationship to LOAD, the treatment with pioglitazone has been shown to reduce glial pro-inflammatory activity and, to decrease Aβ peptide levels due to the phagocytic activity of microglia [92]. In 3xTg-AD mice treated with pioglitazone for 4 months, this drug improves brain spatial learning impairment, TAU hyperphosphorylation, and neuroinflammation [93]. In a recent preclinical study Fernandez-Martos and co-workers reported that the association of pioglitazone with leptin showed beneficial effects on the preclinical APPswe/PS1dE9 mice model of familial AD improving cognition and decreasing Aβ levels [102]. Recent studies indicate a very relevant effect

of the drug reversing the damage that neuroinflammation causes in the structural plasticity of the dendrites. Thus, it has been observed that treatment with pioglitazone can reverse the loss of synaptic density induced by Aβ peptide generation [91]. Although preclinical data gives support to the potential beneficial effects of pioglitazone in AD, clinical data reported until now shows conflicting results regarding efficacy due to the many limitations of these trials [100,101]. Therefore, further clinical trials on the potential use of pioglitazone for the treatment of LOAD are necessary. Phase II clinical trials of the drug demonstrate that it is a safe and well tolerated molecule. Two Phase III trials are currently under way, of which conclusions regarding their effectiveness against AD cannot yet be obtained [91,100,101].

8.2. Intranasal Insulin for LOAD Treatment

In previous preclinical studies, intranasal treatments with insulin or insulin analogues have afforded some degree of memory improvement or of protection against cognitive deterioration in mice models of AD [101–109]. However, in a recent reported study (NCT01595646), Craft and co-workers reported that intranasal-administered insulin improves memory for adults with mild cognitive impairment and LOAD [108–110]. Furthermore, authors suggest that insulin could improve and modify the AD-related pathophysiologic processes. Another interesting point is that the therapeutic effects of insulin are modulated by APOE genotype. Accordingly, the study gives support to the continued investigation on potential stimulation of insulin receptor as a therapy for LOAD [108].

8.3. Rapalogs

It is well known that the PI3K/AKT/mTOR dysregulation may decrease the autophagic process leading to the accumulation of Aβ42 deposition and protein aggregation [44]. Likewise, mTOR is involved in the modulation of IRS1 activity, representing one of the best-characterized events leading to insulin resistance [44]. Therefore, alteration or better activation of the mTOR pathway could represent an important link between Aβ and insulin signalling, providing new insights into the relationship between insulin resistance and incidence of AD.

mTOR is a kinase involved in energy and protein homeostasis in cells. Both rapamycin and its derivatives prevent the formation of the mTORC1 complex, acting as allosteric inhibitors [109–111]. However, the main limitations of rapamycin are its solubility, long half-life and the poor oral absorption making it necessary for the development of analog molecules, among them temsirolimus, which is an ester derived from rapamycin, soluble in water and suitable for administration both oral and intravenously. Its use was approved in 1977 by the FDA and the European Medicines Agency for the treatment of renal carcinoma [111]. Both drugs have the same mechanism of action. Jiang and co-workers recently reported that temsirolimus promotes autophagic clearance of Aβ, exerts protective effects accompanied by an improvement in spatial cognitive functions in APP/PS1 model of familial AD [102]. This study give support to the therapeutic potentials of temsirolimus in preclinical models of AD.

9. Conclusions

Given the amount of data of which we are in possession now, it can be concluded that the pathology hereby described as LOAD is very closely related to the alterations derived of insulin resistance. Effective energy metabolism is the base on the proper functioning of cellular types and, when disrupted, affects negatively their function. In the case of neurons, which are having glucose as its main energy source, this situation can be utterly disastrous leading to their ineffective activity and consequently cognitive decline. That is why the IR has such a prominent role.

It is now well established Aβ could bind to the IR in the hippocampus, revealing important cognitive loss, when the receptor is inhibited and enhancing the neurodegenerative process in this brain region. Moreover, Aβ bind to the liver IR in the preclinical APPswe/PS1E9 mice model of familial AD, suggesting the possibility that decreases of Aβ production may be a novel potential

treatment for use in T2DM (Figure 2) [73,74,112]. Lastly, the therapeutic potential of Aβ inhibitors (for example BACE 1 inhibitors) has not yet been verified in clinical trials. However, antidiabetic therapies such as pioglitazone or intranasal insulin are more likely to be effective in individuals with LOAD. Therefore, we suggest that the future timing of a more effective LOAD therapy should be the key factor in determining if T2DM drugs shown beneficial actions in LOAD. Targeting the early stages of LOAD, before widespread cognitive loss due to synapses and neurons degeneration has occurred is likely to produce the best clinical outcome, but identification of individuals at this stage of LOAD is difficult. Accordingly, the modulation of brain IR preventing its inactivation could be a suitable strategy in a combinatory strategy therapy for LOAD.

Acknowledgments: This work was supported by the Spanish Ministry of Science and Innovation SAF2017-84283-R, PI2016/01, CB06/05/0024 (CIBERNED), the European Regional Development Founds and MAT 2014-59134-R project. Research team from UB and URV belongs to 2014SGR-525 from Generalitat de Catalunya. ESL and MLG belong to 2014SGR-1023 and ESL acknowledges the PhD scholarship FPI-MICINN (BES-2012-026083). CBZ is supported by grants from CONACyT Mexico (No. 0177594) and RDCT from Postdoctoral fellowship CONACYT No. 298337 and the Doctoral Program in Sciences in Molecular Biology in Medicine, LGAC Molecular Bases of Chronic Diseases-Degenerative and its Applications (000091, PNPC, CONACyT). PRM is supported by grants 2015/26084-1 and 2017/13224-5, São Paulo Research Foundation (FAPESP)—Brazil.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Alzheimer's Association. 2016 Alzheimer's disease facts and figures. *Alzheimer's Dement.* **2016**, *12*, 459–509.
2. Kamat, P.K.; Kalani, A.; Rai, S.; Swarnkar, S.; Tota, S.; Nath, C.; Tyagi, N. Mechanism of Oxidative Stress and Synapse Dysfunction in the Pathogenesis of Alzheimer's Disease: Understanding the Therapeutics Strategies. *Mol. Neurobiol.* **2016**, *53*, 648–661. [CrossRef] [PubMed]
3. Alzheimer, A.; Stelzmann, R.A.; Schnitzlein, H.N.; Murtagh, F.R. An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkrankung der Hirnrinde". *Clin. Anat.* **1995**, *8*, 429–431. [PubMed]
4. Vishal, S.; Sourabh, A.; Harkirat, S. Alois Alzheimer (1864–1915) and the Alzheimer syndrome. *J. Med. Biogr.* **2011**, *19*, 32–33. [CrossRef] [PubMed]
5. Masters, C.L.; Bateman, R.; Blennow, K.; Rowe, C.C.; Spearling, R.A.; Cummings, J.L. Alzheimer's disease. *Nat. Rev. Dis. Primers* **2015**, *1*, 15056. [CrossRef] [PubMed]
6. Craft, S. Alzheimer disease: Insulin resistance and AD: Extending the translational path. *Nat. Rev. Neurol.* **2012**, *8*, 360–362. [CrossRef] [PubMed]
7. Ritchie, C.W.; Molinuevo, J.L.; Truyen, L.; Satlin, A.; Van der Geyten, S.; Lovestone, S. Development of interventions for the secondary prevention of Alzheimer's dementia: The European Prevention of Alzheimer's Dementia (EPAD) project. *Lancet Psychiatry* **2016**, *3*, 179–186. [CrossRef]
8. Querfurth, H.W.; La Ferla, F.M. Alzheimer's disease. *N. Engl. J. Med.* **2010**, *362*, 329–344. [CrossRef] [PubMed]
9. Mangialasche, F.; Solomon, A.; Winblad, B.; Mecocci, P.; Kivipelto, M. Alzheimer's disease: Clinical trials and drug development. *Lancet Neurol.* **2010**, *9*, 702–716. [CrossRef]
10. Selkoe, D.J. Resolving controversies on the path to Alzheimer's therapeutics. *Nat. Med.* **2011**, *17*, 1060–1065. [CrossRef] [PubMed]
11. Selkoe, D.J.; Hardy, J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol. Med.* **2016**, *8*, 595–608. [CrossRef] [PubMed]
12. Viola, K.L.; Klein, W.L. Amyloid β oligomers in Alzheimer's disease pathogenesis, treatment, and diagnosis. *Acta Neuropathol.* **2015**, *129*, 183–206. [CrossRef] [PubMed]
13. Moser, V.A.; Pike, C.J. Obesity Accelerates Alzheimer-Related Pathology in APOE4but not APOE3Mice. *eNeuro* **2017**, *4*. [CrossRef] [PubMed]
14. Craft, S.; Peskind, E.; Schwartz, M.W.; Schellenberg, G.D.; Raskind, M.; Porte, D. Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: Relationship to severity of dementia and apolipoprotein E genotype. *Neurology* **1998**, *50*, 164–168. [CrossRef] [PubMed]

15. Liu, C.C.; Liu, C.C.; Kanekiyo, T.; Xu, H.; Bu, G. Apolipoprotein E and Alzheimer disease: Risk, mechanisms and therapy. *Nat. Rev. Neurol.* **2013**, *9*, 106–118. [CrossRef] [PubMed]
16. Zhao, N.; Liu, C.C.; Van Ingelgom, A.J.; Martens, Y.A.; Linares, C.; Knight, J.A.; Painter, M.M.; Sullivan, P.M.; Bu, G. Apolipoprotein E4 Impairs Neuronal Insulin Signaling by Trapping Insulin Receptor in the Endosomes. *Neuron* **2017**, *96*, 115–129. [CrossRef] [PubMed]
17. Neth, B.J.; Craft, S. Insulin Resistance and Alzheimer's Disease: Bioenergetic Linkages. *Front. Aging Neurosci.* **2017**. [CrossRef] [PubMed]
18. De la Monte, S.M. Insulin Resistance and Neurodegeneration: Progress towards the Development of New Therapeutics for Alzheimer's disease. *Drugs* **2017**, *77*, 47–65. [CrossRef] [PubMed]
19. De Felice, F.G.; Benedict, C. A Key Role of Insulin Receptors in Memory. *Diabetes* **2015**, *64*, 3653–3655. [CrossRef] [PubMed]
20. Chneider, L.S.; Mangialasche, F.; Andreasen, N.; Feldman, H.; Giacobini, E.; Jones, R.; Mantua, V.; Mecocci, P.; Pani, L.; Winblad, B.; et al. Clinical trials and late-stage drug development for Alzheimer's disease: An appraisal from 1984 to 2014. *J. Intern. Med.* **2014**, *275*, 251–283. [CrossRef] [PubMed]
21. Lipton, S.A. Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. *Nat. Rev. Drug Discov.* **2006**, *5*, 160–170. [CrossRef] [PubMed]
22. Allgaier, M.; Allgaier, C. An update on drug treatment options of Alzheimer's disease. *Front. Biosci.* **2014**, *19*, 1345–1354. [CrossRef]
23. Sevigny, J.; Chiao, P.; Bussière, T.; Weinreb, P.H.; Williams, L.; Maier, M.; Dunstan, R.; Salloway, S.; Chen, T.; Ling, Y.; et al. The antibody aducanumab reduces Aβ plaques in Alzheimer's disease. *Nature* **2016**, *537*, 50–56. [CrossRef] [PubMed]
24. Frolich, L.; Blum-Degen, H.G.; Bernstein, S.; Engelsberger, J.; Humrich, S.; Laufer, D.; Muschner, A.; Thalheimer, A.; Turk, S.; Hoyer, P.; et al. Insulin and insulin receptors in the brain in aging and in sporadic Alzheimer's disease. *J. Neural Transm.* **1998**, *105*, 423–438. [CrossRef] [PubMed]
25. Frolich, L.; Blum-Degen, D.; Riederer, P.; Hoyer, S. A disturbance of the neuronal insulin receptor signal transduction in sporadic Alzheimer's disease. *Ann. N. Y. Acad. Sci.* **1999**, *893*, 290–294. [CrossRef] [PubMed]
26. Hoyer, S. Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. *Eur. J. Pharmacol.* **2004**, *490*, 115–125. [CrossRef] [PubMed]
27. Maurer, K.; Hoyer, S. Alois Alzheimer revisited: Differences in origin of the disease carrying his name. *J. Neural Transm.* **2006**, *113*, 1645–1658. [CrossRef] [PubMed]
28. Hoyer, S. Neurodegeneration, Alzheimer's disease, and beta-amyloid toxicity. *Life Sci.* **1994**, *55*, 1977–1983. [CrossRef]
29. De Felice, F.G.; Lourenco, M.V.; Ferreira, S.T. How does brain insulin resistance develop in Alzheimer's disease? *Alzheimer's Dement.* **2014**, *10*, S26–S32. [CrossRef] [PubMed]
30. De Felice, F.G.; Ferreira, S.T. Inflammation, defective insulin signaling, and mitochondrial dysfunction as common molecular denominators connecting type 2 diabetes to Alzheimer Disease. *Diabetes* **2014**, *63*, 2262–2272. [CrossRef] [PubMed]
31. De la Monte, S.M.; Wands, J.R. Review of insulin and insulin-like growth factor expresion, signaling, and malfunction in the central nervous system: Relevance to alzheimer's disease. *J. Alzheimer Dis.* **2005**, *7*, 45–61. [CrossRef]
32. De la Monte, S.M. Brain insulin resistance and deficiency as therapeutic targets in Alzheimer's disease. *Curr. Alzheimer Res.* **2012**, *9*, 35–66. [CrossRef] [PubMed]
33. Ott, A.; Stolk, R.P.; van Harskamp, F.; Pols, H.A.; Hofman, A.; Breteler, M.M. Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology* **1999**, *53*, 1937–1942. [CrossRef] [PubMed]
34. Schrijvers, E.M.; Witteman, J.C.; Sijbrands, E.J.; Hofman, A.; Koudstaal, P.J.; Breteler, M.M. Insulin metabolism and the risk of Alzheimer disease: The Rotterdam Study. *Neurology* **2010**, *75*, 1982–1987. [CrossRef] [PubMed]
35. Talbot, K.; Wang, H.; Kazi, H.; Han, L.; Bakshi, K.P.; Stucky, A.; Fuino, R.L.; Kawaguchi, K.R.; Samoyedny, A.J.; Wilson, R.S.; et al. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF1 resistance, IRS1 dysregulation, and cognitive decline. *J. Clin. Investig.* **2012**, *122*, 1316–1338. [CrossRef] [PubMed]
36. Biessels, G.J.; Reagan, L.P. Hippocampal insulin resistance and cognitive dysfunction. *Nat. Rev. Neurosci.* **2015**, *16*, 660–671. [CrossRef] [PubMed]
37. Schwartz, M.W.; Porte, D., Jr. Diabetes, obesity, and the brain. *Science* **2005**, *307*, 375–379. [CrossRef] [PubMed]

38. McNay, E.C.; Recknagel, A.K. Brain insulin signaling a key component of cognitive processes and a potential basis for cognitive impairment in type 2 diabetes. *Neurobiol. Learn. Mem.* **2011**, *96*, 432–442. [CrossRef] [PubMed]
39. Schiöth, H.B.; Craft, S.; Brooks, S.K.; Frey, W.H.; Benedict, C. Brain insulin signaling and Alzheimer's disease: Current evidente and future direction. *Mol. Neurobiol.* **2011**, *46*, 4–10. [CrossRef] [PubMed]
40. Pearson-Leary, J.; McNay, E.C. Intrahippocampal administration of amyloid-β(1-42) oligomers acutely impairs spatial working memory, insulin signaling, and hippocampal metabolism. *J. Alzheimers Dis.* **2012**, *30*, 413–422. [PubMed]
41. Walker, J.M.; Harrison, F. Shared Neuropathological Characteristics of Obesity, Type 2 Diabetes and Alzheimer's Disease: Impacts on Cognitive Decline. *Nutrients* **2015**, *7*, 7332–7357. [CrossRef] [PubMed]
42. Grillo, C.A.; Piroli, G.G.; Lawrence, R.C.; Wrighten, S.A.; Green, A.J.; Wilson, S.P.; Sakai, R.R.; Kelly, S.J.; Wilson, M.A.; Mott, D.D.; et al. Hippocampal Insulin Resistance Impairs Spatial Learning and Synaptic Plasticity. *Diabetes* **2015**, *64*, 3927–3936. [CrossRef] [PubMed]
43. Craft, S. Insulin resistance syndrome and Alzheimer's disease: Age- and obesity-related effects on memory, amyloid, and inflammation. *Neurobiol. Aging* **2005**, *26*, 65–69. [CrossRef] [PubMed]
44. Di Domenico, F.; Barone, E.; Perluigi, M.; Butterfield, D.A. The Triangle of Death in Alzheimer's Disease Brain: The Aberrant Cross-Talk Among Energy Metabolism, Mammalian Target of Rapamycin Signaling, and Protein Homeostasis Revealed by Redox Proteomics. *Antioxid. Redox Signal.* **2017**, *26*, 364–387. [CrossRef] [PubMed]
45. Steen, E.; Terry, B.M.; Rivera, E.J.; Cannon, J.L.; Neely, T.R.; Tavares, R.; Xu, X.J.; Wands, J.R.; de la Monte, S.M. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—Is this type 3 diabetes? *J. Alzheimer's Dis.* **2005**, *7*, 63–80. [CrossRef]
46. Nuzzo, D.; Picone, P.; Baldassano, S.; Caruana, L.; Messina, E.; Marino Gammazza, A.; Cappello, F.; Mulè, F.; Di Carlo, M. Insulin Resistance as Common Molecular Denominator Linking Obesity to Alzheimer's Disease. *Curr. Alzheimer Res.* **2015**, *12*, 723–735. [CrossRef] [PubMed]
47. Cholerton, B.; Baker, L.D.; Montine, T.J.; Craft, S. Type 2 Diabetes, Cognition, and Dementia in Older Adults: Toward a Precision Health Approach. *Diabetes Spectr.* **2016**, *29*, 210–219. [CrossRef] [PubMed]
48. McCrimmon, R.J.; Ryan, C.M.; Frier, B.M. Diabetes and cognitive dysfunction. *Lancet* **2012**, *379*, 2291–2299. [CrossRef]
49. Xu, H.; Moore, E.; Meiri, N.; Quon, M.J.; Alkon, D.L. Brain insulin receptors and spatial memory. *J. Biol. Chem.* **1999**, *274*, 34839–34842.
50. Chami, B.; Steel, A.J.; De La Monte, S.M.; Sutherland, G.T. The rise and fall of insulin signaling in Alzheimer's disease. *Metab Brain Dis.* **2016**, *31*, 497–515. [CrossRef] [PubMed]
51. Hokama, M.; Oka, S.; Leon, J.; Ninomiya, T.; Honda, H.; Sasaki, K.; Iwaki, T.; Ohara, T.; Sasaki, T.; LaFerla, F.M.; et al. Altered expression of diabetes-related genes in Alzheimer's disease brains: The Hisayama study. *Cereb. Cortex* **2014**, *24*, 2476–2488. [CrossRef] [PubMed]
52. De Felice, F.G. Connecting type 2 diabetes to Alzheimer's disease. *Expert Rev. Neurother.* **2013**, *13*, 1297–1299. [CrossRef] [PubMed]
53. Ma, Q.L.; Yang, F.; Rosario, E.R.; Ubeda, O.J.; Beech, W.; Gant, D.J.; Chen, P.P.; Hudspeth, B.; Chen, C.; Zhao, Y. Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: Suppression by omega-3 fatty acids and curcumin. *J. Neurosci.* **2009**, *29*, 9078–9089. [CrossRef] [PubMed]
54. Yarchoan, M.; Toledo, J.B.; Lee, E.B.; Arvanitakis, Z.; Kazi, H.; Han, L.Y.; Louneva, N.; Lee, V.M.; Kim, S.F.; Trojanowski, J.Q.; et al. Abnormal serine phosphorylation of insulin receptor substrate 1 is associated with tau pathology in Alzheimer's disease and tauopathies. *Acta Neuropathol.* **2014**, *128*, 679–689. [CrossRef] [PubMed]
55. Havrankova, J.; Roth, J.; Brownstein, M. Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* **1978**, *5656*, 827–829. [CrossRef]
56. Ribe, E.M.; Lovestone, S. Insulin signalling in Alzheimer's disease and diabetes: From epidemiology to molecular links. *J. Intern. Med.* **2016**, *280*, 430–442. [CrossRef] [PubMed]
57. Kandimalla, R.; Thirumala, V.; Reddy, P.H. Is Alzheimer's disease a Type 3 Diabetes? A critical appraisal. *Biochim. Biophys. Acta* **2017**, *1863*, 1078–1089. [CrossRef] [PubMed]

58. Diehl, T.; Mullins, R.; Kapogiannis, D. Insulin resistance in Alzheimer's disease. *Transl. Res.* **2017**, *183*, 26–40. [CrossRef] [PubMed]
59. Liu, Y.; Liu, F.; Grundke-Iqbal, I.; Iqbal, K.; Gong, C.X. Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. *J. Pathol.* **2011**, *225*, 54–62. [CrossRef] [PubMed]
60. Biessels, G.J.; Kappelle, L.J. Increased risk of Alzheimer's disease in Type II diabetes: insulin resistance of the brain or insulin-induced amyloid pathology? *Biochem. Soc. Trans.* **2005**, *33*, 1041–1044. [CrossRef] [PubMed]
61. Stanley, M.; Macauley, S.L.; Holtzman, D.M. Changes in insulin and insulin signaling in Alzheimer's disease: Cause or consequence? *J. Exp. Med.* **2016**, *213*, 1375–1385. [CrossRef] [PubMed]
62. Pardeshi, R.; Bolshette, N.; Gadhave, K.; Ahire, A.; Ahmed, S.; Cassano, T.; Gupta, V.B.; Lahkar, M. Insulin signaling: An opportunistic target to minify the risk of Alzheimer's disease. *Psychoneuroendocrinology* **2017**, *83*, 159–171. [CrossRef] [PubMed]
63. Schubert, M.; Gautam, D.; Surjo, D.; Ueki, K.; Baudler, S.; Schubert, D.; Kondo, T.; Alber, J.; Galldiks, N.; Küstermann, E.; et al. Role for neuronal insulin resistance in neurodegenerative diseases. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 3100–3105. [CrossRef] [PubMed]
64. Freude, S.; Plum, L.; Schnitker, J.; Leeser, U.; Udelhoven, M.; Krone, W.; Bruning, J.C.; Schubert, M. Peripheral hyperinsulinemia promotes tau phosphorylation in vivo. *Diabetes* **2005**, *54*, 3343–3348. [CrossRef] [PubMed]
65. Guan, J.; Bennet, L.; Gluckman, P.D.; Gunn, A.J. Insulin-like growth factor-1 and post-ischemic brain injury. *Prog. Neurobiol.* **2003**, *70*, 443–462. [CrossRef] [PubMed]
66. Rodriguez-Rodriguez, P.; Sandebring-Matton, A.; Merino-Serrais, P.; Parrado-Fernandez, C.; Rabano, A.; Winblad, B.; Ávila, J.; Ferrer, I.; Cedazo-Minguez, A. Tau hyperphosphorylation induces oligomeric insulin accumulation and insulin resistance in neurons. *Brain* **2017**, *140*, 3269–3285. [CrossRef] [PubMed]
67. Ashrafi, G.; Wu, Z.; Farrell, R.J.; Ryan, T.A. GLUT4 Mobilization Supports Energetic Demands of Active Synapses. *Neuron* **2017**, *93*, 606–615. [CrossRef] [PubMed]
68. Pearson-Leary, J.; McNay, E.C. Novel Roles for the Insulin-Regulated Glucose Transporter-4 in Hippocampally Dependent Memory. *J. Neurosci.* **2016**, *36*, 11851–11864. [CrossRef] [PubMed]
69. Mehta, V.; Parashar, A.; Sharma, A.; Singh, T.R.; Udayabanu, M. Quercetin ameliorates chronic unpredicted stress-mediated memory dysfunction in male Swiss albino mice by attenuating insulin resistance and elevating hippocampal GLUT4 levels independent of insulin receptor expression. *Horm. Behav.* **2017**, *89*, 13–22. [CrossRef] [PubMed]
70. Shinohara, M.; Sato, N. Bidirectional interactions between diabetes and Alzheimer's disease. *Neurochem. Int.* **2017**, *108*, 296–302. [CrossRef] [PubMed]
71. Kang, S.; Lee, Y.H.; Lee, J.E. Metabolism-Centric Overview of the Pathogenesis of Alzheimer's disease. *Yonsei Med. J.* **2017**, *58*, 479–488. [CrossRef] [PubMed]
72. Wu, J.; Fu, B.; Lei, H.; Tang, H.; Wang, Y. Gender differences of peripheral plasma and liver metabolic profiling in APP/PS1 transgenic AD mice. *Neuroscience* **2016**, *332*, 160–169. [CrossRef] [PubMed]
73. Zhang, Y.; Zhou, B.; Deng, B.; Zhang, F.; Wu, J.; Wang, Y.; Le, Y.; Zhai, Q. Amyloid-β induces hepatic insulin resistance in vivo via JAK2. *Diabetes* **2013**, *62*, 1159–1166. [CrossRef] [PubMed]
74. Zhang, Y.; Zhou, B.; Zhang, F.; Wu, J.; Hu, Y.; Liu, Y.; Zhai, Q. Amyloid-β induces hepatic insulin resistance by activating JAK2/STAT3/SOCS-1 signaling pathway. *Diabetes* **2012**, *61*, 1434–1443. [CrossRef] [PubMed]
75. Forny-Germano, L.; Lyra e Silva, N.M.; Batista, A.F.; Brito-Moreira, J.; Gralle, M.; Boehnke, S.E.; Coe, B.C.; Lablans, A.; Marques, S.A.; Martinez, A.M.; et al. Alzheimer's disease-like pathology induced by amyloid-β oligomers in nonhuman primates. *J. Neurosci.* **2014**, *34*, 13629–13643. [CrossRef] [PubMed]
76. Clarke, J.R.; Lyra, E.; Silva, N.M.; Figueiredo, C.P.; Frozza, R.L.; Ledo, J.H.; Beckman, D.; Katashima, C.K.; Razolli, D.; Carvalho, B.M.; et al. Alzheimer-associated Aβ oligomers impact the central nervous system to induce peripheral metabolic deregulation. *EMBO Mol. Med.* **2015**, *7*, 190–210.
77. Arrieta-Cruz, I.; Knight, C.M.; Gutiérrez-Juárez, R. Acute Exposure of the Mediobasal Hypothalamus to Amyloid-β25–35 Perturbs Hepatic Glucose Metabolism. *J. Alzheimers Dis.* **2015**, *46*, 843–848. [CrossRef] [PubMed]
78. Arrieta-Cruz, I.; Gutiérrez-Juárez, R. The Role of Insulin Resistance and Glucose Metabolism Dysregulation in the Development of Alzheimer's Disease. *Rev. Investig. Clin.* **2016**, *68*, 53–58.
79. Roher, A.E.; Esh, C.L.; Kokjohn, T.A.; Castaño, E.M.; Van Vickle, G.D.; Kalback, W.M.; Patton, R.L.; Luehrs, D.C.; Daugs, I.D.; Kuo, Y.M.; et al. Amyloid beta peptides in human plasma and tissues and their significance for Alzheimer's disease. *Alzheimers Dement.* **2009**, *5*, 18–29. [CrossRef] [PubMed]

80. Guglielmotto, M.; Monteleone, D.; Giliberto, L.; Fornaro, M.; Borghi, R.; Tamagno, E.; Tabaton, M. Amyloid-β42 activates the expression of BACE1 through the JNK pathway. *J. Alzheimers Dis.* **2011**, *27*, 871–883. [PubMed]
81. Guglielmotto, M.; Monteleone, D.; Boido, M.; Piras, A.; Giliberto, L.; Borgh, R.; Vercelli, A.; Fornaro, M.; Tabaton, M.; Tamagno, E. Aβ1–42-mediated downregulation of Uch-L1 is dependent on NF-κB activation and impaired BACE1 lysosomal degradation. *Aging Cell* **2012**, *11*, 834–844. [CrossRef] [PubMed]
82. Guglielmotto, M.; Monteleone, D.; Piras, A.; Valsecchi, V.; Tropiano, M.; Ariano, S.; Fornaro, M.; Vercelli, A.; Puyal, J.; Arancio, O.; et al. Aβ1–42 monomers or oligomers have different effects on autophagy and apoptosis. *Autophagy* **2014**, *10*, 1827–1843. [CrossRef] [PubMed]
83. Plucińska, K.; Crouch, B.; Koss, D.; Robinson, L.; Siebrecht, M.; Riedel, G.; Platt, B. Knock-in of human BACE1 cleaves murine APP and reiterates Alzheimer-like phenotypes. *J. Neurosci.* **2014**, *34*, 10710–10728. [CrossRef] [PubMed]
84. Plucińska, K.; Dekeryte, R.; Koss, D.; Shearer, K.; Mody, N.; Whitfield, P.D.; Doherty, M.K.L.; Mingarelli, M.; Welch, A.; Riedel, G.; et al. Neuronal human BACE1 knockin induces systemic diabetes in mice. *Diabetologia* **2016**, *59*, 1513–1523. [CrossRef] [PubMed]
85. Meakin, P.J.; Harper, A.J.; Hamilton, D.L.; Gallagher, J.; McNeilly, A.D.; Burgess, L.A.; Vaanholt, L.M.; Bannon, K.A.; Latcham, J.; Hussain, I.; et al. Reduction in BACE1 decreases body weight, protects against diet-induced obesity and enhances insulin sensitivity in mice. *Biochem. J.* **2012**, *441*, 285–296. [CrossRef] [PubMed]
86. De la Monte, S.M. Type 3 diabetes is sporadic Alzheimer's disease: Mini-review. *Eur. Neuropsychopharmacol.* **2014**, *24*, 1954–1960. [CrossRef] [PubMed]
87. Cardoso, S.; Correia, S.; Santos, R.X.; Carvalho, C.; Santos, M.S.; Oliveira, C.R.; Perry, G.; Smith, M.A.; Zhu, X.; Moreira, P.I. Insulin is a two-edged knife on the brain. *J. Alzheimers Dis.* **2009**, *18*, 483–507. [CrossRef] [PubMed]
88. De la Monte, S.M.; Tong, M.; Lester-Coll, N.; Plater, M., Jr.; Wands, J.R. Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: Relevance to Alzheimer's disease. *J. Alzheimer's Dis.* **2006**, *10*, 89–109. [CrossRef]
89. Ott, V.; Benedict, C.; Schultes, B.; Born, J.; Hallschmid, M. Intranasal administration of insulin to the brain impacts cognitive function and peripheral metabolism. *Diabetes Obes. Metab.* **2012**, *14*, 214–221. [CrossRef] [PubMed]
90. De la Monte, S.M. Early intranasal insulin therapy halts progression of neurodegeneration: Progress in Alzheimer's disease therapeutics. *Aging Health* **2012**, *8*, 61–64. [CrossRef] [PubMed]
91. Galimberti, D.; Scarpini, E. Pioglitazone for the treatment of Alzheimer's disease. *Expert Opin. Investig. Drugs* **2017**, *26*, 97–101. [CrossRef] [PubMed]
92. Zhang, H.B.; Zhang, Y.; Chen, C.; Li, Y.Q.; Ma, C.; Wang, Z.J. Pioglitazone inhibits advanced glycation end product-induced matrix metalloproteinases and apoptosis by suppressing the activation of MAPK and NF-κB. *Apoptosis* **2016**, *21*, 1082–1093. [CrossRef] [PubMed]
93. Sato, T.; Hanyu, H.; Hirao, K.; Kanetaka, H.; Sakurai, H.; Iwamoto, T. Efficacy of PPAR-gamma agonist pioglitazone in mild Alzheimer disease. *Neurobiol. Aging* **2011**, *32*, 1626–1633. [CrossRef] [PubMed]
94. Hölscher, C. Drugs developed for treatment of diabetes show protective effects in Alzheimer's and Parkinson's diseases. *Sheng Li Xue Bao* **2014**, *66*, 497–510. [PubMed]
95. Lambert, M.P.; Barlow, A.K.; Chromy, B.A.; Edwards, C.; Freed, R.; Liosatos, M.; Morgan, T.E.; Rozovsky, I.; Trommer, B.; Viola, K.L.; et al. Diffusible, nonfibrillar ligands derived from Abeta1–42 are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6448–6453. [CrossRef] [PubMed]
96. Pimplikar, S.W. Neuroinflammation in Alzheimer's disease: From pathogenesis to a therapeutic target. *J. Clin. Immunol.* **2014**, *34*, S64–S69. [CrossRef] [PubMed]
97. Zhu, X.C.; Yu, J.T.; Jiang, T.; Tan, L. Autophagy modulation for Alzheimer's disease therapy. *Mol. Neurobiol.* **2013**, *48*, 702–714. [CrossRef] [PubMed]
98. Jiang, T.; Yu, J.T.; Zhu, X.C.; Tan, M.S.; Wang, H.F.; Cao, L.; Zhang, Q.Q.; Shi, J.Q.; Gao, L.; Qin, H.; et al. Temsirolimus promotes autophagic clearance of amyloid-β and provides protective effects in cellular and animal models of Alzheimer's disease. *Pharmacol. Res.* **2014**, *81*, 54–63. [CrossRef] [PubMed]
99. Tramutola, A.; Lanzillotta, C.; Di Domenico, T. Targeting mTOR to reduce Alzheimer-related cognitive decline: From current hits to future therapies. *Expert Rev. Neurother.* **2017**, *17*, 33–45. [CrossRef] [PubMed]

Pharmaceuticals **2018**, *11*, 11

100. Hanyu, H.; Sato, T.; Kiuchi, A.; Sakurai, H.; Iwamoto, T. Pioglitazone improved cognition in a pilot study on patients with Alzheimer's disease and mild cognitive impairment with diabetes mellitus. *J. Am. Geriatr. Soc.* **2009**, *57*, 177–179. [CrossRef] [PubMed]
101. Geldmacher, D.S.; Fritsch, T.; McClendon, M.J.; Landreth, G. A randomized pilot clinical trial of the safety of pioglitazone in treatment of patients with Alzheimer disease. *Arch. Neurol.* **2011**, *68*, 45–50. [CrossRef] [PubMed]
102. Fernandez-Martos, C.M.; Atkinson, R.A.K.; Chuah, M.I.; King, A.E.; Vickers, J.C. Combination treatment with leptin and pioglitazone in a mouse model of Alzheimer's disease. *Alzheimers Dement.* **2016**, *3*, 92–106. [CrossRef] [PubMed]
103. Gasparini, L.; Gouras, G.K.; Wang, R.; Gross, R.S.; Beal, M.F.; Greengard, P.; Xu, H. Stimulation of beta-amyloid precursor protein trafficking by insulin reduces intraneuronal beta-amyloid and requires mitogen-activated protein kinase signaling. *J. Neurosci.* **2001**, *21*, 2561–2570. [PubMed]
104. Rivera, E.J.; Goldin, A.; Fulmer, N.; Tavares, R.; Wands, J.R.; de la Monte, S.M. Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: Link to brain reductions in acetylcholine. *J. Alzheimers Dis.* **2005**, *8*, 247–268. [CrossRef] [PubMed]
105. Banks, W.A.; Farr, S.A.; Salameh, T.S.; Niehoff, M.L.; Rhea, E.M.; Morley, J.E.; Hanson, A.J.; Hansen, K.M.; Craft, S. Triglycerides cross the blood-brain barrier and induce central leptin and insulin receptor resistance. *Int. J. Obes.* **2017**. [CrossRef] [PubMed]
106. Baura, G.D.; Foster, D.M.; Porte, D.; Kahn, S.E.; Bergman, R.N.; Cobelli, C.; Schwartz, M.W. Saturable transport of insulin from plasma into the central-nervous-system of dogs in-vivo—A mechanism for regulated insulin delivery to the brain. *J. Clin. Investig.* **1993**, *92*, 1824–1830. [CrossRef] [PubMed]
107. Plum, L.; Schubert, M.; Brüning, J.C. The role of insulin receptor signaling in the brain. *Trends Endocrinol. Metab.* **2005**, *16*, 59–65. [CrossRef] [PubMed]
108. Craft, S.; Claxton, A.; Baker, L.D.; Hanson, A.J.; Cholerton, B.; Trittschuh, E.H.; Dahl, D.; Caulder, E.; Neth, B.; Montine, T.J.; et al. Effects of Regular and Long-Acting Insulin on Cognition and Alzheimer's Disease Biomarkers: A Pilot Clinical Trial. *J. Alzheimers Dis.* **2017**, *57*, 1325–1334. [CrossRef] [PubMed]
109. Craft, S.; Baker, L.D.; Montine, T.J.; Minoshima, S.; Watson, G.S.; Claxton, A.; Arbuckle, M.; Callaghan, M.; Tsai, E.; Plymate, S.R.; et al. Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: A pilot clinical trial. *Arch. Neurol.* **2012**, *69*, 29–38. [CrossRef] [PubMed]
110. Bergmann, L.; Maute, L.; Guschmann, M. Temsirolimus for advanced renal cellcarcinoma. *Expert Rev. Anticancer Ther.* **2014**, *14*, 9–21. [CrossRef] [PubMed]
111. Kwitkowski, V.E.; Prowell, T.M.; Ibrahim, A.; Farrell, A.T.; Justice, R.; Mitchell, S.S.; Sridhara, R.; Pazdur, R. FDA approval summary: Temsirolimus as treatment foradvanced renal cell carcinoma. *Oncologist* **2010**, *15*, 428–435. [CrossRef] [PubMed]
112. Wang, J.; Gu, B.J.; Masters, C.L.; Wang, Y.J. A systemic view of Alzheimer disease—Insights from amyloid-β metabolism beyond the brain. *Nat. Rev. Neurol.* **2017**, *13*, 612–623. [CrossRef] [PubMed]

Annex III

Triple GLP-1/GIP/glucagon receptor agonists, a potential novel treatment strategy in Alzheimer's disease.

Camins A, Ettcheto M, Busquets O, Manzine PR, Castro-Torres RD, Beas-Zarate C, Verdaguer E, Sureda F, Bulló M, Olloquequi J, Auladell C and Folch J.

Expert Opin Investig Drugs. 2018;28(1): 93-97.

EXPERT OPINION ON INVESTIGATIONAL DRUGS
2019, VOL. 28, NO. 1, 93–97
https://doi.org/10.1080/13543784.2019.1552677

Taylor & Francis
Taylor & Francis Group

EDITORIAL

Triple GLP-1/GIP/glucagon receptor agonists, a potential novel treatment strategy in Alzheimer's disease

Antoni Camins[a,b,c], Miren Ettcheto[a,b,c,d], Oriol Busquets[a,b,c,d], Patricia R. Manzine[a,e], Rubén Dario Castro-Torres[a,f,g], Carlos Beas-Zarate[g], Ester Verdaguer[b,c,f], Francesc X. Sureda[d], Monica Bulló[d], Jordi Olloquequi[h], Carme Auladell[b,c,f] and Jaume Folch[b,d]

[a]Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain; [b]Biomedical Research Networking Centre in Neurodegenerative Diseases (CIBERNED), Madrid, Spain; [c]Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain; [d]Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain; [e]Department of Gerontology, Federal University of São Carlos (UFSCar), São Carlos, Brazil; [f]Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain; [g]Departamento de Biología Celular y Molecular, C.U.C.B.A., Universidad de Guadalajara y División de Neurociencias, Guadalajara, México; [h]Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile

ARTICLE HISTORY Received 3 October 2018; Accepted 22 November 2018

KEYWORDS Alzheimer's disease; type 2 diabetes; glucose-dependent insulinotropic peptide; glucagon; glucagon-like peptide

1. Introduction

In the last 30 years, drug development for Alzheimer's disease (AD) has been focused on those hallmarks first described by Alois Alzheimer: amyloid protein level and TAU phosphorylation. However, none of the treatments has been able to alter the development of the disease. Thus, new molecular targets and therapeutic strategies need to be developed.

Dementia is described as a decrease of cognitive capabilities that affects normal daily living activity and social relationships. Although different types of dementias have been described, AD is the most predominant one, specifically its sporadic or late onset form (LOAD). Multiple epidemiological studies have foretold alarming perspectives for the coming years in the incidence of affected patients as a result of increasing population numbers, lack of an effective treatment, and unknown early-stage markers for the disease, which, in the end, are the results of the ignorance on the actual mechanisms that cause for its appearance [1]. The development of drugs for the prevention of cognitive decline is one of the greatest challenges of this century for the scientific community [2].

One of the most important modifiable risk factors involved in the appearance of LOAD is obesity [3]. On 2018, the Whitehall II clinical study established a relationship between body mass index values (>30kg/m^2), midlife aging risk (50–65 years), and the onset of cognitive deficits [4]. In addition, further complications of this situation like Type 2 Diabetes mellitus (T2D) and metabolic syndrome have proved to significantly increase the risk of LOAD [5]. Some authors have proposed a term for this transition called Type 3 Diabetes (T3D) [6].

T3D would be a term coined from the multiple evidences that demonstrate how in the early stages of LOAD pathogenesis there is a reduction of brain insulin signaling and a tendency toward insulin resistance [7]. These events would lead to down-responsiveness of downstream effectors due to a decrease in the activation of the insulin receptor (IR) itself and, consequently, a reduction of glucose uptake. Furthermore, these pathways would also be involved in the expression of amyloid precursor protein (APP), clearance of amyloid β (Aβ), phosphorylation of TAU protein, control of neuroinflammatory states, and synaptic plasticity [8,9]. In addition, insulin regulates the structural and functional integrity of synapses in the central nervous system [9] and, another recent study strengthened this hypothesis by demonstrating significant alterations in mRNA expression profiles of genes related to insulin signaling in the cortex and hippocampus [7].

From a neuropathological point of view, clinical experts in LOAD have suggested that the disease begins approximately 15–-20 years before cognitive loss begins to show [10]. Thereby, when initial pathological metabolic biomarkers appear, there is still a wide time window for effective preventive therapeutic approaches. For example, analogues of the incretin hormones glucagon-like peptide (GLPI) and glucose-dependent insulinotropic peptide (GIP) have shown to have anti-diabetic properties and positive effects against pathophysiological characteristics of AD-like synaptic plasticity and Aβ levels in the brain [11,12].

In this editorial, we discuss the discovery and development of the first rationally designed approach to unimolecular co-agonism, which recruits the well-established pharmacology of GLPI, GIP, and glucagon. We will be focusing on these agonists for the potential treatment of LOAD in preclinical and clinical studies.

2. Body

2.1. GLPI/GIP/Glucagon receptor triagonists for LOAD treatment

2.1.1. Preclinical data

Previously reported data have shown that some drugs used for the treatment of T2D also exhibit neuroprotective effects in preclinical models of AD [13]. These drugs include metformin, peroxisome proliferator-activated receptor gamma (PPARγ)

CONTACT Antoni Camins camins@ub.edu Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Av. Joan XXIII 27/31, Barcelona, E-08028, Spain

agonists, sulfonylureas, and GLPI receptor agonists such as exendin-4, liraglutide and lixisenatide [13,14].

GLPI agonist drugs have become an attractive possibility considering the wide distribution of GLPI receptors in hippocampal and cortical brain areas. It has been proven that cognitive loss appears in mice deficient for the GLPI receptor [15]. Also, other studies have showed how intraperitoneal injections of liraglutide or lixisenatide in transgenic mice models of AD causes for a decrease in the number of plaque load, reduces neuroinflammatory responses, and improves cognitive learning through an increase in the density of synapses [12]. Likewise, positive effects of liraglutide on cognition were found in the brains of non-human primates [16]. Moreover, GIP analogues such as D-Ala2-GIP also showed memory synapse improvement in AβPP/PS1 mice, a transgenic familial model of AD [12]. Thus, after the encouraging data on the activity of these analogues, current strategies have focused on the synthesis and testing of a tripeptide that contain agonists for the GLPI, GPI, and glucagon receptors. HM15211 and MAR423 are single-molecule peptides that reduce diabetic complications in rodent models of obesity [15].

For now, it has been proven that these molecules would reduce body weight and food intake further than previous tested drugs in experimental models of T2DM and non-alcoholic steatohepatitis [11]. Also, Tai and colleagues recently reported neuroprotective effects when treating AβPP/PS1 mice [17]. The triple combination improved cognitive function and decreased the number of plaques in the cortex and hippocampus. Furthermore, this treatment decreased the levels of oxidative stress biomarkers and increased the levels of brain-derived neurotrophic factor (BDNF). In addition, the triagonist compound decreased neuroinflammatory states in the brain. Further data from Li and colleagues reported improvements in long-term spatial memory evaluated through the Morris water maze test and synaptic plasticity through the evaluation of long-term potentiation (LTP) [18]. Likewise, this compound also decreased hippocampal levels of TAU hyperphosphorylation. Nonetheless, even though these results seem promising, it must be highlighted that these studies have been performed in AD rodent models that carry mutations associated with early-onset familial forms of human AD, thus representing only 3–5% of all cases (Figure 1).

Finally, it has been reported that HM15211 also improved MPTP-induced mice motor impairments and nigrostriatal neurodegeneration by attenuating microglial activation and release of cytokines and reduced lipid peroxidation [19].

2.1.2. Clinical data

Several clinical studies are now evaluating the potential efficacy and safety of GLPI mimetics in AD patients. Gejl and colleagues reported encouraging results with 6-month liraglutide (VICTOZA®, Novo Nordisk, Denmark) treatment in a randomized, placebo-controlled, double-blinded intervention with or without placebo [20]. Authors demonstrated improved brain glucose metabolism related with potential improvement in cognitive performance and synaptic plasticity. There is currently another multicenter randomized double-blind placebo-controlled Phase II study with liraglutide with mild AD patients but, so far, no results are available.

For exendin-4 (Table 1), the safety of the compound was evaluated in early-stage and mild cognitive impaired individuals while trying to infer its effects on clinical progression of dementia (NCT01255163). Unfortunately, the reported results are not conclusive in the conditions of the clinical trial. No differences were observed in parameters like the cognitive capacity affectation evaluated by Mini Mental State Examination (MMSE) and for AD Assessment Scale-cognitive subscale (ADAS-cog70).

The first human study for HM15211 started by evaluating different pharmacokinetic parameters after a single ascending dose of the drug in healthy obese subjects (NCT03374241). This drug is currently being tested in Phase I clinical trials for obesity and non-alcoholic steatohepatitis.

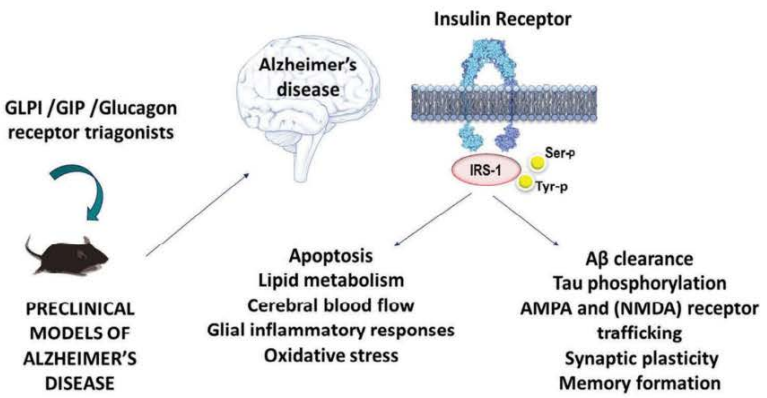


Figure 1. Mechanisms involved in Alzheimer's disease that are likely promoted by brain insulin receptor resistance and counteracted by the administration of GLPI/GIP/glucagon receptor triagonists therapy.

Table 1. Specific antidiabetic and anti-obesity agents currently tested for the treatment of Alzheimer's disease and neurodegenerative disorders.

Drug	Phase	CT number	Disease
Rosiglitazone	II	NCT00381238	Mild-to-moderate Alzheimer's disease
Rosiglitazone	II	NCT00334568	Examine the drug response of patients with AD
Rosiglitazone	I	NCT00688207	Alzheimer's disease (the study is designed to assess the pharmacokinetics of Rosiglitazone)
Rosiglitazone	III	NCT00428090	Mild-to-moderate Alzheimer's disease
Pioglitazone	II	NCT00982202	Alzheimer's disease
Intranasal insulin glulisine	II	NCT01436045	Mild-to-moderate Alzheimer's Disease
Intranasal insulin detemir	II	NCT01595646	AD or amnestic mild cognitive impairment
Metformin	II	NCT00620191	Amnestic mild cognitive impairment
Liraglutide	Not applicable	NCT01469351	Alzheimer's Disease
Exendin-4	II	NCT01255163	Alzheimer's disease
Exendin-4	III	NCT02847403	Cognitive decline in dysglycemic patients
HM15211	I	NCT03374241	Obesity
GIP/GLP-1 Co-Activity	Not applicable	NCT03526289	Overweight and Type 2 diabetes

3. Conclusion

New advances in molecular pharmacology are bringing new perspectives into the AD research field. Obesity and metabolic alterations, including cholesterol levels, would have important roles in the development of LOAD. Thus, modulators of the brain IR and peripheral metabolism might be interesting approaches for new treatments. These compounds, which have been classically used for the treatment of T2D, seem to improve pathological parameters of AD in preclinical models. Among these compounds, the GLPI receptor agonist liraglutide has recently shown promising results in clinical studies. Furthermore, a new pharmacological group of drugs combining triagonists may be a promising approach when used in early stage of pathology development. These molecules have shown *in vivo* benefits in obesity treatment and preclinical diabetes models and seem to have potential therapeutic value in neurodegenerative diseases [14,15]. Considering HM15211 and MAR423, preclinical results would indicate their effectiveness in the treatment of obesity and in the improvement of the state of familial AD mice models. In addition, some of these compounds show potentially effective disease-modifying effects when adequately administered in patients [20]. To conclude, it is our belief that a multiple therapeutic strategy with three or four different drugs cannot be ruled out for a definitive treatment of LOAD. This idea would be in accordance with the other many research lines that are being undertaken that evaluate other mechanisms like Aβ production, TAU hyperphosphorylation, NMDA receptors activity, and inflammatory processes among others.

4. Expert opinion

Since 2005, only memantine has been approved for human use. Its effects have shown to be only temporary and limited in ameliorating memory loss for moderate or severe patients [2]. Thus, there is an urgency in the need to find new molecules and approaches for the treatment of AD.

There are many reasons for the failure of clinical trials in the treatment of this pathology. Among them, we can highlight its complex nature and the lack of understanding of the multifactorial mechanisms involved. Moreover, the continuous use of ineffective models to reproduce the features of LOAD has become another problem. On the one hand, experimental mice specimens such as the AβPP/PS1 only mimic some of the features of the familial form of AD and, thus, do not allow for proper study of the sporadic form of the disease, which accounts for 95–97% of all cases [2,17,18,21]. In addition, variables related to the aging process in humans are not depicted [9]. On the other hand, neuronal cell culture experiments are limited to basic analysis and can be only useful to study specific cellular pathways involved in AD neuropathology.

The amyloid cascade hypothesis has been dominant for many years and almost every molecule that has been produced focused its target on classical hallmarks of the disease, which, although important in its progression, there is controversy on whether they are actually its cause [1–3].

Dr. Hoyer was the first scientist who reported that alterations in brain IR signaling pathway in LOAD could be a key event in the process of memory loss [22]. Many years later, several research groups suggested that LOAD should be addressed as a metabolic disease [8]. In T2D, insulin resistance leads to both Aβ plaque formation and TAU hyperphosphorylation [23]. The increase in brain plaque formation could be originated, in part, through the increase of brain insulin concentrations. The saturation of the insulin degrading enzyme (IDE) by insulin would reduce its capacity to degrade Aβ monomers and, thus promote their accumulation. The process of TAU alteration is mediated by a decrease in IR signaling that, eventually, leads to the inhibition of Protein Kinase B (AKT) and activation of the glycogen synthase kinase 3β (GSK-3β) [6,8]. Moreover, T2D and AD are also characterized with deficits in mitochondrial activity associated with a dysregulation of calcium homeostasis and an increase in oxidative stress [6]. Several research groups have described that soluble Aβ fragments bind directly to IRs on neuronal dendrites and impair their synaptic activity [21,24]. These results would be common ground for those within the controversy on the origin of the disease as it may be a mechanism of how insulin signaling in the brain becomes impaired. In addition, Aβ in peripheral tissues such as the liver would favor T2D appearance [21].

Initial ideas indicated that the use of drugs that stimulate or activate the brain IR would improve cognitive processing [5]. Correspondingly, intranasal insulin was used to treat cognitive loss but it was observed that prolonged exposure to this treatment caused for brain IR desensitization by the appearance of resistances to the effects of this hormone [21,25]. Nowadays, researchers are investigating alternative strategies like the use of a GLPI/GIP/Glucagon receptor triagonist, which has shown to be useful in overcoming primary resistance by having different receptor targets.

The pharmacological combination of GLPI/GIP/Glucagon receptor triagonist prevented the decline of brain glucose metabolism and significantly reduced obesity [26]. Furthermore, these multi-receptor agonists slowed the neurodegenerative process in preclinical models of neurological diseases, such as Parkinson's disease (PD) and AD [27]. At a molecular level, the triagonist upregulated the phosphorylation rates of the cAMP Response Element-Binding (P-CREB) at Ser133, which has been described to be critical for memory formation and LTP maintenance. It has also been described that its activity is involved in the control of expression of genes related to synaptic formation, neuronal plasticity, and neurogenesis [17,18]. Its administration in AD patients has been relatively limited because clinical trials in human studies have focused in the obesity treatment. However, it can expected that these triagonist compounds will improve peripheral and central insulin resistances and have beneficial roles in the course of AD based on: (i) the activation of signaling pathways that converge with and facilitate insulin signaling leading to the activation of AKT, therefore preventing the phosphorylation of TAU and also modulation of apoptotic processes, (ii) the decrease of brain insulin levels and the up-regulation of Aβ metabolism through IDE liberation, (iii) reduction of brain oxidative stress, neuroinflammation, and endoplasmic reticulum stress with a prevention against unfolded protein response, (iv) improvement of cognitive processes through synaptogenesis and neurogenesis increase, as well as, vasodilation and cerebral blood flow after decline of brain Aβ, and (v) finally, it is expected to have peripheral beneficial effects on the overall consequences of obesity.

Even though the obtained clinical results obtained from Gejl and colleagues with liraglutide seem hopeful, it is important to remember that AD is a severely complex disease [20]. Thus, it is possible that, in the end, an effective treatment of the pathology will require the administration of several drugs from different pharmacological families, in order to slow down effectively the neurodegenerative process. Similar methods are being used for the treatment of patients with coronary diseases which need the administration of drugs for the lowering of cholesterol levels (statins), antihypertensive drugs, and the administration of aspirin at low doses to prevent thrombi formation. Additionally, patients require taking on themselves non-pharmacological measures such as periodical moderate exercise, an adequate diet, and a reduction of stress factors.

Finally, seeing how the idea of the bases of the diseases is slowly changing, hopefully, in the coming years, new data from all the ongoing studies will provide us with enough information to support the use of novel compounds, as well as prove the importance of the roles of metabolic parameters and their relevance in the development of treatments for patients with LOAD [28,29].

Funding

This paper was not funded. This work was supported by the Spanish Ministry of Science and Innovation SAF2017-84283-R, PI2016/01 and the European Regional Development Funds. CIBER de Enfermedades Neurodegenrativas (CIBERNED) (Grant CB06/05/2004) is a Project from the Instituto de Salud Carlos III. PR Manzine is supported by grants 2015/26084-1 and 2017/13224-5 from São Paulo Research Foundation (FAPESP), Brazil. RD Castro-Torres received a Postdoctoral Fellowship (No. 298337, CONACYT-MEXICO) in cooperation for consolidating research groups (Doctoral Program in Sciences in Molecular Biology in Medicine, LGAC Molecular Bases of Chronic Degenerative Diseases and its Applications 000091 PNPC, CONACYT-MEXICO).

Declaration of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose

References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Aisen PS, Cummings J, Jack CR Jr, et al. On the path to 2025: understanding the Alzheimer's disease continuum. Alzheimers Res Ther. 2017;9:60.
2. Cummings J, Lee G, Ritter A, et al. Alzheimer's disease drug development pipeline: 2018. Alzheimers Dement (N Y). 2018;4:195–214.
•• **Reviews the current status of all drugs that are in clinical trials for the treatment of Alzheimer's disease.**
3. Alford S, Patel D, Perakakis N, et al. Obesity as a risk factor for Alzheimer's disease: weighing the evidence. Obes Rev. 2018;19:269–280.
4. Singh-Manoux A, Dugravot A, Shipley M, et al. Obesity trajectories and risk of dementia: 28 years of follow-up in the Whitehall II study. Alzheimers Dement. 2018;14:178–186.
5. Vieira MNN, Lima-Filho RAS, De Felice FG. Connecting Alzheimer's disease to diabetes: underlying mechanisms and potential therapeutic targets. Neuropharmacology. 2018;136:160–171.
6. Kandimalla R, Thirumala V, Reddy PH. Is Alzheimer's disease a Type 3 diabetes? A critical appraisal. Biochim Biophys Acta Mol Basis Dis. 2017;1863:1078–1089.
7. Hokama M, Oka S, Leon J, et al. Altered expression of diabetes-related genes in Alzheimer's disease brains: the Hisayama study. Cereb Cortex. 2014;24:2476–2488.
8. Demetrius L, Driver J. Alzheimer's as a metabolic disease. Biogerontology. 2013;14:641–649.
9. Benedict C, Grillo CA. Insulin resistance as a therapeutic target in the treatment of Alzheimer's disease: a state-of-the-art review. Front Neurosci. 2018;12:215.
10. McDade E, Bateman RJ. Stop Alzheimer's before it starts. Nature. 2017;547:153–155.
•• **Suggests the concept of a therapeutic window for the treatment of Alzheimer's disease.**
11. Müller TD, Clemmensen C, Finan B, et al. Anti-Obesity therapy: from rainbow pills to polyagonists. Pharmacol Rev. 2018;70:712–746.
12. Hölscher C. The incretin hormones glucagonlike peptide 1 and glucose-dependent insulinotropic polypeptide are neuroprotective in mouse models of Alzheimer's disease. Alzheimers Dement. 2014;10(1 Suppl):S47–54.
13. Yarchoan M, Arnold SE. Repurposing diabetes drugs for brain insulin resistance in Alzheimer disease. Diabetes. 2014;63:2253–2261.
• **Suggests the application of antidiabetic drugs for the treatment of Alzheimer's disease.**
14. Knerr PJ, Finan B, Gelfanov V, et al. Optimization of peptide-based polyagonists for treatment of diabetes and obesity. Bioorg Med Chem. 2018;26:2873–2881.

15. Capozzi ME, DiMarchi RD, Tschöp MH, et al. Targeting the Incretin/Glucagon system with triagonists to treat diabetes. Endocr Rev. 2018 Jun 13. DOI:10.1210/er.2018-00117.
16. Batista AF, Forny-Germano L, Clarke JR, et al. The diabetes drug liraglutide reverses cognitive impairment in mice and attenuates insulin receptor and synaptic pathology in a non-human primate model of Alzheimer's disease. J Pathol. 2018;245:85–100.
17. Tai J, Liu W, Li Y, et al. Neuroprotective effects of a triple GLP-1/GIP/glucagon receptor agonist in the APP/PS1 transgenic mouse model of Alzheimer's disease. Brain Res. 2018;1678:64–74.
18. Li T, Jiao JJ, Hölscher C, et al. A novel GLP-1/GIP/Gcg triagonist reduces cognitive deficits and pathology in the 3xTg mouse model of Alzheimer's disease. Hippocampus. 2018;28:358–372.
19. Choi IY, Lee JS, Kim JK, et al. Potent body weight loss and efficacy in a NASH animal model by a novel long-acting GLP-1/Glucagon/GIP triple-agonist (HM15211), American Diabetes Association's 77th Scientific Session, 2017; San Diego, CA USA.
20. Gejl M, Gjedde A, Egefjord L, et al. In Alzheimer's disease, 6-Month treatment with GLP-1 analog prevents decline of brain glucose metabolism: randomized, placebo-controlled, double-blind clinical trial. Front Aging Neurosci. 2016;8:108.
    - **Clinical study suggesting the application of liraglutide for the treatment of cognitive loss.**
21. Wijesekara N, Gonçalves RA, De Felice FG, et al. Impaired peripheral glucose homeostasis and Alzheimer's disease. Neuropharmacology. 2018;136:172–181.
22. Hoyer S. The brain insulin signal transduction system and sporadic (type II) Alzheimer disease: an update. J Neural Transm (Vienna). 2002;109:341–360.
23. Arnold SE, Arvanitakis Z, Macauley-Rambach SL, et al. Brain insulin resistance in type 2 diabetes and Alzheimer disease: concepts and conundrums. Nat Rev Neurol. 2018;14:168–181.
24. Bomfim TR, Forny-Germano L, Sathler LB, et al. An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease-associated Abeta oligomers. J Clin Invest. 2012;122:1339–1353.
25. Craft S, Claxton A, Baker LD, et al. Effects of regular and long-acting insulin on cognition and Alzheimer's disease biomarkers: a pilot clinical trial. J Alzheimers Dis. 2017;57:1325–1334.
26. Sánchez-Garrido MA, Brandt SJ, Clemmensen C, et al. GLP-1/glucagon receptor co-agonism for treatment of obesity. Diabetologia. 2017;60:1851–1861.
27. Kim JA, Sangdon L, Sang HL, et al. Neuroprotective effects of HM15211, a novel long-acting GLP-1/GIP/Glucagon triple agonist in the neurodegenerative disease models. Diabetes. 2018;67 (Suppl1):1107-P.
28. Chapman CD, Schiöth HB, Grillo CA, et al. Intranasal insulin in Alzheimer's disease: food for thought. Neuropharmacology. 2018;136:196–201.
29. Li Y, Li L, Hölscher C. Incretin-based therapy for type 2 diabetes mellitus is promising for treating neurodegenerative diseases. Rev Neurosci. 2016;27:689–711.

Annex IV

JNK Isoforms Are Involved in the Control of Adult Hippocampal Neurogenesis in Mice. Both in Physiological Conditions and in an Experimental Model of Temporal Lobe Epilepsy.

Castro-Torres RD, Landa J, Rabaza M, Busquets O, Olloquequi J, Ettcheto M, Beas-Zarate C, Folch J, Camins A, Auladell C and Verdaguer E.

Mol Neurobiol. 2019.

Molecular Neurobiology
https://doi.org/10.1007/s12035-019-1476-7

JNK Isoforms Are Involved in the Control of Adult Hippocampal Neurogenesis in Mice, Both in Physiological Conditions and in an Experimental Model of Temporal Lobe Epilepsy

Rubén D. Castro-Torres[1,2,3] · Jon Landa[1] · Marina Rabaza[1] · Oriol Busquets[2,4,5,6] · Jordi Olloquequi[7] · Miren Ettcheto[2,4,5,6] · Carlos Beas-Zarate[3] · Jaume Folch[4,5] · Antoni Camins[2,5,6] · Carme Auladell[1,5,6] · Ester Verdaguer[1,5,6]

Received: 7 September 2018 / Accepted: 10 January 2019

Abstract
Neurogenesis in the adult dentate gyrus (DG) of the hippocampus allows the continuous generation of new neurons. This cellular process can be disturbed under specific environmental conditions, such as epileptic seizures; however, the underlying mechanisms responsible for their control remain largely unknown. Although different studies have linked the JNK (c-Jun-N-terminal-kinase) activity with the regulation of cell proliferation and differentiation, the specific function of JNK in controlling adult hippocampal neurogenesis is not well known. The purpose of this study was to analyze the role of JNK isoforms (JNK1/JNK2/JNK3) in adult-hippocampal neurogenesis. To achieve this goal, we used JNK-knockout mice (*Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$, and *Jnk3*$^{-/-}$), untreated and treated with intraperitoneal injections of kainic acid (KA), as an experimental model of epilepsy. In each condition, we identified cell subpopulations at different stages of neuronal maturation by immunohistochemical specific markers. In physiological conditions, we evidenced that JNK1 and JNK3 control the levels of one subtype of early progenitor cells (GFAP$^+$/Sox2$^+$) but not the GFAP$^+$/Nestin$^+$ cell subtype. Moreover, the absence of JNK1 induces an increase of immature neurons (Doublecortin$^+$; PSA-NCAM$^+$ cells) compared with wild-type (WT). On the other hand, *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ mice showed an increased capacity to maintain hippocampal homeostasis, since calbindin immunoreactivity is higher than in WT. An important fact is that, after KA injection, *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ mice show no increase in the different neurogenic cell subpopulation analyzed, in contrast to what occurs in WT and *Jnk2*$^{-/-}$ mice. All these data support that JNK isoforms are involved in the adult neurogenesis control.

Keywords JNK isoforms · Knockout mice · Adult hippocampal neurogenesis · Kainic acid

Carme Auladell and Ester Verdaguer contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12035-019-1476-7) contains supplementary material, which is available to authorized users.

✉ Carme Auladell
cauladell@ub.edu

[1] Departament de Biologia Cellular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

[2] Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain

[3] Departamento de Biología Celular y Molecular, Laboratorio de Regeneración Neural, C.U.C.B.A, Universidad de Guadalajara, 44340 Jalisco, Mexico

[4] Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Tarragona, Spain

[5] Networking Research Center on Neurodegenerative Diseases (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain

[6] Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain

[7] Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, 5 Poniente No. 1670, 3460000 Talca, Chile

Published online: 26 January 2019

Abbreviations

ABC	avidin-biotin-peroxidase complex
CB	calbindin
CR	calretinin
CT	control
CBP	Calcium-binding protein
DAB	diaminobenzidine
DCX	doublecortin
DG	dentate gyrus
FBS	fetal bovine serum
GCL	granular cell layer
GC	granule cells
GFAP	glial fibrillary acidic protein
i.p	intraperitoneal injection
IR	immunoreactive
JNKs	c-Jun N-terminal kinases
JNK1, JNK2, and JNK3	JNK isoforms
jnk1	Knockout mice for JNK1
$jnk2^{-/-}$	knockout mice for JNK2
jnk3	knockout mice for JNK3
KA	kainic acid
KO	knockout
NSC	neural stem cells
O/N	overnight
PB	phosphate buffer
PBS	phosphate-buffered saline
PSA-NCAM	polysialic acid neural cell adhesion molecule
RT	room temperature
SD	standard deviation
SDA-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis.
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SGZ	subgranular zone
TLE	temporal lobe epilepsy
WT	wild type

Introduction

The c-Jun N-terminal kinases (JNKs), a subfamily of the mitogen-activated protein kinases (MAPK), are stimulus-response proteins involved in a wide spectrum of cellular processes, including cell proliferation, differentiation, migration, inflammation, and apoptosis [1]. They are encoded by three genes in mammals: *Mapk8 (Jnk1)*, *Mapk9 (Jnk2)*, and *Mapk10 (Jnk3)*, which are expressed differentially in the brain [2]. The activation of JNKs is carried out by two MAPKs: MKK4 and MKK7 [3]. Once activated, JNK can translocate to the nucleus and control the expression of several transcriptional factors and nuclear hormone receptors [2, 4] or phosphorylate other non-nuclear substrates [5]. Moreover, different studies highlighted the important role of JNK in the pathogenesis of several diseases, such as diabetes, lung fibrosis, inflammatory and neurodegenerative disorders, and cancer [6]. Hence, understanding the function of the JNK pathway would be a major step towards developing specific therapeutic strategies for different diseases.

Elucidating the specific function of JNK isoforms is paramount in order to understand the complexity of JNK signaling. An approach to this analysis has been achieved studying JNK knockout (KO) mice ($Jnk1^{-/-}$, $Jnk2^{-/-}$, and $Jnk3^{-/-}$) [7–9]. Eventually, we know that JNK1 and JNK2 are probably essential in normal cell function and, presumably, they have redundant roles, since both show cooperative or synergistic effects [10]. Furthermore, it has been demonstrated that they participate in apoptosis regulation during normal brain development [11]. Nonetheless, specific functions have also been described for JNK1 and JNK2, such as the different and opposite effects on fibroblasts and macrophages proliferation [10, 12]. JNK1 and JNK2 differentially regulate T cell expansion during the viral lymphocytic choriomeningitis [13]. Moreover, $Jnk1^{-/-}$ mice showed greater abnormal cortical neuronal migration, anterior commissure degeneration during brain development [14], and disturbed metabolic regulation [15]. In addition, mice lacking JNK1, or treated with a JNK1 inhibitor (DJNKI-1), display an increased adult hippocampal neurogenesis [16]. Concerning $Jnk2^{-/-}$ mice, they show a less remarkable phenotype, with epidermal hyperplasia and mild immune abnormalities [10]. Lastly, JNK3 is clearly associated with neuronal death and oxidative stress. Thus, $Jnk3^{-/-}$ mice show a decrease in c-Jun phosphorylation in ischemia–hypoxia experimental models [17] and in cytochrome-c (cyt-c) release after spinal cord injury [18]. Furthermore, targeted disruption of *Jnk3* in mice confers a high resistance to kainic acid (KA), an analogue of glutamic acid that has been used to establish excitotoxicity models in vitro and in vivo [8, 19, 20]. KA induces an enhancement of neurogenesis in the dentate gyrus (DG) of the hippocampus by promoting hippocampal hyperexcitability [21], a pathological feature in patients with mesial temporal lobe epilepsy (TLE) [22–25]. In this regard, we have recently demonstrated that $Jnk1^{-/-}$ mice also show neuroprotection against KA, as previously described in $Jnk3^{-/-}$ mice [9, 19].

Specifically, the adult hippocampal neurogenesis in DG occurs in an intermittent zone between the granular cell layer (GCL) and the hilus, called the subgranular zone (SGZ), where a continuous generation of new neurons takes place [26]. These new cells originate from neural stem cells (NSCs) and are structurally integrated into the network of mature granule cells (GCs). This cellular process plays an important role in hippocampal functions related to learning and memory, as well as in mood regulation [27]. Therefore, disturbances in their activity may lead, in addition to epilepsy, to serious cognitive disorders, such as dementia and depression [28, 29].

The aim of the present study was to analyze the role of each JNK isoform (JNK1, JNK2, and JNK3) in the control of hippocampal neurogenesis, both in physiological conditions and under the toxicity of KA. To achieve this goal, we used JNK KO mice (*Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$, and *Jnk3*$^{-/-}$), untreated and treated with intraperitoneal injections of KA. In each condition, we identified cell subpopulations in the SGZ at different stages of neuronal maturation. We determined these subpopulations with the use of specific biomarkers: (i) GFAP (glial fibrillary acidic protein), Nestin protein (neuroectodermal stem cell marker), a type of intermediate filament and Sox2 (transcription factor, SRY-like homeobox 2) which detects NSC or early precursors cells (type 1), (ii) doublecortin (DCX) and polysialic acid-neural cell adhesion molecule (PSA-NCAM), two proteins that target transit-amplifying progenitor cells or immature neurons (types 2a and 2b), and (iii) calretinin (CR), a calcium binding protein (CBP) that helps to identify neuroblasts, which represent the last stage of neuron precursor development (type 3). Since new CR-positive cells modify their expression from CR to calbindin (CB) as they integrate into the existing network, [30], we analyzed the distribution pattern of CB immunoreactive cells in hippocampal areas.

Materials and Methods

Animals

Two-month-old C57BL/6 wild-type (WT) and KO mice for the JNKs isoforms (*jnk1*$^{-/-}$, *jnk2*$^{-/-}$, *jnk3*$^{-/-}$) were used in this study. The generation and characterization of *jnk1*$^{-/-}$, *jnk2*$^{-/-}$, and *jnk3*$^{-/-}$ single knockout mice have been previously described [7–9]. Mice were backcrossed to the C57BL/6 genetic background. Throughout the experiments, all mice were housed in a controlled environment, and food and water were available ad libitum. The experiments were conducted in accordance with the Council of Europe Directive 2010/63. The procedure was registered at the *Department d'Agricultura, Ramaderia i Pesca* of the *Generalitat de Catalunya*. Ref. Number order 8852.

Kainic Acid Treatment and Sample Processing

Animals were divided into two treatments: (i) single intraperitoneal (i.p.) dose (30 mg/kg) of KA (Sigma-Aldrich, USA) dissolved in 0.9% saline and (ii) single i.p. dose of 0.9% saline solution (control group; CT) [31]. After 24 h, mice were anesthetized by i.p. administration of pentobarbital (80 mg/kg) and were perfused with paraformaldehyde (40 g/L) diluted in 0.1 mol/L phosphate buffer (PB). Brains were removed and post-fixed with the same fixative for 24 h; subsequently, they were transferred into the same solution enriched with 300 g/L sucrose for 24 h. Finally, brain samples were frozen to obtain coronal sections of 20 μm in a cryostat (Leica Microsystems, Wetzlar, Germany). Free-floating samples were kept in a cryoprotectant in the freezer until use.

Finally, the following eight experimental groups were established: WT CT, WT KA, *Jnk1*$^{-/-}$ CT, *Jnk1*$^{-/-}$ KA, *Jnk2*$^{-/-}$ CT, *Jnk2*$^{-/-}$ KA, *Jnk3*$^{-/-}$ CT, and *Jnk3*$^{-/-}$ KA.

Immunofluorescences

The primary antibodies used for immunofluorescences were mouse anti-Nestin antibody (1:200, MAB353, Millipore), rabbit anti-Sox2 (1:500, ab97959, Abcam), goat anti-DCX (1:200, sc-8066, Santa Cruz Biotechnology), mouse anti-PSA-NCAM (1:1000, ABC Scientific), rabbit anti-NeuN (1:500, 24307, Cell Signal Technology), rabbit anti-GFAP (1:1000, Z0334, DAKO), and mouse anti-GFAP (1:1000, M0761, DAKO). The secondary antibodies used were donkey anti-goat Alexa 488, goat anti-mouse Alexa 594, goat anti-mouse Alexa 488; goat anti-rabbit Alexa 594, and goat anti-rabbit Alexa 488 (1:200, Life Technology).

Free-floating sections were rinsed in PBS, pH 7.2 prior to pre-incubation in a blocking solution (10% of fetal bovine serum (FBS), 1% of triton X-100 in phosphate-buffered saline (PBS) at room temperature 1 h. Then, they were incubated overnight (O/N) at 4 °C with the corresponding primary antibody and 2 h for the corresponding secondary antibody. We reported two single immunofluorescences against DCX and PSA-NCAM, and three double immunofluorescences against Sox2/GFAP, Nestin/GFAP, and PSA-NCAM/NeuN. The incubation of the primary and secondary antibodies was performed sequentially. At the end, sections were counterstained with 0.1 μg/mL Hoechst 33258 (Sigma-Aldrich, USA) for nuclear stains during 10 min in the dark. Immediately after that, samples were rinsed with PBS and were mounted onto gelatinized slides with Fluoromount medium (Sigma-Aldrich, USA). Stained sections were examined under an epifluorescence microscope (Olympus BX61).

Inmunohistochemistry

The primary antibodies used for enzymatic immunohistochemistry were goat anti-DCX (1:200, sc-8066, Santa Cruz Biotechnology), rabbit anti-Calbindin (1:1000) (Swant Inc., Switzerland), and rabbit anti-Calretinin (1:1000; Swant Inc., Switzerland). Biotinylated secondary antibody horse anti-goat (1:200, Vector Labs), goat anti-rabbit (1:200; Sigma, St. Louis, MO, USA), and horse anti-rabbit (1:200; Sigma-Aldrich, USA) were used.

Enzymatic immunohistochemistry was performed using free-floating technique [31]. First, the sections were rinsed in 0.1 M PB, pH 7.2, and treated with 3% H_2O_2 and 1% methanol in PBS to inactivate endogenous peroxidase for 15 min and then were incubated with the blocking solution as

previously described. Then, they were incubated overnight (O/N) at 4 °C with the corresponding primary antibody and 2 h for the corresponding secondary antibody. After that, the avidin–biotin–peroxidase complex (ABC, Vector Laboratories, USA) was used to develop the chromogenic reaction with 0.05% diaminobenzidine (DAB) in 0.1 M PB and 0.005% H_2O_2. For a double enzymatic immunohistochemistry, a second round of incubation of primary and secondary and ABC complex was performed developing chromogenic reaction using 0.05% DAB-0 with 0.005% H_2O_2, 0.05% cobalt chloride, and 0.05% nickel ammonium sulfate. We reported a single immunohistochemistry against calbidin and a double against calretinin/DCX.

Counting of Immunoreactive Cells

Sections corresponding to the hippocampal levels between Bergman −1.28 and −2.12 mm, in accordance with the Atlas reported by Paxinos et al. [32], were used to perform the cell counting of SGZ of the DG (four to six animals/genotype and age, four to eight sections/animal). In each hippocampus, two field of ×20 magnification were used to count visually spanning from apex towars to the infra and supragranular layers of the dentate gyrus in a total area of 844 × 317.8 μm^2. Then, we report the cell counting as a normalized values per counting area.

Statistical Analysis

All data are presented in box plot graph using the median as the center value; the ends of the box are the lower and upper quartiles, and the ends of the whiskers as minimum and maximum values. The level of significance was fixed at $\alpha = 0.05$. Statistical analyses across the experimental groups were evaluated using individual value per counting area from each animal and performing one-way ANOVA followed by a Holm-Šídák post hoc test for multiple comparisons. Both statistical analyses and graphs were created with the GraphPad InStat software V5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Differential Response of Early Precursor Cells (Nestin$^+$/GFAP$^+$ and Sox2$^+$/GFAP$^+$), in Physiological Conditions and After KA Treatment

The analyses of early precursor cells (type 1) were obtained through double immunofluorescence assays, such as GFAP and Nestin protein and GFAP and Sox2, as a markers of radial glia-like neural stem cells NSC [33, 34].

Nestin$^+$/GFAP$^+$ Cell Progenitors

The levels of GFAP$^+$/Nestin$^+$ radial glia-like cells in the SGZ of the hippocampus were similar among JNK KOs CT and WT CT animals (Fig. 1a, b). However, after 24 h of KA injection, this subpopulation, located in the SGZ, increased in WT and *Jnk2*$^{-/-}$ mice compared with their controls (Fig. 1a, b), whereas no change was detected in *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ mice (Fig. 1a, b).

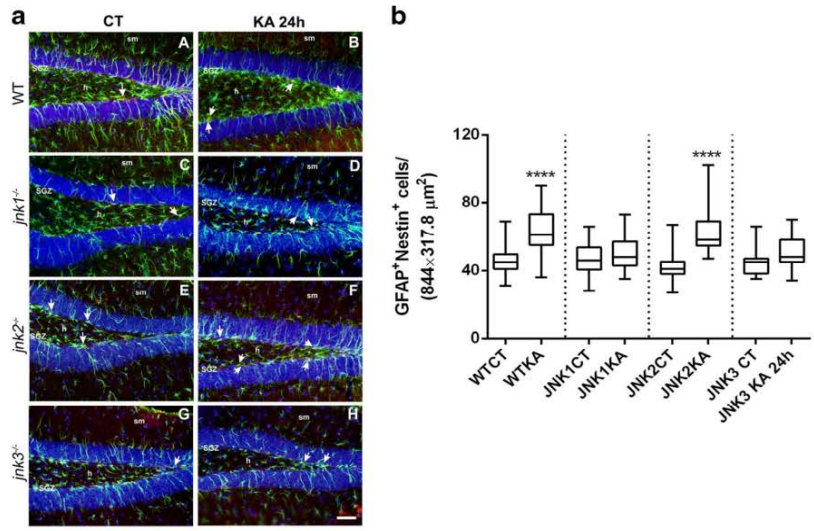


Fig. 1 **a** Representative DG hippocampal images of GFAP/Nestin immunofluorescence (green and red, respectively), from control mice (*A*, *C*, *E*, *G*) and 24 h KA-treated mice (*B*, *D*, *F*, *H*) of WT, *jnk1*$^{-/-}$, *jnk2*$^{-/-}$, and *jnk3*$^{-/-}$. Arrows show double immunofluorescence cells. **b** Quantification of the number of GFAP/Nestin-positive cells and the representative histogram. ****$P > 0.0001$ vs WT CT SGZ: subgranular zone; h: hilus; sm: stratum moleculare. Scale bar 50 µm

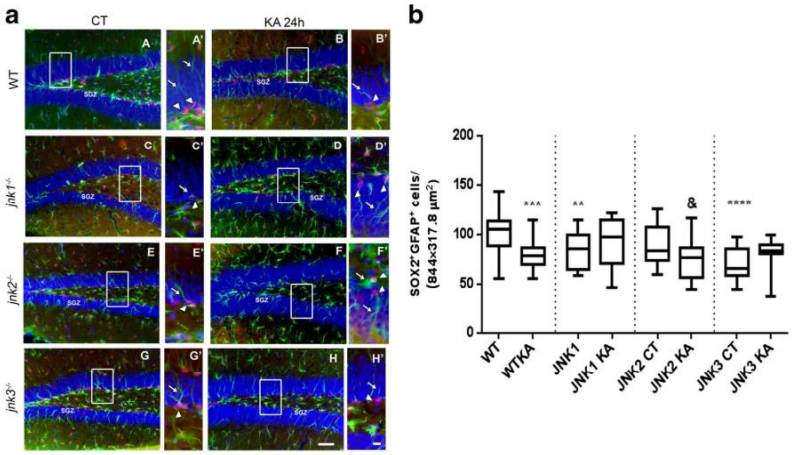


Fig. 2 **a** Representative DG hippocampal images of GFAP/Sox2 immunofluorescence (green and red, respectively), from control mice (*A*, *C*, *E*, *G*) and 24 h KA-treated mice (*B*, *D*, *F*, *H*) of WT, *jnk1*$^{-/-}$, *jnk2*$^{-/-}$, and *jnk3*$^{-/-}$. A′–H′ show high magnification of double-labeled cells. Arrows indicate the cellular processes of radial glia-like cells immunopositive for GFAP; arrowheads signal their nuclei. **b** Quantification of the number of GFAP-Sox2-positive cells and the representative histogram. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs WT CT;; $^{\&}P < 0.05$ vs genotype control. SGZ: subgranular zone. *A–H* Scale bar 50 µm, A′–H′ scale bar 10 µm

GFAP$^+$/Sox2$^+$ Cell Progenitors

In physiological conditions, the number of GFAP$^+$/Sox2$^+$ cells was lower in *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ mice compared with WT CT (Fig. 2a, b). By contrast, there were no differences between WT CT and *Jnk2*$^{-/-}$ CT mice (Fig. 2a, b). After KA injection, a decrease of GFAP$^+$/Sox2$^+$ cells was observed in WT KA compared with WT CT and in *Jnk2*$^{-/-}$ KA versus *Jnk2*$^{-/-}$ CT (Fig. 2a, b).

The high co-localization of GFAP, Sox2, and Nestin hinders the quantification of single immunolabeled Sox2$^+$ or Nestin$^+$ radial glia-like cells. These populations represent a very low pool of cells, and they were unable to quantify with certainty. Further studies should be conducted to clarify the role of these pools of cell in epilepsy.

The Absence of JNK1 Induces an Increase in the Number of Immature Neurons, in Physiological Conditions

DCX Cell Transit-Amplifying Cells

DCX is a microtubule-associated protein expressed in the transiently amplifying progenitor cells and immature neurons (types 2a and 2b) of the adult brain, specifically in areas of continuous neurogenesis [35]. The immunofluorescence against DCX revealed that the number of DCX$^+$ cells in *Jnk1*$^{-/-}$ CT mice was higher than in WT CT (Fig. 3a, b). No differences were detected in *Jnk2*$^{-/-}$ CT and *Jnk3*$^{-/-}$ CT mice compared with WT CT (Fig. 3a, b). After KA treatment, the levels of DCX$^+$ increased in WT mice compared with their CT (Fig. 3a, b). No significant changes were observed in *Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$, and *Jnk3*$^{-/-}$ mice after KA injection compared to their controls (Fig. 3a, b).

In order to identify a more differentiated neuronal cell subpopulation, a co-immunolabeling of DCX and CR was performed. Interestingly, we found high levels of double-positive DCX$^+$/CR$^+$ cells in all genotypes; however, few numbers of single immunolabel DCX$^+$ cells were found (Fig. 4). Moreover, this immunostaining allowed the identification of at least two cellular phenotypes in physiological conditions: (i) cells with positive staining for DCX in plasma membrane and dendritic projections, and double DCX and CR staining in soma (Fig. 4), and (ii) cells with soma exclusively stained for CR however with still DCX stain in dendritic projections. The first DCX$^+$-CR$^+$ cell subtype was mainly located in the SGZ. In physiological conditions, these cells were significantly increased in *Jnk1*$^{-/-}$ CT mice and decreased in *Jnk3*$^{-/-}$ CT mice compared to WT CT (Fig. 4 and Fig. S1). Regarding *Jnk2*$^{-/-}$ CT mice, no difference in the number of these cells was observed when compared to WT CT (Fig. S1). After KA treatment, only WT and *Jnk2*$^{-/-}$ mice showed changes in these cell numbers; however, while an increase was observed in WT (Fig. 4 and Fig. S1), a decrease was detected in *Jnk2*$^{-/-}$ mice (Fig.4 and Fig. S1). No effect was detected in *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ treated mice relative to their CT (Fig. S1). In addition, double immunostaining also showed alterations in the dendritic pattern in all JNK KO CT mice compared to WT CT (Fig. 4). Moreover, the soma of DCX$^+$/CR$^+$ cells in *Jnk3*$^{-/-}$ CT mice was clearly distorted when compared to WT CT (Fig.

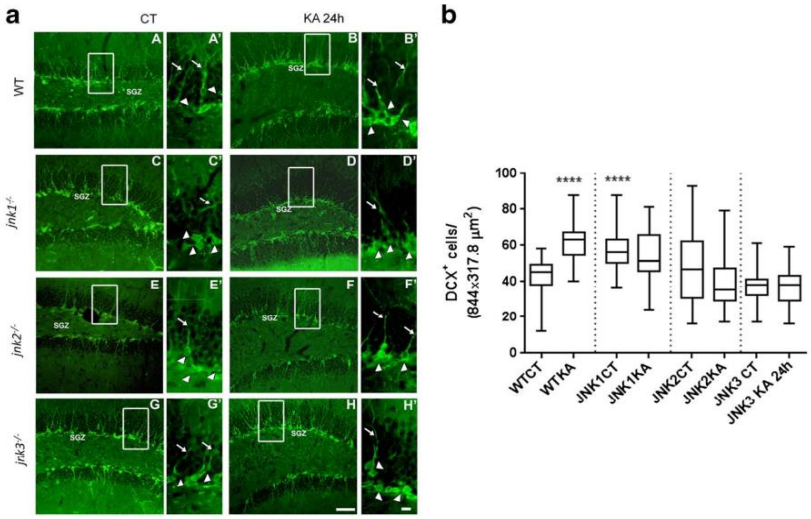


Fig. 3 **a** Representative DG hippocampal images of Doublecortin (DCX) immunofluorescence, from control mice (*A*, *C*, *E*, *G*) and after 24 h of KA injections (*B*, *D*, *F*, *H*). *A*, *B* WT; C-D: *Jnk1*$^{-/-}$; *E*, *F* *Jnk2*$^{-/-}$; *G*, *H* *Jnk3*$^{-/-}$. A detail of DCX-IR cells is shown in panels (A′–H′). The arrows indicate the cell projections and the arrowhead their soma. **b** Bar graphs represent the number of DCX-positive cell and the representative histogram. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$ vs WT CT and $^{\&}P < 0.05$ vs genotype control. SGZ: subgranular zone. *A*–*H* Scale bar 50 µm. A′–H′ Scale bar 10 µm

4). Regarding the second DCX^{+}/CR^{+} cell subtype, it was mainly located above the SGZ, specifically in the granular cell layer (GCL) (Fig. 4).

Most of the second subtype of DCX^{+}/CR^{+} cells were located above the SGZ, in the granular cell layer (GCL) (Fig. 4).

PSA-NCAM Cell Transit-Amplifying Cells

PSA-NCAM plays a role in regulating processes such as migration, survival, and outgrowth of newly generated neurons [36]. The distribution pattern of PSA-NCAM^{+} cells was the same as

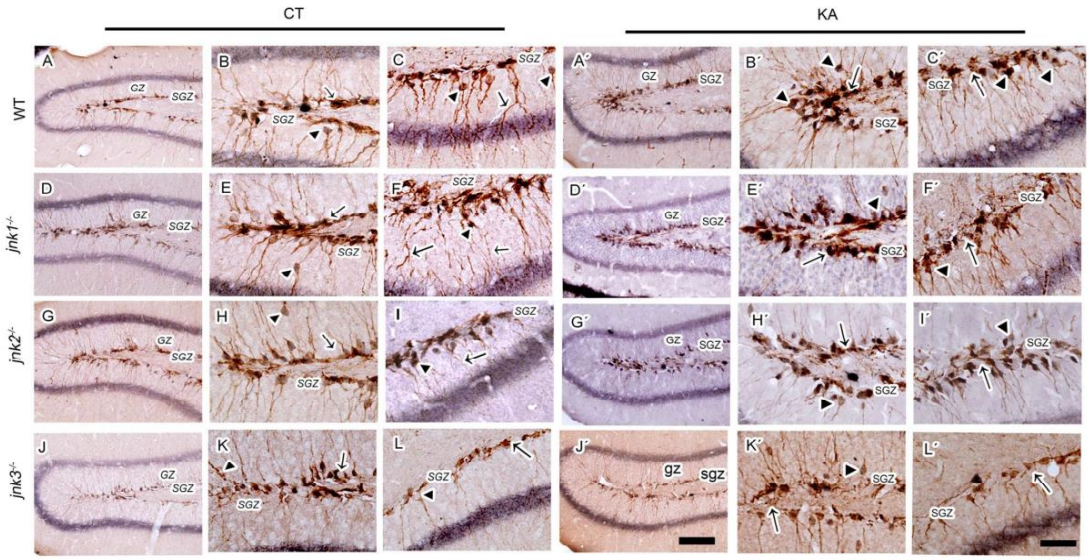


Fig. 4 Representative DG hippocampal images of double immunohistochemistry Doublecortin-calretinin (DCX^{+}/CR^{+}), from control mice (**A**–**L**) and 24 h KA-treated mice (A′–L′) of WT, *jnk1*$^{-/-}$, *jnk2*$^{-/-}$, and *jnk3*$^{-/-}$. WT. D–F′ *Jnk1*$^{-/-}$; G–I′ *Jnk2*$^{-/-}$; J–L′ *Jnk3*$^{-/-}$. Arrowheads mark the soma of cells immunopositive for CR, and arrows the projections immunopositive for DCX. SGZ: subgranular zone; gz: granular zone. Scale bar 50 µm

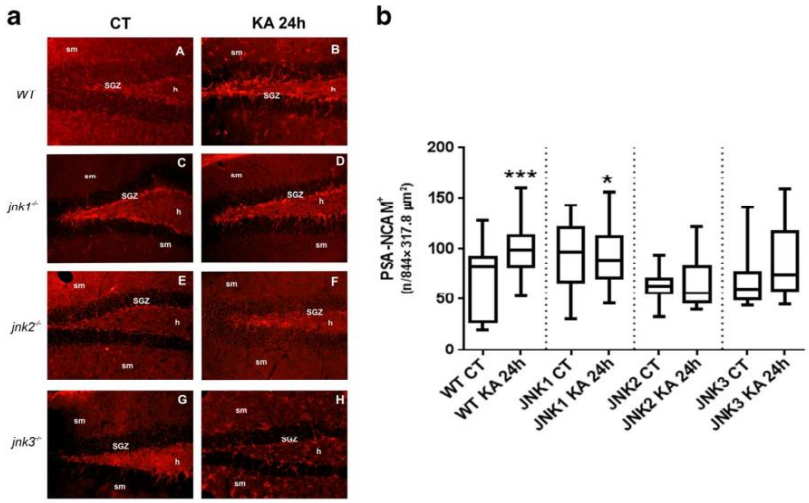


Fig. 5 **a** Representative DG hippocampal images of PSA-NCAM immunopositive cells, obtained from control mice (*A*, *C*, *E*, *G*) and 24 h KA-treated mice (*B*, *D*, *F*, *H*) of WT, *jnk1*$^{-/-}$, *jnk2*$^{-/-}$, and *jnk3*$^{-/-}$ mice. *A*, *B* WT; *C*, *D* *Jnk1*$^{-/-}$; *E*, *F* *Jnk2*$^{-/-}$; *G*, *H* *Jnk3*$^{-/-}$. **b** Graphs represent the number of PSA-NCAM-positive cells in SGZ. *$P < 0.05$, ***$P < 0.0001$ vs WT CT. SGZ: subgranular zone, h: hilus; sm: stratum moleculare. Scale bar 50 µm

the one observed for DCX$^+$ cells. Thus, the number of PSA-NCAM$^+$ cells was higher in *Jnk1*$^{-/-}$ CT mice than in WT CT (Fig. 5a, b). After KA injection, an increase in PSA-NCAM$^+$ cells was observed in WT mice (Fig. 5a, b) and no changes in cell levels were observed in any JNK knockouts mice (Fig. 5b).

In order to identify more differentiated transit-amplifying cells, we performed a double immunostaining for PSA-NCAM and NeuN. This last protein is a nuclear antigen, commonly used as a marker for mature neurons [37, 38]. The counting of PSA-NCAM$^+$/NeuN$^+$ cells evidenced that their number was low in all genotypes (Fig. S2). In addition, no significant differences were observed in JNK KOs compared to WT CT, in physiological conditions. After KA injection, an increase of PSA-NCAM$^+$/NeuN$^+$ cells were observed only in WT CT (Fig. S2).

Calbindin Immunoreactivity Was Higher in *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ Mice than in WT and *Jnk2*$^{-/-}$ Mice, in Physiological Conditions

Mature granule cells in the adult DG are known to express calbindin [39]. Our results revealed higher immunoreactivity in *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ CT mice compared with WT CT (Fig. 6). The immunostaining intensity was slightly lower in *Jnk2*$^{-/-}$ CT mice (Fig. 6). Finally, after KA injection, there

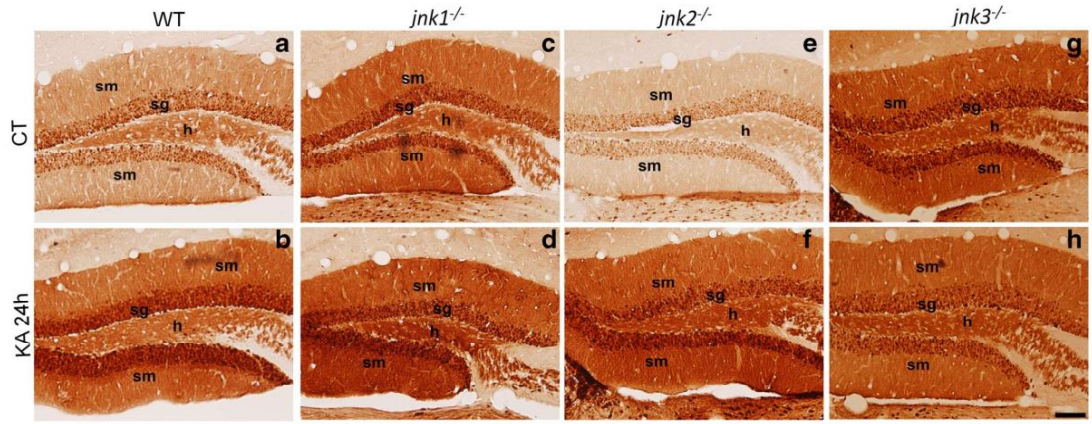


Fig. 6 Calbindin immunofluorescence distribution pattern in the DG of hippocampal coronal sections, obtained from control mice (**a**, **c**, **e**, **g**) and after 24 h of KA injections (**b**, **d**, **f**, **h**). **a**, **b** WT; **c**, **d** *Jnk1*$^{-/-}$; **e**, **f** *Jnk2*$^{-/-}$; **g**, **h** *Jnk3*$^{-/-}$. ZSG: subgranular zone; h: hilus; sm: stratum moleculare. Scale bar 100 µm

was an increase in immunoreactivity in WT mice (GCL layer) and *Jnk2*$^{-/-}$ mice (GCL and hilus) (Fig. 6) but not in *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ mice (Fig. 6).

Discussion

The objective of this study was to evaluate how the absence of JNK isoforms affects the number and the distribution pattern of different neuronal precursor cell subpopulations on the DG, in physiological conditions and in mice treated with KA.

Early Progenitor Cells In physiological conditions, the absence of JNK isoforms has no effect on the levels of GFAP$^+$/Nestin$^+$ cells, compared with WT CT; however, the levels of GFAP$^+$/Sox2$^+$ cells decrease with the lack of JNK1 and JNK3 with respect to WT CT. After KA injection, *Jnk1*$^{-/-}$and *Jnk3*$^{-/-}$ mice have no changes in the number of these cell subtypes with respect to their controls, in contrast with what occurs in WT and *Jnk2*$^{-/-}$ mice, which shows an increase in the number of GFAP$^+$/Nestin$^+$ cells and a decrease in GFAP$^+$/Sox2$^+$ cells.

Transit Amplifying Cells In physiological conditions, the levels of DCX$^+$ and PSA-NCAM$^+$ cells increase with the absence of JNK1, compared with WT CT, while no changes are observed with the lack of JNK2 and JNK3 isoforms. The effect of KA to these cell types is observed only in WT.

The Lack of JNK Isoforms Has a Different Control in Distinct Early Progenitor Cells

Two subtypes of early progenitor cells were analyzed (GFAP/Nestin and GFAP/Sox2), and high levels of co-localization among biomarkers were detected. Thus, we only analyzed the distribution pattern of the double-labeled cells (GFAP$^+$/Nestin$^+$ and GFAP$^+$/Sox2$^+$). In physiological conditions, the results evidenced that JNKs have a different control among these subpopulations. While no JNK isoforms control the levels of GFAP$^+$/Nestin$^+$ cells, the JNK1 and JNK3 isoforms are involved in the regulation of GFAP$^+$/Sox2$^+$ cell levels.

Following KA injection, the examination of these cell subtypes stated that only the lack of JNK2 induces the same changes that are observed in WT. The interesting point is that both subpopulations responded differentially to KA. While an increase was observed in GFAP$^+$/Nestin$^+$ cells, a decrease was visualized in GFAP$^+$/Sox2$^+$ cells.

These results reveal that both subtypes of early progenitor cells are differentially regulated, suggesting that or pointing at that there are cell progenitors of different cell lineages.

The Lack of JNK1 Isoform and JNK3 Prevent Alterations of Neurogenic Cell Subpopulations after KA

In physiological conditions, the transit-amplifying cells (DCX$^+$, DCX$^+$-CR$^+$, and PSA-NCAM$^+$) increase in mice that lack the JNK1 isoform. Thus, the rate of proliferation in *Jnk1*$^{-/-}$ CT mice is higher than in WT CT. These data are in accordance with those reported by Mohammad et al. [16], who showed that mice lacking JNK1, or mice treated with a JNK inhibitor (DJNKI-1), display increased neurogenesis in the hippocampus. Considering that the levels of early progenitor cells (GFAP$^+$/Nestin$^+$ and GFAP$^+$/Sox2$^+$) in adult *Jnk1*$^{-/-}$ mice, in physiological conditions, are lower or equal to WT CT, the higher levels of immature neurons (neuroblasts) in adult *Jnk1*$^{-/-}$ mice, compared to WT CT, could be due to an accumulation of these cells in the SGZ. This can be due to an alteration in their migration into the granular cell layer. It is known that the JNK pathway is linked to the reelin pathway that regulates cortical layering [40]. Moreover, it has been recently described that there are new neuroblasts formed during embryonic development, retained in quiescent state during postnatal period until to be reactivated [41].

Despite this increase in immature neurons in *Jnk1*$^{-/-}$ mice, they have neuroprotection against KA [19]. These data are in accordance with Iyengar et al. [42], who selectively removed newborn neurons and found increased susceptibility to the convulsing effects of KA. By contrast, Cho et al. [43] demonstrated that the ablation of neurogenesis does not affect seizures severity in an animal model treated with pilocarpine. Taking all these data into consideration, we can infer that the neuroprotection in *Jnk1*$^{-/-}$ mice is due to unchanged levels of the different neurogenic cell subtypes after KA, compared to their CT. This effect seems important, because it also occurs in *Jnk3*$^{-/-}$ mice that likewise prevents neurodegeneration or shows neuroprotection against KA [19] and, in physiological conditions, *Jnk3*$^{-/-}$ mice show a reduction in neurogenesis activity, with a significant value in DCX$^+$/CR$^+$ cell subtypes, in comparison with WT CT. Taking into consideration the results of Dominguez et al. [39] who suggest that the rise observed in WT mice, after KA, is due to the existence of a reservoir of pre-existing, not completely differentiated, granule cells, instead of being a direct product of neurogenesis, it is plausible that in *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ mice, the effect of KA is not able to achieve the activation of this reservoir pool, preserving the brain damage induced with this neurotoxin.

The DCX/CR double immunostaining allowed the identification of high co-localization between DCX and CR. This can be explained by the existence of different subtypes of DCX/CR-immunolabeled cells. Thus, the neuronal maturation must be gradual and associated to differences in the amount of proteins. In consequence, the cells with exclusive CR staining in soma but still DCX staining

in dendrites could be the ones that migrate radially into the GCL. The dendritic outgrowth and neuronal motility of these cells justify the presence of DCX in their dendrites [44]. In addition, it is known that CR is transiently expressed and, when it disappears, the expression of CB, which marks the switch to adult-like connectivity in granular cells, starts [45] being difficult to detect cells exclusively immunoreactive to CR.

The data obtained with the double immunostain (DCX/CR), together with the low number of PSA-NCAM$^+$/NeuN$^+$ subtype cells detected, suggest that the transition from transit-amplifying cells to immature neurons takes a long time, and in contrast, the passage to differentiated neuron is faster. All these data could explain why finding a single CR$^+$ or PSA-NCAM$^+$/NeuN$^+$ cell is difficult.

Another data reported with the double immunostaining (DCX/CR) is that the absence of JNK isoforms induces alterations in the dendritic pattern of immature neurons. Hence, all the JNKs take part in the dendritic maturation, without compensatory effects between isoforms, as it is described in brain morphogenesis and axodendritic architecture during development [46]. Therefore, JNKs are involved in the morphology control of cells located in the SGZ and in their neurogenic rate.

CB is a CBP present in mature granule cells that acts as a modulator of calcium homeostasis, calcium channel activity, and neuronal plasticity [30, 47]. Therefore, the high levels of CB-IR in *Jnk1*$^{-/-}$and *Jnk3*$^{-/-}$ mice suggest that they have more capacity to control homeostasis than WT and *Jnk2*$^{-/-}$ CT mice.

In conclusion, the present study shows that *Jnk1*$^{-/-}$ and *Jnk3*$^{-/-}$ CT mice have an enhanced hippocampal homeostasis when compared to WT CT and *Jnk2*$^{-/-}$ CT mice. Moreover, the lack of JNK1 or JNK3 induces different neurogenesis activity in the SGZ, under physiological conditions, but in both genotypes, the increase in early progenitor cells and immature neurons after KA is prevented, and consequently interrupted one of the pathological features of TLE, whereas the absence of JNK2 preserves the increase in immature neurons which does not preserve the alterations in early progenitor cells.

All these findings justify the development of selective inhibitors for JNK1 and JNK3 in order to develop drugs with neuroprotective and cognitive effects.

Acknowledgements This work was supported by Ministerio Español de Ciencia e Innovación, SAF2017-84283-R; Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED) CB06/05/0024; Consejo Nacional de Ciéncia y Tecnologia, CONACYT, 177594; Generalitat de Catalunya, 2014SGR-525; Generalitat de Catalunya, 2017 SGR 625; Postdoctoral Fellowship CONACYT-MEXICO, 298337; Doctoral Program in Sciences in Molecular Biology in Medicine LGAC; and Molecular Bases of Chronic-Degenerative Diseases and its Applications 000091, PNPC, CONACYT-MEXICO.

Thanks to Kyra -Mae Leighton for her technical support.

References

1. Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. Cell 103:239–252
2. Gupta S, Barrett T, Whitmarsh a J et al (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 15:2760–2770
3. Ip YT, Davis RJ (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)–from inflammation to development. Curr Opin Cell Biol 10:205–219
4. Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270:16483–16486. https://doi.org/10.1098/rstb.1996.0008
5. Bogoyevitch MA, Kobe B (2006) Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiol Mol Biol Rev 70:1061–1095. https://doi.org/10.1128/MMBR.00025-06
6. Gkouveris I, Nikitakis NG (2017) Role of JNK signaling in oral cancer: a mini review. Tumor Biol 39:101042831771165
7. Dong C, Yang DD, Wysk M, Whitmarsh AJ, Davis RJ, Flavell RA (1998) Defective T cell differentiation in the absence of Jnk1. Science 282:2092–2095. https://doi.org/10.1126/science.282.5396.2092
8. Yang DD, Conze D, Whitmarsh AJ et al (1998) Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. Immunity 9:575–585
9. Yang DD, Kuan CY, Whitmarsh a J et al (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 389:865–870. https://doi.org/10.1038/39899
10. Zeke A, Misheva M, Reményi A, Bogoyevitch MA (2016) JNK signaling: regulation and functions based on complex protein-protein partnerships. Microbiol Mol Biol Rev 80:793–835. https://doi.org/10.1128/MMBR.00043-14
11. Kuan CY, Yang DD, Samanta Roy DR et al (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron 22:667–676
12. Sabapathy K (2012) Role of the JNK pathway in human diseases. Prog Mol Biol Transl Sci 106:145–169
13. Conze D, Krahl T, Kennedy N, Weiss L, Lumsden J, Hess P, Flavell RA, le Gros G et al (2002) c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 have distinct roles in CD8(+) T cell activation. J Exp Med 195:811–823. https://doi.org/10.1084/JEM.20011508
14. Myers AK, Meechan DW, Adney DR, Tucker ES (2014) Cortical interneurons require Jnk1 to enter and navigate the developing cerebral cortex. J Neurosci 34:7787–7801. https://doi.org/10.1523/JNEUROSCI.4695-13.2014
15. Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS (2002) A central role for JNK in obesity and insulin resistance. Nature 420:333–336. https://doi.org/10.1038/nature01137
16. Mohammad H, Marchisella F, Ortega-Martinez S, Hollos P, Eerola K, Komulainen E, Kulesskaya N, Freemantle E et al (2018) JNK1 controls adult hippocampal neurogenesis and imposes cell-autonomous control of anxiety behaviour from the neurogenic niche. Mol Psychiatry 23:487. https://doi.org/10.1038/mp.2017.21
17. Kuan C-Y, Whitmarsh AJ, Yang DD, Liao G, Schloemer AJ, Dong C, Bao J, Banasiak KJ et al (2003) A critical role of neural-specific JNK3 for ischemic apoptosis. Proc Natl Acad Sci U S A 100:15184–15189. https://doi.org/10.1073/pnas.2336254100
18. Li QM, Tep C, Yune TY, Zhou XZ, Uchida T, Lu KP, Yoon SO (2007) Opposite regulation of oligodendrocyte apoptosis by JNK3

and Pin1 after spinal cord injury. J Neurosci 27:8395–8404. https://doi.org/10.1523/JNEUROSCI.2478-07.2007

19. de Lemos L, Junyent F, Camins A, Castro-Torres RD, Folch J, Olloquequi J, Beas-Zarate C, Verdaguer E et al (2017) Neuroprotective effects of the absence of JNK1 or JNK3 isoforms on kainic acid-induced temporal lobe epilepsy-like symptoms. Mol Neurobiol. https://doi.org/10.1007/s12035-017-0669-1
20. De Lemos L, Junyent F, Verdaguer E et al (2010) Differences in activation of ERK1/2 and p38 kinase in Jnk3 null mice following KA treatment. J Neurochem 114:1315–1322. https://doi.org/10.1111/j.1471-4159.2010.06853.x
21. Raijmakers M, Clynen E, Smisdom N, Nelissen S, Brône B, Rigo JM, Hoogland G, Swijsen A (2016) Experimental febrile seizures increase dendritic complexity of newborn dentate granule cells. Epilepsia 57:717–726. https://doi.org/10.1111/epi.13357
22. Ben-Ari Y, Cossart R (2000) Kainate, a double agent that generates seizures: two decades of progress. Trends Neurosci 23:580–587. https://doi.org/10.1016/S0166-2236(00)01659-3
23. Parent JM (2003) Injury-induced neurogenesis in the adult mammalian brain. Neurosci 9:261–272. https://doi.org/10.1177/1073858403252680
24. Shetty AK, Hattiangady B, Rao MS, Shuai B (2012) Neurogenesis response of middle-aged hippocampus to acute seizure activity. PLoS One 7:e43286. https://doi.org/10.1371/journal.pone.0043286
25. Sakurai M, Suzuki H, Tomita N, Sunden Y, Shimada A, Miyata H, Morita T (2017) Enhanced neurogenesis and possible synaptic reorganization in the piriform cortex of adult rat following kainic acid-induced status epilepticus. Neuropathology 38:135–143. https://doi.org/10.1111/neup.12445
26. Walton RM (2012) Postnatal neurogenesis. Vet Pathol 49:155–165. https://doi.org/10.1177/0300985811414035
27. Kempermann G, Song H, Gage FH (2015) Neurogenesis in the adult hippocampus. Cold Spring Harb Perspect Biol 7:a018812. https://doi.org/10.1101/cshperspect.a018812
28. Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645–660. https://doi.org/10.1016/j.cell.2008.01.033
29. Braun SMG, Jessberger S (2014) Review: adult neurogenesis and its role in neuropsychiatric disease, brain repair and normal brain function. Neuropathol Appl Neurobiol 40:3–12. https://doi.org/10.1111/nan.12107
30. Brandt MD, Jessberger S, Steiner B, Kronenberg G, Reuter K, Bick-Sander A, Behrens W, Kempermann G (2003) Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. Mol Cell Neurosci 24:603–613. https://doi.org/10.1016/S1044-7431(03)00207-0
31. Junyent F, Utrera J, Romero R, Pallàs M, Camins A, Duque D, Auladell C (2009) Prevention of epilepsy by taurine treatments in mice experimental model. J Neurosci Res 87:1500–1508. https://doi.org/10.1002/jnr.21950
32. Paxinos G, Franlin K (2012) The mouse brain in stereotaxic coordinates, 4th edn. Academic Press
33. Zhang J, Jiao J (2015) Molecular biomarkers for embryonic and adult neural stem cell and neurogenesis. Biomed Res Int 2015:727542–727514. https://doi.org/10.1155/2015/727542
34. Brazel CY, Limke TL, Osborne JK, Miura T, Cai J, Pevny L, Rao MS (2005) Sox2 expression defines a heterogeneous population of neurosphere-forming cells in the adult murine brain. Aging Cell 4:197–207. https://doi.org/10.1111/j.1474-9726.2005.00158.x
35. Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N, Bogdahn U, Winkler J et al (2005) Doublecortin expression levels in adult brain reflect neurogenesis. Eur J Neurosci 21:1–14. https://doi.org/10.1111/j.1460-9568.2004.03813.x
36. Gascon E, Vutskits L, Kiss JZ (2010) The role of PSA-NCAM in adult neurogenesis. Springer, New York, pp. 127–136
37. Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. Development 116:201–211
38. Seki T, Arai Y (1993) Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. Neurosci Res 17:265–290
39. Domínguez MI, Blasco-Ibáñez JM, Crespo C et al (2003) Calretinin/PSA-NCAM immunoreactive granule cells after hippocampal damage produced by kainic acid and DEDTC treatment in mouse. Brain Res 966:206–217. https://doi.org/10.1016/S0006-8993(02)04164-1
40. Reiner O, Gdalyahu A, Ghosh I et al (2004) DCX's phosphorylation by not just another kinase (JNK). Cell Cycle 3:747–751
41. Pino A, Fumagalli G, Bifari F, Decimo I (2017) New neurons in adult brain: distribution, molecular mechanisms and therapies. Biochem Pharmacol 141:4–22. https://doi.org/10.1016/j.bcp.2017.07.003
42. Iyengar SS, LaFrancois JJ, Friedman D et al (2015) Suppression of adult neurogenesis increases the acute effects of kainic acid. Exp Neurol 264:135–149. https://doi.org/10.1016/j.expneurol.2014.11.009
43. Cho K-O, Lybrand ZR, Ito N, Brulet R, Tafacory F, Zhang L, Good L, Ure K et al (2015) Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline. Nat Commun 6:6606. https://doi.org/10.1038/ncomms7606
44. Gdalyahu A, Ghosh I, Levy T, Sapir T, Sapoznik S, Fishler Y, Azoulai D, Reiner O (2004) DCX, a new mediator of the JNK pathway. EMBO J 23:823–832. https://doi.org/10.1038/sj.emboj.7600079
45. Todkar K, Scotti AL, Schwaller B (2012) Absence of the calcium-binding protein calretinin, not of calbindin D-28k, causes a permanent impairment of murine adult hippocampal neurogenesis. Front Mol Neurosci 5(56). https://doi.org/10.3389/fnmol.2012.00056
46. Coffey ET (2014) Nuclear and cytosolic JNK signalling in neurons. Nat Rev Neurosci 15:285–299. https://doi.org/10.1038/nrn3729
47. Lledo PM, Somasundaram B, Morton AJ, Emson PC, Mason WT (1992) Stable transfection of calbindin-D28k into the GH3 cell line alters calcium currents and intracellular calcium homeostasis. Neuron 9:943–954

Annex V

A metabolic perspective of late onset Alzheimer's disease.

Ettcheto M, Cano A, Busquets O, Manzine PR, Sánchez-López E, Castro-Torres RD, Beas-Zarate C, Verdaguer E, García ML, Olloquequi J, Auladell C, Folch J and Camins A.

Pharmacol Res. 2019;145.

Pharmacological Research 145 (2019) 104255

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Review

A metabolic perspective of late onset Alzheimer's disease

Miren Ettcheto[a,b,c,d,1], Amanda Cano[d,e,f,1], Oriol Busquets[a,b,c,d], Patricia Regina Manzine[a,c,d,g], Elena Sánchez-López[d,e,f], Rubén D. Castro-Torres[a,c,d,h,i], Carlos Beas-Zarate[i], Ester Verdaguer[c,d,h], María Luisa García[d,e,f], Jordi Olloquequi[j], Carme Auladell[c,d,h], Jaume Folch[b,d], Antoni Camins[a,c,d,*]

[a] Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain
[b] Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain
[c] Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain
[d] Biomedical Research Networking Centre in Neurodegenerative Diseases (CIBERNED), Madrid, Spain
[e] Unitat de Farmàcia, Tecnologia Farmacèutica i Fisico-química, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain
[f] Institute of Nanoscience and Nanotechnology (IN2UB), Universitat de Barcelona, Spain
[g] Department of Gerontology, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil
[h] Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain
[i] Laboratorio de Regeneración y Desarrollo Neural, Instituto de Neurobiología, Departamento de Biología Celular y Molecular, CUCBA, Mexico
[j] Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile

ARTICLE INFO

Keywords:
Alzheimer's disease
Type 2 diabetes mellitus
Insulin
c-Jun N-terminal kinase inhibitors
Licochalcone A
Neuroinflammation
Reticulum stress

ABSTRACT

After decades of research, the molecular neuropathology of Alzheimer's disease (AD) is still one of the hot topics in biomedical sciences. Some studies suggest that soluble amyloid β (Aβ) oligomers act as causative agents in the development of AD and could be initiators of its complex neurodegenerative cascade. On the other hand, there is also evidence pointing to Aβ oligomers as mere aggravators, with an arguable role in the origin of the disease.

In this line of research, the relative contribution of soluble Aβ oligomers to neuronal damage associated with metabolic disorders such as Type 2 Diabetes Mellitus (T2DM) and obesity is being actively investigated. Some authors have proposed the endoplasmic reticulum (ER) stress and the induction of the unfolded protein response (UPR) as important mechanisms leading to an increase in Aβ production and the activation of neuroinflammatory processes. Following this line of thought, these mechanisms could also cause cognitive impairment.

The present review summarizes the current understanding on the neuropathological role of Aβ associated with metabolic alterations induced by an obesogenic high fat diet (HFD) intake. It is believed that the combination of these two elements has a synergic effect, leading to the impairement of ER and mitochondrial functions, glial reactivity status alteration and inhibition of insulin receptor (IR) signalling. All these metabolic alterations would favour neuronal malfunction and, eventually, neuronal death by apoptosis, hence causing cognitive impairment and laying the foundations for late-onset AD (LOAD).

Moreover, since drugs enhancing the activation of cerebral insulin pathway can constitute a suitable strategy

Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; AChEI, anticholinesterase inhibitors; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AβPP, amyloid precursor protein; AβPP/PS1, AβPPswe/PS1dE9; APOE, Apolipoprotein E; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; BACE1, beta-secretase enzyme 1; BDNF, rain-derived neurotrophic factor; BIP, binding immunoglobulin Protein; BMI, body mass index; CHOP, C/EBP homologous protein; CNS, central nervous system; eIF2α, eukaryotic Initiation Factor 2; ER, endoplasmic reticulum; GADD34, growth arrest and DNA damage-inducible protein 34 kDa; GCN2, general control non-depressible 2; GRP78, glucose regulated protein 78; HFD, high fat diet; HRI, heme-regulated inhibitor kinase; HO-1, Heme oxygenase-1; IDE, insulin degrading enzyme; IL-6, interleukin 6; IR, insulin receptor; IRE1α, inositol requiring enzyme 1; IRS1, insulin receptor substrate 1; ISR, integrated stress response; LOAD, Late-onset Alzheimer's disease; MAPs, microtubule-associated proteins; MMP-9, matrix metallopeptidase 9; MnSOD, manganese-dependent superoxide dismutase; MPTP, 1-methyl-4-phenyl-12,3,6-tetrahydropyridine; NFT, neurofibrillary tangles; NMDAR, N-methyl-D-aspartate receptors; NORT, novel object recognition test; NQO1, quinone oxidoreductase 1; p-CREB, phosphorylated cAMP response element-binding; PGC-1α, PPARγ-coactivator-1α; PERK, PKR-like endoplasmic reticulum kinase; PHF, paired helical filaments; PKR, RNA-activated protein kinase; PS1, presenilin; PS2, presenilin 2; PTP1B, protein-tyrosine phosphatase 1B; RAGE, The receptor for advanced glycation end products; T2DM, Type 2 diabetes mellitus; T3D, type 3 diabetes; TLE, temporal lobe epilepsy; TRAF2, tumour necrosis factor receptor associated factor-2; TNFα, tumour necrosis factor α; UPR, unfolded protein response

* Corresponding author at: Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat deBarcelona, Barcelona, Spain. Av. Joan XXIII 27/31, E-08028 Barcelona, Spain.
E-mail address: camins@ub.edu (A. Camins).
[1] Both authors have contributed equally.

https://doi.org/10.1016/j.phrs.2019.104255
Received 16 November 2018; Received in revised form 11 March 2019; Accepted 30 April 2019
Available online 07 May 2019

for the prevention of AD, we also discuss the scope of therapeutic approaches such as intranasal administration of insulin in clinical trials with AD patients.

1. Introduction

The study of Alzheimer's disease (AD) began in the early twentieth century, when the German physician Alois Alzheimer described the first case of this pathology in a 51-year old woman called Auguste Deter [1]. Her symptoms included remarkable memory loss, language difficulties and personality changes. After the post-mortem examination, the brain autopsy revealed specific neuropathological changes in the cerebral cortex [1–4], now known as the classical morphological symptoms of AD: amyloid β (Aβ) peptide plaques derived of the activity of the amyloidogenic pathway and neurofibrillary tangles (NFTs), composed by hyperphosphorylated tau protein [3–8].

At present, the aetiological hypothesis most supported by the scientific community is the "amyloid cascade hypothesis", which is summarized in Fig. 1. According to this hypothesis, under physiological conditions amyloid β protein percussor (AβPP) is cleaved by the enzyme α-secretase, following the non-amyloidogenic pathway. This precludes formation of amyloidogenic peptides and leads to a release of secreted AβPP alpha (sAβPPα), which has neuroprotective properties [5]. On the contrary, in the amyloidogenic pathway, AβPP is cleaved by β-secretase (BACE-1) at N-terminus and, in turn, γ/ε-secretase cleaves it at the C-terminus to yield secreted AβPP (sAβPP), Aβ40/42 fragments (which remain in the extracellular space) and a C-terminal fragment with 99 amino acids (C99) that can be translocated to the nucleus. Here, it may induce expression of genes that promote neuronal death by apoptosis. Moreover, the soluble Aβ oligomers generated in this way affect synapse function, decrease neuronal plasticity, alter energy and glucose metabolism, induce oxidative stress and mitochondrial dysfunction, and disturb celular calcium homeostasis [2,5].

During the early 1990s, some genetic factors -apart from AβPP- were shown to increase the risk of developing the disease, such as apolipoprotein E (APOE) or presenilin 1 and 2 (PS1 and PS2) [7–11]. These factors were also involved in the amyloid cascade. For instance, the allele for APOE ε4 was been shown to impair Aβ clearance and promote its aggregation, leading to increased severity of this amyloid pathology [7–12]. In spite of this, the existence of other factors that can contribute to the pathogenesis of AD emphasizes its complexity. Some of these risk factors are shown in Fig. 2.

Against this background, AD patients were classified into two groups. The first one was formed for those subjects that developed the pathology due to genetic causes leading to the production of classical biomarkers like Aβ. This group covered about 3% of AD patients and was dubbed "familial or early-onset AD". The remaining 97% of patients were categorized as "sporadic or late-onset AD" (LOAD), whose progression was associated with advanced age, hypertension, hyperlipidaemia, coronary disease, obesity and type 2 diabetes mellitus (T2DM) [12–14].

T2DM is a complex disease with a chronic evolution that requires continuous medical care, mainly focused on the reduction of global cardiovascular risk, peripheral complications and cognitive loss [15]. Unlike type 1 diabetes mellitus (T1DM), an autoimmune disorder characterized by the selective destruction of insulin-producing β-cells [16,17], in T2DM there is an alteration in the mechanisms of uptake and/or secretion of insulin. This leads to a chronic increase in blood levels of glucose, resulting in a higher risk of macro and microvascular complications. T2DM is also associated with insulin resistance (IR), which is characterized by lower insulin activity at the cellular level, and affects the metabolism of carbohydrates, lipids and proteins [15]. As we have mentioned, in addition to a risk factor for cardiovascular pathologies, T2DM is also an independent risk factor for LOAD [11–14]. Specifically, it is widely recognized that T2DM and AD share several kinds of abnormalities, including increased oxidative stress, impaired glucose metabolism and insulin resistance characterized by continuous hyperinsulinemia [12,12,13,14,18]. Likewise, some studies have

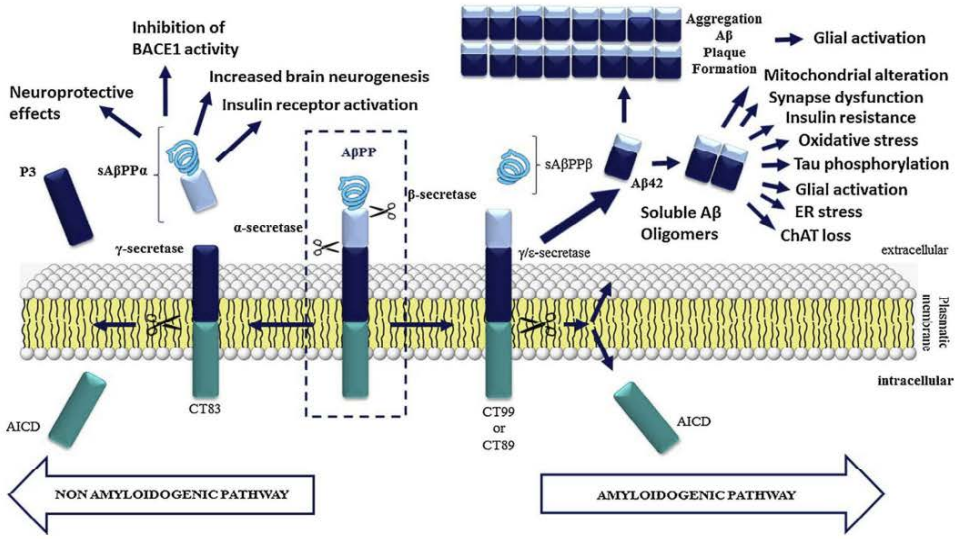


Fig. 1. Schematic representation of the APP's protein processing in the cytoplasmic membrane. Both the amyloidogenic (right) and non-amyloidogenic (left) pathways are depicted. The first one leads to the generation of Aβ oligomers that cause glial activation, mitochondrial alterations, synapse dysfunction as well as ER stress and insulin resistance. The second one favours the generation of the sAβPPβ peptide, which has been described to have neuroprotective effects: it promotes neurogenesis, favours insulin receptor activation and inhibits BACE1.

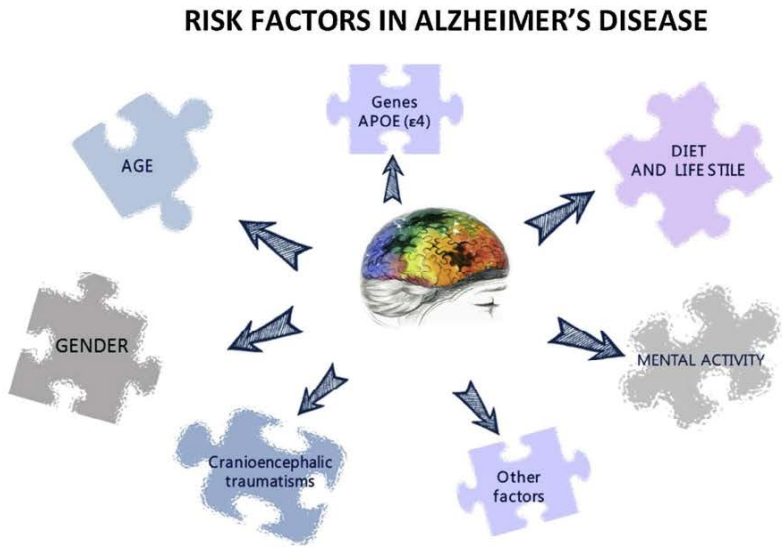


Fig. 2. Some risk factors for the development of sporadic AD. Age, APOE ε4 mutation, diet and lifestyle, gender, mental activity, cranioencephalic traumatisms and others factors such as lack of social engagement have been associated to the onset and/or progression of AD.

focused on the role of insulin receptor (IR), which might be an important player in the pathology of AD, by contributing to the biochemical, molecular and histopathological characteristics of the pathology [13,19–27].

Given that the origin of the pathology is still unknown, and that there seem to be many players involved in its development, AD has been defined as a multifactorial disease (Fig. 2). Consequently, there are different research areas, in addition to neuroscience, trying to elucidate the origin of this pathology. In fact, prospective epidemiological studies have identified metabolic syndrome and T2MD as risk factors for multiple diseases of the nervous system [18–20]. Furthermore, animal studies have shown that hypercaloric diets affect the structure and functions of the hippocampus, although the specific mechanisms are unclear [28,29]. Thus, it has been reported that AβPPswe/PS1dE9 (AβPP/PS1) transgenic mice fed with a diet enriched in palmitic acid, showed reduced IR and increased insoluble Aβ peptide levels, as well as cognitive deficits [30]. Moreover, Ho and colleagues found evidence linking insulin resistance and increased relative risk for AD neuropathology development, by demonstrating that IR signalling can influence Aβ production in the brain [31]. These results evidenced the relationship between metabolic alterations and progression of AD features, thus reinforcing the hypothesis of a metabolic aetiology of AD. Indeed, it has been proposed to re-name AD as "type 3 diabetes mellitus" (T3DM) or brain-specific diabetes [32].

The present review is a state-of-the-art about the relation among obesity, Aβ oligomers and the IR modulation. In addition, we discuss the potential application of drugs modulating the brain insulin receptor pathway as targets for AD prevention.

2. An historical overview of AD's hypotheses and available pharmacological treatments

Altghough over a century has passed since AD was first described, the pathogenesis of this complex disease is still unclear. A number of theories about AD origin have been postulated so far and several drugs have been tested in accordance. The first one, proposed in the 80 s, was the "cholinergic hypothesis", which suggested that a dysfunction of acetylcholine-containing neurons in the brain contributes substantially to the cognitive decline observed in AD patients [33–35]. This paradigm led to the development of the anticholinesterase inhibitors (AChEI), which are currently in the market and provide a symptomatic treatment of the disease [34–38]. However, these drugs only achieve a temporary improvement and they do not slow down or cure AD.

In 1992, a series of discoveries opened the door to the birth of the "amyloid cascade hypothesis", which we have already discussed. This hypothesis stressed the role of Aβ peptide deposition in AD pathogenesis, leading to neurofibrillary tangles, cell loss, vascular damage, and dementia [39–42]. Ostensibly, the appearance of plaques and the onset of AD would be related to the most common isoforms of Aβ, (1–40) and (1–42) [40–43]. However, some current preclinical discoveries pointed to Aβ as a cofactor or aggravator involved in a complex network of pathological changes in the brain, instead of considering Aβ as the main neurotoxin causing AD *per se* [44–48]. In any case, most drugs designed to inhibit amyloid synthesis have failed to modify the evolution of the disease. Notwithstanding, Verubescetat, a drug targeting Aβ or inhibiting the β-site amyloid precursor protein cleaving enzyme 1 (BACE1), was reported to be more effective than placebo in treating AD [47,48].

Besides Aβ plaques, NFTs constitute the other main intracellular hallmark of AD. It was demonstrated that NFTs were composed of tau [48,49], a microtubule-associated protein (MAP) which acts as a major regulator of microtubule formation [50–53]. Interestingly, it was observed that the hyperphosphorylation of tau led to unconstructed microtubules and the appearance of helically crossed filaments that aggregate to form the NFTs [50–53]. In some cases, the presence of NFTs was correlated with the degree of dementia in patients with AD [53]. All these observations paved the way for the "tau hypothesis", which postulates that the origin of AD is associated with an early conformational change in the structure of tau [54]. Indeed, it has been demonstrated that the reduction of hyperphosphorylated tau alleviates the cognitive alterations induced by Aβ in a transgenic mice model of AD [55]. Although in terms of research the tau hypothesis has been a "supporting actor" when compared to the amyloid hypothesis, some anti-tau therapies have shown promising results [48,51]. For instance, TRx0237 (LMTX) is being currently tested in a phase III clinical trial as an inhibitor of tau aggregation, capable of reducing tau-mediated

neuronal damage (clinical trial NT02245568). By this mechanism, this drug could modify the course of AD [48]. On another front, it has been reported that soluble oligomers of Aβ are able to accelerate the hyperphosphorylation of tau [4,50]. Nevertheless, although the two markers are closely related neuropathologically, a mechanism linking both theories is still missing.

Evidence showing inflammatory microglia consistently associated with senile plaques in AD led to the "inflammation hypothesis" [56]. Indeed, it is known that local reactivity responses, such as microgliosis and astrogliosis, could be involved in the development of AD by fostering a severe neuroinflammation through the release of cytokines [42,56–62]. However, while it is accepted that neuroinflammation contributes actively to the development of AD, the fundamental question remains: is it the cause or the consequence of the underlying pathology? [60–64]. In this sense, the drug TTP488 (azeliragon), a receptor for advanced glycation end products (RAGE) antagonist with anti-amyloid and anti-inflammatory properties is in a phase III trail. It has been shown that this compound reduces amyloid uptake in brain and lowers the inflammatory reaction in glial cells (NCT02080364) [48].

Finally, another major hypothesis about AD is the "excitotoxic hypothesis", which describes how over-activation of glutamatergic transmission, especially of N-Methyl-D-aspartate receptors (NMDARs), leads to a massive influx of calcium (Ca^{2+}) that damages neurons [65]. In mammals, NMDARs are distributed throughout the brain and mainly in the hippocampus. The hyperactivation of these receptors leads to the activation of a wide variety of intracellular pathways, inducing homeostatic alterations and diminishing neuronal viability, eventually leading to neuronal death [66–68]. However, the mechanism by which this process is carried out remains unclear since, even though this hypothesis advocates for an over-activation of NMDARs, it has been observed that patients who developed AD actually had a reduction of the number of these receptors in the cellular membrane [68]. Apparently, the lack of receptors leads to an increase in their sensitivity, causing a continuous activation leading to a progressive neurodegeneration due to the excitotoxic damage [68–70]. Interestingly, this also seems to be related to Aβ, since it has been demonstrated that Aβ causes an increase of the endocytosis of the NMDARs in cortical neurons, hence decreasing their expression in the membrane, which leads to an inhibition of synaptic plasticity [70]. Regarding the excitotoxic hypothesis, memantine, a NMDAR antagonist, was approved for the treatment of moderately severe to severe AD in 2002 by the European Agency for the Evaluation of Medical Products (EMEA). Unfortunately, as with any other approved treatment for AD so far, mematine only have shown symptomatic effects.

3. Mechanisms linking obesity and cognitive decline: results from preclinical models

Nearly 20 years ago, the Rotterdam study reported that T2DM patients had increased risk to suffer dementia [71,72]. Today, obesity and diabetes, two T2DM-related disorders, are well established risk factors for AD [11,23,71–74]. In fact, it is of general concern that accumulation of fat in the adipose tissue favours the emergence of metabolic syndrome and T2DM, due to IR signalling deficits in peripheral tissues. What is, perhaps, not so widely known is that obesity also affects the central nervous system (CNS) and it is associated with an exacerbation of cognitive decline [75]. In this respect, data from the clinical study Whitehall II and other research projects established a relation between body mass index (BMI) values (> 30 kg/m2), aging (> 50 years old) and the onset of cognitive deficits [73–78]. Nowadays, the molecular mechanisms at fault are still being studied. For instance, many authors suggest that vascular risk factors and inflammatory responses could also have significant roles [79–82].

Since the pathogenesis of AD remains unclear, it is necessary to use mixed models covering different hypothesis of the disease, evaluating neuronal dysfunction, synapse loss and cell death, as well as potentiating Aβ effects in order to better understand its role in these neuropathological events [80–86]. For now, it seems that Aβ (1–42) oligomers would have a synergistic effect, accelerating the mechanisms related to cognitive impairment [86–89].

From a preclinical level, diet-induced obesity in in vivo models represents a suitable and reproducible method for identifying and understanding potential mechanisms involved in T2DM or IR-induced cognitive loss. Using this experimental approach, the research group of Marta Di Carlo reported that rodents under a high fat diet (HFD) (60% kcal from fat) during 7 months exhibited brain abnormalities similar to the hallmarks of AD, such as the increase in tau phosphorylation, neuroinflammation and memory loss [90]. In addition, they demonstrated that those mice showed an increase in Aβ levels, which was associated with an increase in AβPP and BACE1 expression, both accompanied by an increase in oxidative stress and mitochondrial dysfunction. Our research group also demonstrated that mice fed with a HFD (45% kcal from fat) for 12 months, displayed insoluble Aβ peptide depositions in the brain, thus confirming the relation between the triad obesity, Aβ and cognitive loss [91].

Moreover, other studies showed that HFD-induced brain insulin resistance could decrease synaptic vesicle recycling, thus diminishing synaptic strength and leading to deleterious effects on cognitive behaviour [92]. In a preclinical study, Hao and co-workers demonstrated that only three months of HFD feeding lead to a reversible deterioration of hippocampal synaptic plasticity, dendritic spine density and spatial memory in mice [93]. The authors propose that HFD activated a reversible microglial synaptic phagocytosis process. In this line of research, Osborne and co-workers provided key findings on the implication of Aβ in memory loss in HFD fed rodents [94]. In this study, they showed that intrahippocampal infusion of Aβ33–42 antibodies, with preferential affinity for aggregated Aβ oligomers, improved the memory process in rodents with HFD-induced cognitive loss. In addition, the authors identified a potential mechanism involved in the process of cognitive impairment through the alteration of glutamate transmission by dysregulation of hippocampal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR). However, other authors reported a detrimental cognitive effect of obesogenic HFD in 3xTgAD mice that was not associated with an increase in the brain levels of plaques, Aβ and tau [95]. Contrarily, Vandal and co-workers reported that an obesogenic diet induced insulin resistance in both non-transgenic and 3xTgAD mice, which worsened due to the HFD-amyloid pathology exacerbation [96]. This suggests that HFD exerts a synergistic effect with AD-like pathology in 3xTgAD mice. Moreover, it has been described that the noxious effects of Aβ in brain can also contribute to pancreatic cell degeneration and insulin secretion, and, conversely, abnormal systemic changes might not only develop secondary to brain dysfunction but might also affect AD progression [97]. This suggests that the interactions between the brain and the periphery act as a vicious circle with a crucial role in the development and progression of AD [97]. These data are in line with other studies showing that brain Aβ could bind to IR in the liver and favour peripheral insulin resistance [98,99].

In another study, Ho and colleagues reported that HFD-induced insulin resistance was associated with a decrease in neuronal IR signalling and insulin degrading enzyme (IDE) expression in Tg2576 mice, thus promoting cognitive loss and increased Aβ plaque formation [31]. These results were confirmed by other researchers [100]. In this regard, it is important to emphasize that IDE does not only degrade insulin, but also Aβ [30]. Thus, a decrease in its brain levels may contribute to both insulin signalling dysfunction and accumulation of Aβ. Likewise, other studies using animal models of obesity revealed that HFD induced an alteration in brain GLUT1 and GLUT3/GLUT4 glucose transporters, decreasing its up-take into the CNS. This was linked to cognitive deficits in the CA1 region of the hippocampus, which indicates an impaired synaptic plasticity [101]. Moreover, Ruiz and co-workers added

another interesting point by reporting that HFD increased peripheral glucose levels in AβPP/PS1 mice, probably due to hypothalamic alterations [102]. Other studies provided data about the molecular mechanisms involved in obesity-mediated neurodegeneration. For instance, previous results from our group reported an important down-regulation of PPARγ-coactivator-1α (PGC-1α) in AβPP/PS1 transgenic mice, a preclinical model of AD, under a HFD [30]. HFD caused a dropdown of PGC-1α levels, which was mediated by a decrease of phosphorylated cAMP response element-binding (p-CREB) protein levels [30]. The importance of these results is highlighted by the fact that PGC-1α has a key role in the mitochondrial biogenesis process and, therefore, in the mitochondrial function, which is essential to neuronal activity. Our study also demonstrated acceleration in the process of memory loss in AβPP/PS1 transgenic mice under a hypercaloric diet, as shown by short-term recognition memory values obtained in the novel object recognition test (NORT). This memory impairment was probably mediated by alterations in the brain IR and mitochondria [30]. Interestingly, a correlation between the down-regulation of PGC-1α expression and AD has been also demonstrated, and the low expression of PGC-1α in the brain of transgenic mice models of AD increases the formation of Aβ through the increase in BACE1 activity across the amyloidogenic route [103–107]. Accordingly, Katsouri and co-workers reported that the stereotaxic administration of a lentiviral vector to express human PGC-1α in the hippocampus and cortex of AβPP23 transgenic mice improved memory and showed neuroprotective effects, hence demonstrating the efficacy of this gene therapy as a potential treatment of AD [104]. In turn, Martins and co-workers reported that HFD impairs memory in 3xTgAD mice. This cognitive alteration was associated with changes in mitochondrial energetic balances and loss of synaptic contacts [107]. Since synapses are sites of high-energy demand, some authors correlate the synaptic degeneration process with dysfunctional mitochondria, which are transported to synaptic terminals where high levels of ATP production are required [107].

In the same line, Sah and co-workers found that 3xTg mice fed with HFD suffered from memory impairment associated with a significant decrease in the expression of antioxidant enzymes, such as Heme oxygenase-1 (HO-1) and manganese-dependent superoxide dismutase (MnSOD), through the inactivation of protein kinase B (AKT)- Nuclear factor (erythroid-derived 2 (NRF2) signalling pathway [108]. Therefore, obesity decreases antioxidant enzymes required for brain neuroprotection.

On another front, results obtained by Lin et al. revealed that 5XFAD mice fed with HFD exhibited cognitive impairment and hippocampal oxidative stress. Cognitive impairment is also believed to occur due to decreased levels of brain-derived neurotrophic factor (BDNF), which is a central molecule in synaptic plasticity [109]. Furthermore, this study demonstrated that brain alterations are independent of peripheral metabolic alterations, since glucose tolerance was not impaired in the transgenic mice. In turn, Thériault and co-workers showed that the HFD induces deterioration on cognitive performance in AβPP/PS1 mice, which was associated with a decrease in the enzymatic activity of matrix metallopeptidase (MMP)-9 and a reduction of mRNA and protein levels of BDNF in the brain [110]. These results reinforce the hypothesis of a metabolic aetiology in the molecular basis of the development of AD at the preclinical level [111]. In addition, others demonstrated a down-regulation of hypothalamic BDNF protein levels in HFD-fed transgenic mice compared with wild type HFD-fed mice [112].

In summary, all these preclinical studies showed the existence of an initial memory impairment process associated to obesogenic diets, which could be partially explained by increased levels of soluble Aβ and the inhibition of long term potentiation (LTP) linked to synapse loss. In addition, the down-regulation of BDNF levels mediated by PGC-1α, together with mitochondrial dysfunction, contribute to the decrease of synaptic connections, a marker that correlates with dementia in AD. Overall, these and other studies suggest that some improvement could be achieved through proper lifestyles, by avoiding metabolic alterations and designing therapies targeting one or some of these biomarkers.

4. Are ceramides the bridge between peripheral type II diabetes and neurodegeneration in Alzheimer's disease?

As discussed above, preclinical studies demonstrated that HFDs enhance cognitive loss, stressing its central effects. However, HFDs can also favour cognitive loss through peripheral alterations, specifically in the liver and adipose tissue. In an interesting study, Lyn-Cook and co-workers reported that HFDs induce liver damage (non-alcoholic steatohepatitis), by increasing the levels of pro-inflammatory cytokines (mainly TNFα) and ceramides [113]. Ceramides are toxic lipids which play key roles in brain oxidative stress, insulin resistance and cytoskeletal alterations, mechanisms involved in LOAD apparition [113]. In this respect, Dr. De la Monte suggested that chronic obesity induces insulin resistance associated with non-alcoholic steatohepatitis (NASH) [11–14, 113]. In addition, T2DM-related obesity increases hepatic insulin resistance, thus favouring inflammation and metabolic dysfunction, which leads to dysregulated lipolysis and the generation of ceramides [113]. A significant increase in ceramide levels promotes ER stress in the liver, which exacerbates insulin resistance, inflammation, and oxidative stress [11–13].

In addition to these peripheral harmful effects, ceramides originated in adipose tissue or in the liver reach the bloodstream and pass through the blood-brain barrier, exerting toxic effects in neurons [13]. In this respect, we hypothesize that ceramides in association with Aβ 1–42 could exacerbate brain insulin resistance, oxidative stress, mitochondrial alterations and neuroinflamation. Thus, there could be a vicious circle between brain and peripheral tissues: peripheral damage would increase the risk of cognitive loss and the neurotoxic effects of Aβ 1–42 in the hypothalamus would lead to an altered regulation of peripheral metabolism [114–116]. This idea is supported by the fact that Aβ 1–42 binds directly to the insulin receptor both in the periphery and in the hypothalamus, inducing a peripheral glucose intolerance and insulin resistance and eventually leading to T2DM [116]. Furthermore, it has been demonstrated that Aβ 1–42 induces hepatic insulin resistance through the activation of the Janus Kinase 2 (JAK2) in a preclinical model of familial AD [99]. In addition to the liver, some authors have shown that amyloid can accumulate in other peripheral tissues such as pancreas and skeletal muscle [117,118]. Therefore, the Aβ 1–42 peptide originated at the brain may constitute a key factor involved in insulin resistance and T2DM [97].

Likewise, Shinohara and Sato proposed the involvement of an X Factor underlying the bidirectional interactions between diabetes and LOAD. Hence, they suggest the existence of a vicious circle between diabetes and LOAD, which could be initiated by peripheral ceramides [119]. According to this idea, the authors put forward a potential equation where Aβ1–42 levels would be multiplied by an X factor. This unknown factor could be ceramides, which may amplify the neurotoxicity of Aβ1–42 peptide. Consequently, cognitive loss could be prevented by breaking this circle, and antidiabetic treatments could be beneficial against LOAD.

5. Endoplasmic reticulum (ER) stress: a link between obesity and cognitive loss

Some research studies have indicated that the ER stress is involved in the appearance of degenerative diseases in the brain [120,121]. Obesity seems to exacerbate this situation, favouring cognitive loss and promoting AD-like neuropathology [122–125]. As we have discussed above, the accumulation of disease-specific misfolded proteins is a hallmark of AD, and ER could have a key role in this process.

In eukaryotic cells, such as neurons, the ER is a cellular compartment involved in calcium homeostasis, lipid biosynthesis and protein folding and maturation. Under normal physiological conditions, ER activity is paramount for most cellular mechanisms. Glucose-regulated

protein 78 or binding immunoglobulin protein (GRP78/BiP) molecules are bound to three different ER transmembrane receptor proteins, known as the PKR-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1 (IRE1α) and activating transcription factor 6 (ATF6) [122]. Under ER stress, there is a release of BiP, which triggers the activation of ER stress response. The activation of PERK pathway leads to a signalling cascade which begins with the phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α) and eventually attenuates global protein synthesis [125]. Subsequently, this signalling pathway also promotes the activation of several downstream proteins like the activating transcription factor 4 (ATF4), proapoptotic C/EBP homologous protein (CHOP) and growth arrest and DNA damage-inducible protein 34 KDa (GADD34) [121,122].

In neurons, an appropriate ER response is necessary for the control of protein synthesis and synaptic and neuronal function [125–127]. In AD, alterations in this response could lead to defects to long-term memory formation. Specifically, disruptions on the homeostasis of protein folding lead to the accumulation of misfolded proteins in the ER and upregulation of proapoptotic proteins like CHOP [124]. Hence, conditions inducing a sustained brain ER stress and leading to activation of the unfolded protein response (UPR), such as obesity, could clearly result in deleterious effects, although the underlying mechanisms are still not well understood. Some research groups have reported that palmitate could be one of the elements responsible for the activation of these mechanisms in mice under HFD [128]. Thus, it was hypothesised that the association of palmitate and Aβ could increase even further the burden on the ER (Fig. 3) [129,130].

Other previous studies have shown that phosphorylation of eIF2α is increased in the hipocampus of preclinical mice models of AD. For instance, O'Connor and coworkers reported that PERK-eIF2α signalling activation increases AβPP processing and Aβ formation via direct upregulation of BACE-1 [126]. This data was replicated in the 5xFAD mice model by other groups, which also combined heterogeneous knockouts for $PERK^{+/-}$ and the 5xFAD transgenic model (PERK haploinsufficiency) [131–133]. The results showed improvements in memory deficits in $PERK^{+/-}$ mice compared with 5xFAD mice. Furthermore, under insulin-deficiency conditions, PERK-eIF2α ER stress signalling pathway is dramatically activated in the brain of 5XFAD mice, thus increasing AD risk [133]. Likewise, the PERK pathway effector ATF4 is a repressor of CREB [121], which is required for synaptic plasticity as well as for learning processes.

Complementarity, eIF2α could also be phosphorylated by three different kinases associated with stressors. These kinases are the RNA-activated protein kinase (PKR), the heme-regulated inhibitor kinase (HRI) and the general control non-depressible 2 kinase (GCN2) [87,88,134]. They belong to the integrated stress response (ISR) and some of them have been linked to synapse loss and cognitive deficits in human and preclinical mice models of AD. For example, Mouton-Liger et al. reported that in human AD brains there is significant correlation between increase in PKR and BACE1 protein levels [135]. Strangely, Hwang and co-workers reported that PKR inhibition improves memory without affecting Aβ load in the hippocampus in 12-month-old 5xFAD mice [130]. The authors concluded that this PKR inhibition without affecting Aβ levels might provide a promising alternative strategy for developing a potential AD treatment. In another study, Ma and co-workers crossed PERK and GCN2 knockouts with AβPP/PS1 transgenic mice. They observed that eliminating either enzyme reduced brain levels of Aβ and prevented memory deficits associated with the AβPP/PS1 phenotype [136].

On another research line, Sims-Robinson and co-workers evidenced an increase of ER stress activation on the hippocampus of B6-HF mice fed with HFD. They concluded that this was due to an inhibition of the IR signalling, probably mediated through the activation of the c-Jun N-terminal kinases (JNKs), which are direct inhibitors of the IR substrate 1 (IRS1) [137]. Indeed, an increase in the phosphorylation rates of serine amino acids of the IRS1 would induce desensitization of the hippocampal IR and it is believed that the JNK1 isoform plays a prominent role in this mechanism [138]. In the same line, Liang and co-workers demonstrated a significant increase in the phosphorylation of both PERK and eIF2α, as well as IRE1α and JNK, in hippocampus and frontal cortex of HFD rats [139]. Therefore, they concluded that UPR activation was increased in the brain of obese rats, and that this mechanism was involved in insulin resistance and cognitive loss through JNKs activation and IRS1 inhibitory phosphorylation. This data supports the metabolic hypothesis of the appearance of cognitive deficits independent of Aβ production.

The administration of ursolic acid is a potential strategy to find a therapeutic approach for these alterations. This natural triterpenoid compound exerts many pharmacological actions such as anti-oxidant, anti-inflammatory and anti-tumoral effects. Thus, Lu and co-workers demonstrated that ursolic acid has a protective effect preventing ER activation in the hippocampus, hence improving cognitive responsiveness [128]. Furthermore, quercetin, a polyphenolic flavonoid compound present in a variety of fruits and vegetables, exerts beneficial effects on obesity and cognitive processes by preventing the activation of PERK-eIF2α signalling pathway and avoiding phosphorylation of ATF4 through GADD34 induction [140,141]. Likewise, it has been shown that the administration of the polyunsaturated fatty acid α-linolenic acid to aged female Sprague-Dawley rats fed with a HFD improved the cognitive process [142]. Its beneficial effects are mediated by the inhibition of UPR response through the down-regulation of brain ATF4 levels, thus leading to the increase of p-CREB phosphorylation rates [142].

6. Synaptic loss mediated by neuroinflammation in obesity

Obesity has been associated with chronic inflammatory processes derived from the activity of hypertrophic adipocytes, free fatty acids and reactive oxygen species [143–145]. In the CNS, microglia have significant roles in the control of these mechanisms.

Under physiological conditions, microglial cells present a branched morphology and are responsible for the production of anti-inflammatory and neurotrophic factors (inactive or unreactive state (M2) [146]. However, in some conditions such as obesity, microglial cells become reactive and change their morphology to amoeboid. This is followed by an enhancement of pro-inflammatory responses, both on microglia itself and in other cells such as astrocytes, through the release

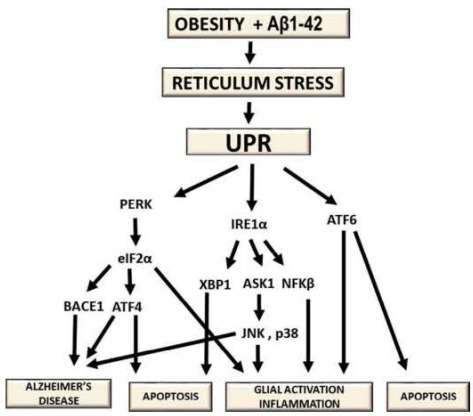


Fig. 3. Chain of events relating obesity alterations, the accumulation of Aβ1–42 and the stress on the reticulum. Activating stimuli on the ER cause release of BiP and the initiation of UPR signalling. These mechanisms have been related to the appearance of pathologies like AD through the upregulation of apoptotic and glial reactivity biomarkers.

of certain cytokines (M1 state) [147]. Eventually, M1 microglia and astrocytic activity lead to neuronal death and pathologies like AD [148,149]. These assumptions have been supported by a meta-analysis of cytokines, which revealed increased concentrations of interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) in peripheral blood of AD patients [150].

As discussed above, when any of the pathways of the UPR activates, there is an up-regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), a key modulator of pro-inflammatory genes [151]. Likewise, several studies indicate that inflammation and abnormal ER activity are critical events in the establishment of hypothalamic and peripheral insulin resistance in metabolic disorders. Thus, in animal models of T2DM and obesity, inflammation in the hypothalamus is an important part of the underlying pathogenic mechanism, especially the activation of TNF-α and the kinase inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ)/NF-kB signalling axis [87,88,116,152]. Specifically, under a HFD, IKKβ becomes activated and phosphorylates IRS1 inhibitory serine sites, resulting in a desensitization of IR signalling [57]. In addition, cortical and hippocampal dysfunction seems to share common pathways in AD.

JNKs constitute another important element regulating inflammation and IR. JNKs pathways control several processes, including insulin resistance in peripheral tissues, such as liver or pancreas, and also in the brain [153–155]. Usually, these pathways are upregulated by cytokines and ER stress. Hence, it is known that neuroinflammation increases brain levels of TNFα, which activates JNKs -mainly JNK1- and leads to a decline of insulin signalling [56,57]. In addition, the activation of ER/UPR response, specifically IRE1α pathway, triggers the splicing of X-box binding protein 1 (XBP1) mRNA. The products of this splicing also activate JNK. This process has important effects on the development of apoptotic processes accounting for cognitive decline [156]. Additionally, IRE1α can also be phosphorylated by JNKs, leading to a recruitment of tumour necrosis factor receptor associated factor-2 (TRAF2), which would enhance further apoptotic responses through caspase 12. Therefore, it seems that the UPR and JNKs are both involved in neuronal apoptosis processes, which are favoured by obesity [125,152].

Interestingly, the expression of protein-tyrosine phosphatase 1B (PTP1B) also becomes up-regulated in obesity models using HFD [157]. This phosphatase is located in the ER membrane and it is a major negative regulator of insulin, leptin and BDNF receptors (Tyrosine Receptor Kinase B; TRKB). Under physiologic conditions, the upregulation of PTP1B is promoted by TNFα and it has significant effects on cognition and memory formation. Elevated levels of PTP1B dephosphorylate tyrosine residues and inhibit downstream signal transduction of the previously mentioned receptors, but activate microglia [158]. In addition, it has been demonstrated that PTP1B is a regulator of the UPR response through the modulation of PERK/eIF1α signalling in peripheral and neuronal cell culture studies [157]. Likewise, PTP1B inhibition attenuates neuronal toxicity in the ER mediated by the mitochondrial neurotoxin rotenone and tunicamycin [159].

Finally, soluble Aβ peptide oligomers can activate microglial cells and increase TNF-α brain levels [56,57]. Therefore, obesity and Aβ can together overstimulate an aberrant TNFα signalling, leading to activation of the stress kinases that block insulin signalling in the brain. Following this line of thought, obesity and neurodegeneration may run in parallel in a complex mechanism where the activation of NF-kB would lead to the expression of inflammatory cytokines, hence promoting a neuroinflammatory state.

7. Pharmacological approaches for metabolic late onset Alzheimer's disease treatment

According to the metabolic hypothesis of AD, therapies capable of restoring normal brain insulin signalling in the CNS may have beneficial effects on brain function. In this sense, a growing body of evidence suggest that insulin receptor have multiple brain functions related to cognition, neuroprotection through the activation of Akt, modulation of AβPP and Aβ levels, neuroinflammation and synapsis formation. Hence, brain insulin dysregulation could contribute to AD pathogenesis, and drugs involved in the modulation of insulin receptor could have potential application in the treatment of AD. This could be achieved not only by improving brain cognition at the hippocampal level, but also modulating the hypothalamus and peripheral tissues such as the liver, which would improve metabolic diseases such as T2DM. Consequently, bearing in mind that AD can be considered a metabolic alteration, its treatment should not only focus in brain processes such as memory, but also in peripheral organs related to insulin resistance (Table 1).

7.1. Intranasal insulin

Mao and co-workers reported that the administration of intranasal insulin for 6 weeks in AβPP/PS1 mice improves cognitive function by activating non-amyloidogenic pathways that favour a decrease in brain Aβ levels and Aβ plaque deposits [160]. Therefore, activation of brain insulin receptors improves Aβ pathology at preclinical level and intranasal insulin may be a potential strategy to modify the course of AD (Table 1).

Several clinical studies have also examined the effects of intranasal-administered insulin in AD patients. For instance, Craft and co-workers

Table 1
Clinical trials that are being conducted with drugs that act by activating the insulin receptor and the signaling pathway for the treatment of Alzheimer's disease.

Drug	Phase	CT number	Disease
Rosiglitazone	II	NCT00381238	Mild to moderate AD
Rosiglitazone	II	NCT00334568	Examine the drug response in patients with AD
Rosiglitazone	I	NCT00688207	AD (the study is designed to assess the pharmacokinetics of Rosiglitazone)
Rosiglitazone	III	NCT00428090	Mild To Moderate AD
Rosiglitazone	III	NCT00550420	Mild to moderate AD
Rosiglitazone	III	NCT00348309	Mild to moderate AD
Pioglitazone	II	NCT00982202	AD
Pioglitazone	III	NCT01931566	Mild to moderate AD
Pioglitazone	III	NCT02284906	AD
Intranasal Insulin Glulisine	II	NCT01436045	Mild To Moderate AD
Intranasal insulin detemir	II	NCT01595646	AD or amnestic mild cognitive impairment
Metformin	II	NCT00620191	Amnestic Mild Cognitive Impairment
Liraglutide	Not Applicable	NCT01469351	AD
Exendin-4	II	NCT01255163	AD
Exendin-4	III	NCT02847403	Cognitive Decline in Dysglycemic Patients
HM15211	I	NCT03374241	Obesity
GIP/GLP-1 Co-Activity	Not Applicable	NCT03526289	Overweight and Type 2 Diabetes
Insulin		NCT00438568	Mild To Moderate AD

evaluated the effects of intranasal administrations of either insulin (10 or 20 IU twice a day for a total dose of 20 or 40 IU per day) or placebo (saline twice a day) for 4 months in AD patients (clinical trial number NCT00438568) [161,162]. They assessed changes in cognitive parameters, brain glucose metabolism and Aβ levels in cerebrospinal fluid (CSF). The results of the trial suggested that the administration of intranasal insulin could improve cognition and brain glucose metabolism in patients with mild cognitive impairment or AD.

After the potential positive effects of intranasal insulin in AD, different pharmaceutical preparations of insulin have been developed to improve its bioavailability. For example, insulin detemir incorporates fatty acids to its chemical structure, thus increasing its binding to albumin and allowing a slower elimination, which lengthens its half-life [162]. Craft and co-workers reported that the positive effects of insulin detemir on memory are associated with an increased volume in brain regions affected by AD neuropathology [162]. However, the authors reported that the efficacy of insulin detemir decreased over longer-term administration in these patients, whereas regular insulin continued to provide beneficial effects on memory. The explanation of these results is still unclear, although it has been proposed that long-acting insulins may desensitize the insulin receptor and thereby increase insulin resistance [56].

Finally, is worth to mention that cognitive processes in AD patients are influenced by gender and APOE4. Interestingly, cognition function in APOE4-negative patients is usually improved after intranasal insulin, while APOE4-positive AD patients showed contradictory results [26,163]. Likewise, men respond better to insulin than women [26].

7.2. Incretins

This group includes some synthetic long-acting analogs such as Glucagon-like peptide-1 (GLP-1), receptor agonists such as exendin-4, as well as liraglutide and lixisenatide, which possess insulinotropic activity, among others.

At preclinical level, intraperitoneally administration of liraglutide or lixisenatide improves neuropathological markers of AD, such as decrease in the number of plaques and glial activation, and also improves cognition parameters in AβPPSwe/PS1dE9 mice [164]. However, several clinical studies are now evaluating the potential efficacy and safety of GLP-1 mimetics in AD, such as liraglutide. For instance, the clinical trial NCT01469351 evaluated the efficacy of liraglutide in AD patients, reporting that 6-month treatment with this drug improved brain glucose metabolism with a slight improvement on cognition (Table 1). Another clinical study, the NCT02140983, evaluated the effects of liraglutide on memory and attention of elder patients with insulin resistance. Worth to mention, the patients had pre-diabetes and half of all subjects had a family history of dementia. However, no data from this study has been reported yet. Exendin-4 is another incretin-mimetic long-acting GLP-1 receptor agonist approved for T2DM treatment, which also have shown neuroprotective effects in preclinical models of AD [165]. Unfortunately, clinical trials with exendin-4 in early-stage AD, such as NCT01255163, did not reported beneficial effects on cognition.

Likewise, glucose-dependent insulinotropic polypeptide (GIP) analogues such as D-Ala2-GIP also showed neuroprotective effects on synaptic plasticity and cognition in animal models. Thus, it has been reported that these compound significantly reduces Aβ42 plaques and neuroinflammation in preclinical AD mice models [166]. Since these peptides were effective at the preclinical level, the next strategy was the development of dual peptide agonists GLP-1/glucagon and GLP-1/GIP combined. Moreover, triagonists GLP-1/GCG/GIP are in early stages of development. Thus far, two studies in murine AD models have reported that triple agonists improve cognitive processes by increasing BDNF levels and p-CREB signalling pathway, and by decreasing plaques and neuroinflammation [167]. However, most research studies with triagonists have been carried out in metabolic disorders such as T2DM and obesity, where they are more effective than the single administration of each peptide alone. Regarding clinical studies, only the triagonist HM15211 is under investigation as a treatment for obesity (NCT03374241). In this sense, the next aim is to evaluate the efficacy of this triagonist, which integrates the actions of the three endogenous hormones, in T2DM and weight decrease, as well as in other potential diseases.

7.3. Metformin

It has been shown that the anti-diabetic drug metformin is effective in preclinical models of AD [168]. Thus, Ou and co-workers reported that metformin improves cognitive process, decrease the Aβ production and neuroinflammatory response in the hippocampus of AβPP/PS1 mice [169]. The authors suggested that the neuroprotective effects of metformin are mediated by the modulation of the AMPK/mTOR/S6K/BACE1 pathway. However, these neuroprotective benefits of metformin in AD are contradictory, since a recent study in transgenic models of tauopathy with cognitive deficits has shown that metformin can aggravate the risk of tauopathy in diabetic patients [170].

In turn, Infante-Garcia and co-workers studied in a mixed murine model of AD and T2DM (AβPP/PS1x db/db mice) the effects of the anti-T2DM polypill (PP), which contains several drugs used to treat T2DM, including metformin, aspirin, a generic statin, and an angiotensin-converting enzyme inhibitor [171]. They reported that PP could be a suitable strategy for the treatment of serious complications of T2DM, such as cognitive alterations.

Recently, Koenig and co-workers published the results of a clinical trial with metformin (NCT01965756) in AD patients with mild cognitive impairment or mild dementia. They reported that metformin slightly improved learning and memory processes [172]. In another clinical trial (NCT00620191), the effects of metformin in Amnestic Mild Cognitive Impairment (MCI) were evaluated. Specifically, the aim of this study was to assess the changes in a memory and general cognitive function test (the Alzheimer's Disease Assessment Scale-cognitive subscale-ADAS-Cog used in clinical trials.). The study also aimed to compare brain function through mean changes in PET scan between the patients treated with metformin and those treated with placebo. So far, no results from this study have been reported yet.

7.4. Thiazolidinediones

Thiazolidinediones are agonists of peroxisome-proliferator activated receptors (PPARs). They possess antidiabetic activity and have also shown neuroprotective effects in preclinical models of AD [48]. These compounds are nuclear hormone receptors that induce physiological responses through the regulation of gene expression, and they are involved in the metabolic regulation of carbohydrates, proteins and lipids. They also reduce neuroinflammation by inhibiting glial activation, since it has been reported that rosiglitazone significantly improves cognition in rats after Aβ injection in the hippocampus through inhibition of microglial cytokine release [173]. According to these results, the regulation of microglia constitutes a key target involved in cognitive improvement in preclinical AD models.

Regarding the use of rosiglitazone in clinical studies, Gold and colleagues reported that administration of 2-mg or 8-mg of this compound during 24 weeks in APOE-ε4 negative patients does not show any statistical difference when compared to placebo treatment [174]. The current clinical study NCT00334568 aims to evaluate the effects of rosiglitazone on i) cerebral glucose utilization measured by [18F] FDG uptake and ii) cognitive process in AD. No results have been reported yet. In turn, the study NCT00348309 assessed the effects of a 54-week treatment of rosiglitazone (extended release tablets) combined with donepezil on cognitive parameters of patients with mild to moderate AD. Again, no clinical improvement was achieved with this treatment [175].

On another front, previous studies have reported neuroprotective effects of pioglitazone in preclinical models of AD, through a decrease in neuroinflammation and also in mRNA and protein levesl of BACE1 [176]. This compound was able to significantly decrease the cerebral Aβ levels, and could hence constitute a potential drug with the capacity to modify the course of the disease. Interestingly, Fernandez-Martos and co-workers reported that acute 2-week treatment with combined leptin and pioglitazone improves cognition and decrease neuropathological parameters of AD in AβPP/PS1 mice [177]. In turn, Geldmacher and colleagues evaluated the safety of long-term treatment with pioglitazone (15-mg tablets) in elderly nondiabetic AD patients [178]. The authors reported that pioglitazone was well tolerated and safe during the 18-month treatment trial. In the same line, the NCT01931566 study evaluated the effects of pioglitazone compared with placebo in AD patients with mild cognitive impairment (MCI). The aim of this study was to delay the onset of memory loss in cognitively normal participants who were at high-risk for developing MCI within the next 5 years. Currently, no results have been reported. However, the clinical trial NCT02284906 aims to evaluate the safety and effectiveness of pioglitazone on cognitive function in participants who have completed the previous study.

Regarding thiazolidinediones, we can summarize that, in spite the promising results observed in preclinical studies, no clinical study has reported successful results yet [179–181]. Therefore, additional clinical studies with PPARγ agonists are still required, either in monotherapy or in combination with other drugs, in order to evaluate the efficacy in improving brain insulin/IGF-1 resistance and thus improving cognition and preventing neurodegeneration in initial stages of AD.

7.5. c-Jun-N-terminal kinase inhibitors

Although SP600125 is the best characterized inhibitor of JNK activity, its application has been impeded by its low target selectivity, and the clinical efficacy of this compound is also limited by its poor aqueous solubility [157].

Chalcones are phenolic naturally compounds highly widespread in fruits, vegetables, spices, tea and soy-based foodstuff. Some of these compounds have been shown to inhibit JNK pathway and they are considered important secondary metabolites, precursors of flavonoids and isoflavonoids in plants [182]. Moreover, these molecules are interesting due to their simple chemistry, easy synthetic procedures, multiplicity of substitutions and diverse pharmacological potentials, such as anti-cancer, antioxidants, anti-inflammatory, adenosine receptor ligands, antimalarial, antimicrobial, anti-HIV or anti-protozoal [183–187]. Over the years, different chalcones have been isolated, such as isoliquiritigenin, echinatin, licochalcone A (Lic-A), licochalcone C and licochalcone E (Lic-E).

Lic-E exhibits cytotoxicity to human tumor cell lines and endothelial cells, as well as cutaneous anti-inflammatory potential [186]. Moreover, Lic-E activates the NRF2-Antioxidant Response Element (ARE) system and up-regulates downstream NAD (P) H: quinone oxidoreductase 1 (NQO1) and HO-1, suggesting a therapeutically relevant effect to oxidative-stress-related neurodegeneration [182]. In addition, it has been evidenced that Lic-E has a neuroprotective effect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induces nigrostriatal dopaminergic neurodegeneration in mice [186].

Lic-A could be a promising molecule for the treatment of obesity-derived complications associated with cognitive impairment. It is one of the major bioactive constituents of the roots of liquorice and it has been shown to have anti-inflammatory and anti-microbial activities, as well as anti-tumour effects [187,188]. Lic-A is a specific inhibitor of JNK1 and studies using JNK1 knockout mice revealed that these animals showed lower body weight and increased insulin sensitivity [(Busquets et al. 2018). Moreover, Lic-A has been identified as a PTP1B inhibitor, hence increasing its potential effect for the treatment of T2DM at peripheral level and enhancing the cognitive process in the brain. Of note, our group has reported beneficial effects of this molecule in a murine model of epilepsy [135,184,185], by demontsrating that JNK1 inhibition by Lic-A reduced cell death derived of excitotoxic damage, as well as neuroinflammation.

Additionally, several research studies have synthesized and evaluated hydroxychalcones as inhibitors of human acetylcholinesterase. These new compounds were found to be effective and could be potential new disease-modifying drug candidates for the treatment of AD. Thus, Jeon and co-workers synthesized hydroxychalcones with potent BACE1 inhibiting effect, preventing the formation of insoluble Aβ

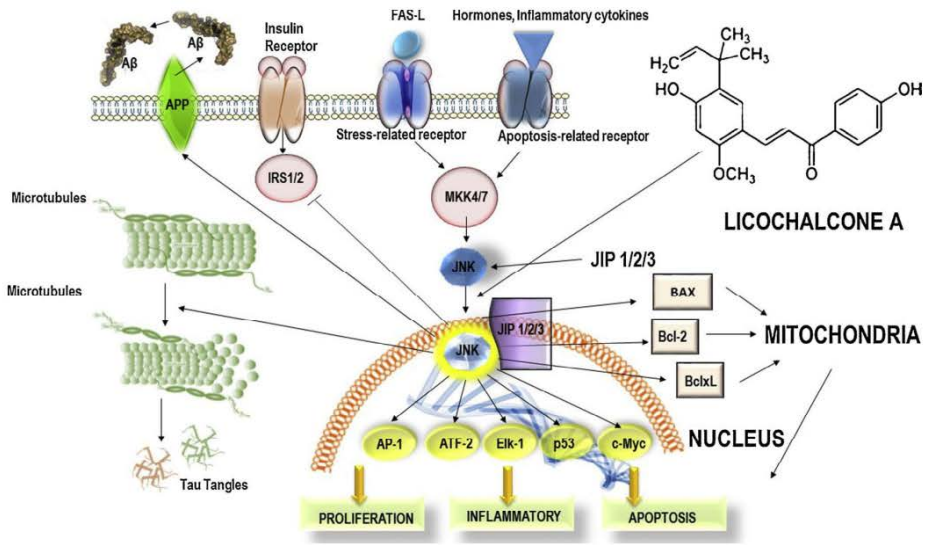


Fig. 4. Graphical representation of some of the mechanisms described. JNKs are mediators of proliferative, inflammatory and apoptotic mechanisms. Lic-A, a chalcone extracted of liquorice roots, behaves as a specific inhibitor of the JNK1.

peptide, as well as inhibition of MAPK signalling and TAU phosphorylation [186,187]. In addition, novel selective water-soluble and brain-penetrant JNK inhibitors have been tested [188]. Likewise, some experiments have evaluated the effects of i.p. and i.c.v. administrations of SR11935 and SR3306, brain-penetrant JNK2/3 isoform-selective inhibitors [188]. The results showed similar anorectic effects for both isoforms, suggesting that JNK2 and JNK3 mediate aspects of the anorectic effect observed in pan-JNK inhibition.

8. Concluding remarks

It has been described that obesity enhances the loss of neurons [189]. Therefore, it is paramount to investigate how this condition is associated with soluble Aβ and how it promotes age-related pathologies [190,191]. Ceramides are generated in peripheral tissues during the obesogenic process. In the brain, these toxic lipids could amplify and potentiate the neurotoxic effects of Aβ1–42 [192]. Hence, drugs with antidiabetic peripheral effects are expected to be capable of preventing the cognitive loss in LOAD, by inhibiting the enhancing effects of ceramides on Aβ1–42 [193–199].

In addition to obesity and altered insulin receptor activity, there is mounting data pointing to ER strees and neuroinflammation as key mechanisms in sporadic AD development [200]. Indeed, obesity causes ER stress by promoting a pro-inflammatory state through the activation of molecules like PTP1B and JNK, as well as by increasing Aβ levels through BACE1 activation in the amyloidogenic pathway [201,202]. Moreover, PTP1B and JNK are also relevant in the regulation of IR signalling, pleading to its inhibition and the appearance of insulin resistance. In this sense, JNKs emerge as molecular targets that could restore homeostasis alterations related to insulin resistance, ER stress and neuroinflammation [203–205]. In order to modulate these mechanisms, different pharmacological approaches that may act in a combined and, potentially, synergistic manner have been proposed. Efforts should be addressed to achieve a better understanding of the precise role of each JNK isoforms in brain and during cognitive impairment, as well as to test effective and specific pharmacological inhibitors.

Finally, drugs modulating one or several of these mechanisms could be a pharmacological strategy to prevent AD. Licochalcones are potential candidates, since these compounds have shown different neuroprotective properties. Specially, Lic-A is a promising compound due to its inhibitory activity on JNK1 and PTP1B [203–205]. Thus, Lic-A is capable of reducing neuroinflammation and insulin resistance, two of the pathological hallmarks of sporadic cognitive loss related to AD (Fig. 4). Likewise, preclinical studies and clinical trials indicate that the intranasal administration of insulin, the administration of incretins and other antidiabetic drugs can also have a therapeutic application in the prevention of AD [206–214]. However, as to other diseases such as AIDS or the prevention of coronary heart disease, a combined treatment could be necessary for the successful treatment of AD.

Conflict of interest

The authors do not have any current or potential conflict of interest, including any financial, personal or other relationships with other people or organizations. All authors have reviewed the contents of the manuscript being submitted and approved its content.

Acknowledgements

This work was supported by the Spanish Ministry of Science and InnovationSAF2017-84283-R, PI2016/01, CB06/05/0024 (CIBERNED), the European Regional Development Founds and MAT2014-59134-R project. NIA 1R15AG050292 from Generalitat de Catalunya. Research team from UB and URV belongs to 2014SGR-525 from Generalitat de Catalunya. ESL and MLG belong to 2014SGR-1023. CBZ is supported by grants from CONACyT Mexico (No. 0177594) and RDCT from Postdoctoral fellowship CONACYT No. 298337 and the Doctoral Program in Sciences in Molecular Biology in Medicine, LGAC Molecular Bases of Chronic Diseases-Degenerative and it's Applications (000091, PNPC, CONACyT).

References

[1] A. Alzheimer, Über eine eigenartige Erkrankung der Hirnrinde, Allgemeine Zeitschrift Fur Psychiatrie Und Psychisch-Gerichtliche Medizin 64 (1907) 146–148.
[2] J. Hardy, N. Bogdanovic, B. Winblad, E. Portelius, N. Andreasen, A. Cedazo-Minguez, H. Zetterberg, Pathways to Alzheimer's disease, J. Intern. Med. 275 (2014) 296–303.
[3] N. Nukina, Y. Ihara, One of the antigenic determinants of paired helical filaments is related to tau protein, J. Biochem 99 (1986) 1541–1544.
[4] H. Zempel, E. Mandelkow, Lost after translation: missorting of tau protein and consequences for Alzheimer disease, Trends Neurosci. 37 (2014) 721–732.
[5] C.A. Lane, J. Hardy, J.M. Schott, Alzheimer's disease, Eur. J. Neurol. 25 (2018) 59–70.
[6] M.A. Pericak-Vance, J.L. Haines, Genetic susceptibility to Alzheimer disease, Trends Genetics 11 (1995) 504–508.
[7] S. Craft, Insulin resistance syndrome and Alzheimer's disease: age- and obesity-related effects on memory, amyloid, and inflammation, Neurobiol Aging, Suppl. 1 (2005) 65–69.
[8] P.G. Ridge, R.B. Hoyt, K. Boehme, S. Mukherjee, P.K. Crane, J.L. Haines, R. Mayeux, L.A. Farrer, M.A. Pericak-Vance, G.D. Schellenberg, J.S.K. Kauwe, Alzheimer's disease genetics consortium (ADGC). Assessment of the genetic variance of late-onset Alzheimer's disease, Neurobiol. Aging 41 (2016) 200 E13-200.e20.
[9] S. Craft, E. Peskind, M.W. Schwartz, G.D. Schellenberg, M. Raskind, Jr.D. Porte, Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype, Neurology 50 (1998) 164–168.
[10] V.A. Moser, C.J. Pike, Obesity accelerates Alzheimer-related pathology in APOE4 but not APOE3 mice, eNeuro 13 (2017) 4.
[11] S.M. de la Monte, Insulin resistance and neurodegeneration: progress towards the Development of New therapeutics for Alzheimer's disease, Drugs 77 (2017) 47–65.
[12] S.M. de la Monte, Insulin resistance and Alzheimer's disease, BMB Rep. 42 (2009) 475–481.
[13] S.M. de la Monte, Type 3 diabetes is sporadic Alzheimer's disease: mini-review, Eur. Neuropsychopharmacol. 24 (2014) 1954–1960.
[14] S.M. De la Monte, M. Tong, N. Lester-Coll, M. Plater Jr, J.R. Wands, Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: relevance to Alzheimer's disease, J. Alzheimer's Dis. 10 (2006) 89–109.
[15] R.A. DeFronzo, E. Ferrannini, L. Groop, R.R. Henry, W.H. Herman, J.J. Holst, F.B. Hu, C.R. Kahn, I. Raz, G.I. Shulman, D.C. Simonson, M.A. Testa, R. Weiss, Type 2 diabetes mellitus, Nat. Rev. Dis. Primers 1 (2015) 15019.
[16] M. Ben Nasr, F. D'Addi, V. Usuelli, S. Tezza, R. Abdi, P. Fiorina, The rise, fall, and resurgence of immunotherapy in type 1 diabetes, Pharmacol. Res. 98 (2015) 31–38.
[17] R. Bassi, P. Fiorina, Impact of islet transplantation on diabetes complications and quality of life, Curr. Diab. Rep. 11 (2011) 355–363.
[18] M. Hokama, S. Oka, J. Leon, T. Ninomiya, H. Honda, K. Sasaki, T. Iwaki, T. Ohara, T. Sasaki, F.M. LaFerla, Y. Kiyohara, Y. Nakabeppu, Altered expression of diabetes-related genes in Alzheimer's disease brains: Hisayama study, Cereb. Cortex 24 (2014) 2476–2488.
[19] S. Kang, Y.H. Lee, J.E. Lee, Metabolism-centric overview of the pathogenesis of Alzheimer's disease, Yonsei Med. J. 58 (2017) 479–488.
[20] W.Q. Zhao, M. Townsend, Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease, Biochim. Biophys. Acta 1792 (2009) 482–496.
[21] Y.F. Chuang, Y. An, M. Bilgel, D.F. Wong, J.C. Troncoso, R.J. O'Brien, J.C. Breitner, L. Ferruci, S.M. Resnick, M. Thambisetty, Midlife adiposity predicts earlier onset of Alzheimer's dementia, neuropathology and presymptomatic cerebral amyloid accumulation, Mol. Psychiatry 21 (2016) 910–915.
[22] L. Devi, M.J. Alldred, S.D. Ginsberg, M. Ohno, Mechanisms underlying insulin deficiency-induced acceleration of β-amyloidosis in a mouse model of Alzheimer's disease, PLoS One 7 (2012) e32792.
[23] S.M. De la Monte, Therapeutic targets of brain insulin resistance in sporadic Alzheimer's disease, Front. Biosci. 4 (2012) 1582–1605.
[24] C.A. Grillo, G.G. Piroli, R.C. Lawrence, S.A. Wrighten, A.J. Green, S.P. Wilson, R.R. Sakai, S.J. Kelly, M.A. Wilson, D.D. Mott, L.P. Reagan, Hippocampal insulin resistance impairs spatial learning and synaptic plasticity, Diabetes 64 (2015) 3927–3936.
[25] E.M. Ribe, S. Lovestone, Insulin signalling in Alzheimer's disease and diabetes: from epidemiology to molecular links, J. Intern. Med. 280 (2016) 430–442.
[26] N. Zhao, C.C. Liu, A.J. Van Ingelgom, Y.A. Martens, C. Linares, J.A. Knight, M.M. Painter, P.M. Sullivan, G. Bu, Apolipoprotein E4 impairs neuronal insulin signaling by trapping insulin receptor in the endosomes, Neuron 96 (2017) 115–129.
[27] S. Hoyer, Glucose metabolism and insulin receptor signal transduction in Alzheimer disease, Eur. J. Pharmacol. 490 (2004) 115–125.

[28] R. Kandimalla, V. Thirumala, P.H. Reddy, Is Alzheimer's disease a type 3 diabetes? A critical appraisal, Biochim. Biophys. Actam. 1863 (2017) 1078–1089.
[29] X. Ma, F. Fang, M. Song, S. Ma, The effect of isoliquiritigenin on learning and memory impairments induced by high-fat diet via inhibiting TNF-α/JNK/IRS signaling, Biochem. Biophys. Res. Commun. 464 (2015) 1090–1095.
[30] D. Petrov, I. Pedrós, G. Artiach, F.X. Sureda, E. Barroso, M. Pallàs, A. Camins, High-fat diet-induced deregulation of hippocampal insulin signalling and mitochondrial homeostasis deficiencies contribute to Alzheimer disease pathology in rodents, Biochim. Biophys. Acta. 1852 (2015) 1687–1699.
[31] L. Ho, W. Qin, P.N. Pompl, Z. Xiang, J. Wang, Z. Zhao, Y. Peng, G. Cambareri, A. Rocher, C.V. Mobbs, P.R. Hof, G.M. Pasinetti, Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease, FASEB J. 18 (2004) 902–904.
[32] F. Formiga, M. Pérez-Maraver, Type 3 diabetes mellitus. The revival of inhaled insulin? Endocrinol. Nutr. 61 (2014) 173–175.
[33] J.T. Coyle, D.L. Price, M.R. DeLong, Alzheimer's disease: a disorder of cortical cholinergic innervation, Science 219 (1983) 1184–1190.
[34] J. Coyle, P. Kershaw, Galantamine, a cholinesterase inhibitor that allosterically modulates nicotinic receptors: effects on the course of Alzheimer's disease, Biol. Psychiatry 49 (2001) 289–299.
[35] A. Contestabile, The history of the cholinergic hypothesis, Behav. Brain Res. 221 (2011) 334–340.
[36] E.K. Perry, B.E. Tomlinson, G. Blessed, K. Bergmann, P.H. Gibson, R.H. Perry, Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia, BMJ 2 (1978) 1457–1459.
[37] P.J. Whitehouse, D.L. Price, A.W. Clark, J.T. Coyle, M.R. De Long, Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis, Ann. Neurol. 10 (1981) 122–126.
[38] M. Sabbagh, J. Cummings, Progressive cholinergic decline in Alzheimer's disease: consideration for treatment with donepezil 23 mg in patients with moderate to severe symptomatology, BMC Neurol. 11 (2011) 21.
[39] J. Hardy, D.J. Selkoe, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, Science 297 (2002) 353–356.
[40] D.J. Selkoe, J. Hardy, The amyloid hypothesis of Alzheimer's disease at 25 years, EMBO Mol. Med. 8 (2016) 595–608.
[41] J.A. Hardy, G.A. Higgins, Alzheimer's disease: the amyloid cascade hypothesis, Science 256 (1992) 184–185.
[42] J. Hardy, The amyloid hypothesis for Alzheimer's disease: a critical reappraisal, J. Neurochem. 110 (2009) 1129–1134.
[43] D.J. Selkoe, Alzheimer's disease is a synaptic failure, Science 298 (2002) 789–791.
[44] J.H. Toyn, M.K. Ahlijanian, Interpreting Alzheimer's disease clinical trials in light of the effects on amyloid-β, Alzheimers Res. Ther. 6 (2014) 14.
[45] J. Sevigny, P. Chiao, T. Bussière, P.H. Weinreb, L. Williams, M. Maier, R. Dunstan, S. Salloway, T. Chen, Y. Ling, J. O'Gorman, F. Qian, M. Arastu, M. Li, S. Chollate, M.S. Brennan, O. Quintero-Monzon, R.H. Scannevin, H.M. Arnold, T. Engber, K. Rhodes, J. Ferrero, Y. Hang, A. Mikulskis, J. Grimm, C. Hock, R.M. Nitsch, A. Sandrock, The antibody aducanumab reduces Aβ plaques in Alzheimer's disease, Nature 537 (2016) 50–56.
[46] M. Shinohara, N. Sato, Bidirectional interactions between diabetes and Alzheimer's disease, Neurochem. Int. 108 (2017) 296–302.
[47] M.F. Egan, J. Kost, P.N. Tariot, P.S. Aisen, J.L. Cummings, B. Vellas, C. Sur, Y. Mukai, T. Voss, C. Furtek, E. Mahoney, L. Harper Mozley, R. Vandenberghe, Y. Mo, D. Michelson, Randomized trial of verubecestat for mild-to-moderate Alzheimer's disease, N. Engl. J. Med. 378 (2018) 1691–1703.
[48] J. Cummings, G. Lee, A. Ritter, K. Zhong, Alzheimer's disease drug development pipeline; 2018, Alzheimers Dement (N Y) 4 (2018) 195–214.
[49] M.D. Weingarten, A.H. Lockwood, S.Y. Hwo, M.W. Kirschner, A protein factor essential for microtubule assembly, Proc. Natl. Acad. Sci. U S A. 72 (1975) 1858–1862.
[50] M. Jin, N. Shepardson, T. Yang, G. Chen, D. Walsh, D.J. Selkoe, Soluble amyloid beta-protein dimers isolated from Alzheimer cortex directly induce tau hyperphosphorylation and neuritic degeneration, Proc. Natl. Acad. Sci. U S A. 108 (2011) 5819–5824.
[51] K. Iqbal, C.X. Gong, F. Liu, Microtubule-associated protein tau as a therapeutic target in Alzheimer's disease, Expert Opinion Ther. Targets 18 (2014) 307–318.
[52] E.H. Corder, M.A. Woodbury, I. Volkmann, D.K. Madsen, N. Bogdanovic, B. Winblad, Density profiles of Alzheimer disease regional brain pathology for the huddinge brain bank: pattern recognition emulates and expands upon braak staging, Exp. Gerontol. 35 (2000) 851–864.
[53] T. Gómez-Isla, R. Hollister, H. West, S. Mui, J.H. Growdon, R.C. Petersen, J.E. Parisi, B.T. Hyman, Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease, Ann. Neurol. 41 (41) (1997) 17–24.
[54] A. Delacourte, A. Defossez, Alzheimer's disease: tau proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments, J. Neurol. Sci. 76 (1986) 173–186.
[55] E.D. Robertson, K. Scearce-Levie, J.J. Palop, F. Yan, I.H. Cheng, T. Wu, H. Gerstein, G.Q. Yu, L. Mucke, Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model, Science 316 (2007) 750–754.
[56] M.T. Heneka, M.J. Carson, J. El Khoury, G.E. Landreth, F. Brosseron, D.L. Feinstein, A.H. Jacobs, T. Wyss-Coray, J. Vitorica, R.M. Ransohoff, K. Herrup, S.A. Frautschy, B. Finsen, G.C. Brown, A. Verkhratsky, K. Yamanaka, J. Koistinaho, E. Latz, A. Halle, G.C. Petzold, T. Town, D. Morgan, M.L. Shinohara, V.H. Perry, C. Holmes, N.G. Bazan, D.J. Brooks, S. Hunot, B. Joseph, N. Deigendesch, O. Garaschuk, E. Boddeke, C.A. Dinarello, J.C. Breitner, G.M. Cole, D.T. Golenbock, M.P. Kummer, Neuroinflammation in Alzheimer's disease, Lancet Neurol. 14 (2015) 388–405.
[57] Benedict C. De Felice, A key role of insulin receptors in memory, Diabetes. 64 (2015) 3653–3655.
[58] F.G. De Felice, M.V. Lourenco, S.T. Ferreira, How does brain insulin resistance develop in Alzheimer's disease? Alzheimers Dement. 10 (2014) S26–32.
[59] Alzheimer's Association Report 2018, Alzheimer's disease facts and figures, Alzheimer's Dement. 14 (2018) 367–429.
[60] H. Akiyama, S. Barger, S. Barnum, B. Bradt, J. Bauer, G.M. Cole, N.R. Cooper, P. Eikelenboom, M. Emmerling, B.L. Fiebich, C.E. Finch, S. Frautschy, W.S. Griffin, H. Hampel, M. Hull, G. Landreth, L. Lue, R. Mrak, I.R. Mackenzie, P.L. McGeer, M.K. O'Banion, J. Pachter, G. Pasinetti, C. Plata-Salaman, J. Rogers, R. Rydel, Y. Shen, W. Streit, R. Strohmeyer, I. Tooyoma, F.L. Van Muiswinkel, R. Veerhuis, D. Walker, S. Webster, B. Wegrzyniak, G. Wenk, T. Wyss-Coray, Inflammation and Alzheimer's disease, Neurobiol. Aging 21 (2000) 383–421.
[61] S.W. Pimplikar, Neuroinflammation in Alzheimer's disease: from pathogenesis to a therapeutic target, J. Clin. Immunol. 34 (Suppl. 1) (2014) S64–S69.
[62] M.T. Heneka, M.P. Kummer, E. Latz, Innate immune activation in neurodegenerative disease, Nat. Rev. Immunol. 14 (2014) 463–477.
[63] M.T. Heneka, M. Sastre, L. Dumitrescu-Ozimek, A. Hanke, I. Dewachter, C. Kuiperi, K. O'Banion, T. Klockgether, F. Van Leuven, G.E. Landreth, Acute treatment with the PPARgamma agonist pioglitazone and ibuprofen reduces glial inflammation and Abeta1-42 levels in APPV717I transgenic mice, Brain 125 (2005) 1442–1453.
[64] P.L. McGeer, J.P. Guo, M. Lee, K. Kennedy, E.G. McGeer, Alzheimer's disease can Be spared by nonsteroidal anti-inflammatory drugs, J. Alzheimers Dis. 62 (2018) 1219–1222.
[65] H. Chen, J. Pellegrini, S. Aggarwal, S. Lei, S. Warach, F. Jensen, S. Lipton, Open-channel block of N-methyl-D-aspartate (NMDA) responses by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity, J. Neurosci. 12 (1992) 4427–4436.
[66] R.H. Porter, J.T. Greenamyre, Regional variations in the pharmacology of NMDA receptor channel blockers: implications for therapeutic potential, J. Neurochem. 64 (1995) 614–623.
[67] W. Danysz, C.G. Parsons, Alzheimer's disease, β-amyloid, glutamate, NMDA receptors and memantine-searching for the connections, British J. Pharmacol. 167 (2012) 324–352.
[68] C.G. Parsons, A. Stöffler, W. Danysz, Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system—too little activation is bad, too much is even worse, Neuropharmacology 53 (2007) 699–723.
[69] J.W. Olney, D.F. Wozniak, N.B. Farber, Excitotoxic neurodegeneration in Alzheimer disease. New hypothesis and new therapeutic strategies, Archives Neurol. 54 (1997) 1234–1240.
[70] E.M. Snyder, Y. Nong, C.G. Almeida, S. Paul, T. Moran, E.Y. Choi, A.C. Nairn, M.W. Salter, P.J. Lombroso, G.K. Gouras, P. Greengard, Regulation of NMDA receptor trafficking by amyloid-beta, Nat. Neurosci. 8 (2005) 1051–1058.
[71] A. Ott, R.P. Stolk, A. Hofman, F. van Harskamp, D.E. Grobbee, N.M. Breteler, Association of diabetes mellitus and dementia: the Rotterdam study, Diabetologia 39 (1996) 1392–1397.
[72] A. Ott, R.P. Stolk, Fvan Harskamp, H.A. Pols, A. Hofman, M.M. Breteler, Diabetes mellitus and the risk of dementia: the Rotterdam study, Neurology 53 (1999) 1937–1942.
[73] E.M. Schrijvers, J.C. Witteman, E.J. Sijbrands, A. Hofman, P.J. Koudstaal, M.M. Breteler, Insulin metabolism and the risk of Alzheimer disease: the Rotterdam study, Neurology 75 (2010) 1982–1987.
[74] W.A. Banks, S.A. Farr, T.S. Salameh, M.L. Niehoff, E.M. Rhea, J.E. Morley, A.J. Hanson, K.M. Hansen, S. Craft, Triglycerides cross the blood-brain barrier and induce central leptin and insulin receptor resistance, Int. J. Obes. 42 (2018) 391–397.
[75] T.I. Emmerzaal, A.J. Kiliaan, D.R. Gustafson, 2003-2013: a decade of body mass index, Alzheimer's disease, and dementia, J. Alzheimers Dis. 43 (2015) 739–755.
[76] A. Rosengren, D. Skoog Gustafson, L. Wilhelmsen, Body mass index, other cardiovascular risk factors, and hospitalization for dementia, Arch. Intern. Med. 165 (2005) 321–326.
[77] E.C. Gorospe, J.K. Dave, The risk of dementia with increased body mass index, Age Ageing 36 (2007) 23–29.
[78] A. Singh-Manoux, A. Dugravot, M. Shipley, E.J. Brunner, A. Elbaz, S. Sabia, M. Kivimaki, Obesity trajectories and risk of dementia: 28 years of follow-up in the whitehall II study, Alzheimers Dement. 14 (2018) 178–186.
[79] M. Kivipelto, T. Ngandu, L. Fratiglioni, M. Viitanen, I. Kareholt, B. Winblad, E.L. Helkala, J. Tuomilehto, H. Soininen, A. Nissinen, Obesity and vascular risk factors at midlife and the risk of dementia and Alzheimer disease, Arch. Neurol. 62 (2005) 1556–1560.
[80] E. Duron, O. Hanon, Vascular risk factors, cognitve decline, and dementia, Vasc Health Risk Manag. 4 (2008) 363–381.
[81] J.T. O'Brien, H.S. Marku, Vascular risk factors and Alzheimer's disease, BMC Med. 12 (2014) 218.
[82] S.T. Ferreira, J.R. Clarke, T.R. Bomfim, F.G. De Felice, Inflammation, defective insulin signaling, and neuronal dysfunction in Alzheimer's disease, Alzheimers Dement. 10 (2014) S76–S83.
[83] M.P. Lambert, A.K. Barlow, B.A. Chromy, C. Edwards, R. Freed, M. Liosatos, T.E. Morgan, I. Rozovsky, B. Trommer, K.L. Viola, P. Wals, C. Zhang, C.E. Finch, G.A. Krafft, W.L. Klein, Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins, Proc. Natl. Acad. Sci. U S A. 95 (1998) 6448–6453.
[84] S.T. Ferreira, W.L. Klein, The aβ oligomer hypothesis for synapse failure and

memory loss in Alzheimer's disease, Neurobiol. Learn Mem. 96 (2011) 529–543.
[85] S.T. Ferreira, M.N. Vieira, F.G. De Felice, Soluble protein oligomers as emerging toxins in Alzheimer's and other amyloid diseases, IUBMB Life 59 (2007) 332–345.
[86] K.L. Viola, W.L. Klein, Amyloid β-oligomers in Alzheimer's disease pathogenesis, treatment, and diagnosis, Acta Neuropathol. 129 (2015) 183–206.
[87] M.V. Lourenco, J.R. Clarke, R.L. Frozza, T.R. Bomfim, L. Forny-Germano, A.F. Batista, L.B. Sathler, J. Brito-Moreira, O.B. Amaral, C.A. Silva, L. Freitas-Correa, S. Espírito-Santo, P. Campello-Costa, J.C. Houzel, W.L. Klein, C. Holscher, J.B. Carvalheira, A.M. Silva, L.A. Velloso, D.P. Munoz, S.T. Ferreira, F.G. De Felice, TNF-α mediates PKR-dependent memory impairment and brain IRS-1 inhibition induced by Alzheimer's β-amyloid oligomers in mice and monkeys, Cell Metab. 18 (2013) 831–843.
[88] M.V. Lourenco, S.T. Ferreira, F.G. De Felice, Neuronal stress signaling and eIF2α phosphorylation as molecular links between Alzheimer's disease and diabetes, Prog. Neurobiol. 129 (2015) 37–57.
[89] E.N. Cline, M.A. Bicca, K.L. Viola, W.L. Klein, The amyloid-β oligomer hypothesis: beginning of the third decade, J. Alzheimers Dis. 64 (2018) S567–S610.
[90] D. Nuzzo, P. Picone, S. Baldassano, L. Caruana, E. Messina, A. Marino Gammazza, F. Cappello, F. Mulè, M. Di Carlo, Insulin resistance as Common molecular denominator linking obesity to Alzheimer's disease, Curr. Alzheimer Res. 12 (2015) 723–735.
[91] O. Busquets, M. Ettcheto, M. Pallàs, C. Beas-Zarate, E. Verdaguer, C. Auladell, J. Folch, A. Camins, Long-term exposition to a high fat diet favors the appearance of β-amyloid depositions in the brain of C57BL/6J mice. A potential model of sporadic Alzheimer's disease, Mech. Ageing Dev. 162 (2017) 38–45.
[92] S.E. Arnold, I. Lucki, B.R. Brookshire, G.C. Carlson, C.A. Browne, H. Kazi, S. Bang, B.R. Choi, Y. Chen, M.F. McMullen, S.F. Kim, High fat diet produces brain insulin resistance, synaptodendritic abnormalities and altered behavior in mice, Neurobiol. Dis. 67 (2014) 79–87.
[93] S. Hao, A. Dey, X. Yu, A.M. Stranahan, Dietary obesity reversibly induces synaptic stripping by microglia and impairs hippocampal plasticity, Brain Behav. Immun. 51 (2016) 230–239.
[94] D.M. Osborne, D.P. Fitzgerald, K.E. O'Leary, B.M. Anderson, C.C. Lee, P.M. Tessier, E.C. McNay, Intrahippocampal administration of a domain antibody that binds aggregated amyloid-β reverses cognitive deficits produced by diet-induced obesity, Biochim. Biophys. Acta 1860 (2016) 1291–1298.
[95] E.M. Knight, I.V. Martins, S. Gümüsgöz, S.M. Allan, C.B. Lawrence, High-fat diet-induced memory impairment in triple-transgenic Alzheimer's disease (3xTgAD) mice is independent of changes in amyloid and tau pathology, Neurobiol. Aging 35 (2014) 1821–1832.
[96] M. Vandal, P.J. White, G. Chevrier, C. Tremblay, I. St-Amour, E. Planel, A. Marette, F. Calon, Age-dependent impairment of glucose tolerance in the 3xTg-AD mouse model of Alzheimer's disease, FASEB J. 29 (2015) 4273–4284.
[97] J. Wang, B.J. Gu, C.L. Masters, Y.J. Wang, A systemic view of Alzheimer disease - insights from amyloid-β metabolism beyond the brain, Nat. Rev. Neurol. 13 (2017) 612–623.
[98] Y. Zhang, B. Zhou, B. Deng, F. Zhang, J. Wu, Y. Wang, Y. Le, Q. Zhai, Amyloid-β induces hepatic insulin resistance in vivo via JAK2, Diabetes 62 (2013) 1159–1166.
[99] Y. Zhang, B. Zhou, F. Zhang, J. Wu, Y. Hu, Y. Liu, Q. Zhai, Amyloid-β induces hepatic insulin resistance by activating JAK2/STAT3/SOCS-1 signaling pathway, Diabetes 61 (2012) 1434–1443.
[100] L. Zhao, B. Teter, T. Morihara, G.P. Lim, S.S. Ambegaokar, O.J. Ubeda, S.A. Frautschy G.M., Cole GM. Insulin-degrading enzyme as a downstream target of insulin receptor signaling cascade: implications for Alzheimer's disease intervention, J. Neurosci. 24 (2004) 11120–11126.
[101] Z. Liu, I.Y. Patil, T. Jiang, H. Sancheti, J.P. Walsh, B.L. Stiles, F. Yin, E. Cadenas, High-fat diet induces hepatic insulin resistance and impairment of synaptic plasticity, PLoS One 10 (2015) 10 e0128274.
[102] H.H. Ruiz, T. Chi, A.C. Shin, C. Lindtner, W. Hsieh, M. Ehrlich, S. Gandy, C. Buettner, Increased susceptibility to metabolic dysregulation in a mouse model of Alzheimer's disease is associated with impaired hypothalamic insulin signaling and elevated BCAA levels, Alzheimers Dement. 12 (2016) 851–861.
[103] L. Katsouri, K. Blondrath, M. Sastre, Peroxisome proliferator-activated receptor-γ cofactors in neurodegeneration, IUBMB Life 64 (2012) 958–964.
[104] L. Katsouri, Y.M. Lim, K. Blondrath, I. Eleftheriadou, L. Lombardero, A.M. Birch, N. Mirzaei, E.E. Irvine, N.D. Mazarakis, M. Sastre, PPARγ-coactivator-1α gene transfer reduces neuronal loss and amyloid-β generation by reducing β-secretase in an Alzheimer's disease model, Proc. Natl. Acad. Sci. U S A. 113 (2016) 12292–12297.
[105] L. Katsouri, C. Parr, N. Bogdanovic, M. Willem, M. Sastre, PPARγ co-activator-1α (PGC-1α) reduces amyloid-β generation through a PPARγ-dependent mechanism, J. Alzheimers Dis. 25 (2011) 151–162.
[106] W. Qin, V. Haroutunian, P. Katsel, C.P. Cardozo, L. Ho, J.D. Buxbaum, G.M. Pasinetti, PGC-1alpha expression decreases in the Alzheimer disease brain as a function of dementia, Archives Neurol. 66 (66) (2009) 352–361.
[107] I.V. Martins, J. Rivers-Auty, S.M. Allan, C.B. Lawrence, Mitochondrial abnormalities and synaptic loss underlie memory deficits seen in mouse models of obesity and Alzheimer's disease, J. Alzheimers Dis. 55 (2017) 915–932.
[108] S.K. Sah, C. Lee, J.H. Jang, G.H. Park, Effect of high-fat diet on cognitive impairment in triple-transgenic mice model of Alzheimer's disease, Biochem. Biophys. Res. Commun. 493 (2017) 731–736.
[109] B. Lin, Y. Hasegawa, K. Takane, N. Koibuchi, C. Cao, S. Kim-Mitsuyama, High-fat-diet intake enhances cerebral amyloid angiopathy and cognitive impairment in a mouse model of Alzheimer's disease, independently of metabolic disorders, J. Am. Heart Assoc. 5 (2016) 5 pii: e003154.
[110] P. Thériault, A. ElAli, S. Rivest, High fat diet exacerbates Alzheimer's disease-related pathology in APPswe/PS1 mice, Oncotarget 7 (2016) 67808–67827.
[111] V. Kothari, Y. Luo, T. Tornabene, A.M. O'Neill, M.W. Greene, T. Geetha, J.R. Babu, High fat diet induces brain insulin resistance and cognitive impairment in mice, Biochim. Biophys. Acta 1863 (2017) 499–508.
[112] M. Kohjima, Y. Sun, L. Chan, Increased food intake leads to obesity and insulin resistance in the tg2576 Alzheimer's disease mouse model, Endocrinology 151 (2010) 1532–1540.
[113] L.E. Lyn-Cook, M.Jr. Lawton, M. Tong, E. Silbermann, L. Longato, P. Jiao, P. Mark, J.R. Wands, H. Xu, S.M. de la Monte, Hepatic ceramide May mediate brain insulin resistance and neurodegeneration in type 2 diabetes and Non-alcoholic steatohepatitis, J. Alzheimers Dis. 16 (2009) 715–729.
[114] I. Arrieta-Cruz, R. Gutiérrez-Juárez, The role of insulin resistance and glucose metabolism dysregulation in the development of Alzheimer's disease, Rev. Investig. Clin. 68 (2016) 53–58.
[115] I. Arrieta-Cruz, C.M. Knight, R. Gutiérrez-Juárez, Acute exposure of the mediobasal hypothalamus to amyloid-β25–35 perturbs hepatic glucose metabolism, J. Alzheimers Dis. 46 (2015) 843–848.
[116] J.R. Clarke, E. Lyra, N.M. Silva, C.P. Figueiredo, R.L. Frozza, J.H. Ledo, D. Beckman, C.K. Katashima, D. Razolli, B.M. Carvalho, R. Frazão, M.A. Silveira, F.C. Ribeiro, T.R. Bomfim, F.S. Neves, W.L. Klein, R. Medeiros, F.M. LaFerla, J.B. Carvalheira, M.J. Saad, D.P. Munoz, L.A. Velloso, S.T. Ferreira, F.G. De Felice, Alzheimer-associated aβ oligomers impact the central nervous system to induce peripheral metabolic deregulation, EMBO Mol. Med. 7 (2015) 190–210.
[117] A.E. Roher, C.L. Esh, T.A. Kokjohn, E.M. Castaño, G.D. Van Vickle, W.M. Kalback, R.L. Patton, D.C. Luehrs, I.D. Daugs, Y.M. Kuo, M.R. Emmerling, H. Soares, J.F. Quinn, J. Kaye, D.J. Connor, N.B. Silverberg, C.H. Adler, J.D. Seward, T.G. Beach, M.N. Sabbagh, Amyloid beta peptides in human plasma and tissues and their significance for Alzheimer's disease, Alzheimers Dement. 5 (2009) 18–29.
[118] J. Miklossy, H. Qing, A. Radenovic, A. Kis, B. Vileno, F. Làszló, L. Miller, R.N. Martins, G. Waeber, V. Mooser, F. Bosman, K. Khalili, N. Darbinian, P.L. McGeer, Beta amyloid and hyperphosphorylated tau deposits in the pancreas in type 2 diabetes, Neurobiol. Aging 31 (2010) 1503–1515.
[119] M. Shinohara, N. Sato, Bidirectional interactions between diabetes and Alzheimer's disease, Neurochem Int. 108 (2017) 296–302.
[120] C. Duran-Aniotz, V.H. Cornejo, S. Espinoza, Á.O. Ardiles, D.B. Medinas, C. Salazar, A. Foley, I. Gajardo, P. Thielen, T. Iwawaki, W. Scheper, C. Soto, A.G. Palacios, J.J.M. Hoozemans, C. Hetz, IRE1 signaling exacerbates Alzheimer's disease pathogenesis, Acta Neuropathol. 134 (2017) 489–506.
[121] C. Duran-Aniotz, G. Martínez, C. Hetz, Memory loss in Alzheimer' s disease: are the alterations in the UPR network involved in the cognitive impairment? Front. Aging Neurosci. 6 (2014) 8.
[122] V.H. Cornejo, C. Hetz, The unfolded protein response in Alzheimer's disease, Semin Immunopathol. 35 (2013) 277–292.
[123] R.C. Chang, K.C. Suen, C.H. Ma, W. Elyaman, H.K. Ng, J. Hugon, Involvement of double-stranded RNA-dependent protein kinase and phosphorylation of eukaryotic initiation factor-2alpha in neuronal degeneration, J. Neurochem. 83 (2002) 1215–1225.
[124] Y. Gerakis, C. Hetz, Emerging roles of ER stress in the etiology and pathogenesis of Alzheimer's disease, FEBS J. 285 (2018) 995–1011.
[125] C. Hetz, S. Saxena, ER stress and the unfolded protein response in neurodegeneration, Nat. Rev. Neurol. 13 (2017) 477–491.
[126] T. O'Connor, K.R. Sadleir, E. Maus, R.A. Velliquette, J. Zhao, S.L. Cole, W.A. Eimer, B. Hitt, L. Bembinster, S. Lammich, S.F. Lichtenthaler, S.S. Hébert, B. De Strooper, C. Haass, D.A. Bennett, R. Vassar, Phosphorylation of the translation initiation factor eIF2a increases BACE1 levels and promotes amyloidogenesis, Neuron 60 (2008) 988–1009.
[127] V. Valenzuela, G. Martínez, C. Duran-Aniotz, C. Hetz, Gene therapy to target ER stress in brain diseases, Brain Res. 1648 (2016) 561–570.
[128] J. Lu, D.M. Wu, Y.L. Zheng, B. Hu, W. Cheng, Z.F. Zhang, Q. Shan, Ursolic acid improves high fat diet-induced cognitive impairments by blocking endoplasmic reticulum stress and IκB kinase β/nuclear factor-κB-mediated inflammatory pathways in mice, Brain Behav. Immun. 25 (201) (2011) 1658–1667.
[129] J. Hugon, F. Mouton-Liger, J. Dumurgier, C. Paquet, PKR involvement in Alzheimer's disease, Alzheimers Res. Ther. 9 (2017) 83.
[130] K.D. Hwang, M.S. Bak, S.J. Kim, S. Rhee, Y.S. Lee, Restoring synaptic plasticity and memory in mouse models of Alzheimer's disease by PKR inhibition, Mol. Brain 10 (2017) 57.
[131] L. Devi, M. Ohno, Phospho-eIF2a level is important for determining abilities of BACE1 reduction to rescue cholinergic neurodegeneration and memory defects in 5XFAD mice, PLoS One 5 (2010) e12974.
[132] L. Devi, M. Ohno, Mechanisms that lessen benefits of β-secretase reduction in a mouse model of Alzheimer's disease, Transl. Psychiatry 3 (2013) e284.
[133] L. Devi, M. Ohno, PERK mediates eIF2α phosphorylation responsible for BACE1 elevation, CREB dysfunction and neurodegeneration in a mouse model of Alzheimer's disease, Neurobiol. Aging 35 (2014) 2272–2281.
[134] W. Yang, X. Zhou, H.R. Zimmermann, D.R. Cavener, E. Klann, T. Ma, Repression of the eIF2α kinase PERK alleviates mGluR-LTD impairments in a mouse model of Alzheimer's disease, Neurobiol. Aging 41 (2016) 19–24.
[135] F. Mouton-Liger, C. Paquet, J. Dumurgier, C. Bouras, L. Pradier, F. Gray, J. Hugon, Oxidative stress increases BACE1 protein levels through activation of the PKR-eIF2α pathway, Biochim. Biophys. Acta 1822 (2012) 885–896.
[136] T. Ma, M.A. Trinh, A.J. Wexler, C. Bourbon, E. Gatti, P. Pierre, D.R. Cavener, E. Klann, Suppression of eIF2α kinases alleviates Alzheimer's disease-related plasticity and memory deficits, Nat. Neurosci. 16 (2013) 1299–1305.

[137] C. Sims-Robinson, A. Bakeman, R. Glasser, J. Boggs, C. Pacut, E.L. Feldman, The role of endoplasmic reticulum stress in hippocampal insulin resistance, Exp. Neurol. 277 (2016) 261–267.
[138] O. Busquets, M. Ettcheto, E. Verdaguer, R.D. Castro-Torres, C. Auladell, C. Beas-Zarate, J. Folch, A. Camins, JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid, Neuropharmacology 131 (2018) 440–452.
[139] L. Liang, J. Chen, L. Zhan, X. Lu, X. Sun, H. Sui, L. Zheng, H. Xiang, F. Zhang, Endoplasmic reticulum stress impairs insulin receptor signaling in the brains of obese rats, PLoS One 10 (5) (2015) e0126384.
[140] K. Ohta, A. Mizuno, S. Li, M. Itoh, M. Ueda, E. Ohta, Y. Hida, M.X. Wang, M. Furoi, Y. Tsuzuki, M. Sobajima, Y. Bohmoto, T. Fukushima, M. Kobori, T. Inuzuka, T. Nakagawa, Endoplasmic reticulum stress enhances γ-secretase activity, Biochem. Biophys. Res. Commun. 416 (2011) 362–366.
[141] M. Hayakawa, M. Itoh, K. Ohta, S. Li, M. Ueda, M.X. Wang, E. Nishida, S. Islam, C.S. Suzuki, K. Ohzawa, M. Kobori, T. Inuzuka, T. Nakagawa, Quercetin reduces eIF2α phosphorylation by GADD34 induction, Neurobiol. Aging 36 (2015) 2509–2518.
[142] H. Gao, P. Yan, S. Zhang, S. Nie, F. Huang, H. Han, Q. Deng, Q. Huang, W. Yang, H. Wu, P. Yao, K. Ye, J. Xu, L. Liu, Chronic alpha-linolenic acid treatment alleviates age-associated neuropathology: roles of PERK/eIF2α signaling pathway, Brain Behav Immun. 57 (2016) 314–325.
[143] T. Wyss-Coray, Inflammation in Alzheimer disease: driving force, bystander or beneficial response? Nat. Med. 12 (2006) 1005–1015.
[144] O. Guillemot-Legris, G.G. Muccioli, Obesity-induced neuroinflammation: beyond the hypothalamus, Trends Neurosci. 40 (2017) 237–253.
[145] U.K. Hanisch, H. Kettenmann, Microglia: active sensor and versatile effector cells in the normal and pathologic brain, Nat. Neurosci. 10 (2007) 1387–1394.
[146] W.J. Streit, Q.S. Xue, J. Tischer, I. Bechmann, Microglial pathology, Acta Neuropathol. Commun. 26 (2014) 2 142.
[147] M.L. Block, L. Calderón-Garcidueñas, Air pollution: mechanisms of neuroinflammation and CNS disease, Trends Neurosci. 32 (2009) 506–516.
[148] M. Schwartz, O. Butovsky, W. Brück, U.K. Hanisch, Microglial phenotype: is the commitment reversible? Trends Neurosci. 29 (2006) 68–74.
[149] F. Zhang, L. Jiang, Neuroinflammation in Alzheimer's disease, Neuropsychiatr. Dis. Treat. 11 (2015) 243–245.
[150] W. Swardfager, K. Lanctôt, L. Rothenburg, A. Wong, J. Cappell, N. Herrmann, A meta-analysis of cytokines in Alzheimer's disease, Biol. Psychiatry 68 (2010) 930–941.
[151] A. Carreras-Sureda, P. Pihán, C. Hetz, Calcium signaling at the endoplasmic reticulum: fine-tuning stress responses, Cell Calcium 70 (2018) 24–31.
[152] G. Castro, M.F. Areias, L. Weissmann, P.G. Quaresma, C.K. Katashima, M.J. Saad, P.O. Prada, Diet-induced obesity induces endoplasmic reticulum stress and insulin resistance in the amygdala of rats, FEBS Open Bio. 11 (2013) 443–449.
[153] G.S. Hotamisligil, Endoplasmic reticulum stress and the inflammatory basis of metabolic disease, Cell 140 (2010) 900–917.
[154] J. Benzler, G.K. Ganjam, K. Legler, S. Stöhr, M. Krüger, J. Steger, A. Tups, Acute inhibition of central c-Jun N-terminal kinase restores hypothalamic insulin signalling and alleviates glucose intolerance in diabetic mice, J. Neuroendocrinol. 25 (2013) 446–454.
[155] U. Ozcan, Q. Cao, E. Yilmaz, A.H. Lee, N.N. Iwakoshi, E. Ozdelen, G. Tuncman, C. Görgün, L.H. Glimcher, G.S. Hotamisligil, Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes, Science 306 (2004) 457–461.
[156] M. Cissé, E. Duplan, F. Checler, The transcription factor XBP1 in memory and cognition: implications in Alzheimer disease, Mol. Med. 4 (2017) 22.
[157] D. Popov, Endoplasmic reticulum stress and the onsite function of resident PTP1B, Biochem. Biophys. Res. Commun. 422 (2012) 535–538.
[158] G.J. Song, M. Jung, J.H. Kim, H. Park, M.H. Rahman, S. Zhang, Z.Y. Zhang, D.H. Park, I.K. Lee, K. Suk, A novel role for protein tyrosine phosphatase 1B as a positive regulator of neuroinflammation, J. Neuroinflammation 13 (2016) 86.
[159] Y.M. Jeon, S. Lee, S. Kim, Y. Kwon, K. Kim, C.G. Chung, S. Lee, S.B. Lee, H.J. Kim, Neuroprotective effects of protein tyrosine phosphatase 1B inhibition against ER stress- induced toxicity, Mol. Cells 40 (2017) 1e11.
[160] Y.F. Mao, Z. Guo, T. Zheng, Y. Jiang, Y. Yan, X. Yin, Y. Chen, B. Zhang, Intranasal insulin alleviates cognitive deficits and amyloid pathology in young adult APPswe/PS1dE9 mice, Aging Cell 15 (2016) 893–902.
[161] S. Craft, L.D. Baker, T.J. Montine, S. Minoshima, G.S. Watson, A. Claxton, M. Arbuckle, M. Callaghan, E. Tsai, S.R. Plymate, P.S. Green, J. Leverenz, D. Cross, B. Gerton, Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial, Arch. Neurol. 69 (2012) 29–38.
[162] S. Craft, A. Claxton, L.D. Baker, A.J. Hanson, B. Cholerton, E.H. Trittschuh, D. Dahl, E. Caulder, B. Neth, T.J. Montine, Y. Jung, J. Maldjian, C. Whitlow, S. Friedman, Effects of regular and long-acting insulin on cognition and Alzheimer's disease biomarkers: a pilot clinical trial, J. Alzheimers Dis. 57 (2017) 1325–1334.
[163] V. Ott, C. Benedict, B. Schultes, J. Born, M. Hallschmid, Intranasal administration of insulin to the brain impacts cognitive function and peripheral metabolism, Diabetes Obes. Metab. 14 (2012) 214–221.
[164] P.L. McClean, Jalewa J, C. Hölscher, Prophylactic liraglutide treatment prevents amyloid plaque deposition, chronic inflammation and memory impairment in APP/PS1 mice, Behav. Brain Res. 293 (2015) 96–106.
[165] M. Bomba, D. Ciavardelli, E. Silvestri, L.-M. Canzoniero, R. Lattanzio, P. Chiappini, M. Piantelli, C. Di Ilio, A. Consoli, S.L. Sensi, Exenatide promotes cognitive enhancement and positive brain metabolic changes in PS1-KI mice but has no effects in 3xTg-AD animals, Cell Death Dis. 2 (2013) 4 e612.
[166] A.M. Duffy, C. Hölscher, The incretin analogue D-Ala2GIP reduces plaque load, astrogliosis and oxidative stress in an APP/PS1 mouse model of Alzheimer's disease, Neuroscience 228 (2013) 294–300.
[167] T. Li, J.J. Jiao, C. Hölscher, M.N. Wu, J. Zhang, J.Q. Tong, X.F. Dong, X.S. Qu, Y. Cao, H.Y. Cai, Q. Su, J.S. Qi, A novel GLP-1/GIP/Gcg triagonist reduces cognitive deficits and pathology in the 3xTg mouse model of Alzheimer's disease, Hippocampus 28 (2018) 358–372.
[168] C. Rotermund, G. Machetanz, J.C. Fitzgerald, The therapeutic potential of metformin in neurodegenerative diseases, Front. Endocrinol. 19 (2018) 9 400.
[169] Z. Ou, X. Kong, X. Sun, X. He, L. Zhang, Z. Gong, J. Huang, B. Xu, D. Long, J. Li, Q. Li, L. Xu, A. Xuan, Metformin treatment prevents amyloid plaque deposition and memory impairment in APP/PS1 mice, Brain Behav. Immun. 69 (2018) 351–363.
[170] E. Barini, O. Antico, Y. Zhao, F. Asta, V. Tucci, T. Catelani, R. Marotta, H. Xu, L. Gasparini, Metformin promotes tau aggregation and exacerbates abnormal behavior in a mouse model of tauopathy, Mol. Neurodegener. 11 (2016) 16.
[171] C. Infante-Garcia, J.J. Ramos-Rodriguez, C. Hierro-Bujalance, E. Ortegon, E. Pickett, R. Jackson, F. Hernandez-Pacho, T. Spires-Jones, M. Garcia-Alloza, Antidiabetic polypill improves Central pathology and cognitive impairment in a mixed model of Alzheimer's disease and type 2 diabetes, Mol. Neurobiol. 55 (2018) 6130–6144.
[172] A.M. Koenig, D. Mechanic-Hamilton, S.X. Xie, M.F. Combs, A.R. Cappola, L. Xie, J.A. Detre, D.A. Wolk, S.E. Arnold, Effects of the insulin sensitizer metformin in Alzheimer disease: pilot data from a randomized placebo-controlled crossover study, Alzheimer Dis. Assoc. Disord. 31 (2017) 107–113.
[173] S. Xu, G. Liu, X. Bao, J. Wu, S. Li, B. Zheng, R. Anwyl, Q. Wang, Rosiglitazone prevents amyloid-β oligomer-induced impairment of synapse formation and plasticity via increasing dendrite and spine mitochondrial number, J. Alzheimers Dis. (2014) 239–251.
[174] M. Gold, C. Alderton, M. Zvartau-Hind, S. Egginton, Saunders A.M, M. Irizarry, S. Craft, G. Landreth, U. Linnamägi, S. Sawchak, Rosiglitazone monotherapy in mild-to-moderate Alzheimer's disease: results from a randomized, double-blind, placebo-controlled phase III study, Dement. Geriatr. Cogn. Disord. 30 (2010) 131–146.
[175] C. Harrington, S. Sawchak, C. Chiang, J. Davies, C. Donovan, A.M. Saunders, M. Irizarry, B. Jeter, M. Zvartau-Hind, C.H. van Dyck, M. Gold, Rosiglitazone does not improve cognition or global function when used as adjunctive therapy to AChE inhibitors in mild-to-moderate Alzheimer's disease: two phase 3 studies, Curr. Alzheimer Res. 8 (2011) 592–606.
[176] M.T. Heneka, A. Fink, G. Doblhamme, Effect of pioglitazone medication on the incidence of dementia, Ann. Neurol. 78 (2015) 284–294.
[177] C.M. Fernandez-Martos, R.A.K. Atkinson, M.I. Chuah, A.E. King, J.C. Vickers, Combination treatment with leptin and pioglitazone in a mouse model of Alzheimer's disease, Alzheimers Dement. 3 (2016) 92–106.
[178] D.S. Geldmacher, T. Fritsch, M.J. McClendon, G. Landreth, A randomized pilot clinical trial of the safety of pioglitazone in treatment of patients with Alzheimer disease, Arch. Neurol. 68 (68) (2011) 45–50.
[179] B.W. Miller, K.C. Willett, A.R. Desilets, Rosiglitazone and pioglitazone for the treatment of Alzheimer's disease, Ann. Pharmacother. 45 (2011) 1416–1424.
[180] T. Sato, K. Hanyu, H. Kanetaka, H. Sakurai, T. Iwamoto, Efficacy of PPAR-gamma agonist pioglitazone in mild Alzheimer disease, Neurobiol. Aging 32 (2011) 1626–1633.
[181] J. Toba, M. Nikkuni, M. Ishizeki, A. Yoshii, N. Watamura, T. Inoue, T. Ohshima, PPARγ agonist pioglitazone improves cerebellar dysfunction at pre-aβ deposition stage in APPswe/PS1dE9 Alzheimer's disease model mice, Biochem. Biophys. Res. Commun. 473 (2016) 1039–1044.
[182] Z. Nowakowska, A review of anti-infective and anti-inflammatory chalcones, Eur. J. Med. Chem. 42 (2007) 125–137.
[183] M.J. Matos, S. Vazquez-Rodriguez, E. Uriarte, L. Santana, Potential pharmacological uses of chalcones: a patent review (from June 2011-2014), Expert Opin. Ther. Pat. 25 (2015) 351–366.
[184] H.N. Lee, H.J. Cho, D.Y. Lim, Y.H. Kang, K.W. Lee, J.H. Park, Mechanisms by which licochalcone e exhibits potent anti-inflammatory properties: studies with phorbol ester-treated mouse skin and lipopolysaccharide-stimulated murine macrophagesv, Int. J. Mol. Sci. 14 (2013) 10926–10943.
[185] L. Wang, R. Yang, B. Yuan, Y. Liu, C. Liu, The antiviral and antimicrobial activities of licorice, a widely used Chinese herb, Acta Pharm. Sin. B 5 (2015) 310–315.
[186] S.S. Kim, J. Lim, Y. Bang, J. Gal, S.U. Lee, Y.C. Cho, G. Yoon, B.Y. Kang, S.H. Cheon, H.J. Choi, Licochalcone E activates Nrf2/antioxidant response element signaling pathway in both neuronal and microglial cells: therapeutic relevance to neurodegenerative disease, J. Nutr. Biochem. 23 (2012) 1314–1323.
[187] X. Chu, L. Jiang, M. Wei, X. Yang, M. Guan, X. Xie, J. Wei, D. Liu, D. Wang, Attenuation of allergic airway inflammation in A murine model of asthma by licochalcone A, Immunopharmacol. Immunotoxicol. 35 (2013) 653–661.
[188] M.M. Rafi, R.T. Rosen, A. Vassil, C.T. Ho, H. Zhang, G. Ghai, G. Lambert, R.S. Di Paola, Modulation of bcl-2 and cytotoxicity by licochalcone-A, a novel estrogenic flavonoid, Anticancer Res. 20 (2000) 2653–2658.
[189] K.H. Jeon, E. Lee, K.Y. Jun, J.E. Eom, S.Y. Kwak, Y. Na, Y. Kwon, Neuroprotective effect of synthetic chalcone derivatives as competitive dual inhibitors against μ-calpain and cathepsin B through the downregulation of tau phosphorylation and insoluble aβ peptide formation, Eur. J. Med. Chem. 121 (2016) 433–444.
[190] Z. Cao, J. Yang, R. Xu, Q. Song, X. Zhang, H. Liu, X. Qiang, Y. Li, Z. Tan, Y. Deng, Design, synthesis and evaluation of 4'-OH-flurbiprofen-chalcone hybrids as potential multifunctional agents for Alzheimer's disease treatment, Bioorg. Med. Chem. 26 (2018) 1102–1115.
[191] S. Gao, S. Howard, P.V. LoGrasso, Pharmacological inhibition of c-jun N-terminal kinase reduces food intake and sensitizes leptin's anorectic signaling actions, Sci.

Rep. 7 (2017) 41795.
[192] S.L. Macauley, M. Stanley, E.E. Caesar, S.A. Yamada, M.E. Raichle, R. Perez, T.E. Mahan, C.L. Sutphen, D.M. Holtzman, Hyperglycemia modulates extracellular amyloid-β concentrations and neuronal activity in vivo, J. Clin. Invest. 125 (2015) 2463–2467.
[193] G.J. Biessels, L.P. Reagan, Hippocampal insulin resistance and cognitive dysfunction, Nat. Rev. Neurosci. 16 (2015) 660–671.
[194] C.L. Masters, R. Bateman, K. Blennow, C.C. Rowe, R.A. Sperling, J.L. Cummings, Alzheimer's disease, Nat. Rev. Dis. Primers. 1 (2015) 15056.
[195] M.M. Mielke, V.V. Bandaru, N.J. Haughey, J. Xia, L.P. Fried, S. Yasar, M. Albert, V. Varma, G. Harris, E.B. Schneider, P.V. Rabins, K. Bandeen-Roche, C.G. Lyketsos, M.C. Carlson, Serum ceramides increase the risk of Alzheimer disease: the women's health and aging study II, Neurology 79 (2012) 633–641.
[196] T.A. Dar, I.A. Sheikh, S.A. Ganie, R. Ali, L.R. Singh, S.H. Gan, M.A. Kamal, M.A. Zargar, Molecular linkages between diabetes and Alzheimer's disease: current scenario and future prospects, CNS Neurol. Disord. Drug Targets 13 (2014) 290–298.
[197] C.D. Chapman, H.B. Schiöth, C.A. Grillo, C. Benedict, Intranasal insulin in Alzheimer's disease: food for thought, Neuropharmacology 136 (2018) 196–201.
[198] A. Claxton, L.D. Baker, A. Hanson, E.H. Trittschuh, B. Cholerton, A. Morgan, M. Callaghan, Arbuckle M, C. Behl, S. Craft, Long-acting intranasal insulin detemir improves cognition for adults with mild cognitive impairment or early-stage Alzheimer's disease dementia, J. Alzheimers Dis. 44 (2015) 897–906.
[199] Q. Jiang, M. Heneka, G.E. Landreth, The role of peroxisome proliferator-activated receptor-gamma (PPARgamma) in Alzheimer's disease: therapeutic implications, CNS Drugs 22 (2008) 1–14.
[200] T. Ma, X. Du, J.E. Pick, G. Sui, M. Brownlee, E. Klann, Glucagon-like peptide-1 cleavage product GLP-1(9-36) amide rescues synaptic plasticity and memory deficits in Alzheimer's disease model mice, J. Neurosci. 32 (2012) 13701–13708.
[201] I. Moreno-Gonzalez, G. Edwards, N. Salvadores, M. Shahnawaz, R. Diaz-Espinoza, C. Soto, Molecular interaction between type 2 diabetes and Alzheimer's disease through cross-seeding of protein misfolding, Mol. Psychiatry 22 (2017) 1327–1334.
[202] S. Merlo, S. Spampinato, P.L. Canonico, A. Copani, M.A. Sortino, Alzheimer's disease: brain expression of a metabolic disorder? Trends Endocrinol. Metab. 21 (2010) 537–544.
[203] A. Salminen, A. Kauppinen, T. Suuronen, K. Kaarniranta, J. Ojala, ER stress in alzheimer's disease: a novel neuronal trigger for inflammation and alzheimer's pathology, J Neuroinflammation 6 (2009) 41.
[204] Q.L. Ma, F. Yang, E.R. Rosario, O.J. Ubeda, W. Beech, D.J. Gant, P.P. Chen, B. Hudspeth, C. Chen, Y. Zhao, H.V. Vinters, S.A. Frautschy, G.M. Cole, Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin, J. Neurosci. 29 (2009) 9078–9089.
[205] J. Furusawa, M. Funakoshi-Tago, T. Mashino, K. Tago, H. Inoue, Y. Sonoda, T. Kasahara, Glycyrrhiza inflata-derived chalcones, licochalcone A, licochalcone B and licochalcone D, inhibit phosphorylation of NF-kappaB p65 in LPS signaling pathway, Int. Immunopharmacol. 9 (2009) 499–507.
[206] K. Yao, H. Chen, M.H. Lee, H. Li, W. Ma, C. Peng, N.R. Song, K.W. Lee, A.M. Bode, Z. Dong, Z. Dong, A. Licochalcone, A natural inhibitor of c-jun N-terminal kinase 1, Cancer Prev. Res. (Phila) 7 (2014) 139–149.
[207] E.J. Yang, G.H. Park, K.S. Song, Neuroprotective effects of liquiritigenin isolated from licorice roots on glutamate-induced apoptosis in hippocampal neuronal cells, Neurotoxicology 39 (2013) 114–123.
[208] C. Zou, Y. Shi, J. Ohli, U. Schüller, M.M. Dorostkar, J. Herms, Neuroinflammation impairs adaptive structural plasticity of dendritic spines in a preclinical model of Alzheimer's disease, Acta Neuropathol. 131 (2016) 235–246.
[209] L.S. Honig, B. Vellas, M. Woodward, M. Boada, R. Bullock, M. Borrie, K. Hager, V. Andreasen, E. Scarpini, H. Liu-Seifert, M. Case, R.A. Dean, A. Hake, K. Sundell, V. Poole Hoffmann, C. Carlson, R. Khanna, M. Mintun, R. DeMattos, K.J. Selzler, E. Siemers, Trial of solanezumab for mild dementia due to Alzheimer's disease, N. Engl. J. Med. 378 (2018) 321–330.
[210] M. Yarchoan, J.B. Toledo, E.B. Lee, Z. Arvanitakis, H. Kazi, L.Y. Han, N. Louneva, V.M. Lee, S.F. Kim, J.Q. Trojanowski, S.E. Arnold, Abnormal serine phosphorylation of insulin receptor substrate 1 is associated with tau pathology in Alzheimer's disease and tauopathies, Acta Neuropathol. 128 (2014) 679–689.
[211] A. Claxton, L.D. Baker, C.W. Wilkinson, E.H. Trittschuh, D. Chapman, G.S. Watson, B. Cholerton, S.R. Plymate, M. Arbuckle, S. Craft, Sex and ApoE genotype differences in treatment response to two doses of intranasal insulin in adults with mild cognitive impairment or Alzheimer's disease, J. Alzheimers Dis. 35 (2013) 789–797.
[212] M.A. Reger, G.S. Watson, W.H. Frey 2nd, L.D. Baker, B. Cholerton, M.L. Keeling, D.A. Belongia, M.A. Fishel, S.R. Plymate, G.D. Schellenberg, M.M. Cherrier, S. Craft, Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype, Neurobiol. Aging (2006) 451–458.
[213] M.A. Reger, G.S. Watson, P.S. Green, L.D. Baker, B. Cholerton, M.A. Fishe, S.R. Plymate, N.M. Cherrier, G.D. Schellenberg, W.H. Frey 2nd, S. Craft, Intranasal insulin administration dose-dependently modulates verbal memory and plasma amyloid-beta in memoryimpaired older adults, J. Alzheimer Dis. 13 (2008) 323–331.
[214] M.A. Reger, G.S. Watson, P.S. Green, C.W. Wilkinson, L.D. Baker, B. Cholerton, M.A. Fishel, S.R. Plymate, J.C. Breitner, W. DeGroodt, P. Mehta, S. Craft, Intranasal insulin improves cognition and modulates beta-amyloid in early AD, Neurology 70 (2008) 440–448.

Annex VI

Potential preventive disease-modifying pharmacological strategies to delay late onset Alzheimer' s disease.

Ettcheto M, Busquets O and Camins A.

Neuro Reg Res. 2019;14(10):1721–5.

NEURAL REGENERATION RESEARCH www.nrronline.org

REVIEW

Potential preventive disease-modifying pharmacological strategies to delay late onset Alzheimer's disease

Miren Ettcheto[1, 2, 3, 4], Oriol Busquets[1, 2, 3, 4], Antoni Camins[1, 3, 4, *]

1 Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain

2 Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain

3 Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain

4 Biomedical Research Networking Centre in Neurodegenerative Diseases (CIBERNED), Madrid, Spain

Funding: *The work was supported by the Spanish Ministry of Science and Innovation SAF2017-84283-R, PI2016/01, CB06/05/0024 (Biomedical Research Networking Centre in Neurodegenerative Diseases), and the European Regional Development Founds (all to AC).*

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease that was histopathologically characterized in the brain by the presence of extracellular senile plaques made of amyloid β peptides and intracellular neurofibrillary tangles composed of hyperphosphorylated Tau protein. Over the years, AD has been classified in two subgroups: early onset or familial AD and late onset or sporadic AD. On the one hand, familial AD has been described to be the result of genetic mutations that cause, in some cases, for the overproduction of amyloid β. On the other, the cause of late onset or sporadic AD is still unclear even though several hypotheses have been proposed to explain the process of severe and progressive memory and cognitive loss. In the present review, some of the current hypotheses that try to explain the origin of late onset or sporadic AD have been summarized. Also, their potential implication in the development of new drugs for the presymptomatic treatment of late onset or sporadic AD has been considered.

Key Words: *Alzheimer's disease; beta-secretase; neuroinflammation; Tau; amyloid; N-methyl-D-aspartate; glutamate*

***Correspondence to:**
Antoni Camins, PhD,
camins@ub.edu.

orcid:
0000-0002-1229-5956
(Antoni Camins)

doi: *10.4103/1673-5374.257513*

Received: *January 15, 2019*
Accepted: *March 27, 2019*

Introduction

After more than 100 years since Alzheimer's disease (AD) was first described, the scientific community has not been able to discover an effective molecule to modify or stop its progression (Hardy et al., 2014). Thus, the development of compounds to improve or alleviate neurodegeneration has become one of the major challenges for the future in the field of biomedicine (Cao et al., 2018).

In a recent review, all the drugs that are currently postulated as potentially effective to modify the evolution of AD have been summarized. From those, 26 are already on Phase III trials (Cummings et al., 2018) and, at least 50% of the total number of molecules that are currently under research are directly involved in the modulation of the amyloidogenic pathway. In **Figure 1**, some of the mechanisms in which amyloid beta (Aβ) is involved are depicted. It has been described that the oligomers promote microglial activation and promote neuroinflammation. Also, they can affect many other different intracellular organelles and pathways (mitochondria, endoplasmic reticulum, cell cycle, *etc*) that eventually lead to neuronal death and cognitive loss (Cline et al., 2018). The objective of Aβ-related treatments is the reduction of the levels of soluble oligomers by decreasing their production through the inhibition of beta-secretase (BACE-1) or gamma secretase or, by increasing the degradation and clearance of the oligomers. However, clinical trials with compounds like solanezumab, a humanized monoclonal antibody that recognizes soluble Aβ and increases its clearance from the brain, has failed to slow the clinical progression of mild-to-moderate AD patients (Honig et al., 2018). This data suggests that Aβ is not the only element involved in AD and it may actually be a cofactor that behaves as an aggravator while other alterations are the actual cause (Tse and Herrup 2017). Consequently, the fact that half of the research efforts are single-mindedly focused on the study of the amyloidogenic hypothesis does not favour the discovery of new beneficial therapies and strategies to treat and understand the disease. It is important to emphasise that 95% of AD cases are classified as sporadic or late-onset (LOAD) and, therefore, the previously mentioned drugs would only be potentially effective for the subtype labelled as genetic AD which accounts for the remaining 5%.

New research studies suggest that LOAD should be considered a disease with a multifactorial origin (Wijesekara et al., 2018). It is well known that aging is the main risk factor in its development, however, other mechanisms are involved in this process such as the production of reactive oxygen species in the mitochondria, the activation of the unfolded protein response in the endoplasmic reticulum and the upregulation of proinflammatory responses (Cummings et al., 2018; Swerdlow 2018). These alterations would

Ettcheto M, Busquets O, Camins A (2019) Potential preventive disease-modifying pharmacological strategies to delay late onset Alzheimer's disease. Neural Regen Res 14(10):1721-1725. doi:10.4103/1673-5374.257513

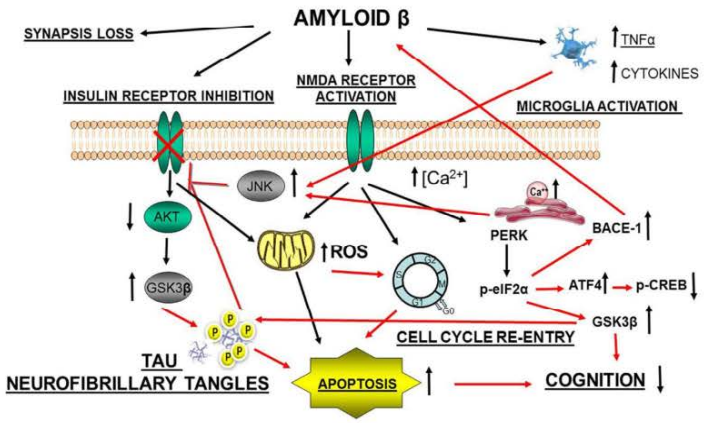


Figure 1 Schematic representation of potential molecular pathways involved in Aβ-induced brain neuronal apoptosis, microglial activation and cognitive affectation.
Aβ induces glial reactivity coupled with increased cytokine levels. TNFα activates JNK favoring IRS1 serine-inhibitory phosphorylation and posterior impaired insulin signaling. Alterations on normal activity of this axis also affects other downstream effectors like PI3K, PDK and AKT which are closely involved with synaptic function. Inhibition of AKT allows for activation of GSK3β, increasing abnormal Tau phosphorylation and posterior neuronal apoptosis. In addition, tyrosine residue phosphorylation by Aβ promotes insulin receptor internalization; exacerbating insulin signaling defects and promoting AD-related cognitive decline. Furthermore, Aβ induces prolonged endoplasmic reticulum stress and activation of the unfolded protein response (PERK/eIF2α/ATF4/CREB), which is also involved in cognitive loss phenomena. Finally, Aβ also promotes NMDA receptor activation, increase in cytosolic calcium levels and mitochondria and ER stress. In the end, all these alterations favor neuronal re-entry into the cell cycle and apoptosis. The relationship between the previously described mechanisms has been indicated in the figure by complete black and red arrows. Aβ: Amyloid beta; TNFα: tumor necrosis factor α; JNK: c-Jun N-terminal kinases; IRS-1: insulin receptor substrate; PI3K: phosphoinositide 3-kinase; PDK: phosphoinositide-dependent protein kinase; AKT: protein kinase B; GSK3β: glycogen synthase kinase 3β; PERK: protein kinase R-like endoplasmic reticulum kinase; eIF2α: eukaryotic initiation factor 2α; ATF4: activating transcription factor 4; CREB: cyclic adenosine monophosphate response element-binding protein; NMDA: N-methyl-D-aspartic acid.

favour cell cycle re-entry and the inhibition of autophagy, which would increase the accumulation of Aβ and the hyperphosphorylation of Tau. Thus, the key to treating LOAD will possibly be the result of the combination of multiple drugs that will modulate more than one molecular pathway at the same time (McDade and Bateman, 2017; Wenzel and Klegeris, 2018).

In the present review, we summarized available literature reviews found on the PubMed database. The search was not limited by any time period and was conducted between November 2018 and February 2019, with the objective to identify published peer-reviewed articles related to the topic in question in English. The collocated database search keywords were as follows: Alzheimer's disease AND type 2 diabetes, Neuroinflammation AND Alzheimer's disease, insulin AND cognitive decline, Alzheimer's disease treatments AND cognitive decline, Tau AND cognitive impairment, Tau AND Alzheimer's disease. The terms were searched using AND to combine the keywords listed and using OR to remove search duplication where possible.

The inclusion criteria were as follows: i) The period of the publishing of the article was limited by February, 2019. ii) Studies were only included from scientific journals in English. iii) The primary interest was focused on information about the delay of cognitive decline and Alzheimer's disease.

Alzheimer's Disease Treatments

Currently, acetylcholinesterase inhibitors and memantine are the only US Food and Drug Administration-approved drugs for the treatment of AD (Hampel et al., 2018). Acetylcholinesterase inhibitors increase acetylcholine levels by inhibiting the enzyme responsible of its degradation. This mechanism is expected to improve cognitive symptoms of AD by favouring the activation of cholinergic transmission even though, in advanced stages of the disease, accumulation of Aβ oligomers and glutamate cause overstimulation of glutamatergic receptors causing a massive entry of calcium into the cells and, as a result, excitotoxicity (Kodis et al., 2018). Recent studies have linked this pathological overstimulation with neuronal re-entry into the cell cycle by the activation of cyclins and cyclin-dependent kinases and posterior induction of apoptosis through the E2F-1 transcription factor. Memantine acts on the ionotropic receptors of glutamate, specifically the N-methyl-D-aspartic acid receptors, eliciting neuroprotective effects through its low-affinity antagonism. Nonetheless, neither of these drugs are effective on stopping the progression of the disease and only show temporary symptomatic improvements (Cao et al., 2018; Cummings et al., 2018). One of the hypothesis to explain the failure of the treatments until now is that their administration begins when the pathology

Ettcheto M, Busquets O, Camins A (2019) Potential preventive disease-modifying pharmacological strategies to delay late onset Alzheimer's disease. Neural Regen Res 14(10):1721-1725. doi:10.4103/1673-5374.257513

has already developed and, consequently, it is too late for it to have significant effects. Therefore, it is possible that a pre-emptive administration would have the desired effects that have been described in pre-clinical trials (McDade and Bateman, 2017).

Metabolic Hypothesis of Alzheimer's Disease

Other research groups have focused on the study of other hypotheses to understand the genesis of sporadic AD. For example, it has been described how resistance to insulin in the hippocampus favours cognitive loss (Ferreira et al., 2018). Thus, some research studies have postulated that LOAD should be considered a metabolic disease (Cardoso et al., 2017; Kang et al., 2017; Ferreira et al., 2018).

The first indications of this relationship were observed in the Rotterdam study where it was determined that type 2 diabetes mellitus patients showed a higher risk to suffer dementia than non-diabetic patients (Ott et al., 1996, 1999; Schrijvers et al., 2010). Also, research by Hoyer and colleagues had already described that the desensitization of the insulin receptor (IR) might be the reason for the onset of LOAD (Hoyer, 2002). This hypothesis states that the development of the pathology is due to the appearance of peripheral and central insulin resistance and it may be related to phenomena like obesity, which has been clearly associated with the appearance of cognitive decline. Likewise, it was reported that insulin resistance is associated with a decrease in brain glucose utilization in a late middle-age and can be used as a predictor for the eventual presence of Aβ deposition in multiple brain regions in middle-aged AD patients (Willette et al., 2015; McLimans et al., 2017). Other authors have described that Aβ can bind directly to hippocampal IR localized in postsynaptic terminals. These effects would further reinforce the idea that Aβ oligomers behave as pathology aggravators by inhibiting the activation of the IR or favoring its internalization (Bomfim et al., 2012; De Felice, 2013).

Thus, the amelioration of resistance in the IR would improve the cognitive process and synaptic function through the activation of protein kinase B (AKT), the inhibition of apoptosis and the modulation of mechanisms like the production of Aβ and neuroinflammation. Interestingly, several research studies have described beneficial effects on cognitive function after the use of antidiabetic drugs (De Felice et al., 2014; Ferreira et al., 2018).

For example, some studies administered intranasal insulin in AD patients. The aim was to improve cognition and brain glucose metabolism in people suffering from mild cognitive impairment or AD. However, results from that research study were not successful because continuous activation of the receptor by the hormone caused for its insensitization (De Felice et al., 2014, Clarke et al., 2015; Cardoso et al., 2017). Hence, several research groups concluded that the key is not in stimulating further the receptor but actually to modulate its activation through its regulatory mechanisms. The glucagon-like peptide-1 (GLP-1) receptor agonists such as exendin-4, liraglutide and lixisenatide, could be potential drugs able to improve the cognitive process in AD (Cardoso et al., 2017). The efficacy of liraglutide treatments have been evaluated in clinical trials and hopeful results in cognition activity have been reported. Glucose-dependent insulinotropic polypeptide (GIP) analogues such as D-Ala2-GIP are also under investigation and recently, it has been published that the development of dual peptide agonists GLP-1/glucagon and GLP-1/GIP and triagonists GLP-1/GCG/GIP is underway (Batista et al., 2019). Moreover, the anti-diabetic drug metformin has been described to be another suitable strategy to improve the cognitive process in LOAD (Markowicz-Piasecka et al., 2017). Several clinical trials are currently evaluating the potential efficacy of this drug in patients with mild cognitive impairment and AD (Cummings et al., 2018). It seems that metformin could improve slightly the cognitive process by favouring hepatic activity and improving insulin activity. Finally, thioazolidinediones are agonists of the peroxisome-proliferator activated receptors. Rosiglitazone and pioglitazone are examples of this group of drugs, which are also under investigation for the treatment of this disease (Cummings et al., 2018). Although preclinical studies suggest promising results, clinical studies with peroxisome-proliferator activated receptor γ agonists are required to confirm the potential efficacy of these molecules in improving brain insulin and insulin growth factor 1 resistance, as well as cognition in LOAD patients (Ferreira et al., 2018).

Neuroinflammatory Hypothesis of Alzheimer's Disease

Over the years, chronic neuroinflammation has been involved in synaptic dysfunctions. In preclinical models, it has been demonstrated that an obesogenic diet leads to loss of hippocampal synaptic plasticity, dendritic spine density and spatial memory through the phagocytosis of synaptic contacts due to increased microglia activity (De Felice et al., 2014; Hao et al., 2016; Rajendran and Paolicelli 2018). Furthermore, the release of cytokines such as tumor necrosis factor α favours cognitive loss through the activation of stress kinases such as the c-Jun N-terminal kinases (Vieira et al., 2018). These enzymes also favour the inactivation of the IR through the phosphorylation of the IR substrate at serine residues, blocking downstream insulin signalling.

Consequently, non-steroidal anti-inflammatory drugs should be considered as a potential strategy to treat neurodegenerative pathologies due to their relevant effect on systemic inflammatory processes (McGeer et al., 2018). These molecules are able to reduce microglial activation *via* canonical antiinflammatory pathways within the brain, decreasing cytokine levels and preventing synaptic loss by phagocytosis. Furthermore, non-steroidal anti-inflam-

Ettcheto M, Busquets O, Camins A (2019) Potential preventive disease-modifying pharmacological strategies to delay late onset Alzheimer's disease. Neural Regen Res 14(10):1721-1725. doi:10.4103/1673-5374.257513

matory drugs can modulate Aβ peptide formation in the brain through peroxisome-proliferator activated receptor γ activation and BACE-1 inhibition, as well as reduce Tau hyperphosphorylation (Ettcheto et al., 2017). Among the most studied non-steroidal anti-inflammatory drugs for the treatment of LOAD, ibuprofen, flurbiprofen and dexibuprofen should be considered while taking into account that neuroprotective effects through microglial inactivation only appear when drugs are administered before the appearance of clinical symptoms (in t' Veld et al., 2001).

Currently, a phase III clinical study on the combination of Cromolyn-Ibuprofen by AZ Therapies (Boston, MA, USA) is underway. The drug, namely ALZT-OP1, has a safe and tolerable profile and is aimed at treating patients with an early cognitive impairment (Cummings et al., 2018).

Tau Hypothesis of Alzheimer's Disease

Targeting Tau phosphorylation is another viable strategy for AD treatment. Preclinical studies suggest that cognitive loss is well related with an abnormal increase in Tau phosphorylation which, has been reported to have negative effects on metabolism (Marciniak et al., 2017). These results would further reinforce the hypotheses that define LOAD as a metabolic disease. Also, it supports other hypotheses that believe that these mechanisms work on systems based on positive feed-back cycles which would worsen progressively over time.

In a recent study, Preische and co-workers reported that the neurofilament light chain, a component of the axonal cytoskeleton expressed in myelinated axons, could be a suitable marker of brain damage and atrophy in preclinical models and neurodegenerative diseases (Preische et al., 2019). They suggest that neurofilament light chain levels could be mainly a fluid serum biomarker of disease progression and brain neurodegeneration at the early presymptomatic stages of familial AD. However, its clinically potential utility as biomarker in LOAD is unclear and may lead to confusion since it can also be altered by other pathologies.

TRx0237 is a Tau-related disease modifying drug in phase III clinical trials that is supposed to decrease neuronal damage mediated by Tau through the inhibition of its aggregation (Cummings et al., 2018).

Discussion

After taking into account some of this information, when considering the paradigm to understand LOAD, it is easy to picture that since the pathology has a multifactorial origin, it will require of more than one drug to treat it. Some researchers believe that the key will be found on the use of cocktails of different drugs, thus allowing for the modulation of different molecular mechanisms. Recent reports have also suggested that the administration of new hybrid compounds called multi-target-directed ligands might be an interesting approach (Wenzel and Klegeris 2018). New drug formulations may include N-methyl-D-aspartic acid antagonists, BACE-1 and acetylcholinesterase inhibitors and other modulators of the IR signalling pathway, neuroinflammatory responses and Tau aggregation (de la Monte et al., 2017; Cummings et al., 2018).

In addition, it is important to insist that current drugs show no significant therapeutic effects because they may be administered too late in the development of the pathology. So, it would be of high interest to determine early stage markers and indicators of the development of the pathology. As an example, recent studies have indicated that an increase of Aβ levels in saliva in LOAD patients can be detected (Lee et al., 2017). Some researchers have reported that treatments may need to be started pre-emptively 15 to 20 years before the actual appearance of clinical symptoms. Midlife may be a critical period for initiating treatments to improve peripheral IR signalling in order to maintain neural metabolism and cognitive function (Willete et al., 2015; Singh-Manoux et al., 2018).

To conclude, it is our belief that an effective AD preventive treatment shall include at least four drugs in a similar strategy to heart attack prevention.

Author contributions: *All the authors have participated in the drafting of the manuscript and have reviewed it.*
Conflicts of interest: *None of the authors has a conflict of interest.*
Financial support: *The work was supported by the Spanish Ministry of Science and Innovation SAF2017-84283-R, PI2016/01, CB06/05/0024 (Biomedical Research Networking Centre in Neurodegenerative Diseases), and the European Regional Development Founds (all to AC).*

Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer reviewers: *Beatrice Macchi, Universita degli Studi di Roma Tor Vergata, Italy; Ian Macreadie, RMIT University, Australia.*
Additional file: *Open peer review reports 1 and 2.*

References

Batista AF, Bodart-Santos V, De Felice FG, Ferreira ST (2019) Neuroprotective actions of glucagon-like peptide-1 (GLP-1) analogues in Alzheimer's and Parkinson's diseases. CNS Drugs 33:209-223.

Bomfim TR, Forny-Germano L, Sathler LB, Brito-Moreira J, Houzel JC, Decker H, Silverman MA, Kazi H, Melo HM, McClean PL, Holscher C, Arnold SE, Talbot K, Klein WL, Munoz DP, Ferreira ST, De Felice FG (2012) An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease- associated Aβ oligomers. J Clin Invest 122:1339-1353.

Cao J, Hou J, Ping J, Cai D (2018) Advances in developing novel therapeutic strategies for Alzheimer's disease. Mol Neurodegener 13:64.

Cardoso S, Seiça R, Moreira PI (2017) Diabesity and brain energy metabolism: the case of Alzheimer's disease. Adv Neurobiol 19:117-150.

Ettcheto M, Busquets O, Camins A (2019) Potential preventive disease-modifying pharmacological strategies to delay late onset Alzheimer's disease. Neural Regen Res 14(10):1721-1725. doi:10.4103/1673-5374.257513

Clarke JR, Lyra E Silva NM, Figueiredo CP, Frozza RL, Ledo JH, Beckman D, Katashima CK, Razolli D, Carvalho BM, Frazão R, Silveira MA, Ribeiro FC, Bomfim TR, Neves FS, Klein WL, Medeiros R, LaFerla FM, Carvalheira JB, Saad MJ, Munoz DP, et al. (2015) Alzheimer-associated Aβ oligomers impact the central nervous system to induce peripheral metabolic deregulation. EMBO Mol Med 7:190-210.

Cline EN, Bicca MA, Viola KL, Klein WL (2018) The amyloid-β oligomer hypothesis: beginning of the third decade. J Alzheimers Dis 64:S567-610.

Cummings J, Lee G, Ritter A, Zhong K (2018) Alzheimer's disease drug development pipeline: 2018. Alzheimers Dement (N Y) 4:195-214.

De Felice FG, Lourenco MV, Ferreira ST (2014) How does brain insulin resistance develop in Alzheimer's disease? Alzheimers Dement 10:S26-32.

De Felice FG (2013) Alzheimer's disease and insulin resistance: translating basic science into clinical applications. J Clin Invest 123:531-539.

de la Monte SM (2017). Insulin resistance and neurodegeneration: progress towards the development of new therapeutics for Alzheimer's disease. Drugs 77:47-65.

Ettcheto M, Sánchez-López E, Pons L, Busquets O, Olloquequi J, Beas-Zarate C, Pallas M, García ML, Auladell C, Folch J, Camins A (2017) Dexibuprofen prevents neurodegeneration and cognitive decline in APPswe/PS1dE9 through multiple signalling pathways. Redox Biol 13:345-352.

Ferreira LSS, Fernandes CS, Vieira MNN, De Felice FG (2018) Insulin resistance in Alzheimer's disease. Front Neurosci 12:830.

Hardy J, Bogdanovic N, Winblad B, Portelius E, Andreasen N, Cedazo-Minguez A, Zetterberg H (2014) Pathways to Alzheimer's disease. J Intern Med 275:296-303.

Hampel H, Vergallo A, Aguilar LF, Benda N, Broich K, Cuello AC, Cummings J, Dubois B, Federoff HJ, Fiandaca M, Genthon R, Haberkamp M, Karran E, Mapstone M, Perry G, Schneider LS, Welikovitch LA, Woodcock J, Baldacci F, Lista S, et al. (2018) Precision pharmacology for Alzheimer's disease. Pharmacol Res 130:331-365.

Hao S, Dey A, Yu X, Stranahan AM (2016) Dietary obesity reversibly induces synaptic stripping by microglia and impairs hippocampal plasticity. Brain Behav Immun 51:230-239.

Hoyer S (2002) The brain insulin signal transduction system and sporadic (type II) Alzheimer disease: an update. J Neural Transm 109:341-360.

Honig LS, Vellas B, Woodward M, Boada M, Bullock R, Borrie M, Hager K, Andreasen N, Scarpini E, Liu-Seifert H, Case M, Dean RA, Hake A, Sundell K, Poole Hoffmann V, Carlson C, Khanna R, Mintun M, DeMattos R, Selzler KJ, et al. (2018) Trial of Solanezumab for mild dementia due to Alzheimer's disease. N Engl J Med 378:321-330.

in t' Veld BA, Ruitenberg A, Hofman A, Launer LJ, van Duijn CM, Stijnen T, Breteler MM, Stricker BH (2001) Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. N Engl J Med 345:1515-1521.

Kang S, Lee YH, Lee JE (2017) Metabolism-centric overview of the pathogenesis of Alzheimer's disease. Yonsei Med J 58:479-488.

Kodis EJ, Choi S, Swanson E, Ferreira G, Bloom GS (2018) N-Methyl-D-aspartate receptor-mediated calcium influx connects amyloid-β oligomers to ectopic neuronal cell cycle reentry in Alzheimer's disease. Alzheimers Dement 14:1302-1312.

Lee M, Guo JP, Kennedy K, McGeer EG, McGeer PL (2017) A method for diagnosing Alzheimer's disease based on salivary amyloid-β protein 42 levels. J Alzheimers Dis 55:1175-1182.

Marciniak E, Leboucher A, Caron E, Ahmed T, Tailleux A, Dumont J, Issad T, Gerhardt E, Pagesy P, Vileno M, Bournonville C, Hamdane M, Bantubungi K, Lancel S, Demeyer D, Eddarkaoui S, Vallez E, Vieau D, Humez S, Faivre E, et al. (2017) Tau deletion promotes brain insulin resistance. J Exp Med 214:2257-2269.

Markowicz-Piasecka M, Sikora J, Szydłowska A, Skupień A, Mikiciuk-Olasik E, Huttunen KM (2017) Metformin - a future therapy for neurodegenerative diseases. Pharm Res 34:614-2627.

McDade E, Bateman RJ (2017) Stop Alzheimer's before it starts. Nature 547:153-155.

McGeer PL, Guo JP, Lee M, Kennedy K, McGeer EG (2018) Alzheimer's disease can be spared by nonsteroidal anti-inflammatory drugs. J Alzheimers Dis 62:1219-1222.

McLimans KE, Webb JL, Anantharam V, Kanthasamy A, Willette AA (2017) Alzheimer's disease neuroimaging initiative. Peripheral versus central index of metabolic dysfunction and associations with clinical and pathological outcomes in Alzheimer's disease. J Alzheimers Dis 60:1313-1324

Ott A, Stolk RP, Hofman A, van Harskamp F, Grobbee DE, Breteler MM (1996) Association of diabetes mellitus and dementia: The Rotterdam Study. Diabetologia 39:1392-1397.

Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM (1999) Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology 53:1937-1942.

Preische O, Schultz SA, Apel A, Kuhle J, Kaeser SA, Barro C, Gräber S, Kuder-Buletta E, LaFougere C, Laske C, Vöglein J, Levin J, Masters CL, Martins R, Schofield PR, Rossor MN, Graff-Radford NR, Salloway S, Ghetti B, Ringman JM, et al. (2019) Dominantly Inherited Alzheimer Network. Serum neurofilament dynamics predicts neurodegeneration and clinical progression in presymptomatic Alzheimer's disease. Nat Med 25:277-283.

Rajendran L, Paolicelli RC (2018) Microglia-mediated synapse loss in Alzheimer's disease. J Neurosci 38:2911-2919.

Schrijvers EM, Witteman JC, Sijbrands EJ, Hofman A, Koudstaal PJ, Breteler MM (2010) Insulin metabolism and the risk of Alzheimer disease: The Rotterdam Study. Neurology 75:1982-1987.

Singh-Manoux A, Dugravot A, Shipley M, Brunner EJ, Elbaz A, Sabia S, Kivimaki M (2018) Obesity trajectories and risk of dementia: 28 years of follow-up in the Whitehall II Study. Alzheimers Dement 14:178-186.

Swerdlow RH (2018) Mitochondria and mitochondrial cascades in Alzheimer's disease. J Alzheimers Dis 62:1403-1416.

Tse KH, Herrup K (2017) Re-imagining Alzheimer's disease - the diminishing importance of amyloid and a glimpse of what lies ahead. J Neurochem 143:432-444.

Vieira MNN, Lima-Filho RAS, De Felice FG (2018) Connecting Alzheimer's disease to diabetes: Underlying mechanisms and potential therapeutic targets. Neuropharmacology 136:160-171.

Wenzel TJ, Klegeris A (2018) Novel multi-target directed ligand-based strategies for reducing neuroinflammation in Alzheimer's disease. Life Sci 207:314-322.

Wijesekara N, Gonçalves RA, De Felice FG, Fraser PE (2018) Impaired peripheral glucose homeostasis and Alzheimer's disease. Neuropharmacology 136:172-181.

Willette AA, Johnson SC, Birdsill AC, Sager MA, Christian B, Baker LD, Craft S, Oh J, Statz E, Hermann BP, Jonaitis EM, Koscik RL, La Rue A, Asthana S, Bendlin BB (2015) Insulin resistance predicts brain amyloid deposition in late middle-aged adults. Alzheimers Dement 11:504-510.

P-Reviewer: Francisco-Morcillo J; C-Editors: Zhao M, Yu J; T-Editor: Jia Y

Annex VII

The Involvement of Peripheral and Brain Insulin Resistance in Late Onset Alzheimer's Dementia.

Folch J, Olloquequi J, Ettcheto M, Busquets O, Sánchez-López E, Cano A, Espinosa-Jiménez T, García ML, Beas-Zarate C, Casadesús G, Bulló M, Auladell C and Camins A.

Front Aging Neurosci. 2019;11(236):1-16.

frontiers in Aging Neuroscience

REVIEW
published: 06 September 2019
doi: 10.3389/fnagi.2019.00236

The Involvement of Peripheral and Brain Insulin Resistance in Late Onset Alzheimer's Dementia

*Jaume Folch[1,2,3], Jordi Olloquequi[4], Miren Ettcheto[1,3,5,6], Oriol Busquets[1,3,5,6], Elena Sánchez-López[3,7,8], Amanda Cano[3,7,8], Triana Espinosa-Jiménez[3,5,6], Maria Luisa García[3,7,8], Carlos Beas-Zarate[9], Gemma Casadesús[10], Mónica Bulló[1,10], Carme Auladell[3,6,11] and Antoni Camins[3,5,6]**

[1]Department of Biochemistry and Biotechnology, Faculty of Medicine and Health Sciences, University Rovira i Virgili (URV), Reus, Spain, [2]Institute of Health Pere i Virgili, IISPV , Reus, Spain, [3]Biomedical Research Networking Centre in Neurodegenerative Diseases (CIBERNED), Institute of Health Carlos III, Madrid, Spain, [4]Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile, [5]Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain, [6]Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain, [7]Unitat de Farmàcia, Tecnologia Farmacèutica i Fisico-Química, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain, [8]Institute of Nanoscience and Nanotechnology (IIN2UB), University of Barcelona, Barcelona, Spain, [9]Laboratorio de Regeneración y Desarrollo Neural, Departamento de Biología Celular y Molecular, Instituto de Neurobiología, CUCBA, Guadalajar, México, [10]Department of Biological Sciences, Kent State University, Kent, OH, United States, [11]Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Institute of Health Carlos III, Madrid, Spain*

OPEN ACCESS

Edited by:
Raymond Chuen-Chung Chang, The University of Hong Kong, Hong Kong

Reviewed by:
Agata Copani, University of Catania, Italy
Alasdair Barr, University of British Columbia, Canada

***Correspondence:**
Antoni Camins
camins@ub.edu

Received: *30 April 2019*
Accepted: *15 August 2019*
Published: *06 September 2019*

Citation:
Folch J, Olloquequi J, Ettcheto M, Busquets O, Sánchez-López E, Cano A, Espinosa-Jiménez T, García ML, Beas-Zarate C, Casadesús G, Bulló M, Auladell C and Camins A (2019) The Involvement of Peripheral and Brain Insulin Resistance in Late Onset Alzheimer's Dementia. Front. Aging Neurosci. 11:236. doi: 10.3389/fnagi.2019.00236

Nowadays, Alzheimer's disease (AD) is a severe sociological and clinical problem. Since it was first described, there has been a constant increase in its incidence and, for now, there are no effective treatments since current approved medications have only shown short-term symptomatic benefits. Therefore, it is imperative to increase efforts in the search for molecules and non-pharmacological strategies that are capable of slowing or stopping the progress of the disease and, ideally, to reverse it. The amyloid cascade hypothesis based on the fundamental role of amyloid has been the central hypothesis in the last 30 years. However, since amyloid-directed treatments have shown no relevant beneficial results other theories have been postulated to explain the origin of the pathology. The brain is a highly metabolically active energy-consuming tissue in the human body. It has an almost complete dependence on the metabolism of glucose and uses most of its energy for synaptic transmission. Thus, alterations on the utilization or availability of glucose may be cause for the appearance of neurodegenerative pathologies like AD. In this review article, the hypothesis known as Type 3 Diabetes (T3D) will be evaluated by summarizing some of the data that has been reported in recent years. According to published research, the adherence over time to low saturated fatty acids diets in the context of the Mediterranean diet would reduce the inflammatory levels in brain, with a decrease in the pro-inflammatory glial activation and mitochondrial oxidative stress. In this situation, the insulin receptor pathway would be able to fine tune the mitochondrial biogenesis in neuronal cells, regulation the adenosine triphosphate/adenosine diphosphate intracellular

balance, and becoming a key factor involved in the preservation of the synaptic connexions and neuronal plasticity. In addition, new targets and strategies for the treatment of AD will be considered in this review for their potential as new pharmacological or non-pharmacological approaches.

Keywords: insulin resistance, obesity, type 2 diabetes mellitus, Alzheimer's disease, Mediterranean diet, neuroinflammation and neurodegeneration

INTRODUCTION

Several theories have been in the headlines of the Alzheimer's disease (AD) research scene in the last 10 years (Folch et al., 2013; Alzheimer's Association, 2016; Dobson, 2017; Hurtado-Puerto et al., 2018) and, in many of them, it was postulated how an alteration in a metabolic mechanism runs in parallel, or is the cause, for the development of the classical features of AD (Frölich et al., 2015). Eventually, it has led to the understanding that pathologies like type 2 Diabetes Mellitus (T2DM) and AD, or conditions like morbid obesity, previously believed to run independently, are actually highly connected through specific molecular interactions that evolve in the same direction (de la Monte, 2012; De Felice et al., 2014).

Despite the hypotheses focus on amyloid and TAU phosphorylation have dominated the neuropathology of AD so far, new theories have come to light, in part, due to the failure of all the developed drugs directed against molecules related to the amyloidogenic pathway (Gauthier et al., 2016). It has been described that this failure may be the result of the complex etiology of AD. As of now, it could be classified into two subgroups. On the one hand, familial AD in which genetic alterations of the amyloid precursor protein (APP) or presenilin-1 (PS-1) are involved, accounting the 3% of cases of the disease. On the other hand, representing the remaining 97%, the late onset form of AD (LOAD) which is associated with advanced age, mutations of the apolipoprotein E (APOE) ε4 allele, hypertension and hyperlipidaemia, as well as with coronary disease, T2DM and obesity among other less determinant factors.

In parallel with AD, obesity has become pandemic in the Western world and it has shown increased prevalence in most countries mainly due to changes in nutritional habits and lifestyle (de la Monte, 2014; Luciano et al., 2015; Kivipelto et al., 2018). Although the interaction between obesity, aging and AD is a complex process, there are evidences demonstrating that obesity and metabolic dysfunctions in middle age substantially increase the risk of developing AD (Kivipelto et al., 2018). Thus, studies conducted in the last 20 years support the hypothesis that deficits in brain insulin and insulin-like growth factor (IGF-1) signaling mediate cognitive impairment and neurodegeneration in AD (de la Monte, 2012). As we will discuss below, the main role of insulin and insulin receptor pathway in different brain regions as hippocampus and cortex is mainly related to the maintenance of intracellular energy levels needed to sustain synaptogenesis and neuronal plasticity. For this reason, some researchers have even referred to AD as Type 3 Diabetes (T3D; de la Monte, 2012, 2014; Chami et al., 2016; Kandimalla et al., 2017; Kang et al., 2017; Tong et al., 2017). This concept describes a brain-specific pathological situation in which insulin and IGF resistance is developed inducing cognitive impairments and neurodegeneration. Insulin resistance is classically defined as the state in which high levels of circulating insulin (hyperinsulinemia) are associated to hyperglycemia, concept that has been extended to other tissues and organs which show reduced activation of the pathways in insulin signaling. Therefore, AD and diabetic pathologies share several common features (Willette et al., 2015), situation in which adherence to good life habits, especially in terms of motor activity and diet, may have beneficial effects on cognitive processes during aging in humans.

From a metabolic outlook, the brain requires a high amount of energy to maintain its functions, especially for the transmission of synaptic impulses (Yun and Hoyer, 2000; Hoyer, 2004; Maurer and Hoyer, 2006). Hence, alterations in brain glucose metabolism lead to severe dysregulations in cellular function. For example, a decrease in ATP production and choline acetyltransferase activity in presynaptic cholinergic neurons reduce the availability of acetylcholine in the brain which is worsened by the depletion of citric acid cycle intermediates that are also required for the synthesis of this neurotransmitter (Hoyer, 2002a,b; Salkovic-Petrisic et al., 2009). Alterations on its availability could also trigger a dysfunction in synaptic transmission and affect proper cognitive function (Hoyer, 2004; Frölich et al., 2015; Fadel and Reagan, 2016).

This review article focuses on the evidence concerning the effects of obesity and T2DM in the process of cognitive loss. Likewise, we discuss throughout the manuscript the close relationship between alterations at the peripheral level related to obesity that may favor the risk of developing AD and the role of amyloid-β (Aβ) generated at the brain level, which could be an inducer of the onset of T2DM. Since obesity and metabolic syndrome are modifiable risk factors that contribute to insulin-resistant diabetes, special emphasis will be put on their relationship with T3D. Finally, pharmacological and environmental interventions, such a suitable diet, will also be discussed as possible strategies to prevent obesity-related cognitive loss.

PHYSIOLOGICAL ROLE OF INSULIN BEYOND PERIPHERAL TISSUES

As we mentioned, the classical amyloid and Tau hypotheses of AD have been challenged by reported evidence that describes how they may not be the source for the pathology in its late onset form (Selkoe and Hardy, 2016). It was Dr. Hoyer

who in 1985 introduced the idea of alterations in brain metabolism as the source for the appearance of LOAD through defects in the oxidative degradation of glucose (Hoyer, 2004; Frölich et al., 2015; Morgen and Frölich, 2015). Specifically, it has been described that oxidative dysregulation triggers cellular downstream signaling pathways promoting neuronal degeneration through the induction of stress in organelles like the mitochondria and the endoplasmic reticulum. As a consequence, Aβ and Tau alterations would emerge contributing to the ongoing cognitive loss. This could explain why drugs focusing on Aβ and Tau have proven useless so far since they would be targeting a consequence or aggravator of the pathology but not its root.

In addition to this, in recent years it has been demonstrated that insulin receptors (IRs) and their ligand hormone play a more relevant role in the brain than was previously thought. Specifically, several studies have reported presence of IRs in hippocampus, cerebral cortex, cerebellum and choroidal plexus of the mammalian brain (Hoyer, 2004; de la Monte et al., 2006) and especially in postsynaptic terminals (Abbott et al., 1999). It has been demonstrated that insulin levels become strongly reduced in these regions during aging and in sporadic AD (Ramalingam and Kim, 2014). Indeed, insulin is preferentially transported to the brain through the blood-brain barrier (BBB) in a receptor-mediated mechanism and its availability is critical for the activation of brain IR. In this aspect, it has been proposed that brain insulin has a neuroprotective capacity against the accumulation of senile plaques, by regulating Aβ peptide levels and preventing the binding of Aβ oligomers to synapses (Zhao et al., 2008; De Felice et al., 2009, 2014; Lyra E Silva et al., 2019). Interestingly, AD preclinical models show that preventing IR inhibition enhances Protein kinase B (AKT) signaling, which is involved in cell proliferation, cell growth, protein synthesis and inhibition of apoptosis, but also in the hyperphosphorylation of Tau through the control of the glycogen synthase kinase 3β (GSK3β; El Khoury et al., 2014). By contrast, insulin deficits have been linked to the inhibition of several phosphatases involved in Tau dephosphorylation (El Khoury et al., 2014). Finally, it has been proven that insulin signaling pathway is also involved in the modulation of neuroinflammatory processes and vascular inflammation (Chen and Zhong, 2013). Likewise, insulin modulation acts through the mitogen-activated protein kinase (MAPK) pathway, which plays an important role in cell differentiation, cell proliferation, apoptosis as well as inflammation. As we will discuss below, the activation of some c-JUN N-terminal Kinases (JNKs) should be a key factor linking insulin signaling to synaptogenesis failure (Huang et al., 2017; Pomytkin et al., 2018).

In 2000, Bru and co-workers published an interesting article demonstrating that brain IR is involved in the metabolic control of peripheral tissues through the generation of a murine model with a specific deletion of the neuronal IR gene (NIRKO mice; Bru et al., 2000). This inactivation resulted in insulin resistance at the central nervous system (CNS) but mice also developed obesity, combined with hyperphagia, an increase of leptin and insulin concentrations in plasma, as well as the development of hypertriglyceridemia. In agreement with this, Obici and co-workers demonstrated that a decrease in the hypothalamic IR expression is enough to induce several key features of metabolic syndrome (Obici, 2009). Hence, insulin resistance in the brain would be involved in the pathophysiology of obesity and T2DM in the peripheral tissues.

All these experimental data indicate that brain IR plays a key role in: (i) regulation of cognitive processes through hippocampal IR; and (ii) regulation of peripheral glucose metabolism. Consequently, restoration of normal insulin levels and prevention of brain insulin resistance may be a therapeutic strategy for delaying cognitive loss in AD (Biessels, 2013; Ramalingam and Kim, 2014).

TOWARDS BRAIN INSULIN RESISTANCE IN LATE ONSET AIZHEIMER'S DISEASE

Recently, Castellani et al. (2019) proposed, in an excellent review on AD therapeutics, that it was very reasonable to conclude that the amyloidogenic pathway is very closely related to AD, however, the important role of other players was suggested into the origin of the pathology since the clearance of Aβ protein was not enough to modify the evolution of the disease. Furthermore, pharmacological strategies targeting Aβ-related biomarkers like BACE1 inhibitors or anti-Aβ antibodies, have failed to cure or halt LOAD (Cummings et al., 2018). Hence, new strategies should be designed to complement the actual therapeutic proposals that, so far, have been mainly focused on the amyloid hypothesis.

One of the first reviews that criticized the paradigm of the amyloid cascade overactivation as the origin of AD was written by one of the co-authors that first described this central hypothesis (Cleary et al., 2005; Hardy, 2009; Selkoe and Hardy, 2016). But, back in 1985, as it was previously mentioned, before the installment of the amyloidogenic hypothesis, Dr. Siegfried Hoyer had already proposed the concept of central insulin resistance and altered insulin signaling in LOAD (Frölich et al., 2015; Hoyer, 2002b, 2004; Morgen and Frölich, 2015). Later on, Dr. de la Monte published a series of articles about the metabolic hypothesis of AD, and became one of the main defenders of the so-called "brain insulin resistance" or T3D hypothesis (de la Monte et al., 2006; de la Monte and Wands, 2008; Chami et al., 2016; de la Monte, 2017). In these experiments, the intracerebral administration of streptozotocin (STZ) was used as an experimental model to induce LOAD-like cognitive impairments in rodents, allowing to study this theory deeper (de la Monte et al., 2006; Correia et al., 2011; Salkovic-Petrisic et al., 2013; Tong et al., 2017). The research by Dr. de la Monte in AD patients also proved significant reductions in insulin and IGF-1 receptor levels in the frontal cortex, hippocampus, and hypothalamus (Steen et al., 2005), reinforcing the idea of T3D. Likewise, Cardoso et al. (2017) suggested the term "diabesity" to explain the presence of both metabolic and cognitive affectations.

In parallel with these observations, results from epidemiologic studies contributed to reinforce the proposed concept of T3D. Doubtlessly, one of the most important is the Rotterdam's study (Ott et al., 1999; Schrijvers et al., 2010;

Luciano et al., 2015). This project has investigated the connection between T2DM and LOAD for almost a decade, revealing that those patients diagnosed with T2DM had higher risk of developing dementia. Subsequent clinical and epidemiological studies have confirmed this association, by demonstrating that alterations of metabolic parameters related to glucose metabolism were associated with cognitive loss (Talbot et al., 2012; Biessels, 2013; Walker and Harrison, 2015). Moreover, in a clinical study in patients with AD and hyperinsulinemia, Willette et al. (2015) demonstrated that insulin resistance increased the number of Aβ depositions in the brain. It has also been reported that neurons from AD patients exhibit an insulin mRNA expression four times lower than normal in the hippocampus and two times lower than normal in the hypothalamus (Chen and Zhong, 2013). This situation would be aggravated by the disruption of the activity of astrocytes forming the BBB. Likewise, patients with AD show alterations in the transport of this hormone into the brain (Molofsky et al., 2012; An et al., 2018). It is noteworthy to point out that, in addition to the lower availability of insulin in the brain, cerebral insulin resistance is another pathophysiological feature of AD. Thus, it seems that insulin signaling could have a key role in cognitive loss.

In 2014, the Hisayama's study showed, through the analysis of microarrays, modifications in the normal expression of genes related to T2DM in AD brains, especially in the hippocampus (Hokama et al., 2014). More recent studies have also reinforced the notion of an insulin resistance having a key role in AD's pathogenesis. For instance, An et al. (2018) reported a strong association between impairments in glucose metabolism and increased glucose concentrations in areas sensible to Aβ deposition and neurofibrillary pathology. The authors suggested that AD is associated with a failure in neuronal glucose utilization, which is mediated by an alteration in glycolysis. The authors argued that three enzymes involved in the glycolysis (hexokinase, phosphofructokinase, and pyruvate kinase) showed significant reduction in their activity in AD. In turn, in the Whitehall II clinical study, Singh-Manoux et al. (2018) reported that obesity (BMI >30 kg/m^2) at 50 years of age is a risk factor for AD. However, the association decreased with increasing age, indicating that this association is modified by age and obesity, being midlife obesity the riskiest stage for dementia (Singh-Manoux et al., 2018). In another interesting clinical study, Ahmed et al. (2017) reported a bidirectional association between T2DM and LOAD. Besides, they reported that Memantine—a drug which is currently used in AD treatment- showed an ameliorating effect on T2DM. This is in accordance with results published by our research group and others, where the benefits of Memantine administration were demonstrated in a mixed murine model of T2DM and AD (Sato and Morishita, 2013; Shinohara and Sato, 2017; Ettcheto et al., 2018b; Deng et al., 2019).

Regarding other preclinical studies, have been shown that hyperglycemia raises Aβ levels in the interstitial fluid (ISF) by altering neuronal activity. It seems that high glucose metabolism can alter ATP-sensitive potassium (KATP) channels, which are the link between changes in metabolism, neuronal activity and ISF Aβ (Macauley et al., 2015; Stanley et al., 2016). In turn, Grillo et al. (2015) reported that the administration of viral vectors expressing an antisense sequence of the rat brain IR caused cognitive impairments. Thus, they generated a specific rat model of altered brain insulin signaling associated to cognitive loss. These results are of great relevance since demonstrated that selective insulin resistance at the hippocampal level contributes directly to the development of cognitive deficits observed in patients with metabolic disorders such as T2DM and obesity (Fadel and Reagan, 2016).

Finally, among other risk factors likely to favor the development of sporadic AD, the mutation of the APOE4 allele has recently shown an intriguing association with insulin resistance. Thus, it has been suggested that APOE4 impairs IR trafficking by trapping it in endosomes, leading to impaired insulin signaling (Zhao et al., 2017). The APOE gene ε4 allele is so far considered the strongest genetic risk factor for AD. These findings are relevant to explain the correlation between T2DM and LOAD (Peila et al., 2002). In addition, this implies that the presence of APOE4 allele and T2DM could act synergistically in AD pathogenesis. Accordingly, the highest risk for AD and the most severe neuropathology is found in individuals with both diabetes and the APOE4 mutation (Peila et al., 2002).

OBESOGENIC DIET AS A RISK FACTOR FOR COGNITIVE IMPAIRMENT

Previous studies reported that obesity is associated to memory impairment through insulin resistance in both, young people (Cheke et al., 2016) and cognitively normal older people although the underlying mechanisms remain unclear (Hargrave et al., 2016; Kivipelto et al., 2018). Likewise, in the older people an association between obesity and brain atrophy has been described (Raji et al., 2010). It is widely accepted that obesity favors the emergence of metabolic syndrome affecting peripheral tissues such as liver, pancreas and adipocytes (Cardoso et al., 2017; Kang et al., 2017; Kothari et al., 2017). We have already discussed the observations based on the experimental use of STZ to induce diabetes in rats (de la Monte et al., 2006; de la Monte, 2017). This toxic compound can trigger Type 1 diabetes mellitus by killing insulin-producing cells in the pancreas and, at lower doses, can lead to T2DM and related alterations (Correia et al., 2011; Salkovic-Petrisic et al., 2013; Tong et al., 2017). Indeed, lower STZ doses have been involved in neuronal loss, neuroinflammation, oxidative stress and accumulations of phospho-TAU and Aβ in cortical-limbic structures that characteristically degenerate in AD, leading to impaired spatial learning and memory (Correia et al., 2011). Since STZ is a nitrosamine, a chemical compound that can be found in many foods and other consumables, the question is: could diet be directly involved in the exacerbation of cognitive decline in LOAD? Indeed, several studies suggest that environmental exposure to food additives may play a critical role in the pathogenesis of AD (de la Monte et al., 2018, 2019). In light of the unstoppable increase in LOAD prevalence rates and the widespread use of nitrites and nitrates in foods and agricultural products over the past

30–40 years, the impact of exposure to dietary components as nitrosamines should be reviewed in relation to T3D (de la Monte et al., 2018).

Several studies suggest that there is a link between calorie intake from diets rich in saturated fats or high fat diets (HFD) and the resulting obesity and cognitive deficits (Kohjima et al., 2010; Yarchoan et al., 2014; Pratchayasakul et al., 2015; Moser and Pike, 2017; Sah et al., 2017). By contrast, moderate dietary restriction has been found to improve cognition and, indeed, life expectancy (Parrella et al., 2013). These findings are the reason why studies on the interaction metabolism-AD have gained a great deal of interest. Thus, it is not surprising that HFD has been associated with a large number of metabolic diseases, such as obesity, systemic insulin resistance, metabolic syndrome and T2DM (de la Monte, 2014; Ferreira et al., 2014; Cardoso et al., 2017). Nuzzo et al. (2015) provided evidence that obesity and insulin resistance are involved to inflammation, adipokine dyshomeostasis, oxidative stress and mitochondrial dysfunction, all of them being mechanisms that favor neurodegeneration. In those experiments, mice fed with an HFD showed elevated levels of APP and Aβ40/Aβ42, BACE, GSK3β and TAU proteins, all involved in APP processing and Aβ accumulation. In light of these results, it is clear that the exposure of rodents to a HFD damages their brain in a similar manner to the hallmarks of AD (Pratchayasakul et al., 2015; Ettcheto et al., 2016; Sah et al., 2017). In another study, Bocarsly et al. (2015) also reported negative consequences of HFD in rats. Specifically, HFD has been associated to alterations in brain cortical dendritic spines and a decrease in presynaptic and postsynaptic protein levels, which was related to behavioral cognitive deficits in working memory and cognition (Bocarsly et al., 2015). In turn, Kothari and colleagues reported that HFD may impair brain insulin signaling promoting neuroinflammation, formation of Aβ plaques and neurofibrillary tangles, as well as loss of synaptic plasticity (Sallam et al., 2015). In the same line, Kohjima et al. (2010) also reported that diet-induced insulin resistance is associated with reduced neuronal insulin receptor signaling, leading to an increase in Aβ levels and cognitive loss in the brain of Tg2576 mice. Likewise, an hypercaloric diet increases brain Aβ levels and cognitive alterations in APPswe/PS1dE9 (APP/PS1) mice (Petrov et al., 2015). Similarly, the same murine model under a HFD at an early pre-symptomatic disease stage (3 months old) showed an increase in Aβ1–42 peptide, a decrease in Protein Kinase A (PKA) levels and alterations in the c-AMP Response Element Binding (CREB)/N-methyl-D-aspartate receptor 2B (NMDAR2B)/Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) pathway (Sheng et al., 2012; Ettcheto et al., 2016; Katsouri et al., 2016; Wang et al., 2017). This mechanism involves the attenuation of the forkhead-like transcription factor 1 (FoxO3a) expression.

There can be found some other several preclinical studies in which the exposure to HFD is associated with a decline in cognitive function. In many of these studies, HFD-induced alterations in peripheral insulin sensitivity lead to a central insulin resistance and biochemical changes related to increased Aβ deposition and neurofibrillary tangle formation (Kothari et al., 2017). For instance, Chua et al. (2012) demonstrated that a reduction in insulin signaling usually precedes the accumulation of Aβ peptide in APP/PS1 mice. In another study developed by our research group, long-term exposure to HFD favored the appearance of Aβ depositions in the brain of C57BL/6J mice (Busquets et al., 2017). This is an intriguing observation because, since these wild-type mice do not develop cognitive loss *per se*, our results implied that HFD maintained for a long time could be enough to damage brain. Moreover, HFD caused alterations in different cell processes, such as normal autophagy and apoptosis, and also enhanced an inflammatory reaction that leads to a decrease in the neuronal precursor cells (Busquets et al., 2017).

Taken altogether, the above-mentioned results reinforce the hypothesis of a metabolic etiology of AD in its sporadic and late onset form. They also confirm that HFD favors Aβ depositions in the brain, a key feature of this disease. In this point, an important question arises: what is the molecular link between diet, T2DM and cognitive impairment? In this regard, Osborne et al. (2016) reported that intrahippocampal infusion of an Aβ33–42 gamma antibody reversed cognitive impairment in rats with HFD-related cognitive loss. Hence, these results stressed the role of soluble Aβ in obesity-mediated cognitive loss and they are in agreement with previous studies hypothesizing that diffusible Aβ oligomers are responsible for neural dysfunction leading to AD (Walsh et al., 2005; Tarasoff-Conway et al., 2015; Xia et al., 2016; Bu et al., 2018; Hurtado-Puerto et al., 2018). Accordingly, Aβ could be a possible connection between the metabolic and the classical amyloid hypotheses of AD, since it binds to the IR and may trigger its internalization at the post-synaptic level, thereby blocking glutamatergic neurotransmission (De Felice et al., 2014; Ribe and Lovestone, 2016; Ahmed et al., 2017). In addition, Aβ oligomers have been found to inactivate IRs *via* the JNKs pathway through a mechanisms that is comparable to the established peripheral effect of JNKs on IRs in T2DM (Zhao et al., 2008; Ma et al., 2009; Freiherr et al., 2013; Lyra E Silva et al., 2019).

WHICH CAME FIRST: OBESITY OR Aβ? THE "CHICKEN OR THE EGG" CAUSALITY DILEMMA IN LOAD

Among the plethora of functions performed by the CNS, it also plays a key role in the glucose homeostasis. Indeed, different glucose sensors signals are integrated and processed by the CNS, involved in regulating glucose production, pancreatic hormonal secretion and glucose uptake, maintaining the balanced glucose levels against changing conditions (Cai, 2013; Zheng et al., 2018). The hypothalamus controls several regulatory mechanisms of peripheral glucose homeostasis through the control of various signals from organs and tissues involved in digestion, absorption and storage of nutrients. Neurons responsible for the CNS metabolic balance are found in a sub-region of the ventromedial hypothalamus, called the arcuate nucleus (aRC). They express anabolic peptides such as the neuropeptide Y and agouti-related peptide (aGRP), as well as proopiomelanocortin, which is the precursor of numerous biologically active peptides, including the α-melanocyte stimulating hormone (αMSH) which

favors catabolism (Obici et al., 2002; Obici, 2009; Sandoval et al., 2009). This complex machinery includes hormones like insulin, adipokines as leptin, molecules like ghrelin or gut peptides as Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP; De Felice et al., 2014; Fasshauer and Blüher, 2015). Insulin decreases blood glucose concentrations by suppressing glucose production and upregulating its absorption by peripheral tissues (such as skeletal muscles and fat). Leptin exerts anorexigenic effect when it is released from fat tissue, whereas GLP1 and GIP are secreted from the pancreas during feeding inducing the increase glucose-dependent insulin secretion. However, the CNS also produces some of these molecules and receptors, which can be found in many of its areas including the aRC. Surprisingly, in addition to be capable of trespassing the BBB, insulin can also be secreted autonomously by the brain (Warren et al., 2012; Folch et al., 2013; Nuzzo et al., 2015).

In light of the above-mentioned, it is crucial to understand the potential mechanisms linking AD to obesity and T2DM. Among the hypotheses suggested, there is mounting data supporting an early involvement of Aβ-mediated alterations in hypothalamus leading to peripheral metabolic dysregulation. This may occur even before the appearance of cognitive loss symptoms. In this line of thought, Arrieta-Cruz and colleagues reported that direct administration of Aβ25–35 in the hypothalamus disrupts the regulation of glucose oxidation (Arrieta-Cruz et al., 2015; Arrieta-Cruz and Gutiérrez-Juárez, 2016). In turn, Clarke and colleagues also reported that intracerebroventricular injections of Aβ oligomers trigger peripheral systemic glucose intolerance and insulin resistance in rodents, through a process related with hypothalamic inflammation (Clarke et al., 2015, 2018; Lourenco et al., 2019). Hence, these data reinforce the notion that Aβ affects hypothalamic function, altering peripheral metabolic control and whole-body homeostasis (Obici et al., 2002; Sandoval et al., 2009; **Figure 1**). Likewise, cerebral Aβ could traverse the BBB and affect peripheral tissues, leading to peripheral insulin resistance. In accordance, it has been demonstrated that plasma Aβ induces insulin resistance in hepatocytes by activating Janus Kinase 2 (JAK2)/STAT3/Suppressor of Cytokine Signaling-1 (SOCS-1) signaling pathway in APP/PS1 mice, suggesting an important role of peripheral Aβ in the regulation of glucose metabolism (Zhang et al., 2013). Moreover, Aβ accumulation also occurs in the pancreas and skeletal muscle which may induce alterations on peripheral glucose metabolism (Roher et al., 2009; Miklossy et al., 2010; Kulas et al., 2017). Likewise, peripheral tissues such as heart, liver, testis, aorta, lung, intestines, skin, as well as the adrenal, salivary, and thyroid glands also produced Aβ peptide (Wang et al., 2017; Wijesekara et al., 2017). Although the implications of peripheral Aβ are still unknown, a contribution of a dynamic maintenance of Aβ levels throughout the body should not be discarded (Selkoe and Hardy, 2016; Bu et al., 2018). Indeed, platelets are a peripheral source of APP and they are able to generate Aβ in a similar manner than neurons, skin fibroblasts, skeletal muscles and cerebrovascular smooth muscle cells (Roher et al., 2009). Hence, peripheral alterations in APP metabolism might constitute a systemic and also a CNS problem in LOAD, hypothesizing that these peripheral Aβ could also contribute to T2DM pathophysiology (Kuo et al., 2000; Shinohara and Sato, 2017). In any case, some recent studies have provided data on the complex mechanistic interactions between T2DM and AD. For instance, Plucińska et al. (2016) demonstrated, using a neuron-specific human BACE1 knock-in mouse model (PLB4) that increased neuronal BACE1 is sufficient to alter systemic glucose metabolism. Therefore, this study also confirms that brain Aβ leads to a peripheral T2DM process. In turn, Sallam and colleagues developed an adipocyte-specific ecto-nucleotide pyrophosphate phosphodiesterase over-expressing transgenic (AtENPP1-Tg) as metabolic syndrome and systemic insulin resistance animal model (Sallam et al., 2015). These mice showed changes in lipid composition of hippocampal synaptosomes, impaired basal synaptic transmission as well as down-regulation of IR expression. The authors concluded that hippocampal molecular and functional integrity become affected by the IR and lipid composition, describing a potential mechanism responsible for the cognitive impairments associated with metabolic syndrome (FFA) and T2DM (Sallam et al., 2015).

Therefore, the process of cognitive impairment could start in peripheral tissues or, at least, could be exacerbated by potential interactions between peripheral tissues and brain. Excessive caloric intake leads to hypertrophy or hyperplasia of adipocytes, resulting in adipose tissue expansion. As a consequence, there is an increased secretion of adipokines, including a broad range of cytokines and chemokines, triggering the recruitment of inflammatory cells to the tissue and a second wave of inflammatory mediators release (Fasshauer and Blüher, 2015). Among these mediators, TNFα and some angiogenic factors could inhibit peripheral insulin signaling. Additionally, other proinflammatory cytokines such as interleukin IL1b and IL6, are able to cross the BBB and exacerbate brain inflammation together with proinflammatory factors produced by microglial cells (Warren et al., 2012; Fasshauer and Blüher, 2015). In turn, high blood insulin concentrations trigger an excessive release of FFA from adipocytes to liver and muscles, leading to exacerbated inflammatory responses and increased accumulation of Aβ. By contrast, hyperinsulinemia decreases insulin transport into the brain. Thus, adipose tissue extension in obesity and T2DM could trigger cognitive loss, reflecting the relationship between the adipose tissue and the brain, thus impacting on its function (Fasshauer and Blüher, 2015). Conversely, hypothalamic soluble Aβ and Tau phosphorylation may contribute to the impairment of the control of peripheral glucose metabolism in patients with AD (Cai, 2013; Chen and Zhong, 2013). This could be mediated by a direct effect of Aβ on central IR and by a mechanism mediated by Tau hyperphosphorylation, which increases intraneuronal insoluble insulin aggregates and downregulates IRs, leading to insulin resistance (El Khoury et al., 2014; Marciniak et al., 2017; Rodriguez-Rodriguez et al., 2017; Gonçalves et al., 2019).

Finally, a study performed by Banks et al. (2018) introduced an additional mechanism whereby metabolic syndrome contributes to cognitive impairment. The authors reported that triglycerides cross the BBB leading to brain leptin and insulin receptors resistance, which has a negative effect on

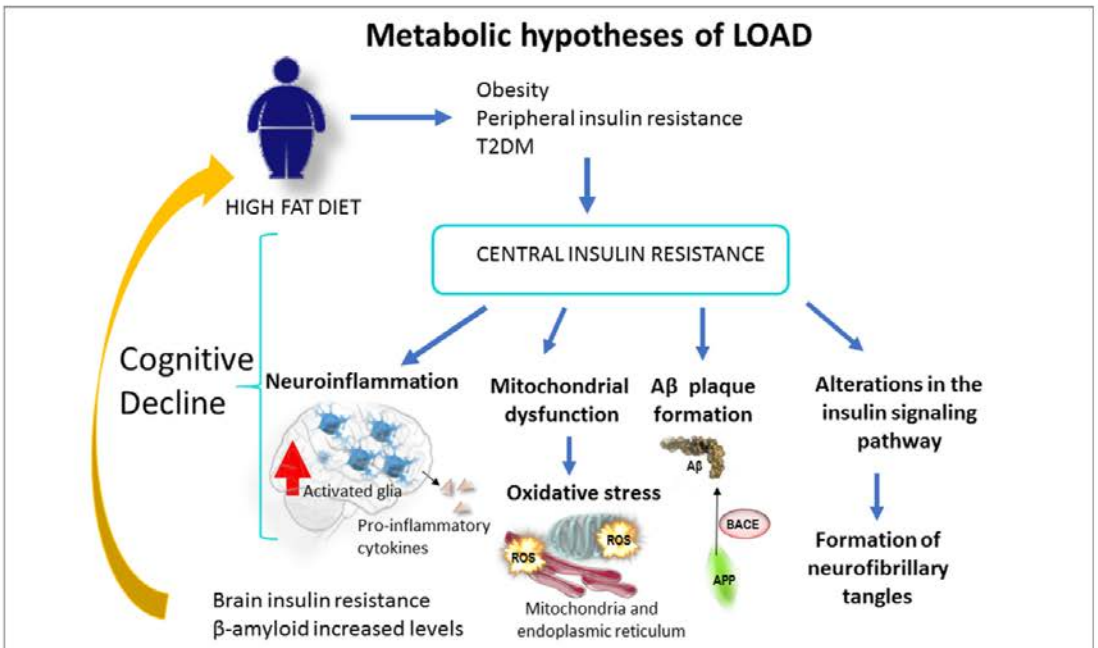


FIGURE 1 | Obesity, caused by a sedentary life or a high fat diet, increases the risk of type 2 Diabetes Mellitus [T2DM; Metabolic hypothesis of Alzheimer's disease (AD)]. This condition increases the risk of cognitive loss and AD through mitochondrial alteration, neuroinflammation, Aβ production and brain insulin resistance. At the brain level Aβ oligomers trigger peripheral systemic glucose intolerance and insulin resistance in rodents, through a process of hypothalamic neuroinflammation (Clarke et al., 2015, 2018; Lourenco et al., 2019). Likewise, cerebral Aβ could traverse the blood-brain barrier (BBB) and affect peripheral tissues, leading to a peripheral insulin resistance. This mechanism would behave in a positive feedback loop that would worsen over time.

cognition (Banks et al., 2018). These results confirm that modulation of peripheral metabolism, for example lowering elevated levels of triglycerides in the blood, could be a strategy to treat obesity and cognitive impairment associated with CNS resistance to leptin and insulin. In another interesting study, Moreno-Gonzalez et al. (2017) demonstrated that IAPP (amylin) aggregates are able to enhance the aggregation of Aβ, providing a potential additional link between AD and T2DM. Clearly, future studies will provide further keys to understand the relation between cognitive loss, obesity and T2DM (Vazquez-Valls et al., 2011; Mukherjee et al., 2017).

ROLE OF JNK1 AS A TARGET FOR DIABETES TYPE 2 AND OBESITY

Obesity triggers inflammatory processes that spread through the human body affecting multiple organs and tissues. Consequently, brains of HFD-exposed mice showed neuroinflammation and glial responses (Busquets et al., 2017). In a very recent review on this topic, authors described how HFD, western diet or sugars cause obesity-derived neuroinflammation, affecting brain structures such as the hippocampus, hypothalamus, cortex, brainstem, or amygdala (Guillemot-Legris et al., 2016). Similarly, AD patients exhibit significantly higher concentrations of IL-6 and TNFα in peripheral blood (Swardfager et al., 2010; Zheng et al., 2018).

Regarding the possibility that peripheral chronic inflammation can contribute to cognitive decline and cause LOAD, data show that IR tyrosine kinases trigger the activation of the RAS/MAPKs pathway. This superfamily includes extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), p38 and c-JNKs (Coffey, 2014). Moreover, it has been reported that ERK1 and ERK2 play a very significant role in the control of synapses in the learning and memory processes, while the contribution of p38 in associative learning has been described marginally (Sherrin et al., 2010, 2011). Likewise, CREB is the main target of ERK and has a pivotal role in long-term memory and synaptic plasticity in the hippocampus (Suzuki et al., 2011; Teich et al., 2015). Regarding to JNK family, it consists of three members, JNK1 (Mapk8), JNK2 (Mapk9) and JNK3 (Mapk10; Sabio and Davis, 2010; Auladell et al., 2017; Solinas and Becattini, 2017). It has been proposed that the JNKs are involved in memory formation during learning under stressful conditions through the regulation of their activity (Coffey, 2014). Short-term activation of JNKs will temporarily boost memory performance, whereas prolonged activation of JNKs may be a contributing factor to memory deficit and even neurodegeneration (Sherrin et al., 2011). The pro-inflammatory

cytokine TNF-α activates JNK1 (Sabio and Davis, 2010; Solinas and Becattini, 2017). Indeed, it has been shown that JNKs are activated in obese humans, thus, JNK1 could be implicated in the mechanism of obesity-induced insulin resistance (Belgardt et al., 2010). Therefore, the characterization of JNK isoforms in the hippocampus and their role in memory processes seems paramount to understand the links among obesity, T2DM and LOAD (Solinas and Becattini, 2017).

In the hippocampus, JNKs have both presynaptic and postsynaptic localizations and can regulate proteins from synaptic vesicles, such as synaptotagmin-4 (Sherrin et al., 2010; Nisticò et al., 2015). JNKs also regulate second messenger systems such as cytosolic phospholipase A, cytoskeletal elements (i.e., MAP2, TAU), nuclear hormone receptors (such as the glucocorticoid receptor) or transcription factors, including c-Jun (a member of the activator protein-1, AP-1), activator transcription factor (ATF)-2, CREB (calcium/cAMP) and Elk-1 (Sabio and Davis, 2010; Kant et al., 2013). Hence, all these substrates are potential JNKs targets during the learning process. Moreover, as previously mentioned, JNKs may be central mediators of many of the deleterious consequences of obesity, such as impaired glucose metabolism and insulin resistance (Belgardt et al., 2010). This hypothesis was proposed after exposing JNK1 knockout mice to an HFD. Surprisingly, these animals were protected from the development of impaired glucose tolerance and insulin resistance (Pal et al., 2016). Indeed, activated JNK1 phosphorylates the insulin receptor substrate 1 (IRS-1) in the serine residues (IRS-1pSer), blocking the insulin pathway and causing a peripheral resistance to this hormone (Solinas and Becattini, 2017). Phosphorylation in S307 is a mechanism by which the activation of JNK can directly antagonize insulin action. Therefore, phosphorylation of IRS1 following the activation of JNK1 has a key role in the insulin resistance mechanism and obesity process in mammals. Likewise, the diabetic status alters the signaling pathway downstream of IR. Among others, it is relevant the energy alteration sensing pathway comprising the AMP-activated protein kinase (AMPK)/sirtuin (SIRT)/peroxisome proliferator-activated receptor-γ coactivator α (PGC-1α; Fernyhough, 2015). In fact, the intracellular ATP/ADP balance is regulated by AMPK, which acts as a master sensor that, in turns, also control the glucose and fatty acids consumption, and the mitochondrial biogenesis through PGC-1α activity. The energy balance inside the hippocampal neurons allows for the formation, maintenance and reorganization of synapses, all of them critical processes for brain development and appropriate responses generation from neuronal circuits to environmental challenges (Cheng et al., 2012). These authors demonstrated how PGC-1α activity increases dendritic spines and enhances the molecular differentiation of synapses in cultured hippocampal cells (**Figure 2**). Then, in light of evidences, it could be hypothesized PGC-1α as a molecular link between metabolic alterations involving brain diabetic status and cognitive impairment.

In addition, other kinases such as nuclear kappa-β kinase inhibitor (IKK) play a crucial role in the development of insulin resistance associated with obesity, in both peripheral and CNS (Sabio and Davis, 2010; Kant et al., 2013; Nisticò et al., 2015). Likewise, recent studies suggest that activated protein RNA kinase, also known as protein kinase R (PKR), plays an important role in insulin resistance induced by a HFD diet (Taga et al., 2018). Based on this activation sequence, JNK1 modulation looks like a promising molecule for future development of novel therapeutic targets aiming to prevent T2DM. Consequently, selective inhibitors like Licochalcone A, may be suitable approaches to treat T2DM-related cognitive loss (Busquets et al., 2018).

Unfortunately, there is a lack of natural or synthetic molecules capable to modulate the activity of JNKs so far. Among the extensive effort in exploring therapeutic interventions focusing on JNK activity, the compound SP600125, a JNK inhibitor, has been the best characterized (Gao et al., 2017). However, the application of SP600125 is very limited due to its low target selectivity, and its clinical efficacy is restricted due to its poor aqueous solubility (Gao et al., 2017). Notwithstanding, selective, water-soluble and brain-penetrant JNK inhibitors have been recently tested (Kumar et al., 2016). I.P. and i.c.v. administrations of SR11935, SR3306 and JNK2/3 isoform-selective inhibitors indicated possible anorectic effects (Ryu et al., 2016).

THE COMPLEX TREATMENT OF LATE ONSET ALZHEIMER'S DISEASE: MORE THAN ANTIDIABETIC DRUGS

At least, a link between cognitive loss and T2DM involves dysregulation of CNS circuits that control hepatic glucose production. Thus, targeting these circuits could be a novel potential strategy for the development of more effective therapies resulting in both, improved glucose regulation and cognitive performance. In this regard, our research group has already discussed the potential cognitive benefits from drugs originally addressed to treat T2DM (Batista et al., 2019; Forny-Germano et al., 2019). Indeed, some molecules used for the treatment of T2DM have shown neuroprotective effects in preclinical models of AD. These drugs included intranasal insulin, sulfonylureas, PPARγ agonists, metformin and GLP-1 receptor agonists such as exendin-4, liraglutide and lixisenatide (de la Monte, 2017; Cummings et al., 2018; Batista et al., 2019). This opens a promising perspective for these antidiabetic drugs. For instance, new formulations of dual GLP-1/GIP, and the triple combination GLP-1/GCG/GIP agonists, which are the most effective drugs for weight loss, have been evaluated to treat LOAD (Camins et al., 2019). Likewise, the pharmacological combination of GLP-1/GCG/GIP has been shown to prevent the decline of brain glucose metabolism in animal models (Capozzi et al., 2018). Molecularly, the triagonist upregulated the expression of CREB, reverted cognitive impairment and enhanced Long-term potentiation (LTP) in preclinical models of AD (Tai et al., 2018). The activation of CREB by phosphorylation at Ser133 (S133p-CREB) is a critical step for memory formation and LTP maintenance since the downstream genes are involved in synaptic formation, neuronal plasticity and neurogenesis (Ettcheto et al., 2018a).

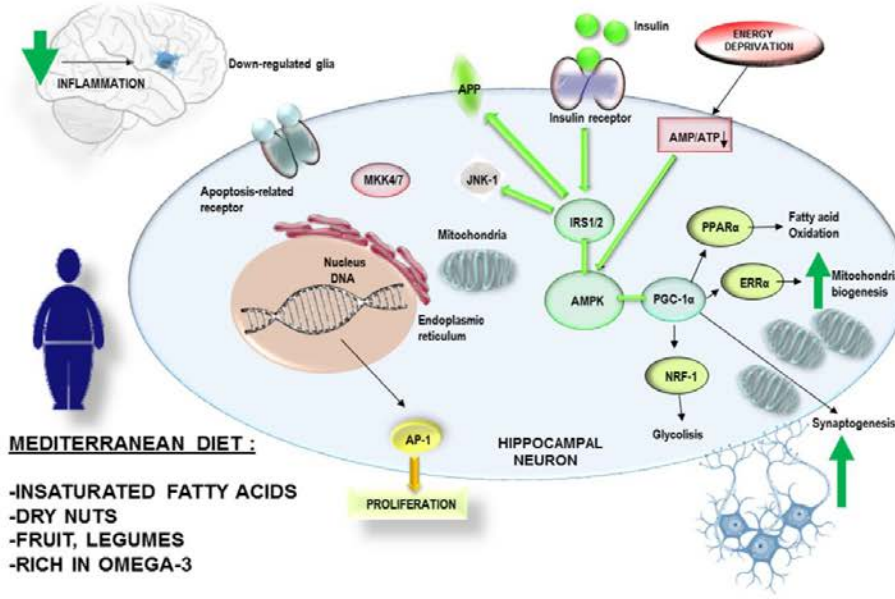


FIGURE 2 | A suitable diet with natural products, for example rich in omega 3, antioxidants et cetera. can be a non-pharmacological complement for the prevention of obesity. Therefore, it would also have effects on the decrease of peripheral and central inflammatory processes. This mechanism would have effects on the prevention of central insulin resistance and improvement of the cognitive processes. The adherence over time to MedDiet would reduce the inflammatory levels in brain, with a decrease in the generation of mitochondrial stress and JUN N-terminal Kinases-1 (JNK-1) activation. It would allow insulin receptor pathway to fine tune the mitochondrial biogenesis according to the ATP/ADP intracellular balance of neuronal cells related to synaptogenesis and neuronal plasticity.

However, taking into account the multidisciplinary nature of AD, more than one drug should be administered to reach a complete treatment capable of slowing the neurodegenerative process. Therefore, a combination of drugs that acts on different pathways involved in the neuropathology of the disease (i.e., amyloidogenic pathway, metabolism disorders, excitotoxicity or neuroinflammation) could be the optimal choice to treat AD. Furthermore, these drugs should be administered as soon as possible to delay the process of cognitive loss.

The antidiabetic drug pioglitazone has been evaluated for AD treatment in the so-called TOMMORROW clinical trial (ClinicalTrials.gov Identifier: NCT01931566). This phase III study assess delay of onset of MCI-AD in cognitively normal subjects between 65 and 83 years of age. The study has two objectives: the first is a new genetic test to determine whether participants are at risk of developing a mild cognitive impairment related to AD (MCI-AD) in the 5 years of study based on a genetic biomarker composed of TOMM40 and APOE genotypes and age used at the time of study incorporation. The second objective is to evaluate the efficacy of pioglitazone to delay the onset of MCI-AD in cognitively normal people who are at high risk of developing MCI-AD (Roses et al., 2014). The study will include 3,500 subjects. However, Takeda Pharmaceutical Company Limited after a preliminary analysis of the results reported that pioglitazone seems that was not effective in MCI-AD prevention.

The studies evaluating the administration of nasal insulin in the fight against forgetting (SNIFF) consists of a multicenter, double-blind, placebo-controlled phase 2/3 trial sponsored by the Cooperative Study of AD (ClinicalTrials.gov Identifier: NCT01767909). The study aims to evaluate the efficacy of intranasal administered insulin on cognition, entorhinal cortex and hippocampal atrophy, and cerebrospinal fluid (CSF) biomarkers in amnestic mild cognitive impairment (aMCI) or mild AD. Thus, it will study AD biomarker profile, gender, or APOE-ε4 allele carriage predict treatment response. According to the hypothesis after 12 months of treatment with nasal insulin compared to placebo, subjects would improve performance on a global measure of cognition, on a memory composite and on daily function. The results of the study have not yet been published.

Finally, as we have previously stated, it has been reported that Memantine improves the metabolic consequences produced by HFD in the APP/PS1 mice model of familial AD (Ettcheto et al., 2018b). These results demonstrate new possibilities into the

role of Memantine not only in the occurrence of AD treatment, but also into its potential application in peripheral metabolic disorders where Aβ could play a role, as is the case of T2DM (Ahmed et al., 2017; Folch et al., 2018).

NON-PHARMACOLOGICAL STRATEGIES TO ENHANCE COGNITIVE PERFORMANCE: DIET INTERVENTIONS

In 2018, the group of Dr. Marta di Carlo published new results in which they discussed the beneficial effects of a natural dietary supplement (NDS) containing *Curcuma longa*, silymarin, guggul, chlorogenic acid and inulin (Nuzzo et al., 2018). They showed that NDS exerts neuroprotective effects in HFD mice by reducing brain fat accumulation, oxidative stress and inflammation, as well as by improving brain insulin resistance (Nuzzo et al., 2018). Hence, it seems that dietary content can enhance or destabilize the delicate machinery that allows neurons to survive, which leads to the following question: could diet influence cognitive performance in human populations?

As it is common in science, the answer goes probably beyond a simple "yes" or "no," but diet is indeed an important modifiable lifestyle factor related to the development of many pathologies and, among them there is all the different subtypes of dementia (Gustafson et al., 2015). The studies leaded by Dr. Mia Kivipelto were the first to show that beneficial midlife dietary changes are associated with a reduced dementia risk later in life (Sindi et al., 2018). Their results highlighted the importance of targeting dietary patterns, describing how the combination of determined food may have synergistic effects, thus further potentiating their benefits. A meta-analysis by Hill et al. (2018, 2019) also revealed an effect of diet on AD biomarkers. With 2,726 records, the review supported the notion that diet and nutrition display potential for non-pharmacological strategies to improve the prognosis and prevent AD (Hill et al., 2018). More recent investigations also showed the potential cerebral benefits of diet interventions in human populations. For instance, results from the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER), which included 1,260 participants at-risk of cognitive decline (60–77 years), allowed to conclude that, in fact, dietary changes seem to play a key role in preventing cognitive loss (Lehtisalo et al., 2019). In this study, the ingestion of a balanced diet was associated with positive changes in executive function, especially in the intervention group, after a 2-years follow-up. Hence, these new approaches would show effects in the long term and would be effective if they were to be followed for a long-term. Thus, becoming complementary and preventive in the long run.

This leads us to the next question: is there any particular diet to adhere in order to prevent cognitive loss? In this regard, a growing body of evidence associate the Mediterranean diet (MedDiet) to preservation of cognitive performance in human populations. MedDiet is characterized by a high intake of fruits, vegetables, legumes, fish, whole grains, nuts, and olive oil, a moderate consumption of dairy products and wine, and a low intake of red and processed meats and foods that contain high amounts of added sugars (Trichopoulou et al., 2003). Indeed, recent results from non-Mediterranean populations suggest that higher MedDiet adherence is associated with higher global cognitive performance and brain structural integrity, as well as decreased risk of AD and vascular dementia (VaD; Karstens et al., 2019). In this line, the geographic location of our research group allows us to describe our own experience from a closer point of view, studying human populations naturally adhered to MedDiet due to cultural reasons. The PREDIMED (in Spanish: *PREvención con DIeta MEDiterránea*) study is a huge project that has published more than 200 articles in indexed journals during the last decade. The results from primary prevention trials reported that long-term adherence to a MedDiet, supplemented with either extra-virgin olive oil or nuts, reduced cardiovascular disease (Hu et al., 2013). Also, other studies on the antioxidant effects of walnuts proved evidence on their effects (Bulló et al., 2010). The results from the PREDIMED project rapidly spread through other metabolic aspects related to a MedDiet adherence. Of note, Salas-Salvadó et al. (2016) reviewed the role of MedDiet on preventing T2DM and stated that the role of the MedDiet on the prevention and management of T2DM and metabolic syndrome proves true according to the data of the study. In turn, metabolomic studies allowed to identify plasma compounds with potential to predict both insulin resistance and incident T2DM (Papandreou et al., 2019). This is relevant since, both in T2DM and LOAD, prevention could be a key factor, and there is still a lack of clear molecular markers allowing to detect at-risk candidates soon enough to reverse the damaging effects of these diseases.

Conclusively, a PREDIMED study examined the effect of T2DM on cognitive performance, specifically executive function tasks, in a large cohort of 6,823 patients above 55 years of age (Mallorquí-Bagué et al., 2018). In this cross-sectional study, T2DM (including illness duration), higher Body Mass Index and lower mood were linked to lower cognitive function in older individuals with conditions like overweight/obesity and metabolic syndrome. Of note, MedDiet includes a moderate intake of red wine and extra virgin olive oil, both of them rich in polyphenolic compounds, such as resveratrol, oleuropein, hydroxytyrosol and their derivatives, which have shown anti-inflammatory effects on microglia on *in vitro* and *in vivo* studies (Hornedo-Ortega et al., 2018). According to these observations, it can be hypothesized the adherence over time to MedDiet would reduce the inflammatory levels in brain and the generation of mitochondrial stress together with JNK-1 activation. It would allow insulin receptor pathway to fine tune the mitochondrial biogenesis according to the ATP/ADP intracellular balance of neuronal cells related to synaptogenesis and neuronal plasticity (**Figure 2**). By contrast, the exposure to high fat diets, enriched in saturated fatty acids, would promote the glial activation and mitochondrial oxidative stress. All those stressing factors would activate JNK-1 resulting in an impairment of insulin receptor pathway, causing and imbalance in ATP/ADP levels and a failure to maintain the synaptic connections (**Figure 3**). This hypothesis would explain how

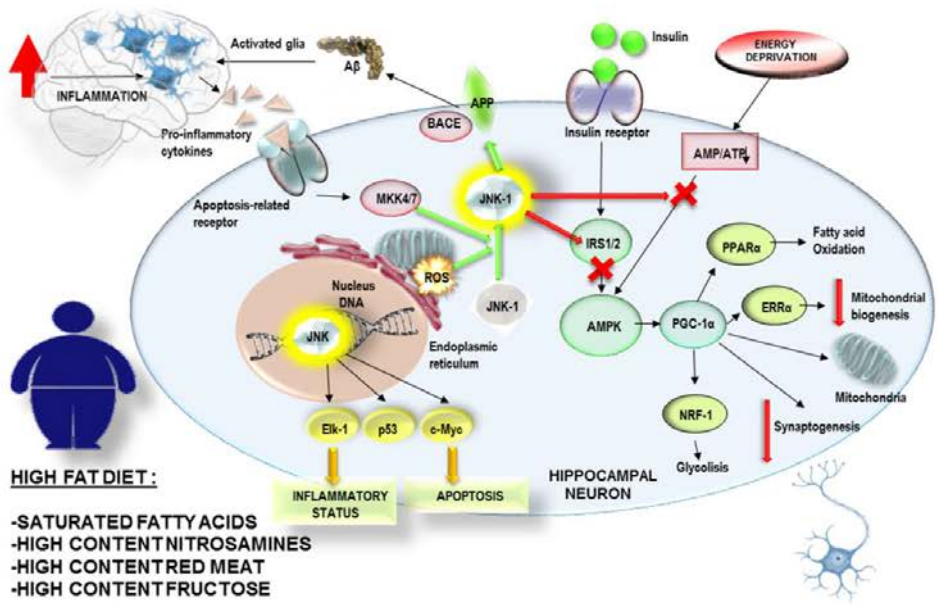


FIGURE 3 | The exposure to high fat diets over time, enriched in saturated fatty acids, would promote the glial activation and an increased mitochondrial oxidative stress. All those stressing factors would activate JNK-1 resulting in an impairment of insulin receptor pathway, causing and imbalance in ATP/ADP levels and a failure to control mitochondrial biogenesis and to maintain the synaptic connexions. This hypothesis would explain how JNK-1 should be a key factor linking diet and insulin signaling to synaptogenesis failure.

JNK-1 should be a key factor linking diet and insulin signaling to synaptogenesis failure.

Interestingly, one of the latest reports from the PREDIMED project revealed changes in circulating MicroRNAs (miRNAs). The GLYNDIET study is a 6-month, parallel, randomized clinical trial conducted on overweight and obese subjects. Results from GLYNDIET recently showed that the intake of an energy-restricted low-glycaemic index diet down-regulates circulating miRNA-361 more than an energy-restricted high-glycaemic index, regardless of the magnitude of the weight loss Giardina et al., 2019). Furthermore, Dr. Inestrosa stated in a seminal review that miRNAs may be a molecular link in the complex relationship between metabolic syndrome and AD (Codocedo et al., 2016) and, a recent systematic review extracted all miRNAs found to be significantly deregulated in peripheral blood and cross-referenced them against the miRNAs deregulated in the brain at Braak Stage III (Swarbrick et al., 2019). This resulted in a group of 10 miRNAs; hsa-mir-107, hsa-mir-26b, hsa-mir-30e, hsa-mir-34a, hsa-mir-485, hsa-mir200c, hsa-mir-210, hsa-mir-146a, hsa-mir-34c, and hsa-mir-125b that could be potentially involved in the regulation of these mechanisms. The authors hypothesized that these molecular markers could be deregulated early in AD, nearly 20 years before the emergence of clinical symptoms (Swarbrick et al., 2019). Yet, PREDIMED results on dysregulated circulating miRNAs in obesity and T2DM do not match with those from AD patients. Despite these negative preliminary observations, undoubtedly further studies on circulating miRNAs could open a new therapeutic perspective for patients affected by LOAD.

NEW PERSPECTIVES IN THE NEAR FUTURE

As it has been discussed in this review article, recent results from multiple studies have contributed to reinforce the proposed concept of T3D. In addition, from all the gathered data, it is clear that targeting LOAD early stages, before widespread neurodegeneration has occurred, is likely to produce the best clinical outcome. However, detection of individuals at this stage is still difficult. Consequently, new and reinforced efforts should be made towards the discovery and description of biomarkers that will allow for the early detection of pre-clinical candidates for T2DM and/or LOAD. Clearly, a huge effort will be necessary to overcome this molecular complexity just like in the new formulation of new and more effective treatments for LOAD. For now, it seems that the testing of more anti-T2DM drugs with beneficial effects against cognitive impairment has a certain promising future.

AUTHOR CONTRIBUTIONS

All the co-authors of this research (JF, JO, ME, OB, ES-L, ACano, TE-J, GC, CB-Z, MG, CA, MB and ACamins) have directly participated in the planning, execution of the manuscript. All authors have read and approved the final version submitted.

FUNDING

The Spanish Ministry of Science and Innovation SAF2017-84283-R, PI2016/01, CB06/05/0024 (CIBERNED), the European Regional Development Funds supported this work. Research team from UB and URV belongs to 2017SGR625 from Generalitat de Catalunya.

REFERENCES

Abbott, M. A., Wells, D. G., and Fallon, J. R. (1999). The insulin receptor tyrosine kinase substrate p58/53 and the insulin receptor are components of CNS synapses. *J. Neurosci.* 19, 7300–7308. doi: 10.1523/jneurosci.19-17-07300.1999

Ahmed, A. S., Elgharabawy, R. M., and Al-Najjar, A. H. (2017). Ameliorating effect of anti-Alzheimer's drugs on the bidirectional association between type 2 diabetes mellitus and Alzheimer's disease. *Exp. Biol. Med.* 242, 1335–1344. doi: 10.1177/1535370217711440

Alzheimer's Association. (2016). 2016 Alzheimer's disease facts and figures. *Alzheimers Dement.* 12, 459–509. doi: 10.1016/j.jalz.2016.03.001

An, Y., Varma, V. R., Varma, S., Casanova, R., Dammer, E., Pletnikova, O., et al. (2018). Evidence for brain glucose dysregulation in Alzheimer's disease. *Alzheimers Dement.* 14, 318–329. doi: 10.1016/j.jalz.2017.09.011

Arrieta-Cruz, I., and Gutiérrez-Juárez, R. (2016). The role of insulin resistance and glucose metabolism dysregulation in the development of Alzheimer's disease. *Rev. Invest. Clin.* 68, 53–58.

Arrieta-Cruz, I., Knight, C. M., and Gutiérrez-Juárez, R. (2015). Acute exposure of the mediobasal hypothalamus to amyloid-β25–35 perturbs hepatic glucose metabolism. *J. Alzheimers Dis.* 46, 843–848. doi: 10.3233/jad-131865

Auladell, C., de Lemos, L., Verdaguer, E., Ettcheto, M., Busquets, O., Lazarowski, A., et al. (2017). Role of JNK isoforms in the kainic acid experimental model of epilepsy and neurodegeneration. *Front. Biosci.* 22, 795–814. doi: 10.2741/4517

Banks, W. A., Farr, S. A., Salameh, T. S., Niehoff, M. L., Rhea, E. M., Morley, J. E., et al. (2018). Triglycerides cross the blood-brain barrier and induce central leptin and insulin receptor resistance. *Int. J. Obes.* 42, 391–397. doi: 10.1038/ijo.2017.231

Batista, A. F., Bodart, V., De Felice, F. G., and Ferreira, S. T. (2019). Neuroprotective actions of glucagon-like peptide-1 (GLP-1) analogues in Alzheimer's and Parkinson's diseases. *CNS Drugs* 33, 209–223. doi: 10.1007/s40263-018-0593-6

Belgardt, B. F., Mauer, J., and Brüning, J. C. (2010). Novel roles for JNK1 in metabolism. *Aging* 2, 621–626. doi: 10.18632/aging.100192

Biessels, G. J. (2013). Sweet memories: 20 years of progress in research on cognitive functioning in diabetes. *Eur. J. Pharmacol.* 719, 153–160. doi: 10.1016/j.ejphar.2013.04.055

Bocarsly, M. E., Fasolino, M., Kane, G. A., LaMarca, E. A., Kirschen, G. W., Karatsoreos, I. N., et al. (2015). Obesity diminishes synaptic markers, alters microglial morphology, and impairs cognitive function. *Proc. Natl. Acad. Sci. U S A* 112, 15731–15736. doi: 10.1073/pnas.1511593112

Bru, J. C., Gautam, D., Burks, D. J., Gillette, J., Schubert, M., Orban, P. C., et al. (2000). Role of brain insulin receptor in control of body weight and reproduction. *Science* 289, 2122–2125. doi: 10.1126/science.289.5487.2122

Bu, X. L., Xiang, Y., Jin, W. S., Wang, J., Shen, L. L., Huang, Z. L., et al. (2018). Blood-derived amyloid-β protein induces Alzheimer's disease pathologies. *Mol. Psychiatry* 23, 1948–1956. doi: 10.1038/mp.2017.204

Bulló, M., Nogués, M. R., López-Uriarte, P., Salas-Salvadó, J., and Romeu, M. (2010). Effect of whole walnuts and walnut-skin extracts on oxidant status in mice. *Nutrition* 26, 823–828. doi: 10.1016/j.nut.2009.09.002

Busquets, O., Ettcheto, M., Pallàs, M., Beas-Zarate, C., Verdaguer, E., Auladell, C., et al. (2017). Long-term exposition to a high fat diet favors the appearance of β-amyloid depositions in the brain of C57BL/6J mice. A potential model of sporadic Alzheimer's disease. *Mech. Ageing Dev.* 162, 38–45. doi: 10.1016/j.mad.2016.11.002

Busquets, O., Ettcheto, M., Verdaguer, E., Castro-Torres, R. D., Auladell, C., Beas-Zarate, C., et al. (2018). JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid. *Neuropharmacology* 131, 440–452. doi: 10.1016/j.neuropharm.2017.10.030

Cai, D. (2013). Neuroinflammation and neurodegeneration in overnutrition-induced diseases. *Trends Endocrinol. Metab.* 24, 40–47. doi: 10.1016/j.tem.2012.11.003

Camins, A., Ettcheto, M., Busquets, O., Manzine, P. R., Castro-Torres, R. D., Beas-Zarate, C., et al. (2019). Triple GLP-1/GIP/glucagon receptor agonists, a potential novel treatment strategy in Alzheimer's disease. *Expert Opin. Investig. Drugs* 28, 93–97. doi: 10.1080/13543784.2019.1552677

Capozzi, M. E., DiMarchi, R. D., Tschöp, M. H., Finan, B., and Campbell, J. E. (2018). Targeting the incretin/glucagon system with triagonists to treat diabetes. *Endocr. Rev.* 39, 719–738. doi: 10.1210/er.2018-00117

Cardoso, S., Seiça, R., and Moreira, P. I. (2017). "Diabesity and brain energy metabolism: the case of Alzheimer's disease," in *Obesity and Brain Function. Advances in Neurobiology*, eds L. Letra and R. Seiça (Cham: Springer), 117–150.

Castellani, R. J., Plascencia-Villa, G., and Perry, G. (2019). The amyloid cascade and Alzheimer's disease therapeutics: theory versus observation. *Lab. Invest.* 99, 958–970. doi: 10.1038/s41374-019-0231-z

Chami, B., Steel, A. J., De La Monte, S. M., and Sutherland, G. T. (2016). The rise and fall of insulin signaling in Alzheimer's disease. *Metab. Brain Dis.* 31, 497–515. doi: 10.1007/s11011-016-9806-1

Cheke, L. G., Simons, J. S., and Clayton, N. S. (2016). Higher body mass index is associated with episodic memory deficits in young adults. *Q. J. Exp. Psychol.* 69, 2305–2316. doi: 10.1080/17470218.2015.1099163

Chen, Z., and Zhong, C. (2013). Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies. *Prog. Neurobiol.* 108, 21–43. doi: 10.1016/j.pneurobio.2013.06.004

Cheng, A., Wan, R., Yang, J. L., Kamimura, N., Son, T. G., Ouyang, X., et al. (2012). Involvement of PGC-1α in the formation and maintenance of neuronal dendritic spines. *Nat. Commun.* 3:1250. doi: 10.1038/ncomms2238

Chua, L. M., Lim, M. L., Chong, P. R., Hu, Z. P., Cheung, N. S., and Wong, B. S. (2012). Impaired neuronal insulin signaling precedes Aβ42 accumulation in female AβPPsw/PS1ΔE9 mice. *J. Alzheimers Dis.* 29, 783–791. doi: 10.3233/jad-2012-111880

Clarke, J. R., Lyra E Silva, N. M., Figueiredo, C. P., Frozza, R. L., Ledo, J. H., Beckman, D., et al. (2015). Alzheimer-associated Aβ oligomers impact the central nervous system to induce peripheral metabolic deregulation. *EMBO Mol. Med.* 7, 190–210. doi: 10.15252/emmm.201404183

Clarke, J. R., Ribeiro, F. C., Frozza, R. L., De Felice, F. G., and Lourenco, M. V. (2018). Metabolic dysfunction in Alzheimer's disease: from basic neurobiology to clinical approaches. *J. Alzheimers Dis.* 64, S405–S426. doi: 10.3233/JAD-179911

Cleary, J. P., Walsh, D. M., Hofmeister, J. J., Shankar, G. M., Kuskowski, M. A., Selkoe, D. J., et al. (2005). Natural oligomers of the amyloid-β protein specifically disrupt cognitive function. *Nat. Neurosci.* 8, 79–84. doi: 10.1038/nn1372

Codocedo, J. F., Ríos, J. A., Godoy, J. A., and Inestrosa, N. C. (2016). Are microRNAs the molecular link between metabolic syndrome and Alzheimer's disease? *Mol. Neurobiol.* 53, 2320–2338. doi: 10.1007/s12035-015-9201-7

Coffey, E. T. (2014). Nuclear and cytosolic JNK signalling in neurons. *Nat. Rev. Neurosci.* 15, 285–299. doi: 10.1038/nrn3729

Correia, S. C., Santos, R. X., Perry, G., Zhu, X., Moreira, P. I., and Smith, M. A. (2011). Insulin-resistant brain state: the culprit in sporadic Alzheimer's disease? *Ageing Res. Rev.* 10, 264–273. doi: 10.1016/j.arr.2011.01.001

Cummings, J., Lee, G., Ritter, A., and Zhong, K. (2018). Alzheimer's disease drug development pipeline: 2018. *Alzheimers Dement.* 4, 195–214. doi: 10.1016/j.trci.2018.03.009

De Felice, F. G., Lourenco, M. V., and Ferreira, S. T. (2014). How does brain insulin resistance develop in Alzheimer's disease? *Alzheimers Dement.* 10, S26–S32. doi: 10.1016/j.jalz.2013.12.004

De Felice, F. G., Vieira, M. N., Bomfim, T. R., Decker, H., Velasco, P. T., Lambert, M. P., et al. (2009). Protection of synapses against Alzheimer's-linked toxins: insulin signaling prevents the pathogenic binding of Aβ oligomers. *Proc. Natl. Acad. Sci. U S A* 106, 1971–1976. doi: 10.1073/pnas.0809158106

de la Monte, M. S. (2012). Brain insulin resistance and deficiency as therapeutic targets in Alzheimers disease. *Curr. Alzheimer Res.* 9, 35–66. doi: 10.2174/156720512799015037

de la Monte, S. M. (2014). Relationships between diabetes and cognitive impairment. *Endocrinol. Metab. Clin. North Am.* 43, 245–267. doi: 10.1016/j.ecl.2013.09.006

de la Monte, S. M. (2017). Insulin resistance and neurodegeneration: progress towards the development of new therapeutics for Alzheimer's disease. *Drugs* 77, 47–65. doi: 10.1007/s40265-016-0674-0

de la Monte, S. M., Tong, M., Daiello, L. A., and Ott, B. R. (2019). Early-stage Alzheimer's disease is associated with simultaneous systemic and central nervous system dysregulation of insulin-linked metabolic pathways. *J. Alzheimers Dis.* 68, 657–668. doi: 10.3233/JAD-180906

de la Monte, S. M., Tong, M., Lester-Coll, N., Plater, M. Jr., and Wands, J. R. (2006). Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: relevance to Alzheimer's disease. *J. Alzheimers Dis.* 10, 89–109. doi: 10.3233/jad-2006-10113

de la Monte, S. M., Tong, M., and Wands, J. R. (2018). The 20-year voyage aboard the journal of Alzheimer's disease: docking at 'type 3 diabetes', environmental/exposure factors, pathogenic mechanisms, and potential treatments. *J. Alzheimers Dis.* 62, 1381–1390. doi: 10.3233/JAD-170829

de la Monte, S. M., and Wands, J. R. (2008). Alzheimer's disease is type 3 diabetes-evidence reviewed. *J. Diabetes Sci. Technol.* 2, 1101–1113. doi: 10.1177/193229680800200619

Deng, S.-N., Yan, Y.-H., Zhu, T.-L., Ma, B.-K., Fan, H.-R., Liu, Y.-M., et al. (2019). Long-term NMDAR antagonism correlates weight loss with less eating. *Front. Psychiatry* 10:15. doi: 10.3389/fpsyt.2019.00015

Dobson, C. M. (2017). The amyloid phenomenon and its links with human disease. *Cold Spring Harb. Perspect. Biol.* 9:a023648. doi: 10.1101/cshperspect.a023648

El Khoury, N. B., Gratuze, M., Papon, M.-A., Bretteville, A., and Planel, E. (2014). Insulin dysfunction and Tau pathology. *Front. Cell. Neurosci.* 8:22. doi: 10.3389/fncel.2014.00022

Ettcheto, M., Abad, S., Petrov, D., Pedrós, I., Busquets, O., Sánchez-López, E., et al. (2018a). Early preclinical changes in hippocampal creb-binding protein expression in a mouse model of familial Alzheimer's disease. *Mol. Neurobiol.* 55, 4885–4895. doi: 10.1007/s12035-017-0690-4

Ettcheto, M., Sánchez-López, E., Gómez-Mínguez, Y., Cabrera, H., Busquets, O., Beas-Zarate, C., et al. (2018b). Peripheral and central effects of memantine in a mixed preclinical mice model of obesity and familial Alzheimer's disease. *Mol. Neurobiol.* 55, 7327–7339. doi: 10.1007/s12035-018-0868-4

Ettcheto, M., Petrov, D., Pedrós, I., Alva, N., Carbonell, T., Beas-Zarate, C., et al. (2016). Evaluation of neuropathological effects of a high-fat diet in a presymptomatic Alzheimer's disease stage in APP/PS1 mice. *J. Alzheimers Dis.* 54, 233–251. doi: 10.3233/JAD-160150

Fadel, J. R., and Reagan, L. P. (2016). Stop signs in hippocampal insulin signaling: the role of insulin resistance in structural, functional and behavioral deficits. *Curr. Opin. Behav. Sci.* 9, 47–54. doi: 10.1016/j.cobeha.2015.12.004

Fasshauer, M., and Blüher, M. (2015). Adipokines in health and disease. *Trends Pharmacol. Sci.* 36, 461–470. doi: 10.1016/j.tips.2015.04.014

Fernyhough, P. (2015). Mitochondrial dysfunction in diabetic neuropathy: a series of unfortunate metabolic events. *Curr. Diab. Rep.* 15:89. doi: 10.1007/s11892-015-0671-9

Ferreira, S. T., Clarke, J. R., Bomfim, T. R., and De Felice, F. G. (2014). Inflammation, defective insulin signaling, and neuronal dysfunction in Alzheimer's disease. *Alzheimers Dement.* 10, S76–S83. doi: 10.1016/j.jalz.2013.12.010

Folch, J., Busquets, O., Ettcheto, M., Sánchez-López, E., Castro-Torres, R. D., Verdaguer, E., et al. (2018). Memantine for the treatment of dementia: a review on its current and future applications. *J. Alzheimers Dis.* 62, 1223–1240. doi: 10.3233/JAD-170672

Folch, J., Pedrós, I., Patraca, I., Martínez, N., Sureda, F., and Camins, A. (2013). Metabolic basis of sporadic Alzeimer's disease. Role of hormones related to energy metabolism. *Curr. Pharm. Des.* 19, 6739–6748. doi: 10.2174/13816128113199990612

Forny-Germano, L., De Felice, F. G., and Vieira, M. N. D. N. (2019). The role of leptin and adiponectin in obesity-associated cognitive decline and Alzheimer's disease. *Front. Neurosci.* 12:1027. doi: 10.3389/fnins.2018.01027

Freiherr, J., Hallschmid, M., Frey, W. H. II., Brünner, Y. F., Chapman, C. D., Hölscher, C., et al. (2013). Intranasal insulin as a treatment for alzheimer's disease: a review of basic research and clinical evidence. *CNS Drugs* 27, 505–514. doi: 10.1007/s40263-013-0076-8

Frölich, L., Müller, W. E., and Riederer, P. (2015). Editorial: siegfried Hoyer's concept of Alzheimer pathophysiology. *J. Neural Transm.* 122, 495–497. doi: 10.1007/s00702-015-1371-y

Gao, S., Howard, S., and Lograsso, P. V. (2017). Pharmacological inhibition of c-Jun N-terminal kinase reduces food intake and sensitizes leptin's anorectic signaling actions. *Sci. Rep.* 7:41795. doi: 10.1038/srep41795

Gauthier, S., Albert, M., Fox, N., Goedert, M., Kivipelto, M., Mestre-Ferrandiz, J., et al. (2016). Why has therapy development for dementia failed in the last two decades? *Alzheimers Dement.* 12, 60–64. doi: 10.1016/j.jalz.2015.12.003

Giardina, S., Hernández-Alonso, P., Díaz-López, A., Salas-Huetos, A., Salas-Salvadó, J., and Bulló, M. (2019). Changes in circulating miRNAs in healthy overweight and obese subjects: effect of diet composition and weight loss. *Clin. Nutr.* 38, 438–443. doi: 10.1016/j.clnu.2017.11.014

Gonçalves, R. A., Wijesekara, N., Fraser, P. E., and De Felice, F. G. (2019). The link between tau and insulin signaling: implications for Alzheimer's disease and other tauopathies. *Front. Cell. Neurosci.* 13:17. doi: 10.3389/fncel.2019.00017

Grillo, C. A., Piroli, G. G., Lawrence, R. C., Wrighten, S. A., Green, A. J., Wilson, S. P., et al. (2015). Hippocampal insulin resistance impairs spatial learning and synaptic plasticity. *Diabetes* 64, 3927–3936. doi: 10.2337/db15-0596

Guillemot-Legris, O., Masquelier, J., Everard, A., Cani, P. D., Alhouayek, M., and Muccioli, G. G. (2016). High-fat diet feeding differentially affects the development of inflammation in the central nervous system. *J. Neuroinflammation* 13:206. doi: 10.1186/s12974-016-0666-8

Gustafson, D. R., Clare Morris, M., Scarmeas, N., Shah, R. C., Sijben, J., Yaffe, K., et al. (2015). New perspectives on Alzheimer's disease and nutrition. *J. Alzheimers Dis.* 46, 1111–1127. doi: 10.3233/JAD-150084

Hardy, J. (2009). The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. *J. Neurochem.* 110, 1129–1134. doi: 10.1111/j.1471-4159.2009.06181.x

Hargrave, S. L., Jones, S., and Davidson, T. L. (2016). The outward spiral: a vicious cycle model of obesity and cognitive dysfunction. *Curr. Opin. Behav. Sci.* 9, 40–46. doi: 10.1016/j.cobeha.2015.12.001

Hill, E., Clifton, P., Goodwill, A. M., Dennerstein, L., Campbell, S., and Szoeke, C. (2018). Dietary patterns and β-amyloid deposition in aging Australian women. *Alzheimers Dement.* 4, 535–541. doi: 10.1016/j.trci.2018.09.007

Hill, E., Goodwill, M. A., Gorelik, A., and Szoeke, C. (2019). Diet and biomarkers of Alzheimer's disease: a systematic review and meta-analysis. *Neurobiol. Aging* 76, 45–52. doi: 10.1016/j.neurobiolaging.2018.12.008

Hokama, M., Oka, S., Leon, J., Ninomiya, T., Honda, H., Sasaki, K., et al. (2014). Altered expression of diabetes-related genes in Alzheimer's disease brains: the Hisayama study. *Cereb. Cortex* 24, 2476–2488. doi: 10.1093/cercor/bht101

Hornedo-Ortega, R., Cerezo, A. B., de Pablos, R. M., Krisa, S., Richard, T., García-Parrilla, M. C., et al. (2018). Phenolic compounds characteristic of the mediterranean diet in mitigating microglia-mediated neuroinflammation. *Front. Cell. Neurosci.* 12:373. doi: 10.3389/fncel.2018.00373

Hoyer, S. (2002a). The aging brain. Changes in the neuronal insulin/insulin receptor signal transduction cascade trigger late-onset sporadic Alzheimer disease (SAD). A mini-review. *J. Neural Transm.* 109, 991–1002. doi: 10.1007/s007020200082

Hoyer, S. (2002b). The brain insulin signal transduction system and sporadic (type II) Alzheimer disease: an update. *J. Neural Transm.* 109, 341–360. doi: 10.1007/s007020200028

Hoyer, S. (2004). Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. *Eur. J. Pharmacol.* 490, 115–125. doi: 10.1016/j.ejphar.2004.02.049

Hu, E. A., Toledo, E., Diez-Espino, J., Estruch, R., Corella, D., Salas-Salvado, J., et al. (2013). Lifestyles and risk factors associated with adherence to the mediterranean diet: a baseline assessment of the PREDIMED trial. *PLoS One* 8:e60166. doi: 10.1371/journal.pone.0060166

Huang, N. Q., Jin, H., Zhou, S. Y., Shi, J. S., and Jin, F. (2017). TLR4 is a link between diabetes and Alzheimer's disease. *Behav. Brain Res.* 316, 234–244. doi: 10.1016/j.bbr.2016.08.047

Hurtado-Puerto, A. M., Russo, C., and Fregni, F. (2018). Alzheimer's disease. *Neuromethods* 138, 297–338. doi: 10.1007/978-1-4939-7880-9_9

Kandimalla, R., Thirumala, V., and Reddy, P. H. (2017). Is Alzheimer's disease a type 3 diabetes? A critical appraisal. *Biochim. Biophys. Acta* 1863, 1078–1089. doi: 10.1016/j.bbadis.2016.08.018

Kang, S., Lee, Y. H., and Lee, J. E. (2017). Metabolism-centric overview of the pathogenesis of Alzheimer's disease. *Yonsei Med. J.* 58, 479–488. doi: 10.3349/ymj.2017.58.3.479

Kant, S., Barrett, T., Vertii, A., Noh, Y. H., Jung, D. Y., Kim, J. K., et al. (2013). Role of the mixed-lineage protein kinase pathway in the metabolic stress response to obesity. *Cell Rep.* 4, 681–688. doi: 10.1016/j.celrep.2013.07.019

Karstens, A. J., Tussing-Humphreys, L., Zhan, L., Rajendran, N., Cohen, J., Dion, C., et al. (2019). Associations of the Mediterranean diet with cognitive and neuroimaging phenotypes of dementia in healthy older adults. *Am. J. Clin. Nutr.* 109, 361–368. doi: 10.1093/ajcn/nqy275

Katsouri, L., Lim, Y. M., Blondrath, K., Eleftheriadou, I., Lombardero, L., Birch, A. M., et al. (2016). PPARγ-coactivator-1α gene transfer reduces neuronal loss and amyloid-β generation by reducing β-secretase in an Alzheimer's disease model. *Proc. Natl. Acad. Sci. U S A* 113, 12292–12297. doi: 10.1073/pnas.1606171113

Kivipelto, M., Mangialasche, F., and Ngandu, T. (2018). Lifestyle interventions to prevent cognitive impairment, dementia and Alzheimer disease. *Nat. Rev. Neurol.* 14, 653–666. doi: 10.1038/s41582-018-0070-3

Kohjima, M., Sun, Y., and Chan, L. (2010). Increased food intake leads to obesity and insulin resistance in the Tg2576 Alzheimer's disease mouse model. *Endocrinology* 151, 1532–1540. doi: 10.1210/en.2009-1196

Kothari, V., Luo, Y., Tornabene, T., O'Neill, A. M., Greene, M. W., Geetha, T., et al. (2017). High fat diet induces brain insulin resistance and cognitive impairment in mice. *Biochim. Biophys. Acta Mol. Basis Dis.* 1863, 499–508. doi: 10.1016/j.bbadis.2016.10.006

Kulas, J. A., Puig, K. L., and Combs, C. K. (2017). Amyloid precursor protein in pancreatic islets. *J. Endocrinol.* 235, 49–67. doi: 10.1530/joe-17-0122

Kumar, V., Kumar, M., Beniwal, V., Gupta, G. K., Kumar, S., and Kataria, R. (2016). Synthesis of some aroylhydrazones and 2,5-disubstituted-1,3,4-oxadiazoles as DNA photocleaving agents. *Med. Chem.* 6, 474–485. doi: 10.4172/2161-0444.1000386

Kuo, Y., Kokjohn, T. A., Watson, M. D., Woods, A. S., Cotter, R. J., Sue, L. I., et al. (2000). Elevated aβ42 in skeletal muscle of Alzheimer disease patients suggests peripheral alterations of AβPP metabolism. *Am. J. Pathol.* 156, 797–805. doi: 10.1016/S0002-9440(10)64947-4

Lehtisalo, J., Levälahti, E., Lindström, J., Hänninen, T., Paajanen, T., Peltonen, M., et al. (2019). Dietary changes and cognition over 2 years within a multidomain intervention trial—the finnish geriatric intervention study to prevent cognitive impairment and disability (FINGER). *Alzheimers Dement.* 15, 410–417. doi: 10.1016/j.jalz.2018.10.001

Lourenco, M. V., Frozza, R. L., de Freitas, G. B., Zhang, H., Kincheski, G. C., Ribeiro, F. C., et al. (2019). Exercise-linked FNDC5/irisin rescues synaptic plasticity and memory defects in Alzheimer's models. *Nat. Med.* 25, 165–175. doi: 10.1038/s41591-018-0275-4

Luciano, R., Barraco, G. M., Muraca, M., Ottino, S., Spreghini, M. R., Sforza, R. W., et al. (2015). Biomarkers of Alzheimer disease, insulin resistance, and obesity in childhood. *Pediatrics* 135, 1074–1081. doi: 10.1542/peds.2014-2391

Lyra E Silva, N. M., Gonçalves, R. A., Boehnke, S. E., Forny-Germano, L., Munoz, D. P., and De Felice, F. G. (2019). Understanding the link between insulin resistance and Alzheimer's disease: insights from animal models. *Exp. Neurol.* 316, 1–11. doi: 10.1016/j.expneurol.2019.03.016

Ma, Q.-L., Yang, F., Rosario, E. R., Ubeda, O. J., Beech, W., Gant, D. J., et al. (2009). β-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin. *J. Neurosci.* 29, 9078–9089. doi: 10.1523/JNEUROSCI.1071-09.2009

Macauley, S. L., Stanley, M., Caesar, E., Yamada, S., Raichle, M., Perez, R., et al. (2015). Hyperglycemia modulates extracellular amyloid-β levels and neuronal activity *in vivo*. *J. Clin. Invest.* 125, 2463–2467. doi: 10.1172/jci79742

Mallorquí-Bagué, N., Lozano-madrid, M., Toledo, E., Corella, D., Salas-Salvadó, J., Cuenca-Royo, A., et al. (2018). Type 2 diabetes and cognitive impairment in an older population with overweight or obesity and metabolic syndrome: baseline cross-sectional analysis of the PREDIMED-plus study. *Sci. Rep.* 8:16128. doi: 10.1038/s41598-018-33843-8

Marciniak, E., Leboucher, A., Caron, E., Ahmed, T., Tailleux, A., Dumont, J., et al. (2017). Tau deletion promotes brain insulin resistance. *J. Exp. Med.* 214, 2257–2269. doi: 10.1084/jem.20161731

Maurer, K., and Hoyer, S. (2006). Alois Alzheimer revisited: differences in origin of the disease carrying his name. *J. Neural Transm.* 113, 1645–1658. doi: 10.1007/s00702-006-0592-5

Miklossy, J., Qing, H., Radenovic, A., Kis, A., Vileno, B., Làszló, F., et al. (2010). β amyloid and hyperphosphorylated tau deposits in the pancreas in type 2 diabetes. *Neurobiol. Aging* 31, 1503–1515. doi: 10.1016/j.neurobiolaging.2008.08.019

Molofsky, A. V., Krenick, R., Ullian, E., Tsai, H., Deneen, B., Richardson, W. D., et al. (2012). Astrocytes and disease: a neurodevelopmental perspective. *Genes Dev.* 26, 891–907. doi: 10.1101/gad.188326.112

Moreno-Gonzalez, I., Edwards III, G., Shahnawaz, M., Salvadores, N., Soto, C., and Diaz-Espinoza, R. (2017). Molecular interaction between type 2 diabetes and Alzheimer's disease through cross-seeding of protein misfolding. *Mol. Psychiatry* 22, 1327–1334. doi: 10.1038/mp.2016.230

Morgen, K., and Frölich, L. (2015). The metabolism hypothesis of Alzheimer's disease: from the concept of central insulin resistance and associated consequences to insulin therapy. *J. Neural Transm.* 122, 499–504. doi: 10.1007/s00702-015-1377-5

Moser, V. A., and Pike, C. J. (2017). Obesity accelerates Alzheimer-related pathology in *APOE4* but not *APOE3* mice. *eNeuro* 4:ENEURO.0077-17.2017. doi: 10.1523/eneuro.0077-17.2017

Mukherjee, A., Morales-Scheihing, D., Salvadores, N., Moreno-Gonzalez, I., Gonzalez, C., Taylor-Presse, K., et al. (2017). Induction of IAPP amyloid deposition and associated diabetic abnormalities by a prion-like mechanism. *J. Exp. Med.* 214, 2591–2610. doi: 10.1084/jem.20161134

Nisticò, R., Florenzano, F., Mango, D., Ferraina, C., Grilli, M., Di Prisco, S., et al. (2015). Presynaptic c-Jun N-terminal Kinase 2 regulates NMDA receptor-dependent glutamate release. *Sci. Rep.* 5:9035. doi: 10.1038/srep09035

Nuzzo, D., Amato, A., Picone, P., Terzo, S., Galizzi, G., Bonina, F. P., et al. (2018). A natural dietary supplement with a combination of nutrients prevents neurodegeneration induced by a high fat diet in mice. *Nutrients* 10:E1130. doi: 10.3390/nu10091130

Nuzzo, D., Picone, P., Baldassano, S., Caruana, L., Messina, E., Gammazza, A., et al. (2015). Insulin resistance as common molecular denominator linking obesity to Alzheimer's disease. *Curr. Alzheimer Res.* 12, 723–735. doi: 10.2174/1567205012666150710115506

Obici, S. (2009). Minireview: molecular targets for obesity therapy in the brain. *Endocrinology* 150, 2512–2517. doi: 10.1210/en.2009-0409

Obici, S., Zhang, B. B., Karkanias, G., and Rossetti, L. (2002). Hypothalamic insulin signaling is required for inhibition of glucose production. *Nat. Med.* 8, 1376–1382. doi: 10.1038/nm798

Osborne, D. M., Fitzgerald, D. P., O'Leary, K. E., Anderson, B. M., Lee, C. C., Tessier, P. M., et al. (2016). Intrahippocampal administration of a domain antibody that binds aggregated amyloid-β reverses cognitive deficits produced by diet-induced obesity. *Biochim. Biophys. Acta* 1860, 1291–1298. doi: 10.1016/j.bbagen.2016.03.005

Ott, A., Stolk, R. P., van Harskamp, F., Pols, H. A., Hofman, A., and Breteler, M. M. (1999). Diabetes mellitus and the risk of dementia: the Rotterdam study. *Neurology* 53, 1937–1942. doi: 10.1212/wnl.53.9.1937

Pal, M., Febbraio, M. A., and Lancaster, G. I. (2016). The roles of c-Jun NH2-terminal kinases (JNKs) in obesity and insulin resistance. *J. Physiol.* 594, 267–279. doi: 10.1113/jp271457

Papandreou, C., Bullo, M., Ruiz-Canela, M., Dennis, C., Deik, A., Wang, D., et al. (2019). Plasma metabolites predict both insulin resistance and incident type 2 diabetes: a metabolomics approach within the Prevencion con Dieta Mediterranea (PREDIMED) study. *Am. J. Clin. Nutr.* 109, 626–634. doi: 10.1093/ajcn/nqy262

Parrella, E., Maxim, T., Maialetti, F., Zhang, L., Wan, J., Wei, M., et al. (2013). Protein restriction cycles reduce IGF-1 and phosphorylated tau and improve behavioral performance in an Alzheimer's disease mouse model. *Aging Cell* 12, 257–268. doi: 10.1111/acel.12049

Peila, R., Rodriguez, B. L., and Launer, L. J. (2002). Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies. *Diabetes* 51, 1256–1262. doi: 10.2337/diabetes.51.4.1256

Petrov, D., Pedrós, I., Artiach, G., Sureda, F. X., Barroso, E., Pallàs, M., et al. (2015). High-fat diet-induced deregulation of hippocampal insulin signaling and mitochondrial homeostasis deficiences contribute to Alzheimer disease pathology in rodents. *Biochim. Biophys. Acta* 1852, 1687–1699. doi: 10.1016/j.bbadis.2015.05.004

Plucińska, K., Dekeryte, R., Koss, D., Shearer, K., Mody, N., Whitfield, P. D., et al. (2016). Neuronal human BACE1 knockin induces systemic diabetes in mice. *Diabetologia* 59, 1513–1523. doi: 10.1007/s00125-016-3960-1

Pomytkin, I., Costa-Nunes, J. P., Kasatkin, V., Veniaminova, E., Demchenko, A., Lyundup, A., et al. (2018). Insulin receptor in the brain: mechanisms of activation and the role in the CNS pathology and treatment. *CNS Neurosci. Ther.* 24, 763–774. doi: 10.1111/cns.12866

Pratchayasakul, W., Sa-nguanmoo, P., Sivasinprasasn, S., Pintana, H., Tawinvisan, R., Sripetchwandee, J., et al. (2015). Obesity accelerates cognitive decline by aggravating mitochondrial dysfunction, insulin resistance and synaptic dysfunction under estrogen-deprived conditions. *Horm. Behav.* 72, 68–77. doi: 10.1016/j.yhbeh.2015.04.023

Raji, C. A., Ho, A. J., Parikshak, N. N., Becker, J. T., Lopez, O. L., Kuller, L. H., et al. (2010). Brain structure and obesity. *Hum. Brain Mapp.* 31, 353–364. doi: 10.1002/hbm.20870

Ramalingam, M., and Kim, S. J. (2014). Mechanisms of action of brain insulin against neurodegenerative diseases. *J. Neural Transm.* 121, 611–626. doi: 10.1007/s00702-013-1147-1

Ribe, E. M., and Lovestone, S. (2016). Insulin signalling in Alzheimer's disease and diabetes: from epidemiology to molecular links. *J. Intern. Med.* 280, 430–442. doi: 10.1111/joim.12534

Rodriguez-Rodriguez, P., Sandebring-Matton, A., Merino-Serrais, P., Parrado-Fernandez, C., Rabano, A., Winblad, B., et al. (2017). Tau hyperphosphorylation induces oligomeric insulin accumulation and insulin resistance in neurons. *Brain* 140, 3269–3285. doi: 10.1093/brain/awx256

Roher, A. E., Esh, C. L., Kokjohn, T. A., Castaño, E. M., Van Vickle, G. D., Kalback, W. M., et al. (2009). Amyloid β peptides in human plasma and tissues and their significance for Alzheimer's disease. *Alzheimers Dement.* 5, 18–29. doi: 10.1016/j.jalz.2008.10.004

Roses, A. D., Saunders, A. M., Lutz, M. W., Zhang, N., Hariri, A. R., Asin, K. E., et al. (2014). New applications of disease genetics and pharmacogenetics to drug development. *Curr. Opin. Pharmacol.* 14, 81–89. doi: 10.1016/j.coph.2013.12.002

Ryu, Y., Jin, L., Kee, H. J., Piao, Z. H., Cho, J. Y., Kim, G. R., et al. (2016). Gallic acid prevents isoproterenol-induced cardiac hypertrophy and fibrosis through regulation of JNK2 signaling and Smad3 binding activity. *Sci. Rep.* 6:34790. doi: 10.1038/srep34790

Sabio, G., and Davis, R. J. (2010). CJun NH2-terminal kinase 1 (JNK1): Roles in metabolic regulation of insulin resistance. *Trends Biochem. Sci.* 35, 490–496. doi: 10.1016/j.tibs.2010.04.004

Sah, S. K., Lee, C., Jang, J. H., and Park, G. H. (2017). Effect of high-fat diet on cognitive impairment in triple-transgenic mice model of Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 493, 731–736. doi: 10.1016/j.bbrc.2017.08.122

Salas-Salvadó, S. J., Guasch-Ferré, M., Lee, C. H., Estruch, R., Clish, C. B., and Ros, E. (2016). Protective effects of the mediterranean diet on type 2 diabetes and metabolic syndrome. *J. Nutr.* 146, 920S–927S. doi: 10.3945/jn.115.218487

Salkovic-Petrisic, M., Knezovic, A., Hoyer, S., and Riederer, P. (2013). What have we learned from the streptozotocin-induced animal model of sporadic Alzheimer's disease, about the therapeutic strategies in Alzheimer's research. *J. Neural Transm.* 120, 233–252. doi: 10.1007/s00702-012-0877-9

Salkovic-Petrisic, M., Osmanovic, J., Grünblatt, E., Riederer, P., and Hoyer, S. (2009). Modeling sporadic Alzheimer's disease: the insulin resistant brain state generates multiple long-term morphobiological abnormalities including hyperphosphorylated tau protein and amyloid-β. *J. Alzheimers Dis.* 18, 729–750. doi: 10.3233/JAD-2009-1184

Sallam, H. S., Tumurbaatar, B., Zhang, W. R., Tuvdendorj, D., Chandalia, M., Tempia, F., et al. (2015). Peripheral adipose tissue insulin resistance alters lipid composition and function of hippocampal synapses. *J. Neurochem.* 133, 125–133. doi: 10.1111/jnc.13043

Sandoval, D. A., Obici, S., and Seeley, R. J. (2009). Targeting the CNS to treat type 2 diabetes. *Nat. Rev. Drug Discov.* 8, 386–398. doi: 10.1038/nrd2874

Sato, N., and Morishita, R. (2013). Plasma Aβ: a possible missing link between alzheimer disease and diabetes. *Diabetes* 62, 1005–1006. doi: 10.2337/db12-1549

Schrijvers, E. M., Witteman, J. C., Sijbrands, E. J., Hofman, A., Koudstaal, P. J., and Breteler, M. M. (2010). Insulin metabolism and the risk of Alzheimer disease: the Rotterdam study. *Neurology* 75, 1982–1987. doi: 10.1212/WNL.0b013e3181ffe4f6

Selkoe, D. J., and Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol. Med.* 8, 595–608. doi: 10.15252/emmm.201606210

Sheng, B., Wang, X., Su, B., Lee, H. G., Casadesus, G., Perry, G., et al. (2012). Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in Alzheimer's disease. *J. Neurochem.* 120, 419–429. doi: 10.1111/j.1471-4159.2011.07581.x

Sherrin, T., Black, T., and Todorovic, C. (2011). C-Jun N-terminal kinases in memory and synaptic plasticity. *Rev. Neurosci.* 22, 403–410. doi: 10.1515/rns.2011.032

Sherrin, T., Blank, T., Hippel, C., Rayner, M., Davis, R. J., and Todorovic, C. (2010). Hippocampal c-Jun-N-terminal kinases serve as negative regulators of associative learning. *J. Neurosci.* 30, 13348–13361. doi: 10.1523/jneurosci.3492-10.2010

Shinohara, M., and Sato, N. (2017). Bidirectional interactions between diabetes and Alzheimer's disease. *Neurochem. Int.* 108, 296–302. doi: 10.1016/j.neuint.2017.04.020

Sindi, S., Kåreholt, I., Eskelinen, M., Hooshmand, B., Lehtisalo, J., Soininen, H., et al. (2018). Healthy dietary changes in midlife are associated with reduced dementia risk later in life. *Nutrients* 10:E1649. doi: 10.3390/nu10111649

Singh-Manoux, A., Dugravot, A., Shipley, M., Brunner, E. J., Elbaz, A., Sabia, S., et al. (2018). Obesity trajectories and risk of dementia: 28 years of follow-up in the whitehall II study. *Alzheimers Dement.* 14, 178–186. doi: 10.1016/j.jalz.2017.06.2637

Solinas, G., and Becattini, B. (2017). JNK at the crossroad of obesity, insulin resistance and cell stress response. *Mol. Metab.* 6, 174–184. doi: 10.1016/j.molmet.2016.12.001

Stanley, M., Macauley, S. L., and Holtzman, D. M. (2016). Changes in insulin and insulin signaling in Alzheimer's disease: cause or consequence? *J. Exp. Med.* 213, 1375–1385. doi: 10.1084/jem.20160493

Steen, E., Terry, B. M., Rivera, E. J., Cannon, J. L., Neely, T. R., Tavares, R., et al. (2005). Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—is this type 3 diabetes?. *J. Alzheimers Dis.* 7, 63–80. doi: 10.3233/jad-2005-7107

Suzuki, A., Fukushima, H., Mukawa, T., Toyoda, H., Wu, L.-J., Zhao, M.-G., et al. (2011). Upregulation of CREB-mediated transcription enhances both short- and long-term memory. *J. Neurosci.* 31, 8786–8802. doi: 10.1523/jneurosci.3257-10.2011

Swarbrick, S., Wragg, N., Ghosh, S., and Stolzing, A. (2019). Systematic review of miRNA as biomarkers in Alzheimer's disease. *Mol. Neurobiol.* 56, 6156–6167. doi: 10.1007/s12035-019-1500-y

Swardfager, W., Lanctt, K., Rothenburg, L., Wong, A., Cappell, J., and Herrmann, N. (2010). A meta-analysis of cytokines in Alzheimer's disease. *Biol. Psychiatry* 68, 930–941. doi: 10.1016/j.biopsych.2010.06.012

Taga, M., Mouton-Liger, F., Sadoune, M., Gourmaud, S., Norman, J., Tible, M., et al. (2018). PKR modulates abnormal brain signaling in experimental obesity. *PLoS One* 13:e0196983. doi: 10.1371/journal.pone.0196983

Tai, J., Liu, W., Li, Y., Li, L., and Hölscher, C. (2018). Neuroprotective effects of a triple GLP-1/GIP/glucagon receptor agonist in the APP/PS1 transgenic mouse model of Alzheimer's disease. *Brain Res.* 1678, 64–74. doi: 10.1016/j.brainres.2017.10.012

Talbot, K., Wang, H. Y., Kazi, H., Han, L. Y., Bakshi, K. P., Stucky, A., et al. (2012). Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation and cognitive decline. *J. Clin. Invest.* 122, 1316–1338. doi: 10.1172/jci59903

Tarasoff-Conway, J. M., Carare, R. O., Osorio, R. S., Glodzik, L., Butler, T., Fieremans, E., et al. (2015). Clearance systems in the brain-implications for Alzheimer disease. *Nat. Rev. Neurol.* 11, 457–470. doi: 10.1038/nrneurol.2015.119

Teich, A. F., Nicholls, R. E., Puzzo, D., Fiorito, J., Purgatorio, R., Fa', M., et al. (2015). Synaptic therapy in Alzheimer's disease: a CREB-centric approach. *Neurotherapeutics* 12, 29–41. doi: 10.1007/s13311-014-0327-5

Tong, M., Leão, R., Vimbela, G. V., Yalcin, E. B., Kay, J., Krotow, A., et al. (2017). Altered temporal lobe white matter lipid ion profiles in an experimental model of sporadic Alzheimer's disease. *Mol. Cell. Neurosci.* 82, 23–34. doi: 10.1016/j.mcn.2017.04.010

Trichopoulou, A., Costacou, T., Bamia, C., and Trichopoulos, D. (2003). Adherence to a mediterranean diet and survival in a Greek population. *N. Engl. J. Med.* 348, 2599–2608. doi: 10.1056/nejmoa025039

Vazquez-Valls, E., Flores-Soto, M. E., Chaparro-Huerta, V., Torres-Mendoza, B. M., Gudiño-Cabrera, G., Rivera-Cervantes, M. C., et al. (2011). HIF-1α expression in the hippocampus and peripheral macrophages after glutamate-induced excitotoxicity. *J. Neuroimmunol.* 238, 12–18. doi: 10.1016/j.jneuroim.2011.06.001

Walker, J. M., and Harrison, F. E. (2015). Shared neuropathological characteristics of obesity, type 2 diabetes and Alzheimer's disease: impacts on cognitive decline. *Nutrients* 7, 7332–7357. doi: 10.3390/nu7095341

Walsh, D. M., Klyubin, I., Shankar, G. M., Townsend, M., Fadeeva, J. V., Betts, V., et al. (2005). The role of cell-derived oligomers of Aβ in Alzheimer's disease and avenues for therapeutic intervention. *Biochem. Soc. Trans.* 33, 1087–1090. doi: 10.1042/BST20051087

Wang, J., Gu, B. J., Masters, C. L., and Wang, Y. J. (2017). A systemic view of Alzheimer disease—insights from amyloid-β metabolism beyond the brain. *Nat. Rev. Neurol.* 13, 612–623. doi: 10.1038/nrneurol.2017.111

Warren, M. W., Hynan, L. S., Weiner, M. F., Doody, R., Rountree, S., Capriles, V., et al. (2012). Lipids and adipokines as risk factors for Alzheimer's disease. *J. Alzheimers Dis.* 29, 151–157. doi: 10.3233/jad-2012-111385

Wijesekara, N., Ahrens, R., Sabale, M., Wu, L., Ha, K., Verdile, G., et al. (2017). Amyloid-β and islet amyloid pathologies link Alzheimer's disease and type 2 diabetes in a transgenic model. *FASEB J.* 31, 5409–5418. doi: 10.1096/fj.201700431r

Willette, A. A., Modanlo, N., and Kapogiannis, D. (2015). Insulin resistance predicts medial temporal hypermetabolism in mild cognitive impairment conversion to Alzheimer disease. *Diabetes* 64, 1933–1940. doi: 10.2337/db14-1507

Xia, M., Cheng, X., Yi, R., Gao, D., and Xiong, J. (2016). The binding receptors of Aβ: an alternative therapeutic target for Alzheimer's disease. *Mol. Neurobiol.* 53, 455–471. doi: 10.1007/s12035-014-8994-0

Yarchoan, M., Toledo, J. B., Lee, E. B., Arvanitakis, Z., Kazi, H., Han, L. Y., et al. (2014). Abnormal serine phosphorylation of insulin receptor substrate 1 is associated with tau pathology in Alzheimer's disease and tauopathies. *Acta Neuropathol.* 128, 679–689. doi: 10.1007/s00401-014-1328-5

Yun, S. W., and Hoyer, S. (2000). Effects of low-level lead on glycolytic enzymes and pyruvate dehydrogenase of rat brain *in vitro*: relevance to sporadic Alzheimer's disease? *J. Neural Transm.* 107, 355–368. doi: 10.1007/s007020050030

Zhang, Y., Zhou, B., Deng, B., Zhang, F., Wu, J., Wang, Y., et al. (2013). Amyloid-β induces hepatic insulin resistance *in vivo* via JAK2. *Diabetes* 62, 1159–1166. doi: 10.2337/db12-0670

Zhao, W.-Q., De Felice, F. G., Fernandez, S., Chen, H., Lambert, M. P., Quon, M. J., et al. (2008). Amyloid β oligomers induce impairment of neuronal insulin receptors. *FASEB J.* 22, 246–260. doi: 10.1096/fj.06-7703com

Zhao, N., Liu, C. C., Van Ingelgom, A. J., Martens, Y. A., Linares, C., Knight, J. A., et al. (2017). Apolipoprotein E4 impairs neuronal insulin signaling by trapping insulin receptor in the endosomes. *Neuron* 96, 115.e5–129.e5. doi: 10.1016/j.neuron.2017.09.003

Zheng, H., Zhou, Q., Du, Y., Li, C., Xu, P., Lin, L., et al. (2018). The hypothalamus as the primary brain region of metabolic abnormalities in APP/PS1 transgenic mouse model of Alzheimer's disease. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864, 263–273. doi: 10.1016/j.bbadis.2017.10.028

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

"*Freedom of thought is best promoted by the gradual illumination of men's minds which follows from the advance of science*"

Charles Darwin