Identification of a risk transcriptome and proteome in Parkinson's disease, Dementia with Lewy bodies and rapidly progressive Dementia with Lewy bodies

Paula Garcia Esparcia

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Doctoral Thesis in Biomedicine specialty Neuroscience
School of Medicine

Institute of Neuropathology; Bellvitge University Hospital
Department of Pathology and Experimental Therapeutics
Faculty of Medicine; University of Barcelona
Bellvitge Biomedical Research Institute (IDIBELL)
Biomedical Reserch Center of Neurodegenerative Diseases (CIBERNED)

Author

Paula Garcia Esparcia

Thesis director

Prof. Isidre Ferrer Abizanda

Barcelona, September 2016

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Thesis presented by **Paula Garcia Esparcia**, graduated in Sanitary Biology and Master in Neuroscience to obtain the Degree of Doctor in Biomedicine specialty Neuroscience by the University of Barcelona.

The present doctoral thesis has been supervised and directed by Prof. **Isidre Ferrer Abizanda**, at the Neuropathology Institute, Bellvitge University Hospital; Department of Pathology and Experimental Therapeutics, University of Barcelona; Bellvitge Biomedical Research Institute (IDIBELL); Biomedical Reserch Center of Neurodegenerative Diseases (CIBERNED), Institute Carlos III, Ministry of Economy and Innovation.

Signatures,

Author	**Thesis director**
Paula Garcia Esparcia	**Prof. Isidre Ferrer Abizanda**

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FOREWORD

The present thesis to obtain the Degree of Doctor in Biomedicine, specialty Neuroscience by the University of Barcelona is the result of a project carried out during four years at the Bellvitge Biomedical Research Institute in the Department of Pathology and Experimental Therapeutics, School of Medicine, University of Barcelona.

This thesis represents an attempt to characterize the mechanisms underlying cognitive impairments in Parkinson's disease, Dementia with Lewy bodies, and rapidly progressive Dementia with Lewy bodies, through transcriptomic and proteomic techniques.

This four-year work resulted in the production of the six scientific papers listed below. The following papers have been published in the 1st quartile of the international journals with a referenced impact factor (IF) and the second and the last paper are currently under review in an indexed journal.

- *Altered machinery of protein synthesis is region- and stage-dependent and is associated with α-synuclein oligomers in Parkinson's disease.* Garcia-Esparcia P, Hernández-Ortega K, Koneti A, Gil L, Delgado-Morales R, Castaño E, Carmona M, Ferrer I. Acta Neuropathologica Communications 2015 Dec 1; 3(1):76. DOI: 10.1186/s40478-015-0257-4. IF: *not available*.
- *Mitochondrial activity in the frontal cortex and angular gyrus in Parkinson's disease and Parkinson's disease with dementia.* Garcia-Esparcia P, Koneti A, Rodríguez-Oroz MC, Schlutter A, Pujol A, del Rio JA, Ferrer I. *Submitted* Brain Pathology. IF: 5.256. Q1.
- *Complex deregulation and expression of cytokines and mediators of the immune response in Parkinson's disease brain is region dependent.* Garcia-Esparcia P, Llorens F, Carmona M, Ferrer I.

Brain Pathology 2014 Nov; 24(6):584-98. DOI: 10.1111/bpa.12137. IF: 5.256. Q1.

- *Purine metabolism gene deregulation in Parkinson's disease.* Garcia-Esparcia P, Hernández-Ortega K, Ansoleaga B, Carmona M, Ferrer I. Neuropathology and Applied Neurobiology 2015 Dec; 41(7):926-40. DOI: 10.1111/nan.12221. IF: 4.483. Q1.
- *Functional genomics reveals dysregulation of cortical olfactory receptors in Parkinson disease: novel putative chemoreceptors in the human brain.* Garcia-Esparcia P, Schlüter A, Carmona M, Moreno J, Ansoleaga B, Torrejón-Escribano B, Gustincich S, Pujol A, Ferrer I. Journal of Neuropathology & Experimental Neurology 2013 Jun; 72(6):524-39. DOI: 10.1097/NEN.0b013e318294fd76. IF: 3.432. Q1.
- *Dementia with Lewy bodies: molecular pathology in the frontal cortex in typical and rapid forms.* Garcia-Esparcia P, López-Gonzalez I, Grau-Rivera Oriol, García-Garrido MF, Koneti A, Llorens F, Zafar S, Carmona M, Del Río JA, Zerr I, Gelpi E, Ferrer I. *Submitted* Frontiers in Neurology. IF: 3.184. Q1.

BACKGROUND

Parkinson's disease (PD) is a progressive degenerative disease of the nervous system (CNS) characterized by *parkinsonism* resulting from neuronal loss in the substantia nigra pars compacta and from consequent dopaminergic denervation in the striatum. However, PD is preceded by other clinical symptoms such as autonomic deficits, impaired olfaction, sleep disorders, and depression. Cognitive impairment and dementia are not uncommon with disease progression. These changes are due to the fact that nuclei of the autonomic nervous system and the olfactory bulb are affected at earlier stages of the neurodegenerative process, whereas other nuclei of the medulla oblongata and pons are involved before the substantia nigra is affected. The limbic system and the neocortex are also involved in the disease progression. Dopaminergic innervation in the basal ganglia is detectable by Positron Emission Tomography (PET). Cognitive disorders and dementia in PD usually occur with prevalence of about 70-80% after 15-20 years of disease progression, thus leading to **Parkinson disease with dementia** (PDD). Some aspects of the cognitive impairment in PD seem to be supported by the association with Alzheimer's disease (AD), but there is also primary cerebral pathology attributable to PD. Additionally, **Dementia with Lewy Bodies** (DLB) is another neurodegenerative disorder characterized by *parkinsonism* and dementia occurring within a time period of less than two years. Fluctuating course and hallucinations, in addition to early cognitive deterioration, are key symptoms in DLB. The neuropathological substrates of DLB are similar to those seen in PDD and AD-related pathology. The main biochemical abnormality in PD and DLB is the accumulation of abnormal α-synuclein in neurons and cell processes forming Lewy bodies and neurites. For this reason, PD and DLB are considered Lewy Body Diseases (LBDs) and both are also considered prototypic α-synucleinopathies. Moreover, **rapidly progressive Dementia with Lewy bodies** (rpDLB) is the rapid form of DLB; it develops more quickly and has been related with an older age. rpDLB may have concomitant AD as well as sudden, sharp course with an average survival time of 9 months.
The use of biochemical and morphological methods has permitted the identification of molecular alterations in different metabolic pathways that may participate in the pathogenesis of PD, DLB, and rpDLB.

ANTECEDENTES

La **enfermedad de Parkinson** (EP) es un trastorno degenerativo progresivo del sistema nervioso central caracterizado por afección del movimiento o *parkinsonismo* resultante de la pérdida neuronal en la sustancia nigra pars compacta (SN), con la consiguiente denervación en el estriado. A pesar de ello, la EP se predice por otros síntomas clínicos como el déficit autonómico, el daño en el sistema olfativo, los desórdenes del sueño y la depresión, además del deterioro cognitivo y la demencia. Estos cambios son debidos al hecho de que tanto el núcleo autonómico del sistema nervioso como el bulbo olfatorio se afectan en estadiajes tempranos del proceso neurodegenerativo, mientras que otros núcleos cómo la medula oblongata o el pons están comprometidos previa afectación de la SN. Referente a los desórdenes cognitivos y la demencia en la EP, se concluye que ocurren generalmente con una prevalencia de alrededor del 70-80% trascurridos 15-20 años de progresión y conducen a una **EP con demencia**. Algunos aspectos del daño cognitivo en dicha enfermedad parecen estar respaldados por su asociación con la enfermedad de Alzheimer (EA), a pesar de que existe una patología cerebral primaria atribuible únicamente a la EP. Por otro lado, la **Demencia con cuerpos de Lewy** (DCL) es otro desorden neurodegenerativo caracterizado por la aparición de *parkinsonismo* y demencia. Un curso fluctuante con alucinaciones, además de un pronto deterioro cognitivo son síntomas claves. Por lo que refiere a los substratos neuropatológicos, se consideran bastante similares a los observados tanto en la EP con demencia como a la EA. Las principales anomalías bioquímicas observadas en la EP y en la DCL son la acumulación de α-synucleína en las neuronas así como la formación de cuerpos de Lewy y de neuritas de Lewy. Por ello, están incluidas dentro de las enfermedades con cuerpos de Lewy y a la vez ambas son consideradas como α-synucleinopatías prototípicas. Por lo que refiere a la **Demencia con cuerpos de Lewy rápidamente progresiva** (DCLrp) es la forma rápida de la DCL; Se desarrolla de manera más rápida que la forma clásica y se relaciona con la edad avanzada. La DCLrp puede presentar EA concomitante y tiene un repentino y agudo curso con un tiempo promedio de supervivencia alrededor de los 9 meses. El uso de métodos bioquímicos y morfológicos ha permitido la identificación de alteraciones moleculares en diferentes rutas metabólicas, las cuales participan en la patogénesis de la EP, la DCL y la DCLrp.

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by movement impairment, or *parkinsonism*, for which there is still no cure. The manifest clinical signs result from neuronal loss of more than 60% in the substantia nigra pars compacta. Cognitive disorders and dementia in PD usually occur, thus leading to **Parkinson disease with dementia** (PDD). Moreover, **Dementia with Lewy bodies** (DLB) is also considered a neurodegenerative disease and one of the most common causes of dementia, with cognitive impairment symptoms similar to Alzheimer-type dementia, and with *parkinsonism*. Its onset is insidious and is characterized by a slow progression in comparison with its fast form, also known as **Dementia with Lewy bodies rapidly progressive** (rpDCL), which appears suddenly and progresses quickly. In these pathologies there occurs a neural degeneration not only related to the accumulation of altered proteins, but more likely as a result of multiple deleterious factors. The *hypothesis* of this work is that the identification of molecular changes analyzed through the application of "-*omics*" techniques will be useful to obtain information about a risk transcriptome/proteome in the aforementioned diseases. Thus, the main *objective* of the present thesis is the identification of molecular alterations underlying functional cerebral changes and anatomical modifications in different brain regions and different Braak stages of PD, as well as DCL and DCLrp, with the use of *post-mortem* human brain samples compared with controls, combining microarray, mRNA, protein and enzyme assays studies. The obtained *results* have identified molecular alterations in PD, DLB, and rpDLB of different metabolic pathways including changes in the machinery of protein synthesis, in the mitochondrial energy metabolism, in neuroinflammation, in the purine pathway, and in new signaling pathways comprising olfactory and taste receptors paths.

RESUMEN

La **enfermedad de Parkinson** (EP) es una patología neurodegenerativa perteneciente al grupo de afecciones conocidas como trastornos del movimiento, o *parkinsonismo*, para la cual actualmente no existe cura. Los signos clínicos que manifiesta son resultado de una pérdida neuronal superior al 60% en el área cerebral más afectada, la sustancia nigra pars compacta. Asimismo, la aparición de demencia y los desórdenes cognitivos en la EP conducen a una **EP con demencia**. A su vez, la **Demencia con cuerpos de Lewy** (DCL) es también una enfermedad neurodegenerativa considerada como una de las causas más comunes de demencia, con una sintomatología de deterioro cognitivo similar a la observable en la demencia de tipo Alzheimer y con la aparición de síntomas de *parkinsonismo*. Su aparición es insidiosa y se caracteriza por presentar una progresión lenta, a diferencia de su forma rápida también conocida como **Demencia con cuerpos de Lewy rápidamente progresiva** (DCLrp), que aparece de forma súbita y evoluciona vertiginosamente. En todas estas enfermedades se produce una degeneración neural debida no únicamente a la acumulación de proteínas alteradas, sino más probablemente consecuencia de múltiples factores deletéreos convergentes. La *hipótesis* de este trabajo es considerar que la identificación de cambios moleculares analizados gracias a la aplicación de métodos "*-ómicos*" servirá para obtener información sobre un trascriptoma/proteoma de riesgo en las anteriormente citadas enfermedades. El principal *objetivo* abordado en la presente tesis es la identificación de las alteraciones moleculares subyacentes a los cambios cerebrales funcionales y anatómicos presentes en diferentes regiones cerebrales y en distintos estadiajes de Braak de la EP, así como en la DCL y en la DCLrp, por medio del uso de muestras de cerebro humano *post-mortem* comparando con controles, combinando estudios de microarrays, mRNA, proteínas y ensayos enzimáticos. Los *resultados* obtenidos por medio de métodos de transcriptómica con su posterior validación y ampliación a proteómica han permitido identificar alteraciones moleculares en la EP, DCL y DCLrp de distintas vías metabólicas incluyendo cambios en la maquinaria de síntesis de proteínas, en el metabolismo mitocondrial y energético, en la neuroinflamación, en la vía de las purinas y en nuevas vías de señalización comprendiendo las vías de receptores olfatorios y gustativos.

GLOSSARY OF ABBREVIATIONS

aa	Amino acids
aaRS	Aminoacyl-tRNA synthetase
AARS	Alanyl-tRNA synthetase
AC	Adenylyl Cyclase
AC3	Adenylyl Cyclase type 3
AChE	Acetylcolinestrease
AD	Alzheimer's Disease
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AG	Angular gyrus
AK1	Adenylate kinase 1
AK2	Adenylate kinase 2
AK4	Adenylate kinase 4
AK5	Adenylate kinase 5
AK7	Adenylate kinase 7
ALP	Autophagy-liposome pathway
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
APOE	Apolipoprotein E
APRT	Adenine phosphoribosyl-transferase
ATF4	Active transcription factor 4
ATF6	Active transcription factor 6
ATP	Adenosine triphosphate
ATP2B3	ATPase, Ca++ transporting, plasma membrane 3
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4
ATP4A	ATPase, H+/K+ exchanging, alpha polypeptide
ATP5D	ATP synthase, H+ transporting, mitochondrial F1 complex, subunit delta
ATP5G2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2 (subunit 9)
ATP5H	ATP synthase, Fo complex, subunit D
ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G
ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, subunit O
ATP6V0A1	ATPase, H+ transporting, lysosomal V0 subunit A1
ATP6V0B	ATPase, H+ transporting,
ATP6V1H	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H
BCHE K	Butyrylcholinesterase K
BCSG1	Breast cancer-associated protein 1
C	Cerebellum
C1QL1	Complement component 1, Q subcomponent 1
C1QTNF7	C1Q and tumour necrosis factor related protein 7
C3AR1	Complement component 3A receptor 1
cAMP	Cyclic adenosine monophosphate
CBD	Corticobasal degeneration
CG	Cingulate gyrus
cGMP	Cyclic guanosine monophosphate
CJD	Creutzfeld-Jacob disease
CLEC7A	C-type lectin domain family 7, member A
CNS	Central nervous system
COMT	catechol-O-methyltransferase
CoQ	Coenzyme Q
COX-2	Cyclooxygenase 2
COX7A2L	Cytochrome c oxidase subunit VIIa polypeptide 2 like
COX7C	Cytochrome C oxidase subunit VIIc

CSF	Colony-stimulating factor
CSF	Cerebrospinal fluid
CSF1R	Colony-stimulating factor 1 receptor
CSF3R	Colony-stimulating factor 1 receptor
CST7	Cystatin F (leukocystatin)
CTSC	Cathepsin C
CTSS	Cathepsin S
CYBA	Cytochrome B-245 alpha chain
Cyt c	Cytochrome c
DA	Dopamine/dopaminergic
DCP1	Dipetidyl carboipeptidase 1
DGUOK	Deoxyguanosine kinase
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid
EEG	Electroencephalography
eIF	Eukaryotic translation initiation factor
ELISA	Enzyme-linked immunosorbent assay
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1
ENTPD2	Ectonucleoside triphosphate diphosphohydrolase 2
ENTPD3	Ectonucleoside triphosphate diphosphohydrolase 3
EOPD	Early-onset Parkinson's disease
ER	Endoplasmic reticulum
ETC	Electronic transport chain
FACS	Fluorescent-activated cell sorter
FAD	Flavin adenine dinucleotide
FAM82A2	Family with sequence similarity 82, member A2
FC	Frontal Cortex
FFI	Fatal familial insomnia
FTD	Fronto-temporal dementia
GBA	Glucorocerebrocidase
GD	Gaucher disease
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GMP	Guanosine monophosphate
GRP78	Glucose-regulated protein 78
GRP94	Glucose-regulated protein 94
GTP	Guanosine triphosphate
GUS-B	β-glucuronidase
GWAS	Genome-wide association studies
$G_{\alpha olf}$	Olfactory G Protein
HD	Huntington's disease
HLA	Human leukocyte antigen
HPRT	Hypoxanthine phosphoribosyltransferase1
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IL10	Interleukin-10
IL10RA	Interleukin-10 receptor A
IL10RB	Interleukin-10 receptor B
IL1B	Interleukin-1B
IL6	Interleukin-6
IL6ST	Interleukin-6 signal transducer
IL8	Interleukin-8
iNOS	Inducible nitric oxide synthase
INPP5D	Inositol polyphosphate-5-phosphatase D
iPD	Incidental Parkinson's disease
IRE1	Inositol requiring kinase 1
ITGB2	Integrin subunit beta 2
JNK	C-Jun N-terminal kinase
L-DOPA	Levodopa
LB	Lewy body
LBD	Lewy body disease
LHPP	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase
LN	Lewy neurite
LPS	Lipopolysaccharide
LR	Lipid Rafts
LRRK2	Leucine-rich repeat kinase 2
	lysosomal 21kDa, V0 subunit B
MA	Middle age case

MAO-B	Enzyme monoamine oxidase B
MCI	Mild cognitive impairment
MDA	Malondialdehyde
MMSE	Mini-mental state examination
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MSA	Multisistemic atrophy
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NCL	Nucleolin
NDD	Neurodegenerative disorders
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa
NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa
NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7, 14.5kDa
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa
NF-κβ	Nuclear factor kappa-B
NFT	Neurofibrillary tangle
NME1	Non-metastatic cells 1, protein expressed in (nucleoside-diphosphate kinase)
NME3	Non-metastatic cells 3, protein expressed in (nucleoside-diphosphate kinase)
NME4	Non-metastatic cells 4, protein expressed in (nucleoside-diphosphate kinase)
NME5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)
NME6	Non-metastatic cells 6, protein expressed in (nucleoside-diphosphate kinase)
NME7	Non-metastatic cells 7, protein expressed in (nucleoside-diphosphate kinase)
NORs	Nuclear organiser regions
NPM1	Nucleophosmin (nucleolar phospho-protein B23, numatrin)
NPM3	Nucleophosmin / nucleoplasmin 3
NT5C	5', 3'-nucleotidase, cytosolic
NT5E	5'-nucleotidase, ecto (CD73)
OBP	Olfactory-binding protein
OMP	Olfactory marker protein
OR	Olfactory Receptor
OR10G8	Olfactory receptor, family 10, subfamily G, member 8
OR11H1	Olfactory receptor, family 11, subfamily H, member 1
OR2D2	Olfactory receptor, family 2, subfamily D, member 2
OR2J3	Olfactory receptor, family 2, subfamily J, member 3
OR2L13	Olfactory receptor, family 2, subfamily L, member 13
OR2T1	Olfactory receptor, family 2, subfamily T, member 1

OR2T33 Olfactory receptor, family 2, subfamily T, member 33
OR4F4 Olfactory receptor, family 4, subfamily F, member 4
OR51E1 Olfactory receptor, family 51, subfamily E, member 1
OR52H1 Olfactory receptor, family 52, subfamily H, member 1
OR52L1 Olfactory receptor, family 52, subfamily L, member 1
OR52M1 Olfactory receptor, family 52, subfamily M, member 1
OR6F1 Olfactory receptor, family 6, subfamily F, member 1
PABP Poly A-binding protein
PC Precuneus
PD Parkinson's disease
PDD Parkinson's disease with Dementia
PERK PKR-like ER kinase
PET Positron emission tomography
PGC1-α Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pi Inorganic phosphate
PiD Pick disease
PINK1 PTEN induced putative kinase 1
PNP Purine nucleoside phosphorylase
POLR3B Polymerase (RNA) III (DNA directed) polypeptide B
PRUNE Prune homolog (Drosophila)
PSP Supranuclear progressive palsy
PUT Putamen
RT-qPCR Real time quantitative polymerase chain reaction
RC Respiratory chain
RT-QuIC Real-time quaking induced conversion
rDNA Ribosomal DNA
REED1 Regulated in development and DNA damage responses 1
REEP Receptor expression enhancing protein 1
REM Rapid eye movement
RNA Ribonucleic acid
RNA Ribonucleic acid
ROS Reactive oxygen species
RPD Rapidly progressive Dementia
rpDLB Rapidly progressive Dementia with Lewy Bodies
RPL21 Ribosomal protein L21
RPL22 Ribosomal protein L22
RPL23A Ribosomal protein L23A
RPL26 Ribosomal protein L26
RPL27 Ribosomal protein L27
RPL30 Ribosomal protein L30
RPL31 Ribosomal protein L31
RPL5 Ribosomal protein L5
RPL7 Ribosomal protein L7
RPS10 Ribosomal protein S10
RPS13 Ribosomal protein S13
RPS16 Ribosomal protein S16
RPS17 Ribosomal protein S17
RPS20 Ribosomal protein S20
RPS3A Ribosomal protein S3A
RPS5 Ribosomal protein S5
RPS6 Ribosomal protein S6
rRNA Ribosomal RNA
rRNA 18S Eukaryotic 18S rRNA
rRNA 28S RNA, 28S ribosomal 5
RT-PCR Reverse transcription polymerase chain reaction
RTP1 Receptor transporter protein 1
RTP2 Receptor transporter protein 2
sAD Sporadic Alzheimer's disease
SD Standard deviation
SDHB Succinate dehydrogenase complex, subunit B, iron sulphur (Ip)
SDS Sodium dodecyl sulfate

SLC25A31 Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31
SLC6A6 Solute carrier family 6 (neurotransmitter transporter, taurine), member 6
SN Substantia nigra
SNCA Synuclein
SOD1 Cu/Zn-superoxidate dismutase I
SOD2 Mn-superoxidate dismutase
TAS2R10 Taste receptor, type 2, member 10
TAS2R13 Taste receptor, type 2, member 13
TAS2R14 Taste receptor, type 2, member 14
TAS2R4 Taste receptor, type 2, member 4
TAS2R5 Taste receptor, type 2, member 5
TAS2R50 Taste receptor, type 2, member 50
TGFA1 Transforming growth factor-A1
TGFA2 Transforming growth factor-A2
TGFβ Transforming growth factor beta
TH Tyrosine hydroxylase
TLR Toll-like receptor
TLR4 Toll-like receptor 4
TLR7 Toll-like receptor 7
TNF-α Tumour necrosis factor alpha
TNFR1 Soluble tumour necrosis factor α receptor-1
TNFRSF1A Tumour necrosis factor receptor superfamily member 1A
TOMM40 Translocase of outer mitochondrial membrane 40 homolog (yeast)
TR Taste receptor
UBTF Upstream binding transcription factor, RNA polymerase
UGT UDP-glucuronosyltransferase
UPS Ubiquitin-proteasome system
UQCR11 Ubiquinol-cytochrome C reductase, complex III subunit 11
UQCRB Ubiquinol-cytochrome C reductase binding protein
VDAC Voltage-dependent anion chanel
VTA Ventral tegmental area
WB Western Blot
XPNPEP1 X-prolylaminopeptidase (aminopeptidase P) 1
ZNF642 Zinc finger protein

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INTRODUCTION

INTRODUCTION

The human brain is the principal organ of the central nervous system (CNS) and one of the most complex and impressive organs in the human body. It processes a huge number of information and gives us inputs about awareness of our environment and ourselves. Among its main features, the brain is involved in the control of homeostatic functions, sensorial information processing and integration of the accurate response, coordination of voluntary movements, organization of higher mental functions and decision-making processes. Further, the human brain is mainly composed not only of neurons but also neuroglia, or glial cells. Neurons are in charge of the communication and the proceedings within the brain, whereas neuroglia operate as helper cells protecting and giving support to the neurons. Both types of cells are tightly related and interconnected. For this reason, when neurons suffer an abnormal process of death, different regions of the brain may malfunction. This is a distinctive characteristic that occurs in neurodegenerative disorders (NDD) affecting the memory, cognition, and movement coordination among other symptoms. Thus, NDD are long-term pathologies with selective loss of neurons as a consequence of the aggregation of some vulnerable proteins located in the cytoplasm, in the nucleus, or in the surroundings of the cells, which may cause diminish or total loss of function. These are called as *proteinopathies*.

1. Neurodegenerative diseases

Neurodegenerative diseases (NDD) are defined as a group of disorders characterized by a progressive degeneration of specific populations of neurons, and distinct involvement of functional systems, determining clinical presentation and course. Neuronal loss is related with extra- and intracellular accumulation and aggregation of misfolded native proteins, considered the hallmarks of numerous 'protein misfolding' diseases or *proteinopathies* (Golde and Miller, 2009; Uversky, 2009). Furthermore, as well as neurons, glial cells also accumulate these proteins, which are the cause of the homeostasis disruption of the endoplasmic reticulum (ER). Most NDD generally occur in the elderly age group, beginning insidiously, developing slowly, and finally, showing irreversible dysfunctions. Thus, these varieties of pathologies have a silent start and a progressive course without remission. The phenotypes observed in these disorders are varied: from movement, speaking, or respiratory dysfunctions, to cardiac malfunction, and many of the most common NDD show cognitive or motor deficits, or both. The major basic mechanisms of neuronal loss, and the basis for selective vulnerability are increasingly understood based upon fundamental molecular abnormalities. These include abnormal protein dynamics due to deficiency of the ubiquitin-proteasome-autophagy system in charge of cell homeostasis, oxidative stress and free radical formation, mitochondrial dysfunction and impaired bioenergetics, deoxyiribonucleic acid (DNA) damage, neuroinflammatory or neuroimmune processes, changes to several metabolic pathways, and disruption of cellular and axonal transport, among other mechanisms (Jellinger, 2010; Nijholt *et al*., 2011). In addition, several molecular and biochemical studies have revealed modifications of proteins, which are intrinsic to the disease (Table I). These modifications range from phosphorylation, nitration, oligomeric species or proteinase resistance, to cleavage products (Kovacs *et al*., 2010). The processes leading to neurodegeneration are considered multifactorial, caused by genetic,

environmental, and endogenous factors related with aging, but their pathogenic role and their molecular mechanisms are not fully comprehended (Jellinger, 2009[b]; Skovronsky *et al*., 2006). Although progress has been made in identifying the genetic and epigenetic causes, several challenges remain. New findings suggest that these alterations may result mainly from somatic genetic events that occur over long periods of time (De Jager *et al*., 2013). These NDD comprise several pathologies such as Alzheimer's disease (AD), Parkinson's disease (PD), Dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), Pick disease (PiD), Creutzfeldt-Jakob disease (CJD), Amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD), among others (Table I).

Amyloid-β protein	• Alzheimer's disease (AD)
α-synuclein	• **Parkinson's disease (PD)** • **Dementia with Lewy bodies (DLB)** • Multisistemic atrophy (MSA)
Hyperphosphorylated tau protein	• Alzheimer's disease (AD) • Fronto-temporal lobar degenration to MAPT gene mutations (FTD) • Corticobasal degeneration (CBD) • Supranuclear progressive palsy (PSP) • Pick disease (PiD)
Prion protein	• Creutzfeldt-Jakob disease (CJD) • Kuru • Fatal familial insomnia (FFI)

Other proteins	• Superoxid dismutase:
	  • Familial amyotrophic lateral sclerosis (ALS)
	• Huntingtin:
	  • Huntington's disease (HD)

Table I. Protein metabolism abnormalities characteristic of major neurodegenerative disorders with protein deposits. Modified from Cummings, 2004[b]; Kovacs and Budka, 2009; Ferrer, 2009.

In the present thesis we have focused on PD, DLB, and its rapidly progressive form rpDLB, which are classified according to the major compound of their protein deposits (α-synuclein) as synucleinopathies, and more specifically categorized as Lewy body diseases (Figure 1).

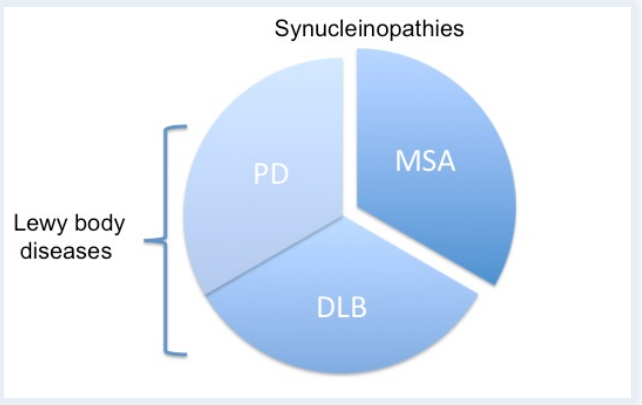


Figure 1. Distinction between synucleinopathies comprising PD, DLB and MSA, and Lewy body diseases, which include PD and DLB.

1.1 Lewy body diseases

Lewy body diseases (LBDs) share α-synuclein aggregation and Lewy body (LB) and Lewy neurite (LN) formation as their key pathogenic features. It was in 1912 that Frierich Heinrick Lewy described for the first time the inclusion bodies, later referred to as Lewy bodies and Lewy neurites by Konstantin Nikolaevich Tretiakoff in 1919 (Jankovic, 2008). Whereas both LBs and LNs are found mainly within the brainstem in PD, their widespread distribution through almost all brain areas is a characteristic feature in DLB. The specific composition of these inclusions remained unknown until 1997, when two essential discoveries were made. First, unequivocal evidence linking abnormal α-synuclein to mechanisms of brain degeneration came from the detection of missense mutations in the gene encoding α-synuclein (*SNCA)*, pathogenic for familial PD in rare kindred groups (Sahay *et al*., 2015; Tagliafierro and Chiba-Falek, 2016). Shortly thereafter, α-synuclein was shown to be a

major fibrillary component of LBs and LNs in sporadic PD, DLB, and the LB variant of AD (Spillantini *et al*., 1998; Polymeropoulos *et al*., 1997; Spillantini., 1997; Duda *et al*., 2000).

To date, the particular mechanisms related with the misfolding and aggregation of α-synuclein, as well as how aggregates produce selective DA neuron loss in synucleinopathies, remain poorly understood. Nevertheless, growing evidence have strengthened the hypothesis that α-synuclein may be transmissible from cell to cell in a *prion-like spreading* process (Guo and Lee, 2014), explained in detail later on (page 59). Neuropathologically, the presence of LBs is accompanied by neurodegeneration in the affected areas. Consequently, the brainstem involvement in PD causes parkinsonian symptoms, and the additional cortical affectation in DLB, dementia (Beyer *et al*., 2009; Kosaka *et al*., 2014). Further, differential expression changes of isoforms corresponding to encoding genes involved in LB formation point to alternative splicing as another important mechanism in the development of LBDs. In conclusion, Lewy bodies and Lewy neurites, composed mainly of α-synuclein, are the defining neuropathological characteristics of PD and DLB.

1.1.1 Lewy bodies

Lewy bodies are considered proteinaceous cytoplasmic inclusions, which represent end-products or reactions to unknown neurodegenerative processes (Jellinger, 2010).

Structure

They may be divided into brainstem and cortical types. The former are located in the brainstem nuclei and diencephalon, and are spherical intraneuronal cytoplasmic inclusions characterized by hyaline eosinophilic cores, concentric lamellar bands, narrow pale halos, and immunoreactivity for α-synuclein and ubiquitin, as explained above (Campbell *et al*., 2001). In contrast, cortical LBs are preferentially

localized in the cerebral cortex and amygdala, and typically lack a halo (Gómez-Tortosa *et al*., 2000) (Figure 2).

Composition

The main component of inclusion bodies in synucleinopathies is α-synuclein; after its initial aggregation, additional proteins are arrested and accumulated in the LBs. More than 76 LB components have been described including structural elements, α-synuclein binding proteins, synphilin-1 binding proteins, and components of the ubiquitin-proteasome system (UPS), among others (Wakabayashi *et al*., 2007); however the precise biochemical composition of these has not yet been clarified (Beyer and Ariza, 2007). α-synuclein aggregation has been accepted as the key procedure preceding LB formation (Baba *et al*., 1998). The first stage starts with aggregation of α-synuclein as revealed by ultrastructural studies, and afterwards LB appearance may be coincidental with increasing ubiquitin immunoreactivity. Final stages of LB formation are characterized by dendrite involvement, LB distortion and degradation, decreased α-synuclein immunoreactivity, and astroglial processes (Irizarry *et al*., 1998; Katsuse *et al*., 2003[b]; Halassa and Haydon, 2010).

Function

For a long time it was thought that LBs played a key role in the neuronal death. However, whereas LBDs are characterized by neuronal loss of DA system, a large number of surviving neurons contain intracellular inclusions in the form of LB. For this reason, nowadays it is said that LBs may protect cells by the up-take of misfolded and un-functional proteins (Olanow *et al*., 2004; Shults, 2006; Tran *et al*., 2014). Furthermore, involvement of the UPS system, as well as autophagy-liposome pathway (ALP), suggests that LBs are structural manifestations of a cytoprotective mechanism.

Neurotoxic or cytoprotective role?

On the one hand, inclusion body formation does not necessarily lead to cell death, and seems to represent a protective mechanism for cell survival (Tompkins and Hill, 1997). Consequently with this proposal, animal and cell models of different NDD develop inclusion bodies that conversely correlate with cell death (Ross and Poirier, 2005), while synphilin-1 and 1A inclusion bodies, which interact with α-synuclein and also play a role in the formation of cytoplasmatic inclusions, may be cytoprotective in PD. (Tanaka *et al*., 2004; Xie *et al*., 2010). Moreover, the possibility has not been ruled out that α-synuclein *per se*, or under some circumstances associated with the α-synuclein aggregation process, may be neurotoxic (Lee *et al*., 2008). On the other hand, inclusions constituted by monoubiquitinated α-synuclein increase cell death, and are characterized as neurotoxic (Periquet *et al*., 2007). This implies that LB may be toxic to cells at their initial stages of formation (Engelender, 2008; Li *et al*., 2008). Nevertheless, to date it is still impossible to prognosticate how LB formation can affect dopaminergic neuron viability, due to their complex protein composition (Rott *et al*., 2008).

1.1.2 Lewy neurites

Together with Lewy bodies in the PD brain we also find Lewy neurites (LN), and both are considered features of α-synucleinopathies. LNs are abnormal neurites in diseased neurons, containing granular material and abnormal α-synuclein, located in the axonal processes with elongated shape (Spillantini *et al*., 1998). Furthermore, they can be found in the absence of LBs, suggesting that they may precede them in time (Figure 2).

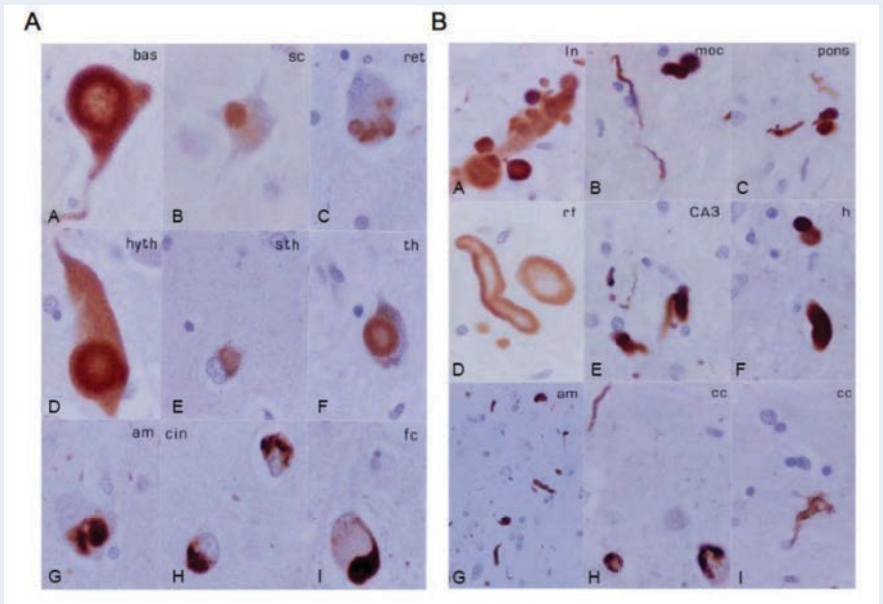


Figure 2. Immunohistochemistry staining of α-synuclein in different brain areas of LBD patients showing: (**A**) Lewy bodies (LB) and (**B**) Lewy neurites (LN). Nomenclature: *am*: amygdala; *bas*: nucleus basalis of Meynert, *cin*: cingulate gyrus; *fc*: frontal cortex; *h*: hypothalamus; *hyth*: hypothalamus; *In*: sunstantia nigra; *pons*: pontine protuberance; *ret*: reticulum formation, *rf*: reticulum formation; *sc*: coeruleus; *sth*: subthalamus; *th*: thalamus. Reproduced with permission from the Institute of Neuropathology of the Bellvitge Hospital.

1.2 α-synuclein

Similar to other *proteinopathies*, the synucleinopathies comprise an important heterogeneous group of disorders with a common injury: aggregates of α-synuclein in several neuronal populations and/or glia (Spillantini *et al*., 1997; Wakabayashi *et al*., 1998). Additionally, α-synuclein is the main component of LBs and LNs, and its aggregation is a key event in the pathogenesis of PD and DLB.

Structure

α-synuclein is an abundant protein of 140 amino acids highly expressed in neurons and presynaptic terminals, where it exists in an equilibrium between a soluble and a membrane-bound state (Fauvet *et al*., 2012).

Interestingly, the specific protein location in the synaptic terminals and in the nuclear envelope yielded the name synuclein (Lewy, 1913; Maroteaux *et al*., 1988). In healthy conditions, α-synuclein adopts defined conformations to function cells, and its α-helical rich conformation has been related with the membrane lipid rafts (LR) (Fortin *et al*., 2004). It lacks a defined secondary structure in α-synuclein, and in situations such as changes in pH and oxidative stress, the protein can suffer a serious conformational transition to a β-sheet-rich structure, adopting a wide range of dynamic structures, including oligomers, fibrils, and aggregates (Danzer *et al*., 2009; Luk *et al*., 2009; Lashuel *et al*., 2013; Stefanis, 2012). Many post-translational modifications have been characterized as promoters of the pathological changes in the protein including ubiquitination, phosphorylation, nitration, oxidation, and C-terminal truncation (Deleersnijder *et al*., 2013).

Composition

Furthermore, α-synuclein is a member of a protein family that includes β- and δ-synucleins, which have also been recognized as playing a role in the pathogenesis of novel axonal lesions in PD and DLB (Kempster *et al*., 2007; Duda *et al*., 2000; Burke *et al*., 2013). β-synuclein inhibits α-synuclein aggregation *in vitro* and *in vivo*, and has been shown to interact directly with α-synuclein regulating its functionality and preventing its oligomerization (Beyer *et al*., 2011). In contrast, δ-synuclein was first described as breast cancer-associated protein 1 (BCSG1) (Ji *et al*., 1997), and there is no genetic evidence linking δ-synuclein to any neurological disease. The first member of this family was identified in 1988 when it was cloned from the electric organ of the fish *Torpedo californica* by screening an expression library.

Human synucleins are encoded by three genes located in different chromosomes: 4 (α), 5 (β), and 10 (δ), respectively (Kempster *et al*., 2007), but only α-synuclein can produce the filaments present in LBDs

and MSA. Although there is a considerable homology sequence common to the three members of the family, only α-synuclein contains three modular, and differentiated domains (Iwai *et al*., 1995; Crowther *et al*., 1998) (Figure 3):

- **Amphipathic α-helical domain** responsible for the lipid-binding, middle hydrophobic non-amyloid-β-component, and composed of seven imperfect repeats (KTKEGV) (aa 1-60).
- **Highly amyloidogenic domain** located in the midregion or NAC region and responsible for the β-sheet secondary conformation required for its aggregation (aa 61-95) (Bodles *et al*., 2000, 2001; El-Agnaf and Irvine, 2002).
- **Acidic COOH-terminal region**, the truncation of which can accelerate its aggregation (aa 96-140).

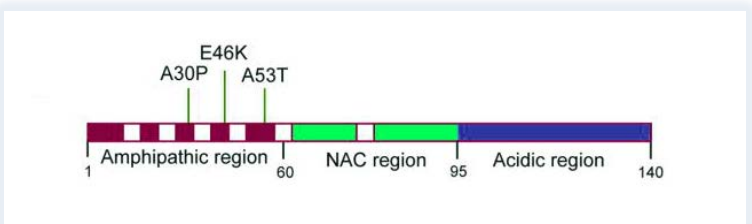


Figure 3. Schematic representation of the domains that constitute α-synuclein. Adapted from Martin *et al*., 2011.

Function

The physiological function of α-synuclein is still unknown but it must be transcendental due to its preservation during the evolutionary process. Some of the proposed functions of α-synuclein include its role as lipid-binding protein, active participant in oxidative stress production with neuroprotective and neurotoxic roles, and involvement in vesicle trafficking and neurotransmitter release in nerve cells (Meredith *et al*., 2008; Dorsey *et al*., 2007; Budd Haeberlein and Harris, 2015). Indeed, mouse models showing either lack or overexpression of α-synuclein,

manifest abnormal changes in synaptic vesicle recycling and mobilization (Abeliovich *et al*., 2000; Cabin *et al*., 2002; Wan and Chung, 2012).

1.2.1 α-synuclein aggregation and oligomerisation

A causative link between protein aggregate formation and neurodegeneration is suggested to be the result of toxic activation of substances produced during early stages (Israeli and Sharon, 2009; Selkoe, 2008; Soto and Estrada, 2008; Kalia *et al*., 2013; Lorenzen and Otzen, 2014; Vekrellis *et al*., 2011). Whether the α-synuclein toxic species are the filaments or the process that leads to their formation is not known, although multiple studies suggest that early aggregates and oligomeric forms of the protein are responsible for its toxicity. Three possible mechanisms for α-synuclein neurotoxicity have been proposed, as follows:

- Direct interaction of α-synuclein with histones inhibiting their acetylation and causing nuclear neurotoxicity (Goers *et al*., 2003; Kontopoulos *et al*., 2006).
- α-synuclein protofibrils with pore-like structure are able to permeabilize membranes, disrupting the ionic and metabolic homeostasis (Volles and Lansbury, 2003; Lashuel *et al*., 2002).
- α-synuclein inhibits the function of the neuronal survival factor MEF2D causing 40% loss of neuronal viability (Yang *et al*., 2009[a, b]).

Thus, abnormal interactions between proteins result in aberrant intra- and extracellular deposition of self-aggregating misfolded proteins with the corresponding formation of high-ordered insoluble fibrils. α-synuclein aggregation is preceded by its oligomerization. In addition to oligomers, which undergo quick transition to a fibrillary state, stable prefibrillar α-synuclein oligomers are identified. Elevated levels of soluble α-synuclein oligomers have been detected in *post-mortem* brains of PD, but

especially in DLB patients (Paleologou *et al*., 2009). Further, α-synuclein protofibrils are toxic and constitute deleterious species of protein producing cell death (Figure 4) (Caughey and Lansbury, 2003; Lashuel and Grillo-Bosch, 2005; Marques and Outeiro, 2012). Interestingly, in murine models there has been demonstrated to be a direct link between membrane-associate α-synuclein oligomers and DA neurodegeneration, strengthening the idea that oligomeric prefibrillar α-synuclein, rather than its fibrils, may represent the pathogenic species of α-synuclein in PD (Winner and Rockenstein, 2008; Kazantsev and Kolchinsky, 2008).

In conclusion, since the aggregation of brain proteins into possible toxic lesions is emerging in sporadic and hereditary neurodegenerative disorders and is considered a pathological hallmark of major NDD (Jellinger, 2009[b]; Skovronsky *et al*., 2006; Herczenik and Gebbink, 2008; Ovádi and Orosz, 2009), clarification of the α-synuclein aggregation mechanism could help to develop new therapies for many neurodegenerative disorders.

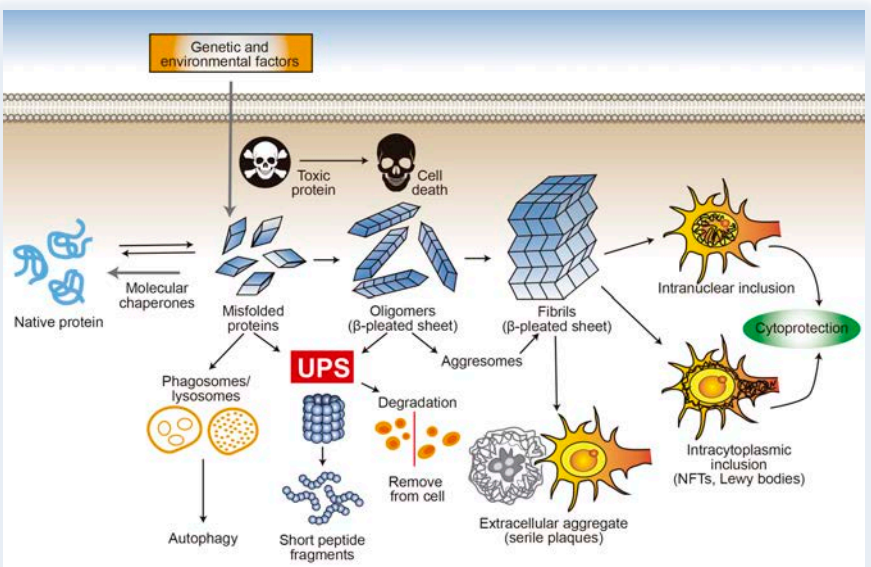


Figure 4. Model of protein misfolding and fibrillation leading to deposition of aggregated proteins in cells and extracellular spaces. Modified from Jellinger, 2009[b].

2. Parkinson's Disease

General definition

Two centuries ago, in 1817, the British physician James Parkinson (1755-1824) described for the first time the essential clinical features of the pathology in his monograph *Essay on the Shaking Palsy* (Parkinson, 1817). However, it was Jean Martin Charcot (1825-1893) who finally recognized the importance of Parkinson's work and named the disease, in honour of him, *malade de Parkinson* or Parkinson's disease (Kempster *et al*., 2007). Afterwards, in 1913, Frederic Heinrich Lewy (1885-1950) first reported the concentric hyaline cytoplasmatic inclusions (Lewy, 1913), and in 1919 Konstantin Trétiakoff (1892-1958) described the characteristic lesions of the SN (Trétiakoff, 1919). Interestingly, in the ancient Ayurvedic literature in India (4500-1000 BCE) a pathology called *kampavata* was described, presenting tremor, rigidity, bradykinesia, and gait disturbance, which may be regarded as an ayurvedic analogue of *parkinsonism* (Ovallath *et al*., 2013).

Parkinson's disease (PD) has been considered a paradigm of degenerative diseases of the nervous system, characterized by motor impairment, called *parkinsonism*, due to the malfunction and loss of dopaminergic/dopamine (DA) neurons of the substantia nigra pars compacta (SN), and the intracellular accumulation of LB and LN (Gibb *et al*., 1988), containing the protein α-synuclein in an aggregated and phosphorylated form (Figure 5). The resulting progressive degeneration of the nigrostriatal dopaminergic system, and other neuronal networks, as well as other biochemical deficiencies, lead to the clinical features of this multisystem and multiorgan disease (Burn *et al*., 2003; Kalia and Lang, 2015). Thus, PD is nowadays considered a systemic disease of the nervous system with variegated clinical symptoms appearing before *parkinsonism* as a result of the involvement of selected nuclei of the

medulla oblongata, pons, autonomic nervous system, and olfactory structures, among others (Ferrer *et al*., 2009).

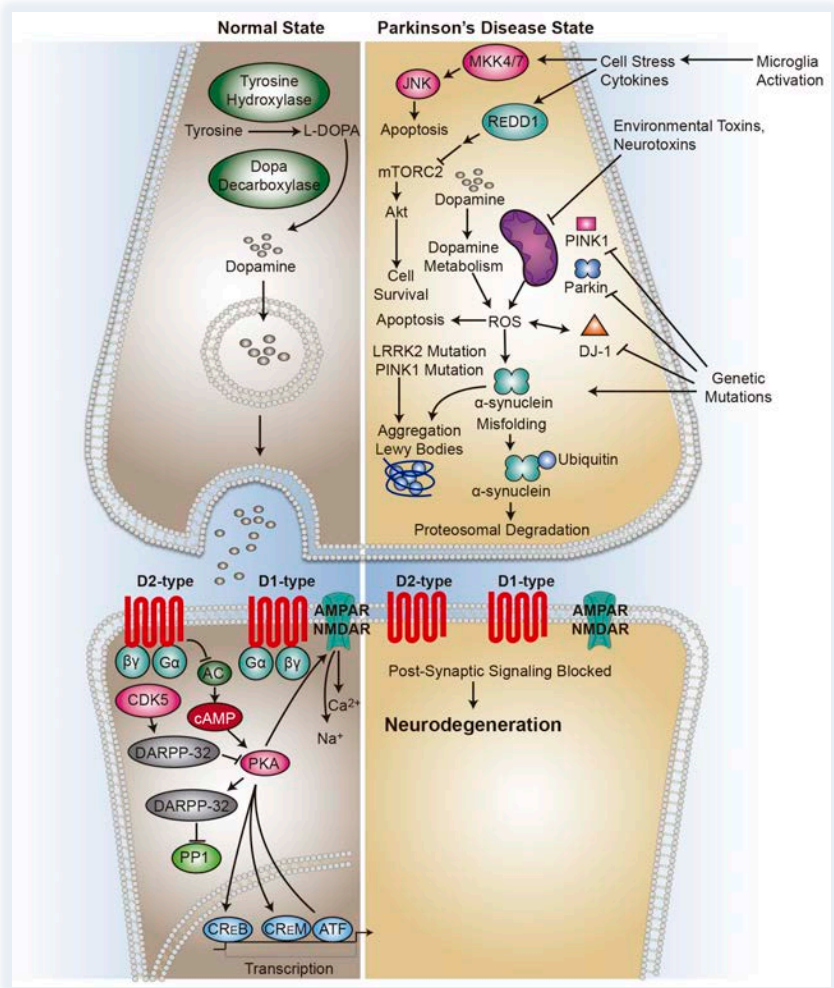


Figure 5. Schematic figure of dopamine signalling comparing neurons in normal state and PD condition. In the normal state, release of the neurotransmitter DA in the presynaptic neuron results in signalling in the postsynaptic neuron through D1- and D2-type dopamine receptors. Mitochondrial dysfunction and protein aggregation in dopaminergic neurons may be responsible for their premature degeneration. There is also an inflammatory component to this disease, resulting from activation of microglia that cause the release of inflammatory cytokines and cell stress. This microglia activation causes apoptosis via the JNK pathway and by blocking the Akt signalling pathway via REDD1. Modified from *Cell signalling*.

Further, PD is the second most common neurodegenerative pathology after Alzheimer's disease, and the most common movement disorder of neurodegenerative origin. It affects about 2% of the population over the age of 60 (Tanner 1992; De Rijk *et al*., 2000), and only about 5% of the patients have disease onset before the age of 50 years. The number of affected individuals is projected to double by the year 2030 (Dorsey *et al*., 2007), and in the coming decades, the prevalence of PD is expected to increase notably due to population aging.

2.1 Epidemiology

Although Parkinson's disease can be diagnosed at any age, the incidence rises steeply after age 60 years and is rarely before age 50 years. Due to the increase in life expectancy, not only the occurrence but also the prevalence of PD is rising exponentially. It occurs throughout the world, but its prevalence is increased among whites, intermediate in Asians, and low among blacks (Yacoubian *et al*., 2009; Alladi *et al*., 2009). A lifetime risk of developing PD of 1.5% exhibits considerable regional variations. PD affects both sexes but with a preponderance of 1.5 in males (Canturi-Castelvetri *et al*., 2007). Further, 0.3% of the entire population and about 0.5-1% of people over 65 years of age are the estimated prevalence of PD in industrialised countries (Nussbaum *et al*., 2003). Reported standardised incidence rates of PD are 4.5 to 21 per 100,000 person-years (De Lau *et al*., 2006), and the mean duration of the disease from diagnosis to death is 15 years (Katzenschlager *et al*., 2008).

2.2 Etiology

The etiology of PD has been presumed to be a complex combination of environmental factors, intrinsic cellular metabolic damage, and vulnerable genetic alleles. Available data indicate that multiple etiologies are more probable than a single factor (Figure 6). The cause still remains as elusive as when it was described for the first time in 1817 (Lees *et al*.,

2009; Shulman and 2011), even though genetic and pathological clues have recently been found (Tanner, 2003).

One the one hand, genetic vulnerability may be determined in part through damaged metabolism of free radicals/ROS or mitochondrial complex I activity injury, which at the same time may be the result of mitochondrial or nuclear genomic deficiency. On the other, environmental factors may include exogenous compounds with uptake and conversion comparable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) targeting the SN or endogenous generated neuroxins (rotenone) related with ROS and disruption of Ca^{2+} homeostasis.

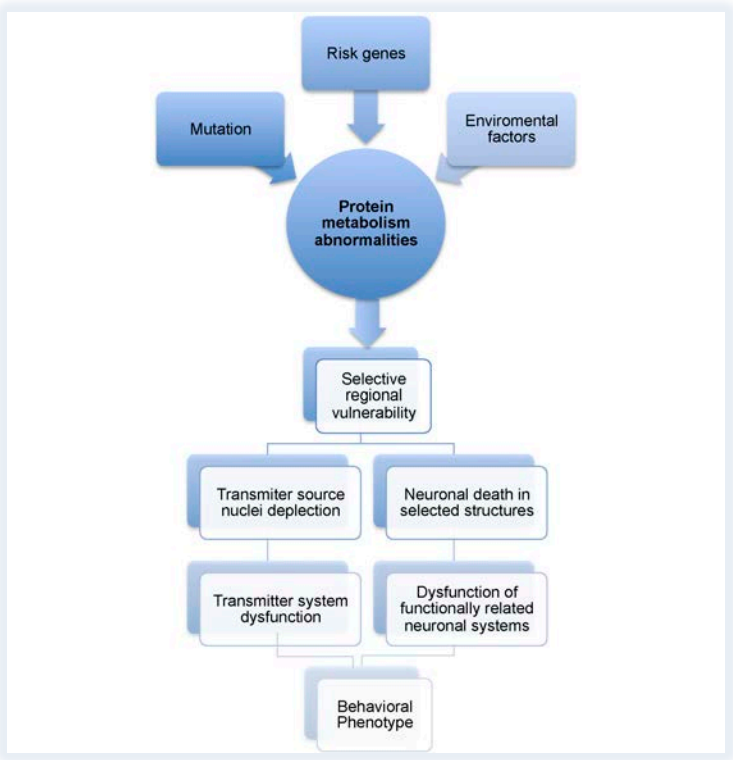


Figure 6. Schema of the relationship between causative factors, prototypes and phenotypes in Parkinson's disease. Modified from Cummings, 2004[b].

2.2.1 Genetics

Most PD cases appear to be sporadic in nature, although there may be genetic risk factors that raise the likelihood of developing PD and distinctly contribute to the sporadic form (Gasser, 2001; Kumar *et al*., 2012). Less than 10% of PD patients have been linked to particular genes, considered rare PD forms with Mendelian inheritance. Interestingly, recent studies report a new autosomal dominant locus in familial PD on the short arm of chromosome 20 (20pter-p12), and identify mutations in TMEM230 as a potential disease-causing gene (Deng *et al*., 2016). Moreover, more than 16 loci, designated as *PARK1* to *PARK16*, and 11 associated genes with inherited forms of *parkinsonism* have been identified (Table II). These genes show that mitochondrial and lysosomal dysfunctions, protein aggregation, and ubiquitin-proteasome system, as well as kinase-signalling pathways, play a major role in the pathogenesis of Parkinson's disease (Budd Haeberlein and Harris, 2015).

Genome-wide association studies (GWAS) have established additional susceptibility loci, and have presented evidence that polymorphic variants in these gens induce sporadic PD (Satake *et al*., 2009; Simón-Sánchez *et al*., 2009); so, the acquired knowledge suggests that inherited and sporadic PD may share the same pathways of neurodegeneration (Corti *et al*., 2011). The possible function of variants in these PD-linked genes is interesting, and greater than previously thought in a considerable number of idiopathic late-onset PD patients without a family history of the disease (Gilks *et al*., 2005; Lesage *et al*., 2007) (Table II).

PARK locus	Gene	Map position	Inheritance	Clinical phenotype	Pathology
PARK1/4	*SNCA*	4q21	Autosomic dominant	Parkinsonism with common dementia	Lewy bodies
PARK2	*parkin*	6q25-q27	Autosomic recesive	Early-onset, slowly progressing parkinsonism	Lewy bodies
PARK3	-	2p13	Autosomic dominant	Late-onset parkinsonism	Lewy bodies
PARK5	*UCHL1*	4p14	Autosomic dominant	Late-onset parkinsonism	-
PARK6	*PINK1*	1p35-p36	Autosomic recesive	Early-onset, slowly progressing parkinsonism	One case exhibiting Lewy bodies
PARK7	*DJ-1*	1p36	Autosomic recesive	Early-onset parkinsonism	-
PARK8	*LRRK2*	12q12	Autosomic dominant	Late-onset parkinsonism	Lewy bodies (usually)
PARK9	*ATP13A2*	1p36	Autosomic recesive	Early-onset parkinsonism with Kufor-Rakeb syndrome	-
PARK10	-	1p32	-	Unclear	-
PARK11	*GIGYF2*	2q36-q37	Autosomic dominant	Late-onset parkinsonism	-
PARK12	-	Xq	-	Unclear	-
PARK13	*Omi/ HTRA2*	2p13	Autosomic dominant	Unclear	-
PARK14	*PLA2G6*	22q13.1	Autosomic recesive	Parkinsonism with additional features	Lewy bodies
PARK15	*FBX07*	22q12-q13	Autosomic recesive	Early-onset parkinsonism	-
PARK16	-	1q32	-	Late-onset parkinsonism	-
FTDP-17	*MAPT*	17q21.1	Autosomic dominant	Dementia, sometimes parkinsonism	Neurofibrillary tangles
SCA2	*Ataxin 2*	12q24.1	-	Usually ataxia, sometimes parkinsonism	-
SCA3	*Ataxin 3*	14q21	-	Usually ataxia, sometimes parkinsonism	-
Gaucher's locus	*GBA*	1q21	-	Late-onset parkinsonism	Lewy bodies

Table II. Summary of Parkinson's disease associated loci and genes. -: Unknown. Adapted from Martin *et al.*, 2011.

There are five missense mutations in p.A53T, p.E46K, p.H50Q and p.G51D (Krüger *et al*., 1998; Zarranz *et al*., 2004; Appel-Cresswell *et al*., 2013; Lesage *et al*., 2013), and duplications and triplications in α-synuclein gene that are genetically associated with clinical *parkinsonism* (Polymeropoulos *et al*., 1997; Ibáñez *et al*., 2004; Chartier-Harlin *et al*., 2004; Singleton *et al*., 2003). It is known that a single point mutation in the α-synuclein gene can cause autosomal dominant PD (Polymeropoulos *et al*., 1997), and that different mutations in this single gene display clinical and neuropathological variables, both within and between lineages. Thus, the genetic relation and pathological linkage establish α-synuclein as a critical player in the development of PD. Common risk loci linked with certain likelihood for sporadic PD (Hardy *et al*., 2009; Bras *et al*., 2009) are the following:

- **Autosomal dominant forms of PD:** Related to *PARK1/4* loci (α-synuclein, *SNCA*) and *PARK8* (Leucine-rich repeat kinase 2, *LRRK2*).

α-synuclein (*SNCA*)

Three point mutations in the *SNCA* gene have been identified including A53T, A30P and E46K, being the first one the most frequent (Figure 7). Patients with A53T mutation can present from a mild to a more severe phenotype, whereas patients carrying A320P show a similar idiopathic PD, with an increase in the protein aggregates due to the association of this mutation with a reduced affinity to binding lipid rafts from the membrane. Familial PD patients due to A30P mutant α-synuclein share similarities with sporadic PD cases, but with more critical pathology (Seidel *et al*., 2010). E46K mutations manifest in those patients an early-age onset with sever *parkinsonism*. Mutations in *SNCA* present cell loss in pigmented brainstem nuclei with extensive LB. Furthermore, patients with *SNCA* gene duplication and triplication, and heterozygous Parkin carriers linked to familial PD, show neuronal loss and gliosis in the hippocampus with α-synuclein positive glial inclusions and extended

neuritic pathology (Farrer *et al*., 2004; Anderson *et al*., 2006). While *SNCA* duplications are more often linked with a typical late-onset PD phenotype, triplications in the same region generate early-onset PD with dementia. Additionally, an evidence link between sporadic PD and gene expression of *SNCA* is corroborated with reduced epigenetic silencing or decreased methylation in the intron 1 of *SNCA* and polymporphisms in its promoter region, increasing the expression of α-synuclein (Jowaed *et al*., 2010; Puschmann *et al*., 2009; Jowaed *et al*., 2010).

Leucine-rich repeat kinase 2 (*LRRK2*)

LRRK2 is a large constitutively expressed complex protein of 2527 amino acids present in different brain regions, as well as in many tissues and organs (Figure 7). It is located throughout the soma and the dendritic processes of neurons, especially in the neuronal populations of the nigrostriatal dopaminergic pathway. Thus *LRRK2* mutations represent the most ordinary cause of familial and sporadic late-onset PD identified to date (Higashi *et al*., 2007; Corti *et al*., 2011; Zimprich *et al*., 2004). The wide range of neuropathology related with *LRRK2*-linked with PD, suggests that *LRRK2* may play a central role in the pathogenic pathways regulating α-synuclein and tau deposition in human brain, indicating common pathogenic pathways. Approximately 80 variants of *LRRK2* gene are linked with PD, most of them missense mutations, and only 7 considered pathogenic: enzymatic GTPase (N1437H, R1441G/C/H), COR (Y1699C), and kinase domains (G2019S and I2020T) (Martin *et al*., 2011). Interestingly, recent investigation supports the idea that mutations in *LRRK2* gene are produced by an increase in *LRKK2* kinase activity and a depression in GTPase activity for the kinase domain, inducing cell damage and death (Cookson, 2010).

❖ **Autosomal-recessive forms:** Related with several *Parkin* genes (*PRKN/PARK2*), *PINK1* (*PARK6*), *DJ1* (*PARK7*) and others (*ATP13A2, PARK9*).

Parkin

Parkin is considered an ubiquitin E3 protein-ligase with 465-amino-acid, primarily situated in the cytoplasm (Figure 7). It is covalently attached to the lysine residue of substrate proteins in a process named ubiquitination, which leads to proteasomal degradation (Zhang *et al*., 2000; Dawson and Dawson, 2010). Furthermore, it is demonstrated that *Parkin* plays a critical role in the regulation of the mitochondria by stimulating mitophagy and mitochondrial biogenesis (Scarffe *et al*., 2014). The *Parkin* gene has been identified as a causative gene for autosomal recessive *parkinsonism* in 50% of cases worldwide, whereas mutations in this gene are the most frequently described in early-onset Parkinson's disease (EOPD), accounting for 10-20% (Lücking *et al*., 2000). Pathologically, *Parkin* mutations are related with the loss of A9 DA neurons, and 50% of patients affected present LB formation (Mori *et al*., 1998).

PTEN induced putative kinase 1 (*PINK1*)

PINK1 is a 581 amino-acid protein kinase located not only in the cytosol but also in the mitochondria, an organelle strongly associated with PD (Valente *et al*., 2004, Zhou *et al*., 2008) (Figure 7). *PINK1*-related PD patients show a clinical phenotype broadly similar to *Parkin* or *DJ-1* related disease, although patients with *PINK1* mutations tend to present a better response to levodopa (L-dopa; the most common treatment for PD), with a severe disease and longer mean disease duration (Kulisevsky *et al*., 1996; Ibáñez *et al*., 2006). *PINK1* is the second most common causative gene in EOPD with a slowly progressive phenotype; patients with *PINK1* mutations show atypical symptoms such as dystonia, dyskinesia, and significant prevalence of psychiatric disorders (Corti *et al*., 2011).

DJ-1

DJ-1 is a member of ThiJ/Pfp family of molecular chaperones, which are induced during oxidative stress (Figure 7). Under these stress conditions, *DJ-1* translocates from the cytoplasm to the outer mitochondrial membrane, where it is thought to play a role in neuroprotection (Canet-Avilés *et al*., 2004). Mutations in *DJ-1* induce rapid proteasomal degradation by destabilizing the protein, probably interfering with the neuroprotective mechanism (Miller *et al*., 2003; Moore *et al*., 2003). It has been described how *DJ-1* plays an important role in the maintenance of mitochondrial function and acts in parallel with *PINK1/Parkin* pathway in order to control autophagy (Thomas *et al*., 2011). The *DJ-1* phenotype is characterized by early-onset and slow disease progression, similar to patients with *PINK1* and *Parkin* mutations.

Lysosomal type 5-P-type ATPase (*ATP13A2*)

The *ATP13A2* gene encodes a large transmembrane protein with putative ATPase activity (Figure 7). It is located in the lysosomes linking abnormal lysosomal function to neurodegeneration, and acts as a cation pump (Corti *et al*., 2011). *ATP13A2* loss of function is related with an increase in neuronal sensitivity to zinc and lysosomal injury leading to α-synuclein accumulation (Perret *et al*., 2015). Interestingly, *ATP13A2*, at *PARK9*, has been associated with Kufor-Rakeb syndrome, a form of recessively inherited atypical *parkinsonism* (Williams *et al*., 2005).

Glucocerebrocidase (GBA)

Gaucher disease (GD), the most frequent lysosomal storage disorder, results from a recessively inherited deficiency of glucocerebrosidase (GBA). Almost 300 GBA mutations have been detected in GD patients including point mutations, insertions and deletions, as well as complex alleles derived from the recombination or gene conversion between the *GBA* gene and its highly homologous pseudogene, which represent 20% of the pathogenic mutations in *GBA1* (Hruska *et al*., 2008; Corti *et al*.,

2011). Heterozygous mutation carriers in the *GBA* gene have a 5- to 6-fold increased risk of suffering PD (Sidransky *et al*., 2009; Tsuang *et al*., 2012). *GBA* mutations, found in about 8-14% of autopsy-confirmed PD, are the most frequent genetic risk factors for PD, especially in familial forms and early age onset PD; being more common than other PD-associated genes as *SNCA* or *LRKK2* (Lesage *et al*., 2011; Mitsui *et al*., 2009). Patients with *GBA1* mutations that are PD-linked exhibit clinical features associated with early-onset, and are clinically and pathologically undistinguishable from idiopathic PD patients with abundant α-synuclein pathology and prominent diffuse Lewy body-type pathology in the neocortex (Neumann *et al*., 2009). Interestingly, recent *in vivo* and *in vitro* experiments demonstrate that GBA mutations promote the aggregation of α-synuclein, and enhance α-synuclein-mediated neurotoxicity. These results are the first evidence directly linking alteration of GBA function to synucleinopathies (Cullen *et al*., 2011; Mazzulli *et al*., 2011; Bohnen *et al*., 2011; Tsuang *et al*., 2012).

❖ **Autosomal-recessive early-onset Parkinson's disease with an onset <40 years:** Caused by mutations in *Parkin* gene, *DJ1*, *PINK1* and *ATP13A2*. There are three groups with an EOPD (Klein *et al*., 2009):

- Atypical features in patients with mutations of one of the *PARK* genes (*PARK2, 6, 7*).
- Classic *parkinsonism* due to mutations in genes other than *PARK* genes, such as *DYT5* and *SCA3*; heterozygous glucocerebrosidase (GBA) mutations, and mitochondrial gene mutations (*PINK1*, *DJ1*, *Omi/HtA2*).
- Atypical *parkinsonism* in other genetic disorders, e.g. *FMRI*, *MAPT*, *GRN*, *ATP7B*, *PANK2*, *FBXO7*, *CHAC*, and *FTL1*.

To summarize, discovered PD genes point to α-synuclein aggregation, mitochondrial dysfunction and oxidative stress, malfunction of proteasomal and lysosomal protein degradation, and alterations in signalling cascades as the main molecular pathways involved in the

pathogenesis of PD (Budd Haeberlein and Harris, 2015). It is necessary to identify how genetic associations converge into populations for epidemiological studies and for future neuroprotective or preventive assays (Gasser, 2009). In addition, mutations may help to define molecular pathways underlying neurodegeneration in PD (Hardy *et al*., 2009).

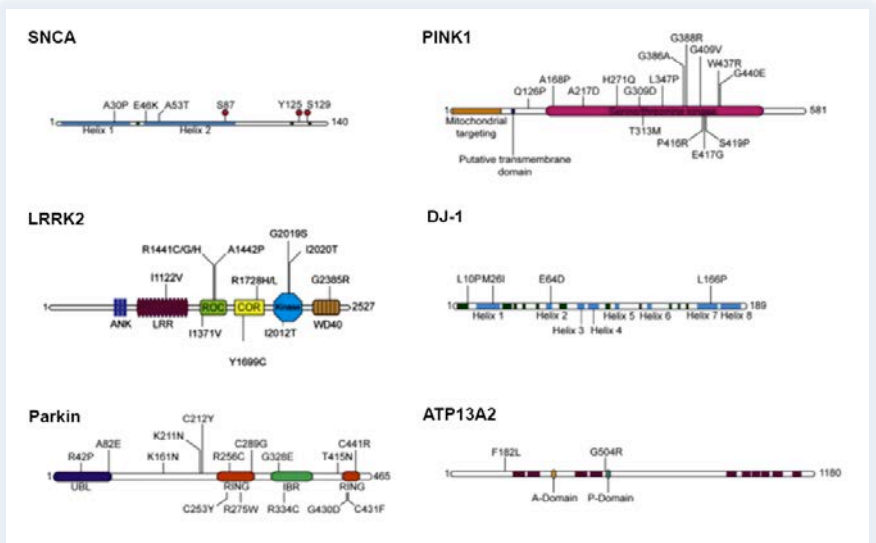


Figure 7. Ideograms of proteins linked to genetic PD, with point mutations indicated. Only mutations with defined segregation, and present in a homozygous state for recessive genes, have been include. Adapted from Hardy *et al*., 2009.

2.2.2 Non-genetic risk factors

Several non-genetic risk factors for PD related with the environment have been suggested on the basis of presumed pathogenic mechanisms of the disease. Similar to other NDD, **ageing** is the major risk factor and the best predictor of PD progression rate (Bennett *et al*., 1996; Tanner 2003; Taylor *et al*., 2005; Dick *et al*., 2007). Ageing has been identified as actively leading to accumulation of unrepaired cellular damage due to the progressive failure of compensatory mechanisms including mitochondrial activity malfunction, decline in proteasome functioning, and autophagy damage (Corti *et al*., 2011). Studies of the effects of ageing in non-human

primates by analysing typical traits of PD patients, such as neurochemical and morphological changes in DA neurons in the brain, identified more susceptibility in those neurons of the ventral tier of the substantia nigra (vtSN) (A9), and more resistance to degeneration in those of the ventral tegmental area (VTA) (A10). Moreover, a decrease in tyrosine hydroxylase (TH) immune-reactivity in ageing monkeys, linked with an increase in α-synuclein staining, as well as the presence of intranuclear ubiquitin-positive inclusions, suggests that the accumulation of α-synuclein may support the ageing-related deterioration in DA neurons phenotypes. Therefore, the pre-parkinsonian state induced by aging mixed with genetic and environmental factors may result in PD phenotypes, and the mechanism could be extrapolated to humans (Collier *et al*., 2011). It is proposed that the effects of ageing may produce a cascade of stressors within the SN, which weakens the neurons and their capacity to react to further insults related with disease progression (Reeve *et al*., 2014).

On the other hand, **gender** is considered another relevant risk factor for suffering PD. There is good evidence that both, incidence and prevalence of PD are 1.5 or even 2 times greater in men than in women, as to developing PD. These gender-specific differences may result from underlying divergences in metabolic attributes of DA neurons in basal ganglia, with greater preservation of dopamine uptake sites in women with PD due to the effect of oestrogens (Kaasinen *et al*., 2001; Cantuti-Castelvetri *et al*., 2007; Haaxma *et al*., 2007). A long fertile life span in woman has been related with iron loss in menses and pregnancies, suggesting minor oxidative stress in nigrostriatal systems and conferring protection. Later menopause also delays the onset age due to the levels of hormones (Haaxma *et al*., 2007). This neuroprotective role has been proposed as a possible explanation for men's higher risk (Saunders-Pullman, 2003).

Other factors have been associated with sporadic PD including exposure to **pesticides** such as paraquat, MPTP, and rotenone (Semchuk *et al*., 1992), **toxin and chemicals** exposure, **non-steroidal anti-inflammatory drug** (NSAID) consumption, and brain injury, although conclusive evidence is still lacking. Moreover, lifestyles and dietary habits have been related with markedly lower risk of developing PD, perhaps associated with reward pathways rather than neuroprotective effects of some substances. These include diets, which contributes to increased levels of plasma urate, smoking, caffeine intake, tea consumption, and a great physical activity in midlife (Gao *et al*., 2008; Thacker *et al*., 2008; Weisskopf *et al*., 2007).

To sum up, several environmental factors contribute considerably to the risk of Parkinson's disease. Some may already be involved in the early stages of life, while others may interact with genetic factors acting as triggers.

2.2.3 Parkinson's disease classification

Regarding the etiology, there are two main forms of PD: the **familial** type represents 5-10% of PD cases with an onset age lower than 45 years, and the **sporadic** kind. The latter form covers the vast majority of PD patients, who constitute approximately 90-95% of all cases without family history. It is thought that sporadic PD is the result of complex interactions among gene susceptibility and environmental factors, although the causes still remain hidden (Martin *et al*., 2011; Corti *et al*., 2011; Dawson and Dawson, 2003; Obeso *et al*., 2010). For this reason, it is assumed that PD is a multifactorial pathology in which age is the most relevant factor, and it occurs when complex mechanisms such as mitochondrial activity, autophagy, and degradation via proteasome, among other pathways treated in the present thesis, are deregulated by environmental pressure or specific mutations. To explore the pathomechanisms, causes, and possible treatment of PD, several genetic and experimental

models have been used, but there is still no ideal animal model for the pathology (Olanow and Kordower, 2009; Meredith *et al*., 2008; Dauer and Przedborski, 2003).

2.3 Pathogenesis

The pathogenesis of PD has been related to several harmful factors, including misfolded aggregated α-synuclein, formation of free radicals/reactive oxygen species (ROS), increased activity of malondialdehyde (MDA) caused by lipid peroxidation, advanced glycation end products (AGEs), oxidative, nitrosactive and proteolytic stress, mitochondrial dysfunction (Banerjee *et al*., 2009; Schapira *et al*., 2009; Ferrer, 2009), energetic damage (Jellinger, 2010), nuclear RNA deficits (Nelson and Keller, 2007), excitotoxicity, transcriptional deregulation, neuroinflammation (Hirsch *et al*., 2009; Tansey and Goldberg, 2010), perturbation of protein degradation systems such as ubiquitin-proteasome system (UPS) (Pan *et al*., 2008), and altered proteosomal functions, among others (Figure 8).

Prion-like spreading

Cumulative evidence has strengthened the idea that α-synuclein may be transmissible from cell to cell, acting as a self-propagating pathogenic protein or *prion-like* protein, and contributing to propagation and extension of PD. In favour of this premise is the Braak staging hypothesis, suggesting an ascending LB formation across the brain, following a caudo-rostral pattern, and explaining the clinical and the progression of most sporadic forms of PD (Braak *et al*., 2003), explained in depth in the Neuropathology of Parkinson's disease chapter (page 63). In addition, recent studies using patients with advanced PD, who received transplantation of fetal nigral mesencephalic cells, confirm that this aberrantly folded protein can be transferred from affected to unaffected nerve cells (Olanow *et al*., 2003; Lindvall *et al*., 1994; Olanow

and Brundin, 2013; Kordower *et al*., 2008). Novel reports demonstrate that the inoculation of misfolded α-synuclein and PD-derived Lewy body extracts can cause Lewy-like pathology in cells that can diffuse to naive cells, and in different regions, inducing not only a progressive neurodegeneration that starts at striatal DA terminals, but also motor dysfunctions in mice and monkeys (Hansen *et al*., 2011; Recasens *et al*., 2014). Importantly, LB-induced pathogenic effects required human α-synuclein present in PD-derived LB extracts, as well as host expression of α-synuclein. Therefore, the exogenous α-synuclein is internalized within host neurons and triggers the pathological conversion of endogenous α-synuclein, resulting in nigrostriatal neurodegeneration (Recasens *et al*., 2014; Angot *et al*., 2012; Roberts and Brown, 2015).

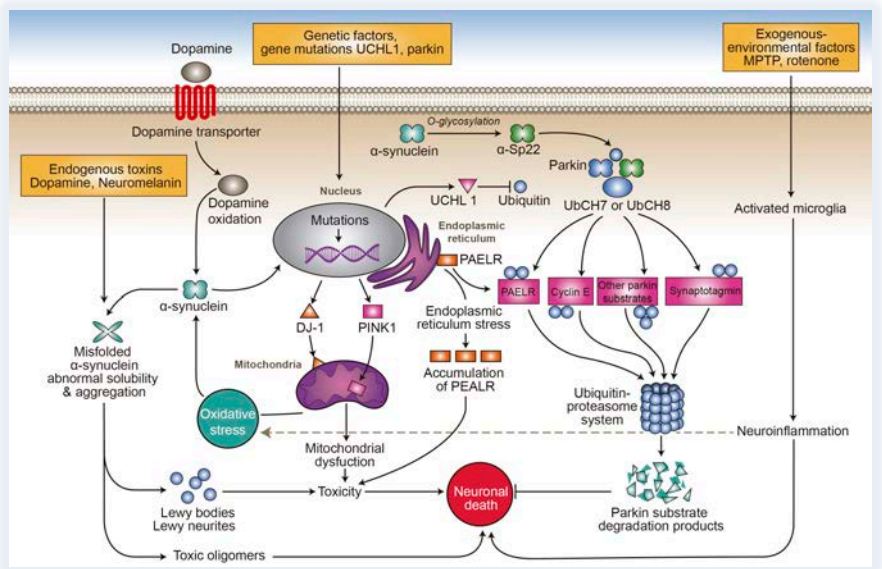


Figure 8. Schematic summary of established etiopathogenic mechanisms and interactions in the dopaminergic cells of the substantia nigra in Parkinson's disease. Cell death may be caused by α-synuclein aggregation, proteosomal and lysosomal system dysfunction, and reduced mitochondrial activity, among others. In addition, secondary changes, as excitotoxicity, endogenous toxins, genetic factors and neuroinflammation are likely to play a relevant role in progressive neuronal degeneration. Modified from Obeso *et al*., 2012, and Ferrer, 2009.

These recent findings give convincing support to the hypothesis that α-synuclein is a *prion-like* protein, which can cause neurodegeneration. However, the mechanism of the pathological conversion, or the start of the seeding prion process, is still not clear.

2.4 Clinical features

Motor symptoms

Despite the fact that Parkinson's disease cannot be considered merely a motor disease, its clinical diagnosis depends on the manifestation of motor signs and symptoms, principally: hypokinesia, bradykinesia, rest tremor (*4-6 Hz range*), and postural instability (Table III). These are considered the four cardinal motor symptoms of PD. Early on, bradykinesia may manifest as loss of spontaneous movements and gesturing, hypomimia, hypophonia, reduced dexterity, small and slow handwriting (micrographia), reduced arm swing during walking, short-stepped gait, and general slowness. Symptom onset is gradual, and the disease progression is relatively slow. Motor signs are typically asymmetrical, or even unilateral at the time of presentation, and a certain degree of asymmetry is maintained throughout the course of PD in many patients (Kovacs, 2015). All these manifestations distinguish the syndrome called *parkinsonism*.

These motor features are highly heterogeneous in PD, which has prompted attempts to categorize different subtypes of the disease (Marras and Lang, 2013; Levy *et al*., 2000). The classical parkinsonian syndromes are ordinarily placed into three categories: Parkinson's disease, atypical parkinsonian disorders, and secondary or symptomatic *parkinsonism*. Furthermore, the subtypes of PD are hypothesised to present different etiology and pathogenesis (Marras and Lang, 2013). Multiple system atrophy (MSA), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), vascular *parkinsonism*, communicating

hydrocephalus, and some drugs and toxics can also develop into parkinsonian syndromes (Rooper and Samuels 2009).

Clinical features of Parkinon's disease
• Resting tremor (4-6 Hz)
• Cogwheel rigidity
• Bradykinesia (akinesia) and related symptoms - Difficulty in initiating movements - Slowness of movements - Micrographia - Small voice
• Loss of righting reflex - Postural instability - Small step, shuffling gait - Start hesitation and freezing
• Loss of automatic movements - Loss of blinking - Loss of automatic ocular movement - Sialorrhea - Difficulty with two motor acts
• Autonomic dysfunction - Constipation, bowel dysfunction - Dysphagia - Dysarthria - Urinary frequency, bladder dysfunction - Orthostatic hypotension - Increased sweating
• Non-motor symptoms - Cognitive impairment, bradyphrenia, tip-of-the-tongue (word finding) phenomenon - Depression, apathy, anhedonia, fatigue, other behavioral and psychiatric problems - Sensory symptoms: anosmia, ageusia, pain (shoulder, back), paresthesias - Dysautonomia: orthostatic hypotension, constipation, urinary and sexual dysfunction, abnormal sweating, weight loss - Sleep disorders (REM behavior disorder, vivid drams, daytime drowsiness, sleep fragmentation, restless legs syndrome

Table III. Clinical features of PD. Adapted from Galasko *et al*., 1996 and Kovacs, 2015.

Non-motor symptoms

In addition to motor symptoms, almost all patients experience non-motor symptoms including olfactory dysfunction, constipation, mood disorders (anxiety, depression), cognitive damage (Kulisevsky and Pascual-Sedano, 1999), psychiatric symptoms, autonomic dysfunction, rapid eye movement (REM) sleep behaviour disorder, fatigue, and pain (Mortimer

et al., 1982) (Table IV). These are common at early stages of PD, and related with reduced life quality, preceding in some cases the onset of the classical motor symptoms (Ferrer *et al*., 2012; Noyce *et al*., 2012; Khoo *et al*., 2013; Chaudhuri and Schapira, 2009; Lim and Lang, 2010). In the absence of classical motor features, this can lead to a substantial diagnostic delay. The premotor phase can be extended 12-14 years as it occurs between the average latency onset of REM sleep behaviour disorder and the appearance of parkinsonian motor symptoms (Postuma *et al*., 2012). For this reason, it is believed that the pathogenic PD course during the premotor phase involves peripheral and CNS areas, in additional to the dopaminergic neurons of the SN.

Premotor PD	Early PD	Advanced PD
Hyposmia	Mild orthostatic hypotension	Severe orthostatic hypotension
Constipation	Mild bladder problems	Urinary incontinence and retention
Depression, anxiety	Siallorrhoea	Erectile dysfunction
REM sleep behaviour disorder	Pain, paresthesias	Disturbed thermoregulation
Fatigue	Anhedonia	Apathy
	Frontal-executive dysfunction	Confusion, dementia

Table IV. Non-motor symptoms of Parkinson's disease grouped by typical time of onset. Primary related to the underlying disease. Adapted from Kovacs, 2015.

2.5 Neuropathology

The hallmark of PD neuropathology is the widespread α-synuclein-immunoreactive deposits in neurons (LB), and also in dystrophic neurites (LN) throughout the CNS, as well as the progressive and marked neuronal loss in different subcortical nuclei, especially in SN, but also in locus coeruleus, nucleus basalis of Meynert, and dorsal motor nucleus of the *vagus* nerve (Gelb *et al*., 1999; Braak and Braak, 2000; Ballard *et al*., 2006). It is demonstrated that in the major sporadic PD, LB and LN

pathology, patients progress through a caudal-rostral path (Braak *et al*., 2003; Del Tredici *et al*., 2002). Cell loss spreads along a caudo-rostral, lateromedial, and ventrodorsal sequence in a somatotropic pattern of dopaminergic terminal loss, which is more acute in the dorsal and caudal putamen than in the caudate nucleus. The DA neurons are importantly depressed in the striatum and correlate with the disease advance (Porritt *et al*., 2006). Thus, SN cell damage, α-synuclein aggregation and accumulation, and reduced tyrosine hydroxylase (TH), which lead to impaired synthesis of DA, all show an evident relationship. The majority of SN pigmented neurons (explained in more detail later in this chapter page 68) carrying α-synuclein aggregates lack TH reactivity, which could represent a cytoprotective mechanism that can be preserved in neurons with primary α-synuclein accumulation (Figure 9) (Mori *et al*., 2006; Kovacs *et al*., 2010).

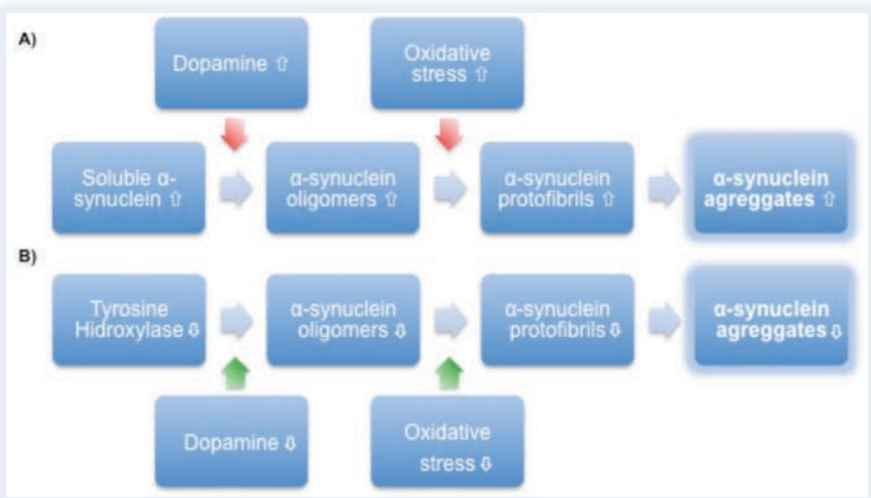


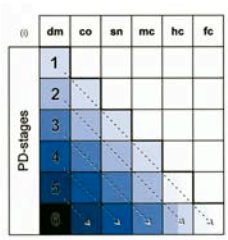
Figure 9. Schematic illustration of (**A**) presumable α-synuclein aggregation mechanism, and (**B**) protective role of depletion of TH immunoreactivity in pigmented neurons. Adapted from Mori *et al*., 2006.

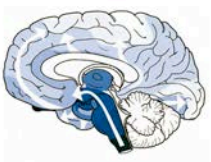
Evolution of Lewy body-related pathology

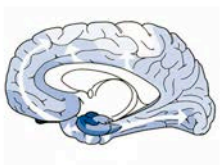
The Braak and Braak staging procedure related to sporadic PD rests on the assumption that incidental Lewy body pathology is the earliest sign in the disease progression (Braak *et al*., 2003, 2004, 2009). PD advances in six neuropathological stages characterized by the constant expansion of spindle-like or thread-like inclusion bodies, and by the presence of LN and LB in the somata of the implicated nerve cells. The intraneuronal lesions in this pathology develop in a sequential manner, beginning at predisposed sites and progressing in a predictable way (Figure 10).

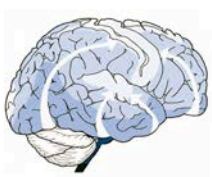
LB pathology at **stage 1** of the disease may begin in the lower brainstem and involves the dorsal motor nucleus of the vagal nerve (DMX), the anterior olfactory nucleus, and the intermediate reticular zone with the preservation of the nucleus basalis of Meynert (NBM), as well as the midbrain regions. The pathology in the DMX worsens at **stage 2** with the injury spreading beyond the limits of this nucleus to involve the caudal raphe nuclei, the gigantocellular reticular nucleus, and the coeruleus-subcoeruleus complex. Thus, during the first two stages of the disease, the pathology in non-olfactory sites is restricted to the medulla oblongata and the pontine tegmentum, with no involvement of the SN, the area that finally leads to the clinical representation. These first stages are considered presymptomatic, and may explain the non-motor symptoms including olfactory and autonomic dysfunction, which precede the somatomotor damage. Cases with LB pathology in the brainstem without *parkinsonism* are considered as incidental PD (iPD), and whether these cases constitute presymptomatic PD has been a matter of controversy for years (Ferrer, 2009; Jellinger, 2009[a]). It is in the **stage 3** that the locus coeruleus, the cholinergic magnocellular nuclei of the basal forebrain, the central nucleus of the amygdala, and the posterolateral and medial SN are considered targets of neuronal depletion and cytoskeletal changes. The first LNs can be observed in the substantia nigra pars compacta also with the presence of granular aggregations, pale bodies, and LBs, all of

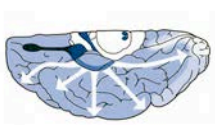
which lead to thin and sparsely myelinated axons (Fearnley and Lees, 1991). Additional brain areas related with this stage include the cholinergic tegmental pedunculopontine nucleus and the upper raphe nuclei (Garcia-Rill 1991; Candy *et al*., 1983; Whitehouse *et al*., 1983). However, one of the main characteristics of stage 3 is the involvement of a subset of melano-neurons in the SN, restricted to melanised projection neurons of the posterolateral and posteromedial subnuclei, but without macroscopically detectable depigmentation of the SN (Braak *et al*., 1986). At **stage 4**, the limbic system, which includes the accessory cortical and basolateral nuclei of amygdala, as well as the thalamus, and the temporal cortex (anteriomedial temporal mesocortex, enthorinal region and hippocampal formation) are especially affected. The anteriomedial temporal mesocortex is important to note due to its relevance in the data transference from the high-order sensory association areas to the prefrontal cortex, using the limbic system. Furthermore, marked devastation of the melano-neurons in the susceptible SN is detected. All this damage produces memory dysfunction and cognitive decline. Thereby, at some point during the intermediate stages 3 and 4, the presymptomatic phase moderately gives way to the clinical phase. For this reason, both stages 3 and 4 of Braak have been associated with clinically symptomatic phases. In the final **stages 5 and 6**, when the patients manifest the extensive range of PD-associated clinical symptoms, the neurodegenerative process reaches the neocortex and attains a vast topographic expansion. Inclusion bodies emerge firstly in the prefrontal cortex and high-order sensory association areas of the neocortex, such as the anterior cingulate cortex and the insula. Afterwards, they reach the premotor and primary sensory association areas, or else involve the whole neocortex (Braak *et al*., 2003; Del Tredici *et al*., 2002). Finally, the SN becomes practically denuded of melanin-laden neurons and has an obvious paleness upon macroscopic inspection (Damier *et al*., 1999; Braak *et al*., 2003) (Figure 11).

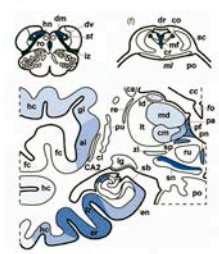


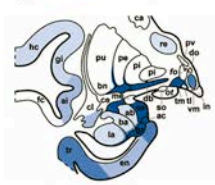












Sensory centers

- Olfactory: early and severe involvement
- Nociceptive: early involvement
- Somatosensory: mostly intact
- Auditory and visual: uninvolved

Motor centers

- Limbic: severe involvement
- Visceromotor: early and severe involvement
- Somatomotor: partial involvement

Stage 1

- Anterior olfactory nucleus
- Olfactory bulb, olfactory tract
- Dorsal motor nucleus of the vagal nerve

Stage 2

- Lower raphe nuclei
- Magnocellular reticular nuclei
- Coeruleus-subcoeruleus complex
- Plexuses of Meissner and Auerbach

Stage 3

- Central subnucleus of the amygdala
- Piriform cortex (olfactory system)
- Periamygdelear cortex (olfactory system)
- Substantia nigra pars compacta
- Upper raphe nuclei
- Pedunculopontine tegmanetal nucleus

Stage 4

- Cortical and basolateral amygdala
- Thalamic nuclei
- Anterior cingulate cortex
- Insular and subgenual cortex

Stage 5

- High order sensory association neocortex
- Prefrontal neocortex
- Enthorinal region, CA1 and CA3

Stage 6

- First order sensory association neocortex
- Premotor neocortex
- Primary sensory areas
- Primary motor field

Figure 10. Overview of the anatomical systems and regions involved in sPD, showing the progression of PD-related intraneuronal pathology. Modified from Braak *et al*., 2003, 2009.

SN pigmentation

The characteristic pigmentation of the SN is due to the accumulation of neuromelanin, a pigment accumulated with advanced age from the 3rd year of life (Fedorow *et al*., 2006; Halliday *et al*., 2005) and composed of lipids, proteins, and products of the DA metabolism, explaining its selective distribution (Figure 11). Neuromelanin has been proposed as a free radical scavenger with a potential protective role against oxidative stress, and implicated in the cell survival. For this reason, changes in neuromelanin contribute to the selective vulnerability of DA neurons in PD. Several studies suggest that this pigment may preserve neurons against intracellular stressors due to the pigmented neurons of the SN containing less neuromelanin in PD brains than in controls (Kastner *et al*., 1992; Zecca *et al*., 2001).

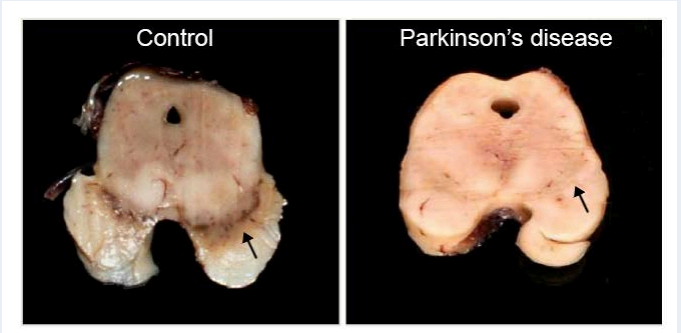


Figure 11. Comparison of pigmented neurons of the SN in control and PD. Pallor of SN (arrow) is observed in PD in comparison to control (arrow). Reproduced with permission from the Institute of Neuropathology of the Bellvitge University Hospital.

2.6 Diagnosis and treatment

Diagnosis

The diagnosis of PD is based on the presence of a slowly progressive parkinsonian syndrome in the absence of evidence for another etiology. Asymmetrical onset and classical resting tremor are particularly helpful pointers towards a diagnosis of PD (Kovacs, 2015). Thus, the diagnosis of PD in around 80% of the cases is performed using the most universal clinical diagnostic criteria, the UK Parkinson's Disease Society Brain Bank Criteria (Daniel and Lees, 1993), although other cognitive rating scales are also used (Paganobarraga *et al*., 2008). Frequently, a significant number of patients require supplementary diagnostic procedures. Clinical criteria at best allow diagnosis of probable PD, while an accurate diagnosis requires *post-mortem* neuropathological verification. In 80-90% of the cases the clinical diagnosis of PD is ratified with the autopsy as several clinicopathological studies have demonstrated (Litvan *et al*., 2003).

Treatment

Worsening of motor qualities is a characteristic of PD progression, which can be treated with symptomatic therapies at the beginning. Nevertheless, the development of difficulties related to long-term symptomatic treatment comprising motor and non-motor fluctuations emerges when the disease advances (Hely *et al*., 2005). In late-stages of PD, treatment-resistance is remarkable, and comprises axial motor symptoms such as postural instability, falls, freezing of gait, dysphagia, and speech dysfunction, which contribute substantially to disability. Thus, to date PD is still an incurable disease and current treatments are focused on the control of the motor symptoms to improve quality of life and functional capacity. The pharmacological substitution of DA with levodopa (L-dopa) is the most widely used treatment for PD (Kulisevsky *et al*., 1996; Kulisevsky and Pascual-Sedano, 1999; Pezzoli and Zini,

2010), although other pharmacological treatments (Enzyme monoamine oxidase B (MAO-B) inhibitors, Catechol-O-methyltransferase (COMT) inhibitors, anticholinergics, and Amantadine, among others), and surgical treatments (*e.g* deep brain stimulation) could also be useful.

2.7 Parkinson's disease with dementia (PDD)

General definition

Dementia is considered a clinical state distinguished by loss of function in several cognitive domains including attention, visuo-spatial, constructional and executive functions, and memory, which cause dysfunction in the patient's life functioning (Aarsland *et al*., 2003[a]). Dementia may complicate PD after years of an otherwise classical course and become the primary origin of disability, eclipsing the motor aspects of *parkinsonism*. It is noted that patients with PDD have a more rapid motor decline, and falls are more frequent than in non-demented PD patients (Emre *et al*., 2007).

Epidemiology

This affects around 40% of patients with Parkinson's disease (Cummings *et al*., 1998) and is less predictive than the common dementia associated with Alzheimer's disease-type pathology. Among PD patients who do not firstly evidence dementia, which is common later in the disease course, the annual incidence range varies from 2.6% to 9.5% (Marder *et al*., 1995; Mayeux *et al*., 1990; Aarsland *et al* 2001; Apaydin *et al* 2002). Further, the frequency of dementia in clinic-hospital cohorts ranges from 6% to 29% (Brown and Marsden, 1984; Taylor *et al*., 1985; Mayeux *et al*., 1988; Friedman and Barcikowska, 1994; Apaydin *et al*., 2002). Thus, the risk of dementia is considerable if PD patients live long enough, and the incidence is up to 6 times more than in healthy people (Apaydin *et al*., 2002; Mayeux *et al*., 1988).

Etiology

Certain factors at the time of PD diagnosis may increase future dementia risk including ageing, greater severity of motor symptoms, in particular rigidity, postural instability and gait disturbance, hallucinations, mild cognitive impairment (MCI) at baseline, and mood disorders (Table V) (Emre, 2003, Emre *et al*., 2007; Aarsland *et al*., 2009). Age and severity of motor symptoms seem to have a combined rather than additive effect on the risk of dementia in Parkinson's disease (Emre *et al*., 2007). According to the neuropsychological assessment, some studies suggest that taking into account the cortical-type cognitive dysfunction in patients with early PD could also help to predict the development of dementia (Song *et al*., 2008). In addition, in one study alternating verbal fluency with delayed verbal memory independently differentiated the PD patients with MCI from the cognitively intact PD patients (Paganobarraga *et al*., 2008).

Risk factors for dementia in patients with PD
• Advanced age
• Advanced age at onset of motor symptoms
• Early occurence of levodopa related confusion or psychosis
• Presence of speech and axial involvement
• Severe motor symptoms, especially bradykinesia
• Poor cognitive test scores, especially verbal fluency
• Depression

Table V. Main risk factors for dementia in patients with PD. Adapted from Emre, 2003.

The effect of genetic predisposition has also been studied on PDD focused in APOE genotypes with conflicting results and has been reported in *PARK1* (Polymeropoulos *et al*., 1997; Singleton *et al*., 2003) and *PARK8* (Wszolek *et al*., 1997).

Pathogenesis

Regarding the pathogenesis, it has been demonstrated that there is a correlation between the duration of *parkinsonism* prior to dementia and the severity of plaques, cortical α-synuclein pathology, and cortical cholinergic deficits (Ballard *et al*., 2006; Rinne *et al*., 2007). Pathologic and neurochemical characteristics are associated with the duration of PD previous to dementia in a correlation analysis. Neurochemical deficits outside of the DA system are the most essential factors for cognitive decline in PDD (Hilker *et al*., 2005). Several studies prove the association between striatal, temporal, and parietal decrease in patients with PDD, pointing toward an interdependent degeneration in mesostriatal dopaminergic and cortical cholinergic neurons, thereby giving support to the premise that, clinically, dementia develops when the disease spreads from nigral neurons to the cortex, leading to a cholinergic dysfunction in impaired patients (Tiraboschi *et al*., 2000; Hilker *et al*., 2005; Ballard *et al*., 2006). In addition, noradrenaline depletion in the locus coeruleus, serotonin loss in the raphe nuclei and the striatopalidal complex, may also be implicated in the pathophysiology of PDD (Scatton *et al*., 1983). Thus, loss of cholinergic, dopaminergic, and noradrenergic innervation has been suggested as the underlying neurochemical deficits of the pathology. Finally, regarding with molecular alterations in PDD, there is an important difference in the subforms of α-synuclein and β-synuclein. While β-synuclein is overexpressed in messenger ribonucleic acid (mRNA) levels in PD, α-synuclein is reduced in PDD in the caudate, suggesting that these molecular mechanisms are importantly involved in these complex diseases (Beyer *et al*., 2011).

Clinical features

A wide variety of cognitive impairments have been reported comprising memory, visuospatial function, and frontal lobe or executive dysfunction in PD and PDD, even early in the course of the disease (Canavan *et al*.,

1989; Cooper *et al*., 1991, 1993; Emre et al., 2007). In comparison with PD, PDD show worse scores in confrontation naming (Pagonabarraga *et al*., 2008), and greater impairment in attention, visuospatial functions, and language and executive functions than non-cognitively impaired PD and MCI-PD (Song *et al*., 2008). The presence of cognitive impairment in PD is usually accompanied by brain atrophy, and structural imaging techniques such as MRI allow accurate identification of global and regional damage. There is converging proof that dementia generates substantial clinical consequences for the demented patients including reduction of life quality, raised burden of caring for patients, risk of psychosis, and increased mortality (Emre *et al*., 2007). Clinically, the prototype of dementia in PD is a dysexecutive syndrome. Numerous patients with PD develop mental dysfunction ranging from slight cognitive deficits to severe dementia (Aarsland *et al*., 2003[a]; Hilker *et al*., 2005). PET studies using the radioligand N-C [11C]-methyl-4-piperidyl propionate (11C-PMP) as a substrate of the cerebral acetylcholine esterase (AChE) evidenced a serious loss of cerebral cholinergic activity in PDD (Bohnen *et al*., 2003). The regional AChE activity in parietal motor, association, and limbic areas was found to be diminished in these patients, whereas the cholinergic dysfunction was more substantial (Bohnen *et al*., 2003; Shinotoh *et al*., 2001). Thus, cholinergic deficits seem to play an important role in the pathogenesis of PD-associated dementia, but the link between cortical cholinergic dysfunction and DA depletion in the basal ganglia is still uncertain (Hilker *et al*., 2005).

Neuropathology

The neuropathological substrate for the dementia that develops in PD patients has been considerably discussed (Hakim *et al*., 1979; Gaspar *et al*., 1984; Rinne *et al*., 1989; Braak and Braak, 1990; Jendroska *et al*., 1996; Mattila *et al*., 1998; Apaydin *et al*., 2002), and there is no common consensus as to whether this dementia is fundamentally a cortical or subcortical process, or whether AD or LBD is the main contributor

(Jellinger and Attems, 2008; Apaydin *et al*., 2002; Irwin *et al*., 2012). Significant clinical heterogeneity could explain some of this lack of consensus. Some neuropathological studies indicate that diffuse cortical LBs are the prime substrate of dementia (Tsuboi *et al*., 2005; Aarsland *et al*., 2003[b]; Ballard *et al*., 2006), affecting about 50% of demented PD. Otherwise, these studies suggest that an extensive cortical Lewy body pathology alone could be enough to produce dementia without high levels of neurofibrillary tangles (NFTs) Braak stage (Horvath *et al*., 2003). High tau burden could also be an important factor to take care since higher Braak tau stages correlate well with cognitive impairment as a Braak NFT stage > 3, and predicted dementia in 100% of the cases (Gold *et al*., 2007; Horvath *et al*., 2013). These results promote tau pathology as an independent and aggravating contributor to the spread of severe dementia in PD. In addition, there has been observed to be a correlation between dementia and the seriousness of cortical tau, β-amyloid, and α-synuclein pathology, supporting a synergistic interaction between tau, Aβ aggregations and α-synuclein (Apaydin *et al*., 2002; Lashley *et al*., 2008; Mandal *et al*., 2006; Pletnikova *et al*., 2005).

Diagnosis

The most ordinary criteria used for the dementia diagnosis has been, for a long time, the Diagnostic and Statistical Manual for Mental Disorders (DMS-IV) (American Psychiatric Association, 2003), prior to the Task Force diagnostic criteria for PDD, established in 2007 by the Movement Disorder Society (Emre *et al*., 2007). In this manner, the diagnosis of PDD as well as other pathologies is based on a combination of clinical, imaging, and biochemical measures.

Treatment

As in Parkinson's disease, there are no treatments to slow or stop the brain injury caused by PDD. Current strategies are focused on treating the symptoms with cholinesterase inhibitors, antipsychotic drugs,

levodopa (L-dopa), antidepressants, and clonazepam, among others (Emre *et al*., 2003).

Relation between PDD and DLB

PDD and DLB are suggested by some to be different features of the same disease and seen by others as two distinct entities, particularly clinically (McKeith, 2009, Revuelta and Lippa, 2009, Aarsland *et al*., 2009). They share similar clinical syndromes with specific neuropathological changes such as deposition of α-synuclein in LB and LN, and loss of dopamine and cholinergic cell populations in the tegmental area and basal forebrain, respectively. Cognitive impairment associated with *parkinsonism*, deficits in attention and wakefulness, and visual hallucinations are differential aspects of clinical features for these pathologies (Kraybill *et al*., 2005). The clinical criteria are of primarily importance and include both motor and non-motor symptoms (McKeith *et al*., 2004; Jankovic 2008; Dubois *et al*., 2007), but is the symptom of cognitive impairment that is the most essential to distinguish between PDD and DLB (Dubois *et al*., 2007). The apparance of dementia is the principal clinical distinction between the pathologies; while early cognitive impairment is related with DLB, PDD begins in the setting of well-established idiopathic Parkinson disease after at least 1 year of motor symptoms (Gomperts, 2016). Several studies directly comparing PDD and DLB have based the distinction between conditions on the 1-year rule explained in the McKeith criteria (McKeith *et al*., 1996; Ballard *et al*., 2006), where patients presenting less than 1 year of *parkinsonism* before dementia are diagnosed with Parkinson's disease with dementia. In addition, different reports propose that DLB patients have significantly greater cortical Lewy body pathology (Richard *et al*., 2002; Ballard *et al*., 2006), and more noticeable atrophy (Tam *et al*., 2005). Nevertheless, concurrent senile plaques and NFTs are common to PDD and DLB (Tsuboi *et al*., 2005; Aarsland *et al*., 2009; Ballard *et al*., 2006).

In conclusion, the most significant differences between the two groups are more severe neuronal loss in the SN in PDD cases, and more current involvement of hippocampal C2/3 area, amygdala, and neocortex with Lewy bodies in DLB patients.

3. Dementia with Lewy bodies

General definition

Dementia with Lewy bodies (DLB) is the second most frequent cause of dementia in the elderly after AD, representing a significant proportion of NDD, accounting for 10-15% of autopsy-proven cases of dementia (McKeith *et al*., 2004). It is clinically characterized by progressive combined cortical and subcortical dementia, in association with neuropsychiatric symptoms such as fluctuating cognition and visual hallucinations, and often accompanied by *parkinsonism*; explained in more detail below (page 78) (Jellinger, 2003; McKeith *et al*., 1996). Repeated falls, syncope, transient loss of consciousness, systematized delusions, and neuroleptic sensitivity are not uncommon (McKeith *et al*., 1996, 2005; Mayo and Bordelon, 2014). In addition, DLB patients present similar motor impairment compared with PD, although there is a higher age onset and shorter disease duration in DLB (Bonelli *et al*., 2004), suggesting a more rapid development of *parkinsonism* in DLB, and more symmetrical in comparison to PD (Ransmayr *et al*., 2001; Jellinger and Mizuno, 2003).

3.1 Epidemiology and etiology

Epidemiology

There is a wide range of prevalence estimated in the published literature depending on the design study and the clinical background. The highest estimates come from specialist secondary dementia referral centres, and range up to 26% of demented patients (Heidebrink, 2002; Lennox *et al*., 1989). Thus, DLB is estimated to be 0.7% of the population over 65 years

of age (Kosaka *et al*., 1976; Kosaka, 1990), and the incidence is 3.5 per 100,000 person-year and increasing with age (Savica *et al*., 2013). Onset prior to 65 years is unknown and there is no established lower age limit. As happens in PD, there is a slightly greater male predominance with a 1.9:1 (male: female) rate (McKeith *et al*., 1994). Advanced age, hypertension, hyperlipidemia, and carrying one or more APOE ε4 alleles, are some of the considered risk factors for DLB (Galasko *et al*., 1996; Gardner *et al*., 2013; Benjamin *et al*., 1994; Benjamin *et al*., 1995; Lamb *et al*., 1998; Lippa *et al*., 1995).

Genetics

No single gene determinant of sporadic DLB has been described. A few families with autosomal dominant inheritance of DLB have been identified (Ishikawa *et al*., 1997), including E46K mutation on *SNCA* gene (Zarranz *et al*., 2004), *UCH-L1* gene I93M mutation (Marx *et al*., 2003), and two β-synuclein mutations (V70M and P123H) (Ohtake *et al*., 2004). Positive associations between DLB and the following genetic factors have also been described: inducible nitric oxide synthase 2 (iNOS 2) (Xu *et al*., 2000), CYP2D6B (Tanaka *et al*., 1998), dipetidyl carboipeptidase 1 (DCP1) (Mattila *et al*., 2000), and butyrylcholinesterase K (BCHE K) (Singleton *et al*., 1998), as well as a greater heterozygous frequency of glucocerebrosidase 1 (GBA1), estimated at 3.5%.

Pathogenesis

The pathological features include a variable burden of α-synuclein immunoreactive neuronal pathology and Alzheimer-type pathology. An important number of DLB cases show, in addition to LB related pathology, characteristic AD changes (Lopez *et al*., 2002; Del Ser *et al*., 2001; Merdes *et al*., 2003; Ferman *et al*., 2006), where high Braak stages of AD-type pathology result in a clinical diagnosis of AD rather than DLB (McKeith *et al*., 2005).

Widespread distribution of LBs in virtually every brain area is a classical feature of DLB, although the frontal cortex, pigmented midbrain and brainstem nuclei, dorsal efferent nucleus of *vagus*, basal forebrain nuclei, and the limbic cortical regions are particularly involved (Neef and Walling, 2006; Beyer *et al*., 2009). Interestingly, cortical LBs contain α-synuclein; however, they may not demonstrate the classic structure of an eosinophilic core with surrounding halo. DLB is characterized by a loss of cholinergic neurons in the nucleus basalis of Meynert, depletion in DA-containing neurons, and decreased cortical choline acetyltransferase (ChAT) (Ballard *et al*., 2000). These neurohistochemical changes potentially account for both the dementia and the motor symptoms of the disease (Mayo and Bordelon, 2014). In the only prospectively diagnosed series with *post-mortem* confirmation, the pathology was heterogeneous (McKeith *et al*., 1996, McKeith *et al*., 2000, McKeith *et al*., 1996); there are no biomarkers, either in the serum or CSF, that have been shown to be useful in the diagnosis of DLB, as explained later (page 80).

3.2 Clinical features and neuropathology

Clinical features

DLB is characterized by both cortical and subcortical dementia, in addition to motor dysfunction, as explained previously. The three core features of this pathology are fluctuations, visual hallucinations, and *parkinsonism.* Any one of them is sufficient for diagnosis of possible DLB, and two for diagnosis of probable DLB, as established by the Consensus Consortium on DLB in 1995, and later revised (McKeith *et al*., 2005; Nelson *et al*., 2010). The pathology begins with dementia marked by fluctuating cognition. These fluctuations can be clinically apparent as periods of reduced consciousness, increased confusion, and cognitive impairment (Cummings, 2004[a]). They are followed by severe visuospatial deficit detected by the performance on copy tasks, shape detection, block design or clock drawing, and also by relative sparing of memory (Allan *et*

al., 2009; Shimomura *et al*., 1998; Collerton *et al*., 2003). Verbal fluency, attention, psychomotor speed, and executive function are also damaged in DLB (Salmon *et al*., 1996). Additionally, visual hallucinations, which are recurrent and tend to occur at night, as well as parkinsonian motor features, are other clinical hallmarks of the pathology. *Parkinsonism* may be present at onset, or develop later. Non-motor features typically develop early in DLB, and may be subtle, such as olfactory dysfunction, sleep disorders, autonomic dysfunction, depression, constipation, sialorrhea or slow waves and temporal sharp waves on electroencephalography (EEG). REM sleep behaviour disorder has been found to precede the diagnosis in PD and DLB (McKeith *et al*., 2005). Using FDG-PET imaging, hypometabolism is present in the parietotemporal, posterior cingulate, frontal association, and occipital cortices. Thus, the hypometabolism in occipital cortices, predominant in the primary visual cortex, is unique in DLB and can differentiate properly between DLB and AD or PD (Minoshima *et al*., 2001; González-Redondo *et al*., 2014).

Neuropathology

The hallmark pathology of DLB is α-synucleinopathy, manifested as LB of both classical and cortical types, and neuritic degeneration. Cortical LBs appear to affect various subgroups of neurons including pyramidal cells and GABA-ergic interneurons (Gómez-Tortosa *et al*., 2000; Smith *et al*., 1995; Wakabayashi *et al*., 1995). In DLB, preliminary studies indicate that Lewy pathology starts in the amygdala, advances to the limbic cortex, and finally advances to the neocortex (Katsuse *et al*., 2003[b], Ballard *et al*., 2006; Kövari *et al*., 2009). However, non-uniformity in the regional pattern of the Lewy pathology has been reported (Yamamoto *et al*., 2005), indicating that the most vulnerable neurons may differ in different patients with LBD.

3.3 Diagnosis and treatment

As explained previously in the PD chapter, the diagnosis in Dementia with Lewy bodies is also based on a combination of different measures including clinical, biochemical and imaging, following the consensus guidelines for the clinical and pathologic diagnosis of DLB (McKeith, 2006). In addition, there are no definitive biomarkers for DLB, though it has been proposed that a relative increase in CSF tau, with decrease in Aβ-42 can distinguish between DLB and PD (Kaerst *et al*., 2014), and that levels of Aβ-42/Aβ-38 can differentiate between DLB and AD (Mulugeta *et al*., 2011). Moreover, there's currently no cure for DLB, but some therapies can help to manage the symptoms. The pharmacological treatment includes L-dopa, antidepressants, clonazepam, and antipsychotics.

3.4 Rapidly progressive Dementia with Lewy bodies (rpDLB)

General definition

Rapidly progressive dementia (RPD) is traditionally defined as the subacute development of cognitive impairment, often associated with behavioural and movement disorders within 2 years (Rosenbloom and Atri, 2011; Geschwind, 2016). Patients develop dementia over weeks to months in comparison to the slowly progressive dementias that occur over a few years. A substantial part of RPDs are caused by prion disease or Creutzfeldt-Jakob disease (CJD), a fatal prion-related neurodegenerative illness, whereas the most frequent origin of RPDs is atypical presentations of other NDD disorders (Figure 12).

Epidemiology and etiology

Aside from prion diseases, neurodegenerative non-CJD disorders represent the most common cause of RPD, and are those that develop in a faster way when compared with the classical form. Whereas the

prognosis is relatively well-defined for the chronic dementias, the prognosis in RPDs is variable depending on the underlying cause of symptoms (Rosenbloom and Atri, 2011). Mean age of RPDs is 60 years, with a high range from 55 to 77 years, and the duration, from onset to RPD, varies from about 2 months to just over 12 months (Geraldes *et al*., 2012; Pasi *et al*., 2014). Specifically, rpDLB has been associated with an older age at onset ~ 80 years (Geschwind *et al*., 2008, 2016; Josephs *et al*., 2009), has an average survival time of 9 months, and for those with better prognosis less than 3 years life span (Williams *et al*., 2006; Geschwind *et al*., 2007, 2008). Several etiologies for RPDs exist including infectious, neurodegeneration, neoplastic, autoimmune, and toxic-metabolic, among others processes, some of which can be identified clinically through history and physical examination. One useful mnemonic device for the differential evaluation of RPDs is the acronym **VITAMINS**: **V**ascular, **I**nfectious, **T**oxic-metabolic, **A**utoimmune, **M**alignancy, **I**atrogenic, **N**eurodegenerative, and **S**ystemic etiologies.

Clinical features

rpDLB often show sharp waves on EEG, with dramatic fluctuations in cognition, features that can mimic CJD. Other predominant symptoms are delirium, visual hallucinations, delusions, *parkinsonism*, and myoclonus. Interestingly, those individuals with DLB that have a sudden course have sometimes been shown to have a history of encephalopathy associated with surgery or illness followed by a minimally symptomatic period before the onset of a withering dementia. Additionally, those with a sudden clinical DLB may also have concomitant AD.

Diagnosis and treatment

RPDs are rather rare and the diagnosis can be difficult. Because of the rapid decline, patients need urgent evaluation and often require an extensive workup with multiple tests performed concurrently. Establishing the time course through a history obtained from family members is a

critical part of the evaluation, and the first symptoms of a dementia can help with the diagnosis (Geschwind, 2016, 2008). The progression rate of RPDs may be based on the decline in psychometric tests as Mini-Mental State Examination (MMSE), or on the survival time. Prion-diseases such as CJD, perhaps the prototypical RPD, are often the first diagnosis many neurologists consider with a rapid cognitive decline patient. Non-prion RPDs are frequently diagnosed by exclusion, requiring comprehensive serum and CSF analyses and gadolinium-enhanced MRI neuroimaging to rule out vascular, toxic-metabolic, infectious, and inflammatory conditions. The distinction between prion and non-prion RPDs has become easier with the use of biomarkers in the CSF, as well as with protein misfolding cyclic amplification assay and real-time quaking induced conversion (RT-QuIC) (Geschwind, 2015). Neuroimaging is a helpful tool in distinguishing prion-related from non-prion-related RPDs (Degnan *et al*., 2014). Hence, electroencephalography (EEG) with high temporal resolution is also useful in determining the locality of the lesions, and assessing the presence of cortical irritability (Rosenbloom and Atri, 2011). Furthermore, as with other RPDs, there is no treatment for rpDLB.

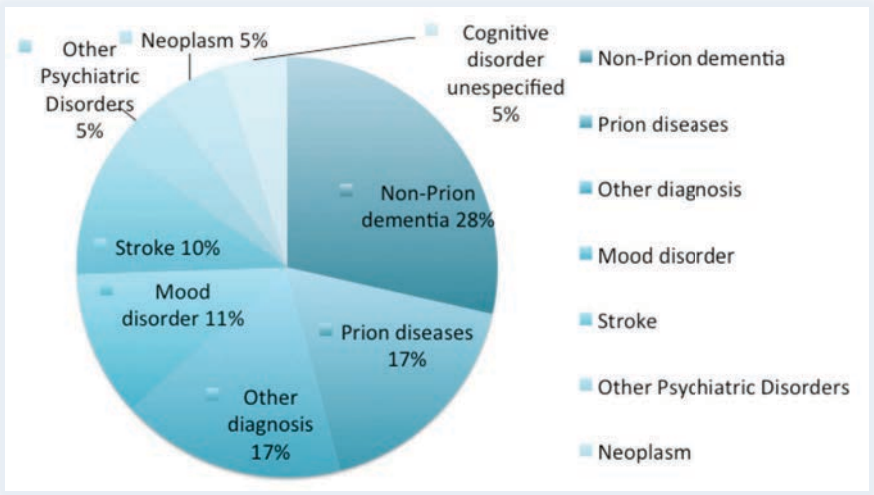


Figure 12. General classification of RPDs including prion and non-prion diseases. Adapted from Appleby, 2009.

4. Metabolic pathways involved in PD, DLB, and rpDLB

In addition to the classical neuropathological hallmarks, including selected neuronal loss, presence of Lewy bodies, altered neurites, and abnormal α-synuclein production, deposition and aggregation, a large number of metabolic defects converge not only in Parkinson's disease brains but also in Dementia with Lewy bodies and rapidly progressive Dementia with Lewy bodies. These include protein synthesis damage, mitochondrial abnormalities, oxidative and reticulum stress injury, neuroinflammation, purine metabolism involvement, altered ubiquitin-proteasome system function and autophagy, neurotransmitter and neuromodulator abnormalities, and altered composition of lipid membranes particularly lipid rafts, among others (Ferrer, 2009; Ferrer *et al*., 2011, 2012). Further, these metabolic alterations occur in the cerebral cortex and other systems at relatively early stages (Tang *et al*., 2010; Ferrer *et al*., 2012; Dunn *et al*., 2014). Some of these pathways, including protein synthesis machinery, mitochondria and energy metabolism, neuroinflammation, purine metabolism, and new signalling pathways (olfactory and taste receptors path), are studied in the present thesis following the clustering results obtained by microarray aproaches, which will be explained in more detail later on.

4.1 Protein synthesis machinery

4.1.1 Protein synthesis pathway

Proteins and translation process

Proteins are one of the elementary components of life and catalyse the majority of reactions sustaining life with a structural, transport, and regulatory role. The process of decoding the mRNA by the ribosome to synthesise a protein in a mechanism called **translation** is fundamental for all forms of life (Mathews *et al*., 2000) (Figure 13).

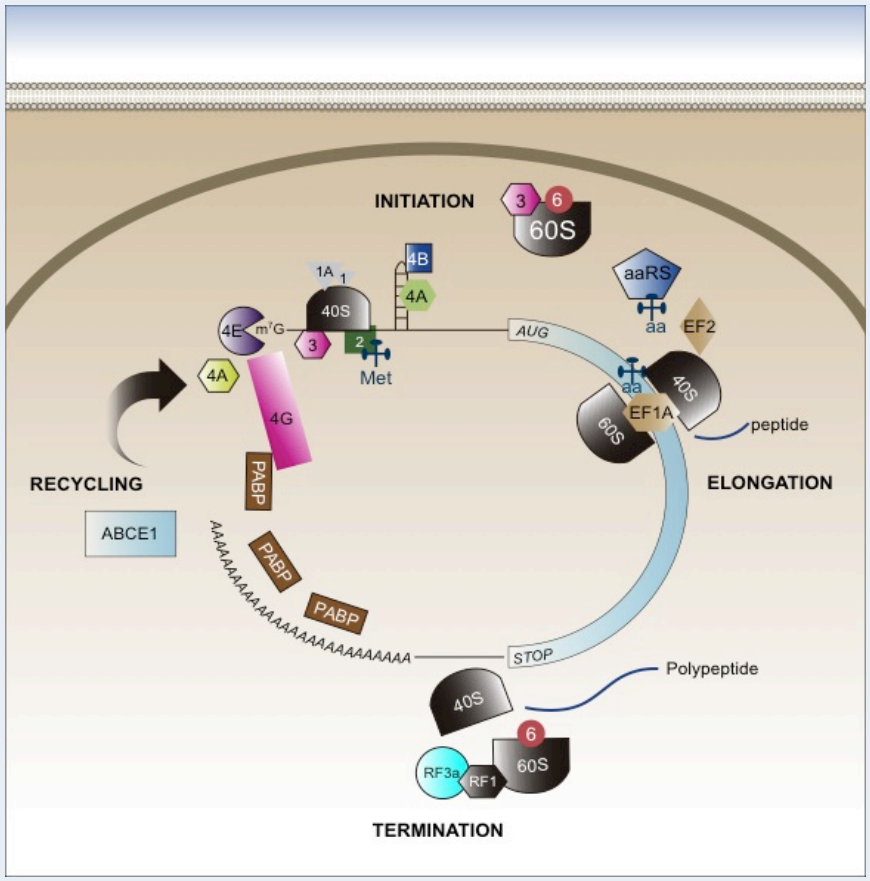


Figure 13. General process of translation in eukaryotes. During **initiation**, most eukaryotic mRNAs are translated by the cap-dependent mechanism, which requires recognition by eIF4E, complexed with eIF4G, and eIF4A -the so-called eIF4F complex- of the cap structure at the 5'end. A 43S pre-initiation complex, consisting in a 40S ribosomal subunit, loaded with eIF3, eIF1 and eIF1A, initiator Met-$tRNA_1^{Met}$, eIF2, and GTP binds the eIF4F-mRNA complex and scans along the 5'-UTR of the mRNA to reach the start codon. In the **scanning**, eIF4A, stimulated by eIF4B, unwinds secondary RNA structure in an ATP-dependent manner. The poly A-binding protein (PABP) binds both the poly (A) tail and eIF4G promoting mRNA circularization. **Elongation** is assisted by elongation factors eEF1A and eEF2. During this step aminoacyl-tRNA synthetases (aaRSs) catalyse the binding of amino acids (aa) to cognate tRNAs. **Termination** is mediated by the release factors eRF1 and eRF3, and happens when a termination codon (*STOP*) of the mRNA is exposed in the A-site of the ribosome. In this step the completed polypeptide is released. Finally, in the **recycling** process, required to allow further rounds of translation, both ribosomal subunits dissociate from the mRNA assisted by ABCE1.Modified from Hernández, 2012.

In spite of the advances in the understanding of specific altered proteins causative of particular diseases, such as specific intra- and extracellular protein aggregates, together with the production and accumulation of abnormal oligomeric species that lead to neurodegeneration and neuronal death, little attention has been paid to the process of total protein synthesis in these disorders. Nevertheless, this information is crucial, as possible alterations in protein synthesis may jeopardize several cellular functions, stimulate neurodegeneration and neuron atrophy, and lead eventually to cell death. Different studies have demonstrated ribosomal dysfunction (De Las Heras-Rubio *et al*., 2014), abnormal expression levels of certain transcription factors, and impaired protein synthesis in AD (Dönmez-Altuntas *et al*., 2005; Langstrom *et al*., 1989; Ferrer, 2002; Ding *et al*., 2005; Honda *et al*., 2005), whereas insufficient information is available about alterations in protein synthesis in PD, DLB, and rpDLB. In AD, the main modifications are reduced rRNA levels, increased RNA oxidation as seen by increased 8-hydroxyguanosine immunoreactivity, and reduced capacity of isolated polyribosomes to incorporate S^{35} methionine into protein (Ding *et al*., 2005, 2006; Honda *et al*., 2005; Jackson *et al*., 2010; Nunomura *et al*., 2006; Shan *et al*., 2003, 2006). Together, these findings suggest a complex scenario in which several pathways, from the nucleus and nucleolus to the ribosome, are altered in the cerebral cortex in AD (Hernández-Ortega *et al*., 2015), and probably in other NDD.

Nucleus and nucleolus

Abnormal morphology and disruption of the nucleolus, and reduced nucleolin expression, have been reported in the SN in PD (Colla *et al*., 2012[a]; Gertz *et al*., 1994; Rieker *et al*., 2011), and related experimental models (Rieker *et al*., 2011; Healy-Stoffel *et al*., 2013). Furthermore, mutations in DJ1 causative of familial PD alter rRNA biogenesis (Vilotti *et al*., 2012). Ribosomal RNA genes are arranged in tandem repeats in the nuclear organizer regions (NORs), and are

transcribed by RNA polymerase I in conjunction with associated factors including upstream binding transcription factor (UBF) encoded by *UBTF*, and SL1 proteins (Bell *et al*., 1988, 1989; Lyon and Lamond, 2000; Lamond and Sleeman, 2003; Fromont-Racine *et al*., 2003; Ishii *et al*., 2006; McKewon and Shaw, 2009; Panov *et al*., 2006; Sylvester *et al*., 1986; Wachtler *et al*., 1991; Ueshima *et al*., 2014). UBF is located in the nucleoli in interphase cells and regulates RNA polymerase I following acetylation (Mais *et al*., 2005; Meraner *et al*., 2006; Roussel *et al*., 1993). In addition, rDNAs encode precursor transcripts, which are processed to form 18S, 28S, and 5.8S RNAs (Brock and Bird, 1997). Regarding the nucleus, nucleolin (NCL) and nucleophosmin (NPM1/B23) are other major nucleolar proteins acting as histone-binding chaperones required for chromatin compaction and regulation of rRNA transcription through the modulation of H3K9m2 and H4K12ac in rRNA genes (Cong *et al*., 2012; Tamada *et al*., 2006), nucleic acid binding, and nuclear re-programming (Vilotti *et al*., 2012; Wachtler *et al*., 1991; Angelov *et al*., 2006; Erard *et al*., 1988; Ginisty *et al*., 1999; Okuwaki *et al*., 2001; Tajrishi *et al*., 2011). In addition, NPM1 is involved in the nuclear protein transport to the nucleolus, and transport of certain ribosomal proteins to the cytoplasm (Borer *et al*., 1989; Szebeni *et al*., 1995, 1997; Yu *et al*., 2006); its importance is demonstrated with the NPM1-null mice, which display altered ribosome biogenesis and premature death (Colombo *et al*., 2005; Grisendi *et al*., 2005). NPM1 interacts with histones H3 and H4, acts as a ribonuclease for the maturation of rRNA transcript (Herrera *et al*., 1995; Savkur *et al*., 1998), and is related to DNA replication, and ribosome biogenesis, transcription, and repair (Lindström, 2011). In addition, silencing NPM1 results in altered processing of 28S RNA (Itahana *et al*., 2003). NPM3 is a nucleolar histone chaperone, which interacts with NPM1 and modulates ribosome biogenesis (Eitoku *et al*., 2008; Huang *et al*., 2005). It is known that altered expression of nucleolar chaperones and factors involved in rRNA synthesis results in nucleolar stress, which in turn leads to impaired ribosomal biogenesis (Kiryk *et al*.,

2013; McKeown and Shaw, 2009; Parlato and Kreiner, 2013; Parlato and Liss, 2014). Thus, cellular and molecular alterations related with impaired nucleolar activity are causative of nucleolar stress (Boulon *et al*., 2010; Hetman and Pietrzak, 2012; Olson, 2004; Mayer and Grummt, 2005), which can lead to the malfunction of the nucleolar machinery, altered rRNA expression, reduced protein synthesis, and lastly, cell death. Nucleolar stress is emerging as an important sensor in several pathological conditions (Marquez-Lona *et al*., 2012; James *et al*., 2014), including ischemic injury, cancer (Avitabile *et al*., 2011; Hein *et al*., 2013; Ruggero *et al*., 2012), and neurodegeneration (Baltanas *et al*., 2011; Becherel *et al*., 2006; Parlato and Kreiner, 2013).

Ribosomal proteins and transcription factors

Ribosomes (80S) are cytoplasmic structures measuring 25-30 nm, composed of 65% RNAs and 35% ribosomal proteins, that form a small subunit (40S) that binds to mRNA, and a large subunit (60S), which binds to tRNAs and amino acids (Graifer and Karpova, 2012, 2015; Korobeinikova *et al*., 2012). In eukaryotes, the small subunit is made of 18S RNA and 33 proteins, whereas the large subunit is formed by 5S RNA, 5.8S RNA, 28S RNA, and 46 ribosomal proteins (Hetman and Pietrzak, 2012; Ben-shem *et al*., 2011; Doudna and Rath, 2002; Freed *et al*., 2010; Granneman and Baserga, 2004; Henras *et al*., 2008; Klein *et al*., 2004; Klinge *et al*., 2011; Korobeinikova *et al*., 2012; Kressler *et al*., 2010; Rabl *et al*., 2011). These ribosomal proteins also participate in protein synthesis initiation and elongation, and they can regulate their own synthesis at a translational level (Connell *et al*., 2002; Dresios *et al*., 2006; Glück and Wool, 2002; Stelzl *et al*., 2001; Wilson *et al*., 2002; Yu *et al*., 2009; Nyborg and Liljas, 1998). The translation initiation in the ribosome is geared by the interactions of 12 eukaryotic translation initiation factors (eIFs), most of them made up of several subunits. The 43S preinitiation complex is constituted by the small 40S ribosomal subunit, the initiating methionyl-tRNA bound to eIF2-GTP, and eIF1,

eIF1a, and eIF3. mRNA is added to the 43S preinitiation complex together with the poly(A) binding protein (PABP), and the eIF4f complex, a heterotrimeric complex composed of eIF4a, eIF4e, and eIF4g, bound to an AUG codon. eIF2B and eIF5 activate eIF2 and regulate eIF2-GDP recycling, respectively, whereas eIF5b and eIF6 participate in ribosomal subunit joining and binding (Hershey *et al*., 2012; Jackson *et al*., 2010; Kapp and Lorsch, 2004; Spilka *et al*., 2013). Elongation occurs when elongating factor eEF1A is activated following GTP-binding, and forms a complex with aminoacyl-tRNA, which recognizes the specific sequence in mRNA at the ribosome. Once the interaction of the codon in mRNA with the anti-codon in tRNA is decoded, eEF1A-GDP is hydrolysed, released from the ribosome, and recycled into its active form by eEF1B. Then, eEF2 assists in the precise codon location at the ribosome (Andersen *et al*., 2000, 2001, 2003; Gómez-Lorenzo *et al*., 2000; Jorgensen *et al*., 2003; Merrick and Nyborg, 2000; Miller and Weissbach, 1977; Nyborg and Liljas, 1998; Sasikumar *et al*., 2012; Voorhees and Ramakrishnan, 2013). Synthesis terminates in the presence of a stop codon in the mRNA sequence, which is recognized by realizing factor that sets the polypeptide chain free (Dever and Green, 2012; Nakamura and Ito, 2011). Phosphorylation of eIF2 on its α subunit prevents the delivery of initiator methionyl-tRNA, resulting in global inhibition of translation of most mRNAs (Harding *et al*., 1999).

4.1.2 Protein synthesis and oligomers

Apart from this limited input of protein synthesis, nothing is known about the possible link between the machinery of protein synthesis and α-synuclein aggregates, particularly α-synuclein oligomers in PD. As we have seen in previous chapters, α-synuclein was first described in the nucleus and presynaptic nerve terminals from *Torpedo* (Lewy, 1913; Maroteaux *et al*., 1988) (page 41). The exactly localization and function of this protein in the nucleus has been a primary focus of study partly due to

the overwhelming information about the accumulation of abnormal α-synuclein in the cytoplasm of neurons in PD, and in neurons and oligodendroglia in multiple system atrophy (MSA). However, α-synuclein is identified in the nucleus in different settings using different methods (Goers *et al*., 2003; Gonçalves and Outeiro, 2013; Ma *et al*., 2014; McLean *et al*., 2000; Nishie *et al*., 2004; Specht *et al*., 2005; Yu *et al*., 2007), and it is especially abundant during development, modulating neurogenesis (Crews *et al*., 2008; Winner *et al*., 2008; Zhong *et al*., 2010). Nuclear α-synuclein levels are increased with oxidative stress *in vitro* and *in vivo* (Monti *et al*., 2010; Xu *et al*., 2006), which seems to facilitate, in turn, oxidative stress (Zhou *et al*., 2013). The mechanism of effect of nuclear α-synuclein is poorly understood, but it is known that α-synuclein binds to histones and inhibits histone acetylation (Goers *et al*., 2003; Kontopoulos *et al*., 2006). In addition, α-synuclein under oxidative stress conditions binds to the promoter of the mitochondrial transcription factor, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), and reduces transcription of mitochondrial genes (Siddiqui *et al*., 2012). Interestingly, increased expression levels of α-synuclein oligomers have been found in the brains of LBDs and related transgenic models (Dalfó *et al*., 2004; Paleologou *et al*., 2009; Sharon *et al*., 2003; Tsika *et al*., 2010).

4.1.3 Reticulum stress response

The unfolded protein response (UPR) designates the cellular response to the accumulation of abnormal proteins in the endoplasmic reticulum (ER). The reaction can also be elicited by other factors such as hypoglycemia, hypoxia, acidosis, calcium, redox reactions, and a variety of natural compounds and drugs (Scönthal, 2012; Oakes and Papa, 2015). Control of protein folding at the ER is modulated by the chaperone glucose-regulated protein 78 (GRP78, also named immunoglobulin binding protein BIP), a member of the HSP70 family which, in non-stressed cells,

binds to three ER transmembrane proteins: PKR-like ER kinase (PERK), inositol requiring kinase 1 (IRE1), and transcription factor 6 (ATF6) (Bernales *et al*., 2012; Lindholm *et al*., 2006; Kohno, 2007; Schröder, 2008; Schröder and Kaufman, 2005) (Figure 14). Glucose-regulated protein 94 is the HSP90-like protein in the lumen of the endoplasmic reticulum, dedicated to protein folding and quality control in the ER (Little *et al*., 1994; Marzec *et al*., 2012). Accumulation of misfolded proteins in the ER activates PERK (Szegezdi *et al*., 2006) and phosphorylates the α-subunit of eukaryotic initiation factor 2 (EIF2-α) at serine 51, resulting in decreased protein synthesis (Donnelly *et al*., 2013). In addition, eIF2α phosphorylation sets off activating transcription factor 4 (ATF4), promoting DNA transcription of specific genes (Ron and Walter, 2007; Sun *et al*., 2013). ER responses also involve the activation of IRE1 by dimerization and phosphorylation that activates the transcription factor Xbox binding protein (XBP1), which in turns activates transcription of stress genes in DNA (Caudle *et al*., 2009; Sidrauski and Walter, 1997; Yoshida *et al*., 2001). Upon ER stress, full activating transcription factor 6 (ATF6) moves to the Golgi complex where it is cleaved to form the active transcription factor (ATF6-50 kDa, ATF6f), which translocates to the nucleus and activates transcription of stress genes (Chen *et al*., 2002). Thus, activation of ATF4, IRE1, and ATF6f increases the production of GRP78, GRP94, PERK, IRE1, XBP1, and ATF6, and stimulates the ER-associated degradation (ERAD) pathway (Braakman and Bulleid *et al*., 2011; Meusser *et al*., 2005), contributing to restoring homeostasis. However, once certain thresholds are passed, ER stress can trigger NF-κβ activation and caspase-mediated apoptosis (Szegezdi *et al*., 2006; Kim *et al*., 2008; Tabas and Ron, 2011; Xu *et al*., 2005).

Interestingly, markers of the unfolded protein response, phosphorylated PERK and phosphorylated eIF2α, have been identified in dopaminergic neurons of the SN containing α-synuclein inclusions at relatively early stages of PD (Ferrer, 2011; Hoozemans *et al*., 2007). Thus, ER stress

has been related with the pathogenesis of NDD, including PD and DLB (Colla *et al*., 2012[b]; Doyle *et al*., 2011; Mercado *et al*., 2013).

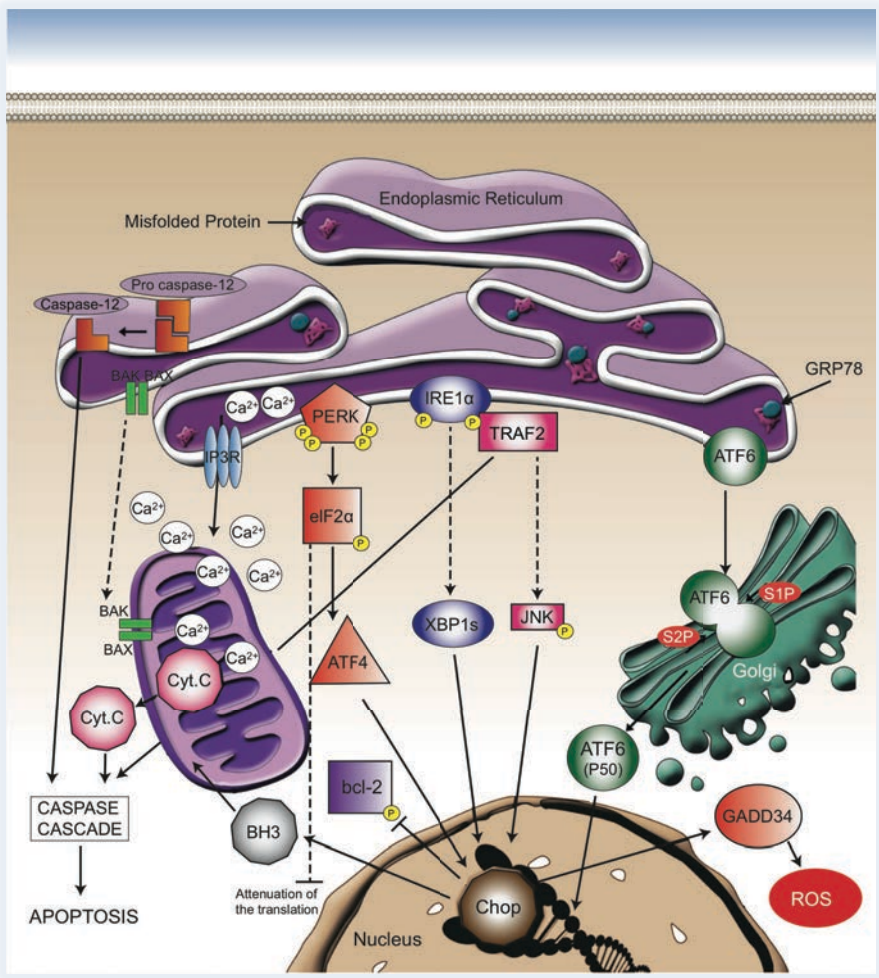


Figure 14. Endoplasmic reticulum (ER) stress-mediated apoptosis. Unresolved ER stress induces apoptotic cell death through different mechanisms. Modified from Plácido *et al*., 2014.

4.2 Mitochondria and energy metabolism system

Mitochondria and respiratory chain

Mitochondria are perhaps the most sophisticated and dynamic responsive sensing system in the cell, and their morphology is constantly modified by fission and fusion events. The function of the mitochondria goes beyond its capacity to create molecular energy and includes the generation of reactive oxygen species (ROS), the regulation of calcium, and the activation of cell death. The respiratory chain (RC) is in charge of oxidative phosphorylation, which comprises four complexes from I to IV present in the inner mitochondrial membrane, and complex V, an ATP synthase that drives the synthesis of ATP and energy for the cell. Electrons (e^-) are donated to complex I (NADH dehydrogenase) from NADH, or to complex II (succinate dehydrogenase) from $FADH_2$. Subsequently, they are moved to coenzyme Q (CoQ or ubiquinone), carrying them to complex III (cytochrome c reductase or cytochrome bc_1 complex), and then the e^- are passed to cytochrome c (Cyt c), which relay them to complex IV (cytochrome c oxidase). In this step, Cyt c transfers e^- from complex III to complex IV, which reduces O_2 in order to generate H_2O. The flow of these e^- is accompanied by proton (H^+) transfer across the membrane at complexes I, III, and IV, generating an electrochemical gradient, and finally by the use of complex V, producing ATP from ADP, and inorganic phosphate (Pi), creating an electrochemical gradient ($\Delta\psi m$) (Figure 15). The optimal balance between biogenesis and mitophagy is crucial to adjust the bioenergetic demand of the cell, and to reduce the mitochondrial damage.

Mitochondrial malfunction

Mitochondrial dysfunction has emerged as one of the main mechanisms underlying the pathogenesis of PD including familial and sporadic PD, but occurs as an early event in many varieties of NDD. The first link between mitochondrial dysfunction and PD was detected a long time ago with the

implication of some inhibitors of the mitochondrial complex I (MPTP) in the appearance of *parkinsonism* (Langston *et al*., 1983). Altered mitochondrial function, mainly characterized by reduced complex I activity and increased oxidative damage, is well documented in the SN in PD where A9 neurons appear to be more vulnerable, although this is not restricted to this area as phosphorous and proton magnetic resonance spectroscopic imaging reveal this in midbrain and putamen as well (Hattingen *et al*., 2009). Thus, oxidative damage and disassembly of complex I subunits, functional impairment of complex I activity, and adaptive responses are found in the frontal cortex as well as other regions at advanced stages of PD, some of them probably having suffered from dementia (Keeney *et al*., 2006; Parker *et al*., 2008). Mitochondrial damage produces oxidative stress, as well as energy metabolism defects and neuronal exhaustion, suggesting a causative involvement in the pathogenesis of Parkinson's disease (Ferrer *et al*., 2009). Interestingly, some toxin-induced models of *parkinsonism* are based on the use of different mitochondrial inhibitors to generate PD-related phenotypes, including MPTP, rotenone, and paraquat (Valadas *et al*., 2015).

In addition, increased expression levels of oxidative markers as a result of oxidation including α-synuclein, β-synuclein, UCHL1, SOD1, SOD2 and DJ-1 (Choi *et al*., 2004, 2005, 2006; Dalfó and Ferrer, 2008), and altered oxidative responses, occur in the frontal cortex of PD (Alam *et al*., 1997; Power and Blumbergs, 2009; Mythri *et al*., 2011; Harish *et al*., 2013). Even though the role of the interaction between α-synuclein and mitochondria is still not well known, in normal conditions α-synuclein probably has a physiological role due to the evidence that demonstrates mitochondrial stability in the electronic transport chain (ETC) by the specific binding of α-synuclein to mitochondria, and their antioxidant capacity (Zhu *et al*., 2006). Finally, SN, hippocampus, and striatum have been shown to contain important mitochondrial α-synuclein content in PD

patients compared to control individuals, suggesting that mitochondrial accumulation of α-synuclein in PD contributes to mitochondrial defects in the disease (Devi *et al*., 2008).

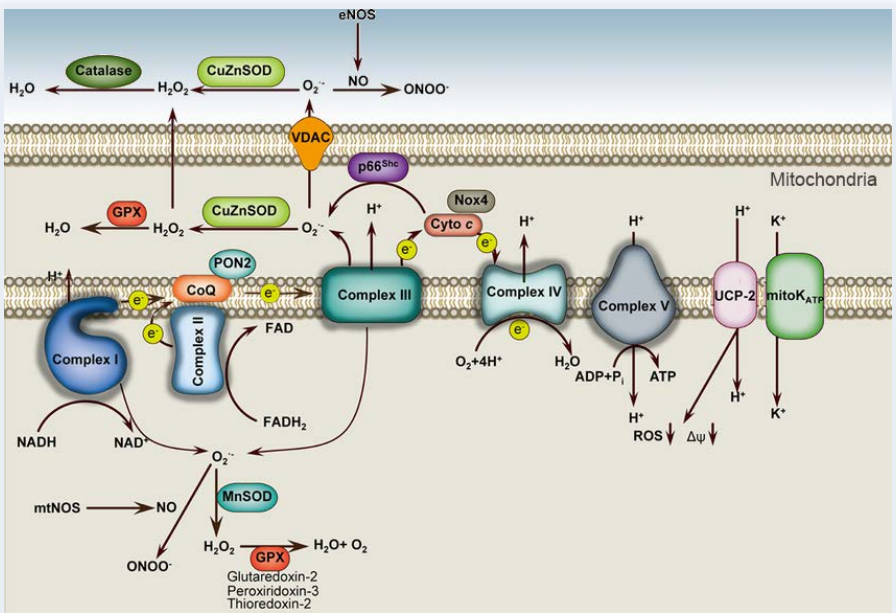


Figure 15. Mitochondrial ROS regulation. Respiratory chain complexes I–IV generate the proton gradient over the mitochondrial inner membrane that drives ATP generation by ATP synthase (complex V). Modified from Tang *et al*., 2014.

4.3 Neuroinflammation and mediators of the immune response

The central nervous system (CNS) is a complex organ composed of neurons, which represent 10% of the total number of CNS cells, and glia, representing 90% of the total cell number. At the same time, glia can be divided into macroglia, with oligodendrocytes and astrocytes of neuroectodermal origin, and microglia, which are of mesenchymal origin. The special function of all glia is to maintain the optimal operation of the CNS involving the active regulation of network operations, ongoing maintenance, and active defence and repair following injury or pathogen attack (Carson et al., 2004). All of them, neurons, macroglia and microglia, participate in these processes in a coordinated form, and interact with CNS-infiltrating immune cells as part of their regulation of inflammatory responses in the CNS. After a CNS injury, glial cells show phenotypic changes referred to as reactive gliosis, one of the most important features in neuroinflammation.

Microglia

Microglia are considered to be the resident macrophages of the CNS and were first described by Del Rio-Hortega (1932), who used silver impregnation techniques to visualize non-neuronal cells. They are located throughout the brain and spinal cord, express the most common macrophage markers and have a stellate morphology in the healthy brain. Microglial cells express monocytic cellular markers such as the αMβ2 integrin (or CD11b/CD18 and MAC-1), IgG receptors (CD16/CD32), ionized calcium-binding adaptor protein-1 (Iba-1), and the major histocompatibility complex (MHC).

Microglia provide the first line of defence in response to pathogens and neuronal injury producing a wide variety of cytokines, chemokines, proteases, reactive oxygen species, and regulating the T cell infiltration. Microglia are known to recognize pathogens using evolutionarily conserved pathogen recognition receptors such as Toll-like receptors

(TLRs). When microglia are activated they acquire an amoeboid morphology and modify their gene expression leading to the production of neurotoxic mediators. However, in chronic neuroinflammation, microglia remain activated during a long period producing more mediators than usual and contributing to neuronal death.

Astrocytes

Astrocytes are the most abundant cellular type in the CNS and play important roles in the function of the healthy CNS, including regulation of blood flow, provision of energy metabolites to neurons, synaptic function and plasticity, and maintenance of extracellular balance of ions and transmitters, among other activities. Astrocytes are also primary responding cells to injury and disease and can acquire characteristics of effector immune cells. Reactive astrocytes become hypertrophic, show increased expression of intermediate filament proteins such as vimentin, nestin, and, in particular, glial fibrillary acidic protein (GFAP), and exhibit potential for resolution if the triggering insult is removed or resolves. In addition, the signalling molecules of astrogliosis that can be released by neurons, microglia, oligodendrocytes, pericytes, endothelial cells, and other astrocytes include ROS, growth factors, cytokines such as IL-6, TNF-α, and interferon gamma (IFN-γ), neurotransmitters (glutamate and noradrenaline), and purines such as ATP.

Cytokines

Cytokines are produced by a broad range of cells including macrophages, lymphocytes and mast cells, among others. The production of the cytokines interleukin IL-1α, IL-1β, and tumour necrosis factor α (TNF-α) in the CNS results in a neuroinflammatory response and neuronal degradation.

Chemokines

Chemokines are a family of small cytokines, classified into CXC, CC, CX3C and XC subfamilies, that are proinflammatory and contribute to the neuroinflammation process. They induce chemotaxis near responsive cells, so they are chemotactic cytokines.

Neuroinflammation in central nervous system (CNS)

It is well known that CNS inflammation and immune activation play a crucial role in the pathophysiology of NDD (Frank-Cannon *et al*., 2009). Recent studies have identified the inflammatory process as being closely linked with multiple neurodegenerative pathways, related with depression, a consequence of NDD. Accordingly, pro-inflammatory cytokines are relevant in the pathophysiology of depression and dementia (reviewed in Chen *et al*., 2016), suggesting that the role of neuroinflammation in neurodegeneration needs to be fully elucidated. This aforementioned neuroinflammation can be a consequence of neurodegeneration; however, some studies have reported that inflammation could also be involved in the neuronal death process (Hirsch and Hunot, 2009). The CNS is an immune-privileged organ with innate and acquired immune responses, closely regulated in relation with the periphery. Immune activation in the CNS involves microglia and astrocytes, which constitute the resident immune cells, and play an important role in the homeostasis regulation of the brain (Perry and Teeling, 2013). Microglia constantly survey the microenvironment by producing factors that influence astrocytes and neurons, especially in response to pathogen invasion and tissue damage, promoting an inflammatory response (Sofroniew and Vinters, 2010; Wyss-Coray and Mucke, 2002). Inflammation in tissue pathology indicates the persistence of inflammatory stimuli or failure of normal resolution mechanisms (Lull and Block, 2010; Das Sarma, 2014). Specific inducers of inflammation converge in mechanisms responsible for sensing, transducing, and amplifying the inflammatory processes, resulting in the production of

neurotoxic mediators such as cytokines and interleukins (Glass *et al*., 2010; Teeling and Perry, 2009) (Figure 16).

Neuroinflammation in Parkinson's disease, Dementia with Lewy bodies and rapidly progressive Dementia with Lewy bodies

Inflammation comprising neuroinflammation and peripheral inflammatory responses with microglia and astrocyte activation are well documented in sporadic PD with *post-mortem*, epidemiological and imaging studies from patients (Ghosh *et al*., 2007; Hirsch *et al*., 1998, 1999, 2003, 2012; Long-Smith *et al*., 2009; McGeer *et al*., 1988, 2002, 2 004; Nagatsu *et al*., 2000; Sawada *et al*., 2006; Tansey and Goldberg, 2010; Teismann *et al*., 2003; Tufekci *et al*., 2012; Whitton, 2007; Cappellano *et al*., 2013). Environmental factors known to promote neuroinflammatory response have been related to idiopathic PD. Neuroinflammation can be triggered by immunological challenges (viral/bacterial infections), neuronal injury (brain trauma, stroke), epigenetic factors (arthritis, multiple sclerosis), and environmental toxins (MPTP, paraquat, rotenone, and metals).

Microglia are activated mainly in the SN and striatum in PD, the most affected areas in this disease, but also in other brain regions including frontal, transentorhinal, cingulate, and temporal cortices, hippocampus, and pons, where neuronal loss is also important (Long-Smith *et al*., 2009; Witte *et al*., 2010; Politis *et al*., 2012). Consequently, inflammatory responses involve not only the SN, but also the putamen and other regions, such as the hippocampus (Imamura *et al*., 2003, 2005; Mogi *et al*., 1994, 1996; Blum-Degen *et al*., 1995; Hunot *et al*., 1997; Nagatsu *et al*., 2005, 2007; Duke *et al*., 2007). These results have been detected *in vivo* using positron emission tomography (PET) with specific ligands, in the CSF, and by histological study in *post-mortem* PD brains (McGeer *et al*., 1988, 2004; Sawada *et al*., 2006; Imamura *et al*., 2003, 2005; Ouchi *et al*., 2005, 2009; Gerhard *et al*., 2006; Brooks, 2010; Choe *et al*., 2013). In addition, limited activation of microglia has been observed in DLB

(Streit and Xue, 2015), whereas other studies have reported increased cytokine expression in glial cells, mostly microglia, in several regions of the brain in DLB (Katsuse *et al*., 2003[a]).

Microglia can be activated by molecules released by dopaminergic neurons such as α-synuclein aggregates, ATP, matrix metalloproteinase-3 (MMP-3), and neuromelanin, as well as by lipopolysaccharides (LPS) (Long-Smith *et al*., 2009). Under appropriate inflammatory conditions, microglia may produce a wide range of pro- and anti-inflammatory cytokines, cytokine receptors, and other immune-related molecules (Kim and Joh, 2006).

Moreover, several studies support the hypothesis that activation of the nuclear factor kappa-B (NF-κβ) plays a relevant role in PD pathogenesis. Nuclear translocation of NF-κβ (p65), an indicator of NF-κβ activation, has been described in the SN in PD (Hunot *et al*., 1997). When NF-κβ is activated, it enters the nucleus and stimulates the gene transcription of several pro-inflammatory factors such as inducible nitric oxide synthase (iNOS), interleukin (IL)-1β, IL-6, cyclooxygenase 2 (COX-2) and tumour necrosis factor-α (TNF-α) with the microglia (Wilms *et al*., 2003; Zhang *et al*., 2010; Bassani *et al*., 2015). Because of this, IL-1β, IL-6, IL-2, colony stimulating factor (CSF), and TNF-α are expressed in higher levels in PD *post-mortem* brains especially in the striatum, in serum, and in CSF, compared to age-matched controls as revealed by enzyme-linked immunosorbent assay (ELISA) (Blum-Degen *et al*., 1995; Mogi *et al*., 1994, 1996, Nagatsu *et al*., 2000; Nagatsu and Sawada, 2005; Long-Smith *et al*., 2009). Furthermore, double immune-fluorescence staining has revealed the production of TNF-α and IL-6 in activated microglia in the putamen of PD patients (Nagatsu and Sawada, 2007), while TNF-α expression levels in blood correlate with altered cognition, depression, and sleep disturbances in PD (Menza *et al*., 2010).

In addition, variable increased levels in serum and peripheral blood mononuclear cells in PD of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, NT-pro-2',3'-cyclic nucleotide 3' phosphodiesterase (NT-proCNP), TNF-α, soluble tumour necrosis factor α receptor-1 (TNFR1), and chemokine (C-C motif) ligand 5 (RANTES) indicate the existence of a systemic inflammation manifested as a peripheral dysregulation of cytokines and related molecules in PD (Bessler *et al*., 1999; Brodacki *et al*., 2008; Chen *et al*., 2008; Dufek *et al*., 2009: Reale *et al*., 2009; Rentzos *et al*., 2007, 2009; Stevens *et al*., 2012).

Interestingly, COX-2 aggravates the degenerative process through proinflammatory mechanisms and generation of ROS.
In relation to mRNA, increased levels of *IL-6* have been reported in the hippocampus (Imamura *et al*., 2005), and an increased expression of several genes encoding cytokines in the SN has also been shown by a microarray study (Duke *et al*., 2007). All these observations point to the activation of innate CNS immune response in PD and DLB.

In spite of this volume of information in humans and additional data provided by several experimental models *in vivo* and *in vitro* indicating the role of inflammation in PD and DLB, not much is known about the regional variability in inflammatory responses in PD, DLB and rpDLB.

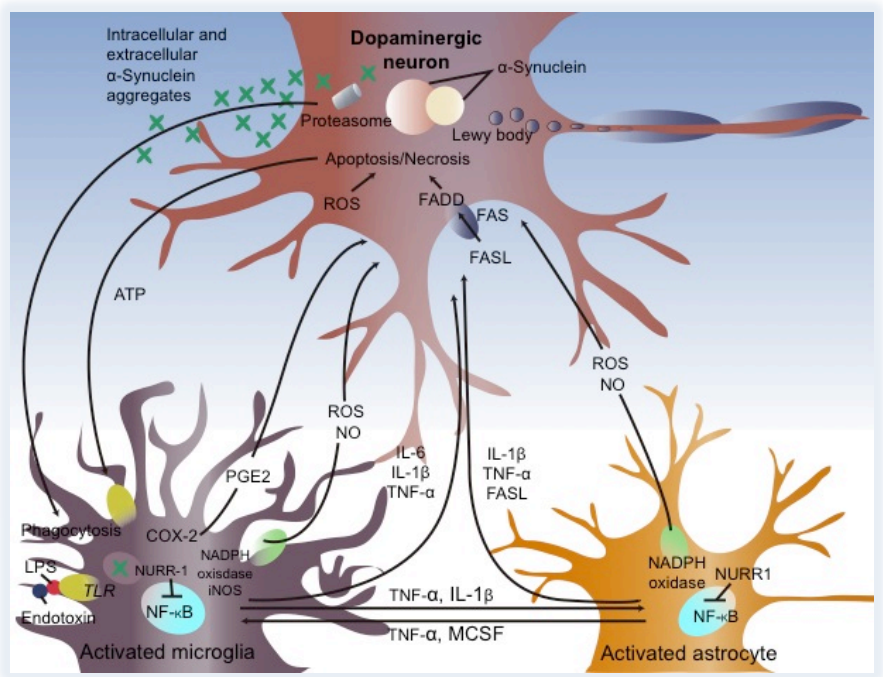


Figure 16. Neuroinflammation in PD. Prominent neuropathological hallmarks of Parkinson's disease (PD) are the loss of dopaminergic neurons in the substantia nigra of the midbrain and the presence of intracellular inclusions containing aggregates of the α-synuclein protein, called Lewy bodies. Besides forming Lewy bodies, aggregates of α-synuclein form intermediate-state oligomers that when released from neurons activate microglia through Toll-like receptor (TLR)-independent mechanisms. This leads to activation of NF-κβ and production of reactive oxygen species (ROS) and proinflammatory mediators. These factors act directly on DA neurons of the SN. These factors also activate microglia, which amplify the inflammatory response in a positive feedback loop, leading to further activation of microglia. Products derived from microglia and astrocytes act in a combinatorial manner to promote neurotoxicity. Bacterial lipopolysaccharide (LPS), acting primarily through TLR4 expressed by microglia, is sufficient to induce an inflammatory response in the substantia nigra that results in loss of dopaminergic neurons. The transcription factor NURR1 acts to suppress inflammatory responses in microglia and astrocytes by inhibiting NF-κB target genes.Modified from Glass *et al*., 2010.

4.3 Purine metabolism

Purines

Purines are small heterocyclic double-ring aromatic organic molecules essential for the cell that play critical roles in neuronal differentiation and function (Figure 17). Their importance is highlighted by several inherited disorders of purine metabolism such as Lesch-Nyhan disease, in which a defective gene causes gaps to appear in their recycling process.

Purine composition

Basically, purine adenine and guanosine nucleobases, as well as one-ring primary pyrimidine nucleobases cytosine, thymidine, and uracil, are the core of DNA, RNA, nucleosides and nucleotides. Adenosine and guanosine are purine ribonucleosides resulting from the β-N9-glycosidic bound to adenine or guanine and ribose, respectively. Nucleotides result from the incorporation of phosphate groups in nucleosides. Thus, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), and cyclic forms cAMP and cGMP are primary purine-derived nucleotides.

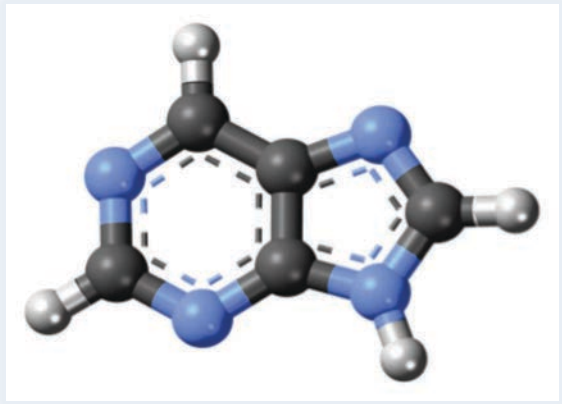


Figure 17. Purine structure. Ball-and stick model of the purine molecule, a nitrogen heterocycle. Many different purine derivatives occur in nature.Colour code: Carbon, C: black Hydrogen, H: white Nitrogen, N: blue. This chemical image is online created with *Discovery Studio Visualizer*.

Purine and nucleotide function

Aside from the relevant roles of purines in the DNA and the RNA, they are also significant components in a number of important biomolecules including ATP, GTP, cAMP, NADH, and coenzyme A as explained above. Its nucleotides participate in an extensive variety of crucial metabolic pathways including energy metabolism and cell signalling, acting as intra- and intercellular signalling messengers. In addition, purine bases are integrated into other molecules to generate not only cofactors of several enzymatic reactions such as coenzyme A, Flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD+), and nicotinamide adenine dinucleotide phosphate ($NADP^+$), but also the complementary reduced forms $FADH_2$, NADH, and NADPH. S-Adenosyl methionine is made from ATP and methionine by methionine adenosyltransferase, and is involved in the transfer of methyl groups to distinct substrates including nucleic acids, proteins, lipids, and metabolites. In addition to intracellular signalling, purines and their products may function as extracellular signals, acting upon other cells, either between neurons or between neurons and glial cells equipped with appropriate receptors (Boison, 2008; Ipata *et al*., 2011; Ansoleaga *et al*., 2015) (Figure 18). Interestingly, adenosine is responsible for regulating, integrating and fine-tuning neuronal activity, and influencing relevant brain functions, including sleep and arousal, cognition and memory, neuronal damage, and degeneration. Adenosine acts as an extracellular molecular via specific adenosine receptors (Rahman, 2009) and modulates permeability of the blood barrier (Carman *et al*., 2011). It exerts its function by binding adenosine receptors, which are G-protein-coupled receptors (A1, A2A, A2B and A3), that can inhibit (A1, A3) or enhance (A2) neuronal communication through neurotransmitter release (Ribeiro *et al*., 2003; Burnstock *et al*., 2011). A2A receptors are abundant in striatopallidal neurons where they bind to dopamine receptors (DA) and reduce their affinity for DA (Fuxe *et al*., 2007). Striatal A2A receptor levels are increased in PD (Calon *et al*., 2004; Mishina *et al*., 2011;

Ramlackhansingh *et al*., 2011), even at early stages of the disease, possibly regulated, in part, by miR-34b (Villar-Menéndez *et al*., 2014). Therefore, A2A receptor agonists are potential therapeutic agents to increase DA uptake in the PD striatum, thereby improving dopamine availability (Jenner *et al*., 2009; Vallano *et al*., 2011; Szabó *et al*., 2011). Regarding purine neurocrine factors, adenosine receptors have attracted interest because of their neuroprotective role in several neurologic disorders (Boison, 2008; Ribeiro *et al*., 2003).

Purine metabolism

Under physiological conditions, nucleotides can undergo chemical modification of the purine bases, especially by deamination. (Sancar and Sancar, 1988; Nguyen *et al*., 1992; Davies *et al*., 2012). Deaminated nucleotides may accumulate in nucleotide pools or become incorporated into DNA and RNA, thus leading to alteration of nucleic acid structure and genetic information (Davies *et al*., 2012). For this reason, organisms possess a huge number of mechanisms to maintain viability, including (deoxy) nucleotide phosphohydrolases, which hydrolyse these nucleotides. However, energy metabolism dependent on mitochondrial function is markedly altered in PD (Schapira, 2008; Navarro and Boveris 2009; Hattingen *et al*., 2009; Navarro *et al*., 2009), and oxidative damage of energy-related enzymes hampers physiological energy balance (Poon *et al*., 2005; Keeney *et al*., 2006; Martínez *et al*., 2010); oxidative damage to nucleic acids is found in the PD brain (Nakabeppu *et al*., 2007; Sanders and Greenamyre, 2013).

Despite the known importance of purines in the nervous system, knowledge regarding their metabolism in neurons is limited. Moreover, little is known about possible alterations in the expression of genes encoding enzymes involved in purine metabolism in PD brains. This information would permit better understanding of the primary regulation of purine-related genes and their possible implications in the pathogenesis

of the disease. Previous reports in AD showed a deregulated cluster of genes involved in purine metabolism, as well as abnormal regulation of several genes with disease progression and with particular regional patterns (Ansoleaga *et al*., 2015).

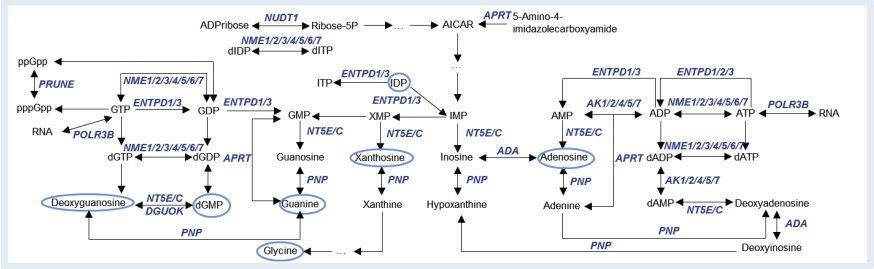


Figure 18. Schematic purine metabolism pathway including all the nucleotides with their main pathways treated in the present thesis. All the analysed genes are remarked in *italics* and blue. Modified from KEGG pathway Homo sapiens *00230* and Ansoleaga *et al*., 2015.

4.5 New signalling pathways: Olfactory and taste receptors and obligate mediators

4.5.1 Olfactory receptors and obligate mediators

Olfactory receptors and obligate components

Chemical signals, including odorant and pheromone signalling, are the most conventional and precise form of communication in both unicellular organisms and complex individuals, allowing an important interaction with the environment. Vital function information about the availability of food, mating, reproduction, orientation, new-born imprinting, and territorial making are some of the crucial information in natural settings, enabling chemical communication among species and individuals of the same or other species (Hanson, 1999; Ryan, 2002; Blomquist and Vogt, 2003; Wyatt, 2003; Brewer *et al*., 2006; Ihara *et al*., 2013). Odorant and pheromone receptors, which occupy a major part of the genome from insects to mammals, are specialized receptors designed to respond to volatile molecules by activating down-stream pathways. Odorant or olfactory receptors (ORs) are located in sensory organs such as the olfactory epithelium in the nasal cavity in mammals, where the olfaction initiates. However, recent evidence has shown that these receptors and down-stream related molecules are also localized in different organs and systems in mammals, designed as *ectopic* olfactory receptors. Germinal cells, testes, embryos, kidney, spleen, lung, and heart, among others, are different organs where ORs have been reported (Parmentier *et al*., 1992; Vanderhaeghen *et al*., 1997; Branscomb *et al*., 2000; Feldmesser *et al*., 2006; Zhang *et al*., 2007; De la Cruz *et al*., 2009). Furthermore, the concomitant occurrence of olfactory protein ($G_{\alpha olf}$) and adenylyl cyclase type III (AC3) may ascribe a function to ORs in mammalian cerebral cortex that has not been documented. General anosmia with altered recognition of several odorants is observed in mice null for the expression of $G_{\alpha olf}$ and AC3, thus indicating that both are

obligatory components of olfactory function in sensory olfactory neurons (Belluscio *et al*., 1998; Wong *et al*., 2000).

The aforementioned ORs have been identified as members of the G-protein-coupled receptors (GPCRs) family, which share characteristic conserved in trans-membrane motifs (Young *et al*., 2003). Approximately 350 different functional ORs are expressed in humans (Niimura and Nei, 2003, 2005; Zhang *et al*., 2007; Malnic *et al*., 2010). The genes encoding ORs are distributed along the genome, with the exception of chromosomes 18 and Y (Malnic *et al*., 2004; Glusman *et al*., 2001; Zozulya *et al*., 2001; Niimura and Nei, 2005). Thus, the odorant activation leads to the dissociation of the olfactory G-protein Gαolf into three subunits: α, β, and γ, inducing the formation of the functional heterotrimeric G protein: Gαolf, Gβ1, and Gγ13 (Von Dannecker *et al*., 2005, 2006; Kerr *et al*., 2008). The activation of the Gαolf and the AC3 induces an increase in the intracellular concentration of cyclic adenosine monophosphate (cAMP), activates cAMP-gated channels, and produce neuron depolarization. The consequent repolarization takes place through the plasma membrane by extruding intracellular Ca^{2+} with the action of a non-potassium-dependent sodium/calcium exchanger modulated by the olfactory marker protein (OMP), a potassium-dependent sodium/calcium exchanger, and a plasma membrane Ca^{2+}-ATPase (Pace *et al*., 1985; Sklar *et al*., 1986; Jones and Reed, 1989; Lowe *et al*., 1989; Firestein *et al*., 1991; Pyrski *et al*., 2007; Kwon *et al*., 2009; Billig *et al*., 2011; Stephan *et al*., 2011).

Furthermore, olfactory-binding proteins (OBPs), characterized as small soluble extracellular proteins, trap and convey odorants and pheromones to specific receptors (Figure 19) (Vogt and Riddiford, 1981; Vogt *et al*., 1985, 1991; Pevsner *et al*., 1986, 1988, 1990).

Other related molecules

Different receptors including receptor transporter proteins 1 and 2 (RTP1 and RTP2), as well as receptor expression enhancing protein 1 (REEP1), allow the proper functioning of ORs by the recruitment of ORs to lipid rafts (LR). UDP-glucuronosyltransferases (UGTs), located in the nuclear membrane and in the ER, facilitate the excretion of glucuronidate xenobiotic and endobiotic compounds and contribute to the detoxification of cells (Saito *et al*., 2004) (Figure 19).

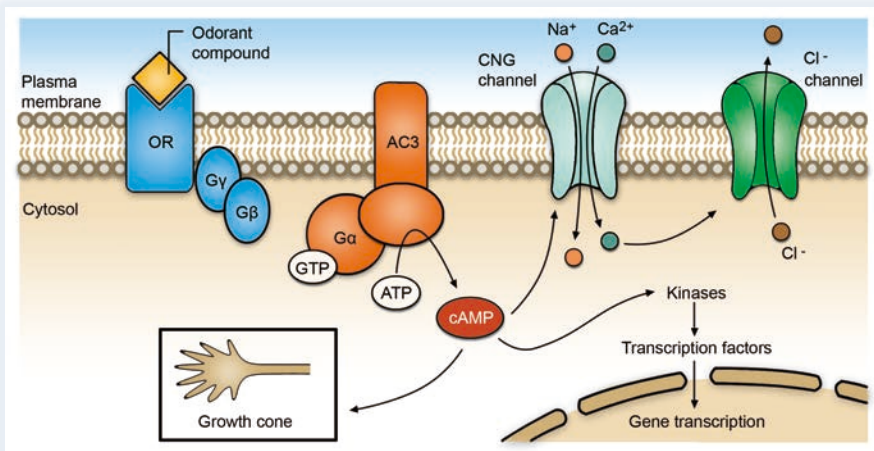


Figure 19. Odorant transduction pathway. Binding of odorant compounds to an OR initiates a transduction cascade involving a G protein and activation of AC3, which in turn produces the second messenger cyclic AMP. cAMP binds to a cyclic nucleotide gated (CNG) channel and results in the influx of cations (Na^+, Ca^{2+}), which depolarize the cell membrane. Olfactory sensory neurons (OSNs) maintain a high intracellular Cl^- concentration, such that this channel supports an efflux of negatively charged Cl^-, producing a further depolarization of the cell membrane. The elevated levels of cAMP in the soma have a crucial role in regulating the phosphorylation of proteins and the transcription of genes important for growth and survival of the axons of OSNs. Modified from Zou *et al*., 2009.

ORs and obligate mediators function

Whether OR and mediators expression occurs in humans is still not known, but increasing interest has recently shed light on these putative non-odorant-related functions of ORs in various non-olfactory regions. Although these ORs in non-sensory tissues and organs are not involved in olfaction, they carry out diverse local functions without connection to

the brain (reviewed in Ferrer *et al*., 2016). As in other non-sensory organs and regions, the function of ORs in brain is not related to odour detection, and differs from the specific perception of molecules by ORs in the nasal cavity. Besides, the perception of odours and their recognition in humans is not the only function of ORs.

Activation of certain ORs switches on complex cellular responses mediated by neuronal networks, neurotransmitters, and hormones which in turn determine sophisticated behaviours. Therefore, ORs located in non-olfactory tissue and organs are able to trigger complex response receptors but not precisely linked to any sensorial perception. Recent findings lend weight to the assumption that they may support novel physiological functions (Grison *et al*., 2014; Ferrer *et al*., 2016).

4.5.2 Taste receptors

Taste receptors

Taste discrimination has facilitated the development of an innate priority for energy-providing nutrients and rejection of injurious substances during the course of evolution. Taste detection occurs at the taste buds of the oral cavity, where the taste receptors (TR), mainly located on the tongue, mediate this perception. Each taste bud contains from 50 to 100 sensory cells with capacity to react and transduce one of the five categories of taste: bitter, sour, sweet, salty, and umami (Iwatsuki and Uneyama, 2012). The most widely studied gustatory signalling events are those related with the transduction of bitter compounds. Bitter taste detection initiates through bitter tastant binding to a TR in the gustatory cells. The bitter TRs involved belong to the taste 2 receptors family (TAS2R), coupled to a gustatory-specific G-protein, α-gustducin (Iwatsuki and Uneyama, 2012). The activation of α-gustducin triggers an intracellular cascade, implicating phospholipase C-β 2 (PLCB2) and inositol 1,4,5-triphosphate receptor type 3 (ITPR3), which consequently opens transient receptor potential channels generating an Na^+ influx, and

resulting in cell depolarization (Ishimaru and Matsunami, 2009; Iwatsuki and Uneyama, 2012). Ultimately, changes in the membrane potential promote the release of ATP toward the first neuron of the gustatory nerve (Iwatsuki and Uneyama, 2012) (Figure 20). Curiously, mice null for α-gustducin present an abnormal detection of sweet and bitter tastes, suggesting the important role of these mediators in the taste transduction (Wong *et al.*, 1996).

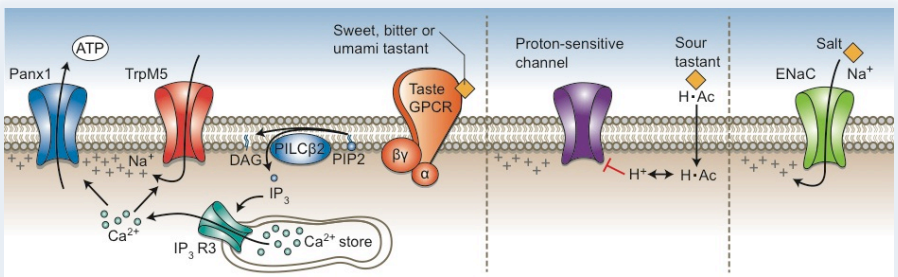


Figure 20. Taste transduction pathway. Mechanisms by which five taste qualities are transduced in taste cells. For sweet, bitter or umami tastes, ligands bind taste GPCRs and activate a phosphoinositide pathway that elevates cytoplasmatic Ca^{2+} and depolarizes the membrane via cation channel and resulting in ATP release. For sour taste, organic acids (HAc) permeate through the plasma membrane and acidify the cytoplasm where they dissociate to acidify the cytosol. Intracellular H^+ is believed to block a proton-sensitive K channel and depolarize the membrane. Finally, the salty taste of Na^+ is detected by direct permeation of Na^+ ions though membrane ion channels, including ENaC, to depolarize the membrane. Adapted from Chaudhari and Roper, 2010.

TR and down-stream mediators function

As for ORs, *ectopic* TASRs are present in several organs and systems as well as the tongue (Behrens and Meyerhof, 2010). Respiratory tract, digestive system, pancreas, liver, kidney, and testes are some of the tissues in which the TASRs are expressed in (Yamamoto and Ishimaru, 2013; Xu *et al.*, 2013). Bitter TASRs together with down-stream functional mediators are also expressed in the brain of mice and rats (Singh *et al.*, 2011; Dehkordi *et al.*, 2012). In contrast to TASRs presents in the mouth, which communicate with the brain, TASRs in the digestive system are probably not connected to the brain, but rather detect local soluble substances and respond by ordering cells to release hormones and other

molecules. A total of 25 genes encoding bitter taste in human have been identified (Behrens *et al*., 2007). It has been demonstrated that these receptors play an important role in the control of food intake (Van Avesaat *et al*., 2015), and their expression seems to be partially modulated by diet (Vegezzi *et al*., 2014).

OBJECTIVES

General objective

To identify molecular alterations underlying functional cerebral changes and anatomical modifications in *post-mortem* human brain samples of Parkinson's disease (PD), Dementia with Lewy Bodies (DLB), and rapidly progressive Dementia with Lewy bodies (rpDLB).

Specific objectives

Objective 1. To identify alterations in concatenated pathways commanding protein synthesis and related molecules from the nucleolus to the ribosome in middle-aged control cases and Parkinson's disease individuals in different regions and stages of the disease.

- To analyse putative alterations of protein synthesis machinery at the mRNA and protein levels in regions with variable vulnerability in PD, including the substantia nigra, the frontal cortex area 8, the angular gyrus, the precuneus, and the putamen, at Braak stages 3-4 and 5-6 of the disease.
- To identify whether alterations are associated with α-synuclein oligomers.

Objective 2. To characterize mitochondria and energy metabolism involvement in the cerebral cortex and their implication in the metabolic process in different stages of Parkinson's disease compared to middle-aged individuals.

- To analyse putative mitochondrial and energy metabolism alterations by using mRNA levels and protein expression.
- To explore regional differences by studying the frontal cortex area 8 and the angular gyrus and to investigate whether these alterations occur with disease progression by studying three stages comprising incidental PD (iPD; Braak stages 3-4), PD (Braak stages 5-6), and PD with dementia (PDD).
- To verify mitochondrial alteration by assessing enzymatic activity of the five enzymatic complexes of the electron transport chain.

Objective 3. To evaluate and compare the vulnerability of the neuroinflammation and immune response in Parkinson's disease *post-mortem* human brain cases in comparison to middle-aged control individuals.

- To analyse putative neuroinflammatory altered response by mRNA and protein levels in different brain areas including the substantia nigra, frontal cortex area 8, angular gyrus, and putamen at progressive stages of the disease.
- To detect implications of α-synuclein extracellular aggregates in the inflammatory response.

Objective 4. To identify the involvement and the regional variability of the purine metabolic pathway in Parkinson's disease cases compared to control samples.

- To explore whether these alterations are region- and stage-dependent by studying three different regions, comprising the substantia nigra, the putamen, and frontal cortex area 8, at different stages of PD-related pathology (stages 3-4 and 5-6).
- To analyse primary regulation of purine-related genes and their implication in the pathogenesis of the disease by mRNA levels.
- To localise the cellular expression of altered genes at the protein level using immunohistochemistry.

Objective 5. To detect, identify, and map a new signalling brain pathway of cortical olfactory and taste receptors, obligate mediators, and specific transporters of these receptors and evaluate putative modifications in PD.

- To identify gene expression of olfactory and bitter taste receptors in frontal cortex area 8 in Parkinson's disease individuals.
- To characterize obligate downstream components of OR function and receptor transporter proteins.
- To localize these receptors and their obligate mediators in the human brain in different regions and stages of the pathology.

Objective 6. To identify biochemical abnormalities underlying the pathogenesis of Dementia with Lewy bodies and rapidly progressive Dementia with Lewy bodies in the cerebral cortex.

- To analyse putative alterations in the frontal cortex of protein synthesis machinery, mitochondrial function, energy and purine metabolism, neuroinflammation, and expression of recently identified ectopic olfactory and taste receptors in DLB and rpDLB *post-mortem* human brain samples compared to control individuals by studying mRNA and protein levels.
- To detect protein levels of α-synuclein aggregates, β-amyloid bound to membranes, β-amyloid 1-40, 1-42, and soluble tau oligomers in middle-aged control cases, DLB, and rpDLB.
- To discriminate by means biochemical alterations between DLB and rpDLB.

RESULTS

The present thesis is the result of the following publications:

Altered machinery of protein synthesis is region- and stage-dependent and is associated with α-synuclein oligomers in Parkinson's disease. Garcia-Esparcia P, Hernández-Ortega K, Koneti A, Gil L, Delgado-Morales R, Castaño E, Carmona M, Ferrer I. Acta Neuropathol Commun. 2015 Dec 1; 3(1):76.

Mitochondrial activity in the frontal cortex and angular gyrus in Parkinson's disease and Parkinson's disease with dementia. Garcia-Esparcia P, Koneti A, Rodríguez-Oroz MC, Schlutter A, Pujol A, del Rio JA, Ferrer I. *Submitted Brain Pathology.*

Complex deregulation and expression of cytokines and mediators of the immune response in Parkinson's disease brain is region dependent. Garcia-Esparcia P, Llorens F, Carmona M, Ferrer I. Brain Pathol. 2014 Nov; 24(6):584-98.

Purine metabolism gene deregulation in Parkinson's disease. Garcia-Esparcia P, Hernández-Ortega K, Ansoleaga B, Carmona M, Ferrer I. Neuropathol Appl Neurobiol. 2015 Dec; 41(7):926-40.

Functional genomics reveals dysregulation of cortical olfactory receptors in Parkinson disease: novel putative chemoreceptors in the human brain. Garcia-Esparcia P, Schlüter A, Carmona M, Moreno J, Ansoleaga B, Torrejón-Escribano B, Gustincich S, Pujol A, Ferrer I. J Neuropathol Exp Neurol. 2013 Jun; 72(6):524-39.

Dementia with Lewy bodies: molecular pathology in the frontal cortex in typical and rapid forms. Garcia-Esparcia P, López-Gonzalez I, Grau-Rivera Oriol, García-Garrido MF, Koneti A, Llorens F, Zafar S, Carmona M, Del Río JA, Zerr I, Gelpi E, Ferrer I. *Submitted Frontiers in Neurology*.

ARTICLE 1

Altered machinery of protein synthesis is region- and stage-dependent and is associated with α-synuclein oligomers in Parkinson's disease.

Garcia-Esparcia P, Hernández-Ortega K, Koneti A, Gil L, Delgado-Morales R, Castaño E, Carmona M, Ferrer I.

Acta Neuropathologica Communications 2015 Dec 1; 3(1):76.

ABSTRACT

Introduction: Parkinson's disease (PD) is characterized by the accumulation of abnormal α-synuclein in selected regions of the brain following a gradient of severity with disease progression. Whether this is accompanied by globally altered protein synthesis is poorly documented. **Methods:** The present study was carried out in PD stages 1-6 of Braak and middle-aged (MA) individuals without alterations in brain in the substantia nigra, frontal cortex area 8, angular gyrus, precuneus and putamen. **Results:** Reduced mRNA expression of nucleolar proteins nucleolin (NCL), nucleophosmin (NPM1), nucleoplasmin 3 (NPM3) and upstream binding transcription factor (UBF), decreased NPM1 but not NPM3 nucleolar protein immunostaining in remaining neurons; diminished 18S rRNA, 28S rRNA; reduced expression of several mRNAs encoding ribosomal protein (RP) subunits; and altered protein levels of initiation factor eIF3 and elongation factor eEF2 of protein synthesis was found in the substantia nigra in PD along with disease progression. Although many of these changes can be related to neuron loss in the substantia nigra, selective alteration of certain factors indicates variable degree of vulnerability of mRNAs, rRNAs and proteins in degenerating sustantia nigra. NPM1 mRNA and 18S rRNA was increased in the frontal cortex area 8 at stage 5-6; modifications were less marked and region-dependent in the angular gyrus and precuneus. Several RPs were abnormally regulated in the frontal cortex area 8 and precuneus, but only one RP in the angular gyrus, in PD. Altered levels of eIF3 and eIF1, and decrease eEF1A and eEF2 protein levels were observed in the frontal cortex in PD. No modifications were found in the putamen at any time of the study except transient modifications in 28S rRNA and only one RP mRNA at stages 5-6. These observations further indicate marked region-dependent and stage-dependent alterations in the cerebral cortex in PD. Altered solubility and α-synuclein oligomer formation, assessed in total homogenate fractions blotted with anti-α-synuclein oligomer-specific

antibody, was demonstrated in the substantia nigra and frontal cortex, but not in the putamen, in PD. Dramatic increase in α-synuclein oligomers was also seen in fluorescent-activated cell sorter (FACS)-isolated nuclei in the frontal cortex in PD. **Conclusions:** Altered machinery of protein synthesis is altered in the substantia nigra and cerebral cortex in PD being the frontal cortex area 8 more affected than the angular gyrus and precuneus; in contrast, pathways of protein synthesis are apparently preserved in the putamen. This is associated with the presence of α-synuclein oligomeric species in total homogenates; substantia nigra and frontal cortex are enriched, albeit with different band patterns, in α-synuclein oligomeric species, whereas α-synuclein oligomers are not detected in the putamen.

Garcia-Esparcia *et al. Acta Neuropathologica Communications*
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Acta Neuropathologica Communications

RESEARCH Open Access

Altered machinery of protein synthesis is region- and stage-dependent and is associated with α-synuclein oligomers in Parkinson's disease

Paula Garcia-Esparcia[1], Karina Hernández-Ortega[1], Anusha Koneti[1], Laura Gil[2], Raul Delgado-Morales[3], Ester Castaño[4], Margarita Carmona[1] and Isidre Ferrer[1,5*]

Abstract

Introduction: Parkinson's disease (PD) is characterized by the accumulation of abnormal α-synuclein in selected regions of the brain following a gradient of severity with disease progression. Whether this is accompanied by globally altered protein synthesis is poorly documented. The present study was carried out in PD stages 1-6 of Braak and middle-aged (MA) individuals without alterations in brain in the substantia nigra, frontal cortex area 8, angular gyrus, precuneus and putamen.

Results: Reduced mRNA expression of nucleolar proteins nucleolin (NCL), nucleophosmin (NPM1), nucleoplasmin 3 (NPM3) and upstream binding transcription factor (UBF), decreased NPM1 but not NPM3 nucleolar protein immunostaining in remaining neurons; diminished 18S rRNA, 28S rRNA; reduced expression of several mRNAs encoding ribosomal protein (RP) subunits; and altered protein levels of initiation factor eIF3 and elongation factor eEF2 of protein synthesis was found in the substantia nigra in PD along with disease progression. Although many of these changes can be related to neuron loss in the substantia nigra, selective alteration of certain factors indicates variable degree of vulnerability of mRNAs, rRNAs and proteins in degenerating sustantia nigra. NPM1 mRNA and 18S rRNA was increased in the frontal cortex area 8 at stage 5-6; modifications were less marked and region-dependent in the angular gyrus and precuneus. Several RPs were abnormally regulated in the frontal cortex area 8 and precuneus, but only one RP in the angular gyrus, in PD. Altered levels of eIF3 and eIF1, and decrease eEF1A and eEF2 protein levels were observed in the frontal cortex in PD. No modifications were found in the putamen at any time of the study except transient modifications in 28S rRNA and only one RP mRNA at stages 5-6. These observations further indicate marked region-dependent and stage-dependent alterations in the cerebral cortex in PD. Altered solubility and α-synuclein oligomer formation, assessed in total homogenate fractions blotted with anti-α-synuclein oligomer-specific antibody, was demonstrated in the substantia nigra and frontal cortex, but not in the putamen, in PD. Dramatic increase in α-synuclein oligomers was also seen in fluorescent-activated cell sorter (FACS)-isolated nuclei in the frontal cortex in PD.

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* Correspondence: 8082ifa@gmail.com
[1]Institute of Neuropathology, Bellvitge University Hospital, University of Barcelona, Bellvitge Biomedical Research Institute (IDIBELL), Hospitalet de Llobregat; Biomedical Research Center of Neurodegenerative Diseases (CIBERNED), Barcelona, Spain
[5]Institute of Neuropathology, Service of Pathologic Anatomy, Bellvitge University Hospital, carrer Feixa Llarga s/n, 08907, Hospitalet de Llobregat, Spain
Full list of author information is available at the end of the article

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Conclusions: Altered machinery of protein synthesis is altered in the substantia nigra and cerebral cortex in PD being the frontal cortex area 8 more affected than the angular gyrus and precuneus; in contrast, pathways of protein synthesis are apparently preserved in the putamen. This is associated with the presence of α-synuclein oligomeric species in total homogenates; substantia nigra and frontal cortex are enriched, albeit with different band patterns, in α-synuclein oligomeric species, whereas α-synuclein oligomers are not detected in the putamen.

Keywords: α-synuclein oligomers, Parkinson's disease, protein synthesis, nucleolar stress, ribosomes

Introduction

Neurodegenerative diseases with abnormal protein aggregates are characterized by post-translational modifications of constitutive proteins which result in abnormal conformation, truncation, and eventual formation of fibrils that impair endoplasmic reticulum function and alter the ubiquitin-proteasome system and autophagy pathways, thereby leading to their accumulation in neurons and, in some conditions, in glial cells. Alzheimer's disease (AD), Parkinson's disease (PD), tauopathies, amyotrophic lateral sclerosis, and Huntington's disease are among this extensive group of disorders in which specific intra- and extracellular protein aggregates, together with the production and accumulation of abnormal oligomeric species, lead to neurodegeneration and neuronal death. In spite of the advances in understanding of specific altered proteins causative of particular diseases, little attention has been paid to the process of total protein synthesis in these disorders. This information is nevertheless crucial, as possible alterations in protein synthesis may jeopardize multiple cellular functions, fuel neurodegeneration and neuron atrophy (i.e. loss of dendrites, synapses and axons), and lead eventually to cell death.

Several studies have demonstrated ribosomal dysfunction and impaired protein synthesis in AD [1–5]. However, little information is available about alterations in protein synthesis in PD. Abnormal morphology and disruption of the nucleolus and reduced nucleolin expression have been reported in the substantia nigra in PD [6–8] and related experimental models [8, 9]. Mutations in DJ1 causative of familial PD alter rRNA biogenesis [10]. Added to this limited input is the fact that nothing is known about the possible link between the machinery of protein synthesis and α-synuclein aggregates, particularly α-synuclein oligomers in PD.

For these reasons, the present study was designed to identify possible alterations in concatenated pathways commanding protein synthesis from the nucleolus to the ribosome in regions with variable vulnerability to PD, including the substantia nigra, frontal cortex area 8, angular gyrus, precuneus, and putamen, at different stages of disease progression. The study includes analysis of selected nucleolar proteins involved in rRNA synthesis, rRNA 18S and rRNA 28S, and mRNAs of ribosomal proteins. This is followed by analysis of protein expression of initiation translation and elongation factors of protein synthesis at the ribosome. Finally, whether alterations are associated with α-synuclein oligomers was assessed in total homogenate fractions and in FACS-isolated nuclei analysed with anti-α-synuclein oligomer-specific antibodies. Post-mortem human brain is not suitable for direct studies of protein synthesis using *in vitro* incorporation of labelled amino acids in proteins because of unpredictable individual variations probably related to pre-mortem status and post-mortem delay in tissue processing. For this reason, the present study does not explore protein synthesis in human PD samples but rather focuses directly on the vulnerability of molecules and pathways involved in protein synthesis in several brain regions at different stages of disease progression in human PD.

Material and methods

Human cases

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank and the Hospital Clinic-IDIBAPS Biobank following the guidelines of the Spanish legislation on this matter and the approval of the local ethics committees. The post-mortem interval between death and tissue processing was between 3 and 20 h. Pathological cases were categorized as having PD pathology (Lewy body disease pathology) stages 1 to 6 according to the nomenclature of Braak et al. [11]. Only typical cases according to the Braak classification were included. Cases with concomitant tauopathies, excepting Alzheimer's disease-related pathology stages I-II/0-B [12], vascular disease, and metabolic syndrome were excluded from the present study. Middle-aged (MA) cases had not suffered from neurologic, psychiatric, or metabolic diseases (including metabolic syndrome), and did not have abnormalities in the neuropathological examination excepting sporadic Alzheimer's disease-related pathology stages I-II/0-B of Braak and Braak.

In total, 122 brains including 44 MA and 78 cases with PD-related pathology were included in the present study.

Table 1 Summary of the number of cases, mean ages, and standard deviation (SD) of each group of samples used in the present study including substantia nigra, frontal cortex area 8, angular gyrus, precuneus, and putamen. MA: middle-aged; PD: Parkinson's disease

	substantia nigra Number (N) mean age ± SD	frontal cortex Number (N) mean age ± SD	angular gyrus Number (N) mean age ± SD	precuneus Number (N) mean age ± SD	putamen Number (N) mean age ± SD
MA	N = 11 65.67 ± 12.76	N = 16 63.88 ± 12.65	N = 10 60.90 ± 10.28	N = 11 64.64 ± 15.09	N = 15 70.27 ± 8.89
PD stages 1-2	N = 6 80.17 ± 9.20	N = 2 73.50 ± 2.12	-	-	-
PD stages 3-4	N = 22 76.55 ± 6.93	N = 17 70.12 ± 8.40	N = 8 66.50 ± 6.52	N = 10 76.80 ± 7.73	N = 7 76.00 ± 13.10
PD stages 5-6	N = 17 78.82 ± 6.15	N = 12 77.83 ± 4.51	N = 4 79.25 ± 3.40	N = 4 81.25 ± 3.86	N = 2 78.00 ± 1.41

Incidental PD (iPD or incidental Lewy Body Disease iLBD) occurred in 13 cases (mostly stages 1, 2, and 3 of Braak). Pre-parkinsonian symptoms in iPD cases were not recorded. Regarding PD cases, all of them had been treated for their motor symptoms. The disease duration ranged from 6 to 16 years. The most common causes of death in the MA and PD cases were infections, neoplasia, and acute cardiac disease.

Five regions were examined for mRNA expression: frontal cortex area 8, substantia nigra, angular gyrus, precuneus, and putamen; the selection of these areas was based on their differing vulnerability to PD and to their accumulative involvement with disease progression. Number of cases, mean ages, and standard deviation for each group are summarized in Table 1. A summary of individual cases and methods used for all cases examined is shown in Additional file 1: Table S1. Most cases here analysed were also the subject of other studies [13–15].

RNA purification

Purification of RNA from the substantia nigra, right frontal cortex area 8, angular gyrus, precuneus, and putamen was carried out using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer and performing the optional DNase I digest to avoid extraction and later amplification of genomic DNA. The concentration of each sample was obtained from A260 measurements with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA).

Retrotranscription reaction

Retrotranscription reaction of RNA samples was carried out with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) following the guidelines provided by the manufacturer, and using a Gene Amp® 9700 PCR System thermocycler (Applied Biosystems). A parallel reaction for one RNA sample was processed in the absence of reverse transcriptase to rule out DNA contamination.

Real Time PCR

RT-qPCR was conducted in duplicate on cDNA samples obtained from the retrotranscription reaction using 1,000ηg of RNA, diluted 1:20 in 384-well optical plates (Kisker Biotech, Steinfurt, Germany) utilizing the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Parallel amplification reactions were carried out using 20x TaqMan Gene Expression Assays and 2x TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probes used in the study are shown in Additional file 2: Table S2. The reactions were performed using the following parameters: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. TaqMan PCR data were captured using the Sequence Detection Software (SDS version 2.2, Applied Biosystems). Subsequently, threshold cycle (CT) data for each sample were analysed with the double delta CT (ΔΔCT) method. First, delta CT (ΔCT) values were calculated as the normalized CT values for each target gene in relation to the endogenous controls β-glucuronidase (GUS-β) and X-prolylaminopeptidase (aminopeptidase P) 1 (XPNPEP1) for normalization [16, 17]. Second, ΔΔCT values were obtained with the ΔCT of each sample minus the mean ΔCT of the population of MA samples (calibrator samples). The fold-change was determined using the equation $2^{-\Delta\Delta CT}$. These housekeeping genes were selected because they show no modifications in several neurodegenerative diseases in human post-mortem brain tissue [16, 17]. Similar results were obtained using GUS-β and XPNPEP1 as correctors; GUS-β was selected for representation.

Statistical analysis

The normality of distribution of the mean fold-change values obtained by RT-qPCR for each region and stage

between MA and PD cases were analysed with the Kolmogorov-Smirnov test. The non-parametric Mann–Whitney test was performed to compare each group when the samples did not follow a normal distribution, while the unpaired *t* test was used for normal variables. *t* test was used instead of one-way ANOVA when analyzing MA and PD cases in parallel in the same optical plate. Statistical analysis was performed with GraphPad Prism version 5.01 (La Jolla, CA, USA) and Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (Warrenton, VA, USA). Differences between groups were considered statistically significant at *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Gel electrophoresis and western blotting

Samples of the substantia nigra including 14 MA and 14 PD cases of frontal cortex area 8 (0.1 g of tissue) were homogenized with a glass homogenizer in Mila lysis buffer (0.5 M Tris at pH 7.4 containing 0.5 methylenediaminetetracetic acid at pH 8.0, 5 M NaCl, 0.5 % Na doxicholic, 0.5 % Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, bi-distilled water) with protease and phosphatase inhibitor cocktails (Roche Molecular Systems, Pleasanton, CA, USA), and then centrifuged at 4 °C for 15 min at 13,000 rpm (ultracentrifuge Beckman with 70Ti rotor, CA, USA). Protein concentration was measured by Smartspect™plus spectrophotometer (Bio-Rad, CA, USA) using the Bradford method (Merck, Darmstadt, Germany). Samples containing 20 μg of protein and the standard Precision Plus Protein™ Dual Color (Bio-Rad) were loaded onto 10 % and 12 % acrylamide gels. Proteins were separated in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes using the Trans-Blot®Turbo™ transfer system (Bio-Rad) at 200 mA/membrane for 20 min. Non-specific bindings were blocked by incubation in 5 % albumin in Tris-buffered saline (TBS) containing 0.2 % Tween for 1 h at room temperature. After washing, the membranes were incubated at 4 °C overnight with several antibodies in TBS containing 5 % albumin and 0.2 % Tween. A list of the antibodies used is shown in Additional file 3: Table S3. Monoclonal antibody anti-β-actin diluted 1:30,000 (β-Actin, A5316; Sigma-Aldrich, St. Louis, MO, USA) was blotted for the control of protein loading. Afterwards, the membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibody (1:1,000, Dako, Glostrup, Denmark), and the immune complexes were visualized with a chemiluminescence reagent (ECL, Amersham, GE Healthcare, Buckinghamshire, UK). Densitometry of western blot bands was assessed with the TotalLab program (TotalLab Quant, Newcastle, UK) and subsequently analysed with GraphPad Prism by one-way ANOVA with *post hoc* Tukey's student range test for multiple comparisons. We used one-way ANOVA instead of *t* test because each gel contained MA and different stages of PD cases. Differences were considered statistically significant with *P*-values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Immunohistochemistry, double-labelling immunofluorescence, and confocal microscopy

Immunohistochemical study of selected nucleolar proteins was performed on 4 μm-thick dewaxed paraffin sections of the substantia nigra. PD cases were analysed including 2 stage 1 cases (1 male and 1 female), 3 stage 3 males, 2 stage 4 males, and 3 stage 5 males. Tissue sections were boiled in citrate buffer for 20 min to retrieve antigenicity. Endogenous peroxidases were blocked with peroxidase (Dako, Glostrup, Denmark) followed by 10 % normal goat serum. The primary antibodies were mouse monoclonal anti-nucleophosmin (NPM1) and rabbit polyclonal anti-nucleoplasmin-3 (NPM3). A few sections of the substantia nigra and frontal cortex area 8 were incubated with anti-α-synuclein oligomer-specific antibody (Agrisera, Vännäs, Sweden) at a dilution of 1:1,000. Following incubation with the primary antibody at room temperature overnight, the sections were incubated with EnVision + system peroxidase (Dako) at room temperature for 15 min. The peroxidise reaction was visualized with diaminobenzidine (DAB) and H_2O_2. The omission of the primary antibody in some sections was used as a control for the immunostaining; no signal was obtained with the incubation only of the secondary antibody. No immunogenic peptides were available for any antibody used. Sections were slightly counterstained with haematoxylin.

Double-labelling immunofluorescence was carried out on de-waxed sections, 4 μm-thick, which were stained with a saturated solution of Sudan black B (Merck, DE) for 15 min to block the autofluorescence of lipofuscin granules present in cell bodies, and then rinsed in 70 % ethanol and washed in distilled water. The sections were boiled in citrate buffer to enhance antigenicity and blocked for 30 min at room temperature with 10 % foetal bovine serum diluted in PBS. Then, the sections were incubated at 4 °C overnight with combinations of primary antibodies. After washing, the sections were incubated with Alexa488 or Alexa546 (1:400, Molecular Probes, US) fluorescence secondary antibodies against the corresponding host species. Nuclei were stained with DRAQ5™ (1:2,000, Biostatus, UK). After washing, the sections were mounted on Immuno-Fluore mounting medium (ICN Biomedicals, US), sealed, and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope. Again, omission of the primary antibody in some sections was used as a control for the immunostaining.

Quantitative studies were carried out in the substantia nigra on serial non-consecutive sections stained with

haematoxylin and eosin or processed for NPM1 and NPM3 immunohistochemistry. Nucleolar counts were performed directly under the ocular of the microscope at a magnification x200 in three areas (0.48 mm^2) selected at random in every one of the eleven cases (2 PD1, 3 PD3, 3 PD4, 3 PD5). Results of NPM1 and NPM3-stained nucleoli per section were expressed as percentage of the total nucleoli visualized in haematoxylin and eosin-stained. Sections of PD at stage 1, stained with haematoxylin and eosin, were used to quantify neurons in which the nucleolus was visualized in that section.

To learn whether reduced NPM1 immunoreactivity in substantia nigra dopaminergic neurons was linked to α-synuclein inclusions, double-labelling immunofluorescence and confocal microscopy using anti-NPM1 and anti-α-synuclein antibodies was used to analyze six cases (1 tissue section per case) of PD at stages 4–5.

Quantification of co-localization of α-synuclein and eIF3 in sections processed for double-labelling immunofluorescence and confocal microscopy was done by counting 31 α-synuclein immunoreactive neurons ($n = 6$ sections) from PD stages 4 and 5, and noting how many of these neurons contained eIF3 immunoreactive inclusions. Results of eIF3 co-localization were expressed as the percentage of neurons with α-synuclein inclusions containing eIF3 deposits.

α-synuclein oligomeric species in total homogenate fractions

Brain samples (0.1 g) of substantia nigra pars compacta, frontal cortex area 8 and putamen from MA ($n = 3$ per group) and stage 5 PD cases ($n = 3$ per group) were homogenized in a glass homogenizer, in 750 μl of ice-cold PBS+ (sodium phosphate buffer pH 7.0, plus protease inhibitors), sonicated, and centrifuged at 2,700 g at 4 °C for 10 min. The pellet was discarded and the resulting supernatant was ultra-centrifuged at 133,000 g at 4 °C for one hour. The supernatant (S2) was kept as the PBS-soluble fraction. The resulting pellet was re-suspended in a solution of PBS, pH 7.0, containing 0.5 % sodium deoxycholate, 1 % Triton, and 0.1 % SDS, and this was ultra-centrifuged at 133,000 g at 4 °C for one hour. The resulting supernatant (S3) was kept as the deoxycholate-soluble fraction. The corresponding pellet was re-suspended in a solution of 2 % SDS in PBS and maintained at room temperature for 30 min. Afterwards, the samples were centrifuged at 133,000 g at 25 °C for one hour and the resulting supernatant (S4) was the SDS-soluble fraction. Equal amounts of each fraction were mixed with reducing sample buffer and processed in parallel for 10 % SDS-PAGE electrophoresis and western blotting. Membranes were incubated with anti-α-synuclein oligomer-specific antibody (Agrisera, Vännäs, Sweden) at a dilution of 1:1,000. The protein bands were visualized with the ECL method (Amersham).

α-synuclein oligomeric species in isolated nuclei

Small pieces of frozen brain samples (0.2 g) of frontal cortex area 8 from MA ($n = 2$, cases 4 and 15 of the Additional file 1: Table S1) and 2 PD cases stages 5–6 ($n = 2$, cases 113 and 120 of the Additional file 1: Table S1) were homogenized in a 6 ml ice-cold Solution D buffer (0.25 M sucrose, 25 mM potassium chloride (KCl), 5 mM magnesium chloride ($MgCl_2$), and 20 mM Tris–HCl pH 7.5) with 0.1 % Triton, and then centrifuged at 1,000 g for 10 minutes at 4 °C (Ultracentrifuge Beckman with 70Ti rotor). The supernatant obtained was discarded and the pellet was re-suspended in 2 ml of Optiprep (D1556, Sigma, St Louis, MO, USA) to allow better separation by density gradient, and centrifuged at 3,200 g for 20 minutes at 4 °C. The new supernatant obtained was discarded again whereas the pellet was re-suspended in 500 μl ice-cold PBS buffer. Isolated nuclei were stained with mouse antibody to NeuN (see Additional file 3: Table S3). Primary antibody was visualized with appropriate secondary antibodies conjugated with Alexa 488. DNA content was determined using DAPI (4',6-diamidino-2-phenylindole). Subsequently, samples were centrifuged at 1,000 g for 10 min at 4 °C, and the solution obtained was re-suspended in 800 μl ice-cold PBS buffer.

Flow cytometry sorting was performed with a Beckman Coulter MoFlo Astrios. Nuclei were sorted at 25 PSI (pounds per square inch) through a 100 micron nozzle. Sample and collection tubes were kept at 10 °C for the duration of the sorting. Afterwards, NeuN+ (neuronal nuclei) and NeuN- (assumed glial nuclei) were collected separately in Optiprep and centrifuged at 3,200 g for 20 minutes at 4 °C. The pellet obtained was mixed with Laemmli buffer (2 % SDS, 10 % Glycerol, 0.002 % bromophenol blue, 6.25 mM Tris–HCl pH 6.8, bidistilled H_2O, 2 % β-mercaptoethanol and phosphatase inhibitor cocktails), and pellets containing NeuN+ and NeuN- nuclei were processed in parallel for 10 % SDS-PAGE electrophoresis and western blotting. Demonstration that this fraction contained only nuclei without cytoplasmic contamination was carried out by western blotting with SOD-1 and histone H3 antibodies as indicated in Additional file 3: Table S3. Anti-α-synuclein oligomer-specific antibody (Agrisera, Vännäs, Sweden) was used to identify the presence of oligomers; the bands were visualized with the ECL method (Amersham).

Results

mRNA expression levels of nucleolar proteins in the substantia nigra, frontal cortex area 8, angular gyrus, precuneus, and putamen

Since nucleolar proteins are implicated in rRNA processing, the first step in the study was to analyse the mRNA

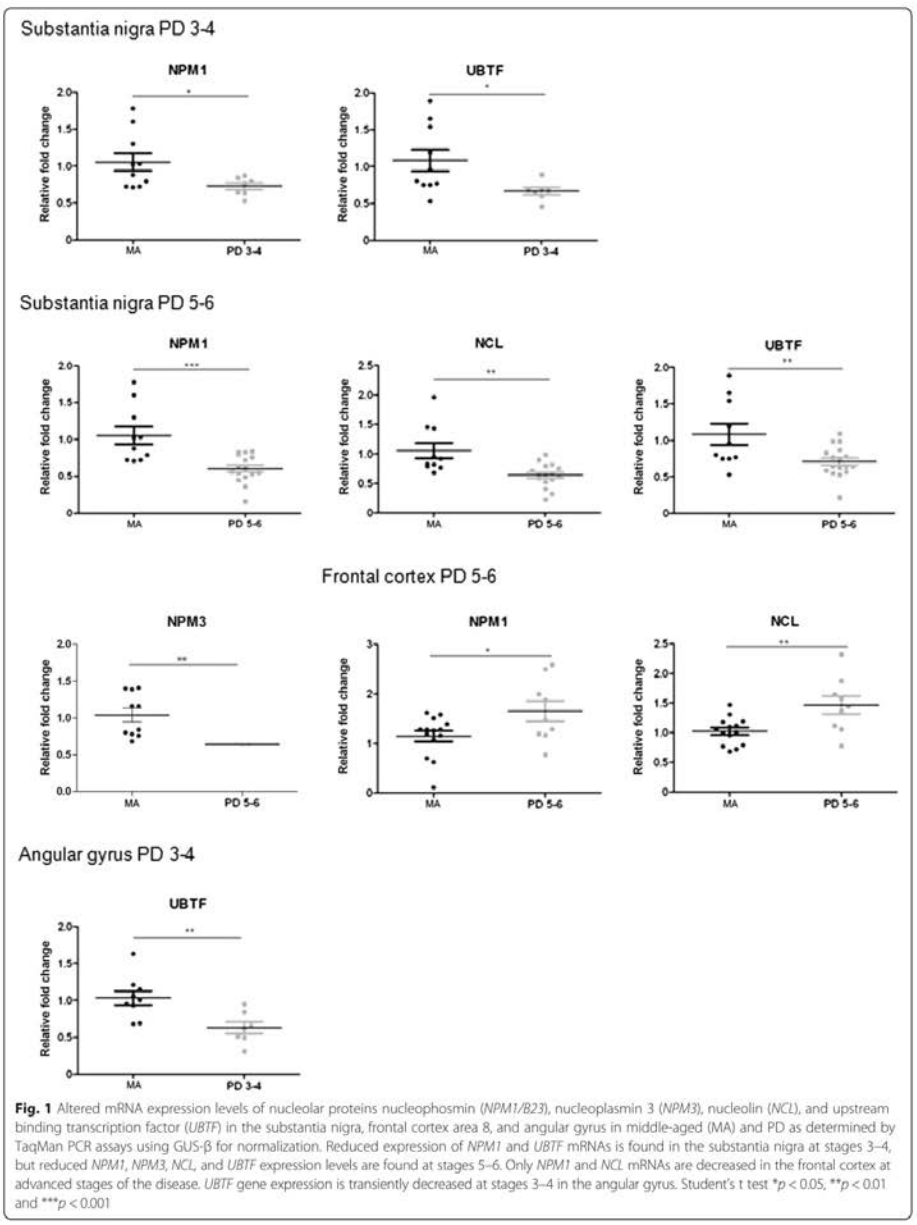

Fig. 1 Altered mRNA expression levels of nucleolar proteins nucleophosmin (*NPM1/B23*), nucleoplasmin 3 (*NPM3*), nucleolin (*NCL*), and upstream binding transcription factor (*UBTF*) in the substantia nigra, frontal cortex area 8, and angular gyrus in middle-aged (MA) and PD as determined by TaqMan PCR assays using GUS-β for normalization. Reduced expression of *NPM1* and *UBTF* mRNAs is found in the substantia nigra at stages 3–4, but reduced *NPM1*, *NPM3*, *NCL*, and *UBTF* expression levels are found at stages 5–6. Only *NPM1* and *NCL* mRNAs are decreased in the frontal cortex at advanced stages of the disease. *UBTF* gene expression is transiently decreased at stages 3–4 in the angular gyrus. Student's t test $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

expression levels of two chaperones and one protein linked to RNA polymerase.

No modification in the mRNA expression levels of nucleophosmin (*NPM1*), nucleoplasmin 3 (*NPM3*), nucleolin (*NCL*), or upstream binding transcription factor (*UBTF*) was observed in the substantia nigra at PD stages 1–2 when compared with the MA group. However, *NPM1* and *UBTF* were significantly down-regulated ($p < 0.05$) at stages 3–4, as were *NPM1*, *NPM3*, *UBTF*, and *NCL* at stages 5–6 ($p < 0.001$) (Fig. 1). No significant differences were observed when comparing PD 3–4 with PD 5–6. Therefore, changes with disease progression were seen between stages 1–2 and stages 3–6 (see Additional file 4: Table S4).

No differences in *NCL*, *NPM1*, *NPM3*, or *UBTF* mRNA expression were observed in frontal cortex area 8 at stages 3–4 when compared with the MA group, but *NPM1* and *NCL* mRNAs were significantly increased ($p < 0.05$, and $p < 0.001$, respectively) at stages 5–6 (Fig. 1).

A transient decrease in *UBTF* mRNA expression was found in the angular gyrus at stages 3–4 (Fig. 1). No modifications in the expression of *NPM1*, *NCL*, *NPM3*, or *UBTF* mRNAs were identified in the precuneus and putamen at any stage analysed.

Details of all genes analyzed are found in Additional files 4, 5, 6, 7 and 8.

18S rRNA and 28S rRNA in the substantia nigra, frontal cortex area 8, angular gyrus, precuneus, and putamen

In the substantia nigra, 18S rRNA levels were significantly reduced at stages 3–4 ($p < 0.01$), as were 28S rRNA and 18S rRNA levels at stages 5–6 ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 2). Therefore, differences

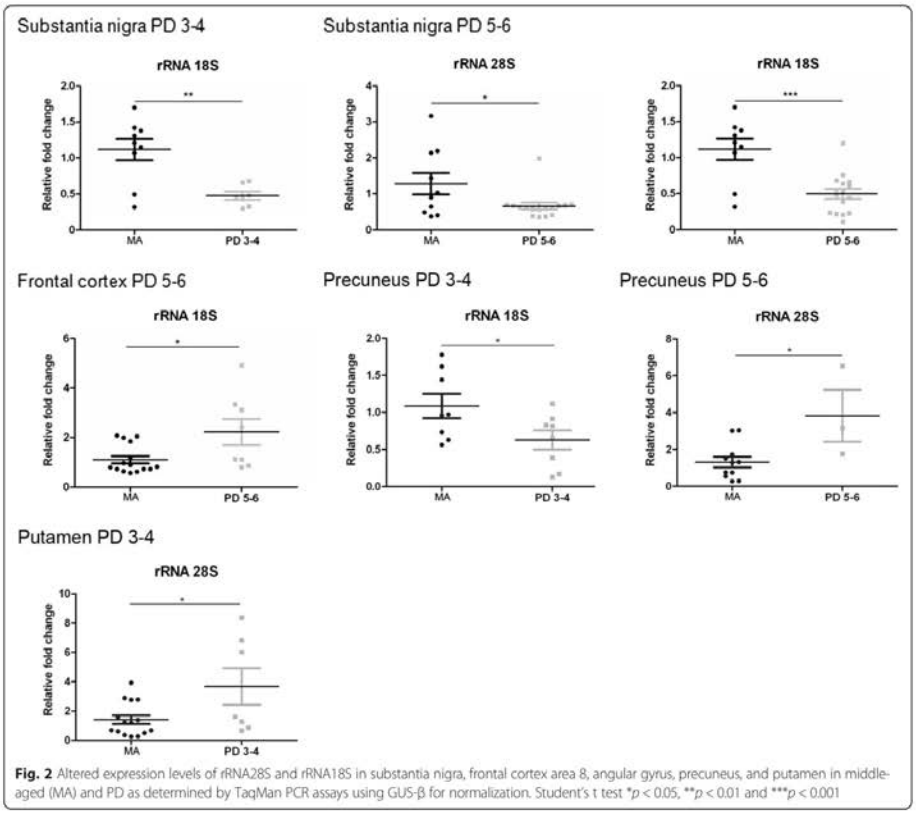

Fig. 2 Altered expression levels of rRNA28S and rRNA18S in substantia nigra, frontal cortex area 8, angular gyrus, precuneus, and putamen in middle-aged (MA) and PD as determined by TaqMan PCR assays using GUS-β for normalization. Student's t test *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

along disease progression were seen between stages 1–2 and 3–6 (Additional file 4: Table S4).

In frontal cortex area 8, a significant increase in 18S rRNA ($p < 0.05$) was found at stages 5–6 (Fig. 2). rRNA expression was not altered in the angular gyrus. A transient 18S rRNA decrease ($p < 0.05$) and a transient 28S rRNA increase ($p < 0.05$) was noted in the precuneus and putamen, respectively, at stages 3–4. 28S rRNA up-regulation ($p < 0.05$) was observed in the precuneus at stages 5–6 (Fig. 2). Therefore, definite up-regulation of 18S rRNA and 28S rRNA was identified in the frontal cortex and precuneus, respectively, at advances stages of PD.

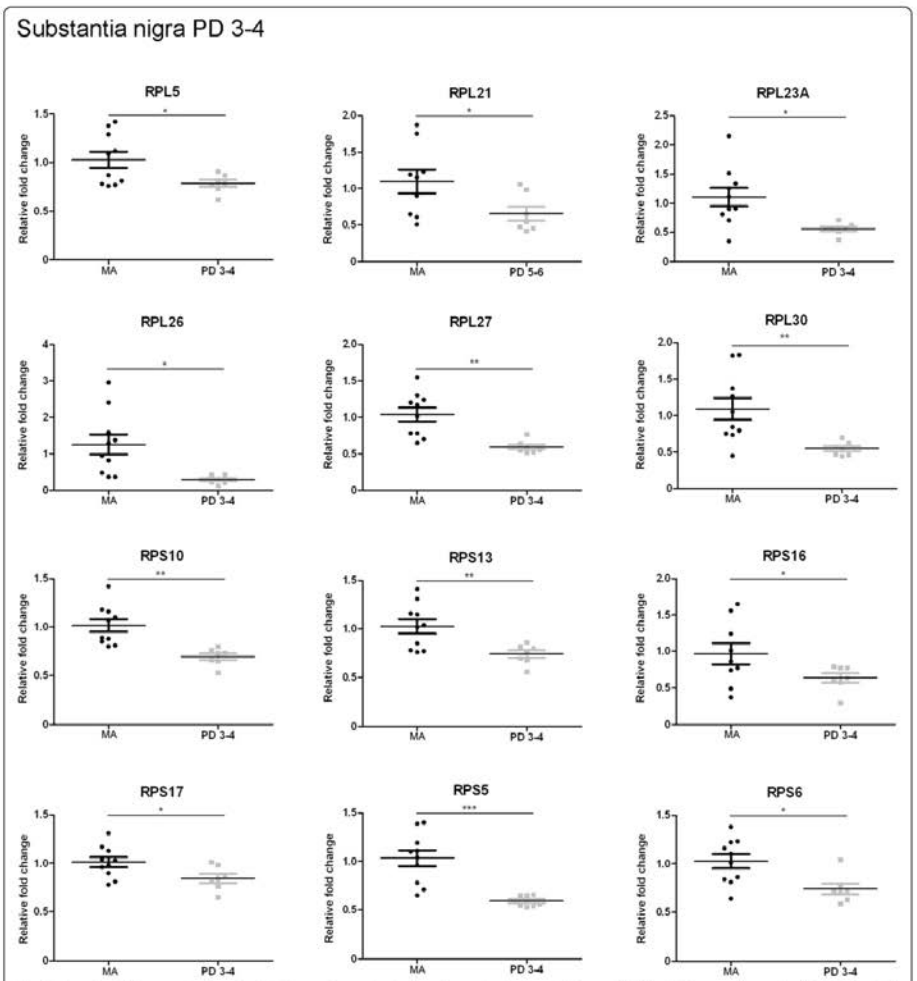

Fig. 3 Altered mRNA expression levels of 16 ribosomal proteins in the substantia nigra in middle-aged (MA) and PD cases determined by TaqMan PCR assays using GUS-β for normalization. Student's t test *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

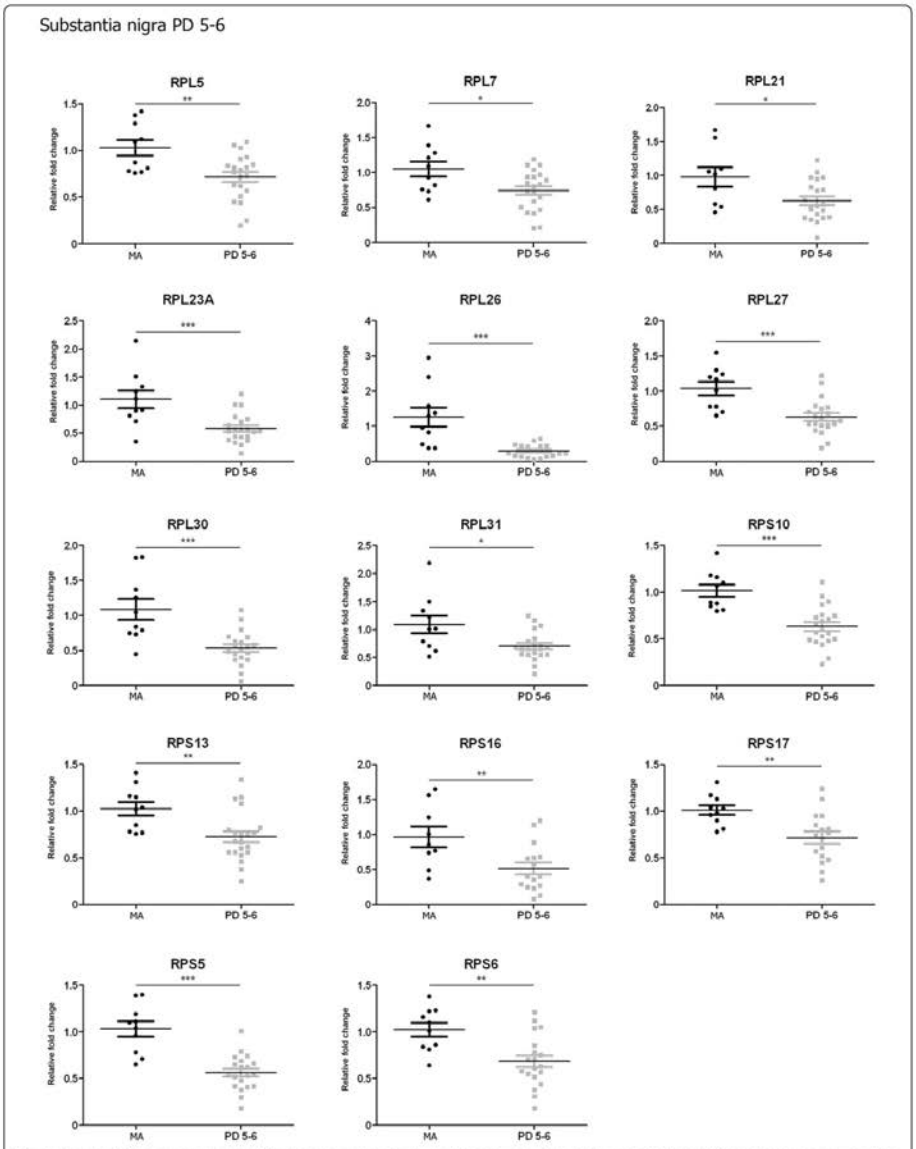

Fig. 4 Altered mRNA expression levels of 16 ribosomal proteins in the frontal cortex area 8 in middle-aged (MA) and PD cases determined by TaqMan PCR assays using GUS-β for normalization. Student's t test *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

Details of rRNA results in all regions and stages are found in Additional files 4, 5, 6, 7 and 8.

mRNA expression levels of genes encoding ribosomal proteins in the substantia nigra

Since ribosomal proteins are essential to the assembly of ribosomal subunits and to the process of protein synthesis, the next step was to analyse gene expression of 9 RPL and 7 RPS genes. Selection of these mRNAs was done at random.

No significant changes were observed in the substantia nigra at stages 1–2. However, twelve of sixteen genes analysed were significantly down-regulated in the substantia nigra at stages 3–4 including *RPL5*, *RPL21*, *RPL23A*, *RPL26*, *RPL27*, *RPL30*, *RPS10*, *RPS13*, *RPS16*, *RPS17*, *RPS5*, and *RPS6* (p values varied from < 0.05 to <0.001) (Fig. 3). Fourteen genes were down-regulated in the substantia at stages 5–6. These included, in addition to those down-regulated at stages 3–4, *RPL7* and *RPL31* (p values ranged from < 0.01 to < 0.001) (Fig. 4). Therefore, major modifications with disease progression were found between stages 1–2 and stages 3–6.

Details of all genes analyzed are found in Additional file 4: Table S4.

mRNA expression levels of genes encoding ribosomal proteins in frontal cortex area 8, angular gyrus, precuneus, and putamen

A transient down-regulation in the expression of *RPL7* ($p < 0.05$), *RPS6* ($p < 0.05$), *RPS10* ($p < 0.05$) and *RPS13* ($p < 0.001$) was found in frontal cortex area 8 at stages 3–4.

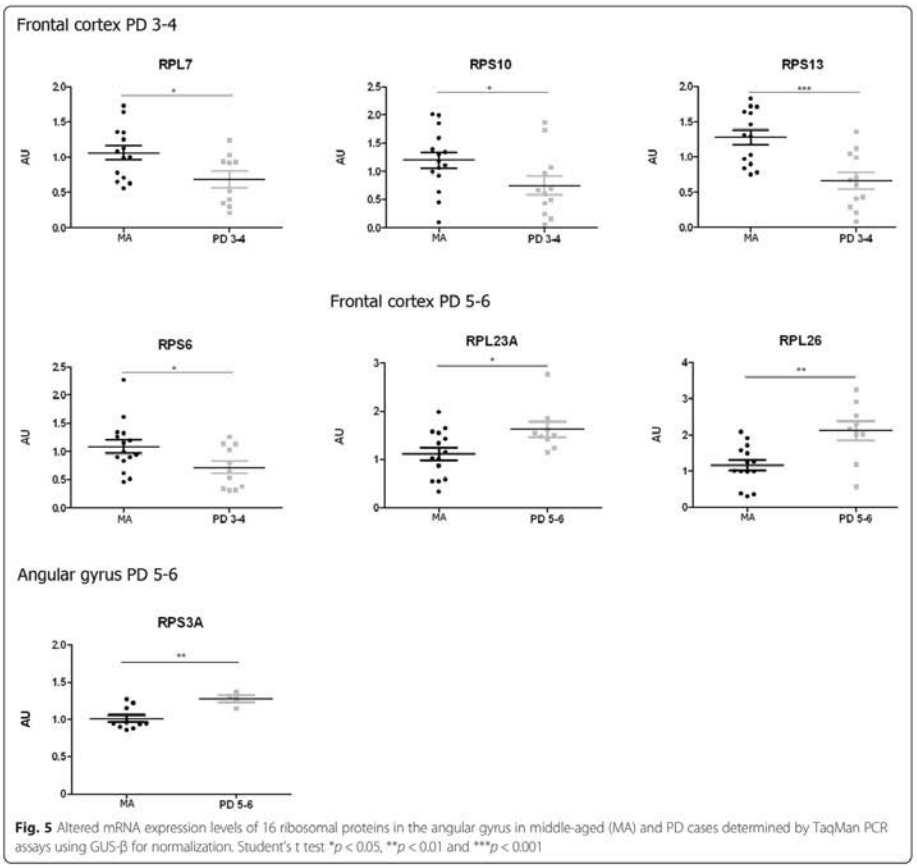

Fig. 5 Altered mRNA expression levels of 16 ribosomal proteins in the angular gyrus in middle-aged (MA) and PD cases determined by TaqMan PCR assays using GUS-β for normalization. Student's t test *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

However, two genes were up-regulated in the frontal cortex at stages 5–6: *RPL23A* ($p < 0.05$) and *RPL26* ($p < 0.01$) (Fig. 5). In short, five genes were significantly up-regulated in the frontal cortex when comparing PD stages 3–4 and PD stages 5–6: *RPL7*, *RPL22*, *RPL23A*, RPL26, *RPS6*, and *RPS17* (see Additional file 5: Table S5).

Only one gene, *RPS3A*, was up-regulated ($p < 0.01$) in the angular gyrus at stages 5–6 (Fig. 5), thus indicating modifications with disease progression (see Additional file 6: Table S6).

The precuneus showed a similar pattern to frontal cortex area 8 with decreased mRNA expression of certain genes at PD stages 3–4 followed by increased expression of other genes at stages 5–6. Yet the genes involved differed in the frontal cortex area 8 from the precuneus. Reduced *RPL5* ($p < 0.05$), *RPS10* ($p < 0.05$), and *RPS16* ($p < 0.01$) mRNA expression occurred at stages 3–4 compared to the MA group. Increased *RPL27* ($p < 0.05$), *RPL30* ($p < 0.001$), *RPL31* ($p < 0.01$), *RPS5* ($p < 0.05$), and *RPS6* ($p < 0.01$) mRNA expression was found at stages 5–6 (Fig. 6).

Only *RPL5* of the sixteen assessed RNAs encoding ribosomal proteins was increased at stages 5–6 in the putamen in PD (see Additional file 8: Table S8).

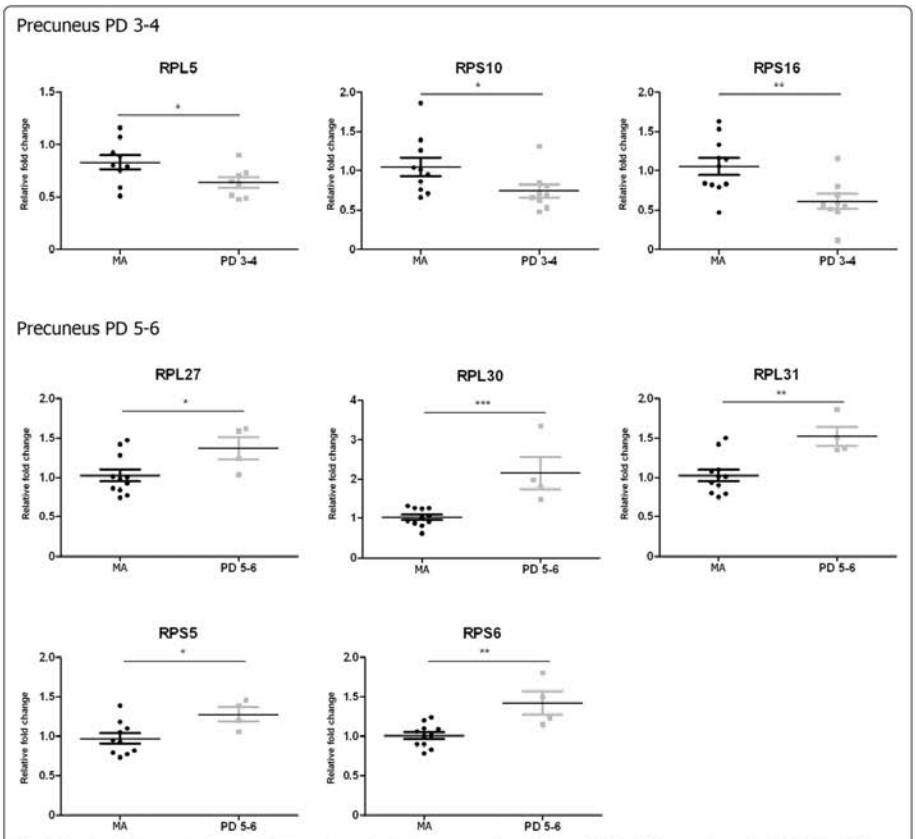

Fig. 6 Altered mRNA expression levels of 16 ribosomal proteins in the precuneus in middle-aged (MA) and PD cases determined by TaqMan PCR assays using GUS-β for normalization. Student's t test *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

Immunohistochemistry of nucleolar proteins in the substantia nigra

To learn whether mRNA changes in nucleolar chaperones translated into altered protein expression, immunohistochemistry and immunofluorescence to NPM1 and NPM3 were performed in the substantia nigra.

A similar approach was tried for the study of ribosomal proteins. After testing more than twelve antibodies, none of them was useful for immunohistochemistry and western blotting.

NPM1 and NMP3 localized in the nucleolus in MA and diseased cases. However, decreased NPM1 immunoreactivity was reduced in pigmented neurons (Fig. 7a-c). In contrast, NPM3 immunoreactivity was preserved in the majority of neurons (Fig. 7d-f).

Quantitative studies were performed to rule out reduced or missing NPM1 immunoreactivity being due to artefacts of nucleolar sectioning or to altered nucleolar immunoreactivity to different nucleolar markers. Counts were made on serial non-consecutive sections of the same cases stained with haematoxylin and eosin, and processed for NPM1 and NPM3 immunohistochemistry without counterstaining. The percentage of neurons in the substantia nigra pars compacta with visualized nucleoli at PD stage 1, as assessed in haematoxylin and eosin sections, and NPM1 and NPM3 immunohistochemistry, was about 50 %. The total number of neurons decreased with disease progression, representing about 40 % neuron loss at stage 5 when compared with PD stage 1. NPM3-immunoreactive nucleoli were found in 44 % of remaining neurons at stage 5, but the percentage of NPM1-immunoreactive nucleoli in remaining neurons was around 34 % at stage 5. Weakly stained nuclei were considered positive nuclei. Quantitative results are shown in Additional file 9: Table S9.

These results suggest that reduced NPM1 immunoreactivity is not merely a reflection of neuron loss but rather indicates selective vulnerability of NPM1 when compared with NPM3 to PD.

Whether decreased NPM1-immunoreactive nucleoli in advanced stages of PD was a reflection of the co-occurrence of Lewy bodies in a particular neuron was assessed using double-labelling immunofluorescence and confocal microscopy with anti-NPM1 and anti-α-synuclein antibodies in six cases (1 tissue section per case) of PD at stages 4–5. Decreased nucleolar NPM1 immunoreactivity occurred independently of the presence or absence of α-synuclein inclusions in the cytoplasm of neurons (data not shown).

Protein expression of initiation and elongation transcription factors in substantia nigra and frontal cortex area 8

The expression levels of initiation factors eIF1, eIF2-α, P-eIF2-α, eIF3, eIF3η, and eIF5, and elongation factors eEF1A and eEF2, were analysed with western blotting. These antibodies did not work for immunohistochemistry.

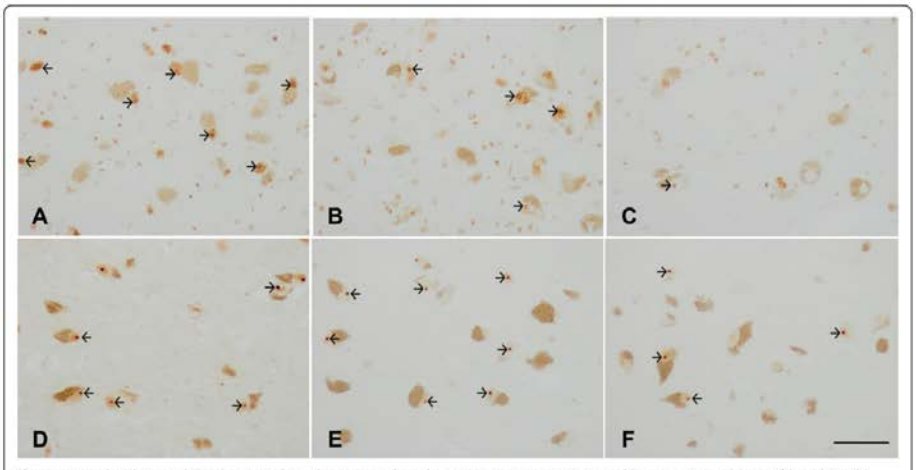

Fig. 7 a-c: Nucleophosmin (NPM1) immunohistochemistry in the substantia nigra pars compacta in PD at stages 1–2 (**a**), 3–4 (**b**), and 5–6 (**c**) showing NPM1 immunoreactivity in the nucleoli (arrows). The number and intensity of NPM1 immunoreactivity decreases at advanced stages of PD. **d-f**: Nucleoplasmin 3 (NPM3) immunohistochemistry in the substantia nigra pars compacta in PD at stages 1–2 (**d**), 3–4 (**e**), and 5–6 (**f**) showing preserved NPM3 immunoreactivity in the nucleolus (arrows). Paraffin section without haematoxylin counterstaining, bar = 50 μm

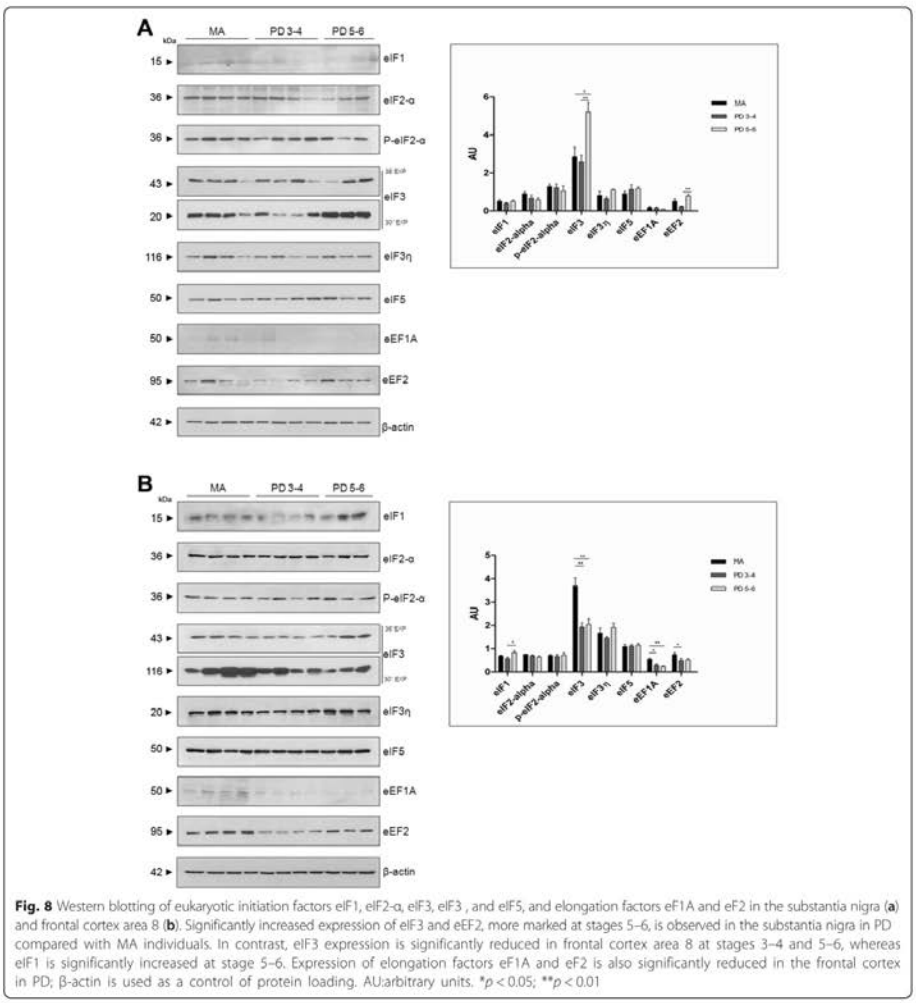

Fig. 8 Western blotting of eukaryotic initiation factors eIF1, eIF2-α, eIF3, eIF3 , and eIF5, and elongation factors eF1A and eF2 in the substantia nigra (**a**) and frontal cortex area 8 (**b**). Significantly increased expression of eIF3 and eEF2, more marked at stages 5–6, is observed in the substantia nigra in PD compared with MA individuals. In contrast, eIF3 expression is significantly reduced in frontal cortex area 8 at stages 3–4 and 5–6, whereas eIF1 is significantly increased at stage 5–6. Expression of elongation factors eF1A and eF2 is also significantly reduced in the frontal cortex in PD; β-actin is used as a control of protein loading. AU:arbitrary units. $*p < 0.05$; $**p < 0.01$

A significant increase in eIF3 and eEF2 was observed in the substantia nigra, especially at stages 5–6, when compared with the values for the MA group (Fig. 8a).

In contrast, eIF3 expression levels were significantly reduced at stages 3–4 ($p < 0.01$) and 5–6 ($p < 0.001$), whereas eIF1 expression levels were significantly increased ($p < 0.05$) at stages 5–6 in frontal cortex area 8. Regarding elongation factors, eEF1A and eEF2 protein expression was significantly decreased at stages 3–4 ($p < 0.05$), and more markedly so for eEF1A ($p < 0.01$) at stages 5–6 (Fig. 8b).

Reticulum stress markers in substantia nigra and frontal cortex area 8

Abnormal protein synthesis and accumulation of abnormal proteins in the endoplasmic reticulum is causative of the endoplasmic reticulum stress response [18]. For

this reason, we also explored the unfolded protein response (UPR) in the two brain areas in PD.

The expression levels of proteins GRP78, GRP94, ATF4, ATF6, XBP1, p54, and P-IRE-1 were analysed in the substantia nigra and frontal cortex area 8 in PD cases at stages 3–4 and 5–6 in comparison with samples from MA individuals.

Increased expression levels of ATF4 and ATF6 90 kDa at stages 5–6 ($p < 0.05$), and reduced GRP94 expression levels at stages 3–4 and 5–6 ($p < 0.01$), were found in the substantia nigra in PD (Fig. 9a). No ATF6f (50 kDa) was identified in any group (data not shown). Reduced expression levels of GRP78 were observed in frontal cortex area 8 at stages 3–4 and 5–6 ($p < 0.01$ and $p < 0.001$, respectively). No changes in the expression of other reticulum stress markers were found in frontal cortex area 8 in PD (Fig. 9b).

Double-labelling immunofluorescence and confocal microscopy in the substantia nigra in PD cases stages 4 and 5 showed α-synuclein in Lewy bodies and neurites co-localizing with eIF3. Quantitative studies showed 27 of 31 α-synuclein immunoreactive neurons co-localizing with eIF3 in Lewy bodies (87 %). In contrast, GRP78, GRP94, IRE-1, and XBP1 did not show co-localization with α-synuclein inclusions (Fig. 10).

α-synuclein oligomeric species in total homogenate fractions

The anti-α-synuclein oligomer-specific antibody stained Lewy bodies and neurites, and small granules in substantia nigra neurons in paraffin sections. The background was clear, and the small dots consistent with small deposits of oligomers in the neuropil were extremely rare (data not shown).

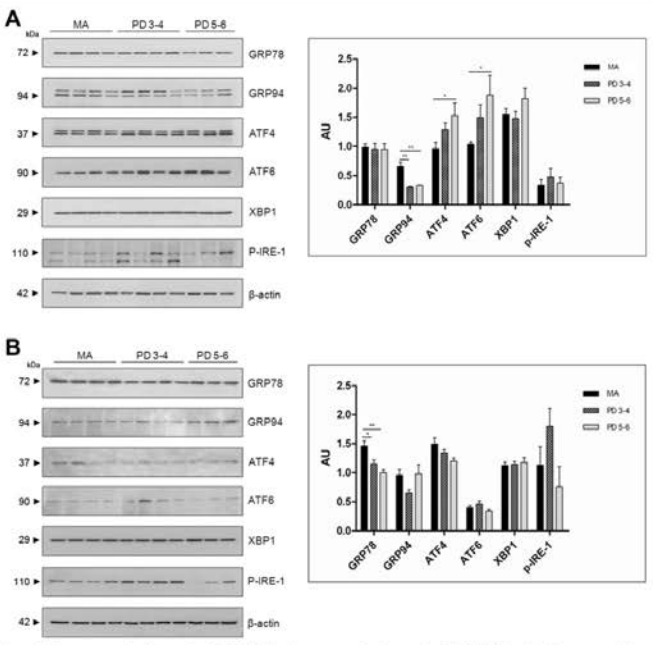

Fig. 9 Western blotting of glucose-regulated protein 78 (GRP78), glucose-regulated protein 94 (GRP94), activating transcription factor 4 (ATF4), activating transcription factor 6 (ATF6), Xbox binding protein 1 (XBP1), and phosphorylated inositol requiring kinase 1 (P-IRE-1) in the substantia nigra (**a**) and frontal cortex area 8 (**b**) shows significantly increased expression of ATF4 and ATF6 90 kDa at stages 5–6 ($p < 0.05$), and reduced expression of GRP94 at stages 3–4 and 5–6 ($p < 0.01$) in the substantia nigra in PD when compared with MA individuals. ATF6f (50 kDa), the cleaved and active form of ATF6, was not identified in any group. In contrast, significant reduction of GRP78 ($p < 0.01$ at stages 3–4, and $p < 0.001$ at stages 5–6) with preservation of other reticulum stress markers was found in frontal cortex area 8 in PD. Note that a double band with GRP94, ATF4, and P-IRE-1 antibodies is observed in the substantia nigra but not in the frontal cortex. The expected molecular weight is marked by an arrowhead in SN western blots. β-actin is used as a control of protein loading. AU: arbitrary units; *$p < 0.05$; **$p < 0.01$

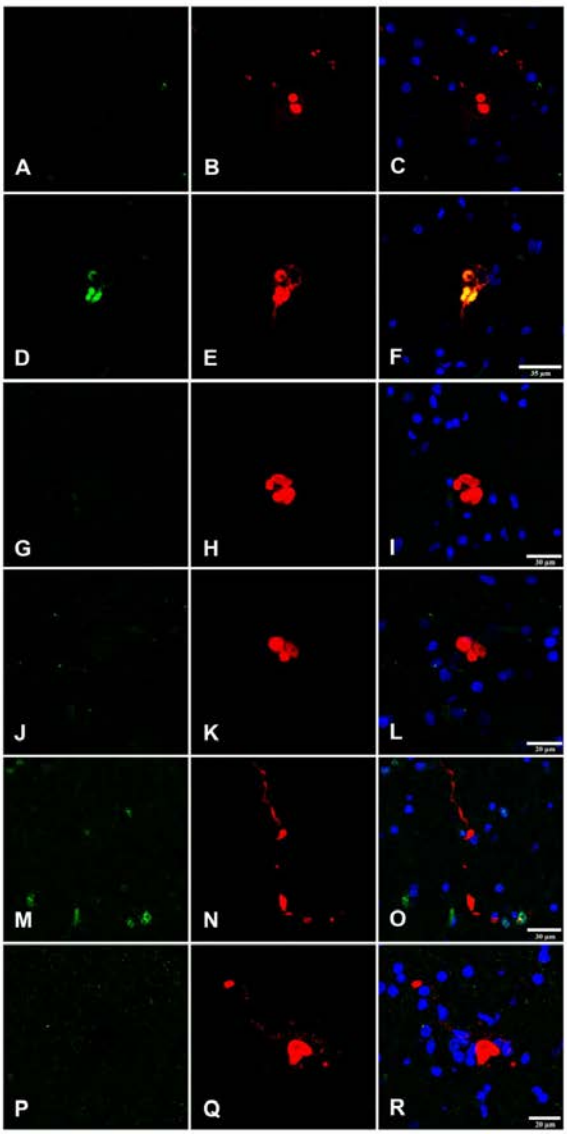

Fig. 10 Double-labelling immunofluorescence and confocal microscopy in the substantia nigra in PD cases stages 4 and 5 to α-synuclein (**b**, **e**, **h**, **k**, **n**, **q**) and p54 (**a**), eIF3 (**d**), GRP78 (**g**), GRP94 (**j**), IRE1 (**m**), and XBP1 (**p**); **c**, **f**, **i**, **l**, **o**, **r**: merge. α-synuclein in Lewy bodies and neurites (red) only co-localizes with eIF3 (green). Paraffin sections, nuclei are stained with DRAQ5™. **a**-**f**, bar = 35 μm; G-l, m-o, bar = 30 μm; **j**-**l**, **p**-**r**, bar = 20 μm

The biochemical analysis of α-synuclein oligomeric species was carried out on frozen samples of the substantia nigra, frontal cortex area 8, and putamen in MA and PD cases in cytosolic (PBS; Cyt), deoxycholate (Dxc), and sodium dodecyl sulfate (SDS) fractions. In the substantia nigra, a band of α-synuclein at the expected molecular weight, about 17 kDa, was observed in MA and PD cases at stages 5–6 in the three fractions, although the density of the band was higher in PD than in MA cases. In addition, two well-defined bands of 50 kDa and approximately 100 kDa were obtained in all three fractions (Cyt, Dxc, and SDS) in the substantia nigra in PD (Fig. 8). α-synuclein oligomeric species were also seen in the frontal cortex in PD at stages 5–6, but the band pattern differed from that seen in substantia nigra. In addition to the band of about 17 kDa found in MA and PD cases, three bands of molecular weight of about 35 kDa, 50 kDa, and 90 kDa, all of them with marked smear, were observed mainly in the cytosolic and deoxycholate fractions in the frontal cortex only in PD (Fig. 8). In contrast, no oligomeric species were detected in the putamen; only the band of about 17 kDa was identified equally in MA and PD cases (Fig. 11).

α-synuclein oligomeric species in isolated nuclei obtained by FACS

Nuclear fractions were obtained by FACS using DAPI and NeuN antibody from the grey matter of frontal cortex area 8 in MA and PD cases (Fig. 12a). DAPI binds strongly to DNA and labels all nuclei in the suspension regardless of cell type. Using this approach, it is not difficult to discriminate single nuclei (R1) from doublets, triplets, and aggregates, as well as debris and background noise. Nuclei selected in R1 were sorted into neuronal (NeuN+) and non-neuronal (NeuN-). To define more accurately the population, NeuN was analysed on the same plot as red fluorescence to eliminate cells not completely lysed, permeable to DAPI, and with more autofluorescence than isolated nuclei. Purity of the nuclear fractions was assessed by demonstrating histone H3 immunoreactivity (a band at 17 kDa) and lack of SOD-1 immunoreactivity in the isolated nuclei in both NeuN- and NeuN+ samples in MA and PD cases. Western blots using specific antibodies to α-synuclein oligomeric species demonstrated in the NeuN+ samples one band at about 20 kDa and two bands of about 50 kDa and 100 kDa in PD cases. One weak band at about 20 kDa was also seen in the NeuN- samples in PD (Fig. 12b). No bands of α-synuclein oligomers were detected in NeuN+ and NeuN- samples in MA cases processed in parallel, excepting weak bands after long exposure in some cases (Additional file 10: Figure S1).

Discussion

Nucleolar proteins and rRNAs are differentially regulated in the *substantia nigra*, frontal cortex area 8, angular gyrus, precuneus, and putamen in PD, and changes accelerate with disease progression

Ribosomal RNA genes are arranged in tandem repeats in the NORs and are transcribed by RNA polymerase I in conjunction with associated factors including UBF (upstream binding transcription factor, encoded by

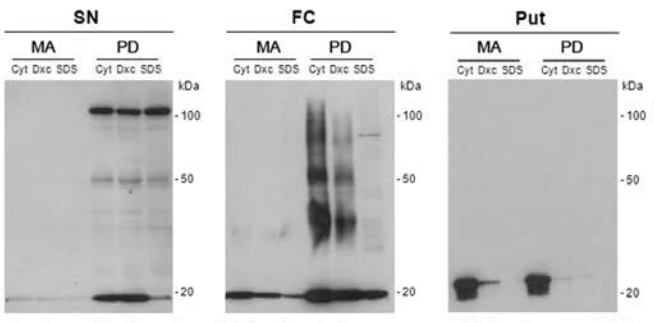

Fig. 11 Western blotting of α-synuclein oligomeric species in the substantia nigra pars compacta (SN), frontal cortex area 8 (FC), and putamen (Put) in total homogenate cytosolic (Cyt), deoxycholate (Dxc), and sodium dodecyl sulphate (SDS) fractions in middle-aged individuals (MA) and Parkinson's disease stages 5–6 (PD). α-synuclein oligomeric species are only observed in the three fractions in PD but not in MA cases, thus indicating abnormal α-synuclein solubility and aggregation of α-synuclein in the substantia nigra and frontal cortex in PD. However, the patterns of α-synuclein oligomers differ in the three regions. Two net bands of 50 kDa and about 100 kDa are seen in the three fractions in the substantia nigra, but three bands with a considerable smear of about 30 kDa, 50 kDa, and 90 kDa are found, mainly in the cytosolic and deoxycholate fractions, in the frontal cortex. In addition, note the higher density of α-synuclein bands in the frontal cortex when compared with the substantia nigra in PD. No α-synuclein oligomers are detected in the putamen, whereas a band of about 17 kDa is found in MA and PD cases

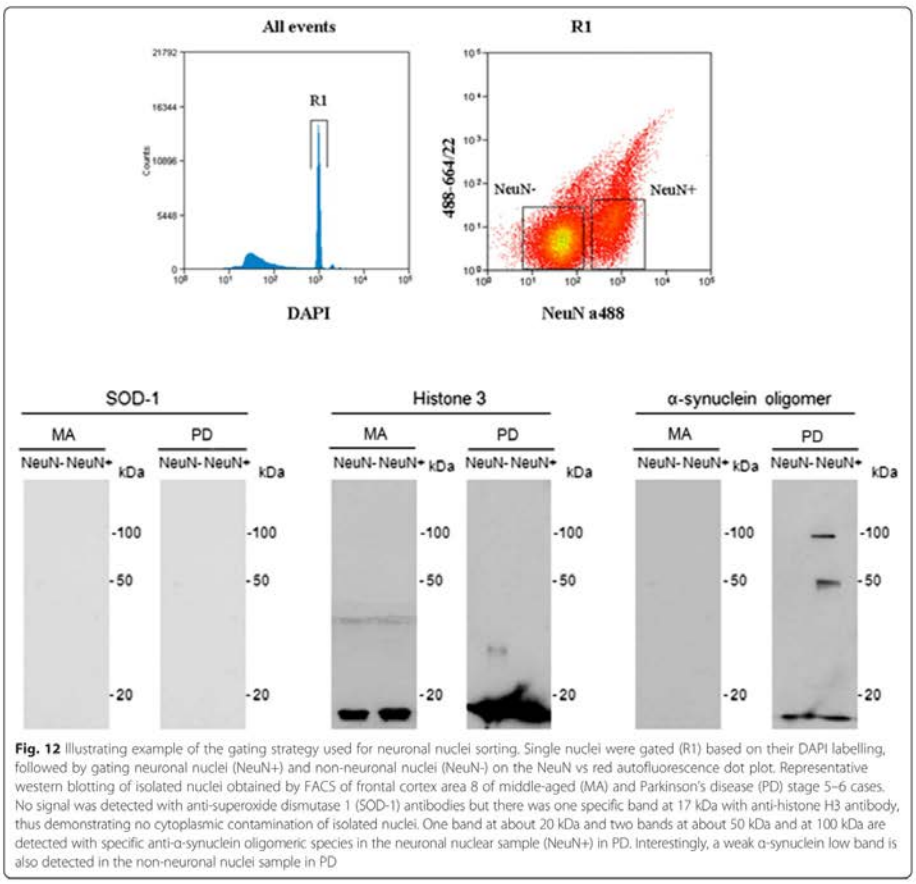

Fig. 12 Illustrating example of the gating strategy used for neuronal nuclei sorting. Single nuclei were gated (R1) based on their DAPI labelling, followed by gating neuronal nuclei (NeuN+) and non-neuronal nuclei (NeuN-) on the NeuN vs red autofluorescence dot plot. Representative western blotting of isolated nuclei obtained by FACS of frontal cortex area 8 of middle-aged (MA) and Parkinson's disease (PD) stage 5–6 cases. No signal was detected with anti-superoxide dismutase 1 (SOD-1) antibodies but there was one specific band at 17 kDa with anti-histone H3 antibody, thus demonstrating no cytoplasmic contamination of isolated nuclei. One band at about 20 kDa and two bands at about 50 kDa and at 100 kDa are detected with specific anti-α-synuclein oligomeric species in the neuronal nuclear sample (NeuN+) in PD. Interestingly, a weak α-synuclein low band is also detected in the non-neuronal nuclei sample in PD

UBTF) and SL1 proteins [19–26]. rDNAs encode precursor transcripts which are processed to form 18S, 28S, and 5.8S RNAs [27]. UBF is localized in the nucleoli in interphase cells and it regulates RNA polymerase 1 following acetylation [28–30].

Nucleolin (NCL) and nucleophosmin (NPM1/B23) are other major nucleolar proteins acting as histone-binding chaperones required for chromatin compaction, regulation of rRNA transcription, nucleic acid binding, and nuclear re-programming [10, 26, 31–42]. In addition, NPM1 is involved in the nuclear transport of proteins to the nucleolus and of certain ribosomal proteins to the cytoplasm [43–46]. NPM1-null mice display altered ribosome biogenesis and premature death at mid-gestation [47, 48]. NPM1 acts as a ribonuclease for the maturation of rRNA transcript [49, 50], silencing NPM1 results in altered processing of 28S RNA [51]. Additional functions of NPM1 are related to DNA replication, transcription, and repair [37]. NPM3 is a nucleolar histone chaperone that interacts with nucleophosmin and modulates ribosome biogenesis [52, 53].

Cellular and molecular alterations associated with impaired nucleolar activity are causative of nucleolar stress [54–56] which can lead to the malfunction of the nucleolar machinery, altered rRNA expression, reduced protein synthesis, and, when extreme, cell death. Nucleolar stress is emerging as an important sensor in several pathological conditions [57] including ischemic damage, cancer

[58–60], and neurodegeneration [61–63]. Nucleolar stress has been documented in AD [64], and in polyglutamine diseases including Huntington's disease and related models [65–68].

The present study identifies reduced *NCL* and *NPM1* mRNA levels and NPM1 immunoreactivity in the substantia nigra in PD cases with disease progression. *NPM3* mRNA expression levels are also reduced in the substantia nigra at advanced stages. Reduced mRNA expression may be related to the progressive loss of dopaminergic neurons in the substantia nigra, but preserved expression of NPM3 protein, in contrast to decreased NPM1 immunoreactivity in remaining dopaminergic neurons, indicates selective vulnerability of NPMs to neurodegeneration. Reduced *NCL* mRNA is in agreement with previous observations showing reduced nucleolin protein expression in the substantia nigra in PD [69]. The reduced *UBTF* here observed is also in line with previous observations of nucleolar disruption in dopaminergic neurons in PD [8]. The reduced nucleophosmin expression in the substantia nigra in PD here observed for the first time may act as an additional cause of neurodegeneration [70].

Reduced expression of 18S rRNA and 28S rRNA gives strong support to the concept that nucleolar stress is a major alteration in the substantia nigra in PD [64]. Although decreased biosynthesis of ribosome subunits may be a response to preserve energy homeostasis in acute stress situations [71], it is less clear that maintained reduced expression of rRNAs has any beneficial effect on cell survival. Rather, perpetuation of nucleolar stress and reduced rRNA synthesis is consistent with parallel cell damage in PD substantia nigra.

In contrast to the substantia nigra, *NCL* and *NPM1* mRNAs are increased in frontal cortex area 8 in PD at stages 5–6, whereas *UBTF* appears to be transiently decreased in the angular gyrus at stages 3–4. No modifications in *NCL*, *NPM1*, and *UBTF* gene expression are seen in the precuneus and putamen at any stage of the disease. In contrast to the substantia nigra, 18S rRNA and 28S rRNA are increased in the frontal cortex and precuneus, respectively, at stages 5–6 of Braak. Overexpression of NCL is neuroprotective against rotenone, a toxicant used to experimentally reproduce some characteristics of PD in animal models [72]. Overexpression of NPM1 is neuroprotective against kainic acid-induced excitotoxicity [73, 74]. Therefore, increased *NCL* and *NPM1* mRNA expression in frontal cortex appears to be a response to PD geared to protecting rRNA synthesis. According to this hypothesis, 18S rRNA expression is increased in frontal cortex at advanced stages of PD. Lack of correlation between preserved *NCL* and *NPM1* expression and increased 28S rRNA in the precuneus may be related to the participation of other regulators of rRNA biosynthesis not examined in the present study.

Altered expression of ribosomal protein mRNAs in PD is region- and stage-dependent

Ribosomes (80S) are cytoplasmic structures measuring 25–30 nm composed of 65 % RNAs and 35 % ribosomal proteins that form a smaller subunit (40S) which binds to mRNA and a larger subunit (60S) which binds to tRNAs and amino acids. In eukaryotes, the smaller subunit is made of 18S RNA and 33 proteins whereas the larger subunit is formed by 5S RNA, 5.8S RNA, 28S RNA, and 46 ribosomal proteins [55, 75–84]. Ribosomal proteins also participate in protein synthesis initiation and elongation, and they can regulate their own synthesis at the translational level [85–90].

The present results show decreased gene expression of 12 of 16 examined ribosomal protein mRNAs in the substantia nigra at stages 3–4 (covering 6 RPL and 6 RPS), and 14 (covering 8 RPL and 6 RPS) of 16 at stages 5–6 of Braak. These changes are progression-dependent and may reflect in part progressive neuronal loss in the substantia nigra. However, altered ribosomal protein gene expression also occurs in cerebral cortex in PD with stage and region peculiarities. Altered mRNA expression of several ribosomal proteins in the cerebral cortex appears to be a plastic process depending on the cortical region and stage of the disease. Decreased expression of a few mRNAs at stages 3–4 followed by up-regulation of a few different ribosomal protein mRNAs at stages 5–6 in frontal cortex and precuneus suggests modifications in the structure and functional capacities of cortical ribosomes with disease progression. It is worth stressing that only 16 of 79 ribosomal protein mRNAs were selected for the present study, and although the number is representative of ribosomal protein mRNA modifications it does not cover the total number of ribosomal proteins and the possible modifications of additional mRNAs.

Expression of initiation and elongation factors in substantia nigra and frontal cortex area 8 in PD

Translation initiation in the ribosome is geared by the interactions of 12 eukaryotic translation initiation factors (eIFs), most of them composed of several subunits. The 43S preinitiation complex is composed of the small 40S ribosomal subunit, the initiating methionyl-tRNA bound to eIF2-GTP, and eIF1, eIF1a, and eIF3. mRNA is added to the 43S preinitiation complex together with the poly(A) binding protein (PABP) and the eIF4f complex (a heterotrimeric complex composed of eIF4a, eIF4e, and eIF4g) bound to an AUG codon. eIF2B and eIF5 activate eIF2 and regulate eIF2-GDP recycling, respectively, whereas eIF5b and eIF6 participate in ribosomal subunit joining and binding [91–94]. Elongation occurs when elongating factor eEF1A is activated following GTP-binding and forms a complex with aminoacyl-tRNA which recognizes the specific sequence in mRNA

at the ribosome. Once the interaction of the codon in mRNA with the anti-codon in tRNA is decoded, eEF1A-GDP is hydrolysed, released from the ribosome, and recycled into its active form by eEF1B. eEF2 assists in the precise codon location at the ribosome [95–104]. Synthesis terminates in the presence of a stop codon in the mRNA sequence which is recognized by a releasing factor that sets the polypeptide chain free [105, 106].

In the substantia nigra, eIF3 and eEF2 expression levels were increased more markedly at stages 5–6 in PD, suggesting activation of peptide synthesis. It can be suggested that activation of peptide synthesis is related to compensatory mechanisms in preserved dopaminergic neurons in the face of the altered expression of genes involved in ribosomal proteins and, consequently, in the assembly of the functional ribosome. Alternatively, since western blots cannot discriminate between neurons and glial cells, increased eIF3 and eEF2 expression levels might be related to increased protein synthesis in reactive astrocytes.

In cerebral cortex area 8, among the five eIFs and subunits examined, only eIF3 was significantly decreased at stages 3–4 and 5–6, suggesting that recruitment of mRNA to the 40S subunit is hampered as a result of lower eIF3 expression. Regarding elongation factors, significantly reduced expression of eEF1A and eEF2 with disease progression lends strong support to the hypothesis of altered polypeptide synthesis in frontal cortex in PD.

Reticulum stress responses in the substantia nigra and frontal cortex in PD with disease progression

The unfolded protein response (UPR) designates the cellular response to the accumulation of abnormal proteins in the endoplasmic reticulum. The reaction can also be elicited by other factors such as hypoglycemia, hypoxia, acidosis, calcium, redox reactions, and a variety of natural compounds and drugs [18]. Control of protein folding at the endoplasmic reticulum (ER) is modulated by the chaperone glucose-regulated protein 78 (GRP78, also named immunoglobulin binding protein BIP), a member of the HSP70 family which, in non-stressed cells, binds to three ER transmembrane proteins: PKR-like ER kinase (PERK), inositol requiring kinase 1 (IRE1), and transcription factor 6 (ATF6) [107–110]. Glucose-regulated protein 94 is the HSP90-like protein in the lumen of the endoplasmic reticulum and therefore it chaperones secreted and membrane proteins [111, 112]. Accumulation of misfolded proteins in the endoplasmic reticulum activates PERK [113] and phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF2-α) at serine 51, resulting in decreased protein synthesis [114]. In addition, eIF2α phosphorylation sets off activating transcription factor 4 (ATF4), promoting DNA transcription of specific genes [115]. ER responses also involve the activation of inositol-requiring kinase 1 (IRE1) by dimerization and phosphorylation which activates the transcription factor Xbox binding protein (XBP1), which in turns activates transcription of stress genes in DNA [69, 116, 117]. Upon ER stress, full activating transcription factor 6 (ATF6) of 90 kDa moves to the Golgi complex where it is cleaved to form the active transcription factor of 50 kDa (ATF6-50 kDa: ATF6f), which translocates to the nucleus and activates transcription of stress genes [118]. Therefore, activation of ATF4, IRE1, and ATF6f increases the production of GRP78, GRP94, PERK, IRE1, XBP1, and ATF6, and stimulates the ER-associated degradation (ERAD) pathway [119, 120], contributing to restoring homeostasis. However, once passed certain thresholds, ER stress can trigger NF-κB activation and caspase-mediated apoptosis [113, 121–123].

ER stress has been implicated in the pathogenesis of neurodegenerative diseases including PD [6, 124, 125]. Markers of the unfolded protein response (phosphorylated PERK and phosphorylated eIF2α) have been identified in dopaminergic neurons of the substantia nigra containing α-synuclein inclusions at relatively early stages of PD [126, 127]. ATF4 and total ATF6 expression levels are elevated in the substantia nigra at advanced stages of the disease, suggesting activation of the UPS response. It is worth noting that ATF4 levels have been reported to be increased in neuromelanin-containing neurons in the substantia nigra in PD, and elevated levels of ATF4 are protective of neurons subjected to noxious stimuli [128].

α-synuclein oligomeric species

α-synuclein was first described in the nucleus and presynaptic nerve terminals from *Torpedo* [129]. The localization and function of this protein in the nucleus has been a primary focus of study partly due to the overwhelming information about the accumulation of abnormal α-synuclein in the cytoplasm of neurons in PD and in neurons and oligodendroglia in multiple system atrophy. However, α-synuclein is identified in the nucleus in different settings using different methods [130–136], and it is especially abundant during development modulating neurogenesis [137–139]. Nuclear α-synuclein levels are increased accompanying oxidative stress *in vitro* and *in vivo* [140, 141], and nuclear α-synuclein seems to facilitate, in turn, oxidative stress [142]. The mechanism of effects of nuclear α-synuclein is poorly understood but α-synuclein binds to histones and inhibits histone acetylation [130, 143]. Moreover, α-synuclein, under conditions of oxidative stress, binds to the promoter of the mitochondrial transcription factor PGC1-α and reduces transcription of mitochondrial genes [144].

α-synuclein is primarily a disordered monomer that binds to and transiently stabilizes different substrates such as lipids and membrane vesicles playing variegated physiological functions [145–147]. However, soluble β-rich oligomers are experimentally promoted *in vitro* by several physical and chemical agents, and they are also naturally produced in disease states influenced by fatty acids, α-synuclein mutations, oxidative stress, phosphorylation, nitration, ubiquitination, and truncation, where they come to be toxic for nerve cells [144, 148–154]. Increased expression levels of α-synuclein oligomers have been found in the brain in Lewy body diseases and related transgenic models [14, 155–158]. Interestingly, the band pattern of α-synuclein oligomers analysed here differs in the substantia nigra and frontal cortex area 8, suggesting regional differences in the composition of oligomeric species in the substantia nigra and cerebral cortex in PD. Importantly, the intensity of oligomeric species in western blots is greater than what is expected following examination of paraformaldehyde-fixed paraffin sections of the frontal cortex in which only a few Lewy bodies and neurites are detected with immunohistochemistry using the same antibody. Therefore, it may be inferred that most α-synuclein oligomers are not identified in paraffin sections processed for immunohistochemistry; only those linked to fibrillary deposits in Lewy bodies and neurites remain. Observations in the putamen are particularly interesting as no oligomeric α-synuclein species are identified in the same PD cases used in substantia nigra and frontal cortex. This means important regional differences in α-synuclein oligomers in PD represent, on the one hand, regional differences in α-synuclein metabolism, and, on the other, these differences may reflect specific regional vulnerability in PD.

A recent study has shown that α-synuclein proximity ligation assay (AS-PLA) permits the visualization of undetected diffuse α-synuclein oligomeric pathology in PD brains [159]. α-synuclein oligomers are detected in the cytoplasm of neurons with α-synuclein inclusions, as revealed with current immunohistochemistry, but also widespread in nerve terminals (consistent with synaptic localization) and in the cytoplasm of vulnerable neurons with no apparent α-synuclein pathology as detected by current immunohistochemical methods. No nuclear α-synuclein oligomers were reported in that study but the accompanying figures showed small immunoreactive dots in certain nuclei. The present observations show unequivocal presence of α-synuclein oligomers in FACS-isolated neuronal nuclei in PD. Interestingly, a weak band of α-synuclein is also detected in NeuN- samples (corresponding to non-neuronal nuclei) in PD. This raises the possibility that α-synuclein is abnormally present in the nuclei of glial cells in PD. In this line, α-synuclein deposition has been reported in protoplasmic astrocytes in PD [160, 161]. It is worth stressing that weak bands of α-synuclein oligomers in isolated neuronal nuclei from certain MA are only visualized after long-term exposure. AS-PLA has also allowed the visualization of α-synuclein oligomers in neuronal nuclei in control cases (see Fig. 12, ref 131) although this fact was not mentioned in the original paper.

Conclusions

Our previous studies, among others, have shown that in spite of the absence or the relatively small numbers of Lewy bodies and neurites in the cerebral cortex in PD until advanced stages of the disease, there is a plethora of molecular alterations including altered synaptic modulation and transmission, mitochondrial dysfunction, oxidative stress damage, reduced energy metabolism, altered purine metabolism, increased inflammatory responses, and abnormal expression of receptors whose function is still poorly understood [13–15, 162, 163]. All these alterations converge in the most vulnerable regions of the cerebral cortex and extend to other regions with disease progression [126, 164–166]. To the list of apparently unrelated deleterious events, we may add altered machinery of protein synthesis targeted not only in the substantia nigra but also in the cerebral cortex in PD at middle and advanced stages of the disease. Frontal cortex area 8 is more affected than the angular gyrus and precuneus. α-synuclein oligomeric species seem to have direct and indirect deleterious effects on mitochondria [152, 167–169], proteasome [170], endoplasmic reticulum [6], and synapses [171–173], among other subcellular structures [153, 174]. Importantly, altered protein machinery in PD relates to the presence of α-synuclein oligomeric species in total homogenates. Substantia nigra and frontal cortex are enriched, albeit with different band patterns, in α-synuclein oligomeric species, whereas α-synuclein oligomers are not detected in the putamen. Unfortunately, rapid blocking of protein synthesis following hypoxia and other insults aimed at not producing abnormal proteins under suboptimal conditions such as those inherent to the process of dying precludes direct and accurate study of protein synthesis in human post-mortem brains (unpublished observations). Therefore, a direct observation of impaired protein synthesis in PD post-mortem brains is technically not possible.

Additional files

Additional file 1: Table S1. Summary of the main individual characteristics of the cases used in this study. M: male; F: female; P-M: post-mortem delay (hours, minutes); PD Braak: Parkinson's disease-related pathology stages 1–6 of Braak; 0: no neurological or neuropathological anomalies; FC: frontal cortex area 8; SN: substantia nigra; AG: angular gyrus; PC: precuneus; PUT: putamen; RIN: RNA integrity number; WB: western blot; IHC: immunohistochemistry; OLI: α-synuclein oligomeric species. (DOC 331 kb)

Additional file 2: Table S2. TaqMan probes used for the study of mRNA expression of nucleolar proteins, ribosomal RNAs, and ribosomal proteins including the probes for normalization (GUS-β and XPNPEP1). (DOC 42 kb)

Additional file 3: Table S3. Summary of the antibodies used for western-blotting (wb) and immunohistochemistry (ihq) or immunofluorescence (if); rb: rabbit polyclonal: m: mouse monoclonal; ip: immunoprecipitation for FACS studies. (DOC 54 kb)

Additional file 4: Table S4. Expression levels of mRNAs encoding nucleolar proteins 18S rRNA and 28S rRNA, and mRNAs encoding ribosomal proteins in the substantia nigra. MA: middle-aged individuals with no PD pathology, 1–6 stages of PD. (DOC 50 kb)

Additional file 5: Table S5. Expression levels of mRNAs encoding nucleolar proteins 18S rRNA and 28S rRNA, and mRNAs encoding ribosomal proteins in frontal cortex area 8. MA: middle-aged individuals with no PD pathology, 1–6 stages of PD. (DOC 58 kb)

Additional file 6: Table S6. Expression levels of mRNAs encoding nucleolar proteins 18S rRNA and 28S rRNA, and mRNAs encoding ribosomal proteins in the angular gyrus. MA: middle-aged individuals with no PD pathology, 1–6 stages of PD (DOC 58 kb)

Additional file 7: Table S7. Expression levels of mRNAs encoding nucleolar proteins 18S rRNA and 28S rRNA, and mRNAs encoding ribosomal proteins in the precuneus. MA: middle-aged individuals with no PD pathology, 1–6 stages of PD (DOC 58 kb)

Additional file 8: Table S8. Expression levels of mRNAs encoding nucleolar proteins 18S rRNA and 28S rRNA, and mRNAs encoding ribosomal proteins in the putamen. MA: middle-aged individuals with no PD pathology, 1–6 stages of PD (DOC 48 kb)

Additional file 9: Table S9. Mean ratio of the number of nucleolar staining and the total number of neurons (ratio SD) visualized with haematoxylin and eosin and immunohistochemistry to NPM1 and NPM3 in the substantia nigra at stages 1, 3, 4, and 5 of PD. Percentage (%) of nucleolus staining and total neurons. No significant differences are seen regarding the ratios of NPM3 nucleolar staining along disease progression. However, NPM1 immunohistochemistry reveals a significant decrease between PD1 and PD5 ($P \leq 0.05$ One-way Anova) (DOC 28 kb)

Additional file 10: Figure S1. Western blotting of isolated nuclei obtained by FACS of frontal cortex area 8 of middle-aged (MA) and Parkinson's disease (PD) stage 5–6 cases subjected to over-exposure (10 min). Several bands of α-synuclein oligomeric species are seen in PD as in Figure 9B (exposure 1 min). In addition, weak bands of α-synuclein oligomers are observed in isolated neuronal nuclei (NeuN+) in MA. (TIF 426 kb)

Abbreviations

ATF4: activating transcription factor 4; ATF6: activating transcription factor 6; eIF1: eukaryotic translation initiation factor 1; eIF2-α: eukaryotic translation initiation factor 2; eIF3: eukaryotic translation initiation factor 3; eIF3η: eukaryotic translation initiation factor 3 η; eIF5: eukaryotic translation initiation factor 5; eEF1A: eukaryotic elongation factor 1A; eEF2: eukaryotic elongation factor 2; GRP94: glucose-regulated protein 94; GRP78: glucose-regulated protein 78; GUS-B: β-glucuronidase; IRE-1: inositol requiring kinase 1; NCL: nucleolin; NPM1: nucleophosmin 1 (nucleolar phosphoprotein B23); NPM3: nucleophosmin/nucleoplasmin 3; P-IRE-1: phosphorylated inositol requiring kinase 1; P-eIF2-α: phosphorylated eukaryotic translation initiation factor 2; RPLS: ribosomal protein L5; RPL7: ribosomal protein L7; RPL21: ribosomal protein L21; RPL22: ribosomal protein L22; RPL23A: ribosomal protein L23A; RPL26: ribosomal protein L26; RPL27: ribosomal protein L27; RPL30: ribosomal protein L30; RPL31: ribosomal protein L31; RPS3A: ribosomal protein S3A; RPS5: ribosomal protein S5; RPS6: ribosomal protein S6; RPS10: ribosomal protein S10; RPS13: ribosomal protein S13; RPS16: ribosomal protein S16; RPS17: ribosomal protein S17; rRNA 28S: ribosomal RNA subunit 28; rRNA18S: ribosomal RNA subunit 18; UBTF: UBF, upstream binding transcription factor, RNA polymerase I; XBP1: Xbox binding protein 1; XPNPEP1: X-prolylaminopeptidase P1.

Competing interest

No relevant data; no conflicts of interest.

Authors'contribution

PGE did the majority of experiments and wrote the preliminary version of material and methods, results, and prepared the figures; KHO and LG helped in the study of nucleolar proteins; AK contributed to the study of gene expression of ribosomal proteins; RD and EC carried out FACS studies; MC gave technical support; IF designed and supervised the study and wrote and circulated the paper for discussion. All the authors read and approved the final manuscript.

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Author details

[1]Institute of Neuropathology, Bellvitge University Hospital, University of Barcelona, Bellvitge Biomedical Research Institute (IDIBELL), Hospitalet de Llobregat; Biomedical Research Center of Neurodegenerative Diseases (CIBERNED), Barcelona, Spain. [2]Department of Genetics, Medical School, Alfonso X el Sabio University, Villanueva de la Cañada, Madrid, Spain. [3]Cancer Epigenetics and Biology Program, IDIBELL, Hospitalet de Llobregat, Barcelona, Spain. [4]Biology-Bellvitge Unit, Scientific and Technological Centers-University of Barcelona (CCiTUB), Hospitalet de Llobregat, Barcelona, Spain. [5]Institute of Neuropathology, Service of Pathologic Anatomy, Bellvitge University Hospital, carrer Feixa Llarga s/n, 08907, Hospitalet de Llobregat, Spain.

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Additional files and supplementary data are present in the following link:
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ARTICLE 2

Mitochondrial activity in the frontal cortex and angular gyrus in Parkinson's disease and Parkinson's disease with dementia.

Garcia-Esparcia P, Koneti A, Rodríguez-Oroz MC, Schlutter A, Pujol A, del Rio JA, Ferrer I.

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ABSTRACT

Introduction: Altered mitochondrial function mainly manifested by reduced complex I activity is characteristic in the substantia nigra in Parkinson's disease (PD). Information about mitochondria in other brain regions such as the cerebral cortex is conflicting mainly because most studies have not contemplated the possibility of variable involvement depending on the region, stage of disease progression, and relevant clinical symptoms such as the presence or absence of dementia. **Methods:** RT-qPCR of 18 mRNAs encoding subunits of mitochondrial complexes and 12 mRNAs encoding energy metabolism-related enzymes; western blotting of mitochondrial proteins; and analysis of enzymatic activities of complexes I, II, II, IV and V of the respiratory chain were assessed in frontal cortex area 8 and the angular gyrus of middle-aged individuals (MA), and those with incidental PD (iPD), long-lasting PD with parkinsonism without dementia (PD) and long-lasting PD with later appearance of dementia (PDD). **Results:** Up-regulation of several genes was found in frontal cortex area 8 in PD when compared with MA and in the angular gyrus in iPD when compared with MA. Marked down-regulation of genes encoding mitochondrial subunits and energy metabolism-related enzymes occurs in frontal cortex but only of genes coding for energy metabolism-related enzymes in the angular gyrus in PDD. Reduced enzymatic activity of complex III in frontal cortex and angular gyrus occurs in PD, but a dramatic reduction in the activity of complexes I, II, II and IV in both regions characterizes PDD. **Conclusions:** Dementia in the context of PD is linked to region-specific deregulation of genomic genes encoding subunits of mitochondrial complexes and to marked reduction in the activity of mitochondrial complexes I, II, III and IV.

Mitochondrial activity in the frontal cortex and angular gyrus in Parkinson's disease and Parkinson's disease with dementia

Paula Garcia-Esparcia MSc[1], Anusha Koneti PhD[1], M Cruz Rodríguez-Oroz, MD, PhD[2, 3,4], Belen Lago, MsC[3,4]; José Antonio del Rio [4,5, 6], Isidro Ferrer MD, PhD[1,4,6, 7,CA]

[1] Institute of Neuropathology, Bellvitge University Hospital, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), Hospitalet de LLobregat, Spain

[2] University Hospital Donostia, San Sebastián, Spain; Ikerbasque (Basque Foundation for Science), Bilbao, Spain

[3] Biodonostia Research Institute, San Sebastián, Spain

[4] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain

[5]Molecular and Cellular Neurobiotechnology, Institute of Bioengineering of Catalonia (IBEC), Parc Científic de Barcelona, Department of Cell Biology, University of Barcelona, Barcelona, Spain

[6] Institute of Neurosciences, University of Barcelona, Barcelona, Spain

[7] Department of Pathology and Experimental Therapeutics, University of Barcelona, Hospitalet de Llobregat, Spain

CA: Corresponding author:

Prof. Isidro Ferrer, Institute of Neuropathology, Service of Pathologic Anatomy, Bellvitge University Hospital, carrer Feixa Llarga s/n, 08907 Hospitalet de Llobregat, Spain; Tel.: +34 93 260 7452; Fax: +34 93 260 7503. Email: *8082ifa@gmail.com*

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No relevant data.

Abstract

Altered mitochondrial function mainly manifested by reduced complex I activity is characteristic in the substantia nigra in Parkinson's disease (PD). Information about mitochondria in other brain regions such as the cerebral cortex is conflicting mainly because most studies have not contemplated the possibility of variable involvement depending on the region, stage of disease progression, and relevant clinical symptoms such as the presence or absence of dementia. RT-qPCR of 18 mRNAs encoding subunits of mitochondrial complexes and 12 mRNAs encoding energy metabolism-related enzymes; western blotting of mitochondrial proteins; and analysis of enzymatic activities of complexes I, II, II, IV and V of the respiratory chain were assessed in frontal cortex area 8 and the angular gyrus of middle-aged individuals (MA), and those with incidental PD (iPD), long-lasting PD with parkinsonism without dementia (PD) and long-lasting PD with later appearance of dementia (PDD). Up-regulation of several genes was found in frontal cortex area 8 in PD when compared with MA and in the angular gyrus in iPD when compared with MA. Marked down-regulation of genes encoding mitochondrial subunits and energy metabolism-related enzymes occurs in frontal cortex but only of genes coding for energy metabolism-related enzymes in the angular gyrus in PDD. Significant decrease in the protein expression levels of several mitochondrial subunits occurs equally in frontal cortex and angular gyrus in PDD. Reduced enzymatic activity of complex III in frontal cortex and angular gyrus is observed in PD, but dramatic reduction in the activity of complexes I, II, II and IV in both regions characterizes PDD. Dementia in the context of PD is linked to region-specific deregulation of genomic genes encoding subunits of mitochondrial complexes and to marked reduction in the activity of mitochondrial complexes I, II, III and IV.

Key words: Parkinson disease, incidental PD, dementia, PDD, mitochondria, energy metabolism, respiratory chain, oxidative phosphorylation

Introduction

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder which in typical cases starts in the autonomic nervous system and olfactory bulb and then progresses to the medulla oblongata, pons and midbrain, and limbic system, eventually involving the neocortex several years after the beginning of symptoms. The classification of disease progression into six stages is based on the presence of the hallmark pathological lesions and is useful to explain clinical manifestations which parallel neuropathological lesions in typical cases, at least at the first and middle stages of the disease (Braak et al., 2003; Dickson et al., 2008; Dickson et al., 2010; Jellinger et al., 2009). Hallmark pathological lesions are Lewy bodies and neurites (LB inclusions) composed of abnormal α-synuclein (Jellinger, 2011).

Altered mitochondrial function mainly characterized by reduced complex I activity and increased oxidative damage is well documented in the substantia nigra in PD where dopaminergic neurons projecting to the striatum degenerate and LB inclusions are abundant. This is accompanied by oxidative damage (Schapira, 2008). Combined phosphorus and proton magnetic resonance spectroscopic imaging have also shown mitochondrial dysfunction in midbrain and putamen in PD (Hattingen *et al*., 2009). Mitochondrial degeneration and oxidation of several mitochondrial-associated proteins have also been observed in α-synuclein transgenic mice used as a model of PD (Poon *et al*., 2005; Martin *et al*., 2006).
Oxidative damage is not restricted to the substantia nigra and striatum in PD. Increased expression of oxidative stress markers and altered oxidative responses occur in the frontal cortex (Alam *et al*., 1997; Power *et al*., 2009; Mythri *et al*., 2011; Harish *et al*., 2013). This is further supported by the identification in the frontal cortex of proteins which are damaged as a result of oxidation, including α-synuclein, β-synuclein, UCHL1, SOD1, SOD2 and DJ-1 (Choi *et al*., 2004; Choi *et al*., 2005; Dalfó *et al*., 2005; Dalfó and Ferrer, 2008; Martínez *et al*., 2010). Moreover, several key proteins are targets of oxidative damage in the frontal cortex even at very early stages of PD, such as aldolase A, enolase 1 and glyceraldehyde dehydrogenase (GAPDH), all of them involved in glycolysis and energy metabolism (Gómez and Ferrer, 2009).

Oxidative damage and disassembly of complex I subunits, functional impairment of complex I activity and adaptive responses are found in the frontal cortex at

advanced stages of PD, in some cases in patients who probably suffered from dementia (Keeney *et al*., 2006; Parker *et al*., 2008; Navarro *et al*., 2009), and in PD plus dementia (Gatt *et al*., 2016). Altered mitochondrial function is also observed in dementia with Lewy bodies (DLB) (Navarro *et al*., 2009). However, little information is available regarding mitochondrial alterations in the cerebral cortex with disease progression from early pre-motor stages of PD to advanced stages without and with associated dementia.

Based on these data, the objective of the present study was to characterize the involvement of mitochondria in the cerebral cortex in PD *post-mortem* samples in three groups: iPD, cases without parkinsonian symptoms categorized neuropathologically as stages 3 and 4 and dying of fatal concurrent events that disrupted life expectancies; PD, cases with a long history of parkinsonism and subjected to anti-parkinsonian therapy for several years without dementia, categorized neuropathologically as stages 5 and 6 without dementia; and PDD, cases with long lasting parkinsonism who developed dementia after several years from the beginning of the parkinsonian symptoms, categorized neuropathologically as stage 6. Two regions, frontal cortex area 8 and the angular gyrus, both progressively involved along with disease duration, were analyzed in parallel.

Material and methods

Human cases

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank following the guidelines of Spanish legislation on this matter and the approval of the local ethics committee. Processing of brain tissue has been detailed elsewhere (Ravid and Ferrer, 2012). The *post-mortem* interval between death and tissue processing was between 2 and 21h 45min. One hemisphere was immediately cut in coronal sections, 1cm thick, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags and stored at -80ºC until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphological studies. Neuropathological diagnosis in all cases was based on the routine study of 20 selected de-waxed paraffin sections comprising different regions of the cerebral cortex, diencephalon, thalamus, brain stem and cerebellum, which were stained with haematoxylin and eosin, and Klüver-Barrera, and for immunohistochemistry for microglia, glial fibrillary acidic protein, β-amyloid, phosphorylated tau (clone AT8), α-synuclein, TDP-43, ubiquitin and p62. Neuropathological staging of PD was based on the classification of Braak et al (Braak *et al*., 2003). Only typical cases according to the Braak classification-those with progressive involvement of the medulla oblongata, pons, substantia nigra, limbic system and neocòrtex-were included. Cases with combined pathologies (i.e., Alzheimer's disease excepting sporadic AD-related pathology stages I-II/0-A (Braak and Braak, 1991), tauopathy, cerebrovascular diseases, and metabolic syndrome) were excluded from the present study. Middle-aged cases (MA) (n=34, 23 men, 11 women; age, 65.4 ± 14.8 years) had not suffered from neurologic, psychiatric, or metabolic diseases (including metabolic syndrome), and did not have abnormalities in the neuropathological examination excepting sporadic AD stages I-II/0-A. PD cases with more advanced stages of sAD-related pathology were not considered in the present series to avoid overlap between PD and AD in the frontal cortex and angular gyrus.

Clinically, iPD cases (n=15, 12 men, 3 women; age 69.6 ± 8.6) at stages 3 and 4 were apparently asymptomatic and were identified at the time of the *post-mortem* neuropathological examination. PD cases (n=20, 11 men, 9 women; age, 78.5 ± 4.4 years) at stages 5 and 6 had suffered from parkinsonism and had received treatment during the duration of the disease but did not have dementia. PDD

cases (n = 4 men, age 80.5 ± 3.4 years), all of them stage 6, had suffered from parkinsonism for more than ten years and later developed dementia. The causes of death in these cases were unrelated events that reduced survival expectancy, including cardiac infarction, disseminated carcinoma and infectious diseases.

Two cortical regions were examined: frontal cortex area 8 and the angular gyrus. A summary of all the cases is shown in Table I. Not all regions were assessed every case due to the limited availability of tissues.

RNA purification

Purification of RNA from right frontal cortex area 8 and the angular gyrus was carried out using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer and performing the optional DNase digest to avoid extraction and later amplification of genomic DNA. The concentration of each sample was obtained from A260 measurements with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values of RNA integrity number (RIN) varied from 6.4 to 8.8 (Table I). *Post-mortem* delay had no effect on RIN values in the present series (Table I).

Retrotranscription reaction

Retrotranscription reaction of RNA samples selected based on their RIN values was carried out with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) following the guidelines provided by the manufacturer, and using Gene Amp® 9700 PCR System thermocycler (Applied Biosystems). A parallel reaction for one RNA sample was processed in the absence of reverse transcriptase to rule out DNA contamination.

Real-time PCR

Real-time quantitative PCR (RT-qPCR) assays were conducted in duplicate on 1,000ηg of cDNA samples obtained from the retrotranscription reaction, diluted 1:20 in 384-well optical plates (Kisker Biotech, Steinfurt, GE) utilizing the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Parallel amplification reactions were carried out using 20x TaqMan Gene Expression Assays and 2x TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probes used in the study are shown in Table II.

Parallel assays for each sample were carried out using probes for β-glucuronidase (GUS-β) and X-prolyl aminopeptidase P1 (XPNPEP1) for normalization. The reactions were performed using the following parameters: 50ºC for 2 min, 95ºC for 10 min, 40 cycles at 95ºC for 15 s, and 60ºC for 1 min. TaqMan PCR data were captured using the Sequence Detection Software (SDS version 2.2, Applied Biosystems). Subsequently, threshold cycle (CT) data for each sample were analyzed with the double delta CT (ΔΔCT) method. First, delta CT (ΔCT) values were calculated as the normalized CT values for each target gene in relation to the endogenous controls GUS-β and XPNPEP1. These housekeeping genes were selected because they show no modifications in several neurodegenerative diseases in human *post-mortem* brain tissue (Barrachina *et al*., 2006; Durrenberger *et al*., 2012). A similar pattern was observed using GUS-β and XPNPEP1 for normalization (data not shown). GUS-β was selected as a housekeeping gene for representation. Finally, ΔΔCT values were obtained with the ΔCT of each sample minus the mean ΔCT of the population of control samples (calibrator samples). The fold-change was determined using the equation $2^{-\Delta\Delta CT}$ (Garcia-Esparcia *et al*., 2013).

Statistical analysis for RT-qPCR

The normality of distribution of the mean fold-change values obtained by RT-qPCR for every region and stage between controls and PD cases was analyzed with the Kolmogorov-Smirnov test. The non-parametric Mann-Whitney test was performed to compare each group when the samples did not follow a normal distribution whereas the unpaired Student's T-test was used for normal variables. Statistical analysis was performed with GraphPad Prism version 5.01 (La Jolla, CA, USA) and Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (Warrenton, VA, USA). Differences between groups were considered statistically significant at *P*-values: *p < 0.05, **p < 0.01 and ***p < 0.001.

Gel electrophoresis and western blotting from total homogenates

0.1g of sample brain tissue from frontal cortex area 8 and the angular gyrus (see Table I) were homogenized with a glass homogenizer in Mila lysis buffer (0.5M Tris at pH 7.4 containing 0.5 methylenediaminetetraacetic acid at pH 8.0, 5M NaCl, 0.5% Na doxicholic, 0.5% Nonidet P-40, 1mM phenylmethylsulfonyl fluoride, bi-distilled water, protease and phosphatase inhibitor cocktails) (Roche Molecular Systems, Pleasanton, CA, USA), and then centrifuged for 15 min at 13,000rpm at

4°C (Ultracentrifuge Beckman with 70Ti rotor, CA, USA). Protein concentration was measured with a Smartspect™plus spectrophotometer (Bio-Rad, CA, USA) using the Bradford method (Merck, Darmstadt, Germany). Samples containing 20µg of protein and the standard Precision Plus Protein™ Dual Color (Bio-Rad) were loaded onto 10-15% acrylamide gels. Proteins were separated in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes using the Trans-Blot®Turbo™ transfer system (Bio-Rad) at 200mA/membrane for 40 min. Non-specific bindings were blocked by incubation in 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween for 1 h at room temperature. After washing, the membranes were incubated at 4°C overnight with each of the following antibodies in TBS containing 5% albumin and 0.1% Tween: monoclonal antibody anti-cytochrome c oxidase subunit VIIa polypeptide 2 like, COX7A2L (1:800; 66062-1-Ig, Proteintech, Chicago, IL, USA); polyclonal antibodies anti-NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 7, NDUFA7 (1:100; 15300-1-AP, Proteintech); anti-NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10, NDUFA10 (1:2,000; GTX114572, GeneTex, CA, USA); anti-NADH dehydrogenase (ubiquinone) 1 beta subcomplex 10, NDUFB10 (1:3,000; 15589-1-AP, Proteintech) and anti-NADH dehydrogenase (ubiquinone) Fe-S protein 8, NDUFS8 (1:1,000; GTX114119, GeneTex). Finally, monoclonal antibody anti-β-actin (1:30,000, A5316; Sigma-Aldrich, St. Louis, MO, USA) was blotted for the control of protein loading. Afterwards, the membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibody (1:2,000, Dako, Glostrup, Denmark), and the immune-complexes were revealed with a chemiluminescence reagent (ECL, Amersham, GE Healthcare, Buckinghamshire, UK). Densitometry of western blot bands was assessed with the TotalLab program (TotalLab Quant, Newcastle, UK) and subsequently analyzed with Student's T-test using the Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (VA, USA); differences were considered statistically significant with *p*-values: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Isolation of mitochondrial-enriched fractions from human brain tissue

Mitochondria were extracted from frozen human brain tissue (100mg) under ice-cold conditions. Tissues were minced in ice-cold isolation buffer (IB) containing 0.25M sucrose, 10mmol/l Tris and 0.5mmol/l EDTA, pH 7.4, and then homogenized and centrifuged at 1,000xg for 10 min. Samples were homogenized

with a micro-pestle using 10 volumes of buffer per mg of tissue and centrifuged at 1,000xg for 10 min at 4ºC. The supernatant (S1) was conserved. The pellet was washed with 2 volumes of IB and centrifuged again under the same conditions. This last supernatant (S2) was combined with S1. Centrifugation at 10,000xg for 10 min at 4ºC resulted in the mitochondria-enriched pellet. The supernatant (S3) was discarded and the pellet was washed with 2 volume of IB. The pellet was finally re-suspended in 1 volume of IB and stored at -80ºC. Protein concentration was measured by Smartspect^{TM}plus spectrophotometer (Bio-Rad, CA, USA) using the Bradford method (Merck, Darmstadt, Germany). Sonication was performed as described (Long *et al*., 2009). Optimal sonication conditions were 10 sec 8 times with minimal intervals at 3W (Zheng *et al*., 1990). The mitochondrial enrichment was used for mitochondrial enzymatic activities and for western blotting using anti-α-synuclein polyclonal antibody (1:10,000; A5316, Chemicon, Darmstadt, Germany); anti-neuroketal (NKT, 1:1,000; AB5611, Chemicon); and anti N-Tyrosine (N-Tyr, 1:500, 32-1900, Thermo Fisher – Invitrogen, Carlsbad, CA, USA). Differences in protein loading were corrected with anti-β-actin (1:30,000, A5316; Sigma-Aldrich); mitochondrial levels were assessed with anti-oxidative phosphorylation-ATP5A (1:10,000; MS601, Mitoscience, Eugene, OR, USA).

Mitochondrial enzymatic activities

The individual activities of mitochondrial complexes I, II, III, IV and V were analysed using commercial kits following the manufacturers' instructions (Mitochondrial complex I, II, IV and V: Novagen, Merck Biosciences, Darmstadt, Germany; Mitochondrial complex III: MyBiosource, CA, USA). 25µg of mitochondria was loaded into each well. Activity of citrate synthase was evaluated following validated protocols (Spinazzi *et al*., 2012) with slight modifications. The activity of citrate synthase was determined as the rate of reduction of DTNB (5', 5'-dithiobis (2-nitrobenzoic acid)) to thionitrobenzoic acid at 412nm. For this purpose, 25µg of mitochondria was added to a 1ml mixture containing 500µl of Tris (200mM, pH8.0) with Triton X-100 (0.2% (vol/vol)), 100µl of DTNB, and 30µl of 10mM Acetyl CoA; the final volume was adjusted to 950µl with distilled water. The reaction was started by the addition of 50µl of 10mM oxalacetic acid. The increase in absorbance at 412 nm was read for 3 min at room temperature with a DU® 800UV/Visible spectrophotometer (Beckman Coulter, CA, USA) in 1ml polystyrene or methacrylate cuvettes.

Statistical analysis for mitochondrial activities

The enzymatic activities for each mitochondrial complex were expressed as a rate of nmol/min per mg of mitochondrial protein per protein concentration normalized with the mitochondrial complex activity rate of citrate synthase activity. Data were presented as mean ± standard error of the mean (SEM) in all the experiments. All the data were analyzed with Student's T-test using GraphPad Prism version 5.01 (La Jolla, CA, USA) and Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (Warrenton, VA, USA). In all experimental procedures the significance level was set at *p < 0.05; **p < 0.01; ***p < 0.001.

Results

mRNA expression levels of selected mitochondrial subunits in frontal cortex area 8

No differences in the expression levels of the majority of assessed genes were observed in iPD when compared with MA excepting *NDUFB3* and *ATP5H,* which decreased in iPD (Table III). However, a significant increase in the expression of *NDUFA7*, *NDUFA10*, *NDUFB7*, *NDUFB10*, *NUDFS8*, *SDHB*, *UQCR11*, *COX7A2L* and *ATP5G2* mRNAs was found in PD compared with MA individuals. *NDUFA2*, *NDUFA7*, *NDUFA10*, *NDUFB3*, *ATP5G2*, *ATP5H* and *ATP5L* were significantly increased when comparing PD with iPD (Table III).Two genes deregulated in arrays, NDUFS7 and ATP5G2, were not validated by RT-qPCR.

In contrast to PD, a marked decrease in the expression of genes encoding several subunits of mitochondrial complexes was found in PDD. Significant down-regulation in *SDHB*, *COX7C* and *ATP5G2* was observed when comparing MA cases to PDD. More dramatic change was recognized when comparing PD and PDD, as the expression of twelve of the eighteen genes analysed was significantly decreased in PDD, including *NDUFA7*, *NDUFA10*, *NDUFB7*, *NDUFB10*, *NDUFS8*, *SDHB*, *UQCRB*, *COX7A2L*, *COX7C*, *ATP5G2*, *ATP5*L and *ATP5O* (Table III). Values were similar using *GUS-β* and *XPNPEP1* for normalization but *GUS-β* was selected as housekeeping for representation.

mRNA expression levels of energy metabolism-related enzymes in frontal cortex area 8

ATP2B4, AT6V0A1 and *TOMM40* were significantly decreased and *ATP4A* increased in iPD when compared with MA. *ZNF642* was up-regulated in PD when

compared with MA. However, the expression levels of *ATP2B4*, *ATP4A*, *ATP6V0A1*, *ATP6VOB*, *TOMM40* and *ZNF642* mRNAs were significantly increased in PD when compared with iPD (Table III). In contrast to PD cases, a marked decrease in the expression of genes related to energy metabolism was found in PDD. *ATP2B4*, *ATP6V0B*, *FAM82A2* and *LHPP* mRNA expression was significantly reduced in PDD when compared with MA. *ATP2B4*, *ATP6V0A1*, *ATP6V0B*, *ATP6V1H* and *ZNF642* were down-regulated in PDD in comparison with PD (Table III).

mRNA expression levels of selected mitochondrial subunits in the angular gyrus

NDUFA7, *NDUFA10*, *NDUFB10*, *NDUFS7*, *UQCRB*, *UQCR11*, *COX7A2L, ATP5D* and *ATP5G2* mRNA expression was up-regulated in iPD when compared with MA (Table IV). However, only NDUFA10 mRNA levels were increased when comparing PD with MA cases (Table IV). This was due to a shift between iPD and PD as *NDUFA7*, *NDUFB10*, *NDUFS7*, *NDUFS8* and *COX7A2L* were significantly decreased in PD when compared with iPD (Table IV).

Interestingly, no apparent changes were found in PDD when compared with MA cases. However, *NDUFA2*, *NDUFA7*, *NDUFA10*, *NDUFB10*, *NDUFS7*, *SDHB*, *UQCR11*, *COX7A2L, ATP5G2* and *AT5O* were significantly down-regulated in PDD when compared with iPD. *COX7A2L* mRNA was decreased in PDD when compared with PD (Table IV).

mRNA expression levels of energy metabolism-related enzymes in the angular gyrus

Two genes out of twelve, *ATP6V0B* and *ATP6V1H1*, encoding energy metabolism-related enzymes assessed in the present study were up-regulated in iPD when compared with PD (Table IV). No significant differences were observed in PD when compared with MA. However, *ATP6V0B* and *ATP6V1H1* were significantly reduced in PD when compared with iPD (Table IV). Gene expression was markedly decreased in PDD cases when compared with MA, iPD and PD. *ATP2B4*, *FAM82A2* and *LHPP* were down-regulated when comparing MA with PDD. *ATP2B4*, *ATP6V0B*, *ATP6V1H*, *SLC6A6* and *ZNF642* were decreased in PDD cases compared with iPD. *ATP2B4*, *ATP6V1H*, *FAM82A2* and *LHPP* were significantly down-regulated in PDD in comparison with PD (Table IV).

Protein expression levels of selected subunits of mitochondrial complexes in frontal cortex area 8 and angular gyrus in iPD, PD and PDD

Western blot was carried out using antibodies to proteins NADH dehydrogenase (ubiquinone) 1 alpha sub-complex, 7 (transcribed by *NDUFA7*), NADH dehydrogenase (ubiquinone) 1 alpha sub-complex, 10 (transcribed by *NDUFA10*), NADH dehydrogenase (ubiquinone) 1 beta sub-complex 10 (transcribed by *NDUFB10*), NADH dehydrogenase (ubiquinone) Fe-S protein 8 (transcribed by *NDUFS8*) and cytochrome c oxidase subunit VIIa polypeptide 2 (transcribed by *COX7A2L*). Expression levels were normalized with β-actin and VDAC. NDUFA7 was significantly decreased in the frontal cortex in iPD in comparison with MA cases, but significantly increased in PD when compared with iPD. NDUFS8 was significantly increased in PD when compared to MA (Figure 1A). NDUFA7 and NDUFS8 protein levels matched corresponding mRNA values (Table III). No variations of NDUFA10, NDUFB10 and COX7A2L protein levels were observed in the frontal cortex in iPD or PD compared with MA (Figure 1A).However, significant decrease in the expression levels of proteins NDUFA7, NDUFB10, NDUFS8 and COX7A2L was found in frontal cortex in PDD cases (Fig. 1A) matching with values of mRNA expression (Table III).

No modifications in NDUFA7, NDUFB10 and NDUFS8 protein expression levels were seen in the angular gyrus in iPD and PD. However, NDUFA10 was significantly increased in PD when compared with MA, and COX7A2L was significantly increased in iPD but not in PD when compared with MA (Figure 1B). NDUFA10, NDUFB10 and COX7A2L protein levels matched corresponding mRNA values obtained with RT-qPCR (Table IV). Significant decrease in NDUFA7, NDUFA10, NDUFB10, NDUFS8 and COX7A2L protein levelos occurred in the angular gyrus in PDD (Fig. 1B) matching with corresponding mRNA levels (Table IV). Results were similar when using β-actin and VDAC for normalisation (Fig 1).

Activity of mitochondrial complexes I, II, III, IV and V in frontal cortex area 8 and angular gyrus in PD

No modifications in the activity of complexes I, II and V were observed in frontal cortex area 8 in PD normalized with citrate synthase activity (Figure 2A). Activity of complex III was decreased in PD while complex IV showed the opposite

pattern, with an increase in the same stages of the pathology; p-values <0.05 (Figure 2A).

Enzyme activities in the angular gyrus showed an equal pattern when comparing MA cases to PD with no variations except in complex III, which showed reduced activity with a significant p-value <0.05 (Figure 2B). All the enzymatic activities of mitochondrial complexes were corrected for citrate synthase activity.

Activity of mitochondrial complexes I, II, III, IV and V in frontal cortex area 8 and angular gyrus in PDD

Mitochondrial enzymatic activities of complexes I, II, III and IV were markedly reduced in frontal cortex area 8 in PDD when compared with MA individuals (p-values between 0.05 and 0.01) (Figure 3A). Similar alterations were seen in the angular gyrus. A marked reduction in the activity of complexes I, II, II and IV was found in PDD when compared with MA (p-values between 0.05 and 0.01) (Figure 3B).

α-synuclein protein expression and NKT protein adduct levels in mitochondrial-enriched fractions

Protein expression levels of α-synuclein were analysed with western blotting in mitochondrial-enriched fractions of frontal cortex area 8 in MA and PD cases. Levels of monomeric α-synuclein (17 kDa) were similar in MA and PD cases. However, protein levels of oligomeric forms of 37 kDa and 50 kDa of α-synuclein were significantly increased in PD when compared with MA (Fig. 4). β-actin was used as a control of protein loading, and ATP5A as a marker of mitochondria, revealing no differences between MA and PD.

Levels of neuroketal (NKT) protein adducts were significantly increased (p<0.01) in mitochondrial-enriched fractions of frontal cortex area 8 in PD when compared with MA cases (Fig. 4B). Levels of N-Tyrosine were similar in mitochondrial-enriched fractions of frontal cortex area 8 in PD when compared with MA cases (Fig. 4C).

Discussion

Molecular studies in human *post-mortem* brain tissue have revealed that metabolic alterations precede neurological symptoms in most neurodegenerative diseases by many years. In particular, multiple defects converge, at least in the frontal cortex, at early stages of PD even in the absence of deficits in cortical function (Ferrer, 2009; Ferrer et al., 2012).

Expression of selected genomic genes encoding mitochondrial protein subunits of the respiratory chain complexes and the activities of complex I, II, III, IV and V was analyzed in the frontal cortex area 8 and the angular gyrus in neuropathologically-verified iPD, PD and PDD cases. The selection of genes was based on parallel studies carried out in several neurodegenerative diseases with abnormal protein aggregates in which the same genes were analyzed including several subunits of complex I-V. Although this approach does not cover all the mitochondrial subunits, it serves to analyze comparative studies among diseases with abnormal protein aggregates in old age. This aspect is founded on the hypothesis that although mitochondria are vulnerable in various neurodegenerative diseases, the alteration of subunits and susceptibility of complexes is disease-dependent. The study of mitochondria is accompanied by gene expression analysis of other energy metabolism-related enzymes.

Previous studies have shown reduced metabolism, as revealed with [(18)F]-fluorodeoxyglucose Positron Emission Tomography (PET), in the frontal cortex, and progression to the angular gyrus, orbital cortex, posterior cingulus and occipital lobes in PD patients with mild cognitive impairment (Lyoo *et al*., 2010; Bohen *et al*., 2011; Garcia-Garcia *et al*., 2012). Neuroimaging analyses have also suggested that cortical hypometabolism is present even at early stages of PD (Borghammer *et al*., 2010; Berti *et al*., 2012). Interestingly, hypometabolism is followed by cortical atrophy in the same areas in PDD (González-Redondo *et al*., 2014).

Present findings show regional differences in the expression of several nuclear genes encoding subunits of mitochondrial complexes and energy metabolism-related enzymes. Most important changes in frontal cortex area 8 are manifested by up-regulation of genes encoding subunits of complexes I, II, III, IV and V in PD when compared with MA, and energy-metabolism related enzymes when comparing iPD with PD. In contrast, gene up-regulation in the angular gyrus

occurs in iPD when compared with MA, and values mostly return to normal levels in PD. Protein levels of selected subunits parallel values of corresponding gene expression. Therefore, altered regulation of genes and proteins related to mitochondria and energy metabolism is an early region-dependent event in the frontal cortex and angular gyrus within the spectrum of PD. However, these alterations have only a moderate impact on the activity of mitochondrial complexes, as only complex III activity is reduced in the frontal cortex and angular gyrus in PD. These changes do not contradict the marked impairment of mitochondria biogenesis, altered mRNA expression, deregulation of several microRNAs predicted to interact with complex I regulators, and alterations of protein levels which also occur in frontal cortex in PD (Thomas *et al*., 2010). Rather, they are consistent with a scenario in which several molecules, including mitochondrial membrane proteins, ion channels and mitochondrial subunits, are altered to determinate thresholds until they produce altered activity. In this line, oxidative damaged of certain protein subunits, rather than reduced protein levels, can jeopardize mitochondrial function in PD (Keeney *et al*., 2006).

Dramatic changes occur in PDD as the expression of genes encoding mitochondrial subunits and energy-related enzymes is markedly down-regulated in frontal cortex area 8, whereas down-regulation mainly involves energy-related enzymes in the angular gyrus in PDD when compared with PD. Nevertheless, protein expression of several mitochondrial subunits falls in frontal cortex and angular gyrus in PDD. Differences between mRNA and protein expression levels are not uncommon as protein translation is modulated by several molecules mainly non-coding RNAs. Changes in protein expression could be the result of mitochondrial loss although decreased protein values are similarly affected when using β-actin and VDAC for normalization. However, reduced mRNA expression corresponds to nuclear-encoded mRNAs therefore indicating that, at least, part of mitochondrial dysfunction in PD is linked to abnormalities in nuclear mRNA processing of genes encoding subunits of mitochondrial complexes. Reduced complex I activity has been reported in frontal cortex area 8 at advanced stages of PD in which unfortunately no clinical information about their cognitive status was available (Keeney *et al*., 2006; Parker *et al*., 2008; Navarro *et al*., 2009; Navarro and Boveris, 2009). More focused studies on PD and dementia have shown reduced complex I activity only in PDD, and not in PD (Gatt *et al*., 2016). The present observations in PDD are in the same line although decreased activity

affects not only complex I but also complexes I, II, III and IV. Moreover, reduced mitochondrial activity is not restricted to the frontal cortex; the angular gyrus, the other region assessed in the present study, has similar mitochondrial functional deficiencies. Curiously, the expression of nuclear genes encoding subunits of mitochondrial complexes is dramatically reduced in the frontal cortex, but not in the angular gyrus, in PDD, thus indicating regional differences in the transcription of nuclear genes encoding mitochondrial subunits between frontal cortex and angular gyrus.

Reduced mitochondrial DNA levels have been reported in the prefrontal cortex in PDD but not in PD, thus suggesting that primary alterations in mitochondrial DNA are causative of mitochondrial dysfunction and dementia in PDD (Gatt *et al*., 2016). However, the expression of mitochondrial subunits encoded by mitochondrial DNA was not examined in that study. Present findings have also shown the presence of α-synuclein oligomers in mitochondrial-enriched fractions in PD thus suggesting that local oligomers may affect mitochondrial function. In this line, abnormal α-synuclein oligomers are detected in the nuclear fractions of FACS-isolated neurons in frontal cortex in PD in association with marked alterations in the expression of genes and nuclear proteins involved in the machinery of protein synthesis (Garcia-Esparcia *et al*., 2015). Moreover, increased levels of NKT protein adducts in mitochondrial-enriched fractions point to increased protein damage derived from docosahexaenoic acid peroxidation end-products (DHA; C22:6 ω3) (Bernoud-Hubac *et al*., 2001).

The present data together with previous studies suggest that the scenario is not simple as mitochondrial dysfunction in cerebral cortex and perhaps in other regions in PD may be due to the combination of altered nuclear and mitochondrial transcription which in turn may depend on the preservation of genomic and mitochondrial DNA, and in post-translational modifications of mitochondrial subunits linked to oxidative damage.

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Abbreviations

ATP2B3: ATPase, Ca++ transporting, plasma membrane 3
ATP2B4: ATPase, Ca++ transporting, plasma membrane 4
ATP4A: ATPase, H+/K+ exchanging, alpha polypeptide
ATP5D: ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit
ATP5G2: ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2 (subunit 9)
ATP5H: ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d
ATP5L: ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G
ATP5O: ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit
ATP6V0A1: ATPase, H+ transporting, lysosomal V0 subunit a1
ATP6V0B: ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b
ATP6V1H1: ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H
COX7A2L: cytochrome c oxidase subunit VIIa polypeptide 2 like
COX7C: cytochrome c oxidase subunit VIIc
FAM82A2: family with sequence similarity 82, member A2
GUS-B: B-glucuronidase
LHPP: phospholysine phosphohistidine inorganic pyrophosphate phosphatase
NDUFA2: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa
NDUFA7: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7, 14.5kDa
NDUFA10: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa
NDUFB3: NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa
NDUFB7: NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa
NDUFB10: NADH dehydrogenase (ubiquinone) 1 beta subcomplex 10, 22kDa
NDUFS7: NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase)
NDUFS8: NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)
SDHB: succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
SLC6A6: solute carrier family 6 (neurotransmitter transporter, taurine), member 6
SLC25A31: solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31
TOMM40: translocase of outer mitochondrial membrane 40 homolog (yeast)
UQCRB: ubiquinol-cytochrome c reductase binding protein
UQCR11: ubiquinol-cytochrome c reductase, complex III subunit XI

XPNPEP1: X-prolyl aminopeptidase P1
ZNF642: zinc finger protein 642

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Tables and Figures

Table I. Summary of cases used for mRNA expression, western blot and mitochondrial activity studies in the frontal cortex area 8 (FC) and in the angular gyrus (AG). PD: Parkinson's disease, 0: Cases 1-34 are middle-aged individuals with no neurological symptoms and without neuropathological evidence of disease; iPD: incidental PD (cases 35-49, stages 3, 4 of Braak without clinical symptoms of parkinsonism), PD: treated parkinsonism of long duration (cases 50-69, stages 5, 6 of Braak); PDD: PD with dementia (cases 70-73, stage 6 with long-lasting parkinsonism and late development of dementia); M: male; F: female; Age in years; *post-mortem* delay in hours and minutes; RIN: values of RNA integrity number; WB: western blot; MA: mitochondrial activity complexes.

Table II. Abbreviated and full names of genes, and TaqMan probes used for the study of mRNA expression including housekeeping genes GUS-β and XPNPEP1 used for normalization.

Table III. mRNA expression levels of genes encoding subunits of the mitochondrial complexes and energy metabolism-related enzymes in the frontal cortex MA, iPD, PD and PDD as determined with TaqMan PCR assays using *GUS-β* for normalization. Note marked differences in gene expression between MA and PD, mostly characterized by up-regulated gene expression, and between PD and PDD, with dramatic down-regulation of the majority of mitochondrial subunits assessed. Subunits are not limited to a single complex but members of all the mitochondrial complexes are affected in PDD. A more complex scenario occurs in the expression of other energy metabolism-related enzymes at different stages of disease. However, important decay in gene expression is also observed in PDD when compared with PD. Student's T-test *p < 0.05, **p < 0.01, ***p < 0.001. [a] No Gaussian distribution (Mann Whitney test *p < 0.05, **p < 0.01, ***p < 0.001.

Table IV. mRNA expression levels of genes encoding subunits of the mitochondrial complexes and energy metabolism-related enzymes in the angular gyrus in MA, iPD, PD and PDD as determined with TaqMan PCR assays using *GUS-β* for normalization. A transient increase in the mRNA expression of several genes encoding subunits of complexes I, IV and V, and encoding energy metabolism-related enzymes is observed in iPD when compared with MA. Although *NDUFA10* mRNA expression remains increased in PD when compared

with MA, a significant decrease of several genes is found in PD when compared with iPD. A dramatic decrease is found in PDD when compared with iPD. *COX7A2L*, *ATP2B4*, *ATP6V1H* and *FAM82A2* mRNA expression is significantly decreased in PDD when compared with PD. Student's T-test *p < 0.05, **p < 0.01, ***p < 0.001. [a] No Gaussian distribution (Mann Whitney test) *p < 0.05, **p < 0.01, ***p < 0.001.

Nº CASE	PD	GENDER	AGE	PM DELAY	RIN FC	RIN AG	WB FC	WB AG	MA FC	MA AG
1	0	MALE	64	8h 30m	7.7	7.7	X	X	-	-
2	0	MALE	56	5h	7.8	6.8	X	X	X	-
3	0	MALE	67	5h	7.4	6.7	X	X	-	-
4	0	MALE	62	3h	8	6.8	X	X	-	-
5	0	MALE	52	4h 40m	8.1	6.7	X	X	-	-
6	0	MALE	30	4h 10m	8.4	-	X	-	-	-
7	0	MALE	53	3h	8.8	6.6	X	X	X	-
8	0	FEMALE	49	7h	8.2	-	X	-	-	-
9	0	FEMALE	75	3h	7.7	8.7	X	X	-	-
10	0	FEMALE	46	9h 35m	6.8	6.8	X	X	-	-
11	0	FEMALE	86	4h 15m	8.1	-	X	-	-	-
12	0	FEMALE	79	3h 35m	7.7	-	X	-	-	-
13	0	FEMALE	79	6h 25m	7.8	-	X	-	-	-
14	0	FEMALE	77	3h 15m	7	-	X	-	-	-
15	0	FEMALE	76	5h 45m	8.3	-	-	-	-	-
16	0	FEMALE	71	8h 30m	7.4	-	-	-	-	-
17	0	MALE	56	5h	6.9	-	-	X	-	X
18	0	FEMALE	78	12h	6.5	-	-	X	-	X
19	0	MALE	71	3h	-	-	-	X	-	X
20	0	MALE	103	3h	-	-	-	X	-	X
21	0	MALE	59	4h 15m	-	-	-	-	-	X
22	0	MALE	61	4h 30m	-	-	-	-	-	X
23	0	MALE	70	13h	-	-	-	-	X	-
24	0	MALE	39	9h 15m	-	-	-	-	X	-
25	0	FEMALE	64	2h 15m	-	-	-	-	X	-
26	0	MALE	52	4h 40m	-	-	-	-	X	-
27	0	MALE	79	7h 45m	7.1	-	-	-	-	-
28	0	MALE	52	3h 30m	8	-	-	-	-	-
29	0	MALE	51	4h	6.5	-	-	-	-	-
30	0	MALE	75	9h	6.6	-	-	-	-	-
31	0	MALE	58	4h	7.7	-	-	-	-	-
32	0	MALE	82	11h	6.6	-	-	-	-	-
33	0	MALE	73	7h	7.2	-	-	-	-	-
34	0	MALE	73	5h 30m	7	-	-	-	-	-
35	iPD	MALE	57	11h	8.7	8.3	X	X	-	-
36	iPD	FEMALE	54	11h 10m	8.2	-	X	X	-	-
37	iPD	FEMALE	70	10h 50m	-	8.7	-	X	-	-
38	iPD	MALE	66	5h	7.3	7.3	X	X	-	-
39	iPD	MALE	72	5h	7.7	-	-	-	-	-
40	iPD	MALE	57	19h	7.6	7	X	X	-	-
41	iPD	MALE	76	4h 30m	7.7	7.9	X	X	-	-
42	iPD	MALE	68	4h 45m	7.3	8.5	X	X	-	-
43	iPD	MALE	79	9h 15m	7.2	-	X	X	-	-
44	iPD	MALE	69	5h 55m	6.4	7.6	X	X	-	-
45	iPD	MALE	68	9h 20m	6.9	-	-	-	-	-
46	iPD	MALE	77	12h	6.9	-	-	-	-	-
47	iPD	FEMALE	84	4h 30m	7.8	-	-	-	-	-
48	iPD	MALE	69	15 h 05 m	8.1	-	-	X	-	-
49	iPD	MALE	78	10h 45m	7.3	-	-	-	-	-
50	PD	FEMALE	70	4h 30m	8.5	-	X	-	X	-
51	PD	FEMALE	77	3h 30m	6.8	-	X	-	-	-
52	PD	FEMALE	81	6h 30m	8.3	-	X	-	-	-
53	PD	FEMALE	69	4h 30 m	8	-	X	-	-	-
54	PD	FEMALE	79	3h 30m	8	-	X	-	X	-
55	PD	MALE	78	13h 30m	6.7	-	-	-	-	-
56	PD	MALE	83	14h	7.7	-	-	-	-	-
57	PD	FEMALE	77	7h 30m	7.1	-	-	-	X	-
58	PD	FEMALE	84	4h	7.3	6.9	-	-	X	X
59	PD	MALE	76	12h	6.5	-	-	-	X	-
60	PD	MALE	78	13h 30m	7.7	-	-	-	X	-
61	PD	MALE	83	14h	7	-	-	-	X	-
62	PD	MALE	76	21h 45m	7.2	6.8	-	-	X	X
63	PD	MALE	79	4h 30m	6.8	7.5	-	-	X	X
64	PD	MALE	77	7h 30m	6.7	7	-	-	X	X
65	PD	FEMALE	78	4h 30m	-	6.6	-	X	-	X
66	PD	FEMALE	79	1h 30m	-	7.2	-	X	-	X
67	PD	MALE	76	4h	-	6.6	-	X	-	X
68	PD	MALE	84	4h	-	7.7	-	X	-	X
69	PD	MALE	87	7h 30m	-	7.5	-	X	-	X
70	PDD	MALE	80	7h 30m	7.2	6.6	X	X	X	X
71	PDD	MALE	82	16h 30m	7.3	6.7	X	X	X	X
72	PDD	MALE	76	9h 40m	6.8	6.4	X	X	X	X
73	PDD	MALE	84	16h 30m	7.1	7.4	X	X	X	X

		Gene	Gene name	Taqman reference
Housekeeping genes		*GUS-B*	β-glucuronidase	Hs00939627_m1
		XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1	Hs00958026_m1
Mitochondria	*Complex I*	*NDUFA2*	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa	Hs00159575_m1
		NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7, 14.5kDa	Hs01561430_m1
		NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	Hs01071117_m1
		NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa	Hs00427185_m1
		NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	Hs00188142_m1
		NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	Hs00605903_m1
		NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase)	Hs00257018_m1
		NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	Hs00159597_m1
	Complex II	*SDHB*	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	Hs00268117_m1
	Complex III	*UQCRB*	ubiquinol-cytochrome c reductase binding protein	Hs00559884_m1
		UQCR11	ubiquinol-cytochrome c reductase, complex III subunit XI	Hs00907747_m1
	Complex IV	*COX7A2L*	cytochrome c oxidase subunit VIIa polypeptide 2 like	Hs00190880_m1
		COX7C	cytochrome c oxidase subunit VIIc	Hs01595220_g1
	Complex V	*ATP5D*	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	Hs00961521_m1
		ATP5G2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2 (subunit 9)	Hs01096582_m1
		ATP5H	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d	Hs01046892_gH
		ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G	Hs00538946_g1
		ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	Hs00426889_m1
Energy metabolism elements		*ATP2B3*	ATPase, Ca++ transporting, plasma membrane 3	Hs00222625_m1
		ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	Hs00608066_m1
		ATP4A	ATPase, H+/K+ exchanging, alpha polypeptide	Hs00167575_m1
		ATP6V0A1	ATPase, H+ transporting, lysosomal V0 subunit a1	Hs00193110_m1
		ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b	Hs01072388_m1
		ATP6V1H	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H	Hs00977530_m1
		FAM82A2	family with sequence similarity 82, member A2	Hs00216746_m1
		LHPP	phospholysine phosphohistidine inorganic pyrophosphate phosphatase	Hs00383379_m1
		SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	Hs00161778_m1
		SLC25A31	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31	Hs00229864_m1
		TOMM40	translocase of outer mitochondrial membrane 40 homolog (yeast)	Hs01587378_mH
		ZNF642	zinc finger protein 642	Hs01372953_m1

		Frontal cortex									
		MA	PD 3-4	PD 5-6	PD+D	MA vs PD 3-4	MA vs PD 5-6	PD 3-4 vs PD 5-6	MA vs PDD	PD 3-4 vs PDD	PD 5-6 vs PDD
Mitochondrial elements											
Complex I	*NDUFA2*	1.08 ± 0.46	0.75 ± 0.49	1.20 ± 0.44	0.60 ± 0.36	-	-	↑*	-	-	-
	NDUFA7	1.08 ± 0.38[a]	0.94 ± 0.56	1.65 ± 0.44	0.62 ± 0.37	-	↑**	↑**	-	-	↓**
	NDUFA10	1.07 ± 0.36	1.09 ± 0.52	1.68 ± 0.53[a]	0.81 ± 0.43	-	↑**	↑*	-	-	↓*
	NDUFB3	1.08 ± 0.42	0.67 ± 0.47	1.33 ± 0.46	0.95 ± 0.45	↓*	-	↑**	-	-	-
	NDUFB7	1.07 ± 0.40	1.15 ± 0.63	1.67 ± 0.76	0.60 ± 0.29	-	↑*	-	-	-	↓*
	NDUFB10	1.10 ± 0.45	1.14 ± 0.74	1.58 ± 0.42	0.74 ± 0.47	-	↑*	-	-	-	↓*
	NDUFS7	1.02 ± 0.19	0.98 ± 0.30	1.17 ± 0.41[a]	0.75 ± 0.33	-	-	-	-	-	-
	NDUFS8	1.10 ± 0.45	1.16 ± 0.62	1.58 ± 0.49	0.82 ± 0.37	-	↑*	-	-	-	↓*
Complex II	*SDHB*	1.04 ± 0.30	1.05 ± 0.67	1.40 ± 0.46	0.49 ± 0.43	-	↑*	-	↓*	-	↓*
Complex III	*UQCRB*	1.07 ± 0.41	0.94 ± 0.66	1.33 ± 0.49	0.55 ± 0.42	-	-	-	-	-	↓*
	UQCR11	1.04 ± 0.31	1.00 ± 0.70	1.44 ± 0.51	0.73 ± 0.40	-	↑*	-	-	-	-
Complex IV	*COX7A2L*	1.17 ± 0.53	1.19 ± 0.87	1.76 ± 0.49	0.70 ± 0.44	-	↑*	-	-	-	↓**
	COX7C	1.10 ± 0.46	0.77 ± 0.59	1.12 ± 0.42	0.50 ± 0.31	-	-	-	↓*	-	↓*
Complex V	*ATP5D*	1.12 ± 0.55	0.91 ± 0.63	1.26 ± 0.62	0.84 ± 0.47	-	-	-	-	-	-
	ATP5G2	1.04 ± 0.28	1.02 ± 0.41	1.37 ± 0.31[a]	0.60 ± 0.43	-	↑*	↑*	↓*	-	↓**
	ATP5H	1.08 ± 0.43	0.64 ± 0.34	1.14 ± 0.41	0.59 ± 0.40	↓**	-	↑**	-	-	-
	ATP5L	1.12 ± 0.66	1.24 ± 0.72	1.66 ± 0.44	0.66 ± 0.36	-	-	↑*	-	-	↓**
	ATP5O	1.06 ± 0.40	0.91 ± 0.52	1.19 ± 0.37	0.60 ± 0.39	-	-	-	-	-	↓*
Energy metabolism-related molecules											
	ATP2B3	1.09 ± 0.43	0.85 ± 0.62	1.30 ± 0.58	0.76 ± 0.49	-	-	-	-	-	-
	ATP2B4	1.06 ± 0.35	0.70 ± 0.39	1.39 ± 0.55	0.59 ± 0.32	↓*	-	↑**	↓*	-	↓*
	ATP4A	1.14 ± 0.78	1.94 ± 0.99	3.04 ± 2.15	0.68 ± 0.24	↑*	-	↑**	-	-	-
	ATP6V0A1	1.10 ± 0.44	0.82 ± 0.42	1.52 ± 0.54	0.65 ± 0.38	-	-	↑**	-	-	↓*
	ATP6V0B	1.10 ± 0.14	0.81 ± 0.20	1.52 ± 0.56	0.72 ± 0.42	↓**	-	↑**	↓**	-	↓*
	ATP6V1H	1.33 ± 0.73	1.34 ± 1.09	1.98 ± 0.72	0.50 ± 0.40	-	-	-	-	-	↓**
	FAM82A2	1.07 ± 0.38	1.05 ± 0.43	1.42 ± 0.70	0.51 ± 0.25	-	-	-	↓*	-	-
	LHPP	1.04 ± 0.31[a]	1.06 ± 0.43	1.15 ± 0.52	0.60 ± 0.23	-	-	-	↓*	-	-
	SLC6A6	1.05 ± 0.45	1.38 ± 0.48	1.15 ± 0.60	0.90 ± 0.31	-	-	-	-	-	-
	SLC25A31	1.11 ± 0.51	1.06 ± 0.49	1.04 ± 0.39	0.73 ± 0.24	-	-	-	-	-	-
	TOMM40	1.09 ± 0.45	0.67 ± 0.36	1.04 ± 0.34	0.60 ± 0.45	↓*	-	↑*	-	-	-
	ZNF642	1.02 ± 0.43	1.94 ± 1.12	1.95 ± 0.53	0.75 ± 0.33	-	↑**	↑***	-	-	↓**

		Angular Gyrus									
		MA	PD 3-4	PD 5-6	PD+D	MA vs PD 3-4	MA vs PD 5-6	PD 3-4 vs PD 5-6	MA vs PDD	PD 3-4 vs PDD	PD 5-6 vs PDD
Mitochondrial elements											
Complex I	*NDUFA2*	1.07 ± 0.40	1.34 ± 0.48	1.01 ± 0.24	0.62 ± 0.36	-	-	-	-	↓*	-
	NDUFA7	1.06 ± 0.38	2.04 ± 0.66	1.02 ± 0.16	0.69 ± 0.50	↑**	-	↓***	-	↓**	-
	NDUFA10	1.14 ± 0.56	1.85 ± 0.53	1.98 ± 0.81	0.89 ± 0.49	↑*	↑*	-	-	↓*	-
	NDUFB3	1.10 ± 0.51	1.59 ± 0.74	1.03 ± 0.28	0.96 ± 0.37	-	-	-	-	-	-
	NDUFB7	1.05 ± 0.34	1.30 ± 0.46	1.01 ± 0.17	0.74 ± 0.42	-	-	-	-	-	-
	NDUFB10	1.39 ± 0.86	2.20 ± 0.65	1.36 ± 0.29	0.88 ± 0.60	↑*	-	↓**	-	↓**	-
	NDUFS7	1.11 ± 0.46	1.62 ± 0.42	1.08 ± 0.12	0.83 ± 0.30	↑*	-	↓**	-	↓**	-
	NDUFS8	1.03 ± 0.24	1.35 ± 0.45	0.97 ± 0.07	0.81 ± 0.24	-	-	↓*	-	-	-
Complex II	*SDHB*	1.09 ± 0.46	1.55 ± 0.66	1.05 ± 0.23	0.56 ± 0.34	-	-	-	-	↓*	-
Complex III	*UQCRB*	1.06 ± 0.37	1.71 ± 0.79	1.16 ± 0.20	0.85 ± 0.48	↑*	-	-	-	-	-
	UQCR11	1.10 ± 0.49	1.70 ± 0.66	1.08 ± 0.19	0.69 ± 0.60	↑*	-	-	-	↓*	-
Complex IV	*COX7A2L*	1.17 ± 0.73	2.06 ± 0.63	1.35 ± 0.16	0.71 ± 0.40	↑*	-	↓**	-	↓**	↓**
	COX7C	1.13 ± 0.58	1.72 ± 0.71	1.03 ± 0.25	0.72 ± 0.50	-	-	-	-	-	-
Complex V	*ATP5D*	1.06 ± 0.39	1.61 ± 0.55	1.15 ± 0.22	0.89 ± 0.49	↑*	-	-	-	-	-
	ATP5G2	1.01 ± 0.19	1.25 ± 0.20	1.06 ± 0.30	0.79 ± 0.62	↑*	-	-	-	↓*	-
	ATP5H	1.05 ± 0.35	1.23 ± 0.47	1.00 ± 0.35	0.77 ± 0.56	-	-	-	-	-	-
	ATP5L	1.08 ± 0.45	1.33 ± 0.47	1.08 ± 0.32	0.73 ± 0.44	-	-	-	-	-	-
	ATP5O	1.12 ± 0.56	1.68 ± 0.62	1.09 ± 0.20	0.68 ± 0.44	-	-	-	-	↓*	-
Energy metabolism-related molecules											
	ATP2B3	1.04 ± 0.31	1.17 ± 0.45	1.04 ± 0.50	0.94 ± 0.68	-	-	-	-	-	-
	ATP2B4	1.05 ± 0.34	0.82 ± 0.27	0.86 ± 0.03	0.46 ± 0.16	-	-	-	↓*	↓*	↓***
	ATP4A	1.16 ± 0.76[a]	1.02 ± 0.78	0.92 ± 0.07	0.83 ± 0.24	-	-	-	-	-	-
	ATP6V0A1	1.04 ± 0.32	1.08 ± 0.38	1.03 ± 0.32	0.73 ± 0.39	-	-	-	-	-	-
	ATP6V0B	1.11 ± 0.49	2.32 ± 1.01	1.30 ± 0.30	0.82 ± 0.41	↑**	-	↓**	-	↓*	-
	ATP6V1H	1.28 ± 0.84	3.09 ± 0.72	1.54 ± 0.46	0.58 ± 0.43	↑***	-	↓***	-	↓***	↓**
	FAM82A2	1.02 ± 0.24	1.36 ± 0.90	1.03 ± 0.18	0.66 ± 0.32	-	-	-	↓*	-	↓**
	LHPP	1.03 ± 0.26	1.04 ± 0.39	1.23 ± 0.26	0.62 ± 0.20	-	-	-	↓*	-	↓**
	SLC6A6	1.04 ± 0.31	1.19 ± 0.24	1.10 ± 0.58	0.81 ± 0.25	-	-	-	-	↓*	-
	SLC25A31	1.17 ± 0.66	1.27 ± 0.82	1.22 ± 0.21	0.88 ± 0.43	-	-	-	-	-	-
	TOMM40	1.17 ± 0.75	1.72 ± 0.75	0.93 ± 0.16	0.70 ± 0.64	-	-	-	-	-	-
	ZNF642	1.11 ± 0.55[a]	1.34 ± 0.32	1.01 ± 0.53	0.68 ± 0.34	-	-	-	-	↓**	-

Figure 1. Representative western blots of NDUFA7, NDUFA10, NDUFB10, NDUFS8 and COX7A2L protein expression in frontal cortex area 8 (**A**) and the angular gyrus (**B**) in MA, iPD, PD and PDD. Expression values are normalized with β-actin and VDAC. Graphs represent the total number of cases examined (see Table I). Data are represented as mean ± SEM. Student's T-test: *p < 0.05, **p < 0.01, ***p < 0.001, significant differences in iPD, PD or PDD when compared with MA cases. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, differences between PD or PDD versus iPD cases. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, observed differences when comparing PD to PDD individuals. [a] No Gaussian distribution (Mann Whitney test).

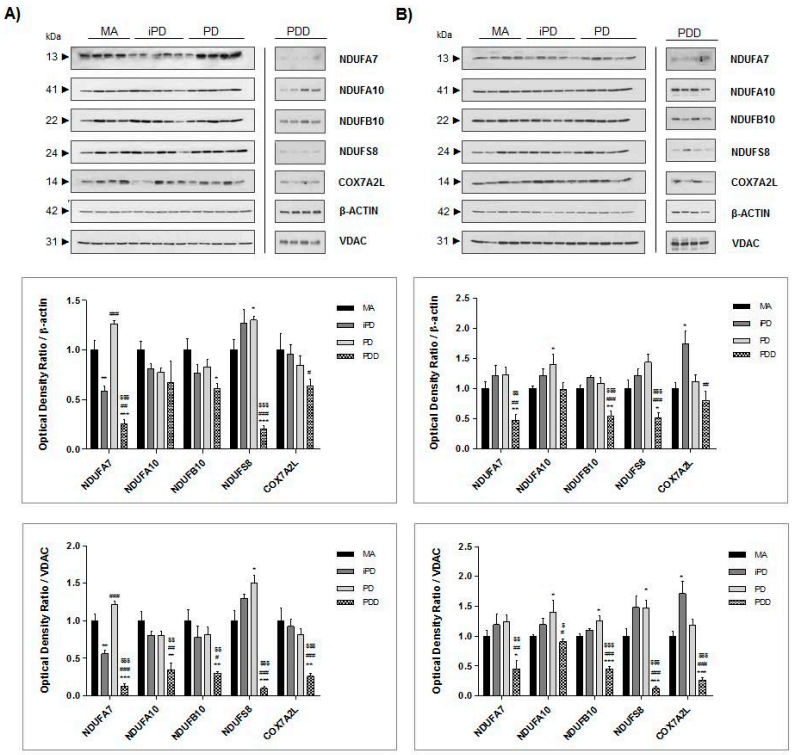


Figure 2. Enzyme activities of complexes I, II, III, IV and V normalized with citrate synthase activity in frontal cortex (**A**) and angular gyrus (**B**) in MA and PD. Activity of complex I, II and V is preserved in the frontal cortex in PD, whereas activity of complex II is decreased and activity of complex IV augmented. Enzymatic activities of complex I, II, IV and V are preserved in the angular gyrus but complex III activity is decreased in PD when compared with MA. Student's T-test *p < 0.05, **p < 0.01, ***p < 0.001. [a] No Gaussian distribution (Mann Whitney test) *p < 0.05, **p < 0.01, ***p < 0.001.

A) Frontal cortex area 8

B) Angular gyrus

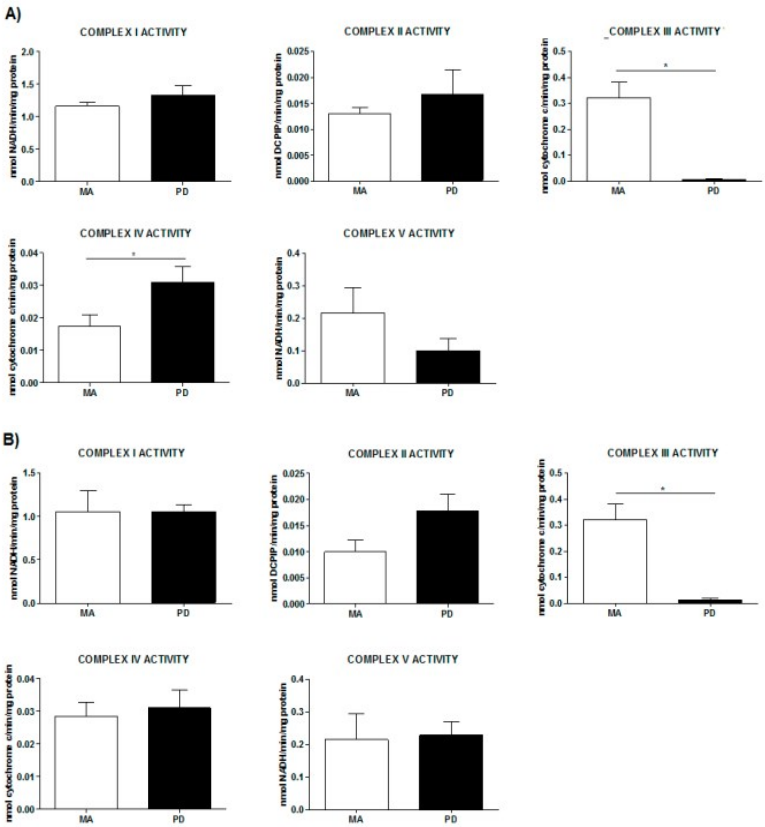


Figure 3. Enzyme activities of complexes I, II, III, IV and V normalized with citrate synthase activity in frontal cortex (**A**) and angular gyrus (**B**) in middle-aged (MA) and PDD. Significantly decreased activity of complex I, II, III and IV is found in frontal cortex and angular gyrus (p-values between 0.05 and 0.01) in PDD when compared with MA. Student's T-test *p < 0.05, **p < 0.01, ***p < 0.001. [a] No Gaussian distribution (Mann Whitney test) *p < 0.05, **p < 0.01, ***p < 0.001.

A) Frontal cortex area 8

B) Angular gyrus

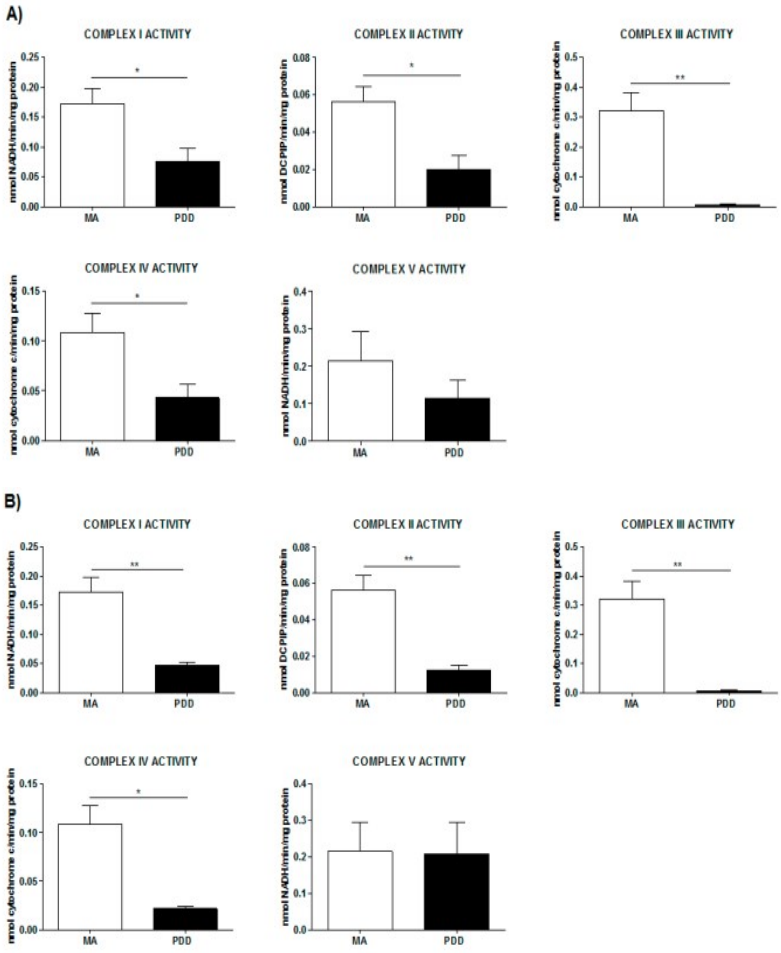
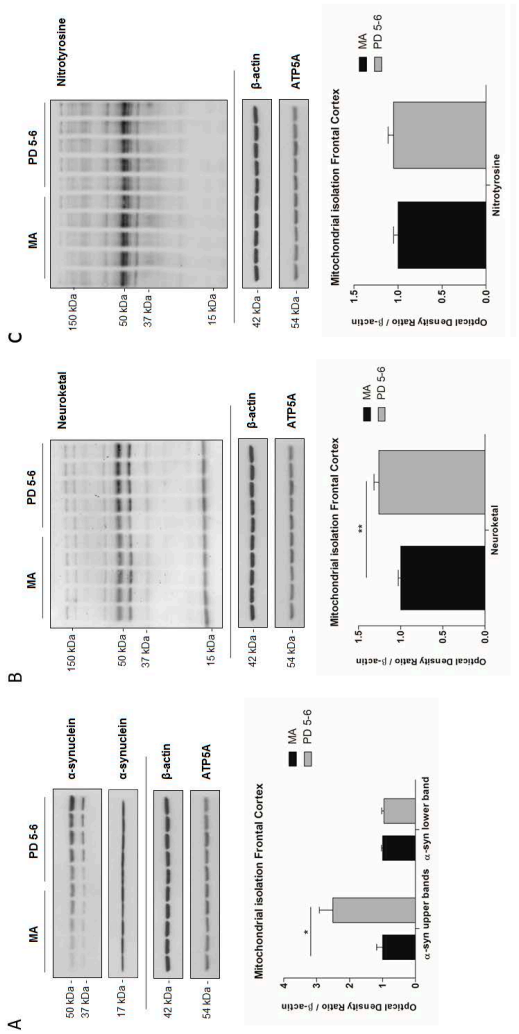


Figure 4. Western blot of mitochondrial-enriched fractions in frontal cortex (FC) in MA cases and PD. **A)** No differences in protein expression levels of monomeric α-synuclein 17 kDa are seen between MA and PD. However, significant expression of oligomeric α-synuclein of 37 kDa and 50 kDa is found in PD when compared with MA. β-actin is used as a marker of protein loading, and ATP5A as a mitochondrial marker. **B**) Increased levels of neuroketal (NKT) protein adducts in mitochondrial-enriched fractions in PD when compared with MA cases. **C**) No significant differences of N-Tyrosine levels are seen between the two groups. Student's T-test *p < 0.05, **p < 0.01.



ARTICLE 3

Complex deregulation and expression of cytokines and mediators of the immune response in Parkinson's disease brain is region dependent.

Garcia-Esparcia P, Llorens F, Carmona M, Ferrer I.

Brain Pathology 2014 Nov; 24(6):584-98.

ABSTRACT

Introduction: Neuroinflammation is common in neurodegenerative diseases including Parkinson's disease (PD). **Methods:** Expression of 25 mRNAs was assessed with TaqMan-PCR including members of the complement system, colony stimulating factors, Toll family, cytokines *IL-8*, *IL-6*, *IL-6ST*, *IL-1B*, *TNF-α* family, *IL-10*, TGFβ family, cathepsins and integrin family, in the substantia nigra pars compacta, putamen, frontal cortex area 8 and angular gyrus area 39, in a total of 43 controls and 56 cases with PD-related pathology covering stages 1–6 of Braak. **Results:** Up-regulation of *IL-6ST* was the only change in the substantia nigra at stages 1-2. Down-regulation of the majority of members examined occurred in the substantia nigra from stage 4 onwards. However, region-dependent down- and up-regulation of selected mRNAs occurred in the putamen and frontal cortex, whereas only mRNA up-regulated mRNAs were identified in the angular cortex from stage 3 onwards in PD cases. Protein studies in frontal cortex revealed increased IL-6 expression and reduced IL-10 with ELISA, and increased IL-6 with western blotting in PD. Immunohistochemistry revealed localization of IL-5, IL-6 and IL-17 receptors in glial cells, mainly microglia; IL-5, IL-10 and M-CSF in neurons; TNF-α in neurons and microglia; and active NF-κβ in the nucleus of subpopulations of neurons and glial cells in PD. **Conclusions:** Distinct inflammatory responses, involving pro- and anti-inflammatory cytokines, and variegated mediators of the immune response occur in different brain regions at the same time in particular individuals. Available information shows that altered α-synuclein solubility and aggregation, Lewy body formation, oxidative damage and neuroinflammation converge in the pathogenesis of PD.

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RESEARCH ARTICLE

Complex Deregulation and Expression of Cytokines and Mediators of the Immune Response in Parkinson's Disease Brain is Region Dependent

Paula Garcia-Esparcia[1]; Franc Llorens[1]; Margarita Carmona[1]; Isidre Ferrer[1,2,3]

[1] Institute of Neuropathology, IDIBELL, Bellvitge University Hospital, Hospitalet de Llobregat, Barcelona, Spain.
[2] Hospitalet de Llobregat, University of Barcelona, Hospitalet de Llobregat, Barcelona, Spain.
[3] CIBERNED (Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas), Institute Carlos III, Hospitalet de Llobregat, Barcelona, Spain.

Keywords
cytokines, immune responses, neuroinflammation, Parkinson's disease, α-synuclein.

Corresponding author:
Isidre Ferrer, MD, PhD, Institute of Neuropathology, Pathologic Anatomy Service, Bellvitge University Hospital, Feixa Llarga sn, Hospitalet de Llobregat, Barcelona 08907, Spain (E-mail: *8082ifa@gmail.com*)

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Abstract

Neuroinflammation is common in neurodegenerative diseases including Parkinson disease (PD). Expression of 25 mRNAs was assessed with TaqMan-PCR including members of the complement system, colony stimulating factors, Toll family, cytokines IL-8, IL-6, IL-6ST, IL-1B, TNF-α family, IL-10, TGFβ family, cathepsins and integrin family, in the substantia nigra pars compacta, putamen, frontal cortex area 8 and angular gyrus area 39, in a total of 43 controls and 56 cases with PD-related pathology covering stages 1–6 of Braak. Up-regulation of IL-6ST was the only change in the substantia nigra at stages 1–2. Down-regulation of the majority of members examined occurred in the substantia nigra from stage 4 onwards. However, region-dependent down- and up-regulation of selected mRNAs occurred in the putamen and frontal cortex, whereas only mRNA up-regulated mRNAs were identified in the angular cortex from stage 3 onwards in PD cases. Protein studies in frontal cortex revealed increased IL6 expression and reduced IL-10 with ELISA, and increased IL-6 with western blotting in PD. Immunohistochemistry revealed localization of IL-5, IL-6 and IL-17 receptors in glial cells, mainly microglia; IL-5, IL-10 and M-CSF in neurons; TNF-α in neurons and microglia; and active NF-κB in the nucleus of subpopulations of neurons and glial cells in PD. Distinct inflammatory responses, involving pro- and anti-inflammatory cytokines, and variegated mediators of the immune response occur in different brain regions at the same time in particular individuals. Available information shows that altered α-synuclein solubility and aggregation, Lewy body formation, oxidative damage and neuroinflammation converge in the pathogenesis of PD.

INTRODUCTION

Inflammation, comprising neuroinflammation and peripheral inflammatory responses are documented in Parkinson's disease (PD; 31, 34–37, 50, 54–56, 62, 78, 85, 86, 88, 91). Microglia are activated in the *substantia nigra* and other brain regions in PD including striatum, frontal and temporal cortex, and pons, as detected by histological and positron emission tomography with specific ligands (9, 13, 30, 40, 41, 54, 55, 66, 67, 78, 86). Increased levels of interleukins (IL)-1β, IL-2, IL-6, and tumor necrosis factor (TNF) α, as revealed by enzyme-linked immunosorbent assay (ELISA), are found in the striatum and colony stimulating factor (CSF; 5, 59, 60, 62, 63). A microarray study has also shown increased expression of genes encoding cytokines in the *substantia nigra* in PD (22), whereas increased IL-6 mRNA has been reported in the hippocampus (40). Under appropriate inflammatory conditions, microglia may produce a wide range of pro-inflammatory and anti-inflammatory cytokines, cytokine receptors, and other immune-related molecules (46). In this line, double immunofluorescence staining has revealed the production of TNF-α and IL-6 in activated microglia in the putamen of PD patients (64), and increased cytokine expression has been reported in glial cells (mostly microglia) in several brain regions in dementia with Lewy bodies (45). Moreover, nuclear translocation of NF-κB (p65), an indicator of NF-κB activation, has been described in the *substantia nigra* in PD (39). All these observations point to the activation of innate central nervous system (CNS) immune responses in PD.

Variable increase of serum and peripheral blood mononuclear cells levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, NT-proCNP, TNF-α, soluble TNF-α receptor-1 (TNFR1) and chemokine (C-C motif) ligand 5 (RANTES) in PD (3, 8, 12, 21, 38, 48, 73–75, 79, 83), indicates the existence of a systemic inflammation manifested as a peripheral dysregulation of cytokines and related molecules in PD. Curiously, increased TNF-α levels in blood have been reported to be significantly correlated with altered cognition, depression and sleep disturbances in PD (57).

In addition, lymphocytes infiltrate the *substantia nigra* in PD (54, 58, 71), and CD+4 and CD+8 T-cells are recruited to the *substantia nigra* in PD (7). Examination of peripheral blood lymphocytes has shown altered percentages of peripheral T and B lymphocytes (83). Increased CD45RO+ and FAS+ CD4+ T-cells, and decreased CD31+ and α4β7+ CD4+ T, further supports the concept that chronic immune stimulation is associated with PD pathobiology (77).

Finally, various studies have shown polymorphisms in TNF-α, IL-1β and IL-6 as risk factors of PD (33, 49, 53, 88, 89).

In spite of this volume of information in humans, and additional data provided by several experimental models *in vivo* and *in vitro* (which are not the subject of the present study) indicating the role of inflammation in PD, little is known about the regional variability in inflammatory responses revealed by a large-scale study of brain-produced cytokines and mediators of immune responses. Moreover, the possible role of other cells in addition to microglia, which may establish interactions between cytokines and their receptors, has not been properly assessed in the CNS, in which immune responses are expected to be triggered locally in primary neurodegenerative diseases.

MATERIALS AND METHODS

Selection of cases and general processing

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL and Clinic Hospital-IDIBAPS Biobanks following the guidelines of Spanish legislation on this matter and the approval of the local ethics committees. The post-mortem interval between death and tissue processing was between 3 and 16 h. One hemisphere was immediately cut in coronal sections, 1-cm thick, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual airtight plastic bags, numbered with water-resistant ink, and stored at −80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphological studies. In addition, samples of *substantia nigra*, putamen, frontal cortex and angular gyrus were fixed in 4% paraformaldehyde for 24 h and then cryoprotected with 30% saccharose for 48 h, frozen in liquid nitrogen, and maintained at −80°C until use.

Neuropathological diagnosis of PD was based on the classification of Braak, but excluding atypical cases in which no graded pathology from the medulla oblongata, pons and midbrain to limbic structures was confirmed (6). The majority of cases had evidence of classical parkinsonism and had received pharmacological treatment for variable periods ($n = 40$). Six of these had suffered from dementia. Others did not complain of parkinsonism, and the diagnosis of incidental PD (iPD) was based on the post-mortem neuropathological study based on the presence of Lewy bodies in selected nuclei of the medulla oblongata, pons and *substantia nigra*, accompanied by variable neuronal loss in the *substantia nigra* not exceeding 50% ($n = 16$). Cases with combined pathologies [i.e. Alzheimer's disease, excepting stages I–II (A) of Braak and Braak; tauopathy; vascular diseases; and metabolic syndrome] were excluded from the present study. Age-matched control cases had not suffered from neurologic, psychiatric diseases, or metabolic diseases (including metabolic syndrome) and did not have abnormalities in the neuropathological examination (excepting Braak and Braak stages I–II). The total number of cases was 43 controls and 56 cases with PD-related pathology. The regions analyzed were *substantia nigra pars compacta*, putamen, frontal cortex area 8 and angular gyrus area 39. However, not all cases had four regions available for biochemical study. The *substantia nigra* was studied in 14 PD cases stages 1–2 ($n = 4$), 3 ($n = 1$), 4 ($n = 3$) and 5 ($n = 6$), and 12 controls; the putamen in 7 PD cases stages 3 ($n = 1$), 4 ($n = 4$) and 5 ($n = 2$) and 8 controls; the frontal cortex (area 8) in 26 PD cases stages 3 ($n = 1$), 4 ($n = 14$), 5 ($n = 10$) and 6 ($n = 1$), and 21 controls; and the angular gyrus (area 39) in 13 PD cases stages 3 ($n = 1$), 4 ($n = 8$) and 5 ($n = 4$), and 9 controls. Representation of genders was as follows: controls: 27 men and 16 women; PD cases: 40 men and 16 women. A summary of cases, and samples and methods available for study in the present series are shown in Table 1.

RNA purification

The purification of RNA was carried out with RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer and following the optional DNase digest. The concentration of each sample was obtained from A260 measurements with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values of RNA quality, or RNA integrity number (RIN), were from 6.6 to 8.8.

Retrotranscription reaction

The retrotranscriptase reaction of all regions—*substantia nigra*, putamen, frontal cortex and angular gyrus—was carried out using the high-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) following the protocol provided by the supplier, and using Gene Amp® 9700 PCR System thermocycler (Applied Biosystems). Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the degree of contaminating genomic DNA.

TaqMan polymerase chain reaction (PCR)

Cases analyzed included *substantia nigra pars compacta* (12 control and 14 PD cases), putamen (8 control and 7 PD cases), frontal cortex, area 8 (16 control and 20 PD cases) and angular gyrus (9 control and 12 PD cases). TaqMan PCR assays for each gene were performed in duplicate on cDNA samples in 384-well optical plates using an ABI Prism 7900 Sequence Detection system (Applied Biosystems). For each 10 µL TaqMan reaction, 4.5 µL cDNA was mixed with 0.5 µL 20× TaqMan Gene Expression Assays and 5 µL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Parallel assays for each sample were carried out using probes for β-glucuronidase (GUS-B) and X-prolyl aminopeptidase (aminopeptidase P) 1 (XPNPEP1) for normalization. The reactions were carried out using the following parameters: 50°C for 2-minute, 95°C for 10-minute, and 40 cycles of 95°C 15 s, and 60°C for 1-minute. Finally, all TaqMan PCR data were captured using the Sequence Detector Software (SDS version

Table 1. Clinical and pathologic characteristics of cases and regions examined.

CASE	GENDER	AGE	P-M	PD BRAAK	FC	SN	AG	PUT	DIAG
1	M	61	4 h 30 minutes	0	X			X	C
2	M	59	4 h 15 minutes	0	X			X	C
3	M	77	6 h 50 minutes	0	X			X	C
4	M	68	10 h 55 minutes	0	X				C
5	M	64	8 h 30 minutes	0	X		X		C
6	M	67	14 h 40 minutes	0	X				C
7	M	66	5 h	PD4	X				iPD
8	M	53	3 h	PD4	X		X		PD
9	M	76	4 h 30 minutes	PD4	X				PD
10	M	68	4 h 45 minutes	PD5	X				PD
11	M	79	9 h 15 minutes	PD5	X				PD
12	M	69	5 h 55 minutes	PD5	X				PD
13	M	56	5 h	0	X		X		C
14	M	67	5 h	0	X		X		C
15	M	62	3 h	0	X		X		C
16	M	52	4 h 40 minutes	0	X		X		C
17	M	30	4 h 10 minutes	0	X				C
18	M	53	3 h	0	X				C
19	F	49	7 h	0	X				C
20	F	75	3 h	0	X		X		C
21	F	46	9 h 35 minutes	0	X		X		C
22	F	86	4 h 15 minutes	0	X				C
23	F	79	3 h 35 minutes	0	X				C
24	F	79	6 h 25 minutes	0	X				C
25	F	77	3 h 15 minutes	0	X				C
26	F	76	5 h 45 minutes	0	X				C
27	F	71	8 h 30 minutes	0	X				C
28	F	70	4 h 30 minutes	PD5	X				PD
29	F	77	3 h 30 minutes	PD4	X				PD
30	M	66	5 h	PD4	X		X		iPD
31	F	81	6 h 30 minutes	PD5	X				PD
32	F	69	4 h 30 minutes	PD5	X				PD
33	F	79	3 h 30 minutes	PD4	X				PD
34	M	57	11 h	PD4	X		X		PD
35	M	57	19 h	PD5	X		X		PD
36	M	76	4 h 30 minutes	PD4	X		X		PD
37	M	68	4 h 45 minutes	PD4	X				PD
38	M	79	9 h 15 minutes	PD4	X				iPD
39	M	69	5 h 55 minutes	PD4	X		X		iPD
40	F	54	11 h 10 minutes	PD3	X				iPD
41	M	78	13 h 30 minutes	PD5	X				PD
42	M	83	14 h	PD5	X				PD
43	F	77	7 h 30 minutes	PD5	X				PD
44	M	80	7 h 30 minutes	PD6	X				PDD
45	F	84	4 h 30 minutes	PD4	X				PD
46	M	68	9 h 20 minutes	PD4	X				PD
47	M	77	12 h	PD4	X				PD
48	M	59	4 h 15 minutes	0		X			C
49	M	67	14 h 40 minutes	0		X			C
50	M	70	2 h	0		X			C
51	M	61	4 h 30 h	0		X			C
52	M	63	8 h 5 h	0		X			C
53	M	30	4 h 10 minutes	0		X			C
54	M	57	4 h 30 minutes	0		X			C
55	M	60	4 h 15 minutes	0		X			C
56	F	68	04 h 30 minutes	0		X			C
57	F	64	2 h 15 minutes	0		X			C
58	F	46	9 h 35 minutes	0		X			C
59	F	79	6 h 25 minutes	0		X			C

Table 1. *Continued*

CASE	GENDER	AGE	P-M	PD BRAAK	FC	SN	AG	PUT	DIAG
60	M	78	13 h 30 minutes	PD5		X			PD
61	M	83	14 h	PD5		X			PD
62	M	76	12 h	PD5		X			PD
63	M	68	9 h 20 minutes	PD4		X			PD
64	M	80	7 h 30 minutes	PD4		X			PD
65	M	85	11 h 45 minutes	PD4		X			iPD
66	M	81	4 h 55 minutes	PD3		X			iPD
67	M	84	16 h 30 minutes	PD5		X			PD
68	F	77	7 h 30 minutes	PD5		X			PD
69	F	84	4 h 30 minutes	PD5		X			PD
70	M	56	5 h	0			X		C
71	F	78	12 h	0			X		C
72	F	78	4 h 30 minutes	PD5			X		PDD
73	F	79	1 h 30 minutes	PD5			X		PDD
74	M	76	4 h	PD5			X		PDD
75	M	84	4 h	PD4			X		PDD
76	M	68	4 h 45 minutes	PD4			X		PD
77	M	69	15 h 05 minutes	PD4			X		PD
78	F	70	10 h 50 minutes	PD3			X		iPD
79	M	78	12 h	0				X	C
80	F	72	4 h	0				X	C
81	M	75	5 h 15 minutes	0				X	C
82	M	57	4 h 30 minutes	0				X	C
83	M	60	4 h 15 minutes	0				X	C
84	F	70	4 h 40 minutes	PD4				X	PD
85	M	81	4 h 55 minutes	PD3				X	iPD
86	M	84	9 h	PD4				X	iPD
87	M	74	6 h 45 minutes	PD4				X	iPD
88	M	79	4 h 30 minutes	PD5				X	PD
89	M	77	7 h 30 minutes	PD5				X	PD
90	F	88	11 h 50 minutes	PD4				X	iPD
91	M	80	15 h 50 minutes	0					C
92	M	66	5 h 45 minutes	0					C
93	M	69	15 h 05 minutes	PD4					PDD
94	F	73	15 h 45 minutes	0					C
95	M	50	15 h 30 minutes	PD5					PD
96	M	74	10 h 50 minutes	PD1		X			iPD
97	M	83	3 h 30 minutes	PD2		X			iPD
98	F	97	3 h 40 minutes	PD2		X			iPD
99	M	80	6 h	PD1		X			iPD

P-M = post-mortem delay (hours, minutes); PD Braak = Parkinson's disease-related pathology stages of Braak; FC = frontal cortex area 8; SN = *substantia nigra pars compacta*; AG = angular gyrus, area 39; Put = putamen; DIAG = neuropathological diagnosis; C = control (no neurological and neuropathological anomalies); iPD = incidental Parkinson's disease (no clinical history of parkinsonism); PD = clinical manifestation of parkinsonism; PDD = PD plus dementia. Cases with additional pathology, excepting stages I–II of neurofibrillary pathology and A of senile plaques following Braak and Braak nomenclature, were not considered in the present series.

1.9, Applied Biosystems). The probes, the housekeeping and all context sequences are listed in Table 2.

Samples were analyzed with the double delta cycle threshold (CT) (ΔΔCT) method. Delta CT (ΔCT) values represent normalized target gene levels with respect to the internal controls (GUS-B and XPNPEP1). These novel reference genes were selected because XPNPEP1 is the most efficient in replicating microarray target gene expression in human post-mortem brain tissue (2, 23).

ΔΔCT values were calculated as the ΔCT of each test sample minus the mean ΔCT of the calibrator samples for each target gene. The fold change was calculated using the equation $2^{(-\Delta\Delta CT)}$. Results were analyzed with one-way analysis of variance (ANOVA) followed by Student's *t*-test when required and checked with the Tukey's method. Differences between mean values were considered statistically significant $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2. Context sequence of TaqMan probes.

GUS-B: GCTACTACTTGAAGATGGTGATCGC
XPNPEP1: CAAAGAGTGCGACTGGCTCAACAAT
C1QL1: CTGCAAGAATGGCCAGGTGCGGGCC
C1QTNF7: GGGAACTGCAGGTTTGAGAGGTAAG
C3AR1: TCTCAGTTTTTTGAAGTTTAGCAAT
CLEC7A: TCTAACTTATTTCAGATCAGAACCA
CSF1R: CCAAAGAATATATACAGCATCATGC
CSF3R: GCTGCTCCCCGGAAGTCTGGAGGAG
CST7: GGCCCTTCCCCAGATACTTGTTCCC
CTSC: CGGTTATGGGACCACAAGAAAAAAA
CTSS: AAAGCCATGGATCAGAAATGTCAAT
CYBA: ATCTCCTGCTCTCGGTGCCCGCCGG
IL-1B: CAGATGAAGTGCTCCTTCCAGGACC
IL-6: TCAGCCCTGAGAAAGGAGACATGTA
IL-6ST: CAAAGTTTGCTCAAGGAGAAATTGA
IL-8: GTGTGAAGGTGCAGTTTTGCCAAGG
IL-10: AATAAGCTCCAAGAGAAAGGCATCT
IL-10RA: CAGTGTCCTGCTCTTCAAGAAGCCC
IL-10RB: TCCACAGCACCTGAAAGAGTTTTTG
INPP5D: GACGAATCCTATGGCGAGGGCTGCA
ITGB2: GCGACCAGGCCAGGCAGCAGCGTTC
TGFB1: AGTACAGCAAGGTCCTGGCCCTGTA
TGFB2: GCACAGCAGGGTCCTGAGCTTATAT
TLR4: GGAGCCCTGCGTGGAGGTGGTTCCT
TLR7: AGACTAAAAATGGTGTTTCCAATGT
TNF: TGGCCCAGGCAGTCAGATCATCTTC
TNFRSF1A: CTCCTGTAGTAACTGTAAGAAAAGC

C1QL1 = complement component 1, q subcomponent-like 1; C1QTNF7 = C1q and tumor necrosis factor related protein 7; C3AR1 = complement component 3a receptor 1; CLEC7A = C-type lectin domain family 7, member A; CSF1R = colony stimulating factor 1 receptor; CSF3R = colony stimulating factor 3 receptor; CST7 = cystatin F (leukocystatin); CTSC = cathepsin C; CTSS = cathepsin S; CYBA = cytochrome b-245, α polypeptide; GUS-B = β-glucuronidase; IL-10 = interleukin 10; IL-10RA = interleukin 10 receptor α; IL-10RB = interleukin 10 receptor β; IL-17R = interleukin 17 receptor A; IL-1B = interleukin 1β; IL-5 = interleukin 5; IL-6 = interleukin 6; IL-6ST = interleukin 6 signal transducer; IL-8 = interleukin 8; INPP5D = inositol polyphosphate-5-phosphatase; ITGB2 = integrin β2; TGFB1 = transforming growth factor β1; TGFB2 = transforming growth factor β2; TLR4 = toll-like receptor 4; TLR7 = toll-like receptor 7; TNF, TNFα = tumor necrosis factor α; TNFRSF1A = TNF receptor superfamily, member 1A; XPNPEP1 = X-prolyl aminopeptidase (aminopeptidase P) 1.

ELISA

Seven control samples and seven stage 4 and 5 PD cases from frontal cortex area 8 were used to measure the levels of protein concentration with ELISA. For each essay, we used different Peprotech kits following the instructions provided by the manufacturer: anti-IL-6 (human IL-6, 900-M16, Mini ELISA Development Kit of Peprotech, London, UK), anti-IL10 (human IL-10, 900-M21, Mini ELISA Development Kit), and anti-TNF-α (human TNF-α, 900-M25, Mini ELISA Development Kit). These kits of sandwich ELISA format, within the range of 24–1500 pg/mL, recognize natural and/or recombinant IL-6, IL-10 and TNF-α. All the results obtained were analyzed with one-way ANOVA followed by Student's *t*-test when required and checked with the Tukey's method. Differences between mean values were considered statistically significant: *P*-values $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Gel electrophoresis and Western blotting

Samples of the frontal cortex area 8 (0.1 g) of 12 controls cases and 12 PD cases were homogenized with a glass homogenizer in Mila lysis buffer (0,5 M Tris at pH 7.4 containing 0.5 M ethylenediaminetetraacetic acid at pH 8.0, 5 M NaCl, 0.5% Na doxicholic, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, bi-distilled water and protease and phosphatase inhibitor cocktails (Roche Molecular Systems, Pleasanton, CA, USA), and then centrifuged at 4°C for 15 minutes at 13 000 rpm (Ultracentrifuge Beckman with 70Ti rotor, CA, USA). Protein concentration was determined with the Bradford method (Merck, Darmstadt, Germany). Samples containing 20 µg of protein were loaded onto 10% acrylamide gels. Proteins were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes (200 mA/membrane, 20 minutes). Nonspecific bindings were blocked by incubation in 5% albumin in phosphate buffered saline (PBS) containing 0.2% Tween for 1 h at room temperature. After washing, the membranes were incubated at 4°C overnight with one of the following rabbit polyclonal antibodies in PBS containing 5% albumin and 0.2% Tween: anti-IL-10 diluted 1:200 (IL-10, AP52181PU-N; Acris Antibodies, Herford, Germany), anti-IκBα diluted 1:1000 (IκBα, 9242; Cell Signalling Technology, Beverly, CA, USA), anti-IKK-α (IKK-α, 2682; Cell Signalling Technology), and anti-IL-6 (IL-6, sc-7920; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-β-actin diluted 1:30 000 (β-Actin, A5316; Sigma-Aldrich, St. Louis, MO, USA) was blotted for the control of protein loading. Membranes were then incubated for 1 h with the appropriate HRP conjugated secondary antibody (1:1000, Dako, Glostrup, Denmark), and the immune-complexes were revealed with a chemiluminescence reagent (ECL, Amersham, GE Healthcare, Buckinghamshire, UK). Densitometry of Western blot bands was assessed with the TotalLab program (TotalLab Quant, Newcastle, UK) and subsequently analyzed with the Tukey's method; differences were considered statistically significant with *P*-values: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Immunohistochemistry

De-waxed sections of the *substantia nigra* and frontal cortex of 8 controls and 12 PD cases were stained with hematoxylin and eosin, or processed for immunohistochemistry, following the En Vision+ system method. After incubation with methanol and normal serum, the sections were incubated with one of the primary antibodies overnight at 4°C. Monoclonal antibodies against α-synuclein (Menarini, Florence, Italy), and clones CD68 and CD20 (Ventana, Roche) were used at a dilution of 1/200 or pre-diluted, respectively. Rabbit polyclonal antibodies CD8 and CD4 (Ventana, Roche, Basel, Switzerland) were used pre-diluted as recommended by the supplier. Following incubation with the primary antibody, the sections were incubated with EnVision + system peroxidase (Dako) for 15 minutes at room temperature. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Control of the

immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody. Sections were slightly counterstained with hematoxylin. Following incubation with the primary antibody, the sections were incubated with EnVision + system peroxidase (Dako) for 15 minutes at room temperature. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Control of the immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody. Sections were slightly counterstained with hematoxylin.

Cryoprotected sections of the *substantia nigra*, frontal cortex, and angular gyrus (controls, $n = 5$; PD cases $n = 6$ *substantia nigra*, $n = 8$ frontal cortex and $n = 6$ angular gyrus) were processed for free-floating immunohistochemistry. Rabbit polyclonal antibodies against IL-10 (AP52181PU-N, Acris) diluted 1/1000; IL-5 (IL-5, ab22448, Abcam, Cambridge, UK) diluted 1/1000; IL-6 (IL-6, ab6672, Abcam) diluted 1/100; macrophage CSF [H300; macrophage CSF (M-CSF), sc13103 Santa Cruz Biotechnology] diluted 1/100; nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) phosphorylated (3033, Cell Signalling Technology) diluted 1/300; and mouse monoclonal antibodies against TNF-α (ab1793, Abcam) diluted 1/10, and IL-17 receptor B (ab13653, Abcam) diluted 1/200 were used. The peroxidase reaction was visualized with diaminobenzidine, NH_4NiSO_4, and H_2O_2. The immunoreaction results in a blue-grey precipitate. This procedure permits the visualization of subtle staining properties often missed by more commonly used protocols. Control of the immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody.

Semi-quantitative studies of neurons, astrocytes [as stained with glial fibrillary acidic protein (GFAP) antibodies], microglia (as recognized with CD68 antibodies) and CD8 T lymphocytes were carried out in the *substantia nigra pars compacta*, putamen, frontal cortex area 8 and angular gyrus at PD stages 3–4 and 5–6, and controls. For the *substantia nigra*, all neurons in a given section were counted in every case, whereas for the putamen, frontal cortex and angular cortex, counts were made on three different fields per section and case at a magnification of ×200. The average number of a defined cell type in control cases was considered 100, and the decrease or increase compared with the control values was expressed as a percentage of increase or lost. Lymphocytes in the brain parenchyma were counted as present or absent because no lymphocytes were found in control cases.

α-Synuclein solubility and aggregation

Brain samples (0.1 g) of *substantia nigra pars compacta and* frontal cortex area 8 from controls ($n = 3$ per group) and PD cases stage 5 ($n = 3$ per group) were homogenized in a glass homogenizer, in 750 µL of ice-cold PBS+ (sodium phosphate buffer pH 7.0, plus protease inhibitors), sonicated, and centrifuged at 5200 rpm at 4°C for 10-minutes. The pellet was discarded and the resulting supernatant was ultra-centrifuged at 43 000 rpm at 4°C for 1 h. The supernatant (S2) was kept as the PBS-soluble fraction. The resulting pellet was re-suspended in a solution of PBS, pH 7.0, containing 0.5% sodium deoxycholate, 1% Triton, and 0.1% SDS, and this was ultra-centrifuged at 43 000 × rpm at 4°C for 1 h. The resulting supernatant (S3) was kept as the deoxycholate-soluble fraction. The corresponding pellet was re-suspended in a solution of 2% SDS in PBS and maintained at room temperature for 30-minute. Immediately afterward, the samples were centrifuged at 43 000 rpm at 25°C for 1 h and the resulting supernatant (S4) was the SDS-soluble fraction. Equal amounts of each fraction were mixed with reducing sample buffer and processed in parallel for 10% SDS-PAGE and Western blotting. Membranes were incubated with anti-α-synuclein (Chemicon, Temecula, CA, USA) at a dilution of 1:30 00. The protein bands were visualized with the ECL method (Amersham).

RESULTS

mRNA expression of cytokines and mediators of the immune response in the *substantia nigra*, putamen, frontal cortex and angular cortex in PD

Analyzed mRNAs

Twenty-five mRNAs were assessed in the present study including members of the complement system [complement component 1, q subcomponent-like 1 (C1QL1), C1q and tumor necrosis factor related protein 7 (C1QTNF7), complement component 3a receptor 1 (C3AR1)], CSFs [cytochrome b-245, α polypeptide (CYBA), cystatin F (leukocystatin) (CST7), INPP5D inositol polyphosphate-5-phosphatase, CSF 1 receptor and CSF 3 receptor (CSF3R)], Toll family [Toll-like receptors (TLR) 4, TLR7], cytokines IL-8, IL-6, IL-6 signal transducer (IL-6ST), IL-1B, TNF-α family (TNF receptor superfamily, member 1A and TNF), IL-10 [IL-10, IL-10 receptor α (IL-10RA), IL-10 receptor β (IL-10RB)], transforming growth factor (TGF) β family (TGFβ1, TGFβ2), cathepsins S (CTSS) and cathepsin C (CTSC) and integrin family [C-type lectin domain family 7, member A (CLEC7A), integrin β2 (ITGB2)]. Similar results were obtained using two different housekeeping genes: GUS-B and XPNPEP1. mRNA expression was examined in the *substantia nigra pars compacta*, putamen, frontal cortex area 8, and angular cortex, in 39 PD samples Braak stages 3–6 with no distinction between cases with and without clinical signs of parkinsonism, compared with corresponding regional controls.

Substantia nigra pars compacta

Down-regulation of C1QL1, C1QTNF7, C3AR1, TLR7, IL-1B, IL-6, IL-6ST, TNF-α, IL-10, IL-10RB, TGFB2 and CTSC mRNAs was found in the *substantia nigra* at Braak stages 3–6 whereas no modifications were found in the mRNA expression levels of the remaining cytokines and related molecules. Interestingly, not a single gene was up-regulated in the series examined (Table 3).

We also analyzed mRNA expression of cytokines and mediators of the immune response in the *substantia nigra* at stages 1 and 2 of Braak—that is before the appearance of any deposition of α-synuclein in the *substantia nigra pars compacta*, but with variable deposits in the motor nucleus of the vagus nerve and *locus coeruleus*. The number of samples was limited to four PD cases and six age-matched controls. Only IL-6ST was significantly up-regulated ($P > 0.05$) in PD whereas no differences in the expression of the other genes were observed between control and PD cases.

Table 3. Regional differences in mRNA expression levels of cytokines and mediators of the immune response in Parkinson's disease.

		Substantia nigra		Frontal cortex area 8		Angular gyrus		Putamen	
		C	PD	C	PD	C	PD	C	PD
Complement system	C1QL1	0.73 ± 0.33	0.38 ± 0.22**	0.98 ± 0.56	0.80 ± 0.47	1.04 ± 0.31	1.02 ± 0.23	1.06 ± 0.38	1.04 ± 0.33
	C1QTNF7	1.16 ± 0.82	0.50 ± 0.54*	1.06 ± 0.72	1.33 ± 0.96	1.05 ± 0.36	0.86 ± 0.44	1.03 ± 0.25	1.33 ± 0.16*
	C3AR1	0.69 ± 0.26	0.37 ± 0.11**	1.06 ± 0.57	1.13 ± 0.90	1.12 ± 0.52	1.61 ± 0.52*	1.06 ± 0.37	0.67 ± 0.26*
Colony stimulating factors	CYBA	0.90 ± 035	0.96 ± 0.39	1.00 ± 0.66	1.74 ± 0.86*	1.17 ± 0.62	1.49 ± 0.66	1.10 ± 0.44	1.45 ± 0.47
	CST7	0.99 ± 0.62	0.45 ± 029*	0.99 ± 0.65	1.14 ± 0.79	0.67 ± 0.32	1.54 ± 0.92*	1.15 ± 0.66	1.62 ± 0.83
	INPP5D	1.07 ± 0.44	0.82 ± 0.35	1.05 ± 0.30	1.25 ± 0.48	1.20 ± 0.21	1.14 ± 0.30	1.01 ± 0.15	1.14 ± 0.37
	CSF1R	1.01 ± 0.38	0.67 ± 0.46	1.07 ± 0.37	1.06 ± 0.32	1.10 ± 0.47	1.36 ± 0.38	0.93 ± 0.17	1.07 ± 0.24
	CSF3R	1.19 ± 0.39	0.86 ± 0.49	1.16 ± 0.66	0.78 ± 0.46*	0.85 ± 0.32	1.57 ± 0.72*	1.04 ± 031	1.26 ± 0.57
Toll-like receptors	TLR4	1.01 ± 0.37	1.10 ± 0.61	1.22 ± 0.70	0.69 ± 0.52*	1.04 ± 0.29	0.91 ± 0.39	1.09 ± 0.46	0.76 ± 0.31
	TLR7	0.96 ± 0.34	0.48 ± 0.22***	1.03 ± 0.48	1.03 ± 0.68	1.25 ± 075	1.54 ± 0.43	1.05 ± 0.36	0.69 ± 0.23*
Cytokines	IL-8	0.53 ± 0.33	0.51 ± 0.53	0.93 ± 0.75	1.06 ± 0.91	1.29 ± 0.92	0.79 ± 0.55	0.97 ± 0.79	1.29 ± 0.54
	IL-1B	1.38 ± 1.13	0.59 ± 0.50*	0.78 ± 0.70	0.69 ± 0.60	1.17 ± 1.07	1.75 ± 1.34	0.89 ± 0.33	0.65 ± 0.44
	IL-6	0.75 ± 0.38	0.32 ± 0.28**	1.09 ± 0.74	1.56 ± 0.92	1.10 ± 0.83	1.10 ± 0.79	0.86 ± 0.44	1.25 ± 0.76
	IL-6ST	1.07 ± 0.44	0.66 ± 0.30*	1.01 ± 0.35	1.22 ± 0.69	1.02 ± 0.20	1.03 ± 0.19	1.03 ± 0.25	1.07 ± 0.27
TNF family	TNF-α	1.11 ± 0.48	0.32 ± 0.22***	0.87 ± 0.44	0.87 ± 0.64	1.07 ± 0.28	1.52 ± 1.11	1.22 ± 0.85	0.51 ± 0.19*
	TNFRS1A	0.80 ± 0.64	0.62 ± 0.16	1.02 ± 0.50	1.03 ± 0.71	1.11 ± 0.47	1.25 ± 0.57	1.04 ± 0.29	1.02 ± 0.16
IL-10 family	IL-10	1.13 ± 0.51	0.59 ± 0.37**	1.24 ± 0.72	1.02 ± 0.44	1.37 ± 1.04	1.57 ± 0.92	1.04 ± 0.31	0.93 ± 0.30
	IL-10RA	0.87 ± 0.46	0.73 ± 0.42	0.67 ± 0.17	1.60 ± 1.11**	1.08 ± 0.38	1.58 ± 0.57*	1.02 ± 0.25	1.78 ± 0.92*
	IL-10RB	1.03 ± 0.20	0.77 ± 0.27*	1.08 ± 0.68	1.23 ± 0.58	1.06 ± 0.38	1.10 ± 0.25	1.02 ± 0.22	1.02 ± 0.23
TGF-β	TGFB1	1.10 ± 0.54	0.72 ± 0.32	1.01 ± 0.38	1.07 ± 0.48	1.02 ± 0.24	1.26 ± 0.33	1.01 ± 0.17	0.90 ± 0.39
	TGFB2	1.00 ± 0.52	0.55 ± 0.25*	1.12 ± 0.73	0.99 ± 0.64	1.01 ± 0.47	1.01 ± 0.30	1.15 ± 0.42	1.13 ± 0.17
Cathepsins	CTSS	1.04 ± 0.30	0.84 ± 0.57	0.97 ± 0.47	1.41 ± 0.66*	1.13 ± 0.52	1.38 ± 0.61	1.06 ± 0.37	0.88 ± 0.48
	CTSC	0.98 ± 0.33	0.54 ± 0.35**	0.99 ± 0.52	1.13 ± 0.55	1.11 ± 0.48	1.72 ± 0.84	1.04 ± 0.29	1.18 ± 0.67
Integrins	CLEC7A	0.95 ± 0.30	0.66 ± 0.43	0.96 ± 0.29	1.75 ± 0.96**	0.81 ± 0.47	0.70 ± 0.33	1.04 ± 0.31	1.26 ± 0.57
	ITGB2	1.01 ± 0.42	1.01 ± 0.53	1.16 ± 0.62	1.36 ± 0.60	1.18 ± 0.65	1.95 ± 0.97*	1.04 ± 0.31	0.97 ± 0.56

mRNA expression levels of selected genes of complement system (C1QL1, C1QTNF7, C3AR1), colony stimulation factors [(CSF1R, CSF3R, CYBA, CS7, INPP5D inositol polyphosphate-5-phosphatase (INPP5D)], TLRs (TLR4, TLR7), IL-8, pro-inflammatory cytokines (IL-6, IL-6ST, IL-1B), TNF-α family (TNFRS1A, TNF-α), IL-10 family (IL-10, IL-10RA, IL-10RB), TGF-β family (TGFB1, TGFB2), cathepsins (CTSS, CTSC), and integrin family (CLEC7A, ITGB2) in the *substantia nigra pars compacta*, frontal cortex area 8 and angular gyrus in Parkinson's disease (PD) cases stages 3–5 and age-matched controls (C), analyzed with TaqMan PCR assays. One-way analysis of variance followed by Student's *t*-test when required and checked with the Tukey's method. Differences between mean values were considered statistically significant $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. For the meaning of the abbreviations, please refer to Tables 1 and 2 footnotes.

Putamen

Down-regulation of C3AR1, TLR7, and TNF-α, and up-regulation of C1QTNF7 and IL-10RA were observed in the putamen (Table 3).

Frontal cortex and angular gyrus

Down-regulation of CSF3R and TLR4 mRNAs, and up-regulation of CTSS, CYBA, IL-10RA and CLEC7A mRNAs were observed in the frontal cortex area 8, whereas no modifications in gene expression were noted in the rest of the mRNAs assessed albeit with a trend of up-regulation of IL-6 (Table 3).

Up-regulation of C3AR1, CST7, CSF3R, IL-10RA and ITGB2 mRNAs was found in the angular cortex (area 39) in PD cases. Other genes had expression levels similar to controls, and none of them was down-regulated (Table 3).

Then we analyzed whether differences were seen in frontal cortex area 8 in cases with incidental PD stages 3 and 4 ($n = 5$) and cases with PD and parkinsonism stages 4 and 5 ($n = 20$). No significant differences were found between the two groups (data not shown) thus suggesting that inflammatory changes in the frontal cortex were not modified with disease progression or that treatment of parkinsonism stabilized inflammatory responses in the frontal cortex.

Finally, no differences between cases with ($n = 6$) and without dementia ($n = 4$) were seen in the angular cortex, the only region tested because of the small number of samples covering both conditions.

ELISA in frontal cortex

A limited study of proteins was performed to correlate the modifications observed in mRNA expression with protein levels. A significant increase in IL-6 protein expression levels ($***P < 0.001$ Tukey's method) was seen in the frontal cortex area 8 in PD cases ($n = 7$, stages 4 and 5) compared with controls ($n = 7$), whereas a slight significant reduction of IL-10 ($*P < 0.05$ Tukey's method) occurred in PD. TNF-α protein levels did not differ in PD cases from controls (Figure 1A).

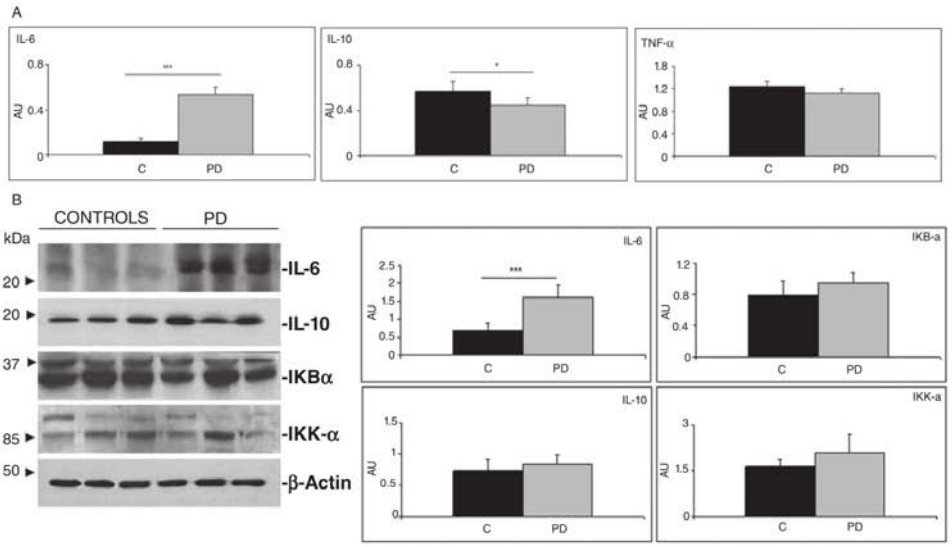

Figure 1. A. Protein expression levels of interleukins (IL)-6, IL-10 and tumor necrosis factor (TNF)-α, as revealed by enzyme-linked immunosorbent assay (ELISA), in the frontal cortex area 8 in control and Parkinson's disease (PD) cases. IL-10 is down-regulated whereas IL-6 is up-regulated. No significant differences are seen in TNF-α expression levels between control and PD cases. Significant differences are seen using the Tukey's method; *P*-value: $*P<0.05$; $**P<0.01$; $***P<0.001$. **B**. Protein expression levels, as revealed with Western blotting, of IL-6, IL-10, IKBα and IKK-α corrected with β-actin measured by Total Lab (1D Gel analysis) and analyzed with the Tukey's method; $P<0.05$; $**P<0.01$; $***P<0.001$. Only IL-6 expression levels are significantly increased in frontal cortex.

Western blotting

Protein expression levels of IL-6, IL-10, IκBα (inhibitor of nuclear factor-kappa-B subunit alpha) and IKK-α (inhibitor of nuclear factor kappa-B kinase subunit alpha) using Western blotting, all of them corrected by β-actin, measured with Total Lab (1D Gel analysis) and subsequently analyzed with the Tukey's method, revealed significant increments of IL-6 ($P < 0.001$) in the frontal cortex area 8 in PD cases ($n = 12$) compared with controls ($n = 12$). No differences were observed regarding expression levels of IL-10, IκBα and IKK-α (Figure 1B).

Immunohistochemistry

Increased numbers of ramified and amoeboid microglial cells were observed in the *substantia nigra* in PD cases, with the number of amoeboid microglial cells correlating with neuron loss and debris accumulation. This was accompanied by increased numbers of astrocytes in the *substantia nigra pars compacta* correlating with dopaminergic neuron loss. A slight increase in microglial cells, most of them ramified, and slight increase in astrocytes occurred in the putamen and cerebral cortex.

Microglia and macrophages with neuromelanin were present in the *substantia nigra pars compacta* from stage 3 onward. Lesser amounts of CD68-immunoreactive microglia were observed in the frontal cortex at early and advanced stages of PD. A few lymphocytes expressing CD8 were found in the *substantia nigra* from stage 3 onward but they were extremely rare or most often absent in the cerebral cortex at all stages of PD (Figure 2). Only a few CD4+ and no CD20+ lymphocytes were seen at any time. A semi-quantitative study of neurons, astrocytes, microglia and T lymphocytes in the different regions is shown in Table 4.

The study with immunohistochemistry of cytokines and mediators of the immune response in *substantia nigra* and frontal cortex was limited to ILs IL-5, IL-6, IL-10; IL-17 receptor; M-CSF; phosphorylated (active) NFκB; and TNF-α. IL-5, IL-6 and IL-17 receptor were expressed in glial cells, mainly microglia, whereas IL-5, IL-10, M-CSF and TNF-α were expressed in neurons; TNF-α was expressed in neurons and microglia. Active NFκB was found in the nucleus of subpopulations of neurons and glial cells mainly in *substantia nigra* and less frequently in putamen and cerebral cortex (Figure 3).

α-Synuclein solubility and aggregation

α-Synuclein solubility and aggregation were assessed in samples of the *substantia nigra pars compacta* and frontal cortex area 8 in cytosolic, deoxycholate and SDS fractions. In the *substantia nigra*,

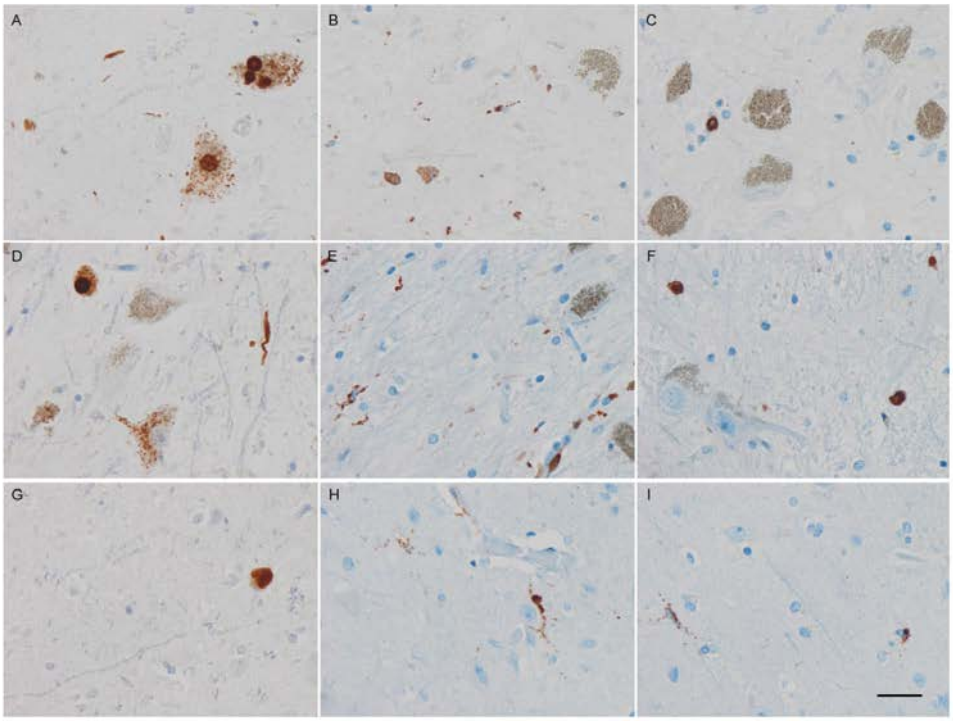

Figure 2. Immunohistochemistry of α-synuclein (**A**, **D**, **G**), CD68 (**B**, **E**, **H**, **I**), and CD8 (**C** and **F**) in the *substantia nigra* stage 3 (**A**–**C**), stage 5 (**D**–**F**), frontal cortex stage 5 (**G**, **H**), and stage 3 (i). **A, D, G.** α-synuclein decorates Lewy bodies and neurites in the *substantia nigra* and frontal cortex; CD68 is found in microglia and macrophages in the *substantia nigra* **B, E.** and frontal cortex at stages with **H** and without **I** concomitant Lewy bodies. Lymphocytes C8 are found in the *substantia nigra pars compacta* at early **C** and late stages **F** of PD. Paraffin sections, visualized with diaminobenzidine and H_2O_2 with hematoxylin counterstaining. Neuromelanin is distinguished by the moderately dense accumulation of regular granules in contrast to 3, 3'-diaminobenzidine (DAB)-positive inclusions, which are darker and commonly homogeneous, as seen in **B**, **C**, **D** and **F**; however, differences are more subtle regarding fine granular α-synuclein inclusions in addition to Lewy bodies, that may deposit together with neuromelanion as seen in **A** and **B**. Bar = 25 µm.

a band of α-synuclein at the expected molecular weight (about 20 kDa) was observed in control and PD cases in the cytosolic, deoxycholate and SDS fractions. α-Synuclein bands of variable molecular weight (between 40 and 80 kDa) were observed in deoxycholate fractions in control and PD cases, but multiple bands between 40 and 80 kD were only found in PD. Altered α-synuclein solubility and aggregation was also seen in the frontal cortex with increased numbers of bands between 50 and 100 kDa in SDS fractions from PD cases (Figure 4). It is worth stressing that the patterns, and more importantly, the exposure time to reveal the bands of high molecular weight corresponding to α-synuclein aggregates differed in the *substantia nigra* and frontal cortex in samples run in parallel under the same conditions. α-synuclein aggregates were clearly identified after 1 min of exposure in samples of the *substantia nigra* whereas α-synuclein aggregates were only seen after 10 minutes of exposure, accompanied by a grey background, in frontal cortex samples.

DISCUSSION

The study was designed to compare and evaluate the expression of several cytokines and mediators of the immune response in different brain regions at progressive stages of PD. The selection of molecules was performed taking into consideration to include members of the complement system, CSFs, pro- and anti-inflammatory cytokines, TGF-β, cathepsins and integrins which have been found abnormally regulated in the cerebral cortex in a parallel study in progress of Alzheimer's disease. Therefore, the

Table 4. Semi-quantitative values of neurons, astrocytes, microglia and CD8 T lymphocytes in the *substantia nigra pars compacta*, putamen, frontal cortex area 8 and angular gyrus at stages 3–4 and 5–6 of Braak.

Region	Braak stages	Neurons	Astrocytes	Microglia	CD8 T
Substantia nigra	3–4	– –	* *	* *	+
	5–6	– – –	* *	* *	+
Putamen	3–4	=	=	*	0
	5–6	=	*	*	0
Frontal cortex area 8	3–4	=	=	=	0
	5–6	=	*	*	0
Angular gyrus	3–4	=	=	=	0
	5–6	–	*	*	0

Loss of neurons in the *substantia nigra* and cortex is represented as follows: – less than 20%; – – between 40% and 60%, and – – – more than 50% neuron loss compared with controls. Increased numbers of astrocytes, as revealed with GFAP immunostaining, and microglia, as revealed by CD68 immunoreactivity, are represented as follows: * between 10% and 20%; * * more than 20% when compared with controls. Presence or absence of CD8 lymphocytes is represented as + and 0, respectively. Lack of differences between control and diseased cases is marked as =. No attempts to discriminate between iPD and PD, and PD and PDD were made. iPD = incidental Parkinson's disease (no clinical history of parkinsonism); PD = clinical manifestation of parkinsonism; PDD = PD plus dementia.

study of the same molecules will serve, not only to increase understanding about neuroinflammation in PD but also to compare disease-dependent, region-dependent and stage-dependent neuroinflammatory responses in selected neurodegenerative diseases in the near future. Regarding the choice of regions in the present study, the *substantia nigra* and the putamen are obliged areas because they are primary substrates of parkinsonism (22). The study of the cerebral cortex is due to its involvement in PD even at early stages of the neurodegenerative process (25, 26). The selection of the frontal cortex area 8 and angular gyrus is based on neuroimaging observations showing that these areas are particularly vulnerable to PD (13, 87).

The present findings showing increased microglia and macrophages, and T lymphocytes in the *substantia nigra*, and increased microglia in the cerebral cortex, are in line with previous observations in PD, and they further support neuroinflammation in the pathogenesis of PD. Deregulation of several genes involved in the inflammatory and immune responses has also been demonstrated by using quantitative reverse transcription-polymerase chain reaction (RT-PCR). However, the diversity of inflammatory responses in the *substantia nigra pars compacta*, putamen, and cortical regions frontal cortex area 8 and angular gyrus at the same stages of disease progression reveals a new scenario that is more complex than formerly envisaged.

Increased IL-6st mRNA expression has been observed at early stages of PD-related pathology (stages 1–2 of Braak). Yet mRNA expression levels of several mediators of inflammation and immune response are significantly decreased in the *substantia nigra* at stages 3–5 of Braak; these include members of the complement system C1QL1, C1QTNF7 and C3AR1; TLR7; IL-1B, IL-6, IL-6ST, TNF-α; IL-10; IL-10β; TGFβ2; and cathepsin C when compared with age-matched controls. This contrasts with previous observations reporting up-regulation of pro-inflammatory cytokines such as IL-1β in the *substantia nigra pars compacta* in PD (71, 72). Down-regulation of C3AR1, TLR7 and TNF-α, and up-regulation of C1QTNF7 and IL-RA have been observed in the putamen at stages 5 and 6, which does not match with the transient profile of increased mRNA expression of inflammatory mediators in the striatum of mice following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (68). Yet chronic responses in the putamen in PD cases are barely comparable with acute changes in the striatum of mice following MPTP administration.

In contrast, down-regulation of CSF3R and TLR4 mRNAs, together with up-regulation of CTSS, CYBA, IL-10RA, and CLEC7A mRNAs, is found in the frontal cortex. Finally, up-regulation of C3AR1, CST7, CSF3R, IL-10RA, and ITGB2 mRNAs without accompanying modifications in the expression of genes which were down-regulated in other brain regions occurs in the angular gyrus area 39.

Taken together, the results of the study of the mRNA expression profile of cytokines and mediators of the immune response in PD reveal marked regional variations, with expression levels markedly reduced in the *substantia nigra pars compacta*, variably down-regulated or up-regulated in the putamen and frontal cortex area 8, and up-regulated in the angular gyrus.

Interestingly mRNA and protein expression did not correlate, at least in the frontal region—the only one examined for mRNA and protein. ELISA and Western blotting showed a marked increase in IL-6 protein expression in PD, whereas IL-10 was reduced with ELISA, but not with Western blots. TNF-α, IκBα, and IKK-α protein levels did not differ in PD from controls.

Finally, immunohistochemistry reveals the presence of IL-5, IL-6, IL-10; IL-17 receptor; M-CSF and TNF-α protein expression in the PD *substantia nigra* and frontal cortex cytokines and mediators of the immune response are localized in neurons, astrocytes and microglia. Interleukines IL-5, IL-6, IL-17 receptor, and TNF-α are expressed in glial cells, mainly microglia, whereas IL-5, IL-10, M-CSF, and TNF-α are expressed in neurons. Active NFκB is localized in the nucleus of subpopulations of neurons and glial cells mainly in *substantia nigra* and less frequently in putamen and cerebral cortex. These findings confirm and expand previous information describing the localization of cytokines and mediators of the immune response, including nuclear translocation of NF-κB in the *substantia nigra* in PD (39, 45, 63). The presence of isolated lymphocytes in the *substantia nigra* in PD here observed is also in line with previous observations (7, 54, 58, 71).

Differences in mRNA levels as revealed by qRT-PCR and protein expression as seen with immunohistochemistry is particularly notorious in the *substantia nigra*. Down-regulation of the majority of genes is accompanied by increased expression of cytokine and mediators of the immune response in subpopulations of neurons, microglia and astrocytes, thus indicating a complex modulation of neuroinflammation in the *substantia nigra* in PD.

An important difficulty in the study of putative signaling in the human brain under physiological and pathological conditions is the information about dynamics and mechanistic aspects. Therefore, the significance of the complex scenario of neuroinflammation in PD still remains elusive. For example, IL-6 in the nervous system acts mainly as a neurotrophic factor in normal

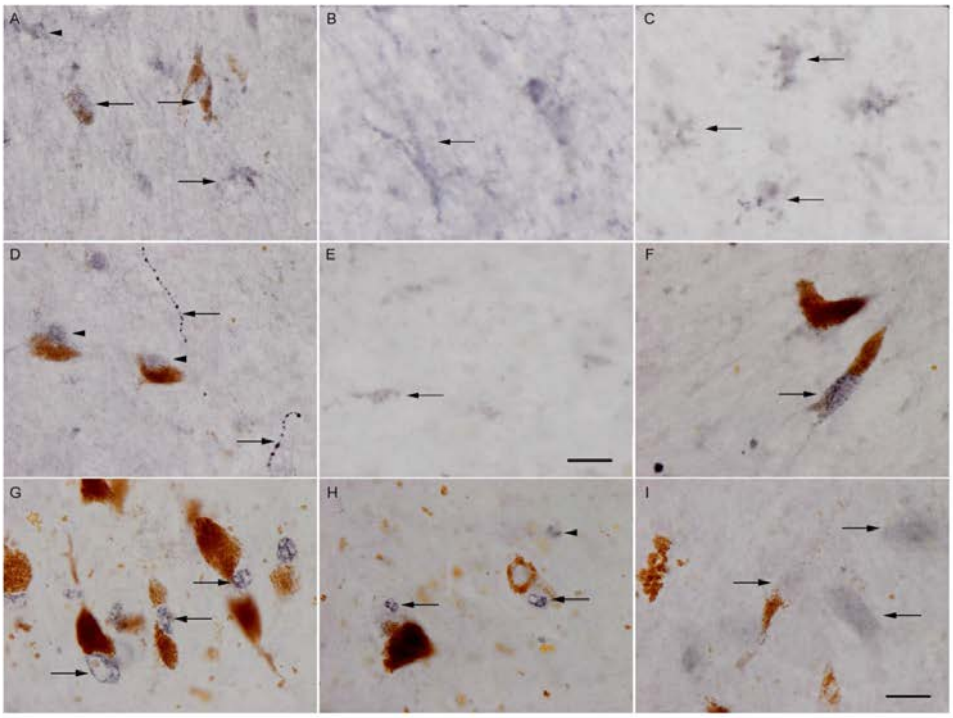

Figure 3. Immunohistochemistry of interleukins (IL)-5 (**A**, **B**), IL-6 (**C**), IL-10 (**D**), IL-17R (**E**), M-CSF (**F**) NFκB phosphorylated (**G**, **H**), and TNF-α (**I**) in the *substantia nigra pars compacta* (**A**, **D–I**) in PD stages 3–4, and frontal cortex (**B**, **C**) in PD staged 4–5. **A.** IL-5 immunoreactivity is seen in neurons (arrows) and astrocytes (arrowhead); and **B.** in astrocytes in the subcortical white matter (arrows); **C.** IL-6 is mainly expressed in astrocytes (arrows); **D.** IL-10 immunoreactivity occurs in neurons (arrowheads) and in beaded cell processes (arrows) in the *substantia nigra*; **E.** IL-17 receptor is expressed in glial cells (probably microglia); **F.** Macrophage colony stimulating factor (M-CSF) is expressed in neurons; whereas **G, H.** phosphorylated (active) NFκB immunoreactivity is found in the nucleus of neurons (arrows) and glial cells (arrowheads); **I.** weak TNF-α immunoreactivity is found in neurons. Cryostat sections processed free-floating visualized with diaminobenzidine, NH_4NiSO_4, and H_2O_2 without haematoxylin counterstaining. **A**, **D**, **F–I**, bar in **I** = 25 μm; **B**, **C**, **E**, bar in **E** = 10 μm.

conditions and in several experimental models, although it can also play a role as a pro-inflammatory cytokine (24). It may be speculated that the trend of increased IL-6 mRNA in frontal cortex, accompanied by a significant increase in IL-6 protein as revealed by ELISA and Western blotting, is consistent with a protective role of this IL in PD progression, but the opposite is also a possibility depending on the region and the accompanying responses. As another example, in spite of the reduced IL-1β mRNA expression here observed and the presumed lower levels of the encoded protein, phosphorylated (active) NFκB is found in the nucleus of several neurons and glial cells in the *substantia nigra pars compacta* in PD at stages 3–5 of Braak, thus indicating that NFκB can play a role in neuroinflammation in the *substantia nigra* in PD (84). However, NFκB is regulated by oxidative stress (61), and reactive oxygen species may activate or inhibit NF-κB activity (70). It has been reported that inhibition of NFκB activity by injection of modifier-binding domain of IκB kinase α (IKKα) or IKKβ promotes cell survival in MPTP-induced dopaminergic cell death in mice (31); however, we cannot merely translate observations in an acute model to those that work in a chronic state such as PD.

The reasons for increased neuroinflammatory responses in PD are not known, but it has been shown that regional-specific microglial activation together with increased levels of Tnf-α mRNA, but not of Il-1β, Tgf-β, and certain toll-like receptors, occurs in the *substantia nigra* but not in the cerebral cortex in young mice overexpressing human wild-type α-synuclein (90). This is in line with pioneering studies showing that α-synuclein

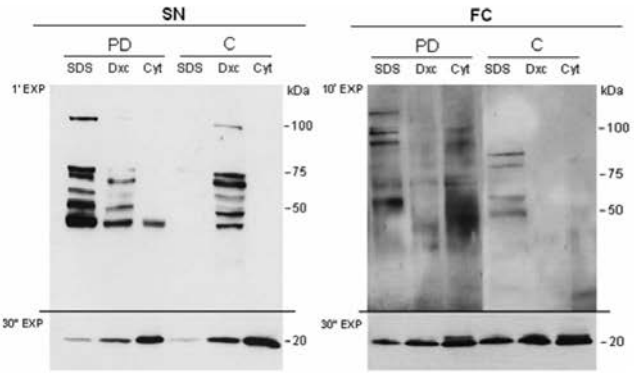

Figure 4. Representative figure of α-synuclein solubility and aggregation in the *substantia nigra* (SN) and frontal cortex area 8 (FC) in Parkinson's disease (PD) stage 4 and age-matched control (C). Cyt, cytosolic fraction, DXC, deoxycholate-soluble fraction, SDS: SDS-soluble fraction. α-synuclein at the appropriate molecular weight (about 20 kDa) is observed at 30" of exposure of the membrane in the *substantia nigra* and frontal cortex in control and diseased samples in all the fractions. Altered solubility and presence of multiple bands of aggregated α-synuclein of molecular masses between 40 and 80 kDa are seen in PD *substantia nigra* when compared with *substantia nigra* in control after 1-minute of exposure. A different pattern of α-synuclein solubility and aggregation is seen in the frontal cortex in PD when compared with control. Note that the exposure time needed to visualize the bands is greater (10-minute) in the frontal cortex than in the *substantia nigra* under exactly the same protein loading conditions.

extracellular aggregates activate microglia in primary mesencephalic neuron-glia culture system and that microglial activation increases dopaminergic neurodegeneration induced by aggregated α-synuclein (94). Astrocytes are also stimulated by α-synuclein *in vitro* (47). The present studies in human brains have shown a relationship between the presence of Lewy bodies and neurites, α-synuclein aggregates of variable solubility, astrocyte and microglial proliferation, and deregulated mRNA expression of cytokines and mediators of the immune response in the *substantia nigra* in PD. Microglial and astrocytic responses are much more limited in the putamen, frontal cortex and angular gyrus in PD in parallel with the absence of Lewy bodies and the lower formation of α-synuclein aggregates in these regions when compared with the *substantia nigra* at similar Braak stages. Therefore, the present findings support the idea that altered α-synuclein including nitrated α-synuclein can trigger neuroinflammation in PD (4). However, it is clear from the present data that α-synuclein cannot be considered simply as an activator of cytokine expression in PD, as the region with largest numbers of Lewy bodies and abnormal α-synuclein aggregates (i.e. *substantia nigra pars compacta*) has the lowest up-regulation of cytokines and immune response mediators when compared with other brain regions (putamen, frontal cortex area 8, and angular gyrus).

Other factors that can activate neuroinflammation in PD are related to the production of reactive oxygen and nitrogen species by neurons. Studies in the *substantia nigra* in PD have shown decreased levels of reduced glutathione (69, 82, 93), increased Cu/Zn-superoxide dismutase I (SOD1), and Mn-superoxide dismutase (SOD2) protein and mRNA levels (11, 52, 76); and increased levels of protein carbonyls (1, 28), lipid hydroperoxides (19, 43), 4-hydroxy-2-nonenal (81, 92), as well as advanced glycation end products (10). Reduced gluthatione levels are decreased in the *substantia nigra* in cases with incidental PD (20, 42) indicating that alterations in glutathione function in the *substantia nigra* are an early marker of nigral pathology in PD (20, 44, 93). Oxidative damage in the *substantia nigra* and proteins related to oxidative stress responses are already present at stages 2 and 3 of Braak (18, 28). Lipoxidative damage of α-synuclein is found in incidental PD (18). Oxidative damage of DNA and RNA has also been reported in PD (27, 29, 65, 80).

Oxidative damage is not restricted to the *substantia nigra* in PD. Increased oxidative damage and increased expression of advanced glycation end products have been observed in the amygdala and cerebral cortex in incidental PD, and in parkinsonian stages of PD without cognitive impairment (18, 25, 28). Redox proteomics has been useful in identifying protein targets of oxidative damage in PD (51). In addition to α-synuclein (17), several key proteins have been identified as targets of oxidative damage including β-synuclein and SOD2 (17, 18, 25), UCHL1, SOD1 and DJ-1 (14–16), and aldolase A, enolase 1 and glyceraldehyde dehydrogenase, all of them involved in glycolysis and energy metabolism (32) in the frontal cortex in pre-motor stages of PD and in established PD. Other proteins have also been identified as lipoxidatively damaged using the malonaldehyde-Lysine marker: phosphoprotein enriched in astrocytes, SH3 domain binding glutamic acid-rich protein-like, ubiquitin-conjugating enzyme E2N-like, proteasome subunit Y, and thioredoxin (26).

Together, available information shows that altered α-synuclein solubility and aggregation, and Lewy body formation, oxidative damage, and neuroinflammation converge in the pathogenesis of PD, but it is difficult to reconcile a simple scenario of direct cause–effect mechanisms. Regarding neuroinflammation, the

present findings have important implications not only in the pathogenesis, but also in the therapeutics of PD, as neuroinflammation involves pro- and anti-inflammatory cytokines, and variegated mediators of the immune response which, at least in the immune system, have distinct often opposing functions. Moreover, neuroinflammatory responses are subject to regional variations at the same stages of PD-related pathology, implying that distinct inflammatory responses occur in different brain regions at the same time in a particular individual.

Concluding comments

The added value of the present comprehensive study using complementary methods and different brain regions in a large series of PD cases when compared with previous studies can be summarized as follows: (i) genes encoding cytokines and mediators of the immune response are markedly deregulated in PD; (ii) deregulation is subject to regional differences, certain genes being oppositely regulated in the *substantia nigra* and cerebral cortex at the same PD stages; (iii) the majority of analyzed genes are downregulated in the *substantia nigra* from stage 4 onward; (iv) mRNA gene expression levels do not always correlate with protein expression levels, suggesting that altered gene regulation is accompanied by modified protein expression either because of altered translation or modified protein degradation; (v) immunohistochemistry localizes intrinsic neuroinflammatory responses either in neurons and glial cells, or in both, mainly in neurons and microglia; (vi) particular protein expression in neurons and in glial cells suggests intrinsic neuroinflammatory cross-information among neurons, astrocytes, and microglia in PD with limited participation of the peripheral immune system excepting the scant infiltration of isolated lymphocytes in the *substantia nigra*; (vii) in line with the previous point, neurons may be considered as active trigger agents of neuroinflammation in PD expressing certain cytokines and mediators of the immune response. Available information shows that altered α-synuclein solubility and aggregation; and (viii) Lewy body formation, oxidative damage, and neuroinflammation converge in the pathogenesis of PD.

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ARTICLE 4

Purine metabolism gene deregulation in Parkinson's disease.

Garcia-Esparcia P, Hernández-Ortega K, Ansoleaga B, Carmona M, Ferrer I.

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ABSTRACT

Introduction and aims: To explore alterations in the expression of genes encoding enzymes involved in purine metabolism in Parkinson's disease (PD) brains as purines are the core of the DNA, RNA, nucleosides and nucleotides which participate in a wide variety of crucial metabolic pathways. **Methods:** Analysis of mRNA using real-time quantitative PCR of 22 genes involved in purine metabolism in the substantia nigra, putamen and cerebral cortex area 8 in PD at different stages of disease progression, and localization of selected purine metabolism-related enzymes with immunohistochemistry. **Results:** Reduced expression of adenylate kinase 2 (*AKA2*), *AK3*, *AK4*, adenine phosphoribosyltransferase, ectonucleoside triphosphate diphospho-hydrolase 1 (*ENTPD1*), *ENTPD3*, nonmetastatic cells 3, nucleoside-diphosphatese kinase 3 (*NME1*), *NME7* and purine nucleoside phosphorylase 1 (*PNP1*) mRNA in the substantia nigra at stages 3–6; up-regulation of *ADA* mRNA in the frontal cortex area 8 at stages 3–4 and of *AK1*, *AK5*, *NME4*, *NME5*, *NME6*, 5′-nucleotidase (*NT5E*), *PNP1* and prune homolog *Drosophila* at stages 5–6. There is no modification in the expression of these genes in the putamen at stages 3–5. **Conclusions:** Gene downregulation in the *substantia nigra* may be interpreted as a consequence of dopaminergic cell death as *ENTPD3*, *NME1, NME7*, *AK1* and *PNP1* are mainly expressed in neurons. Yet *ENTPD1* and *NT5E*, also down-regulated in the substantia nigra, are expressed in astrocytes, probably pericytes and microglia, respectively. In contrast, gene up-regulation in the frontal cortex area 8 at advanced stages of the disease suggests a primary manifestation or a compensation of altered purine metabolism in this region.

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Purine metabolism gene deregulation in Parkinson's disease

P. Garcia-Esparcia*†, K. Hernández-Ortega*, B. Ansoleaga*, M. Carmona*† and I. Ferrer*†‡

**Institute of Neuropathology, Bellvitge University Hospital-Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet de Llobregat, †Center for Networked Biomedical Research in Neurodegenerative Diseases (CIBERNED), Institute Carlos III, Ministry of Health, and ‡University of Barcelona, Barcelona, Spain*

P. Garcia-Esparcia, K. Hernández-Ortega, B. Ansoleaga, M. Carmona and I. Ferrer (2015) *Neuropathology and Applied Neurobiology*

Purine metabolism gene deregulation in Parkinson's disease

Aims: To explore alterations in the expression of genes encoding enzymes involved in purine metabolism in Parkinson's disease (PD) brains as purines are the core of the DNA, RNA, nucleosides and nucleotides which participate in a wide variety of crucial metabolic pathways. **Methods:** Analysis of mRNA using real-time quantitative PCR of 22 genes involved in purine metabolism in the *substantia nigra*, putamen and cerebral cortex area 8 in PD at different stages of disease progression, and localization of selected purine metabolism-related enzymes with immunohistochemistry. **Results:** Reduced expression of adenylate kinase 2 (*AKA2*), *AK3*, *AK4*, adenine phosphoribosyltransferase, ectonucleoside triphosphate diphosphohydrolase 1 (*ENTPD1*), *ENTPD3*, nonmetastatic cells 3, nucleoside-diphosphatese kinase 3 (*NME1*), *NME7* and purine nucleoside phosphorylase 1 (*PNP1*) mRNA in the *substantia nigra* at stages 3–6; up-regulation of *ADA* mRNA in the frontal cortex area 8 at stages 3–4 and of *AK1*, *AK5*, *NME4*, *NME5*, *NME6*, 5′-nucleotidase (*NT5E*), *PNP1* and prune homolog *Drosophila* at stages 5–6. There is no modification in the expression of these genes in the putamen at stages 3–5. **Conclusions:** Gene down-regulation in the *substantia nigra* may be interpreted as a consequence of dopaminergic cell death as *ENTPD3*, *NME1*, *NME7*, *AK1* and *PNP1* are mainly expressed in neurons. Yet *ENTPD1* and *NT5E*, also down-regulated in the *substantia nigra*, are expressed in astrocytes, probably pericytes and microglia, respectively. In contrast, gene up-regulation in the frontal cortex area 8 at advanced stages of the disease suggests a primary manifestation or a compensation of altered purine metabolism in this region.

Keywords: frontal cortex, Parkinson's disease, purine metabolism enzymes, putamen, *substantia nigra*

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative illness, clinically characterized by a motor dysfunction known as parkinsonism resulting from dopaminergic neuron loss in the *substantia nigra pars compacta*. This is preceded by several years by a plethora of premotor symptoms including loss of olfaction, sleep disorders, depression and dysautonomia, and which is followed by worsening of motor symptoms, cognitive impairment and dementia over the years. PD is neuropathologically distinguished by the production and accumulation of abnormal α-synuclein eventually in the form of cytoplasmic inclusions named Lewy bodies and dystrophic neurites [1–3]. The systematic post-mortem study of large series of cases with Lewy bodies has permitted the categorization of PD progression into six stages from stage 1 (medulla oblongata), 2 (pons), 3 (*substantia nigra*), 4

Correspondence: Isidre Ferrer, Institute of Neuropathology, Service of Pathology, Bellvitge University Hospital, carrer Feixa Llarga sn, 08907 Hospitalet de Llobregat, Spain. Tel: +34 93 260 7452; Fax: +34 93 2607503; E-mail: 8082ifa@gmail.com

(limbic system), to stages 5 and 6 (cortical involvement) in about 70% of cases [4,5]. Neuropathological findings correlate with neurological deficits in typical cases which represent about 70% of the total [6–10].

In addition to the classical neuropathological hallmarks (selected neuron loss, Lewy bodies, altered neurites, and abnormal α-synuclein production and deposition), a large number of metabolic defects converge in PD brains. These include mitochondrial abnormalities, oxidative stress damage, reticulum stress damage, altered ubiquitin-proteasome system function and autophagy, and neurotransmitter and neuromodulator abnormalities, altered composition of lipid membranes, particularly lipid rafts, and neuroinflammation, among many others (see Ferrer and Ferrer *et al.* [11,12] for references and review). Importantly, metabolic alterations in the cerebral cortex and other systems occur at relatively early stages of PD [13–15].

Purines are heterocyclic double-ring aromatic organic molecules: primary purine adenine and guanosine nucleobases, together with one-ring primary pyrimidine nucleobases cytosine, thymidine and uracil, are the core of DNA, RNA, nucleosides and nucleotides. AMP, ADP, ATP, GMP, GDP, GTP, and cyclic forms cAMP and cGMP are primary purine-derived nucleotides. Nucleotides participate in a wide variety of crucial metabolic pathways including energy metabolism and cell signalling. In addition, purine bases are incorporated into other molecules to form cofactors of several enzymatic reactions such as coenzyme A, flavin adenine dinucleotide, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate and the corresponding reduced forms. S-Adenosyl methionine is made from ATP and methionine by methionine adenosyltransferase and is involved in the transfer of methyl groups to distinct substrates including nucleic acids, proteins, lipids and metabolites. In addition to intracellular signalling, purines and their products may function as extracellular signals acting upon other cells, either between neurons, or between neurons and glial cells equipped with appropriate receptors [16–18]. Adenosine exerts its function by binding adenosine receptors which are G-protein-coupled receptors (A1, A2A, A2B and A3) that can inhibit (A1, A3) or enhance (A2) neuronal communication through neurotransmitter release [19,20].

A2A receptors are abundant in striatopallidal neurons where they bind to D2 dopamine receptors and reduce their affinity for dopamine [21]. Striatal A2A receptor levels are increased in PD [22–24], even at early stages of the disease, and they are possibly regulated, in part, by miR-34b [25]. Therefore, A2A receptor antagonists are potential therapeutic agents to increase dopamine uptake in the PD striatum, thereby improving dopamine availability [26–28].

Energy metabolism dependent on mitochondrial function and ATP production is markedly altered in PD [29–32], and oxidative damage of energy-related enzymes hampers physiological energy balance [33–35]. In addition, oxidative damage to nucleic acids is found in the brain in PD [36,37].

However, little is known about possible alterations in the expression of genes encoding enzymes involved in purine metabolism in PD brains. This information would permit a better understanding of the primary regulation of purine-related genes and their possible implications in the pathogenesis of the disease. For this purpose, we have analysed mRNA expression of selected genes involved in purine metabolism in three regions of the brain at different stages of PD-related pathology: substantia nigra, putamen and frontal cortex area 8. The selection of these particular genes was based on previous observations in Alzheimer's disease (AD) in which the expression of these genes was abnormally regulated with disease progression with particular regional patterns [18], and with the intention to identify commonalities and differences in gene expression between these prevalent neurodegenerative diseases. Immunohistochemistry was also used to localize the cellular expression of altered genes at the protein level.

Material and methods

Human cases

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank and the Biobank of Hospital Clinic-IDIBAPS following the guidelines of Spanish legislation on this matter and the approval of the local ethics committee. The post-mortem interval between death and tissue processing was between 3 and 20 h. One hemisphere was immediately cut in coronal sections, 1cm thick, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags, numbered with water-resistant ink and stored at −80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphological

studies. Neuropathological study to categorize the present cases was routinely performed on a series of 20 de-waxed paraffin sections comprising different regions of the cerebral cortex, diencephalon, thalamus, brain stem and cerebellum which were stained with haematoxylin and eosin, Klüver-Barrera and for immunohistochemistry to microglia (clone CD68), glial fibrillary acidic protein, β-amyloid, phosphorylated tau (clone AT8), α-synuclein, TDP-43, ubiquitin and p62. Neuropathological diagnosis of PD was based on the classification of Braak *et al.* [5]. Only typical cases according to the Braak classification were included. Cases with combined pathologies (i.e., AD, tauopathy, cerebrovascular diseases and metabolic syndrome) were excluded from the present study. Age-matched control cases had not suffered from neurologic, psychiatric diseases or metabolic diseases (including metabolic syndrome), and did not have abnormalities in the neuropathological examination excepting sporadic Alzheimer's disease (sAD)-related pathology stages I–II/0-B (Braak and Braak [38]). PD cases with more advanced stages of sAD-related pathology were not considered in the present series to avoid overlap between PD and AD in the frontal cortex.

In total, 72 brains including 29 controls and 43 cases with PD-related pathology were included in the present studs. Incidental PD (iPD or incidental Lewy body disease) occurred in 15 cases (most stages 1, 2 and 3 of Braak), and 28 had suffered from parkinsonism. Clinical preparkinsonian symptoms in iPD cases were not available. Regarding PD cases, all of them were treated for their motor symptoms. The disease duration was variable from 6 years to 16 years. The most common causes of death in the control and PD cases were infections, neoplasia and acute cardiac disease.

Three regions were examined: *substantia nigra pars compacta*, putamen and frontal cortex area 8. Cases studied for mRNA expression in *substantia nigra* were 12 controls (eight males, four females), six PD stages 1–2 (four males, two females) and 17 PD stages 3–6 (14 males, three females). The putamen was analysed in eight controls (seven males, one female) and seven PD stages 3–5 (five males, two females). Gene expression in frontal cortex area 8 was analysed in 16 controls (seven males, nine females), two PD stages 1–2 (a male, one female),16 PD stages 3–4 (13 males, three females), and 12 PD stages 5–6 (six males, six females). Not all regions were available in every case; this deficiency was compensated for by studying a large number of individuals. A summary of all the cases examined in this study is shown in Table 1; mean ages and standard deviation of every group are shown in Table 2.

RNA purification

Purification of RNA from the right *substantia nigra*, putamen and frontal cortex area 8 was carried out with RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer after DNase digest to avoid extraction and later amplification of genomic DNA. The concentration of each sample was obtained from A260 measurements with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values of RNA integrity number (RIN) varied from 6.4 to 8.8 (Table 1). Post-mortem delay had no implications for RIN values in the present series (Table 1).

Retrotranscription reaction

Retrotranscription reaction of RNA samples selected based on their RIN values was carried out with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) following the guidelines provided by the manufacturer and using Gene Amp® 9700 PCR System thermocycler (Applied Biosystems). A parallel reaction for one RNA sample was processed in parallel in the absence of reverse transcriptase to rule out DNA contamination.

Real-time PCR

Real-time quantitative PCR (RT-qPCR) assays were conducted in duplicate on cDNA samples obtained from the retrotranscription reaction, diluted 1:20 in 384-well optical plates (Kisker Biotech, Steinfurt, Germany) utilizing the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Parallel amplification reactions were carried out using 20× TaqMan Gene Expression Assays for purine metabolism enzymes and 2× TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probes used in the study are shown in Table 3. The selection of these probes was based on previous data obtained in Alzheimer's disease in which several genes recognized by these probes were deregulated in different regions at different stages of disease progression [18]. The selection of these particular probes was considered advantageous in order to

Table 1. Summary of cases for mRNA expression and immunohistochemistry studies in the SN, PUT and FC

°*Case*	*CLIN*	*PD Braak*	*Gender*	*Age*	*PM delay*	*RIN SN*	*RIN PUT*	*RIN FC*	*IHC SN*	*IHC FC*
1	0	0	Male	64	8 h 30			7.7		
2	0	0	Male	56	5 h			7.8	x	x
3	0	0	Male	67	5 h			7.4		
4	0	0	Male	62	3 h			8	x	
5	0	0	Male	52	4 h 40 m			8.1		
6	0	0	Male	30	4 h 10 m	7.4		8.4		x
7	0	0	Male	53	3 h			8.8		
8	0	0	Female	49	7 h			8.2		
9	0	0	Female	75	3 h			7.7		
10	0	0	Female	46	9 h 35 m	7.2		6.8	x	x
11	0	0	Female	86	4 h 15 m			8.1		
12	0	0	Female	79	3 h 35 m			7.7	x	
13	0	0	Female	79	6 h 25 m	6.4		7.8		
14	0	0	Female	77	3 h 15 m			7		
15	0	0	Female	76	5 h 45 m			8.3		
16	0	0	Female	71	8 h 30 m			7.4		
17	0	0	Male	59	4 h 15 m	6.8	6.7			
18	0	0	Male	67	14 h 40 m	6.4				
19	0	0	Male	70	2 h	6.5				
20	0	0	Male	61	4 h 30 m	7.1	6.5			
21	0	0	Male	63	8 h 05 m	6.4				
22	0	0	Male	57	4 h 30 m	6.6	6.9			
23	0	0	Male	60	4 h 15 m	6.6	7.3			
24	0	0	Female	68	4 h 30 m	6.4				
25	0	0	Female	64	2 h 15 m	7.3				
26	0	0	Male	77	6 h 55 m		6.5			
27	0	0	Male	78	12 h		6.9			
28	0	0	Female	72	4 h		6.7			
29	0	0	Male	75	5 h 15 m		7			
30	0	PD 1	Male	72	8 h 55 m				X	X
31	0	PD 1	Female	75	23 h				X	X
32	0	PD 1	Male	74	10 h 50 m	6.4				
33	0	PD 1	Male	80	6 h	6.6				
34	0	PD 2	Male	83	3 h 30 m	6.4				
35	0	PD 2	Female	97	3 h 40 m	6.5				
36	0	PD 3	Male	57	11 h			8.7		
37	0	PD 3	Female	54	11 h 10 m			8.2		
38	0	PD 3	Male	71	18 h 45 m				X	
39	0	PD 3	Male	73	4 h 15 m				X	X
40	0	PD 3	Male	81	4 h 55 m	6.9	6.7		X	X
41	0	PD 3	Male	73	15 h 30 m				X	
42	PD	PD 4	Male	66	5 h			7.3		
43	0	PD 4	Male	72	5 h			7.7		
44	0	PD 4	Male	57	19 h			7.6		
45	PD	PD 4	Male	76	4 h 30 m			7.7		
46	PD	PD 4	Male	68	4 h 45 m			7.3		
47	0	PD 4	Male	79	9 h 15 m			7.2		
48	PD	PD 4	Male	69	5 h 55 m			6.4		
49	PD	PD 4	Male	68	9 h 20 m	8.2		6.9		
50	PD	PD 4	Male	77	12 h			6.9		
51	PD	PD 4	Female	84	4 h 30 m	6.8		7.8		
52	PD	PD 4	Male	69	5 h 55 m				X	X
53	PD	PD 4	Male	69	15 h 05 m				X	
54	PD	PD 4	Female	70	4 h 40 m		6.5			
55	PD	PD 4	Male	84	9 h		6.5			
56	PD	PD 4	Male	74	6 h 45 m		7.3		X	X
57	PD	PD 4	Female	88	11 h 50 m		7.2		X	X

Table 1. (*Continued*)

°Case	CLIN	PD Braak	Gender	Age	PM delay	RIN SN	RIN PUT	RIN FC	IHC SN	IHC FC
58	PD	PD 5	Female	70	4 h 30 m			8.5		
59	PD	PD 5	Female	77	3 h 30 m			6.8		
60	PD	PD 5	Female	81	6 h 30 m			8.3		
61	PD	PD 5	Female	69	4 h 30 m			8		
62	PD	PD 5	Female	79	3 h 30 m			8		
63	PD	PD 5	Male	78	13 h 30 m	6.4		6.7		
64	PD	PD 5	Male	83	14 h	7.6		7.7		
65	PD	PD 5	Female	77	7 h 30 m	6.4		7.1		
66	PD	PD 5	Male	76	12 h	6.7				
67	PD	PD 5	Male	80	7 h 30 m	7.1				
68	PD	PD 5	Male	85	11 h 45 m	7.7				
69	PD	PD 5	Male	84	16 h 30 m	6.4			X	X
70	PD	PD 5	Male	79	4 h 30 m		6.5		X	X
71	PD	PD 5	Male	77	7 h 30 m		6.6		X	X
72	PD	PD 6	Male	80	7 h 30 m	7.9		7.5		

CLIN: neurological manifestations (0: refers to the lack of parkinsonism, and PD to parkinsonism). PD Braak: stage of PD- related pathology following the nomenclature of Braak; Age in years; post-mortem delay in hours and minutes.
F, female; FC, frontal cortex area 8; IHC: immunohistochemistry; M, male; PUT, putamen; RIN: values of RNA integrity number; SN, substantia nigra.

Table 2. Summary of mean ages and standard deviation of each group of samples used in the present study including *substantia nigra*, putamen and frontal cortex area 8

	Substantia nigra	*Putamen*	*Frontal cortex*
	Mean age ± SD	*Mean age ± SD*	*Mean age ± SD*
Controls	60.33 ± 12.45	67.38 ± 8.93	63.88 ± 15.20
PD stages 1–2	80.17 ± 9.20	–	73.50 ± 2.12
PD stages 3–4	75.00 ± 6.93	79.40 ± 7.33	71.25 ± 8.69
PD stages 5–6	79.90 ± 3.14	78.00 ± 1.41	77.83 ± 4.51

compare commonalities and differences between AD and PD regarding purine gene metabolism deregulation.

Parallel assays for each sample were carried out using probes for β-glucoronidase (GUS-β) and X-prolyl aminopeptidase (aminopeptidase P) 1 (XPNPEP1) for normalization. The reactions were performed using the following parameters: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. TaqMan PCR data were captured using the Sequence Detection Software (SDS version 2.2, Applied Biosystems). Subsequently, threshold cycle (CT) data for each sample were analysed with the double delta CT (ΔΔCT) method. First, delta CT (ΔCT) values were calculated as the normalized CT values for each target gene in relation to the endogenous controls GUS-β and XPNPEP1. These housekeeping genes were selected because they show no modifications in several neurodegenerative diseases in human post-mortem brain tissue [39,40]. Second, ΔΔCT values were obtained with the ΔCT of each sample minus the mean ΔCT of the population of control samples (calibrator samples). The fold change was determined using the equation $2^{-\Delta\Delta CT}$.

Statistical analysis

The normality of distribution of the mean fold change values obtained by RT-qPCR for every region and stage between controls and PD cases were analysed with the Kolmogorov–Smirnov test. The nonparametric Mann–Whitney test was performed to compare each group when the samples did not follow a normal distribution, whereas the unpaired *t*-test was used for normal variables. Differences between groups were considered statistically significant at *P* values: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Statistical analysis to detect association of some variables including gender, age, post-mortem delay and RIN was carried out by using Spearman correlations for nonparametric variables. Gender was converted into a quantitative variable to perform the corresponding analysis.

Statistical analysis was performed with GraphPad Prism version 5.01 (La Jolla, CA, USA) and Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (Warrenton, VA, USA).

Table 3. Abbreviated names of genes, full names and TaqMan probes used for the study of mRNA expression of purine metabolism enzymes including GUS-β and XPNPEP1 used for normalisation

Gene	*Full name*	*Sequence of TaqMan probes*
ADA	Adenosine deaminase	AGTTTAAAAGGCTGAACATCAATGC
AK1	Adenylate kinase 1	AGTTTGAGCGACGGATTGGACAGCC
AK2	Adenylate kinase 2	GGCAGGCAGAAATGCTCGATGACCT
AK4	Adenylate kinase 4	ACTGGCTCCTTGATGGTTTTCCTAG
AK5	Adenylate kinase 5	CTTTTGAGGACCAAATCTGTACCCC
AK7	Adenylate kinase 7	GGAGACGTACTCTGCCATCTCTCAA
APRT	Adenine phosphoribosyltransferase	CGCATCGACTACATCGCAGGCCTAG
DGUOK	Deoxyguanosine kinase	GCAACATTGCTGTGGGAAAGTCCAC
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1	TCATTGGCAAGATCCAGGGCAGCGA
ENTPD2	Ectonucleoside triphosphate diphosphohydrolase 2	CCCGCCCTCAAGTATGGCATCGTCC
ENTPD3	Ectonucleoside triphosphate diphosphohydrolase 3	TGAAAAAGAAGTGGGGAATAGCAGC
GUS-B	β-glucuronidase	GCTACTACTTGAAGATGGTGATCGC
NME1	Nonmetastatic cells 1, protein (NM23A) expressed in (nucleoside-diphosphate kinase)	TGTTACCATCCCCGACCATCTGATT
NME3	Nonmetastatic cells 3, protein expressed in (nucleoside-diphosphate kinase)	ATCGAGGTTGGCAAGAACCTGATTC
NME4	Nonmetastatic cells 4, protein expressed in (nucleoside-diphosphate kinase)	GTCCACATCAGCAGGAATGTCATCC
NME5	Nonmetastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	TTTTATGTTTCCTGAAGTGATTGTT
NME6	Nonmetastatic cells 6, protein expressed in (nucleoside-diphosphate kinase)	CCACTGATTCTGGAGGCTGTTCATC
NME7	Nonmetastatic cells 7, protein expressed in (nucleoside-diphosphate kinase)	CTGATCCTGAAATTGCCCGGCATTT
NT5C	5′, 3′-nucleotidase, cytosolic	TGCGGCCCGACCTGGCGGATAAAGT
NT5E	5′-nucleotidase, ecto (CD73)	TTCTTTACACAGGCAATCCACCTTC
PNP	Purine nucleoside phosphorylase	GGGAGCAGACGCTGTTGGCATGAGT
POLR3B	Polymerase (RNA) III (DNA directed) polypeptide B	GGTATTCTGGCTGGTGCCATTACTG
PRUNE	Prune homolog (Drosophila)	CCCTTCTGCATGGAACCATCATCCT
XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1	CAAAGAGTGCGACTGGCTCAACAAT

Immunohistochemistry

The immunohistochemical study of purine metabolism was performed in 4-µm thick dewaxed paraffin sections of the *substantia nigra* and frontal cortex area 8 of controls ($n = 6$) and PD cases (three PD stages 3–4; five PD stages 5–6). The sections were boiled in citrate buffer for 20 min to retrieve antigenicity. Endogenous peroxidases were blocked with Peroxidase (Dako, Glostrup, Denmark) followed by 10% normal goat serum solution. The monoclonal antibodies used were anti-adenylate kinase 1, AK1 (1:100; ab54825, Abcam, Cambride, UK) and anti-ecto-5-prime-nucleotidase, NT5E (1:200; H00004907-M01, Abnova, Taipei, Taiwan). The polyclonal antibodies were anti-nucleoside phosphorylase 1, PNP (1:700; GTX117364, GeneTex, CA, USA); anti-ectonucleoside triphosphate diphosphohydrolase 1, ENTPD1 (1:400; GTX118055, GeneTex); anti-nucleoside diphosphate kinase 7, NME7 (1:100; AP15032PU-N, Acris, CA, USA); anti-nucleoside diphosphate kinase 1, NME1 (1:700; AM2209B, Abgent, Majdenhead, UK); and anti-ectonucleoside triphosphate diphosphohydrolase 3, ENTPD3 (1:50; ab67263, Abcam). Immediately afterwards, the sections were incubated with EnVision + system peroxidise (Dako) at room temperature for 15 min. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. The omission of the primary antibody in some sections was used as a control of the immunostaining: no signal was obtained with the only incubation being the secondary antibody. No antigenic peptides were available for preabsorption studies. Sections were lightly counterstained with haematoxylin.

Results

mRNA expression of purine metabolism enzymes in the *substantia nigra pars compacta* in PD at stages 1–2 and 3–6

Expression of 22 genes implicated in the purine metabolism was performed with mRNA assay using TaqMan PCR. GUS-β was employed to normalize mRNA expression levels.

Table 4. mRNA expression levels of purine metabolism enzymes in control and PD cases in the *substantia nigra* stages 1–2 and 3–6 as determined by TaqMan PCR assays using *GUS-β* for normalization

Substantia nigra pars compacta

	C	*PD 1–2*	*C* vs. *PD 1–2*	*C*	*PD 3–6*	*C* vs. *PD 3–6*
POLR3B	1.02 ± 0.22	0.77 ± 0.21	–	0.98 ± 0.38	0.81 ± 0.38	–
NT5C	1.07 ± 0.38	0.74 ± 0.40	–	1.07 ± 0.39	0.72 ± 0.38	–
PRUNE	NA	NA	–	0.93 ± 0.42	0.98 ± 0.49	–
APRT	NA	NA	–	1.10 ± 0.50	0.68 ± 0.37†	↓*
DGUOK	1.08 ± 0.47	0.84 ± 0.20	–	1.10 ± 0.50	0.75 ± 0.35	–
NT5E	1.07 ± 0.45	1.36 ± 0.58	–	1.03 ± 0.49	1.17 ± 0.95	–
ENTPD1	1.06 ± 0.40	1.21 ± 0.32†	–	0.86 ± 0.23	0.60 ± 0.25	↓*
ENTPD2	1.07 ± 0.39†	1.13 ± 0.28†	–	1.12 ± 0.58	0.83 ± 0.40†	–
ENTPD3	1.03 ± 0.31	1.00 ± 0.26	–	1.04 ± 0.66	0.41 ± 0.52	↓*
ADA	1.14 ± 0.71	1.05 ± 0.24	–	1.12 ± 0.52	0.86 ± 0.60	–
AK1	1.07 ± 0.41	0.70 ± 0.11	–	0.96 ± 0.44	0.59 ± 0.37	–
AK2	1.04 ± 0.28	0.75 ± 0.05	–	0.95 ± 0.35	0.63 ± 0.26	↓*
AK4	1.05 ± 0.31	0.91 ± 0.30	–	1.04 ± 0.51	0.59 ± 0.36	↓*
AK5	1.02 ± 0.19	0.89 ± 0.20	–	1.08 ± 0.42	0.66 ± 0.22	↓*
AK7	1.05 ± 0.35	0.80 ± 0.21	–	1.09 ± 0.50	0.90 ± 0.47	–
NME1	1.11 ± 0.52	0.58 ± 0.16	–	1.14 ± 0.57	0.32 ± 0.26	↓***
NME3	1.04 ± 0.34	0.72 ± 0.18	–	1.13 ± 0.56	0.69 ± 0.56	–
NME4	1.02 ± 0.23	0.96 ± 0.26	–	1.04 ± 0.46	0.53 ± 0.35	–
NME5	1.12 ± 0.54	0.76 ± 0.42	–	1.15 ± 0.80	0.73 ± 0.63†	–
NME6	1.02 ± 0.21	0.88 ± 0.26	–	0.97 ± 0.29	0.89 ± 0.37	–
NME7	1.03 ± 0.28	0.68 ± 0.22	–	1.02 ± 0.46	0.59 ± 0.32	↓*
PNP	1.08 ± 0.48	0.87 ± 0.71†	–	1.10 ± 0.42	0.67 ± 0.36	↓*

Student's *t*-test **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.
†No Gaussian distribution (Mann–Whitney test) **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.
NA, not available.

No significant differences in expression levels were found between controls ($n = 5$) and stages 1–2 of PD ($n = 4$), but a general tendency to decrease was observed in the most of the probes analysed. These results could have been masked due to the low number of cases available (Table 4).

However, nine of the 22 genes studied were significantly down-regulated in PD stages 3–6 ($n = 11$) compared with controls ($n = 14$), including *APRT*, *ENTPD1*, *ENTPD2*, *AK2*, *AK4*, *AK5*, *NME1*, *NME7* and *PNP*. Fold changes of significantly down-regulated genes varied from 0.28 to 0.70 and *P* values from <0.05 to <0.001.

The remaining genes showed a trend to decline in this region without statistical significance (Table 4 and Figure S1).

Similar trends were observed when using *XPNPEP1* as the housekeeping gene (Table S1).

Assessment of covariates study showed no correlation between gene expression and gender, age, post-mortem delay and RIN values, excepting *NME1* and *NME4* expression which significantly correlated with age at stages 3–6 (Tables S2 and S3).

mRNA expression of purine metabolism enzymes in the putamen in PD at stages 3–5

No differences were observed in the putamen in PD stages 3–5 ($n = 7$) in relation to controls (n = 8) regarding the expression levels of the 22 genes under study. Results using GUS-β for normalization are shown in Table 5.

Similar trends were seen using *XPNPEP1* for normalization (Table S4).

ENTPD1 and *AK2* showed correlations with age in the covariate analysis. *AK1* was correlated with RIN and *AK7* with gender. The rest of the genes did not show statistically significant correlations with age, gender, post-mortem delay and RIN values (Table S5).

Table 5. mRNA expression levels of purine metabolism enzymes in control and PD cases in the putamen, determined by TaqMan PCR assays

	C	*PD 3–5*	*C* vs. *PD 3–5*
POLR3B	1.02 ± 0.25	1.03 ± 0.32	–
NT5C	1.05 ± 0.31	1.27 ± 0.34	–
PRUNE	1.02 ± 0.24	1.08 ± 0.35	–
APRT	NA	NA	–
DGUOK	1.06 ± 0.35	0.91 ± 0.24	–
NT5E	1.04 ± 0.29	0.96 ± 0.49	–
ENTPD1	1.02 ± 0.23	0.84 ± 0.22	–
ENTPD2	1.23 ± 0.75	1.67 ± 0.90	–
ENTPD3	1.03 ± 0.26	0.93 ± 0.31	–
ADA	1.10 ± 0.49	1.42 ± 0.96	–
AK1	1.02 ± 0.19	1.15 ± 0.36	–
AK2	1.00 ± 0.10	0.87 ± 0.34†	–
AK4	1.07 ± 0.42	0.78 ± 0.25	–
AK5	1.10 ± 0.50	0.96 ± 0.44	–
AK7	1.06 ± 0.38	1.24 ± 0.35	–
NME1	1.07 ± 0.39	0.97 ± 0.40	–
NME3	1.03 ± 0.28	1.07 ± 0.65	–
NME4	1.04 ± 0.34	1.14 ± 0.27	–
NME5	1.07 ± 0.41	1.00 ± 0.42	–
NME6	1.02 ± 0.21	1.06 ± 0.72	–
NME7	1.06 ± 0.38	1.01 ± 0.47	–
PNP	1.06 ± 0.38	1.25 ± 0.66	–

Expression levels of controls and PD stages 3–5 cases using *GUS-β* for normalization. Student's *t*-test $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.
†No Gaussian distribution (Mann–Whitney test) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.
NA, not available.

mRNA expression of purine metabolism enzymes in the frontal cortex area 8 in PD at stages 3–4 and 5–6

Only ADA was up-regulated in the frontal cortex area 8 in PD cases stages 3–4 ($n = 12$) when compared with controls ($n = 16$); fold change = 1.43; P value <0.05. However, the expression levels of *PRUNE*, *NT5E*, *ENTPD1*, *AK1*, *AK2*, *AK5*, *NME4*, *NME5*, *NME6* and *PNP* were up-regulated at PD stages 5–6 ($n = 9$) when compared with controls ($n = 16$), using GUS-β for normalization, as detailed in Table 6 and Figure S2. Fold changes of significantly up-regulated genes varied from 1.28 to 1.70, P values from <0.05 to <0.001.

Similar trends were seen using *XPNPEP1* for normalization (Table S6).

NT5C gene expression showed association with gender variable, *DGUOK* with RIN, *ENTPD1* with age and *AK1* with post-mortem delay (Table S7).

Immunohistochemistry of purine metabolism enzymes

Immunohistochemistry was carried out using antibodies to selected genes whose mRNA was abnormally regulated in PD according to RT-PCR assays and which worked on paraffin sections. The quality of immunostaining varied from one case to another probably due to individual variations in protein vulnerability to the agonal state and/or post-mortem delay. Therefore, immunohistochemistry was only used to identify the localization of the protein in particular cell types: Cellular localization of the different enzymes was the same in control and diseased cases.

The following proteins were examined: ectonucleoside triphosphate diphosphohydrolase 3 (encoded by *ENTPD3*), nucleoside diphosphate kinase 1(encoded by *NME1*), nucleoside diphosphate kinase 7 (encoded by *NME7*), adenylate kinase 1 (encoded by *AK1*), purine nucleoside phosphorylase 1 (encoded by *PNP1*), ectonucleoside triphosphate diphosphohydrolase 1 (encoded by *ENTPD1*) and ecto-5-prime-nucleotidase (encoded by *NT5E*).

Ectonucleoside triphosphate diphosphohydrolase 3, nucleoside diphosphate kinase 1, nucleoside diphosphate kinase 7, adenylate kinase 1 and purine nucleoside phosphorylase 1 are localized only in neurons, whereas nucleoside diphosphate kinase 1, nucleoside diphosphate kinase 7, adenylate kinase 1 and purine nucleoside phosphorylase 1 are also expressed in astrocytes, mainly in the white matter. Ectonucleoside triphosphate diphosphohydrolase 1 was localized in astrocytes, and ecto-5-prime-nucleotidase was localized in vascular cells, probably pericytes, and in microglia (Figure 1).

Discussion

This study was designed to gain understanding about purine metabolism regulation in the *substantia nigra* and frontal cortex area 8 in PD at different stages of disease progression. The putamen was also analysed because of its connections with the *substantia nigra*. This was based on the importance of purine metabolism in normal brain function and also with the purpose to compare observations in PD with our previous studies in sAD [18] to look for commonalities and differences between these two prevalent neurodegenerative diseases.

According to these objectives, the expression of 22 genes embracing different steps of purine metabolism which were altered in our previous studies in AD was

Table 6. mRNA expression levels of purine metabolism enzymes in control and PD cases in the frontal cortex area 8, determined by TaqMan PCR assays

	C	*PD 3–4*	*C* vs. *PD 3–4*	*PD 5–6*	*C* vs. *PD 5–6*
POLR3B	1.18 ± 0.60	0.74 ± 0.61†	–	1.16 ± 0.40	–
NT5C	1.03 ± 0.23	0.85 ± 0.25	–	1.16 ± 0.26	–
PRUNE	1.08 ± 0.40	1.33 ± 0.74	–	1.67 ± 0.58	↑ **
APRT	1.07 ± 0.93	0.93 ± 0.43	–	1.22 ± 0.45	–
DGUOK	1.14 ± 0.56	0.94 ± 0.52	–	1.32 ± 0.42	–
NT5E	1.03 ± 0.36	1.13 ± 0.73	–	1.57 ± 0.68	↑ **
ENTPD1	1.04 ± 0.27	1.08 ± 0.35	–	1.33 ± 0.30	↑ *
ENTPD2	1.10 ± 0.46	0.95 ± 0.58	–	1.35 ± 0.35	–
ENTPD3	1.08 ± 0.43	1.08 ± 0.58	–	1.44 ± 0.77	–
ADA	1.03 ± 0.27	1.47 ± 0.63	↑ *	1.28 ± 0.42	–
AK1	1.12 ± 0.46	1.53 ± 0.79	–	1.83 ± 0.55	↑ **
AK2	1.03 ± 0.28	1.17 ± 0.32	–	1.41 ± 0.65†	↑*
AK4	1.13 ± 0.51	1.06 ± 0.69	–	1.43 ± 0.42	–
AK5	1.06 ± 0.52	1.29 ± 0.72	–	1.57 ± 0.61	↑ *
AK7	1.05 ± 0.31	1.14 ± 0.58	–	1.15 ± 0.43	–
NME1	1.19 ± 0.60	1.22 ± 0.55	–	1.41 ± 0.65	–
NME3	1.07 ± 0.36	1.30 ± 0.61	–	1.48 ± 0.65	–
NME4	1.10 ± 0.40	1.40 ± 0.68	–	1.60 ± 0.45	↑ **
NME5	1.16 ± 0.52	1.10 ± 0.60	–	1.61 ± 0.49	↑ *
NME6	1.08 ± 0.40	1.20 ± 0.62	–	1.61 ± 0.44	↑ **
NME7	1.27 ± 0.69	1.05 ± 0.69	–	1.50 ± 0.54	–
PNP	1.00 ± 0.54†	0.95 ± 0.41	–	1.70 ± 0.65	↑ *

Expression levels of controls and PD stages 3–4 and 5–6 cases using *GUS-β* for normalization. Student's *t*-test $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.
†No Gaussian distribution (Mann–Whitney test) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

selected for the present purposes. Not all regions were available in every case, but this deficiency was circumvented by analysing 72 cases, including 29 controls and 43 PD cases, covering a total of 79 samples.

Purines are heterocyclic aromatic organic compounds present in the core of DNA, RNA, nucleosides and nucleotides [41]. Purines and also pyrimidines constitute the nucleotide bases of deoxyribonucleotides and ribonucleotides. Nucleotides result from the addition of phosphate groups to nucleosides, and this process is carried out by adenylate kinases (AKs) and nucleoside diphosphatase kinases (NMEs). AKs participate in the phosphorylation of AMP to ADP and dAMP to dATP, and they regulate multiple intracellular and extracellular functions such as nuclear transport, DNA synthesis and repair, and energy metabolism [42]. The *AK* family is made up of seven members all of them expressed in different tissues including brain [42,43]. The *NME* family is implicated in the phosphorylation of nucleotide diphosphates to form nucleotide triphosphates (i.e. GTP, ATP, dGTP, dATP and dITP).

In a close relationship with *NME*, *PRUNE* encodes prune exopolyphosphatase which participates in the metabolism of guanosine pentaphosphate and tetraphosphate in bacteria, and it is involved in the ability of RNA polymerases to initiate transcription. The human homolog *h-PRUNE* probably has similar functions. In addition to this metabolic function, it has also been involved in glyogenesis [44] and in brain development [45].

On the other hand, adenine phosphoribosyltransferase (APRT) is related with adenine metabolism and catalyses the phosphorylation reaction, while ADA deaminates adenosine. Ectonucleoside triphosphate diphosphohydrolases (NTPDs) hydrolyse the terminal phosphate group of nucleoside tri- and diphosphates, yielding diphosphates and monophosphates, respectively [46–49]. Purine nucleotide phosphorylase, encoded by *PNP*, reversibly catalyses the phosphorolysis of purine nucleosides. Finally, nucleotidases catalyse the hydrolysis of a nucleotide into a nucleoside liberating phosphate.

The present findings show significant reduced gene expression of *APRT*, *ENTPD1*, *ENTPD3*, *AK2*, *AK4*, *AK5*,

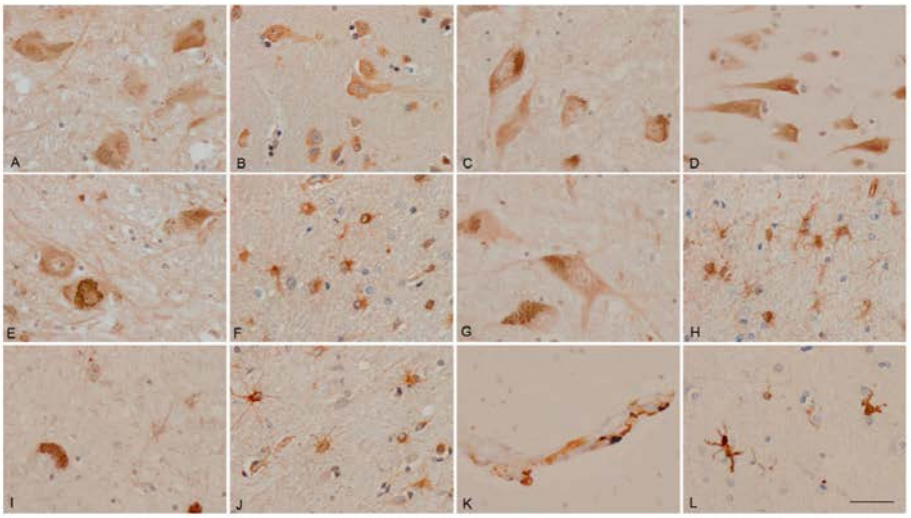

Figure 1. Purine enzyme immunohistochemistry. Immunohistochemistry to purine enzymes in the *substantia nigra* (**A**, **C**, **E**, **G**, **I**, **K**) and frontal cortex area 8 (**B**, **D**, **F**, **H**, **J**, **L**). **A**, **B**: ectonucleoside triphosphate diphosphohydrolase 3; **C**: nucleoside diphosphate kinase 1; **D**: nucleoside diphosphate kinase 7; **E**, **F**: adenylate kinase 1; **G**, **H**: purine nucleoside phosphorylase 1; **I**, **J**: ectonucleoside triphosphate diphosphohydrolase 1; **K**, **L**: ecto-5-prime-nucleotidase. Ectonucleoside triphosphate diphosphohydrolase 3 is localized only in neurons, whereas nucleoside diphosphate kinase 1, nucleoside diphosphate kinase 7, adenylate kinase 1, and purine nucleoside phosphorylase 1 are expressed in neurons and astrocytes, mainly in the white matter. Ectonucleoside triphosphate diphosphohydrolase 1 is localized in astrocytes, and ecto-5-prime-nucleotidase is expressed in vascular cells, probably pericytes, and in microglia. The immunoreaction is identified as a homogeneous cytoplasmic staining in neurons and proximal dendrite branches in neurons and in the cytoplasm and fine cell processes in astrocytes and microglia. Neuromelanin deposition in dopaminergic neurons in the *substantia nigra* is clearly distinguished by its granular morphology. Paraffin sections with slight haematoxylin counterstaining, scale bar = 25 µm.

NME1, *NME7* and *PNP* in the *substantia nigra pars compacta* at stages 3–6 of PD. In contrast, *ADA* mRNA is up-regulated in the frontal cortex area 8 at stages 3–4, and *PRUNE*, *NT5E*, *ENTPD1*, *AK1*, *AK2*, *AK5*, *NME4*, *NME5*, *NME6* and *PNP* are up-regulated in the frontal cortex area 8 at stages 5–6 of PD. No significant modifications in the expression of analysed genes were found in the putamen at PD stages 3–5, the only available stages for this region.

The present findings show region-dependent alterations in the expression of several genes involved in purine metabolism in PD. In addition, stage-dependent deregulation is observed in the frontal cortex area 8, the only area available for the study of disease progression.

Unfortunately, preliminary Western blot studies to analyse protein levels of enzymes encoded by purine metabolism-related genes have shown sub-optimal results in our hands due to individual variations in all groups. The causes of these individual variations are probably related to the susceptibility of these proteins to the agonal state and the post-mortem delay in tissue processing, among other unknown factors. The same occurs when using immunohistochemistry in paraffin sections (and in cryoprotected, free-floating processed sections; data not shown). For these reasons, immunohistochemistry was used only to identify the predominant localization of the enzymes in particular cell types because the intensity of the immunostaining varied from one case to another in all groups and regions, and any attempt at quantification was unrealistic. Yet the localization of the different enzymes analysed was the same in control and pathological cases.

NME1 mRNA is transcribed into nucleoside diphosphate kinase 1, *NME7* mRNA into nucleoside diphosphate

kinase 7, *AK1* mRNA into adenylate kinase 1 and *PNP1* mRNA into nucleoside phosphorylase 1. All these enzymes were detected in neurons in agreement with previous separate reports in different systems [43,50–54]. Astrocytes are considered not only structural and trophic assistants of neurons but also modulators of several environmental elements of neurons [55]. In this line, astrocytes are the essential origin of extracellular adenine-based nucleotides [49,56,57]. Nucleoside diphosphate kinase 1, nucleoside diphosphate kinase 7, adenylate kinase 1 and nucleoside phosphorylase 1 were also expressed in astrocytes, in agreement with previous reports [52,58]. *ENTPD1* mRNA is transcribed into ectonucleoside triphosphate diphosphohydrolase 3 which has been reported as the main ectonucleotidase of microglia [59] although we have not been able to confirm this unique localization. Finally, *NT5E* mRNA is transcribed into ecto-5-prime-nucleotidase which we found was particularly expressed in microglia and vascular cells consistent with pericytes.

Taken together, mRNA and protein studies may help to understand possible implications of modified expression in different cell types. As *ENTPD3*, *NME1*, *NME7*, *AK1* and *PNP1* are mainly expressed in neurons, down-regulation of these genes in the *substantia nigra* may be interpreted as a consequence of dopaminergic cell death. Expression of *NME1*, *NME7*, *AK1* and *PNP1* in reactive astrocytes in the substantia nigra probably does not compensate decreased mRNA expression due to the loss of neurons. On the contrary, gene up-regulation in the frontal cortex area 8 suggests a primary alteration or a compensation of altered purine metabolism in this region affected at advanced stages of PD. As only a few enzymes involved in purine metabolism have been analysed in the present study, altered expression of other factors cannot be excluded.

The expression of the same series of genes was analysed in the entorhinal cortex and frontal cortex in sporadic AD at different Braak and Braak stages [18].

APRT, *DGUOK*, *POLR3B*, *ENTPD3*, *AK5*, *NME1*, *NME3*, *NME5* and *NME7 were* down-regulated, while *ENTPD2* mRNAs were up-regulated in the entorhinal cortex of AD stages V-VI of Braak and Braak; no modifications in the mRNA expression levels of these genes were noted in the entorhinal cortex at stages III-IV.

In contrast to AD entorhinal cortex, *ENTPD2*, *NME3*, *PNP* and *PRUNE* mRNAs were significantly up-regulated in the AD frontal cortex area 8 at stages III-IV, whereas *AK5* mRNA was down-regulated and *PRUNE* mRNA up-regulated in the frontal cortex at AD stages V-VI of Braak and Braak [18].

As the studies in AD and PD have been performed by the same researchers, in the same laboratory and using the same probes, differences in gene expression between AD and PD cannot be related to interlaboratory differences, but rather, they represent disease-dependent alterations of genes encoding purine metabolism-related enzymes.

Taken together, these observations show that (i) genes involved in purine metabolism are differentially regulated in PD and sAD at least in the frontal cortex area 8, the only region analysed in both PD and AD brains; (ii) alteration in the expression of purine-related genes increases with disease progression; (iii) reduced gene expression occurs in the substantia nigra in PD and in the entorhinal cortex in AD which are the most vulnerable analysed regions in these diseases, respectively; and (iv) in contrast, the expression of genes involved in purine metabolism is increased in the frontal cortex although with a different pattern in PD and sAD. As PD cases analysed in the present series have concomitant sAD-related pathology up to stage IIB of Braak and Braak, changes in the frontal cortex in PD cannot be attributed to concomitant sAD-related pathology. As a corollary, purine metabolism changes appear to be a late effect in both diseases as they appear in the frontal cortex only after significant infiltration of pathologies in these regions. Yet mRNA targets related to purine metabolism differ in PD and AD.

Present observations show for the first time modifications in the expression of genes involved in purine metabolism in a selected series of cases with PD-related pathology and minimal or absent concomitant neurological pathologies. No comparative studies between regions in the same individuals have been possible because of the very limited numbers of cases in which the three regions analysed were available. However, comparative analysis between controls and diseased brains has shown regional differences in gene expression among the substantia nigra, putamen and frontal cortex area 8 in PD. Furthermore, changes are modified with disease progression at least in the frontal cortex area 8, the only region with sufficient numbers of samples for study. When comparing these data with those obtained in AD, the affected genes differed from one disease to another, but gene down-regulation occurred in the substantia nigra and entorhinal cortex, two regions with significant cell loss, respectively in PD and AD, whereas gene up-regulation occurred in the

frontal cortex in both conditions. As proteins encoded by affected genes are expressed in neurons or in glial cells or in both neurons and astrocytes, the present findings further support a complex scenario of specific altered regulation of purine metabolism in PD.

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Authors' contributions

PG-E performed qRT-PCR and Western blot studies and wrote the first version of the manuscript; KH-O helped in carrying out qRT-PCR studies; BA, Western blot and parallel studies in Alzheimer's disease cases; MA, immunohistochemistry; and IF designed the study, supervised the results and wrote the final version of the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. mRNA expression levels of deregulated genes of purine metabolism in the *substantia nigra* in controls, and in PD cases stages 3–6, as determined by TaqMan PCR assays. Expression values for the distinct probes are normalized with GUS-β. Mean fold change values for each group are compared with Student's *t*-test when the cases follow a Gaussian distribution. The nonparametric Mann–Whitney test is used to compare controls and PD cases when the samples do not belong to a normal distribution. Differences are considered statistically significant at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. All genes are down-regulated in the *substantia nigra* in PD cases when compared with controls.

Figure S2. mRNA expression levels of deregulated genes of purine metabolism in the frontal cortex area 8 in controls and PD cases stages 3–4 (**A**) and 5–6 (**B**) as determined with TaqMan PCR assays. Expression values for the distinct probes are normalized with GUS-β. Mean fold change values for each group are compared with Student's *t*-test when the cases follow a Gaussian distribution. The nonparametric Mann–Whitney test is used to compare controls and PD cases when the samples do not belong to a normal distribution. Differences are considered statistically significant at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. All genes are up-regulated in the frontal cortex area 8 in PD cases when compared with controls.

Table S1. mRNA expression levels of purine metabolism enzymes in control and PD cases in the *substantia nigra* stages 1–2 and 3–6 as determined by TaqMan PCR assays and corrected by *XPNPEP1*. Student's *t*-test $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. NA: not available. [a]No Gaussian distribution (Mann–Whitney test) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Table S2. Association analysis of variables gender, age, post-mortem delay and RIN in the *substantia nigra* in control cases and PD stages 1–2; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; *r*: Spearman's correlation for nonparametric variables.

Table S3. Association analysis of variables gender, age, post-mortem delay and RIN in the *substantia nigra* in control cases and PD at stages 3–6; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; *r*: Spearman's correlation for nonparametric variables.

Table S4. mRNA expression levels of purine metabolism enzymes in control and PD cases in the putamen, determined by TaqMan PCR assays. Expression levels of controls and PD stages 3–5 cases using *XPNPEP1* for normalization. Student's *t*-test $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. NA: not available. [a]No Gaussian distribution (Mann–Whitney test) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Table S5. Association analysis of variables gender, age, post-mortem delay and RIN in the putamen; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; *r*: Spearman's correlation for nonparametric variables.

Table S6. mRNA expression levels of purine metabolism enzymes in control and PD cases in the frontal cortex area 8, determined by TaqMan PCR assays. Expression levels of controls and PD stages 3–4 and 5–6 cases using *XPNPEP1* for normalization. Student's *t*-test $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. [a]No Gaussian distribution (Mann–Whitney test) $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Table S7. Association analysis of variables gender, age, post-mortem delay and RIN in the frontal cortex area 8: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. *r*: Spearman's correlation for nonparametric variables.

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ARTICLE 5

Functional genomics reveals dysregulation of cortical olfactory receptors in Parkinson disease: novel putative chemoreceptors in the human brain.

Garcia-Esparcia P, Schlüter A, Carmona M, Moreno J, Ansoleaga B, Torrejón-Escribano B, Gustincich S, Pujol A, Ferrer I.

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ABSTRACT

Introduction: Parkinson's disease (PD) is no longer considered a complex motor disorder but rather a systemic disease with variable non-motor deficits that may include impaired olfaction, depression, mood and sleep disorders, and altered cortical function. Increasing evidence indicates that multiple metabolic defects occur in regions outside the substantia nigra, including the cerebral cortex, even at premotor stages of the disease. **Methods:** We investigated changes in gene expression in the frontal cortex in PD patient brains using a transcriptomics approach. **Results:** Functional genomics analysis indicated that cortical olfactory receptors (ORs) and taste receptors (TASRs) are altered in PD patients. Olfactory receptors OR2L13, OR1E1, OR2J3, OR52L1, and OR11H1 and taste receptors TAS2R5 and TAS2R50 were downregulated, but TAS2R10 and TAS2R13 were upregulated at premotor and parkinsonian stages in the frontal cortex area 8 in PD patient brains. Furthermore, we present novel evidence that, in addition to the ORs, obligate downstream components of OR function adenylyl cyclase 3 and olfactory G protein ($G_{\alpha olf}$), OR transporters, receptor transporter proteins 1 and 2 and receptor expression enhancing protein 1, and OR xenobiotic removing UDP-glucuronosyltransferase 1 family polypeptide A6 are widely expressed in neurons of the cerebral cortex and other regions of the adult human brain. **Conclusions:** Together, these findings support the concept that ORs and TASRs in the cerebral cortex may have novel physiologic functions that are affected in PD patients.

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ORIGINAL ARTICLE

Functional Genomics Reveals Dysregulation of Cortical Olfactory Receptors in Parkinson Disease: Novel Putative Chemoreceptors in the Human Brain

Paula Garcia-Esparcia, MSc, Agatha Schlüter, PhD, Margarita Carmona, Jesús Moreno, Belen Ansoleaga, MSc, Benjamín Torrejón-Escribano, Stefano Gustincich, PhD, Aurora Pujol, MD, PhD, and Isidre Ferrer, MD, PhD

Abstract

Parkinson disease (PD) is no longer considered a complex motor disorder but rather a systemic disease with variable nonmotor deficits that may include impaired olfaction, depression, mood and sleep disorders, and altered cortical function. Increasing evidence indicates that multiple metabolic defects occur in regions outside the substantia nigra, including the cerebral cortex, even at premotor stages of the disease. We investigated changes in gene expression in the frontal cortex in PD patient brains using a transcriptomics approach. Functional genomics analysis indicated that cortical olfactory receptors (ORs) and taste receptors (TASRs) are altered in PD patients. Olfactory receptors OR2L13, OR1E1, OR2J3, OR52L1, and OR11H1 and taste receptors TAS2R5 and TAS2R50 were downregulated, but TAS2R10 and TAS2R13 were upregulated at premotor and parkinsonian stages in the frontal cortex area 8 in PD patient brains. Furthermore, we present novel evidence that, in addition to the ORs, obligate downstream components of OR function adenylyl cyclase 3 and olfactory G protein (Gαolf), OR transporters, receptor transporter proteins 1 and 2 and receptor expression enhancing protein 1, and OR xenobiotic removing UDP-glucuronosyltransferase 1 family polypeptide A6 are widely expressed in neurons of the cerebral cortex and other regions of the adult human brain. Together, these findings support the concept that ORs and TASRs in the cerebral cortex may have novel physiologic functions that are affected in PD patients.

Key Words: Adenylyl cyclase 3, Cerebral cortex, Olfactory receptors, Parkinson disease, Protein Gαolf, Taste receptors, UDP-glucuronosyltransferase.

INTRODUCTION

Odorant and pheromone signaling make up a highly complex system that permits chemical communication among species and allows individuals of a particular species to gain information about vital functions such as the availability of food, sexual status, mating, reproduction, breeding, newborn imprinting, territorial marking and homing, and navigation. The repertoire of receptors from insects to fish and mammals occupies a major part of the genome at the time that putative ligands number in the thousands (1–5).

Odorant activation of sensory olfactory neurons in mammals is mediated by activation of G proteins and adenylyl cyclase (AC), increased intracellular concentration of cyclic adenosine monophosphate (cAMP), activation of cAMP-gated channels, and neuron depolarization (6–13). The specific olfactory G protein is Gαolf (14), and the olfactory cyclic nucleotide–gated cation channel is specifically linked to olfaction (15). Mice null for the expression of either G_{olf} or adenylyl cyclase 3 (AC3) are anosmic, thus indicating that both Gαolf and AC3 are obligate components of olfactory function in sensory olfactory neurons (16, 17).

Olfactory or odorant receptors (ORs) have been identified as a large family of G protein–coupled receptors, having conserved transmembrane motifs in common (18–20). The number of functional OR genes and OR pseudo-genes is variable among species, but of about 30,000 genes in the mouse genome, more than 1,000 are for ORs (18). There are approximately 350 functional ORs in humans distributed among all chromosomes, with the exception of 18 and Y (20–24).

Institut de Neuropatologia, IDIBELL-Hospital Universitari de Bellvitge, Hospitalet de Llobregat (PG-E, MC, JM, BE, AP, IF); and Universitat de Barcelona, Hospitalet de Llobregat (IF) , Barcelona; CIBERNED (Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas) (IF); Neurometabolic Diseases Laboratory, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), Hospitalet de Llobregat, Barcelona, Catalonia (AS, AP); Centre for Biomedical Research on Rare Diseases (CIBERER) (AS, AP); Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Catalonia (AP), Spain; Area of Neuroscience, International School for Advanced Studies (SISSA), Trieste, Italy (SG); and Unitat de Biologia, Serveis Cientifics I Tecnics, Universitat de Barcelona, Hospitalet de Llobregat, Barcelona, Spain (BT-E).

Send correspondence and reprint requests to: Isidre Ferrer, MD, PhD, Institut de Neuropatologia, Servei Anatomia Patologica, Hospital Universitari de Bellvitge, Carrer Feixa LLarga sn, 08907 Hospitalet de Llobregat, Spain; E-mail: 8082ifa@gmail.com

Paula Garcia-Esparcia and Agatha Schlüter contributed equally and must be considered as first authors.

Aurora Pujol and Isidre Ferrer should be considered last authors.

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"Ectopic" localization of ORs in nonolfactory tissues has recently become a focus of attention and has raised questions about putative nonodorant-related functions of ORs in various nonolfactory regions. For example, ORs have been detected in germinal cells, testis, kidney, heart, and lung, among others (24–29), and expression of the olfactory signaling system (Gαolf and AC3) has been reported in the kidney (30). A number of ORs (M71, I7, C6, P2, and OR3) have also been detected in mouse cerebral cortex (31), but the concomitant occurrence of Gαolf and AC3 that might ascribe a function to ORs in mammalian cerebral cortex has not been documented. Whether OR expression occurs in the cerebral cortex in species other than the mouse and, particularly, in humans is not known.

A long-standing focus of our laboratory lies in gaining insight into cortical molecular events in early Parkinson disease (PD) (32, 33). Toward this aim, we performed transcriptomic analysis of frontal cerebral cortex of premotor and parkinsonian stages of postmortem brain samples from PD patients and age-matched controls. We encountered unexpected dysregulation of a group of genes corresponding to ORs in PD patients. Therefore, the rationale and objectives of the present work were to 1) perform accurate functional enrichment analysis of microarray results; 2) validate candidate dysregulated OR genes in PD by polymerase chain reaction (PCR); 3) study the expression of other candidate chemoreceptors (i.e. taste receptors [TASRs]) in frontal cortex and their possible modifications in PD (to determine the specificity of OR dysregulation); 4) identify, map, and determine the cellular distribution of several ORs and the obligate mediators Gαolf and AC3 in the frontal cortex; and 5) detect putative OR transporters and specific molecules involved in the detoxification of various xenobiotic and endobiotic compounds linked to OR signaling.

MATERIALS AND METHODS

Case Material

Brain tissue was obtained from the Institute of Neuropathology Brain Bank (HUB-ICO-IDIBELL Biobank) and the Biobank of Hospital Clinic-IDIBAPS following the guidelines both of Spanish legislation on this matter and of the local ethics committee. The postmortem interval between death and tissue processing was between 3 and 14 hours in all cases. One hemisphere was immediately cut in 1-cm-thick coronal sections, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags, numbered with water-resistant ink, and stored at −80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphologic studies. A summary of the cases and their use in the different methods used is shown in Table 1.

The neuropathologic diagnosis of PD was based on the classification of Braak but excluded atypical cases in which no graded pathology from the medulla oblongata, pons, and midbrain to limbic structures was identified (34). The PD series included Braak neuropathologic stages 3 (n = 7), 4 (n = 16), and 5 (n = 12). Approximately 30% of the cases did not have a clinical history of parkinsonism and were categorized as "premotor" stages (7 PD3; 4 PD4) (35). The olfactory bulb was involved in every case, showing α-synuclein deposits in scattered neurons and neurites; α-synuclein deposits in the amygdala were found in cases at stage 4 and 5 and in the entorhinal cortex in cases at stage 5. Only pure cases were chosen for study; cases with mixed pathologies (i.e. associated tauopathies and vascular diseases) were excluded. Finally, control cases (n = 19) did not have histories of a neurologic or mental disorder and the neuropathologic postmortem study revealed no brain lesions, including neurofibrillary tangles with the exception of a few (if any) that were restricted to the entorhinal cortex; there were no β-amyloid deposits or abnormal protein aggregates in any region. Sex representation was similar in control and diseased brains (8 women and 11 men in the control group and 16 women and 19 men in the PD group).

In addition, to analyze the vulnerability of the proteins under study to postmortem delay, unrelated selected samples of the frontal cortex (area 8) of 3 control cases were processed mimicking an artificial postmortem delay (36). For this purpose, the brains were obtained 3 hours after death, and samples of the left frontal cortex were rapidly frozen at −80°C or stored at 4°C for different periods and then frozen at 6, 9, 24, and 48 hours after death. These samples were only used for Western blot studies, and they were run in parallel in the same gels.

RNA Purification

The purification of RNA was carried out with RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. During purification, samples were treated with RNase-free DNase Set (Qiagen) to avoid later amplification of genomic DNA. The concentration of each sample was obtained from A260 measurements with Nanodrop 1000. RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA). Values of RNA quality (RIN values) were from 7 to 8.8.

Retrotranscription Reaction

The retrotranscriptase reaction was carried out using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) following the protocol provided by the supplier. Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the degree of contaminating genomic DNA.

Whole-Transcript Expression Arrays

RNA samples from the frontal cortex (area 8) of control (n = 7) and PD (n = 11) cases were analyzed using the Affymetrix microarray platform and the Genechip Human Gene 1.1 ST Array. Parkinson disease cases included premotor stages (n = 4) and cases with parkinsonism (n = 7) (Table 1). This array analyzes gene expression patterns on a whole-genome scale on a single array, with probes covering several exons on the target genes. Starting material was 200 ng of total RNA of each sample. Quality of isolated RNA was first measured by Bioanalyzer Assay (Agilent). Sense ssDNA suitable for labeling was generated from total RNA with the Ambion WT Expression Kit from Ambion (Carlsbad, CA), according to the manufacturer's instructions. Sense ssDNA was fragmented, labeled,

TABLE 1. Clinical and Pathologic Characteristics of Patients and Analysis Methods

Case	Sex	Age, years	Postmortem Interval	Neuropathologic Diagnosis	Array	PCR	Other	Clinical Diagnosis
1	F	79	7 hours, 45 minutes	C	X		Ih, if	—
2	F	52	3 hours, 30 minutes	C	X		if	—
3	F	51	4 hours	C	X		if	—
4	F	75	9 hours	C	X		if	—
5	F	58	4 hours	C	X		if	—
6	M	49	7 hours	C	X			—
7	M	82	11 hours	C	X			—
8	M	73	7 hours	C			if	—
9	M	73	5 hours, 30 minutes	C			if	—
10	M	64	8 hours, 30 minutes	C		X	ih; L-R	—
11	M	56	5 hours	C		X	Ih	—
12	M	67	5 hours	C		X	ih; L-R	—
13	M	62	3 hours	C		X	ih; L-R	—
14	F	75	4 hours	C		X	ih; L-R	—
15	M	73	4 hours	C		X	Ih, if	—
16	M	53	3 hours	C		X	if	—
17	F	49	9 hours	C		X	ih; L-R	—
18	F	56	4 hours	C		X		—
19	M	62	4 hours	C		X	ih	—
20	F	63	8 hours	PD3			Ih, if	—
21	M	70	11 hours	PD3			if	—
22	M	77	10 hours	PD3			if	—
23	M	72	9 hours	PD3	X			—
24	F	70	19 hours	PD3	X			—
25	M	54	11 hours	PD3	X	X	ih	—
26	F	77	3 hours	PD3		X	ih	—
27	F	66	5 hours	PD4	X	X	if	—
28	F	57	11 hours	PD4	X	X	if	P
29	F	69	4 hours, 30 minutes	PD4	X	X		P
30	F	70	4 hours	PD4	X			P
31	M	79	3 hours, 30 minutes	PD4	X			P
32	F	84	4 hours, 30 minutes	PD4		X	Ih, if	P
33	M	68	9 hours	PD4		X		P
34	M	77	12 hours	PD4		X		P
35	F	78	11 hours	PD4			if	P
36	M	65	9 hours	PD4		X		P
37	M	69	5 hours	PD4		X	Ih, if	P
38	M	79	9 hours	PD4		X	ih	P
39	M	68	4 hours	PD4		X	ih	—
40	M	76	4 hours, 30 minutes	PD4		X	Ih, if	—
41	F	81	6 hours, 30 minutes	PD4		X		P
42	M	66	5 hours	PD4		X	ih	—
43	F	82	16 hours	PD5	X			P
44	F	60	8 hours	PD5	X	X	L-R	P
45	F	68	12 hours	PD5			L-R	P
46	F	85	12 hours	PD5			L-R, if	P
47	F	64	8 hours, 15 minutes	PD5	X		if	P
48	M	72	3 hours, 30 minutes	PD5			L-R	P
49	M	71	6 hours	PD5			if	P
50	M	77	5 hours, 45 minutes	PD5			if	P

(continued on next page)

TABLE 1. *(Continued)*

Case	Sex	Age, years	Postmortem Interval	Neuropathologic Diagnosis	Array	PCR	Other	Clinical Diagnosis
51	M	86	7 hours	PD5		X	Ih, if	P + D
52	F	77	7 hours	PD5		X	ih	P
53	M	83	14 hours	PD5		X	ih	P
54	M	78	13 hours	PD5		X	ih	P

Array, whole-transcript expression array; C, normal control; F, female; if, immunofluorescence; ih, immunohistochemistry; L-R, lipid rafts; M, male; PCR, TaqMan PCR; PD3–PD5, Braak stage of Parkinson disease–related pathology; X, analysis performed.
Clinical diagnosis: -, no symptoms; P, parkinsonism; D, dementia;

and hybridized to the arrays with the GeneChip WT Terminal Labeling and Hybridization Kit from Affymetrix (Affymetrix, Santa Clara, CA). Chips were processed on an Affymetrix GeneTitan platform.

Microarray Data Normalization and Differential Expression Analysis

Preprocessing of raw data and statistical analyses were performed using Bioconductor packages in R programming environment (37). We read CEL files from Affymetrix arrays, corrected the background, and summarized and normalized the data with the robust microarray A method implemented in the Bioconductor Limma package (38). Furthermore, we estimated the fold change and SEs by fitting a linear model (using the lmFit function in Limma package) for each gene given the groups of arrays. Genes with empirical Bayes *t* test p values at a level of 0.05 were selected and corrected by calculating the false discovery rate $p < 0.05$ (adjusted probability [probadj]). The microarray experiment was deposited in the Array Express Database under accession number E-MTAB-1194.

Functional Enrichment Analysis

To evaluate which pathways or functional categories were enriched in PD samples, we followed a similar strategy as shown elsewhere (39). Briefly, we computed the Gene Set Enrichment Analysis from Limma package and Hypergeometric-based (HYPERG) tests from GOstats package (40). We used $p < 0.05$ as the cutoff point to determine whether Gene Ontology (GO) term or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were significantly enriched.

GeneSetTest function from Limma package tests whether a set of genes is enriched for differential expression. Its principle is the same as that of the Gene Set Enrichment Analysis (41), but the statistical tests used are different. It is based on a set of probe-wise *t* statistics arising for microarray analysis. We computed 3 different tests: 1) upregulated genes with positive *t* statistics (G.UP); 2) downregulated genes with negative *t* statistics (G.DOWN); and 3) upregulated or downregulated genes as a whole (G.MIXED). We used GOstats package to determine gene ontology terms and KEGG pathways enriched in the subset of genes with differential expression in PD compared with control groups at $p < 0.05$ adjusted probability.

TaqMan PCR

Cases analyzed included 10 controls (7 men and 3 women) and 20 PD cases (12 men and 8 women) (Table 1). TaqMan PCR assays for each gene were performed in duplicate on cDNA samples in 384-well optical plates using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). For each 20-μL TaqMan reaction, 9 μL cDNA was mixed with 1 μL 20× TaqMan Gene Expression Assays and 10 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems). The reactions were carried out using the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Finally, all TaqMan PCR data were captured using the Sequence Detection System software (SDS version 1.9, Applied Biosystems). Probes used in this study are shown in Table 2.

Samples were analyzed with the double delta CT (ΔΔCT) method. Delta CT (ΔCT) values represent normalized target gene levels with respect to the internal controls glucuronidase-β (GUSβ), X-prolyl aminopeptidase (aminopeptidase P) 1 (XPNP EP1), and alanyl-tRNA synthetase (AARS). These novel reference genes were selected because they are very efficient in replicating microarray target gene expression in human postmortem brain tissue (42, 43). Double delta CT values were calculated as the ΔCT of each test sample minus the mean ΔCT of the calibrator samples for each target gene. The fold change was calculated using the equation $2^{(-\Delta\Delta CT)}$. Results were analyzed by 2-way analysis of variance followed by Student *t* test when

TABLE 2. TaqMan Probes Used in the This Study

GUSB: GCTACTACTTGAAGATGGTGATCGC)
XPNPEP1: CAAAGAGTGCGACTGGCTCAACAAT)
AARS: GCAAAATTTGGGGCTGGATGACACC)
OR2J3: ACCGCCAAGTAGATCACTTTTTCTG,
OR52L1: CTCAGCAGATCCGCCAGCGAGTGCT,
OR51E1: TACGGTTGAGCCTCTACCTGCCTGG,
OR2L13: CTCCAAGCCCAGTTACAGCAGAAAG,
OR2T33: AACGGTGGCTGGGGACGTGTGTAAA
OR11H1: CACTGGGAGACATAAGGCCTTCTCT
OR2D2: GTGAGGCCCCTGCACTATTGATCTT
OR4F4: TATACACACTGAGGAACAAAGACAT
TAS2R14: TTTGTCCCTGGCAATGTTTCTTCTC
TAS2R50: AGTCCTAGGAGGCTGCGGAATGACC
TAS2R10: ACCACAGCCATCTATCCCTGGGGTC
TAS2R13: CACCATTTACTGTGGCCTTCATCTC
TAS2R4: TACATCACGCTTAGCCAGGCATCAC
TAS2R5: TTTCTTGTTTCCTCTGGGATGCTGA

Probes for normalization were β-glucuronidase (GUSB), X-prolyl aminopeptidase P1 (XPNPEP1), and alanyl-tRNA synthetase (AARS).

required. Differences between mean values were considered statistically significant * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Immunohistochemistry and Immunofluorescence

The immunohistochemical study of ORs was carried out in 4-μm-thick dewaxed paraffin sections and 8-μm-thick paraformaldehyde-fixed cryoprotected sections. Immunofluorescence was conducted only in paraffin sections in 8 controls and 13 PD brains (Table 1). The following regions were examined: hippocampus proper, dentate gyrus, hilus, entorhinal cortex, inferior temporal cortex, frontal cortex area 8, orbitofrontal cortex, dorsomedial thalamus, substantia nigra, motor ocular nucleus, anterior pontine nuclei, and cerebellar vermis. Sections processed for immunofluorescence were pretreated with a saturated solution of Sudan black B (Merck, Darmstadt, Germany) for 15 minutes to block the autofluorescence of lipofuscin granules present in cell bodies and then rinsed in 70% ethanol and washed in distilled water. In all cases, the sections were boiled in citrate buffer (20 minutes) to retrieve antigenicity. Endogenous peroxidases were blocked by incubation in 10% methanol–1% H_2O_2 solution (15 minutes) followed by 3% normal horse serum solution. Anti-OR antibodies were named according to the corresponding targets: rabbit polyclonal anti-OR2H2 (OR2H2, LS-A4840; MBL International, Woburn, MA), anti-OR2A4 (OR2A4, ab 97486; Abcam, Cambridge, UK), anti-OR6K3 (OR6K3; MBL International), all used at a dilution of 1:100; polyclonal rabbit antibody anti–adenylyl cyclase 3 (ADCY3) (ab 14778; Abcam) diluted 1:100; and anti–Protein Gαolf (sc 55545; Santa Cruz Biotechnology, Santa Cruz, CA), used at a dilution of 1:50. Additional sections were immunostained with polyclonal rabbit antibodies against receptor transporter proteins 1 and 2 (anti-RTP1 and anti-RTP2: ab 107963 and ab103637 Abcam, respectively) both diluted at 1:50 and against receptor expression enhancing protein 1 (REEP1, ab105583; Abcam) used at a dilution of 1:50. Rabbit polyclonal antibody against UDP-glucuronosyltransferase 1 family polypeptide A6 (UGT1A6) (309014, OriGene, Rockville, MD) was used at a dilution 1:50. After incubation with the primary antibody, the sections were incubated with EnVision + system peroxidase (Dako, Glostrup, Denmark) for 30 minutes at room temperature. The peroxidase reaction was visualized with diaminobenzidine, NH_4NiSO_4, and H_2O_2. The immunoreaction results in a blue-gray precipitate. Sections were not counterstained with hematoxylin but were dehydrated and coverslipped for microscopic observation. After incubation with the primary antibody, sections processed for immunofluorescence were incubated with Alexa488 (1:400; Molecular Probes, Eugene, OR) fluorescence secondary antibody. Nuclei were stained with DRAQ5 (1:2000; Biostatus, Inc., Leicestershire, UK). After washing, the sections were mounted in Immuno-Fluore mounting medium (ICN Biomedicals, Irvine, CA), sealed, and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope.

The specificity of the immunoreaction was tested by incubating sections without the primary antibodies; these sections were negative. Because no antigenic peptides were available for preabsorption control studies, specificity of the antibodies was tested by the demonstration of unique band patterns at the appropriate molecular weight in Western blots.

Selection of the antibodies was based on their commercial availability and adequate immunostaining. Some tested antibodies did not work on paraffin and cryoprotected sections but on Western blots; others were only useful for Western blotting; others gave several bands and Western blots; others did not function at all and were not used in the present work. Only antibodies exhibiting convincing immunoreactivity in tissue sections were selected for study. Several procedures to enhance antigen retrieval were assessed, including citrate buffer, citrate-EDTA, Tris-buffered saline–EDTA, TRis buffer, and formic acid; optimal results for both paraffin and cryoprotected sections were obtained after boiling the sections in citrate buffer. Optimal concentrations were determined after trying different dilutions for each antibody.

Gel Electrophoresis and Western Blotting

Samples of the frontal cortex (area 8) (0.1 g) of the 3 unrelated control cases with artificial postmortem delay were homogenized with a glass homogenizer in RIPA lysis buffer (50 mmol/L Tris/HCl buffer, pH 7.4 containing 2 mmol/L EDTA, 0.2% Nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoride, protease, and phosphatase inhibitor cocktails (Roche Molecular Systems, Pleasanton, CA) and then centrifuged at 4°C for 15 minutes at 13,000 rpm (ultracentrifuge Beckman with 70Ti rotor). Protein concentration was determined by BCA method (Thermo Scientific, Waltham, MA). Samples containing 25 μg of protein were loaded onto 12% acrylamide gels. Proteins were separated in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (200 mA per membrane, 90 minutes). Nonspecific binding was blocked by incubation in 5% albumin in PBS containing 0.2% Tween for 1 hour at room temperature. After washing, the membranes were incubated at 4°C overnight with one of the following antibodies in PBS containing 5% albumin and 0.2% Tween: anti-OR2H2 (MBL International), anti-OR2A4 (Abcam), anti-OR6K3 (MBL International), all diluted 1:100, and ADCY3 (Abcam). Membranes were then incubated for 1 hour with the appropriate horseradish peroxidase–conjugated secondary antibody (1:2000; Dako), and the immune complexes were revealed with a chemiluminescence reagent (ECL, Amersham, UK).

Lipid Raft Isolation

Lipid raft fractions were isolated from the frontal cortex (area 8) of control (n = 5) and PD (n = 4) cases (Table 1), as described with slight modifications (44, 45). Briefly, 0.1 g of brain cortex was homogenized at 4°C in 8 volumes of isolation buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L $MgCl_2$, 0.15 mol/L NaCl) containing 1% Triton X-100 and 5% glycerol, 20 mmol/L NaF, 1 mmol/L Na_3VO_4, 5 mmol/L β-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, and a cocktail of protease inhibitors (Roche Diagnostics, Barcelona, Spain) in a glass homogenizer for 5 minutes, centrifuged at 500 × *g* for another 5 minutes; the supernatant was then collected and mixed in an orbital rotor for 1 hour at 4°C. Approximately 800 μL of the supernatant was mixed with an equal volume of 80% sucrose in isolation buffer and overlaid with 7.5 mL of a 36% sucrose solution and 2.7 mL of a 15% sucrose solution in isolation buffer in 10 mL ultracentrifuge tubes (Ultraclear, Beckman). Sucrose gradients were centrifuged at 150,000 × *g* for 18 hours at 4°C using a Beckman SW41Ti

rotor. Two-milliliter fractions were collected from the top to the bottom, and the final pellets, corresponding to nonraft fractions, were collected and resuspended in 2 mL of isolation buffer. The 6 fractions obtained were frozen at −80°C until analysis.

The purity of lipid rafts was tested using different lipid raft and nonraft protein markers in Western blot assays. Because the protocol differs from that used in total homogenates, the main differences are described below. Equal amounts of samples of the 6 raft and nonraft fractions were resuspended in SDS loading buffer (625 mmol/L Tris-HCl pH 6.8; 1% SDS, 10% glycerol, 5% β-mercaptoethanol; 0.01% bromophenol blue), heated at 95°C for 5 minutes, and loaded on 10% Mini-Protean precast TGX gels (Bio-Rad) or on 12% acrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes and processed for Western blotting. All antibodies were diluted in 5% BLOTTO in Tris-buffered saline pH 7.2 with 0.05% Tween-20. Membranes were incubated with monoclonal mouse anti–flotillin-1 (BD Biosciences, San Jose, CA) used at a dilution of 1:1000 to identify raft fractions and with monoclonal mouse anti-Cu/Zn superoxide dismutase SOD-1 (Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:1000 to identify cytosolic fractions. Later, the membranes were incubated with anti-OR2H2 (MBL International) diluted 1:100 and with ADCY3 (Abcam) diluted 1:500.

RESULTS

Global Expression Profiles Identify Dysregulation of ORs in the Frontal Cortex (Area 8) in PD

Messenger RNA samples from PD (n = 4 premotor and 7 with parkinsonism) and age-matched controls (n = 7) were hybridized to an Affymetrix Human Gene 1.1 ST Array, including more than 28,000 well-annotated genes with more than 750,000 distinct probes; 529 genes were seen to be dysregulated in PD. Modification of transcriptional profiles in PD was evidenced on the heat map representation of significantly regulated transcripts at adjusted probability of $p < 0.05$ (Fig. 1).

Functional enrichment of metabolic and cell signaling pathways extracted from the KEGG revealed dysregulation of the olfactory transduction pathway (KEGG 04740) in PD compared with control samples. Pathway enrichment was analyzed with HYPERG tests to selected genes having adjusted values of $p < 0.05$ (Table 3). The GO analysis of selected genes of adjusted values of $p < 0.05$ yielded similar results, with GO 0007608 (sensory perception of smell) at p value of 4.39^{e-06} (Table 4). Dysregulation of ORs occurred in both premotor and parkinsonian stages. Olfactory receptors OR2J3 (prob 1.95^{e-05}, probadj 0.00825), OR11H1 (prob 7.84^{e-05}, probadj 0.01508), OR4F17 (prob 5.94^{e-05}, probadj 0.01383), OR4K15 (prob 1.95^{e-05}, probadj 0.00825), and OR8K5 (prob 2.09^{e-05}, probadj 0.00848) were the first downregulated genes located. Olfactory receptor OR4F4 was selected because it was the only putative upregulated gene, although with low significance.

Functional enrichment also revealed other families of putative chemoreceptors that were affected in the frontal cortex in PD; KEGG analysis also revealed dysregulation of taste transduction pathways (KEGG 04742) and GO 0050909, sensory perception of taste, at a value of $p = 1.01^{e-05}$ in PD (Tables 3, 4). Taste receptors TAS2R4 (prob 4.47^{e-06}, probadj 0.00414), TAS2R5 (prob 0.0001948, probadj 0.022271), TAS2R14 (prob 0.0002424, probadj 0.02376), TAS2R50 (prob 0.000522, probadj 0.032900), TAS2R10 prob 0.000525, probadj 0.033001), and TAS2R13 (prob 0.0005801, probadj 0.034955) were the major dysregulated TASRs. Interestingly, dysregulated TASRs in PD are located on chromosomes 12 and 7, and all of them are type 2 TASRs, that is, involved in the reception of bitterness.

Validation of OR and TASR Dysregulation in PD Using TaqMan Quantitative PCR

Assessment of putative dysregulated genes was conducted using cDNA from some of the same cases used for the arrays and additional cDNAs from other PD and control cases (Table 1). This was done to increase robustness of the results obtained in the arrays. The rationale was based on the assumption that identification of dysregulated genes common to different groups of PD cases determined by different methods further strengthens the validity of the observations. Selection of probes was carried out depending on the results of the arrays together with their commercial availability.

Eight OR genes were selected for TaqMan PCR examination: OR2J3 and OR11H1 to validate downregulated genes observed in the arrays; OR4F4 to check the only gene apparently upregulated in the arrays; and OR2L13, OR51E1, OR52L1, OR2T33, and OR2D2 as additional genes with doubtful significance in the arrays. Olfactory receptors OR52L1, OR2L13, OR51E1, OR2J3, OR52L1, and OR11H1 mRNAs were downregulated in the frontal cortex area 8 in PD cases, whereas OR2T33 and OR2D2 mRNA expression levels were similar in control and diseased samples; OR4F4 mRNA showed a significant augmentation in females with PD (Fig. 2A). Interestingly, most receptors were downregulated in men but not in women, with the exception of OR11H1, which was downregulated equally in both sexes and was significantly decreased in the total population analyzed (Fig. 2A).

Six taste receptors were also analyzed with TaqMan PCR. All of them were selected according to their putative relevance as shown in arrays: TAS2R4, TAS2R5, TAS2R14, TAS2R50, TAS2R10, and TAS2R13. Polymerase chain reaction assessment showed TAS2R4, TAS2R50, and TAS2R14 mRNA expression levels similar in control and diseased cases; however, TAS2R5 was downregulated and TAS2R10 and TAS2R13 were upregulated in the frontal cortex in PD (Fig. 2B). Upregulation affected mainly males and downregulation occurred in males in 1 gene and females in another. Similar results were obtained using GUSβ, XPNPEP1, and AARS as housekeeping genes for PCR validation.

Immunohistochemistry of ORs in Normal Human Brain

Immunohistochemistry was used to detect the presence of selected ORs for which commercial antibodies were available in paraffin sections. No commercial antibodies raised against dysregulated genes in PD were available or suitable for immunohistochemistry. Therefore, immunohistochemistry of ORs and related molecules was directed to demonstrate the presence of ORs and related proteins in the human frontal cortex rather than to complement the results of RNA arrays followed by PCR

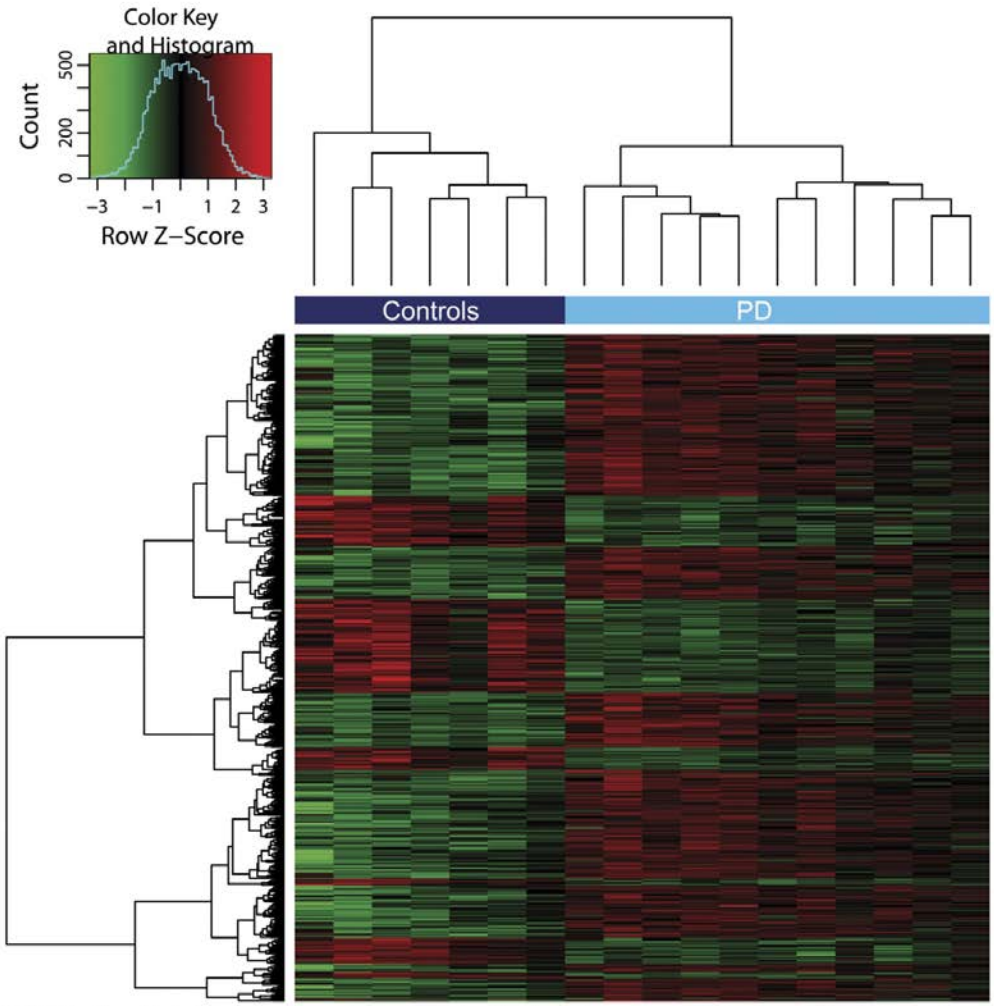

FIGURE 1. Hierarchical clustering heat map of expression intensities of mRNA array transcripts (on the y axis) in Parkinson disease (PD) and controls (on the x axis). A clear separation between PD and control profiles indicates modification of the transcriptional profile in PD.

validation. Olfactory receptors OR2H2, OR2A4, and OR6K3 immunoreactivity mapped neurons of the cerebral cortex, dorsomedial thalamus, selected nuclei of the brainstem, and Purkinje cells. The intensity of the immunoreaction varied from 1 region to another. In the telencephalon, neurons of the CA1 area of the hippocampus and hilus had stronger immunoreactivity than those of the granular layer of the dentate gyrus. Moderate immunostaining was found in neurons of the entorhinal cortex, inferior temporal cortex, frontal cortex (area 8), and orbitofrontal cortex (Fig. 3A–F). In the cerebral cortex, all layers were immunostained, the intensity largely depending on the size of neurons. Olfactory receptor immunoreactivity was also

TABLE 3. Analysis Showing Pathway Enrichment in Parkinson Disease Cases

KEGG ID	Pathway	G.MIXED	G.UP	G.DOWN	HYPERG
04740	Olfactory transduction	7.04^{e-27}	1	4.02^{e-76}	2.72^{e-06}
04742	Taste transduction	0.00015	0.00055	0.99944	0.00037

GSEA analysis includes 3 different tests: G. MIXED, G. UP, and G. DOWN (upregulated, downregulated, and total dysregulated genes, respectively). Kyoto Encyclopedia of Genes and Genomes (KEGG) ID: KEGG identification number Either Gene set Enrichment Analysis (GSEA) or Hypergeometric (HYPERG) distribution function statistical analyses were used.

seen in the dorsomedial nucleus of the thalamus (Fig. 3G); OR immunoreactivity decorated the soma of Purkinje cells and granule cells to a lesser degree (Fig. 3H). Strong OR immunoreactivity was found in selected nuclei of the brainstem including the nucleus of the motor ocular nerve in the mesencephalon, substantia nigra pars compacta and pars reticularis, and pontine nuclei (Fig. 3I–K). In addition to neuronal soma, fine granular immunostaining was also observed in the molecular layer of the cerebellar cortex and neuropil of brainstem nuclei. Optimal staining was obtained using immunofluorescence and confocal microscopy. Similar results were obtained with the antibodies OR2H2, OR2A4, and OR6K3, although immunoreactivity was higher with antibodies anti-OR6K3 and anti-ORH2. No immunoreaction was found in parallel sections processed in parallel but without the primary antibodies (Fig. 3L).

Olfactory receptor expression was not restricted to a single cell population, but no further attempt was made to analyze possible coexpression of different ORs in the same neuron because all the available antibodies against ORs were raised in rabbits. The general distribution of the immunostaining for every antibody was not consistent with the putative expression of a single OR per neuron, as occurs in sensory olfactory neurons.

The distribution of positive neurons was not related to any specific structure and, in particular, no relationship was observed between positive cells and blood vessels, including capillaries, and subpial and periventricular localizations. In contrast to neurons, few, if any, glial cells were immunostained in the different regions (Fig. 3K).

Obligate Functional Components of OR Signaling in Normal Human Brain

Adenylyl cyclase 3 and Gαolf immunoreactivity was low in paraffin sections but was clearly recognizable in sections processed for immunofluorescence and confocal microscopy. Fine granular immunoreactivity was seen in the cytoplasm of neurons of the hippocampus, including CA1 and hilus, dentate gyrus, neocortex, including frontal cortex, and Purkinje cells of the cerebellum (Fig. 4). As for ORs, glial cells were rarely immunostained with anti-AC3 and anti-Gαolf antibodies (data not shown).

Gel Electrophoresis and Western Blotting and Subcellular Localization of ORs, AC3, and Gαolf

The possibility that postmortem delay might have interfered with protein preservation was analyzed in a paradigm of artificial postmortem delay of the same tissue samples from which small pieces were taken and stored at -80°C at different intervals. No differences in expression levels were seen at up to 24 hours of artificial postmortem delay for anti-OR2H2 and anti-AC3 antibodies, thus indicating relative preservation with postmortem delay. Each antibody recognized a net particular band pattern at the appropriate molecular weight; however, antibodies against OR2A4, OR6K3, Gαolf, RTP1, RTP2, and REEP1 were not suitable for Western blotting.

Western blotting was also used to analyze the subcellular localization of ORs and AC3 in control frontal cortex area 8 following a protocol to enrich lipid rafts (Fig. 5A). Flotillin, used as a marker of lipid rafts, was restricted to lanes 1 and 2, whereas SOD1, used as a marker of cytosolic-enriched fraction, was recovered in lane 6. Interestingly, antibodies against OR2H2 revealed an expected single band of about 40 kd, whereas antibodies against AC3 showed an expected triple band between 65 and 100 kd, both restricted to lane 6 (Fig. 5A). Cytosolic localization of ORs was further supported by immunofluorescence and confocal microscopy showing granular cytoplasmic anti-OR2H2 and anti-OR6K3 immunoreactivity (Fig. 5B).

Localization of OR Transporters and UDP-Glucuronosyltransferase

To learn whether neurons are equipped with OR transporters that may act as shuttles to transport ORs to the membranes, immunohistochemistry, immunofluorescence, and confocal microscopy to RTP1, RTP2, and REEP1 were assessed. Best results were obtained in paraffin sections processed for immunofluorescence and, although RTP1 and RTP2 were expressed in neurons, the expression of REEP1 was stronger. Receptor Expression Enhancing Protein 1 immunofluorescence was observed in the cytoplasm of neurons of the hippocampus, dentate gyrus, entorhinal cortex, frontal cortex area 8, and inferior temporal cortex.

TABLE 4. Gene Ontology Terms of Biologic Function Enrichment in Parkinson Disease Cases Using a Hypergeometric Distribution Function Statistical Analysis

GO ID	p	Odds Ratio	Exp Count	Count	Size	Term
GO:0007608	4.39^{e-06}	3.32446451	7.31126639	22	383	Sensory perception of smell
GO:0050909	0.00010177	9.25482625	0.76357874	6	40	Sensory perception of taste

GO ID, gene ontology term identification number; p, p values for each GO term tested; odds ratio, odds ratio for each GO term tested; Exp Count, expected number of differentially expressed genes; count, number of genes differentially expressed that are annotated at the GO term; size, number of genes from the Genechip Array that are annotated at the GO term; term, the GO term name.

Highest expression levels were seen in selected nuclei of the mesencephalon, including the substantia nigra and Purkinje cells of the cerebellum (Fig. 6A–H).

Finally, immunofluorescence and confocal microscopy to UGT1A6 revealed a discrete and generalized immunoreactivity in the cytoplasm of neurons, including those of the

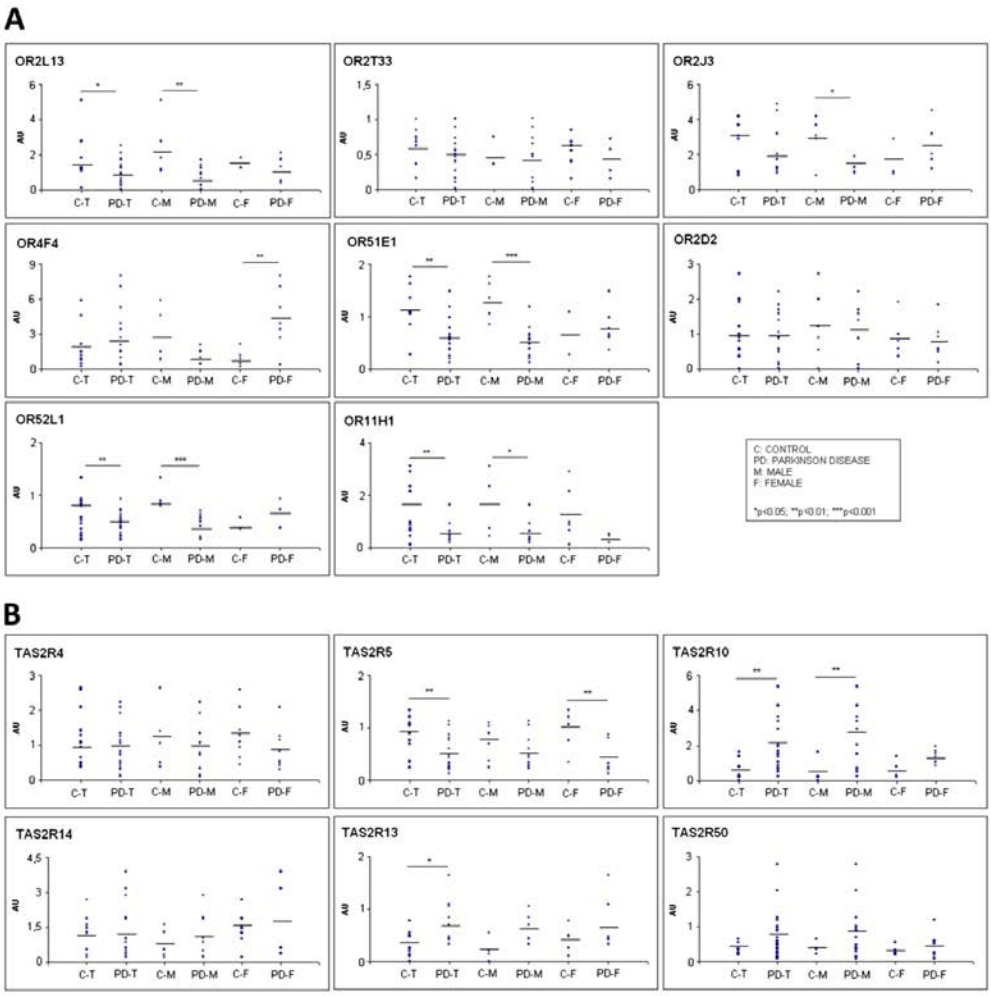

FIGURE 2. Messenger RNA expression levels of selected olfactory (odorant) receptors (ORs) **(A)** and taste receptors (TASRs) **(B)** in the frontal cortex (area 8) in Parkinson disease (PD) and control cases as determined by TaqMan PCR assays. Significant differences are seen in the expression of OR2L13, OR51E1, OR2J3, OR52L1, and OR11H1 mRNA in PD cases compared with those in controls. All these genes are downregulated, mainly in males, in PD. Yet, expression levels of OR2T33, OR2D2, and OR4F4 mRNA in PD do not differ from levels in controls. TAS2R4 and TAS2R14 expression is not modified in PD, but TAS2R5 and TAS2R50 are downregulated, whereas TAS2R10 and TAS2R13 are upregulated in PD when compared with those in controls. TASR dysregulated expression in PD is also subjected to sex differences. C, control; PD, Parkinson disease; T, total population; M, male; F, female; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

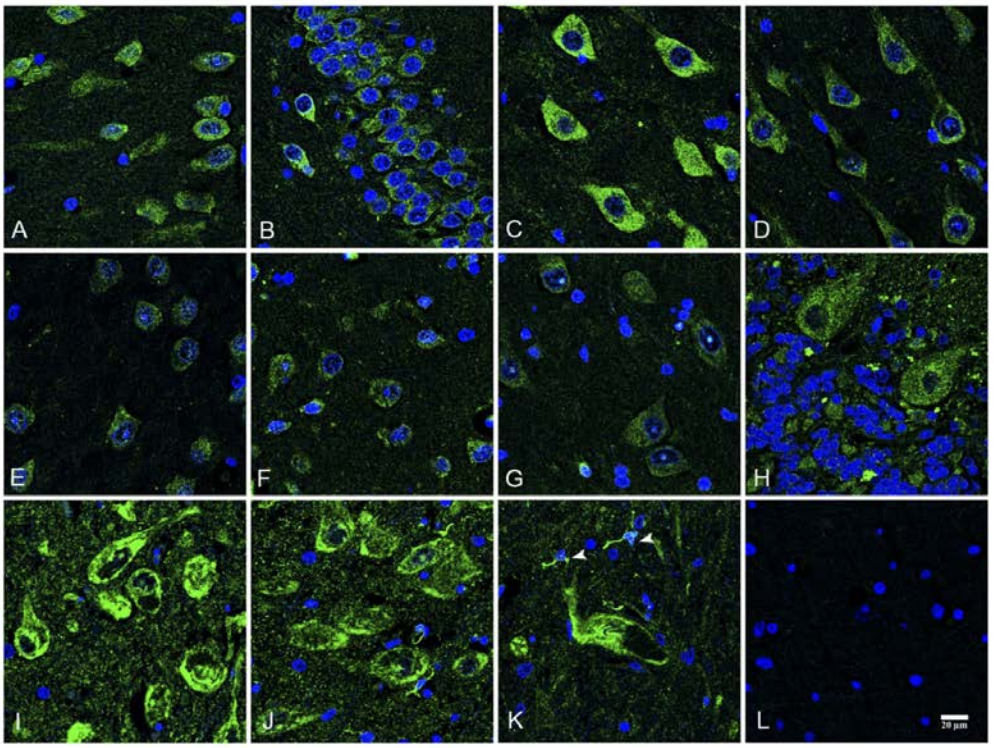

FIGURE 3. Immunofluorescence and confocal microscopy of OR6K3 in entorhinal cortex **(A)**, dentate gyrus **(B)**, hilus of the hippocampus **(C)**, CA1 area of the hippocampus **(D)**, inferior temporal cortex **(E)**, frontal cortex area 8 **(F)**, dorsomedial thalamus **(G)**, molecular layer and Purkinje cells of the cerebellum **(H)**, motor ocular nucleus **(I)**, pontine nuclei **(J)**, and substantia nigra pars compacta **(K)**. Olfactory receptor OR6K3 immunoreactivity is found in the cytoplasm of the vast majority of neurons in all of these regions, although with variable intensity. A very few glial cells (arrows in **[K]**) also exhibit OR6K3 immunoreactivity in the cytoplasm. **(L)** Negative control incubated without the primary antibody shows negative staining. Paraffin sections are preincubated with Sudan black to block lipofuscin autofluorescence. Nuclei are stained with DRAQ5. Scale bar = 20 µm.

hippocampus proper, dentate gyrus, neocortex (area 8), and Purkinje cells (Fig. 6I–L).

Distribution of ORs, Obligate Functional Components of OR Signaling, and Transporters in PD

Immunohistochemistry to OR2H2, OR2A4, and OR6K3, AC3 and Gαolf, and the transporters RTP1, RTP2, and REEP1 showed similar localization and distribution of all of these molecules in control and PD brains. No differences in the subcellular distribution, as revealed with the analysis of lipid rafts, were observed between control and PD cases regarding OR2H2 and AC3 localization in the same cytosolic fractions (data not shown). As stated above, the lack of appropriate antibodies did not permit a validation of OR dysregulation in PD at a protein level.

DISCUSSION

Tissue samples used in the present study correspond to optimally selected cases on the basis of protein and mRNA preservation; neuropathologic diagnoses were verified following the studies carried out and consensus was reached in the context of the European Brain Bank Network (European Brain Net Consortium) (36, 46–48). Because mixed pathologies are very common in old age (49), special attention was paid to excluding cases with combined lesions, excepting mild small blood vessel disease. More specifically, cases with PD selected for study did not have concomitant AD-like pathology, with the exception of a few neurofibrillary tangles

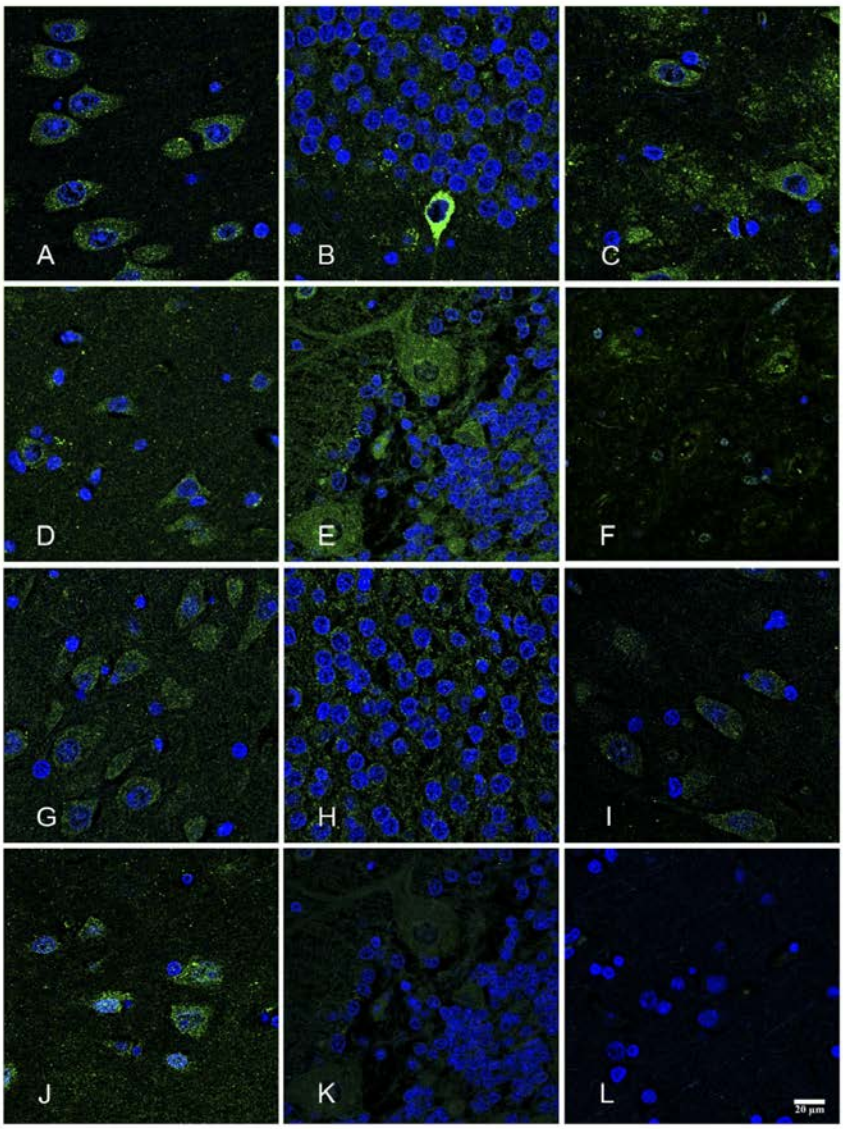

FIGURE 4. Immunofluorescence and confocal microscopy of adenylyl cyclase 3 (AC3) **(A–F)** and olfactory G protein (Gαolf) **(G–K)** in the CA1 area of the hippocampus **(A, G)**, dentate gyrus **(B, H)**, hilus of the hippocampus **(C)**, frontal cortex area 8 **(D, I)**, dorsomedial thalamus **(J)**, molecular layer and Purkinje cells of the cerebellum **(E, K)**, and substantia nigra pars compacta **(F)**. Fine granular AC3 and Gαolf immunoreactivity is found in the cytoplasm neurons in all these regions, although with variable intensity. **(L)** Negative control incubated without the primary antibody shows lack of immunostaining. Paraffin sections were preincubated with Sudan black to block lipofuscin autofluorescence. Nuclei are stained with DRAQ5. Scale bar = 20 µm.

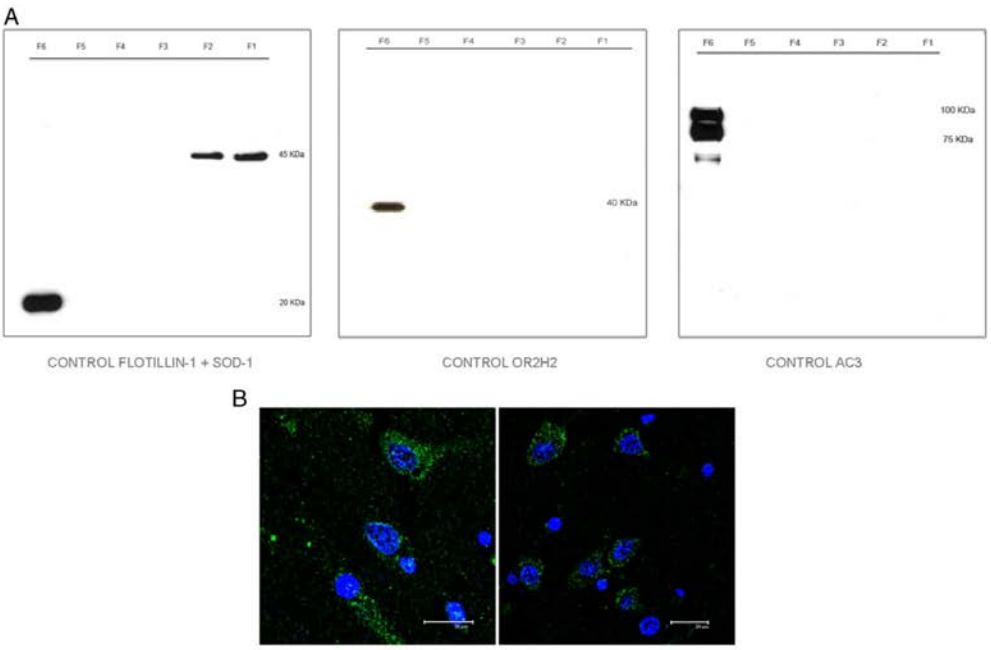

FIGURE 5. (A) Gel electrophoresis and Western blotting of lipid raft fractions from the frontal cortex of control brains. Antibodies to flotillin and SOD-1 are used to identify the lipid raft–enriched fractions (lanes F1 and F2) and the cytosolic fraction (lane F6), respectively. These antibodies show unique bands at 45 kd and 20 kd, which correspond to the molecular weights of flotillin and SOD-1. The OR2H2 antibody recognizes a unique band with the appropriate molecular weight (40 kd) in the cytosolic fraction (lane F6). The anti–adenyl cyclase 3 antibody ADY3 recognizes 3 bands of molecular weights between 75 and 100 kd only in the cytosolic fraction (lane F6). Different gels (10%–12% acrylamide) were used; therefore, the appropriate molecular weight detected by each antibody in the transferred membranes is represented by the corresponding mark and is not necessarily aligned with the antibodies analyzed in the same series. **(B)** Immunofluorescence to OR2H2 (left panel) and OR6K3 (right panel) in the frontal cortex of control human brain. Immunoreactivity (green) is visualized as small granules in the cytoplasm of neurons. Nuclei are stained with DRAQ5. Scale bar = 20 μm.

in the entorhinal cortex (corresponding to stage I of Braak) in a few cases. Beta-amyloid pathology was absent in PD cases and control cases did not have neurologic histories or neuropathologic lesions, with the exception of a few neurofibrillary tangles in the entorhinal cortex in a few cases. This allowed for the conclusion that observed alterations were caused by particular disease states and not to the result of the sum or potentiation of associated pathologies. The frontal cortex (area 8) in PD was selected because of the amount of newly identified molecular alterations in this area in PD and related α-synucleinopathies (32, 33, 35).

The present study reveals that ORs are widely distributed in the human CNS, including cerebral cortex, thalamus, selected nuclei of the brainstem, and Purkinje cells of the cerebellum. Interestingly, certain nuclei of the brainstem as the motor nuclei of the third cranial nerve, substantia nigra, and pontine nuclei, together with Purkinje cells, showed strong OR expression (at least for the ORs examined OR2H2, OR2A4, and OR6K3); within the cerebral cortex, the major expression occurred not only in the CA1 area and hilus of the hippocampus but also in the entorhinal cortex and neocortical areas examined. Central OR neuronal expression is accompanied by the obliged functional molecules of the OR pathway in sensory olfactory neurons, AC3, and Gαolf, indicating that the OR machinery is also able to operate, at least, in selected regions of the CNS and, particularly, in the cerebral cortex. Olfactory receptor and associated molecules were overwhelmingly localized in neurons; their presence in few glial cells was unusual.

Olfactory receptor OR2H2 and AC3 (the only molecules for which antibodies were suitable for Western blot studies) are localized in cytosolic fractions in the human frontal cortex. This localization is consistent with the idea that cortical ORs may require specific mechanisms to facilitate OR translocation from the cytosol to the membranes (50, 51). According to this rationale, cofactors of G protein–coupled receptors bind to specific domains of receptors, thus facilitating or inhibiting cell surface

expression (52). Receptor transporters RTP1 and RTP2, and REEP1, facilitate OR recruitment to lipid rafts (50). The present observations show RTP1, RTP2, and REEP1 in the cytoplasm of the vast majority if not all neurons in parallel with the expression of ORs in different regions, thereby revealing that molecules specifically expressed in sensory olfactory neurons are also present in cerebral cortex and other regions of the human brain, thus enabling the traffic of ORs from the cytosol to the cellular membranes.

UDP-glucuronosyltransferases are enzymes localized in the endoplasmic reticulum and nuclear membranes involved in the detoxification of various xenobiotic and endobiotic compounds through glucuronidation of substrates, thereby facilitating their excretion (53, 54). Several UGTs, including UGT1A6, have been reported in the olfactory epithelium and olfactory bulb (55) and, more recently, in the olfactory cortex of rodents, where they appear to participate in both detoxification and olfactory signal termination (56). Here, we show that UGT1A6 is expressed in neurons of the human cerebral cortex and may be considered as a candidate for assisting in the detoxification of chemicals triggering cortical OR activation.

Bitter taste receptors are also expressed in several brain regions of the rat, and they appear to be functional (57, 58). Several taste receptor mRNAs are also detected in the present study in the human frontal cortex. However, evidence of protein expression of taste receptors and expression of the specific gustatory specific G protein, α-gustducin, in the human brain is still needed but is beyond the scope of the present study. Alpha-gustducin plays a crucial role in peripheral taste signaling transduction in sensory gustatory cells (59, 60).

As of now, we have no precise idea about the role of ORs in the human cerebral cortex and what might be the nature of

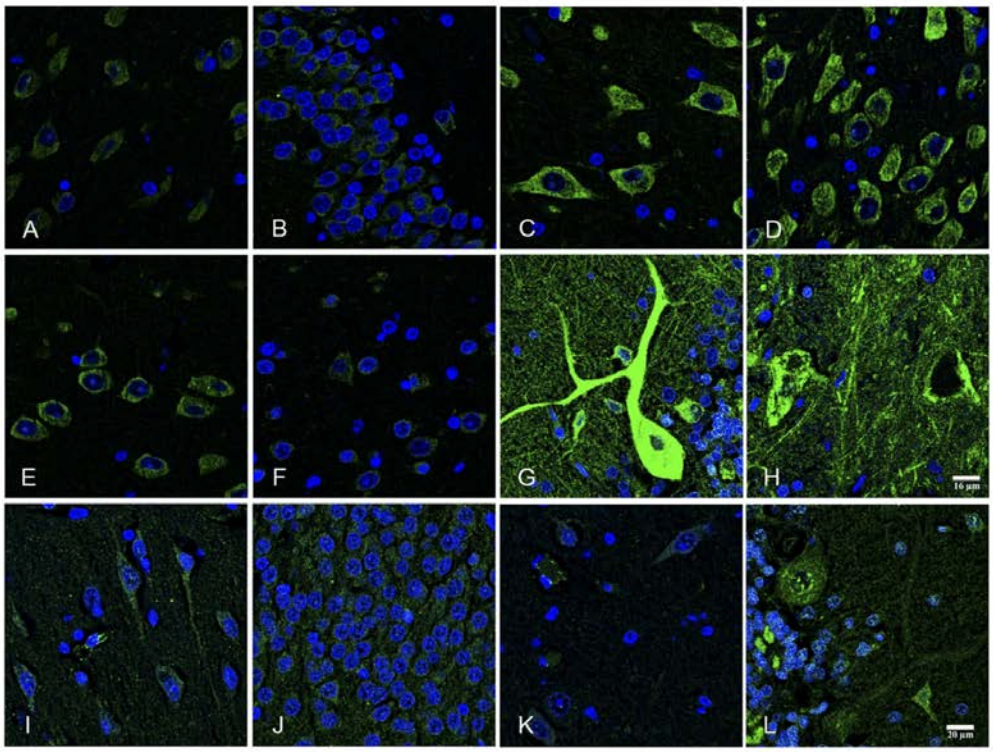

FIGURE 6. (A–H) Immunofluorescence and confocal microscopy of Receptor Expression Enhancing Protein 1 (REEP1) in entorhinal cortex **(A)**, dentate gyrus **(B)**, hilus of the hippocampus **(C)**, CA1 area of the hippocampus **(D)**, frontal cortex area 8 **(E)**, dorsomedial thalamus **(F)**, Purkinje cell of the cerebellum **(G)**, and substantia nigra pars compacta **(H)**. REEP1 immunoreactivity is found in the cytoplasm of the vast majority of neurons in all these regions, although with variable intensity. **(I–L)** UDP-glucuronosyltransferase 1 family polypeptide A6 (UGT1A6) immunoreactivity is found, among other regions, in the CA1 area of the hippocampus **(I)**, dentate gyrus **(J)**, frontal cortex area 8 **(K)**, and Purkinje cells **(L)**. Paraffin sections are preincubated with Sudan black to block lipofuscin autofluorescence. Nuclei are stained with DRAQ5. Scale bars = **(A–H)** 20 µm; **(I–L)** 20 µm.

the putative ligands of cortical ORs or the routes that ligands may use to contact the corresponding ORs in the cerebral cortex. Several natural ligands have been identified in mammals (61–67). These are small molecules of approximately 200 to 400 molecular weight related to steroids, fatty acid derivatives, and various terpenoid compounds (67–79). However, it is not known whether central ORs are functionally related to their putative counterparts within the sensory olfactory neurons. It is possible that putative exogenous ligands to ORs in the human brain are transported through the blood vessels and the extracellular space. Alternatively, brain ORs might be stimulated by local chemicals from neighboring cells, or even from the same cells, thus having a self-stimulating mechanism regulating internal trafficking.

In line with these hypotheses, ORs have been involved in functions other than olfactory signal recognition, including axonal guidance (80–82), and cell assembly functions in the embryonic period (83), muscle regeneration, and regulation of cell adhesion and migration (84, 85). Olfactory receptor expression in the testis has been implicated in sperm chemotaxis (86–88). Regarding the nervous system, expression of ORs in ganglia of the autonomic nervous system has lent support to the idea that they serve functions other than odorant detection (89), including detection of endogenously produced chemicals in their vicinity (31). Gαolf is expressed in different brain regions in the murine brain (16, 90). It is worth noting that surviving homozygous mice with null mutation in Gαolf exhibit, in addition to reduced olfaction, hyperactive behaviors. These behavioral phenotypes suggest that Gαolf may also function as an essential signaling molecule more centrally in the brain (16).

A particularly exciting and fortuitous observation of the present study is that ORs are dysregulated in the PD frontal cortex. It is worth stressing that PD cases did not (with 1 exception) experience gross cognitive deficits, and Lewy bodies in frontal cortex were absent (cases categorized as stage 3 and 4) or present in small numbers (cases categorized as stage 5). Importantly, OR dysregulation occurred in cases at premotor and at parkinsonian stages of the disease. This suggests that OR dysregulation may occur at relatively early stages of PD pathology and that it has no relation with drug therapy because the premotor cases had not received any anti-parkinsonian medication.

Results from the present mRNA arrays and TaqMan PCR analyses have relevant implications. First, dysregulation of ORs in the frontal cortex area 8 appears at relatively early stages of the neurodegenerative processes, along with other molecular changes (32, 33, 35), and precedes the presence of α-synuclein aggregates. Second, OR dysregulation is not related to medication because it was found in patients who had not received drug therapy. Third, there is a predominant OR downregulation in PD, which seems not to be a consequence of a more general downregulation of other central chemoreceptors because certain TASRs appeared to be upregulated in the same region at the same stages of the process in the same cases. Fourth, downregulation of certain ORs in PD is probably sex-dependent because a significant decrease in the expression of, at least, certain genes was found mostly in men but not in women. Unfortunately, lack of appropriate antibodies against the products of dysregulated genes did not permit the validation of RNA dysregulation at the protein levels.

Sex differences have been largely recognized in relation to olfactory signaling (91). These are partly caused by sexually dimorphic olfactory response despite similar olfactory circuits, suggesting that sex-specific initial signals prompt divergent behaviors (92). This may be caused by specific sex responses to sexually dimorphic specific ligands (93–95) and to the imprinting of sex-dimorphic sensory olfactory modulation by hormones (96–101). The present findings indicate sex-specific vulnerability of certain ORs in PD, thus suggesting that at least a subpopulation of cortical ORs is modulated by steroid hormones or their derivatives.

Olfaction is altered in PD even at early premotor stages of the degenerative process (102, 103). Most information regarding smell disorders in neurodegenerative diseases, and particularly PD, is focused on the presence of altered molecules in the olfactory tract (i.e. α-synuclein in PD) and the altered complex functional connectivity of primary and secondary central olfactory areas (102). Interestingly, although less frequent than impairment of smell, taste function is also impaired in PD, and altered taste mainly involves perception of bitter (104, 105). Unfortunately, nothing is known at present about the vulnerability of peripheral sensory olfactory and taste receptors in the nasal cavity and tongue, respectively. Therefore, it may be premature and perhaps incorrect to suggest that impaired olfaction and taste perception constitute a part of a more extended dysregulation of OR and taste pathways involving specific sensory peripheral and random central cortical neurons. Rather, the consequences of dysregulation of ORs and TASRs in the frontal cortex area 8 in PD might not have any relation with sensory organs. It is clear that further study is needed to unveil the function of these new putative central chemoreceptors or signaling systems in the CNS.

In summary, the present observations demonstrate for the first time the expression of several ORs and TASRs in the human brain. For the present purposes, the presence of ORs has been demonstrated at mRNA and protein levels in frontal cortex, and it is accompanied by the expression of obligate functional molecules of the OR pathway, AC3 and Gαolf, and putative OR transporters RTP1, RTP2, and REEP1. These findings lend weight to the idea that ORs (and probably TASRs) in the human brain may support key physiologic roles, and they suggest a new scenario in the still poorly understood chemical signaling system of the brain. Regarding the vulnerability of ORs and TASRs in pathologic conditions, the present findings show dysregulation of certain members in the frontal cortex area 8 in PD. Studies are needed to prove regional differences in PD and assessment of OR and TASR expression in other neurodegenerative disorders, in addition to gaining knowledge about the functional implications of these central neurochemical receptors. This represents a stimulating insight in the field of human neuropathology.

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ARTICLE 6

Dementia with Lewy bodies: molecular pathology in the frontal cortex in typical and rapid forms.

Garcia-Esparcia P, López-Gonzalez I, Grau-Rivera Oriol, García-Garrido MF, Koneti A, Llorens F, Zafar S, Carmona M, Del Río JA, Zerr I, Gelpi E, Ferrer I.

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ABSTRACT

Introduction and Methods: Mitochondrial function, energy and purine metabolism, protein synthesis machinery from the nucleolus to the ribosome, inflammation, and expression of newly identified ectopic olfactory and taste receptors in brain were assessed in the frontal cortex of typical cases of Dementia with Lewy Bodies (DLB) and cases with rapid clinical course (rpDLB) compared with middle-aged non-affected individuals, in order to learn about the biochemical abnormalities underlying the pathogenesis of this disease. **Results:** The main alterations in DLB and rpDLB, which are more marked in the rapidly progressive forms, include: i) deregulated expression of several mRNAs and proteins of mitochondrial subunits, and reduced activity of complexes I, II, III and IV of the mitochondrial respiratory chain; ii) reduced expression of selected molecules involved in energy metabolism and increased expression of enzymes involved in purine metabolism; iii) abnormal expression of nucleolar proteins, rRNA18S, genes encoding ribosomal proteins and initiation factors of the transcription at the ribosome; iv) discrete inflammation; and v) marked deregulation of brain olfactory and taste receptors (ORs and TASRs, respectively). **Conclusions:** Severe mitochondrial dysfunction involving activity of four complexes, minimal inflammatory responses, and dramatic altered expression of ORs and TASRs discriminate DLB from Alzheimer's disease. Altered solubility and aggregation of α-synuclein, increased β-amyloid bound to membranes and absence of soluble tau oligomers are common in DLB and rpDLB. Low levels of soluble β-amyloid are found in DLB. However, increased soluble β-amyloid 1-40 and β-amyloid 1-42, and increased *TNF-*α mRNA and protein expression, distinguish rpDLB.

Dementia with Lewy bodies: molecular pathology in the frontal cortex in typical and rapidly progressive forms

Paula Garcia-Esparcia MSc [1, 3], Irene López Gonzalez MSc [1, 3], Oriol Grau-Rivera MD [4], María Francisca García-Garrido MSc [1], Anusha Konetti [1], Franc Llorens PhD [5], Saima Zafar MD [5], Margarita Carmona Tech [1, 3], José Antonio del Rio PhD [3, 6], Inga Zerr MD, PhD [5], Ellen Gelpi MD [4], Isidro Ferrer MD, PhD [1, 2, 3, CA]

1 Institute of Neuropathology, Service of Pathologic Anatomy, IDIBELL-Hospital Universitari de Bellvitge, Hospitalet de Llobregat, Spain
2 Department of Pathology and Experimental Therapeutics, University of Barcelona, L'Hospitalet de Llobregat, Spain
3 CIBERNED, Network Centre for Biomedical Research of Neurodegenerative Diseases, Institute Carlos III, Spain
4 Neurological Tissue Bank of the Biobanc-Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Barcelona, Spain
5 Department of Neurology, Clinical Dementia Center, University Medical School, Georg-August University and German Center for Neurodegenerative Diseases (DZNE), Göttingen, Germany
6 Molecular and Cellular Neurobiotechnology, Institute of Bioengineering of Catalonia (IBEC), Parc Científic de Barcelona, Department of Cell Biology, University of Barcelona, Barcelona, Spain

CA: Corresponding author
Professor Isidro Ferrer, Institute of Neuropathology, Service of Pathologic Anatomy, Bellvitge University Hospital, carrer Feixa Llarga s/n, 08907 Hospitalet de Llobregat, Spain; Tel.: +34 93 260 7452; Fax: +34 93 260 7503. Email: 8082ifa@gmail.com

Compliance with ethical standards

No relevant data; no conflicts of interest

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Highlights

• Severe mitochondrial dysfunction, minimal inflammatory responses, and dramatic altered expression of ORs and TASRs in frontal cortex distinguish DLB from Alzheimer's disease (AD).

• Altered solubility and aggregation of α-synuclein, increased β-amyloid bound to membranes and absence of soluble tau oligomers are common in DLB and rpDLB.

• Low levels of soluble β-amyloid are found in DLB in comparison to AD at similar stage of AD-related pathology.

• Increased soluble β-amyloid 1-40 and β-amyloid 1-42, and increased TNF-α mRNA and protein expression, distinguish rpDLB from DLB.

Abstract

Mitochondrial function, energy and purine metabolism, protein synthesis machinery from the nucleolus to the ribosome, inflammation, and expression of newly identified ectopic olfactory and taste receptors in brain were assessed in the frontal cortex of typical cases of Dementia with Lewy Bodies (DLB) and cases with rapid clinical course (rpDLB) compared with middle-aged non-affected individuals, in order to learn about the biochemical abnormalities underlying the pathogenesis of this disease. The main alterations in DLB and rpDLB, which are more marked in the rapidly progressive forms, include: i) deregulated expression of several mRNAs and proteins of mitochondrial subunits, and reduced activity of complexes I, II, III and IV of the mitochondrial respiratory chain; ii) reduced expression of selected molecules involved in energy metabolism and increased expression of enzymes involved in purine metabolism; iii) abnormal expression of nucleolar proteins, rRNA18S, genes encoding ribosomal proteins and initiation factors of the transcription at the ribosome; iv) discrete inflammation; and v) marked deregulation of brain olfactory and taste receptors (ORs and TASRs, respectively). Severe mitochondrial dysfunction involving activity of four complexes, minimal inflammatory responses, and dramatic altered expression of ORs and TASRs discriminate DLB from Alzheimer's disease. Altered solubility and aggregation of α-synuclein, increased β-amyloid bound to membranes and absence of soluble tau oligomers are common in DLB and rpDLB. Low levels of soluble β-amyloid are found in DLB. However, increased soluble β-amyloid 1-40 and β-amyloid 1-42, and increased TNF-α mRNA and protein expression, distinguish rpDLB.

Key words: Dementia with Lewy bodies, Alzheimer's disease, α-synuclein, mitochondria, protein synthesis, purine metabolism, inflammation, microglia, β-amyloid, olfactory receptors, taste receptors.

1. Introduction

Dementia with Lewy bodies (DLB) is the second most common neurodegenerative dementia in the elderly, clinically manifested by fluctuating cognition with pronounced variation in attention and alertness, recurrent visual hallucinations which are typically well formed and detailed, and spontaneous motor features of parkinsonism; repeated falls, syncope, transient loss of consciousness, systematized delusions, hallucinations in other modalities and neuroleptic sensitivity are not uncommon (McKeith *et al*., 1996; McKeith *et al*., 2004; McKeith *et al*., 2005; Mayo and Bordelon, 2014). These symptoms are preceded by rapid eye movement sleep behavior disorder, psychiatric symptoms, loss of smell and dysautonomia, together with occipital hypo-metabolism, hallucinations and cognitive impairment (Fujishiro *et al*., 2015; Donaghy *et al*., 2015). DLB is pathologically characterized by Lewy bodies and Lewy neurites in the brainstem, limbic system and cortical areas (McKeith *et al*., 2004; Fujishiro *et al*., 2008; Ince, 2011). The main pathological change is the production and accumulation, in Lewy bodies and neurites, of abnormal α-synuclein, which is phosphorylated, nitrated and truncated, has abnormal solubility, prompts the production of oligomeric species, aggregates into fibrils and is ubiquitinated (Spillantini *et al*., 1998; Serpell *et al*., 2000; Goedert, 2001; Fujiwara *et al*., 2002; Kaplan *et al*., 2003; Tofaris *et al*., 2003; Hashimoto *et al*., 2004; Li *et al*., 2005; Anderson *et al*., 2006; Mukaetova-Ladinska and McKeith, 2006; Tofaris and Spillantini, 2007). For these reasons, DLB is classified among α-synucleinopathies with Lewy bodies or Lewy body diseases (LBDs), together with Parkinson's disease (PD) (Goedert, 2001). Other changes in DLB are neuron loss, microvacuolation, and Alzheimer's disease (AD) pathology distinguished by β-amyloid deposition in the form of diffuse and senile plaques, as well as early changes of neurofibrillary tangle (NFT) pathology (Ince, 2011). Whether the amount of α-synuclein pathology (i.e., Lewy bodies and neurites) in cerebral cortex is predictable of dementia in LBDs is a matter of controversy (Parkinnen *et al*., 2005; Braak *et al*., 2006; Parkinnen *et al*., 2008; Jellinger, 2009). Cholinergic and dopaminergic denervation of the neocortex probably accounts at least in part for cognitive deficits in LBDs (Shimada *et al*., 2009; Lim *et al*., 2009; Kim *et al*., 2011; Grothe *et al*., 2014; Huang *et al*., 2015; Budhala *et al*., 2015). Concomitant pathologies have also been suggested to explain variations in the degree of cognitive impairment in DLB (Jellinger, 2007; Jellinger and Attems, 2008; Colom-

Cadena *et al*., 2013). However, deficits in neurotransmission are not limited to dopaminergic and cholinergic systems. Synapses are primarily damaged in the neocortex in DLB (Hashimoto and Masliah, 2003; Dalfó *et al*., 2004b; Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010; Mukaetova-Ladinska *et al*., 2013; Overk and Masliah, 2014). Synaptic damage is probably related to toxic α-synuclein oligomers and pore formation (Conway *et al*., 2000; Lashuel *et al*., 2002). Moreover, synaptic alterations are accompanied by abnormalities in neurotransmitter signaling (Dalfó *et al*., 2004[a]; Albasanz *et al*., 2005) in a way similar to that reported in other α-synucleinopathies (Nemani *et al*., 2010; Scott *et al*., 2010; Garcia-Reitböck *et al*., 2010). Additional molecular alterations converge in the pathogenesis of DLB, including impaired autophagy and ubiquitin-proteasome system of protein (MacInnes *et al*., 2008; Tanji *et al*., 2011; Kragh *et al*., 2012; Poehler *et al*., 2014; Miki *et al*., 2015), as well as altered responses to protein misfolding (Baek *et al*., 2015). Preliminary studies have shown impaired mitochondrial activity and oxidative damage involving proteins, lipids and DNA in the neocortex in DLB (Lyras *et al*., 1998; Navarro *et al*., 2009); α-synuclein is one of the targets of oxidative damage in the frontal cortex in DLB (Dalfó and Ferrer, 2008). The average survival time for typical DLB from the beginning of symptoms is about 5-8 years (Williams *et al*., 2006). However, some cases have a rapid course and are considered clinically to be in the group of rapidly progressive dementias (Geshwind *et al*., 2008; Josephs *et al*., 2009). DLB with rapid progression is called rapid DLB (rpDLB) (Williams *et al*., 2006; Gaig *et al*., 2011). The mean duration of rpDLB is about 9 months; delirium, visual hallucinations, delusions and fluctuating cognitive impairment, followed by *parkinsonism* and myoclonus, are the predominant symptoms (Gaig *et al*., 2011). Our hypothesis is that alterations of several metabolic pathways converge in the pathogenesis of DLB and that impaired mitochondria and energy metabolism, purine metabolism, protein synthesis and inflammation may be important factors in the pathogenesis of DLB. In the same line, although neuropathological studies have not shown differences between DLB and rpDLB (Gaig *et al*. 2011), biochemical alterations probably discriminate between DLB and rpDLB.

The present DLB-centered study was undertaken to analyze: i.) levels of selected mRNAs and proteins of subunits of the five mitochondrial complexes, and genes linked to energy metabolism; ii.) expression of genes encoding enzymes involved in purine metabolism; iii.) mRNA and protein expression of selected nucleolar

proteins, rRNAs, and genes encoding ribosomal proteins, and protein expression of initiation and elongation factors of protein transcription at the ribosome; iv.) cytokines and mediators of the inflammatory response; and v.) gene expression of particular brain receptors involved in olfaction and taste known to be altered in other neurodegenerative diseases with abnormal protein aggregates. All these pathways were assessed in the frontal cortex of cases with typical course (DLB) and in cases with rapid course (rpDLB) in order to identify additional factors linked to short disease duration.

2. Material and methods

2.1. Human cases

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank and the Hospital Clinic-IDIBAPS Biobank following the guidelines of Spanish legislation on this matter and approval of the local ethics committees. Processing of brain tissue has been detailed elsewhere (Ferrer *et al*., 2008; Ravid and Ferrer, 2012). The *post-mortem* interval between death and tissue processing was between 2 and 15 h. One hemisphere was immediately cut in coronal sections, 1cm thick, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags and stored at -80ºC until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphological studies. Neuropathological diagnosis in all cases was based on the routine study of 20 selected de-waxed paraffin sections of representative regions of the cerebral cortex, diencephalon, thalamus, brain stem and cerebellum which were stained with haematoxylin and eosin, and Klüver-Barrera, or processed for immunohistochemistry for microglia (antibodies Iba1 and CD68), glial fibrillary acidic protein, β-amyloid with antibodies Aβ clone 6 F/3D (diluted 1:50, Dako, Carpinteria, CA, USA), Aβ40 (diluted 1:100, Merck Millipore, Billerica, MA) and Aβ42 (diluted 1:50, Merck Millipore), phospho-tau (clone AT8), α-synuclein, TDP-43, ubiquitin and p62 using EnVision+ System peroxidase (Dako), and diaminobenzidine and H2O2. DLB cases (n = 13) were neuropathologically categorized following current staging classifications for LBD (Braak *et al*., 2003; Parkinnen *et al*., 2008; Alafuzoff *et al*., 2009), Alzheimer's disease (AD) neurofibrillary tangles (Braak and Braak, 1991; Braak *et al*., 2006) and phases of AD-related β-amyloid plaques (Thal *et al*., 2002) and a final ABC score was

assigned according to current consensus guidelines (Montine *et al*., 2012). Based on clinical criteria DLB cases were categorized as typical DLB (DLB, n = 9) or rapid DLB (rpDLB, n = 4) on the basis of the natural clinical course. rpDLB was defined as 2 years or less of disease duration from the first symptom to death (Gaig *et al*., 2011). Cases with associated pathologies such as vascular diseases excepting mild atherosclerosis and arteriolosclerosis, TDP-43 proteinopathy, infections of the nervous system, brain neoplasms, systemic and central immune diseases, metabolic syndrome and hypoxia were excluded from the present study. Middle-aged cases (MA) (n = 12) had not suffered from neurologic, psychiatric or metabolic diseases (including metabolic syndrome), and did not have abnormalities in the neuropathological examination excepting sporadic AD stages I-II/0-A and phases 1-2 of β-amyloid plaques. Quantification of β-amyloid burden was carried out with a Nikon Eclipse E800 microscope (4x objective; Nikon Imaging Inc, Tokyo, Japan). The cortical total Aβ burden was calculated as the percentage of the area of Aβ deposition in plaques with respect to the total area in 9-10 representative pictures taken from frontal cortex in every case. β-amyloid quantification was assessed using the Adobe Photoshop CS5 software (Adobe Systems Inc, San Jose, CA). A summary of cases is shown in Table I.

2.2. RNA purification

Purification of RNA from the right frontal cortex area 8 was carried out using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer combined with DNase digestion to avoid extraction and later amplification of genomic DNA. The concentration of each sample was obtained from A260 measurements with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA) (Garcia-Esparcia *et al*., 2015). Values of RNA integrity number (RIN) are shown in Table I. Special care was taken to assess pre-mortem and *post-mortem* factors which may interfere with RNA processing (Durrenberg *et al*, 2010). Bivariate analyses were carried out to detect association of our variables with potential confounding factors (age, *post-mortem* delay and RIN) using Spearman or Pearson correlations for quantitative variables. Stastical analysis was performed with GraphPad Prism version 5.00 and SPSS 19. *Post-mortem* delay, which was longer in rpDLB, had no effect on RIN values in the present series.

2.3. Retrotranscription reaction

Retrotranscription reaction of RNA samples was carried out with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) following the guidelines provided by the supplier, and using Gene Amp® 9700 PCR System thermocycler (Applied Biosystems). A parallel reaction for one RNA sample was processed in the absence of reverse transcriptase to rule out DNA contamination.

2.4. Real Time PCR

Real Time quantitative PCR (RT-qPCR) assays were conducted in duplicate on 1,000ηg of cDNA samples obtained from the retro-transcription reaction, diluted 1:20 in 384-well optical plates (Kisker Biotech, Steinfurt, GE) utilizing the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Parallel amplification reactions were carried out using 20x TaqMan Gene Expression Assays and 2x TaqMan Universal PCR Master Mix (Applied Biosystems) (Garcia-Esparcia *et al*., 2015). Genes analyzed with the corresponding abbreviations and TaqMan probes used in the study are shown in Table II. The selection of probes was based on criteria covering a larger project geared to analyzing similar expression of the same molecules related to inflammation, mitochondria, energy metabolism, purine metabolism, protein synthesis and selected receptors in several diseases including Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob's disease, certain tauopathies, in addition to DLB, in all cases using the same probes and methods in an attempt to learn about commonalities and differences among these diseases in a particular brain region. Our idea is that all these pathways are altered in most neurodegenerative diseases with abnormal protein aggregates, but alterations are region-, stage- and disease-specific. Parallel assays for each sample were carried out using β-glucuronidase (GUS-β), X-prolyl aminopeptidase (aminopeptidase P) 1 (XPNPEP1), AARS (alanyl-transfer RNA synthase) and HPRT (hypoxanthine-guanine phosphoribosyltransferase) probes for normalization. The reactions were performed using the following parameters: 50ºC for 2 min, 95ºC for 10 min, 40 cycles at 95ºC for 15 s, and 60ºC for 1 min. TaqMan PCR data were captured using the Sequence Detection Software (SDS version 2.2, Applied Biosystems). Subsequently, threshold cycle (CT) data for each sample were analyzed with the double-delta CT (ΔΔCT) method (Garcia-Esparcia *et al*., 2015). First, delta CT

(ΔCT) values were calculated as the normalized CT values for each target gene in relation to the mean values of GUS-β, XPNPEP1, AARS and HPRT. The selection of these housekeeping genes was based on previous data showing low vulnerability in the brain of several human neurodegenerative diseases (Barrachina *et al*., 2006; Durrenberger *et al*., 2012). Second, ΔΔCT values were obtained with the ΔCT of each sample minus the mean ΔCT of the population of control samples (calibrator samples). The fold-change was determined using the equation 2-ΔΔCT.

2.5. Statistical analysis for RT-qPCR

The normality of distribution of the mean fold-change values obtained by RT-qPCR for every region and stage between controls and DLB cases was analyzed with the Kolmogorov-Smirnov test. The non-parametric Mann-Whitney test was performed to compare each group when the samples did not follow a normal distribution whereas the unpaired t test was used for normal variables. Statistical analysis was performed with GraphPad Prism version 5.01 (La Jolla, CA, USA) and Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (Warrenton, VA, USA). Differences between groups were considered statistically significant at p-values: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

2.6. Gel electrophoresis and western blotting from total homogenate

Tissue was processed as reported elsewhere (Garcia-Esparcia *et al*., 2015). 0.1g of tissue of samples from frontal cortex area 8 were lysed with a glass homogenizer in Mila lysis buffer (0.5M Tris at pH 7.4 containing 0.5 methylenediaminetetraacetic acid at pH 8.0, 5M NaCl, 0.5% Na deoxycholic acid, 0.5% Nonidet P-40, 1mM phenylmethylsulfonyl fluoride, bi-distilled water, and protease and phosphatase inhibitor cocktails (Roche Molecular Systems, Pleasanton, CA, USA), and then centrifuged for 15 min at 13,000rpm at 4°C (Ultracentrifuge Beckman with 70Ti rotor, CA, USA). Protein concentration was measured with SmartspectTMplus spectrophotometer (Bio-Rad, CA, USA) using the Bradford method (Merck, Darmstadt, Germany). Samples containing 15µg protein and the standard Precision Plus ProteinTM Dual Color (Bio-Rad) were loaded onto 10-15% acrylamide gels. Proteins were separated with sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes using the Trans-Blot®TurboTM transfer system (Bio-Rad) at 200mA/membrane for 40 min. Non-

specific bindings were blocked by incubation with 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween for 1 h at room temperature. After washing, the membranes were incubated at 4°C overnight with one of the primary antibodies (Supplementary table I) in TBS containing 3% albumin and 0.1% Tween. These membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibody (1:2,000, Dako, Glostrup, Denmark), and the immune-complexes were revealed with a chemiluminescence reagent (ECL, Amersham, GE Healthcare, Buckinghamshire, UK). Monoclonal anti-β-actin antibody diluted 1:30,000 (β-Actin, A5316; Sigma-Aldrich, St. Louis, MO, USA) was blotted to control protein loading. Densitometry of western blot bands was assessed with the TotalLab program (TotalLab Quant, Newcastle, UK), and then subsequently analysed with GraphPad Prism, Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (VA, USA) by one-way ANOVA with post hoc Tukey's range test for multiple comparisons. Differences were considered statistically significant at p-values: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

2.7. Mitochondrial enzymatic activities

The activities of mitochondrial complexes I, II, III, IV and V were analysed using commercial kits following the manufacturers' instructions (Mitochondrial complex I, II, IV and V: Novagen, Merck Biosciences, Darmstadt, Germany; Mitochondrial complex III: MyBiosource, CA, USA). 25µg of mitochondria was loaded into each well. Activity of citrate synthase was evaluated following validated protocols (Spinazzi *et al*., 2012) with slight modifications. The activity of citrate synthase was determined as the rate of reduction of DTNB (5', 5'-dithiobis (2-nitrobenzoic acid)) to thionitrobenzoic acid at 412nm. For this purpose, 25µg of mitochondria was added to a 1ml mixture containing 500µl of Tris (200mM, pH8.0) with Triton X-100 (0.2% (vol/vol)), 100µl of DTNB, and 30µl of 10mM Acetyl CoA, and then the final volume was adjusted to 950µl with distilled water. The reaction was started by the addition of 50µl of 10mM oxalacetic acid. The increase in absorbance at 412 nm was read for 3 min at room temperature with a DU® 800UV/Visible spectrophotometer (Beckman Coulter, CA, USA) in 1ml polystyrene or methacrylate cuvettes (Armand-Ugon *et al*., 2016).

2.8. Statistical analysis

The enzymatic activities for each mitochondrial complex were expressed as a rate of nmol/min per mg of mitochondrial protein per protein concentration

normalized with the mitochondrial complex activity rate of citrate synthase activity. Data were presented as mean ± standard error of the mean (SEM) for all the experiments. All the data were analysed with Student's t-test using GraphPad Prism version 5.01 (La Jolla, CA, USA) and Statgraphics Statistical Analysis and Data Visualization Software version 5.1 (Warrenton, VA, USA). In all experimental procedures the significance level was set at *p < 0.05; **p < 0.01; ***p < 0.001.

2.9. Concentration of β-amyloid 1-40 (Aβ40) and β-amyloid 1-42 (Aβ42)

Frozen brain samples were homogenized in Tris-buffered saline (TBS) buffer composed of 140 mM NaCl, 3 mM KCl, 25 mM Tris-HCl pH 7.4 and 5 mM ethylene-diamine-tetra-acetic acid (EDTA) with a cocktail of protease inhibitors (Roche Molecular Systems, Pleasanton, CA, USA), and ultra-centrifuged at 100,000xg for 1 h at 4°C. The supernatant was the soluble fraction used for amyloid quantification, and the protein of this fraction was measured with BCA. The detection and measurement of β-amyloid 1-40 (Aβ40) and β-amyloid 1-42 (Aβ42) were carried out by enzyme-linked immune-absorbent assay (ELISA) with the corresponding detection kits (Invitrogen, Camarillo, CA, USA), following the instructions of the supplier. Aβ40 and Aβ42 levels were normalized to the total amount of protein from each individual sample (López-González *et al*., 2015[b]).

2.10. Quantification of membrane-associated β-amyloid

Frozen samples were homogenized in TBS with a cocktail of protease and phosphatase inhibitors (Roche Molecular Systems). Homogenates were centrifuged at 14,000xg for 30 min at 4ºC. The pellet was re-suspended in 2% SDS and centrifuged at 14,000xg for 30 min at 4ºC. The supernatant was membrane-associated Aβ and the protein of this fraction was measured with BCA method (Thermo Scientific, US). Proteins were separated in sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). 35µg of protein was loaded onto a precast NuPAGE 4-12 % Bis-Tris gel system (Invitrogen, MA, USA) with MES buffer (Invitrogen, MA, USA). The proteins were transferred to nitrocellulose membranes, 200 mA/membrane, for 90 min. Then membranes were boiled with PBS for 6 min, and non-specific bindings were blocked by incubation in 5% non-fat dry milk in TBS containing 0.2% Tween for 1 h at room temperature. After washing, the membranes were incubated at 4ºC overnight with the primary antibody to human amyloid-beta protein, clone: 4G8 (1:500, Signet, MA, USA) in TBS containing 5% albumin and 0.2% Tween. Membranes were

then incubated for 1h with the appropriate HRP conjugated secondary antibody (1:2,000, Dako, DK), and the immune-complexes were visualized with a chemiluminescence reagent (ECL, Amershan, GB). β-actin was used as a control of protein loading (López-González *et al*., 2015[b]).

2.11. Oligomeric tau species

Frozen samples were homogenized in lysis buffer: 100mM Tris (pH 7.0), 100mM NaCl, 10mM ethylene-diamine-tetra-acetic acid, 0.5% NP-40, and 0.5% sodium deoxycholate plus protease and phosphatase inhibitors (Roche Molecular Systems). After centrifugation at 14,000 g for 20 min at 4ºC (Ultracentrifuge Beckman with 70Ti rotor), supernatants were quantified for protein concentration (BCA), mixed with SDS-PAGE sample buffer, boiled and separated to 8% SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes, 200mA/membrane, for 90min. The membranes were blocked with 5% non-fat milk in TBS containing 0.2% Tween for 1 h at room temperature. After washing, the membranes were incubated at 4º C overnight with the primary antibody anti-tau-5 (1:1,000, Thermo-Fisher, MA, USA) in TBS containing 5% albumin and 0.2% Tween. Membranes were then incubated for 1h with the HRP-conjugated secondary anti-mouse antibody (1:2,000, Dako, DK), and the immune complexes were visualized with ECL. β-actin was used as a control of protein loading (López-González *et al*., 2015[a]).

2.12. α-synuclein oligomeric species in total homogenate fractions

Frozen samples were homogenized in a glass homogenizer, in 750µl of ice-cold PBS+ (sodium phosphate buffer pH 7.0, plus protease inhibitors), sonicated, and centrifuged at 2,700g at 4°C for 10 min. The pellet was discarded and the resulting supernatant was ultra-centrifuged at 43,000 rpm at 4°C for 1 h. The supernatant (S2) was kept as the PBS-soluble fraction. The resulting pellet was re-suspended in a solution of PBS, pH 7.0, containing 0.5% sodium deoxycholate, 1% Triton and 0.1% SDS, and this was ultra-centrifuged at 43,000 rpm at 4°C for 1 h. The resulting supernatant (S3) was kept as the deoxycholate-soluble fraction. The corresponding pellet was re-suspended in a solution of 2% SDS in PBS and maintained at room temperature for 30 min. Afterwards, the samples were centrifuged at 133,000g at 25°C for 1 h and the resulting supernatant (S4) was the SDS-soluble fraction. Equal amounts of each fraction were mixed with reducing sample buffer and processed in parallel for 10% SDS-

PAGE electrophoresis and western blotting. Membranes were incubated with anti-α-synuclein oligomer-specific antibody (Agrisera, Vännäs, Sweden) at a dilution of 1:1,000. The protein bands were visualized with the ECL method (Garcia-Esparcia *et al*., 2015).

3. Results

3.1. General neuropathological findings

Brain weight and neuropathological characteristics in the frontal cortex including neuron loss, astrocytic gliosis, microgliosis, spongiosis, diffuse plaques, senile plaques, β-amyloid angiopathy and α-synuclein aggregates (Lewy bodies and Lewy neurites) were assessed in DLB and rpDLB cases. Thal phase of β–amyloid deposition, Braak stages of neurofibrillary tangle pathology, CERAD global, ABC classification and LBD stage were also considered to frame AD- and LBD-related pathology in every case. Results are summarized in Table III. A remarkable observation was the discrete microglial response, as revealed with Iba1 and CD68 antibodies, in the frontal cortex in DLB and rpDLB. No differences were observed in the majority of neuropathological parameters although LBD limbic stage was found in three of the four cases with rpDLB in contrast to two of nine cases with classical DLB course. The number of cortical Lewy bodies and neurites was higher in DLB than in rpDLB in consonance with LBD staging. Importantly, no differences in the amount of fibrillar β-amyloid, considering diffuse and senile plaques, were observed between the two groups. The average percentage of Aβ40 of the total β-amyloid in plaques was 7.24% in DLB and 6.03% in rpDLB, and the percentage of Aβ42 was 68.49% in DLB and 67.70% in rpDLB.

3.2. mRNA expression levels of selected mitochondrial subunits and energy metabolism-related molecules in MA, DLB and rpDLB

The expression levels of three of twenty-seven genes analyzed were diminished in DLB when compared with MA cases including ATP5G2 and ATP5H involved in mitochondrial complex V, and ATP6VOB in energy metabolism ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively). However, the expression levels of ATP4A and SLC6A6, involved in energy metabolism, were increased in DLB ($p < 0.01$) (Table IV). Regarding rpDLB, the expression of NDUFS8 in mitochondrial complex I, UQCR11 in complex III and SLC6A6 in energy metabolism was increased in

rpDLB when compared with MA cases ($p < 0.001$, $p < 0.05$ and $p < 0.05$, respectively). Only one gene, ATP6VOB, was down-regulated in rpDLB ($p < 0.01$) (Table IV). Since the trend of altered gene expression was in the same direction in DLB and rpDLB, no significant differences were observed when comparing DLB and rpDLB (Table IV).

3.3. Protein expression of mitochondrial subunits of the respiratory chain in MA, DLB and rpDLB

Western blots showed marked alterations in DLB when compared with MA cases. The expression levels of complex I subunits NDUFA7, NDUFA10, NDUFB10 and NDUFB8 were significantly reduced (p ranging from < 0.05 to < 0.001) when compared with MA cases and using β-actin for protein normalization, expression of which was maintained in DLB and rpDLB when compared with MA cases. NDUFS8 protein expression was not modified in DLB when compared with DLB. Protein levels of SDHB (complex II), UQCRC2 (complex III), MTCO1 (complex IV) and ATP5A and ATP50 (complex V) were also significantly decreased in DLB when compared with MA ($p < 0.05$) using β-actin for protein loading normalization (Fig. 1). In contrast, only NDUFA7 was significantly reduced in rpDLB when compared with MA ($p < 0.01$). However, the expression of several subunits showed a trend toward decrease in rpDLB; therefore, significant differences between DLB and rpDLB were restricted to NDUFB8, SDHB, ATP5A and ATP50 ($p < 0.01$) (Fig. 1).

VDAC expression was reduced in DLB and less markedly in rpDLB when compared with controls, thus suggesting a reduced number of mitochondria or reduced mitochondria size. Nevertheless, NDUFA10, NDUFB10, NDUFB8, UQCRC2, MTCO1, and AT5A protein expression was also reduced in DLB cases when using VDAC for normalization (p ranging from < 0.05 to < 0.001). NDUFA17, NDUFS8, and ATP50 levels were similar in DLB and MA cases using VDAC for normalization. Curiously, expression levels of all the assessed subunits were preserved in rpDLB when normalized with VDAC. As a result, levels of NDUFA10, NDUFB10, NDUFB8, SDHB, UQCRC2, ATP5A, and ATP50 were significantly higher in rpDLB when compared with DLB (p values ranging from < 0.05 to < 0.001) (Fig. 1).

3.4. Activity of mitochondrial complexes I, II, III, IV and V in frontal cortex area 8 in DLB and rpDLB cases

Significant decrease in the activity of complexes I, II, III and IV was detected in frontal cortex area 8 in DLB and rpDLB when compared with MA cases and normalized with the ratio of citrate synthase activity ($p < 0.05$) (Fig. 2). No differences were observed when comparing DLB and rpDLB cases. The activity of complex V showed a trend toward decrease in DLB and more clearly in rpDLB but without statistical significance (Fig. 2).

3.5. mRNA expression of genes involved in purine metabolism in MA, DLB and rpDLB

The expression of the majority of assessed genes involved in purine metabolism was altered in the same direction in DLB and rpDLB when compared with MA cases. Changes were greater in rpDLB than in DLB. NME1, NME6 and PNP were significantly increased in DLB when compared with MA ($p < 0.05$), whereas ENTPD2 was significantly reduced ($p < 0.001$). However, ADA, AK1, AK2, AK4, ENTPD1, NME1, NME3, NME4 (all of them with $p < 0.001$), NME6 and PRUNE ($p < 0.01$), and PNP ($p < 0.05$) were up-regulated in rpDLB when compared with MA (Table IV). Finally, only two genes, DGUOK and NME3, were significantly up-regulated in rpDLB when compared with DLB ($p < 0.05$ and $p < 0.01$, respectively (Table V).

3.6. mRNA expression levels of genes encoding nucleolar proteins and ribosomal proteins, and rRNAs18S and 28S in MA, DLB and rpDLB

The expression of NPM1, NCL and UBTF was significantly decreased in DLB when compared with MA ($p < 0.05$) (Table VI). Seven of seventeen genes encoding ribosomal proteins were up-regulated in DLB including RPL7, RPL21, RPL23A, RPL30, RPL31, RPS3A and RPS5 (p values varied from < 0.05 to < 0.001). Two genes were down-regulated, RPL22 and RPS17 ($p < 0.05$), in DLB (Table V). RPL7, RPL21, RPL23A, RPL30, RPL31, RPS5 and RPS6 were up-regulated in rpDLB (p values varied from < 0.01 to < 0.001), whereas RPL26 and RPS16 were down-regulated ($p < 0.05$) (Table VI). rRNA 18S expression was increased in rpDLB ($p < 0.001$) but levels of rRNA 28S were preserved (Table VI). Due to similar trends of gene expression in DLB and rpDLB, only rRNA 18S,

RPS6 and RPS13 were significantly up-regulated ($p < 0.05$) in rpDLB when compared with DLB (Table VI).

3.7. Expression of proteins involved in ribosomal transcription in MA, DLB and rpDLB

Only eIF5 was significantly reduced in DLB when compared with MA cases ($p < 0.05$). However, the expression levels of initiation factors eIF2α and eIF5 were significantly decreased in rpDLB when compared with MA cases ($p < 0.01$ and $p < 0.05$, respectively). eIF2α, p-eIF2α and eIF3η were significantly decreased ($p < 0.05$) in rpDLB when compared with DLB (Fig. 3). In contrast, no alterations in the expression levels of elongation factors eEF1A and eEF2 were observed in DLB and rpDLB when compared with MA cases (Fig. 3).

3.8. mRNA expression levels of cytokines and mediators of the innate inflammatory response in MA, DLB and rpDLB

No differences in the expression of twenty-three genes encoding cytokines and mediators of the inflammatory response were detected in DLB when compared with rpDLB. However, a significant increase in the levels of TNF-α and CST7 was found in rpDLB cases when compared with MA ($p < 0.01$) (Table VII). IL6ST, TNF-α and CSF3R were significantly up-regulated in rpDLB when compared with DLB ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively) (Table VII).

3.9. Microglia and TNFα in MA, DLB and rpDLB

Western blotting revealed a significant increase in GFAP protein levels in DLB and rpDLB when compared with MA cases ($p < 0.01$), whereas the levels of Iba-1 were maintained when compared with MA cases (Fig. 4). TNF-α protein levels were also significantly increased in rpDLB when compared with MA ($p < 0.05$) (Fig. 4).

3.10. mRNA expression levels of olfactory and taste receptors in MA, DLB and rpDLB

Increased expression of OR2D2, OR4F4, OR11H1 and OR52H1 was observed in DLB when compared with MA cases ($p < 0.05$ and $p < 0.01$ depending on the gene). Up-regulation of ORs was higher in rpDLB when compared with MA, as the expression of OR2D2, OR2T33, OR11H1, OR52H1 and OR52M1 was significantly higher in rpDLB compared with MA (p ranging from < 0.05 to <

0.001). However, significant differences between DLB and rpDLB were restricted to OR2T33 ($p < 0.001$) (Table VIII). On the other hand, up-regulation of TAS2R4, TAS2R5, TAS2R10, TAS2R13 and TAS2R14 mRNA was observed in DLB and rpDLB when compared with MA cases; values were higher in rpDLB than in DLB when compared with MA (mainly $p < 0.05$ and $p < 0.01$ in DLB, and $p < 0.01$ and $p < 0.001$ in rpDLB). TAS2R50 was also significantly over-expressed in rpDLB when compared with MA ($p < 0.01$). However, no significant differences between DLB and rpDLB were seen (Table VIII).

3.11. Soluble Aβ40 and Aβ42, membrane-associated β-amyloid, tau oligomeric species and α-synuclein oligomeric species in total homogenate fractions

In spite of there being no apparent differences in β-amyloid plaque burden in DLB and rpDLB, significant increases in soluble Aβ40 and Aβ42 levels were seen in rpDLB when compared with DLB ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 5A). Curiously, levels in DLB were similar to those in controls. However, no differences between DLB and rpDLB were seen regarding β-amyloid associated with membranes (Fig. 5B) which in AD has a close correlation with fibrillar β-amyloid in amyloid plaques (Thal *et al*., 2015). Regarding tau pathology, western blots of total homogenates disclosed no tau oligomers in frontal cortex in DLB and rpDLB (Fig. 5C) thus being in accordance with the small quantity of NFTs in the frontal cortex in the present series. Finally, α-synuclein oligomers were observed in DLB and rpDLB in contrast with the lack of α-synuclein oligomers in MA cases. Importantly, the band pattern and the density of the bands of oligomers were similar in DLB and rpDLB (Fig. 5D).

4. Discussion

The present study was undertaken to identify alterations of several metabolic pathways which may participate in the pathogenesis of DLB including mitochondrial function and energy metabolism, purine metabolism, protein synthesis machinery, inflammation and certain recently discovered new ectopic olfactory and taste receptors expressed in brain. The focus of the study was to learn about biochemical alterations beyond the well-known modifications of target proteins in DLB such as α-synuclein and β-amyloid. Present biochemical studies have shown similar percentages of Aβ40 and Aβ42 in plaques in DLB and rpDLB

abnormal solubility and aggregation of α-synuclein and increased β-amyloid bound to membranes in the frontal cortex in DLB and rpDLB. In contrast, no differences in tau oligomers were found between control and DLB cases. Whether levels of soluble Aβ40 and Aβ42 are within control values in DLB in spite of the presence of plaques and in contrast with the expected increase at similar plaque stages in AD (López-González *et al*., 2015) needs further study.

4.1. Mitochondria and energy metabolism

Mitochondrial alterations in the frontal cortex are prominent in DLB. ATP5G2 and ATP5H expression is decreased in DLB when compared with MA individuals; protein expression of NDUFA7, NDUFA10, NDUFB8, SDHB, UQCRC2, MTCO1 ATP5A and ATP50 is reduced; rpDLB shows distinct profiles as NDUFS8 and UQCRB mRNAS are up-regulated in rpDLB. NDUFA7 protein levels are significantly reduced in rpDLB and significant differences between DLB and rpDLB are restricted to NDUFB8, SDHB, ATP5A and ATP50. It is worth stressing that these alterations were not the mere consequence of mitochondrial loss in DLB. Expression levels of VDAC are decreased in DLB but the expression levels of the mentioned subunits are reduced even considering VDAC for normalization of protein levels. It can be argued that the rapid course of the disease results in a reduced harmful impact on mitochondria in rpDLB when compared with DLB. Despite differences in gene and protein expression, mitochondrial enzymatic activity of complexes I, II, III and IV is significantly decreased in frontal cortex area 8 in DLB and rpDLB. Therefore, the present observations point to altered mitochondrial function in frontal cortex as a major factor in the pathogenesis of DLB and rpDLB. Regarding energy metabolism, only ATP4A and SLC6A6 are up-regulated in DLB, while ATP6V0B is down-regulated in DLB and rpDLB. ATP4A encodes a membrane-bound P-type ATPase, which permits ion transport through cell membranes (Singh *et al*., 2013). SLC6A6 encodes a taurine transporter (SLc6a6/TauT) involved in the uptake of gamma-aminobutyric acid (GABA) (Tomi *et al*., 2008). Whether these changes have implications in GABA metabolism in DLB is not known although GABA levels in the CSF in DLB are not altered when compared with normal individuals (Molina *et al*., 2005). ATP6V0B encodes ATPase H+ transporting Vo subunit b, which is involved in protein sorting, zymogen activation, receptor-mediated endocytosis and synaptic vesicle proton gradient generation (Lu *et al*., 2007). Together, these results suggest energy metabolism impairment in frontal cortex in DLB and rpDLB.

4.2. Purine metabolism

Several genes encoding enzymes linked to purine metabolism are up-regulated in DLB and rpDB although significant values are only obtained when comparing rpDLB with MA cases. These include ADA, AK1, AK2, AK4, ENTPD1, NME1, NME4, NME6, NT5E and PRUNE. However, only two genes, DGUOK and NME1, are significantly up-regulated in rpDLB when compared with DLB. ENTPD2 is down-regulated in DLB but not in rpDLB. Purines and pyrimidines are the core of DNA, RNA, nucleosides and nucleotides. Nucleotides are involved in cell signalling and energy metabolism, and purine bases are also cofactors of several enzymatic reactions (Ribeiro *et al*., 2003; Boison, 2008; Burnstock *et al*., 2011; Ipata *et al*., 2011). Adenylate kinases (AKs) participate in the phosphorylation of AMP to ADP and dAMP to dATP (Noma 2005; Dzeja and Terzic 2009). The NME gene family encodes nucleotide diphosphate kinases which are involved in the phosphorylation of nucleotide diphosphates to form nucleotide triphosphates. These enzymes are enriched in synapses. Increased NME mRNA expression in DLB contrasts with its decrease in AD (Kim *et al*., 2002; Ansoleaga *et al*., 2015) and suggests a compensatory role of NME in response to reduced synapses in DLB. The product of PRUNE participates in the metabolism of guanosine pentaphosphate and tetraphosphate and is linked to NME in memory conservation (Li *et al*., 2012). Deoxyguanosine kinase (encoded by DGUOK) phosphorylates purine deoxyribonucleosides in the mitochondrial matrix (Jüllig and Eriksson 2000; Johansson *et al*., 2001; Desvignes *et al*., 2009). Adenine phosphoribosyltransferase is related to adenine metabolism and catalyses the phosphorylation reaction, whereas ADA de-aminates adenosine. Further, ecto-nucleoside triphosphate diphosphohydrolases, encoded by ENTPD genes, hydrolyze the terminal phosphate group of nucleoside tri- and di-phosphates to form di-and mono-phosphates, thus controlling extracellular ATP concentrations (Yegutkin, 2008), adenosine-activated type I receptors, nucleotide-activated type 2 ligand-gated ion channels, and metabotropic P2Y receptors (Al-Rashida *et al*., 2013). Finally, ecto 5'-nucleotidase (NT5E) catalyzes the generation of adenosine from degradation of AMP in the extracellular space. In brain, ectonucleotidases are involved in variegated functions including modulation of synaptic transmission, ATP-mediated propagation of calcium waves in glial cells, neurogenesis, microglial function and blood flow (Zimmermann, 2006). Present

findings show marked alterations in the expression of enzymes involved in purine metabolism in the frontal cortex in DLB and rpDLB.

4.3. Protein synthesis

Decreased expression of nuclear chaperones as nucleolin (NCL), nucleoplasmin 3 (NPM3) and upstream binding transcription factor (UBTF) is found in the frontal cortex in DLB. Altered expression of NCL, NPM3 and UBTF is indicative of nucleolar stress which may alter ribosomal biogenesis (Olson *et al*., 2004; Boulon *et al*., 2010; Baltanas *et al*., 2011; Hetman and Pietrak, 2012; Parlatto and Kreiner, 2013). Alterations in the expression of genes encoding ribosomal proteins are seen in DLB and rpDLB, including up- and down-regulation of RPLs, thus suggesting impaired ribosome biogenesis (Granneman and Baserga, 2004; Klein *et al*., 2004; Kressleer *et al*., 2010; Korobeinikova *et al*., 2012). This is accompanied by reduced protein expression of several initiation factors of transcription at the ribosome which are more marked in rpDLB than in DLB. However, the expression levels of elongation factors eEF1A and eEF2 are preserved in DLB and rpDLB. Although the direct study of protein synthesis is not possible in human *post-mortem* samples due to *post-mortem* delay between death and tissue processing, the present findings indicate that the machinery of protein synthesis is altered in DLB (Kapp and Lorsch, 2004; Jackson *et al*., 2010; Voorhees and Ramakrishnan, 2013). Importantly, altered expression of proteins involved in transcription at the ribosome is more severe in rpDLB than in DLB.

4.4. Inflammatory responses

No significant differences in gene expression of several cytokines and mediators of the inflammatory response are seen in DLB. These data are in line with the observation of no major increase in Iba1 protein levels and CD68 expression in frontal cortex in DLB when compared with MA individuals. They are also in agreement with previous observations showing very limited activation of microglia in DLB (Shepherd *et al*., 2000; Streit and Xue, 2015). Subtle change refers to *TNF-*α gene expression in rpDLB. Whereas *TNF-*α mRNA is not altered in DLB, significant *TNF-*α mRNA up-regulation and increased TNF-α protein levels occur in the frontal cortex in rpDLB. TNF-α is involved in several metabolic pathways, particularly facilitating gene transcription, activating of the JNK pathway and promoting apoptosis via caspase-dependent and caspase-independent signaling (Thomson and Lotze, 2003). Therefore, TNF-α up-regulation in rpDLB may have

functional implications. Further molecular studies are needed to elucidate activated molecules of the TNF-α pathways in rpDLB.

4. 5. Olfactory and taste brain receptors

Olfactory and taste receptors (ORs and TASRs, respectively) are widely expressed in human and rodent brain including the cerebral cortex. ORs and TASRs in brain are accompanied by all downstream molecules that permit a functional signaling pathway, and they are functional, as revealed in culture neurons under appropriate stimuli (Garcia-Esparcia *et al*., 2013; Grison *et al*., 2014). The expression of several genes encoding olfactory receptors is increased in DLB and rpDLB, and is more marked in the rapid form of the disease in which six of thirteen assessed genes are up-regulated. A similar up-regulation occurred for TASRs; five of six genes in DLB and all six genes are up-regulated. The function of ORs and TASRs in brain is not known although it has been postulated that they may participate in intra- and extra-cellular signaling in association, or not, with other receptors. Identification of natural ligands of brain Ors and TASRs should yield insights about the function of these ectopic receptors (Garcia-Esparcia *et al*., 2013).

4.6. Mitochondrial function, inflammation and deregulation of olfactory and taste receptors in frontal cortex discriminate DLB and AD

Several studies have shown common pathogenic pathways in AD and DLB (Guerreiro *et al*., 2016; Sanchez-Mut *et al*., 2016). Commonly affected pathways include mitochondrial function and energy metabolism, oxidative stress damage, protein synthesis, altered responses to aggregated proteins, impaired protein degradation by the ubiquitin-protesaome system and autophagy, and inflammation, among others. However, the present study reveals disease-specific alterations when comparing the present results in DLB with available data for AD in the same region, the frontal cortex, at similar stages of disease progression. Mitochondrial alterations and impaired activity of complex V are early events in the entorhinal cortex in AD (Terni *et al*., 2010). NDUFA2, NDUFB3, UQCR11, COX7C, ATPD, ATP5L and ATP50 gene expression is reduced in the entorhinal cortex with disease progression and this is accompanied by impaired activity of complexes I, II and V (Armand-Ugon *et al*., 2016). In contrast, RNA expression levels of several subunits of complexes I-V and enzymatic activities of complex I, II, IV and V are preserved in frontal cortex in AD even at stages V-VI of Braak

(Armand-Ugon *et al*., 2016). Therefore, mitochondrial dysfunction markedly differs in AD and DLB, with detrimental effects in most mitochondrial complexes in DLB in comparison with AD. There is a large body of information demonstrating microglial responses and increased expression of inflammatory markers in the cerebral cortex in AD (Griffin *et al*., 1998; McGeer and McGeer, 2001; Streit *et al*., 2001; Akiyama *et al*., 2010; Graeber and Streit, 2010; Lee *et al*., 2010; Eikelenboom *et al*., 2010; Rubio-Perez and Morillas-Ruiz, 2011; Leung *et al*., 2011; Morimoto *et al*., 2011; McGeer and McGeer, 2013; Prokop *et al*., 2013; Heppner *et al*., 2015; Heneka *et al*., 2015; López-González *et al*., 2015; López-González *et al*., 2016). In contrast, inflammatory responses are very limited in DLB. Therefore, inflammation is characteristically an important factor in AD pathogenesis whereas it has low impact in DLB. Regarding purine metabolism, ENTPD2, NME3, PNP and PRUNE RNAs are deregulated in the frontal cortex in AD cases at the same stages of AD-related pathology as those found in the present DLB series (Ansoleaga *et al*., 2015). Severe alterations of the machinery involved in protein synthesis from the nucleolus to the ribosome have been observed in the entorhinal cortex in AD (Hernández-Ortega *et al*., 2016), but no similar data are available in AD frontal cortex at β-amyloid phases and AD Braak stages of NFT pathology corresponding to those assessed in DLB. Finally, OR4F4 mRNA expression levels are increased in frontal cortex area 8 at stages III-IV, and OR52L1 mRNA at stages III-IV and V-VI (Ansoleaga *et al*., 2013). Regarding TASRs, no modification in the mRNA expression levels is observed in frontal cortex area 8 at any stage of AD (Ansoleaga *et al*., 2013). These changes are in contrast with the extensive deregulation of Ors and TASRs in the frontal cortex in DLB, thus indicating marked differences in the regulation of these already poorly understood brain receptors in DLB.

4.7. Specific traits in rpDLB compared with DLB

No neuropathological differences are seen in DLB and rpDLB, as have been reported in other studies (Gaig *et al*., 2011). rpDLB has similar biochemical profiles regarding mitochondrial gene and protein expression, purine metabolism, protein synthesis, and expression of olfactory and taste receptors. Gene and protein expression of subunits of mitochondrial respiratory complexes are more pronounced in DLB when compared with rpDLB whereas alterations in purine metabolism and initiation of protein translation are more pronounced in rpDLB when compared with DLB. However, two traits are substantially different in rpDLB

when compared with DLB. The first of these is the higher levels of soluble Aβ40 and Aβ42 in rpDLB in spite of similar plaque burden and β-amyloid associated with membranes in rpDLB and DLB. In this line, modified characteristics of Aβ42 have been described in rapidly progressive AD (Cohen *et al*., 2015), thus suggesting that β-amyloid conformers pace disease progression in AD. Higher levels of soluble β-amyloid oligomeric species, known to be toxic to nerve cells (Lambert *et al*., 1998; Selkoe, 2002; Klein, 2002; Gandy *et al*., 2010; Hayden *et al*., 2013; Viola and Klein, 2015), can precipitate disease progression in rpDLB. Therefore, procedures geared to reducing soluble β-amyloid species (Klein *et al*., 2001; Hefti *et al*., 2013) seem to be appropriate methods to slow down disease progression in DLB cases with rapid course. The second differential aspect is the higher levels of TNF-α in rpDLB when compared with DLB. Whether this factor influences disease progression needs clinical confirmation. Following this rationale, the use of specific TNF-α inhibitors (Murdaca *et al*., 2009; Ozer and Ozbalkan, 2010) can be considered a putative instrument to mitigate TNF-α-associated harmful effects in rpDLB that were once considered to be pre-treatment conditions and possible side-effects.

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6. Acknowledgements

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Tables and Figures

Table I. Summary of cases used in the present study. NL: no lesions; these cases were considered as middle-aged individuals and served as controls; the mean age +/-SD was 59.92 ± 15.60. DLB cases were older with no differences between DLB (76.44 ± 5.77) and rpDLB (73.75 ± 2.22). DLB. The term DLB is used to name clinically- and neuropathologically-verified Dementia with Lewy Bodies, whereas rpDLB indicates DLB with rapid course. PM delay: *post-mortem* delay; RNA integrity number (RIN); PCR: implies the use of these samples for gene expression studies; WB: cases analyzed with western blotting; ELISA: Enzyme-Linked ImmunoSorbent Assay; MA: Mitochondrial enzyme activities; MI: Mitochondrial isolation**.**

N°:	Diagnosis:	Gender:	Age:	PM delay:	RIN:	PCR	WB	ELISA	MA	MI
1	SLS	MALE	64	8 h 30	7,7	X	X	X		
2	SLS	MALE	56	5h	7,1	X	X	X	X	X
3	SLS	MALE	67	5h	7	X	X	X	X	
4	SLS	MALE	62	3h	7,2	X	X	X	X	X
5	SLS	MALE	52	4h 40m	7,9	X	X	X		X
6	SLS	MALE	30	4h 10m	8,4	X	X	X	X	
7	SLS	MALE	53	3h	7,7	X	X	X		X
8	SLS	FEMALE	49	7h	8,2	X	X	X		
9	SLS	FEMALE	75	3h	6,5	X	X	X		
10	SLS	FEMALE	46	9h 35m	7,2	X	X	X	X	
11	SLS	FEMALE	86	4h 15m	8,4	X	X	X		
12	SLS	FEMALE	79	3h 35m	8	X	X	X		
13	DLB	MALE	81	7h	5,3	X	X	X		
14	DLB	FEMALE	78	5h	5,7	X	X	X		X
15	DLB	MALE	76	5h 10m	5,2	X	X	X		X
16	DLB	MALE	83	9h	5,2	X	X	X	X	X
17	DLB	MALE	78	8h 30m	6,3	X	X	X		
18	DLB	MALE	64	8h 15m	7	X	X	X	X	
19	DLB	MALE	80	8h	6,1	X	X	X		X
20	DLB	MALE	77	7h 20m	6,4	X	X	X		
21	DLB	MALE	71	9h	7	X	X	X	X	
22	rpDLB	FEMALE	75	13h 30m	5,6	X	X	X	X	X
23	rpDLB	MALE	76	6h 30m	5,5	X	X	X	X	X
24	rpDLB	MALE	71	5h	5	X	X	X	X	X
25	rpDLB	FEMALE	73	15h 30m	6,1	X	X	X		X

Table II. Abbreviations, full names and TaqMan probes used to assess gene expression in the frontal cortex of middle-aged and DLB cases in the present study.

Gene	Full Name	Reference
Housekeeping genes		
GUS-B	β-glucuronidase	Hs00939627_m1
XPNPEP1	X-prolylaminopeptidase (aminopeptidase P) 1	Hs00958026_m1
AARS	alanyl-tRNA synthetase	Hs00609836_m1
HPRT	hypoxanthine phosphoribosyltransferase 1	Hs_02800695_m1
Genes encoding proteins of mitochondria and energy metabolism-related molecules		
NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa	Hs00159575_m1
NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7, 14.5kDa	Hs01561430_m1
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	Hs01071117_m1
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa	Hs00427185_m1
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	Hs00188142_m1
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	Hs00605903_m1
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa	Hs00257018_m1
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa	Hs00159597_m1
SDHB	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	Hs00268117_m1
UQCRB	ubiquinol-cytochrome c reductase binding protein	Hs00559884_m1
UQCR11	ubiquinol-cytochrome c reductase, complex III subunit XI	Hs00907747_m1
COX7A2L	cytochrome c oxidase subunit VIIa polypeptide 2 like	Hs00190880_m1
COX7C	cytochrome c oxidase subunit VIIc	Hs01595220_g1
ATP5D	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	Hs00961521_m1
ATP5G2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2	Hs01096582_m1
ATP5H	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d	Hs01046892_gH
ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G	Hs00538946_g1
ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	Hs00426889_m1
ATP2B3	ATPase, Ca++ transporting, plasma membrane 3	Hs00222625_m1
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	Hs00608066_m1

ATP4A	ATPase, H+/K+ exchanging, alpha polypeptide	Hs00167575_m1
ATP6V0A1	ATPase, H+ transporting, lysosomal V0 subunit a1	Hs00193110_m1
ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b	Hs01072388_m1
ATP6V1H	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H	Hs00977530_m1
FAM82A2	family with sequence similarity 82, member A2	Hs00216746_m1
LHPP	phospholysine phosphohistidine inorganic pyrophosphate phosphatase	Hs00383379_m1
SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	Hs00161778_m1
SLC25A31	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31	Hs00229864_m1
TOMM40	translocase of outer mitochondrial membrane 40 homolog (yeast)	Hs01587378_mH
ZNF642	zinc finger protein 642	Hs01372953_m1

Purine metabolism genes

ADA	Adenosine deaminase	Hs01110945_m1
AK1	Adenylate kinase 1	Hs00176119_m1
AK2	Adenylate kinase 2	Hs01123132_g1
AK4	Adenylate kinase 4	Hs03405743_g1
AK5	Adenylate kinase 5	Hs00952786_m1
AK7	Adenylate kinase 7	Hs00330574_m1
APRT	Adenine phosphoribosyltransferase	Hs00975725_m1
DGUOK	Deoxyguanosine kinase	Hs00176514_m1
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1	Hs00969559_m1
ENTPD2	Ectonucleoside triphosphate diphosphohydrolase 2	Hs00154301_m1
ENTPD3	Ectonucleoside triphosphate diphosphohydrolase 3	Hs00928977_m1
NME1	Non-metastatic cells 1, protein expressed in (nucleoside-diphosphate kinase)	Hs02621161_s1
NME3	Non-metastatic cells 3, protein expressed in (nucleoside-diphosphate kinase)	Hs01573874_g1
NME4	Non-metastatic cells 4, protein expressed in (nucleoside-diphosphate kinase)	Hs00359037_m1
NME5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	Hs00177499_m1
NME6	Non-metastatic cells 6, protein expressed in (nucleoside-diphosphate kinase)	Hs00195083_m1
NME7	Non-metastatic cells 7, protein expressed in (nucleoside-diphosphate kinase)	Hs00273690_m1
NT5C	5', 3'-nucleotidase, cytosolic	Hs00274359_m1
NT5E	5'-nucleotidase, ecto (CD73)	Hs00159686_m1

PNP	Purine nucleoside phosphorylase	Hs01002926_m1
POLR3B	Polymerase (RNA) III (DNA directed) polypeptide B	Hs00932002_m1
PRUNE	Prune homolog (Drosophila)	Hs00535700_m1
Nucleolar, rRNAs and genes encoding ribosomal protein		
NCL	nucleolin	Hs01066668_m1
NPM1	nucleophosmin (nucleolar phospho-protein B23, numatrin)	Hs02339479_m1
NPM3	nucleophosmin / nucleoplasmin 3	Hs00199625_m1
rRNA 28S	RNA, 28S ribosomal 5	Hs03654441_s1
rRNA 18S	eukaryotic 18S rRNA	Hs99999901_s1
UBTF	upstream binding transcription factor, RNA polymerase I	Hs01115792_g1
RPL5	ribosomal protein L5	Hs_03044958_g1
RPL7	ribosomal protein L7	Hs_02596927_g1
RPL21	ribosomal protein L21	Hs_00823333_s1
RPL22	ribosomal protein L22	Hs_01865331_s1
RPL23A	ribosomal protein L23A	Hs_01921329_g1
RPL26	ribosomal protein L26	Hs_00864008_m1
RPL27	ribosomal protein L27	Hs_03044961_g1
RPL30	ribosomal protein L30	Hs_00265497_m1
RPL31	ribosomal protein L31	Hs_01015491_g1
RPS3A	ribosomal protein S3A	Hs_00832893_sH
RPS5	ribosomal protein S5	Hs_00734849_g1
RPS6	ribosomal protein S6	Hs_04195024_g1
RPS10	ribosomal protein S10	Hs_01652370_gH
RPS13	ribosomal protein S13	Hs_01011487_g1
RPS16	ribosomal protein S16	Hs_01598516_g1
RPS17	ribosomal protein S17	Hs_00734303_g1
Cytoquines and mediators of the inflammatory responses		
C1QL1	Complement component 1, q subcomponent 1	Hs00198578_m1
C1QTNF7	C1q and tumor necrosis factorY related protein 7	Hs00230467_m1
C3AR1	Complement component 3a receptor 1	Hs00377780_m1

CLEC7A	C-type lectin domain family 7, member A	Hs01124746_m1
CSF1R	Colony-stimulating factor 1 receptor	Hs00911250_m1
CSF3R	Colony-stimulating factor 1 receptor	Hs00167918_m1
CST7	Cystatin F (leukocystatin)	Hs00175361_m1
CTSC	Cathepsin C	Hs00175188_m1
CTSS	Cathepsin S	Hs00356423_m1
IL1B	Interleukin-1B	Hs01555410_m1
IL6	Interleukin-6	Hs00985639_m1
IL6ST	Interleukin-6 signal transducer	Hs00174360_m1
IL8	Interleukin-8	Hs00174103_m1
IL10	Interleukin-10	Hs00961622_m1
IL10RA	Interleukin-10 receptor A	Hs00155485_m1
IL10RB	Interleukin-10 receptor B	Hs00609836_m1
TGFA1	Transforming growth factor-A1	Hs00998133_m1
TGFA2	Transforming growth factor-A2	Hs00234244_m1
TLR4	Toll-like receptor 4	Hs01060206_m1
TLR7	Toll-like receptor 7	Hs00152971_m1
TNF	Tumor necrosis factor	Hs01113624_m1
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1a	Hs01042313_m1
Olfactory and taste receptors genes		
OR2D2	olfactory receptor, family 2, subfamily D, member 2	Hs00999189_s1
OR2J3	olfactory receptor, family 2, subfamily J, member 3	Hs01943871_g1
OR2L13	olfactory receptor, family 2, subfamily L, member 13	Hs00380097_m1
OR2T1	olfactory receptor, family 2, subfamily T, member 1	Hs01661970_s1
OR2T33	olfactory receptor, family 2, subfamily T, member 33	Hs04230793_gH
OR4F4	olfactory receptor, family 4, subfamily F, member 4	Hs03406040_gH
OR6F1	olfactory receptor, family 6, subfamily F, member 1	Hs01054972_s1
OR10G8	olfactory receptor, family 10, subfamily G, member 8	Hs01943074_g1
OR11H1	olfactory receptor, family 11, subfamily H, member 1	Hs03406084_gH

OR51E 1	olfactory receptor, family 51, subfamily E, member 1	Hs02339849 _s1
OR52H 1	olfactory receptor, family 52, subfamily H, member 1	Hs01661724 _s1
OR52L1	olfactory receptor, family 52, subfamily L, member 1	Hs02339119 _g1
OR52M 1	olfactory receptor, family 52, subfamily M, member 1	Hs01098608 _s1
TAS2R 4	taste receptor, type 2, member 4	Hs00249946 _s1
TAS2R 5	taste receptor, type 2, member 5	Hs01549633 _s1
TAS2R 10	taste receptor, type 2, member 10	Hs00256794 _s1
TAS2R 13	taste receptor, type 2, member 13	Hs01059805 _s1
TAS2R 14	taste receptor, type 2, member 14	Hs00256800 _s1
TAS2R 50	taste receptor, type 2, member 50	Hs00604351 _s1

Table III. Summary of neuropathological findings in the present series. MA cases summarized in Table I do not have brain lesions and are not considered in the present Table.

Case	Brain weight	rDLB	Nloss	Astrocytosis	Microglia	Spongiosis	Diffuse plaques	Senile plaques	LB	Lewy bodies	Thal phase	NFT Braak stage	CERAD	ABC	LBD stage
13	1295		moderate	moderate	moderate	mild	frequent	moderate	isolated	frequent	4	III	moderate	A3B2C2	neocortical
14	795		severe	severe	severe	mild	frequent	frequent	frequent	moderate	5	VI	frequent	A3B3C3	neocortical
15	1230		mild	mild	mild	mild	frequent	moderate	sparse	moderate	5	V incipient	frequent	A3B3C3	neocortical
16	1300		mild	moderate	moderate	mild	frequent	moderate	moderate	frequent	5	IV	moderate	A3B2C2	neocortical
17	1220		mild	mild	mild	mild	severe	moderate	sparse	frequent	4	IV	moderate	A3B2C2	neocortical
18	1370		mild	mild	mild	mild	absent	absent	absent	sparse	0	I	0	A0B1C0	limbic
19	1280		mild	moderate	mild	mild	moderate	moderate	isolated	moderate	3	II	moderate	A2B1C2	neocortical
20	1365		mild	mild	mild	mild	absent	absent	absent	sparse	0	III	0	A0B2C0	limbic
21	1300		moderate	moderate	moderate	mild	moderate	frequent	isolated	frequent	4	II	moderate	A3B1C2	neocortical
22	1120	Yes	moderate	moderate	moderate	mild	moderate	moderate	sparse	sparse	4	II	moderate	A3B1C2	limbic
23	1300	Yes	mild	mild	mild	mild	moderate	moderate	moderate	sparse	5	V	moderate	A3B3C2	limbic
24	1300	Yes	mild	moderate	moderate	mild	absent	absent	absent	sparse	0	0	0	-	limbic
25	1240	Yes	mild	mild	mild	mild	moderate	frequent	sparse	frequent	5	V incipient	frequent	A3B3C3	neocortical

Table IV. RNA expression of selected subunits of the mitochondrial respiratory chain and genes encoding proteins linked to energy metabolism in MA, DLB and rpDLB. Similar trends are found in DLB and rpDLB when compared with MA cases; *p < 0.05; **p < 0.01; ***p < 0.001.

Probes	MA	DLB	rpDLB	MA vs DLB	MA vs rpDLB	DLB vs rpDLB
Mitochondria						
Complex I ***NDUFA2***	1.02 ± 0.22	1.01 ± 0.09	0.94 ± 0.08	-	-	-
NDUFA7	1.27 ± 0.58	0.89 ± 0.25	1.00 ± 0.11	-	-	-
NDUFA10	1.03 ± 0.27	1.11 ± 0.26	1.19 ± 0.31	-	-	-
NDUFB3	1.02 ± 0.23	0.95 ± 0.24	0.99 ± 0.20	-	-	-
NDUFB7	1.02 ± 0.23	1.19 ± 0.53	1.25 ± 0.13	-	-	-
NDUFB10	1.05 ± 0.35	0.87 ± 0.30	1.19 ± 0.20	-	-	-
NDUFS7	1.09 ± 0.47	0.98 ± 0.26	0.71 ± 0.14	-	-	-
NDUFS8	1.03 ± 0.25	1.19 ± 0.66	1.64 ± 0.25	-	*** ↑	-
Complex II ***SDHB***	1.05 ± 0.38	0.84 ± 0.16	0.84 ± 0.16	-	-	-
Complex III ***UQCRB***	1.06 ± 0.40	1.09 ± 0.44	1.00 ± 0.24	-	-	-
UQCR11	1.02 ± 0.19	1.10 ± 0.26	1.28 ± 0.27	-	* ↑	-
Complex IV ***COX7A2L***	1.08 ± 0.41	0.93 ± 0.39	1.07 ± 0.38	-	-	-
COX7C	1.03 ± 0.24	0.89 ± 0.38	0.85 ± 0.24	-	-	-
Complex V ***ATP5D***	1.03 ± 0.24	0.98 ± 0.18	0.93 ± 0.13	-	-	-
ATP5G2	1.07 ± 0.42	0.61 ± 0.23	0.69 ± 0.02	** ↓	-	-
ATP5H	1.02 ± 0.21	0.80 ± 0.18	0.82 ± 0.05	* ↓	-	-
ATP5L	1.03 ± 0.26	1.00 ± 0.23	1.01 ± 0.06	-	-	-
ATP5O	1.02 ± 0.18	0.86 ± 0.22	0.95 ± 0.14	-	-	-
Energy metabolism-related molecules						
ATP2B3	1.03 ± 0.25	0.99 ± 0.45	1.03 ± 0.33	-	-	-
ATP2B4	1.01 ± 0.18	0.97 ± 0.27	0.83 ± 0.30	-	-	-
ATP4A	1.11 ± 0.51	2.15 ± 1.00	1.75 ± 1.24	** ↑	-	-
ATP6VOB	1.04 ± 0.29	0.72 ± 0.25	0.54 ± 0.10	* ↓	** ↓	-
FAM 82A2	1.02 ± 0.22	1.05 ± 0.09	1.09 ± 0.09	-	-	-
SLC6A6	1.02 ± 0.19	1.37 ± 0.27	1.39 ± 0.36	** ↑	* ↑	-
SLC25A3	1.05 ± 0.35	1.09 ± 0.43	1.01 ± 0.19	-	-	-
TOM M 40	1.05 ± 0.38	0.88 ± 0.30	0.56 ± 0.15	-	-	-
ZNF642	1.02 ± 0.22	0.94 ± 0.32	1.03 ± 0.04	-	-	-

Table V. RNA expression of genes encoding proteins linked to purine metabolism in MA, DLB and rpDLB. Similar trends are found in DLB and rpDLB when compared with MA cases; *p < 0.05; **p < 0.01; ***p < 0.001.

Probes	MA	DLB	rpDLB	MA vs DLB	MA vs rpDLB	DLB vs rpDLB
ADA	1.12 ± 0.55	2.09 ± 1.41	2.83 ± 0.57	-	*** ↑	-
AK1	1.03 ± 0.27	1.13 ± 0.53	1.64 ± 0.17	-	*** ↑	-
AK2	1.06 ± 0.35	1.26 ± 0.62	1.72 ± 0.35	-	** ↑	-
AK4	1.06 ± 0.35	1.96 ± 1.52	2.22 ± 0.73	-	*** ↑	-
AK5	1.09 ± 0.49	0.83 ± 0.37	1.27 ± 0.53	-	-	-
AK7	1.08 ± 0.48	1.05 ± 0.31	1.29 ± 0.31	-	-	-
APRT	1.04 ± 0.31	0.79 ± 0.25	0.86 ± 0.28	-	-	-
DGUOK	1.03 ± 0.26	0.90 ± 0.32	1.31 ± 0.23	-	-	* ↑
ENTPD1	1.09 ± 0.45	1.55 ± 1.04	2.50 ± 0.09	-	*** ↑	-
ENTPD2	1.01 ± 0.17	0.60 ± 0.19	1.37 ± 0.94	*** ↓	-	-
ENTPD3	1.07 ± 0.42	0.83 ± 0.27	0.92 ± 0.30	-	-	-
NME1	1.05 ± 0.35	1.66 ± 0.95	2.79 ± 1.11	-	*** ↑	-
NME3	1.04 ± 0.29	1.07 ± 0.38	1.89 ± 0.49	-	*** ↑	** ↑
NME4	1.01 ± 0.18	1.68 ± 1.03	2.55 ± 0.84	* ↑	*** ↑	-
NME5	1.05 ± 0.36	0.71 ± 0.41	1.15 ± 0.19	-	-	-
NME6	1.03 ± 0.24	1.31 ± 0.19	1.49 ± 0.33	* ↑	** ↑	-
NME7	1.05 ± 0.34	0.82 ± 0.40	1.08 ± 0.32	-	-	-
NT5C	1.04 ± 0.30	0.94 ± 0.46	1.24 ± 0.36	-	-	-
NT5E	1.05 ± 0.34	0.82 ± 0.40	1.08 ± 0.32	-	-	-
PNP	1.16 ± 0.74	1.94 ± 0.82	2.91 ± 1.72	* ↑	* ↑	-
POLR3B	1.04 ± 0.29	1.03 ± 0.29	0.98 ± 0.19	-	-	-
PRUNE	1.01 ± 0.14	1.18 ± 0.26	1.62 ± 0.44	-	** ↑	-

Table VI. mRNA expression levels of genes encoding nucleolar proteins, ribosomal proteins, and 18S and 28S rRNAs in MA, DLB and rpDLB. Similar trends are found in DLB and rpDLB when compared with MA cases. Nucleolar changes are more marked in DLB, whereas up-regulation of rRNA 18S and of the majority of genes encoding ribosomal proteins occurs in rpDLB; *p < 0.05; **p < 0.01; ***p < 0.001.

	Probes	MA	DLB	rpDLB	MA vs DLB	MA vs rpDLB	DLB vs rpDLB
Nucleolar proteins							
	NPM 1	1.07± 0.45	0.60 ± 0.11	0.71 ± 0.24	* ↓	-	-
	NCL	1.075± 0.446	0.70 ± 0.17	0.78 ± 0.27	* ↓	-	-
	UBTF	1.07± 0.43	0.72 ± 0.17	0.83 ± 0.40	* ↓	-	-
rRNA							
	rRNA18S	1.04± 0.28	1.22 ± 0.41	2.12 ± 0.66	-	*** ↑	* ↑
	rRNA28S	1.12± 0.62	1.01 ± 0.25	1.34 ± 1.12	-	-	-
Ribosomal proteins							
Large subunit	***RPL5***	1.10± 0.14	0.99 ± 0.16	1.23 ± 0.25	-	-	-
	RPL7	1.01± 0.12	1.19 ± 0.15	1.38 ± 0.20	** ↑	** ↑	-
	RPL21	1.03± 0.29	2.10 ± 0.65	2.72 ± 0.77	*** ↑	*** ↑	-
	RPL22	1.01± 0.17	0.78 ± 0.18	0.83 ± 0.20	* ↓	-	-
	RPL23A	1.04± 0.31	1.41 ± 0.28	1.71 ± 0.46	* ↑	* ↑	-
	RPL26	1.02± 0.21	0.76 ± 0.34	0.71 ± 0.22	-	* ↓	-
	RPL27	1.01± 0.15	1.04 ± 0.11	1.23 ± 0.34	-	-	-
	RPL30	1.03± 0.26	1.55 ± 0.34	2.05 ± 0.31	** ↑	*** ↑	-
	RPL31	1.01± 0.15	1.21 ± 0.25	1.55 ± 0.29	* ↑	*** ↑	-
Small subunit	***RPS3A***	1.03± 0.25	1.36 ± 0.40	1.26 ± 0.22	* ↑	-	-
	RPS5	1.01± 0.11	1.20 ± 0.17	1.31 ± 0.19	** ↑	** ↑	-
	RPS6	1.01± 0.17	1.14 ± 0.10	1.70 ± 0.40	-	*** ↑	** ↑
	RPS10	1.02± 0.23	1.04 ± 0.19	1.17 ± 0.30	-	-	-
	RPS13	1.01± 0.16	1.04 ± 0.11	1.35 ± 0.30	-	* ↑	* ↑
	RPS16	1.04± 0.29	0.75 ± 0.29	0.52 ± 0.01	-	* ↓	-
	RPS17	1.01± 0.11	0.90 ± 0.06	1.10 ± 0.28	* ↓	-	-
	RPS20	1.01± 0.13	0.92 ± 0.14	0.96 ± 0.20	-	-	-

Table VII. mRNA expression levels of genes encoding cytokines and mediators of the innate inflammatory response in MA, DLB and rpDLB. Significant increase in the expression of *TNF-α* and *CST7* is found in rpDLB when compared with MA cases ($p < 0.01$). *TNF-α* is also differentially up-regulated in rpDLB when compared with DLB ($p < 0.01$). IL6ST and CSFR3 gene expression is also increased in rpDLB when compared with DLB ($p < 0.05$).

Probes	MA	DLB	rpDLB	MA vs DLB	MA vs rpDLB	DLB vs rpDLB
Anti-inflammatory cytokines						
IL10 family ***IL10***	1.36 ± 0.99	0.70 ± 0.38	1.37 ± 0.97	-	-	-
IL10RA	1.13 ± 0.61	0.84 ± 0.29	1.40 ± 0.66	-	-	-
IL10RB	1.04 ± 0.29	0.85 ± 0.29	1.24 ± 0.60	-	-	-
TGF family ***TGFB1***	1.11 ± 0.50	0.93 ± 0.39	1.40 ± 0.55	-	-	-
TGFB2	1.08 ± 0.46	1.28 ± 0.46	1.40 ± 0.48	-	-	-
Pro-inflammatory cytokines						
IL6	1.20 ± 0.78	1.18 ± 0.99	3.25 ± 2.87	-	-	-
IL6ST	1.04 ± 0.31	0.92 ± 0.18	1.33 ± 0.44	-	-	*†
IL8	1.12 ± 0.60	1.37 ± 0.83	1.39 ± 0.73	-	-	-
IL1β	1.61 ± 1.57	1.50 ± 1.81	1.22 ± 0.71	-	-	-
TNFα family ***TNFα***	1.14 ± 0.57	1.52 ± 0.51	4.28 ± 1.28	-	**†	**†
TNFRSF1A	1.08 ± 0.41	1.57 ± 1.06	1.60 ± 0.84	-	-	-
Inflammation mediators						
TLRs ***TLR4***	1.06 ± 0.38	1.56 ± 1.11	1.74 ± 0.99	-	-	-
TLR7	1.36 ± 1.04	0.83 ± 0.57	1.21 ± 0.52	-	-	-
Colony stimulating ***CSF1R*** *factors*	1.15 ± 0.55	0.80 ± 0.43	1.12 ± 0.29	-	-	-
CSF3R	1.24 ± 0.79	1.14 ± 0.65	2.21 ± 0.44	-	-	*†
Complement system ***C1QL1***	1.04 ± 0.31	0.95 ± 0.27	1.74 ± 0.99	-	-	-
C3AR1	1.27 ± 0.87	0.97 ± 0.66	2.22 ± 1.61	-	-	-
C1QTNF7	1.15 ± 0.64	0.85 ± 0.37	0.97 ± 0.49	-	-	-
Cathepsins ***CTSC***	1.37 ± 1.25	0.86 ± 0.54	0.92 ± 0.09	-	-	-
CTSS	1.36 ± 1.24	0.91 ± 0.53	1.11 ± 0.46	-	-	-
Integrin family & ***ITGB2***	1.33 ± 0.97	0.98 ± 0.62	1.73 ± 1.04	-	-	-
CTL/CTLD superfamily ***CLEC7A***	1.24 ± 0.76	0.95 ± 0.46	1.11 ± 0.39	-	-	-
CST7	1.44 ± 1.17	2.77 ± 1.89	4.46 ± 1.99	-	**†	-

Table VIII. mRNA expression levels of genes encoding olfactory receptors (OR) and taste receptors (TASR) in MA, DLB and rpDLB. Significant increase in the expression of several ORs occurs in DLB and rpDLB. Up-regulation of TASRs is prominent in DLB and rpDLB. Significant differences between DLB and rpDLB are restricted to one gene in rpDLB in spite of the up-regulation trend in rpDLB when compared with DLB; *p < 0.05; **p < 0.01; ***p < 0.001.

Probes	MA	DLB	rpDLB	MA vs DLB	MA vs rpDLB	DLB vs rpDLB
Olfactory receptors						
OR2D2	1.30 ± 0.77	3.39 ± 2.57	4.94 ± 0.89	* ↑	*** ↑	-
OR2J3	1.13 ± 0.54	1.10 ± 0.55	1.55 ± 0.83	-	-	-
OR2L13	1.36 ± 0.86	1.22 ± 0.88	1.09 ± 0.92	-	-	-
OR2T1	1.09 ± 0.49	1.63 ± 0.97	1.71 ± 0.35	-	-	-
OR2T33	1.24 ± 0.83	0.80 ± 0.38	3.60 ± 1.44	-	** ↑	*** ↑
OR4F4	1.20 ± 0.52	2.22 ± 1.09	0.80 ± 0.32	* ↑	-	-
OR6F1	1.02 ± 0.22	1.63 ± 1.40	2.14 ± 0.86	-	* ↑	-
OR10G8	1.11 ± 0.53	1.23 ± 0.44	0.67 ± 0.21	-	-	-
OR11H1	1.07 ± 0.37	3.15 ± 2.37	2.26 ± 0.74	** ↑	** ↑	-
OR51E1	1.11 ± 0.49	1.94 ± 1.57	1.83 ± 0.96	-	-	-
OR52H1	1.18 ± 0.65	3.60 ± 3.00	4.25 ± 1.25	* ↑	*** ↑	-
OR52L1	1.05 ± 0.35	1.66 ± 1.14	1.06 ± 1.02	-	-	-
OR52M 1	1.09 ± 0.52	1.10 ± 0.87	1.84 ± 0.36	-	* ↑	-
Taste receptors						
TAS2R4	1.03 ± 0.27	1.82 ± 0.78	2.16 ± 0.78	** ↑	*** ↑	-
TAS2R5	1.03 ± 0.25	2.25 ± 1.03	2.12 ± 0.33	** ↑	*** ↑	-
TAS2R10	1.13 ± 0.50	1.68 ± 0.64	1.88 ± 0.44	* ↑	* ↑	-
TAS2R13	1.11 ± 0.49	1.63 ± 0.62	1.99 ± 0.06	* ↑	** ↑	-
TAS2R14	1.15 ± 0.57	2.51 ± 1.59	4.14 ± 1.16	* ↑	*** ↑	-
TAS2R50	1.08 ± 0.40	1.54 ± 1.15	2.34 ± 1.16	-	** ↑	-

Figure 1. Protein expression of subunits of mitochondrial complexes I (NDUFFA7, NDUFA10, NDUFB10, NDUFS8, NDUFB8), II (SDHB), III (UQCRC2), IV (MTCO1) and V (ATP5A, ATP50) normalized with β-actin and voltage dependent anion channel (VDAC). Significant decrease in the expression levels of the majority of these subunits is seen in the frontal cortex in DLB and less markedly in rpDLB when compared with MA cases when normalized with β-actin and in most cases with VDAC. Decreased expression of VDAC in DLB probably reflects decrease in the number of mitochondria; *p < 0.05; **p < 0.01; ***p < 0.001.

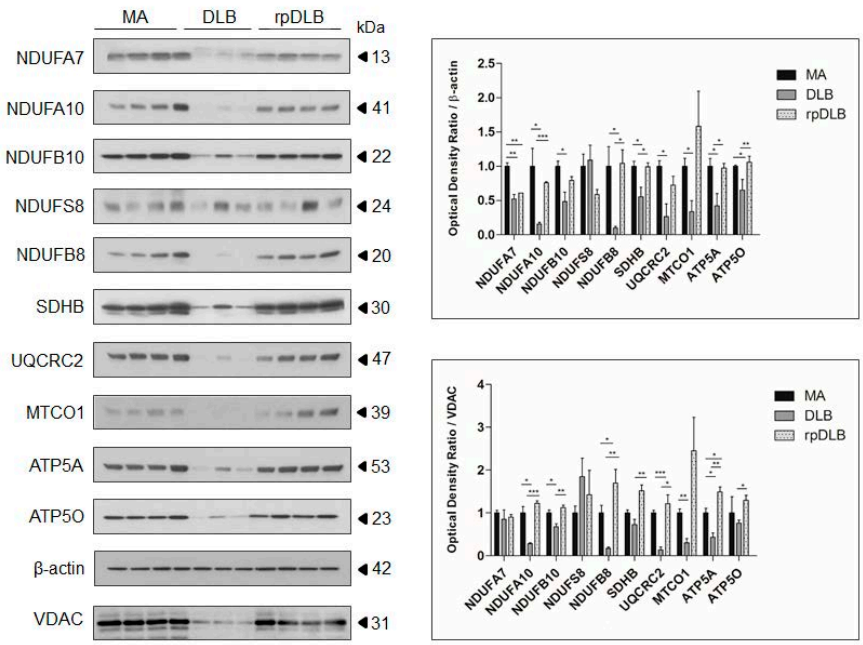


Figure 2. Mitochondrial enzymatic activities in complex I, II, III, IV and V in middle age cases (MA), Dementia with Lewy Bodies (DLB) and rapidly progressive Dementia with Lewy Bodies (rpDLB). All the mitochondrial activities are corrected with the appropriate values of citrate synthase for each sample. Significant decreased activity of complex I, II, III and IV is observed in DLB and rpDLB when compared with MA. Complex V activity showed a trend toward reduction in DLB and rpDLB.

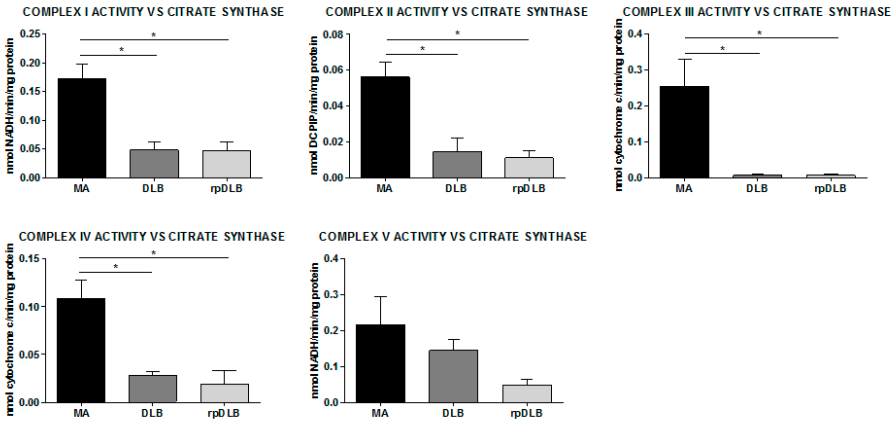


Figure 3. Protein expression, as revealed by western blotting, of initiation and elongation factors of protein transcription at the ribosome in MA, DLB and rpDLB. Reduced expression of initiation factors is more marked in rpDLB than in DLB, whereas the expression of elongation factors eEF1A and eEF2 is not modified in DLB and rpDLB; *p < 0.05; **p < 0.01; ***p < 0.001.

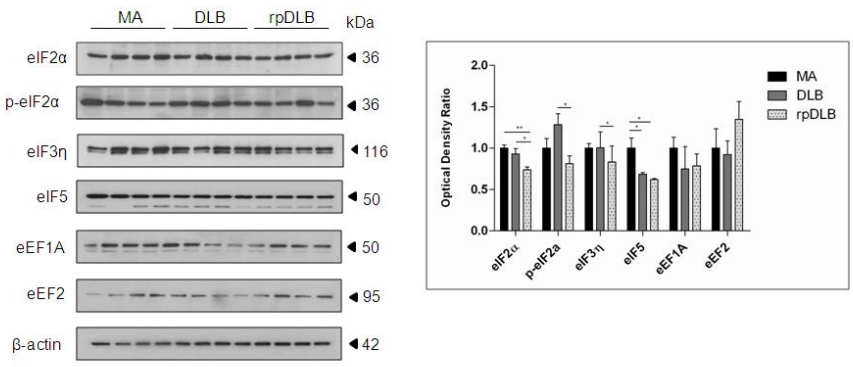


Figure 4. TNF-α, GFAP and Iba-1 protein levels in MA, DLB and rpDLB as revealed by western blotting. Significant increased GFAP expression occurs in DLB and rpDLB when compared with MA ($p < 0.01$). A significant increase also occurs in TNF-α levels in rpDLB when compared with MA ($p < 0.05$).

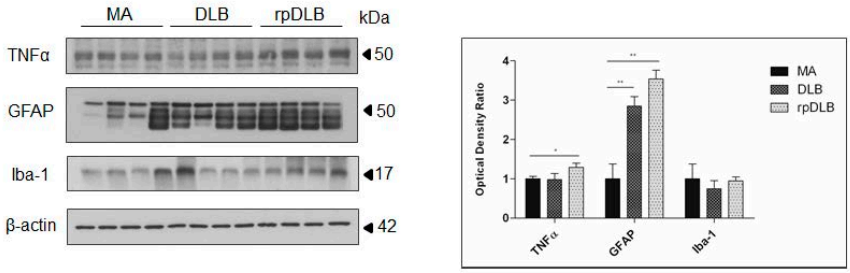
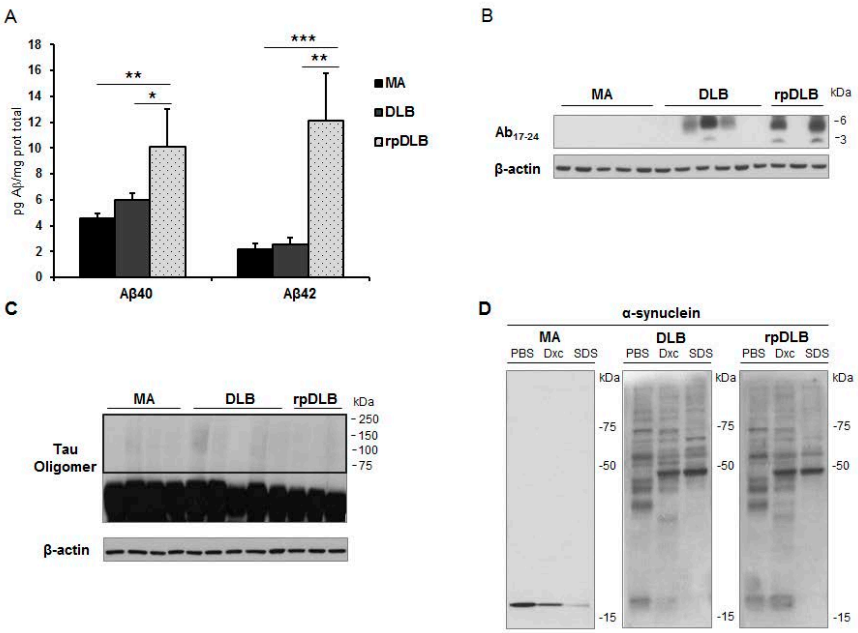


Figure 5. Soluble Aβ40 and Aβ42, membrane-associated β-amyloid, tau oligomers and α-synuclein oligomeric species in total homogenate fractions. A) Soluble Aβ40 and Aβ42 levels are similar in MA and DLB cases but soluble Aβ40 and Aβ42 are significantly increased in rpDLB when compared with MA and DLB; *p < 0.05; **p < 0.01; ***p < 0.001; B) Membrane-associated β-amyloid is detected in DLB and rpDLB but not in MA cases; C) No tau oligomers are detected in DLB and rpDLB even after membrane over-exposition; D) α-synuclein oligomers are present equally in DLB and rpDLB which is in contrast with the lack of α-synuclein oligomers in MA.



SUMMARY OF RESULTS

ARTICLE 1

Altered machinery of protein synthesis is region- and stage-dependent and is associated with α-synuclein oligomers in Parkinson's disease. Garcia-Esparcia P, Hernández-Ortega K, Koneti A, Gil L, Delgado-Morales R, Castaño E, Carmona M, Ferrer I. *Acta Neuropathol Commun. 2015 Dec 1; 3(1):76.*

The study of protein synthesis machinery was performed analysing the expression of 21 genes from the nucleolus to the ribosome in middle-aged individuals (MA) and Parkinson's disease (PD) patients at different Braak stages of disease progression. With this purpose we selected different brain areas with high vulnerability including substantia nigra (SN), frontal cortex area 8 (FC), angular gyrus (AG), precuneus (PC), and putamen (PUT). The study included the analysis of certain nucleolar proteins related to the synthesis of rRNA, mRNA expression of ribosomal RNAs (18S rRNA and 28S rRNA), and ribosomal proteins of the two subunits, the long subunit (RPL) and the short subunit (RPS), selecting specific housekeeping genes to correct (Barrachina *et al*., 2006; Durrenberger *et al*., 2012). This was accompanied by protein expression analysis of initiation and elongation transcription factors, and reticulum stress responses. Finally, we analysed α-synuclein oligomers in total homogenates with western blotting as well as in nuclear fractions isolated by FACS (Fluorescence-activated cell sorting) (Table VI).

Nucleolar proteins mRNA

Since nucleolar proteins are implicated in rRNA processing, the first step in the study was to analyse the mRNA expression levels of two chaperones and one protein linked to RNA polymerase. Reduced expression of mRNA, as revealed by RT-qPCR, was found in the nucleolar proteins as nucleolin (*NCL*), nucleophosmin 1 and 3 (*NPM1 / NPM3*), and upstream binding transcription factor (*UBTF*) at PD Braak stages 5-6 in the substantia nigra, which was accompanied by decreased protein expression as revealed by immunohistochemistry.

No modifications of *NPM1*, *NPM3*, *NCL*, or *UBTF* were detected at PD stages 1-2 in the same region, whereas significant down-regulation of *NPM1* and *UBTF* was observed at stages 3-4. Interestingly, in frontal cortex area 8 at stages 5-6, *NPM1* and *NCL* mRNAs were significantly increased, and in the angular gyrus a reduction in *UBTF* mRNA expression at stages 3-4 was detected. No modifications were identified in the precuneus and putamen at any stage analysed.

mRNA of rRNA

Reduction of 18S rRNA levels at stages 3-4 as well as 18S and 28S at stages 5-6 was observed in the SN, whereas in FC, a significant increase in 18S rRNA was found at stages 5-6. rRNA expression was not altered in the AG. A transient 18S rRNA decrease in the PC and an important increase in the PUT of 28S rRNA was noted at stages 3-4.

Ribosomal proteins mRNA

Ribosomal proteins are essential to the assembly of ribosomal subunits and to the process of protein synthesis; for this reason the next step was to analyse gene expression of 9 RPL and 7 RPS genes. Selection of these mRNA was done at random. Some genes encoding ribosomal proteins were abnormally deregulated in FC area 8 and PC showing a general decrease at stages 3-4 and an increase at stages 5-6. One gene showed significant changes in the AG (*RPS3A*) in PD at stages 5-6, and another gene (*RPL5*) at PD stages 5-6 in the PUT. Interestingly, twelve of sixteen genes analysed in the SN were down-regulated at stages 3-4 and fourteen at stages 5-6.

Immunohistochemistry, immunofluorescence and confocal microscopy

To learn whether mRNA changes in nucleolar chaperones translated into altered protein expression, immunohistochemistry and immunofluoresece to NPM1 and NPM3 were performed in the SN. Decreased NPM1 immunoreactivity was observed in pigmented neurons, whereas NPM3 was preserved in the majority of neurons. Double-labelling

immunofluorescence and confocal microscopy in the SN in PD cases stages 4 and 5 showed α-synuclein in Lewy bodies and Lewy neurites co-localizing with eIF3.

Protein expression

Increased protein levels of initiation factor eIF3 and elongation factor eEF2 were observed in the SN. Although many of these observed changes in the SN may be attributable to neuronal loss, the selective alteration of certain factors points to particular vulnerability of certain molecules. Altered protein levels of eIF3 and eIF1, and reduced eEF1A and eEF2 protein expression, were observed in the FC. Increased levels of ATF4 and ATF6 at stages 5-6 and reduced GRP94 expression levels at stages 3-4 and 5-6 were found in the SN in PD. Reduced expression levels of GRP78 were observed in FC area 8 at stages 3-4 and 5-6.

α-synuclein oligomeric species

Abnormal solubility and aggregation were identified in the SN and in the FC, but not in the PUT using the same cases of PD at stages 5-6. In addition, oligomeric species in the nuclei of PD were detected by FACS isolation.

ARTICLE 2

Mitochondrial activity in the frontal cortex and angular gyrus in Parkinson's disease and Parkinson's disease with dementia. Garcia-Esparcia P, Koneti A, Rodríguez-Oroz MC, Rodríguez-Oroz MC, Lago B, del Rio JA, Ferrer I. *Submitted Brain Pathology*.

Reduced complex I activity and increased oxidative damage are well-known alterations in the SN in PD. However, little is known about mitochondrial alterations in other brain regions. Random selection of oxidative pathway probes has been made in order to compare between middle-aged individuals (MA) and PD cases including the genes: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7 (*NDUFA7*), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10 (*NDUFA10*), NADH

dehydrogenase (ubiquinone) 1 beta subcomplex 10 (*NDUFB10*), NADH dehydrogenase (ubiquinone) Fe-S protein 7 (*NDUFS7*), NADH dehydrogenase (ubiquinone) Fe-S protein 8 (*NDUFS8*), cytochrome c oxidase subunit VIIa polypeptide 2 like (*COX7A2L*), ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2 (*ATP5G2*), ATPase, H+ transporting, lysosomal V0 subunit a1 (*ATP6VOB*), ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H (*ATP6V1H*), and phospholysine phosphohistidine inorganic pyrophosphate phosphatase (*LHPP*). RT-qPCR of 18 mRNAs encoding subunits of mitochondrial complexes and 12 mRNAs encoding energy metabolism-related enzymes, western blotting of mitochondrial proteins, and enzymatic activities of complexes I, II, III, IV, and V of the respiratory chain were assessed in the FC and in the AG of middle-aged individuals (MA), and those with incidental PD (iPD), long-lasting PD with parkinsonism without dementia (PD), and long-lasting PD with later appearance of dementia (PDD) (Table VI).

Mitochondrial subunits mRNA

Thirty possible susceptible genes including mitochondrial subunits and energy metabolism-related molecules were studied with RT-qPCR. No differences were observed in the majority of assessed genes at Braak stages 3-4 when compared to MA in the FC. However, significantly increased expression of several genes was detected at advanced stages of the disease, stages 5-6, when compared with MA but also with PD cases at stages 3-4. An opposite pattern was detected in the AG, whereas an important increase was observed in the majority of the genes at stages 3-4 of the disease, followed by a tendency to decrease at advanced stages with significant differences when comparing PD 3-4 with PD 5-6. Interestingly, marked down-regulation of genes encoding mitochondrial subunits and energy metabolism-related enzymes occured in FC but only in genes coding for energy metabolism-related enzymes in the AG in PDD.

Protein expression

At the protein level, a total of five proteins studied in the FC and the AG matched with results obtained in the mRNA studies. A significant decrease in the protein expression levels of several mitochondrial subunits occurs equally in frontal cortex and angular gyrus in PDD.

Enzymatic activities

Regarding mitochondrial enzymatic activities, reduced activity of mitochondrial complexes was only observed in Parkinson's disease with dementia. Cases without dementia did not have significant alterations in mitochondrial function, at least as detected by measuring enzymatic activity of the distinct complexes.

α-synuclein

Interestingly, increased α-synuclein was detected in mitochondrial-enriched fractions in the FC in PD as well as Neuroketal, suggesting a close relationship.

ARTICLE 3

Complex deregulation and expression of cytokines and mediators of the immune response in Parkinson's disease brain is region dependent. Garcia-Esparcia P, Llorens F, Carmona M, Ferrer I. *Brain Pathol.* 2014 Nov; 24(6):584-98.

Neuroinflammation processes are common in many neurodegenerative diseases as Parkinson's disease. Twenty-five genes of different families related to inflammatory response including members of the complement system, colony stimulating factors, Toll-like receptor family, cytokines *IL-8*, *IL-6*, *IL-6ST*, *IL-1B*, *TNF-α* family, *IL-10*, *TGFβ* family, cathepsins, and integrin family were analysed with RT-qPCR in the SN, PUT, FC area 8, and AG area 39 in middle-aged individuals (MA) and PD Braak stages 1-6. Additionally, protein studies including western blotting (WB), ELISA, and immunohistochemistry were performed in this study (Table VI).

Mediators of the immune response mRNA levels

The SN showed decreased mRNA expression of *C1QL1*, *C1QTNF7*, *C3AR1*, *TLR7*, *IL-1B*, *IL-6*, *IL-6ST*, *TNF-α*, *IL-10*, *IL-10RB*, *CTSC*, and *TGFB2*, but an increase in *IL-6ST* at early stages of PD (1-2 Braak). The FC and the PUT showed up-regulation of certain genes and down-regulation of others, whereas the AG exhibited little inflammatory responses. mRNA levels in the FC did not differ when comparing intermediate stages 3-4 with advanced stages of PD 5-6, suggesting that inflammatory alterations in this region were not modified with disease progression, or that *parkinsonism* treatment stabilized inflammatory response.

Protein expression

Immunoassay techniques (ELISA) were performed in the FC showing increased IL-6, and reduced IL-10 levels in PD; western blotting confirmed the result for IL-6. No changes were observed in TNF-α expression; moreover, elements of this pathway, including NF-κβ, IKβα, and IKK-α, were not modified. Immunohistochemistry in the FC and the SN showed that IL-5, IL-6, and IL-17 were localized in glial cells, mainly in microglia, whereas IL-5, IL-10, and M-CSF were in neurons. TNF-α was found in neurons and microglia, and NF-κβ in the nucleus of subpopulations of neurons and glial cells.

α-synuclein

The presence of α-synuclein aggregates was demonstrated in the SN and in the FC in several studied fractions (cytosolic, deoxycholate and sodium dodecyl sulphate (SDS) fractions).

ARTICLE 4

Purine metabolism gene deregulation in Parkinson's disease. Garcia-Esparcia P, Hernández-Ortega K, Ansoleaga B, Carmona M, Ferrer I. *Neuropathol Appl Neurobiol.* 2015 Dec; 41(7):926-40.

Purines are present in the molecules of DNA, RNA, nucleosides, and nucleotides, all of them involved in a variety of crucial metabolic pathways. In the present study, twenty-two genes implicated in the metabolism of purines were examined with RT-qPCR and an interesting deregulation was validated in the SN, the PUT, and the FC area 8 at different stages of the disease progression. The PUT region was selected due to its important connections with the SN, an area of great relevance and significance in PD (Table VI).

mRNA and protein purine levels

mRNA expression levels in the SN of adenylate kinase (*AK 2, 3, 4*), adenine phosphoribosyltransferase (*APRT*), ectonucleoside triphosphate diphosphohydrolase (*ENTPD1*, *ENTPD3*), nucleoside diphosphate kinase (*NME1*, *NME7*), and purine nucleoside phosphorylase (*PNP*) were reduced at Braak stages from 3 to 6. In contrast, adenosine deaminase (*ADA*) gene was up-regulated at stage 3-4, while *AK1*, *AK5*, *NME4*, *NME5*, *NME6*, 5'-nucleotidase (*NT5E*), *PNP* and *PRUNE* at stages 5-6 showed over-expression. Significant changes were not detected in the PUT region in the considered stages. The down-regulation of the SN was interpreted as a result, in part, of damage to the dopaminergic cells, resulting in neuronal cell death, as ENTPD3, NME7, AK1, and PNP1 were expressed in neurons detected with immunohistochemistry. Furthermore, NT5E and ENTPD1, two of the genes also down-expressed in the SN, were observed in astrocytes, pericytes, and possible microglia, respectively. Gene over-regulation in the FC suggested a primary manifestation or compensation of the altered purine metabolism in this region in advanced stages of PD. Comparing the observations of the

present work to parallel studies in AD, both pathologies showed altered enzymes involved with regional variation.

ARTICLE 5

Functional genomics reveals dysregulation of cortical olfactory receptors in Parkinson disease: novel putative chemoreceptors in the human brain. Garcia-Esparcia P, Schlüter A, Carmona M, Moreno J, Ansoleaga B, Torrejón-Escribano B, Gustincich S, Pujol A, Ferrer I. *J Neuropathol Exp Neurol*. 2013 Jun; 72(6):524-39.

The use of functional genomics with Whole-Transcript Expression Array and the subsequent bioinformatics analysis facilitated the cluster identification of olfactory (ORs) and taste receptors (TRs) in human brain *post-mortem* samples. The selection of some of these deregulated ORs, TRs, and other obligate mediators, as well as their validation using RT-qPCR techniques, served to identify the presence of a large number of elements linked to what could be a new signalling pathway in the human brain (Table VI).

mRNA and protein levels

Evidence of a clear decrease in the mRNA expression levels in most of the ORs analysed in PD cases such as *OR2L13*, *OR2J3*, *OR51E1*, *OR52L1*, or *OR11H1*, and manifested overexpression levels of TRs as *TAS2R10*, and *TAS2R13*, were noted in the FC, revealing the susceptibility of this region to disease progression. Moreover, a considerable impact on males compared to females was observed. The separation of lipid-raft by cell subfractionation allowed verifying that those receptors and obligate mediators for its functionality, such as adenylyl cyclase 3 (AC3), or protein G olfactory ($G_{\alpha olf}$), were located at a cytosolic level. The result was further contrasted with immunofluorescence techniques and confocal microscopy, revealing the widespread presence of all elements referred to throughout the human brain. These studies were also used to verify the existence in the

neuronal cytoplasm of UDPglucoronosyltransferase and carriers such as RTP1, RTP2, and REEP1, responsible for carring the ORs to the neuronal membranes.

ARTICLE 6

Dementia with Lewy bodies: molecular pathology in the frontal cortex in typical and rapid forms. Garcia-Esparcia P, López-Gonzalez I, Grau-Rivera Oriol, García-Garrido MF, Koneti A, Llorens F, Zafar S, Carmona M, Del Río JA, Zerr I, Gelpi E, Ferrer I. *Submitted Frontiers in Neurology.*

This work, related to DLB and their rapidly progressive form, rpDLB, is an attempt to understand how the alterations of several metabolic pathways could converge to the pathogenesis of these pathologies, made by studying the impairment in the protein synthesis machinery, mitochondria and energy metabolism, purine metabolism, neuroinflammation, and other signalling pathways such as olfactory and taste receptors. In the same line, although neuropathological studies have not shown differences between the two pathologies, we planned to study the possible biochemical alterations in order to discriminate between DLB and rpDLB in FC area 8, and to identify additional factors linked to short disease duration. For this reason, selected mRNAs and proteins of the different metabolic pathways mentioned and previously studied in Parkinson's disease were used to identify the possible changes that occur in both pathologies compared with MA cases. Besides, a wide-ranging study of the presence of oligomeric species of tau and α-synuclein, the concentration of β-amyloid 40 and 42, and the quantification of membrane-associated β-amyloid was also performed in the same human samples to better understand the whole mechanism that is acting in the brain (Table VI).

mRNA and protein levels

At the mRNA level, the most important changes were observed in the protein synthesis machinery with important variations between DLB, as well as rpDLB, with MA cases, followed by alterations in the purine metabolic pathway and encoding genes of olfactory and taste receptors. No dramatic changes were detected in mitochondrial respiratory chain genes or neuroinflammatory response elements, with the interesting exception of *TNF*-α. Regarding protein levels, acute reduction in DLB was detected in all of the proteins analysed with a corresponding mitochondrial activity reduction in all of the studied complexes. rpDLB presented the same behavioural pattern as DLB in mitochondrial enzymatic values. Protein changes observed in the initiation and elongation elements, as well as in the neuroinflammation systems, showed important variations in these two pathologies when compared with MA individuals.

Oligomeric species

Interestingly, overexpression of β-amyloid 40, β-amyloid 42, membrane-associate β-amyloid, and α-synuclein oligomers was verified in DLB and rpDLB when compared with MA cases.

	Parkinson's disease (PD)		**Parkinson's disease with dementia (PDD)**		**Dementia with Lewy bodies (DLB)**		**Rapidly progressive Dementia with Lewy bodies (rpDLB)**	
	Regions	Methods	Regions	Methods	Regions	Methods	Regions	Methods
Protein synthesis machinery	SN FC AG PC PUT	RT-qPCR WB IHC-IF-CM Oligomeric species*	-	-	FC	RT-qPCR WB Oligomeric species*	FC	RT-qPCR WB Oligomeric species*
Mitochondria and energy metabolism	FC AG	RT-qPCR WB EA Mitochondria isolation	FC AG	RT-qPCR WB EA	FC	RT-qPCR WB EA	FC	RT-qPCR WB EA
Neuroinflammation	SN FC AG PUT	RT-qPCR WB ELISA IHC Solubility & aggregation	-	-	FC	RT-qPCR WB IHC	FC	RT-qPCR WB IHC
Purine metabolism	SN FC PUT	RT-qPCR IHC	-	-	FC	RT-qPCR	FC	RT-qPCR
Olfactory receptors, Taste receptors and obliged functional mediators	FC	Microarray RT-qPCR WB IHC-IF Lipid-Raft isolation	-	-	FC	RT-qPCR	FC	RT-qPCR
Oligomeric species*	SN FC PUT	Nuclei FACs isolation Subraction-ation	-	-	FC	WB ELISA Subraction-ation	FC	WB ELISA Subraction-ation

Table VI. Summary of all the materials and methods used for the present thesis. For *post-mortem* human brain tissue regions: Frontal cortex area 8 (FC), substantia nigra (SN), angular gyrus area 39 - parietal lobe (AG), putamen, (PUT), and precuneus (PC). For techniques or methods: real time-quantitative protein chain reaction (RT-qPCR), western blot (WB), immunohistochemistry (IHC), immunofluorescence (IF), confocal microscopy (CM), mitochondrial enzymatic activity (EA), nuclei Fluorescent activated cell sorter isolation (FACs), and enzyme-linked immunosorbent assay (ELISA).

DISCUSSION

DISCUSSION

The present thesis describes the molecular alterations underlying the cerebral changes that occur in Parkinson's disease (PD), Dementia with Lewy bodies (DLB), and rapidly progressive Dementia with Lewy bodies (rpDLB). These alterations include impairment in the machinery of protein synthesis, mitochondrial and energy metabolism malfunction, neuroinflammatory response, purine metabolism involvement, and deregulation of olfactory (OR) and taste (TR) brain receptors.

1. Altered machinery of protein synthesis in Parkinson's disease

The present study tried to identify the alteration of protein synthesis machinery in PD. The results obtained with RT-qPCR studies indicate a dramatic region- and stage- dependent alteration. Furthermore, studies using total homogenate fractions with western blotting (WB) demonstrate a distinctive change not only in the solubility but also in the formation of the α-synuclein oligomers, both in the substantia nigra (SN) and in frontal cortex area 8 (FC), but not in the putamen (PUT), where scarcely any differences were seen between middle-aged cases and PD patients. Previous studies demonstrated direct and indirect deleterious effects of α-synuclein oligomers in the mitochondria, proteasome, endoplasmic reticulum, synapses, as well as other subcellular structures. The presence of α-synuclein oligomer aggregates was demonstrated in the nucleus, thanks to the nuclear isolation by FACS in the cerebral cortex of PD patients and to a lesser extent in middle-aged individuals (MA). Thus, the results obtained showed a close relationship between the alteration of the protein synthesis pathways from the nucleolus to the ribosome, and the presence of α-synuclein oligomers in total homogenate and nuclear fractions in PD, suggesting a link between α-synuclein oligomers and altered protein synthesis machinery in PD. Accordingly, these analyses helped to identify many molecular modifications including alteration in the machinery of protein synthesis in the SN and the cerebral cortex, and to a

lesser extent in the AG, PC, and PUT. Finally, the present findings suggest a close relationship between the presence of oligomers and the nucleolar and cytoplasmic dysfunction with functional damage to the protein synthesis machinery.

1.1 Nucleolar proteins and rRNAs are deregulated in the SN, FC, AG, PC, and PUT in PD, and changes accelerate with disease progression.

Nucleolar stress is emerging as an important sensor in several pathological conditions (James *et al*., 2014) including ischemic damage, cancer (Avitabile *et al*., 2011; Hein *et al*., 2013; Ruggero, 2012), and neurodegeneration (Baltanas *et al*., 2011; Becherel *et al*., 2006; Parlato and Kreiner, 2013). The present study identifies reduced nucleolin (*NCL*) and nucleophosmin 1 (*NPM1*) mRNA levels and NPM1 immunoreactivity in the SN in PD cases with disease progression. Nucleophosmin 3 (*NPM3*) mRNA expression levels are also reduced in the SN at advanced stages. Reduced mRNA expression may be related to the progressive loss of dopaminergic (DA) neurons in the SN, but preserved expression of NPM3 protein, in contrast to decreased NPM1 immunoreactivity in remaining DA neurons, indicating selective vulnerability of NPMs to neurodegeneration. Reduced *NCL* mRNA is in agreement with previous observations showing reduced nucleolin protein expression in the SN in PD (Caudle *et al*., 2009). The decreased upstream binding transcription factor (*UBTF*) here observed is also in line with previous observations of nucleolar disruption in DA neurons in PD (Rieker *et al*., 2011). The reduced nucleophosmin expression in the SN in PD here observed for the first time may act as an additional cause of neurodegeneration (Marquez-Lona *et al*., 2012). Decreased expression of 18S rRNA and 28S rRNA gives strong support to the concept that nucleolar stress is a major alteration in the SN in PD (Parlato and Liss, 2014). Although decreased biosynthesis of ribosome subunits may be a response to preserve energy homeostasis in acute stress situations (Sengupta *et al*.,

2010), it is less clear that maintained reduced expression of rRNAs has any beneficial effect on cell survival. Rather, perpetuation of nucleolar stress and reduced rRNA synthesis is consistent with parallel cell damage in PD SN. In contrast to this area, *NCL* and *NPM1* mRNAs are increased in FC area 8 in PD at stages 5-6, whereas *UBTF* appears to be transiently decreased in the AG at stages 3-4. No modifications in *NCL*, *NPM1*, or *UBTF* gene expression are seen in the PC and PUT at any stage of the disease. Furthermore, 18S rRNA and 28S rRNA are increased in the FC and PC, respectively, at stages 5-6 of Braak. Overexpression of NCL is neuroprotective against rotenone, a toxicant used to experimentally reproduce some characteristics of PD in animal models (Betarbet *et al*., 2006). Therefore, increased *NCL* and *NPM1* mRNA expression in FC appears to be a response to PD geared to protecting rRNA synthesis. According to this hypothesis, 18S rRNA expression is increased in FC at advanced stages of PD. Lack of correlation between preserved *NCL* and *NPM1* expression and increased 28S rRNA in the precuneus may be related to the participation of other regulators of rRNA biosynthesis not examined in the present study.

1.2 Altered expression of ribosomal protein mRNAs in PD is region- and stage-dependent

The present results show decreased gene expression of 12 out of 16 examined ribosomal protein mRNAs in the SN at stages 3-4 (covering 6 RPL and 6 RPS), and 14 of 16 (covering 8 RPL and 6 RPS) at stages 5-6 of Braak. These changes are progression-dependent and may reflect in part progressive neuronal loss in the SN. However, altered ribosomal protein gene expression also occurs in the cerebral cortex in PD with stage and region peculiarities. Altered mRNA expression of several ribosomal proteins in the cerebral cortex appears to be a plastic process depending on the cortical region and stage of the disease. Decreased expression of a few mRNAs at stages 3-4 followed by up-regulation of a few different ribosomal protein mRNAs at stages 5-6 in FC and PC

suggests modifications in the structure and functional capacities of cortical ribosomes with disease progression. It is worth stressing that only 16 of 79 ribosomal protein mRNAs were selected for the present study, and although the number is representative it does not cover the total number of ribosomal proteins and the possible modifications of additional mRNAs.

1.3 Different expression of initiation and elongation factors in the SN and FC in Parkinson's disease

In the SN, eIF3 and eEF2 expression levels were increased more markedly at stages 5-6 in PD, suggesting activation of peptide synthesis. It can be suggested that activation of peptide synthesis is related to compensatory mechanisms in preserved DA neurons in the face of the altered expression of genes involved in ribosomal proteins and, consequently, in the assembly of the functional ribosome. Alternatively, since WB cannot discriminate between neurons and glial cells, increased eIF3 and eEF2 expression levels might be related to increased protein synthesis in reactive astrocytes. In cerebral cortex area 8, among the five eIFs and subunits examined, only eIF3 was significantly decreased at stages 3-4 and 5-6, suggesting that recruitment of mRNA to the 40S subunit is hampered as a result of lower eIF3 expression. Regarding elongation factors, significantly reduced expression of eEF1A and eEF2 with disease progression lends strong support to the hypothesis of altered polypeptide synthesis in FC in PD.

1.4 Reticulum stress responses in the SN and FC in PD

The unfolded protein response (UPR) designates the cellular response to the accumulation of abnormal proteins in the endoplasmic reticulum (ER). ER stress has been implicated in the pathogenesis of neurodegenerative diseases including PD (Colla *et al*., 2012; Doyle *et al*., 2011; Mercado *et al*., 2013). Markers of the unfolded protein response (phosphorylated PERK and phosphorylated eIF2α) have been identified in dopaminergic neurons of the SN containing α-synuclein inclusions at relatively early

stages of PD (Ferrer, 2011; Hoozemans *et al*., 2007). ATF4 and total ATF6 expression levels are elevated in the SN at advanced stages of the disease, suggesting activation of the UPS response. It is worth noting that ATF4 levels have been reported to be increased in neuromelanin-containing neurons in the SN in PD, and elevated levels of ATF4 are protective of neurons subjected to noxious stimuli (Sun *et al*., 2013).

1.5 α-synuclein oligomeric species in Parkinson's disease

The localization and function of the α-synuclein protein in the nucleus has been a primary focus of study partly due to the overwhelming information about the accumulation of abnormal α-synuclein in the cytoplasm of neurons in PD, and in neurons and oligodendroglia in multiple system atrophy (MSA). However, α-synuclein is identified in the nucleus in different settings using different methods (Goers *et al*., 2003; Gonçalves and Outeiro, 2013; Ma *et al*., 2014; McLean *et al*., 2000; Nishie *et al*., 2004; Specht *et al*., 2005; Yu *et al*., 2007), and it is especially abundant during development, modulating neurogenesis (Crews *et al*., 2008; Winner *et al*., 2008; Zhong *et al*., 2010). Nuclear α-synuclein levels are increased accompanying oxidative stress *in vitro* and *in vivo* (Monti *et al*., 2010; Xu *et al*., 2006), and nuclear α-synuclein seems to facilitate, in turn, oxidative stress (Zhou *et al*., 2013). The mechanism of effects of nuclear α-synuclein is poorly understood but α-synuclein binds to histones and inhibits histone acetylation (Goers *et al*., 2003; Kontopoulos *et al*., 2006). Moreover, α-synuclein, under conditions of oxidative stress, binds to the promoter of the mitochondrial transcription factor PGC1-α, and reduces transcription of mitochondrial genes (Siddiqui *et al*., 2012).

Increased expression levels of α-synuclein oligomers have been found in the brain in Lewy body diseases and related transgenic models (Garcia-Esparcia *et al*., 2014; Dalfó *et al*., 2004; Paleologou *et al*., 2009; Sharon *et al*., 2003; Tsika *et al*., 2010). Interestingly, the band pattern of α-synuclein oligomers analysed in the present study differs in the SN and

FC area 8, suggesting regional differences in the composition of oligomeric species in PD. Importantly, the intensity of oligomeric species in WB is greater than what is expected following examination of paraformaldehyde-fixed paraffin sections of the FC in which only a few Lewy bodies and neurites are detected with immunohistochemistry using the same antibody. Therefore, it may be inferred that most α-synuclein oligomers are not identified in paraffin sections processed for immunohistochemistry; only those linked to fibrillary deposits in Lewy bodies and neurites remain. Observations in the PUT are particularly interesting as no oligomeric α-synuclein species are identified in the same PD cases used in SN and FC. This means important regional differences in α-synuclein oligomers in PD represents, on the one hand, regional differences in α-synuclein metabolism, and, on the other, putative specific regional vulnerability in PD. A recent study has shown that α-synuclein proximity ligation assay (AS-PLA) permits the visualization of undetected diffuse α-synuclein oligomeric pathology in PD brains (Roberts *et al*., 2015). α-synuclein oligomers are detected in the cytoplasm of neurons with α-synuclein inclusions, but also widespread in nerve terminals (consistent with synaptic localization) and in the cytoplasm of vulnerable neurons with no apparent α-synuclein pathology, as detected with current immunohistochemical methods. The present observations show the unequivocal presence of α-synuclein oligomers in FACS-isolated neuronal nuclei in PD. Interestingly, a weak low band of α-synuclein is also detected in NeuN- samples (corresponding to non-neuronal nuclei) in PD. This raises the possibility that α-synuclein is abnormally present in the nuclei of glial cells. In this line, α-synuclein deposition has been reported in protoplasmic astrocytes in PD (Braak *et al*., 2007; Halliday and Sevens, 2011).

2. Mitochondria and energy metabolism in Parkinson's disease

This study is an attempt to understand more deeply the role of mitochondria and energy metabolism in PD. Expression of selected genomic genes encoding mitochondrial protein subunits of the respiratory chain complexes and the activities of complex I, II, III, IV, and V were analysed in frontal cortex area 8 (FC) and the angular gyrus (AG) in neuropathologically-verified incidental PD (iPD), long-lasting PD with parkinsonism without dementia (PD), and long-lasting PD with later appearance of dementia (PDD) cases. Previous studies have shown reduced metabolism, as revealed with [(18)F]-fluorodeoxyglucose Positron Emission Tomography (PET), in the frontal cortex, and progression to the angular gyrus, orbital cortex, posterior cingulus, and occipital lobes in PD patients with mild cognitive impairment (Lyoo *et al*., 2010; Bohen *et al*., 2011; Garcia-Garcia *et al*., 2012). Neuroimaging analyses have also suggested that cortical hypometabolism is present even at early stages of PD (Borghammer *et al*., 2010; Berti *et al*., 2012). Interestingly, hypometabolism is followed by cortical atrophy in the same areas in PDD (González-Redondo *et al*., 2014). The selection of genes was based on parallel studies carried out in several neurodegenerative diseases (NDD) with abnormal protein aggregates in which the same genes were analysed including several subunits of complex I-V. Although this approach does not cover all the mitochondrial subunits, it serves to analyse comparative studies among diseases with abnormal protein aggregates in old age. This aspect is founded on the hypothesis that although mitochondria are vulnerable in various NDD, the alteration of subunits and susceptibility of complexes is disease-dependent. The study of mitochondria is accompanied by gene expression analysis of other energy metabolism-related enzymes. The results obtained showed regional differences in the expression of certain genes, which vary with disease progression. Regarding the protein levels of mitochondrial subunits, two of the proteins analysed in the FC matched perfectly with the results obtained by RT-qPCR, as well as three out of the five proteins

studied in the AG fitted with the mRNA results. This valuable information gave us the opportunity to continue examining the mitochondrial enzymatic activities of complexes I, II, III, IV, and V, not only in iPD and PD, but also in PDD. The most important variations were detected in the cases with PDD and more significantly in the AG. Activity of complexes I, II, III, and IV were reduced in PD with dementia in the FC and more dramatically decreased in the AG with the exception of complex V, which did not present any statistical difference in comparison with middle-aged (MA) cases. These results are in consonance with previously reported studies, and they are consistent with a scenario in which other molecules, including mitochondrial membrane proteins, ion channels, and other mitochondrial subunits, are primarily damaged (Berndt *et al*., 2013). It is worth noting that oxidatively damaged protein subunits may endanger mitochondrial function.

2.1 Mitochondria and energy metabolism alterations

Present findings show regional differences in the expression of several nuclear genes encoding subunits of mitochondrial complexes and energy metabolism-related enzymes. The most important changes in FC area 8 are manifested by up-regulation of genes encoding subunits of complexes I, II, III, IV, and V in PD when compared with MA, and energy-metabolism related enzymes when comparing iPD with PD. In contrast, gene up-regulation in the AG occurs in iPD when compared with MA, and values mostly return to normal levels in PD. Protein levels of selected subunits parallel values of corresponding gene expression. Therefore, altered regulation of genes and proteins related to mitochondria and energy metabolism is an early region-dependent event in the FC and AG within the spectrum of PD. However, these alterations have only a moderate impact on the activity of mitochondrial complexes, as only complex III activity is reduced in both regions in PD. These changes do not contradict the marked impairment of mitochondria biogenesis, altered mRNA expression, deregulation of several microRNAs predicted to

interact with complex I regulators, and alterations of protein levels which also occur in FC in PD (Thomas *et al*., 2012). Rather, they are consistent with a scenario in which several molecules, including mitochondrial membrane proteins, ion channels, and mitochondrial subunits, are altered to determinate thresholds until they produce altered activity. In this line, oxidative damage to certain protein subunits, rather than reduced protein levels, can jeopardize mitochondrial function in PD (Keeney *et al*., 2006).

2.2 Parkinson's disease with dementia and mitochondria

Dramatic changes occur in PDD as the expression of genes encoding mitochondrial subunits and energy-related enzymes is markedly down-regulated in FC area 8, whereas down-regulation mainly involves energy-related enzymes in the AG in PDD when compared with PD. Nevertheless, protein expression of several mitochondrial subunits falls in FC and AG in PDD. Differences between mRNA and protein expression levels are not uncommon as protein translation is modulated by several molecules mainly non-coding RNAs. Changes in protein expression could be the result of mitochondrial loss although decreased protein values are similarly affected when using β-actin and VDAC for normalization. However, reduced mRNA expression corresponds to nuclear-encoded mRNAs, thereby indicating that at least part of mitochondrial dysfunction in PD is linked to abnormalities in nuclear mRNA processing of genes encoding subunits of mitochondrial complexes. Reduced complex I activity has been reported in FC area 8 at advanced stages of PD in which, unfortunately, no clinical information about their cognitive status was available (Keeney *et al*., 2006; Parker *et al*., 2008; Navarro *et al*., 2009; Navarro and Boveris, 2009). More focused studies on PD and dementia have shown reduced complex I activity only in PDD, and not in PD (Gatt *et al*., 2016). The present observations in PDD are in the same line although decreased activity affects not only complex I but also complexes II, III, and IV. Moreover, reduced mitochondrial activity is not restricted to the FC; the AG has similar mitochondrial functional

deficiencies. Curiously, the expression of nuclear genes encoding subunits of mitochondrial complexes is dramatically reduced in the FC, but not in the AG, in PDD, thus indicating regional differences between the two regions in the transcription of nuclear genes encoding mitochondrial subunits. Reduced mitochondrial DNA levels have been reported in the prefrontal cortex in PDD but not in PD, thus suggesting that primary alterations in mitochondrial DNA are causative of mitochondrial dysfunction and dementia in PDD (Gatt *et al*., 2016). However, the expression of mitochondrial subunits encoded by mitochondrial DNA was not examined in this study.

2.3 α-synuclein oligomers and mitochondria

Present findings have also shown the presence of α-synuclein oligomers in mitochondrial-enriched fractions in PD, suggesting that local oligomers may affect mitochondrial function. In this line, abnormal α-synuclein oligomers are detected in the nuclear fractions of FACS-isolated neurons in FC in PD in association with marked alterations in the expression of genes and nuclear proteins involved in protein synthesis machinery (Garcia-Esparcia *et al*., 2015). The present data together with previous studies suggest that the scenario is not simple, as mitochondrial dysfunction in cerebral cortex, and perhaps in other regions in PD may be due to the combination of altered nuclear and mitochondrial transcription, which in turn may depend on the preservation of genomic and mitochondrial DNA, and on post-translational modifications of mitochondrial subunits linked to oxidative damage.

3. Neuroinflammation in Parkinson's disease

The neuroinflammation study was designed to compare and evaluate the expression of several cytokines and mediators of the immune response in different brain regions at progressive stages of PD. The selection of molecules was performed taking into consideration members of the complement system, CSFs, pro- and anti-inflammatory cytokines, TGF-β, cathepsins and integrins which have been found to be abnormally

regulated in the cerebral cortex in a parallel study in progress of Alzheimer's disease (AD) in order to give supplementary information on what was happening in various NDD, and also to compare the dependence of disease, region, and stage in neuroinflammatory processes. Regarding the choice of regions in this study, the SN and the PUT were obligatory areas because they are primary substrates of *parkinsonism* (Duke *et al*., 2007). The study of the cerebral cortex was due to its involvement in PD even at early stages of the neurodegenerative process (Ferrer, 2009; Ferrer *et al*., 2011). The selection of FC area 8 and AG was based on neuroimaging observations showing that these areas are particularly vulnerable to PD (Choe *et al*., 2013; Tessa *et al*., 2012). The results obtained confirmed increased microglia, macrophages, and T cells in the SN, and an increase in microglia in the cerebral cortex in PD, matching previous observations and corroborating the importance of neuroinflammation in the pathogenesis of PD. The few predictable changes in mRNA expression levels in different tested regions showed a new, much more complex scenario than known until now. The different inflammatory responses including expression of pro- and anti- inflammatory cytokines and a considerable variety of mediators of the immune response were occurred simultaneously in different brain areas. One of the major difficulties that we found in this study of possible signalling in the human brain under physiological and pathological conditions was the information regarding the dynamics and mechanistic aspects of the disease and its progression. We are able to say with all the information obtained including previous studies that solubility alteration and α-synuclein aggregation, formation of Lewy bodies (LB), oxidative damage and neuroinflammation converged in the pathogenesis of PD. Furthermore, there was demonstrated to be concordance between the presence of LB and neurites, the variable solubility in the aggregates of α-synuclein, the proliferation of astrocytes and microglia, and the deregulation in the mRNA expression of cytokines and mediators of the immune response in

the SN in PD. The astrocytic and microglial responses were much more limited in the regions of the PUT, the FC, and the AG in PD, in parallel with the absence of LB and low formation of α-synuclein aggregates in these regions when they were compared with the SN in similar Braak stages. Despite all this, the meaning of the complex scenario of neuroinflammation that occurs in the human brain still remains hidden.

3.1 Neuroinflammatory mRNA levels

Deregulation of several genes involved in the inflammatory and immune responses has been demonstrated by using RT-qPCR. However, the diversity of inflammatory responses in the substantia nigra pars compacta, PUT, and cortical regions FC area 8 and AG at the same stages of disease progression reveals a new scenario that is more complex than formerly envisaged. Increased *IL-6ST* mRNA expression has been observed at early stages of PD-related pathology (stages 1-2 of Braak). Yet mRNA expression levels of several mediators of inflammation and immune response are significantly decreased in the SN at stages 3-5 of Braak; these include members of the complement system *C1QL1*, *C1QTNF7*, and *C3AR1*; *TLR7*; *IL-1B*, *IL-6*, *IL-6ST*, and *TNF-α*; *IL-10* and *IL-10β*; *TGF-β2*; and cathepsin C (*CTSC*) when compared with age-matched controls. This contrasts with previous observations reporting up-regulation of proinflammatory cytokines such as IL-1β in the SN in PD (Phani *et al*., 2012; Przedborski, 2007). Down-regulation of *C3AR1*, *TLR7*, and *TNF-α*, and up-regulation of *C1QTNF7* and *IL-RA*, have been observed in the PUT at stages 5 and 6. In contrast, down-regulation of *CSF3R* and *TLR4* mRNAs, together with up-regulation of *CTSS*, *CYBA*, *IL-10RA*, and *CLEC7A* mRNAs, is found in the FC. Finally, up-regulation of *C3AR1*, *CST7*, *CSF3R*, *IL-10RA*, and *ITGB2* mRNAs without accompanying modifications in the expression of genes which were down-regulated in other brain regions occurs in AG area 39. Taken together, the results of the study of the mRNA expression profile of cytokines and mediators of the immune response in PD reveal marked

regional variations, with expression levels markedly reduced in the SN, variably downregulated or up-regulated in the PUT and FC area 8, and up-regulated in the AG.

3.2 Uncorrelated mRNA and protein expression levels

Interestingly mRNA and protein expression did not correlate, at least in the FC, the only area examined for mRNA and protein. ELISA and WB showed a marked increase in IL-6 protein expression in PD, whereas IL-10 was reduced with ELISA, but not with WB. TNF-α, Iκβα, and IKK-α protein levels did not differ in PD from middle aged controls. Finally, immunohistochemistry reveals the presence of IL-5, IL-6, and IL-10; IL-17 receptor; and M-CSF and TNF-α protein expression in the PD substantia nigra and frontal cortex, while cytokines and mediators of the immune response are localized in neurons, astrocytes and microglia. Interleukines IL-5, IL-6, IL-17 receptor, and TNF-α are expressed in glial cells, mainly microglia, whereas IL-5, IL-10, M-CSF, and TNF-α are expressed in neurons. Active NFκβ is localized in the nucleus of subpopulations of neurons and glial cells mainly in SN and less frequently in PUT and cerebral cortex. These findings confirm and expand previous information describing the localization of cytokines and mediators of the immune response, including nuclear translocation of NF-κβ, in the SN in PD (Hunot *et al*., Katsuse *et al*., 2003; 1997; Nagatsu and Sawada, 2005).

3.3 Differences in mRNA levels and protein expression as seen with immunohistochemistry are particularly noteworthy in the substantia nigra

Down-regulation of the majority of genes is accompanied by increased expression of cytokines and mediators of the immune response in subpopulations of neurons, microglia, and astrocytes, thus indicating a complex modulation of neuroinflammation in the SN in PD. An important difficulty in the study of putative signaling in the human brain under physiological and pathological conditions is the information about dynamics and mechanistic aspects. Therefore, the significance of the

complex scenario of neuroinflammation in PD still remains elusive. For example, IL-6 in the nervous system acts mainly as a neurotrophic factor in normal conditions and in several experimental models, although it can also play a role as a pro-inflammatory cytokine (Erta *et al*., 2012). It may be speculated that the trend of increased *IL-6* mRNA in FC, accompanied by a significant increase in IL-6 protein as revealed by ELISA and WB, is consistent with a protective role of this interleukin in PD progression, but the opposite is also a possibility depending on the region and the accompanying responses. As another example, in spite of the reduced *IL-1β* mRNA expression here observed and the presumed lower levels of the encoded protein, phosphorylated (active) NFκβ is found in the nucleus of several neurons and glial cells in the SN in PD at stages 3-5 of Braak, thus indicating that NFκβ can play a role in neuroinflammation in the SN in PD (Sun and Andersson, 2002). However, NF-κβ is regulated by oxidative stress (Morgan and Liu, 2011), and reactive oxygen species may activate or inhibit NF-κβ activity (Peterson and Flood, 2012).

3.4 α-synuclein and oxidative damage role in neuroinflammation

The reasons for increased neuroinflammatory responses in PD are not known, but it has been shown that regional-specific microglial activation together with increased levels of *TNF-α* mRNA, but not of *IL-1β*, *TGF-β*, and certain toll-like receptors, occurs in the SN but not in the cerebral cortex in young mice overexpressing human wild-type α-synuclein (Watson *et al*., 2012). This is in line with pioneering studies showing that α-synuclein extracellular aggregates activate microglia in a primary mesencephalic neuron-glia culture system, and that microglial activation increases dopaminergic neurodegeneration induced by aggregated α-synuclein (Zhang *et al*., 2005). Astrocytes are also stimulated by α-synuclein *in vitro* (Klegeris et al., 2006).

The present studies in human brains have shown a relationship between the presence of LB and neurites, α-synuclein aggregates of variable

solubility, astrocyte and microglial proliferation, and deregulated mRNA expression of cytokines and mediators of the immune response in the SN in PD. Microglial and astrocytic responses are much more limited in the PUT, FC, and AG in PD in parallel with the absence of LB and the lower formation of α-synuclein aggregates in these regions when compared with the SN at similar Braak stages. Therefore, the present findings support the idea that altered α-synuclein including nitrated α-synuclein can trigger neuroinflammation in PD (Block and Hong, 2005). However, it is clear from the present data that α-synuclein cannot be considered simply as an activator of cytokine expression in PD, as the region with largest numbers of LB and abnormal α-synuclein aggregates (i.e. substantia nigra pars compacta) has the lowest up-regulation of cytokines and immune response mediators when compared with other brain regions (PUT, FC area 8, and AG). Other factors that can activate neuroinflammation in PD are related to the production of reactive oxygen and nitrogen species by neurons. Studies in the SN in PD have shown decreased levels of reduced glutathione (Perry *et al*., 1982; Sian *et al*., 1994; Zeevalk *et al*., 2008), increased Cu/Zn-superoxide dismutase I (SOD1), and Mn-superoxide dismutase (SOD2) protein and mRNA levels (Ceballos *et al*., 1990; Marttila *et al*., 1990), and increased levels of protein carbonyls (Alam *et al*., 1997; Floor and Wetzel, 1998), lipid hydroperoxides (Dexter *et al*., 1989), 4-hydroxy-2-nonenal (Shelley, 1998), as well as advanced glycation end products. Oxidative damage is not restricted to the SN in PD. Increased oxidative damage has been observed in the amygdala and cerebral cortex in inicidental PD and parkinsonian stages of PD without cognitive impairment (Dalfó *et al*., 2005; Ferrer, 2009; Floor and Wetzel, 1998).

Together, available information shows that altered α-synuclein solubility and aggregation, and LB formation, oxidative damage, and neuroinflammation, converge in the pathogenesis of PD, but it is difficult to reconcile a simple scenario of direct cause-effect mechanisms.

Regarding neuroinflammation, the present findings have important implications not only in the pathogenesis, but also in the therapeutics, as neuroinflammation involves pro- and anti-inflammatory cytokines, and variegated mediators of the immune response which, at least in the immune system, have distinct, often opposing functions. Moreover, neuroinflammatory responses are subject to regional variations at the same stages of PD-related pathology, implying that distinct inflammatory responses occur in different brain regions at the same time in particular individuals.

4. Purine metabolism deregulation in Parkinson's disease

This study was performed in order to gain understanding about purine metabolism regulation in different regions of the human brain (substantia nigra (SN), frontal cortex area 8 (FC) and putamen (PUT)) in PD based on the importance of purines in the normal, proper functioning of the brain, as well as to compare the results with those from previous studies done in AD (Ansoleaga *et al*., 2015) (Annex). According to these, the expression of 22 genes embracing different steps of purine metabolism that were altered in our previous studies in AD was selected for the present purposes. The results showed region- and stage- dependence in a large number of enzymes involved in the metabolism of purines in PD brains. Furthermore, the majority of the affected genes in the SN were not the same as those deregulated in the FC, which highlights the fact that in addition to regional and temporal differences in the vulnerability in PD, the altered patterns in the purine enzymes differed when comparing the variations observed in PD and in AD. Regarding the preliminary studies of WB to analyse protein levels of the encoding enzymes by the genes of purine metabolism, observed results were suboptimal, with significant intra-individual variation, probably related to the susceptibility of these proteins to the agonic state and the *post-mortem* delay in tissue processing, among other factors. The same was the case using immunohistochemical techniques in paraffin sections; for this reason this

technique was only used to determine the predominant localization of enzymes in specific cell types, because the staining intensity varied greatly from case to case in all groups and regions.

4.1 Deregulated purine metabolism genes is stage-dependent

The present findings show significantly reduced gene expression of *APRT*, *ENTPD1*, *ENTPD3*, *AK2*, *AK4*, *AK5*, *NME1*, *NME7*, and *PNP* in the SN at stages 3-6 of PD. In contrast, *ADA* mRNA is up-regulated in the FC area 8 at stages 3-4, and *PRUNE*, *NT5E*, *ENTPD1*, *AK1*, *AK2*, *AK5*, *NME4*, *NME5*, *NME6*, and *PNP* are up-regulated in FC area 8 at stages 5-6 of PD. No significant modifications in the expression of analysed genes were found in the PUT at PD stages 3-5, the only available stages for this region. The present findings show region-dependent alterations in the expression of several genes involved in purine metabolism in PD. In addition, stage-dependent deregulation is observed in the FC, the only area available for the study of disease progression.

4.2 Purine enzymes localization

NME1 mRNA is transcribed into nucleoside diphosphate kinase 1, *NME7* mRNA into nucleoside diphosphate kinase 7, *AK1* mRNA into adenylate kinase 1, and *PNP1* mRNA into nucleoside phosphorylase 1. All these enzymes were detected in neurons, in agreement with previous separate reports in different systems (Noma, 2005; Inouye *et al*., 1998; Lacombe *et al*., 2000; Janssen *et al*., 2004; Belcher *et al*., 2006; Appelbaum *et al*., 2007). Astrocytes are considered not only structural and trophic assistants of neurons but also modulators of several environmental elements of neurons (Kast, 2001). In this line, astrocytes are the essential origin of extracellular adenine-based nucleotides (Wink *et al*., 2006; Rathbone *et al*., 1999). Nucleoside diphosphate kinase 1, nucleoside diphosphate kinase 7, adenylate kinase 1, and nucleoside phosphorylase 1 were also expressed in astrocytes, in agreement with previous reports (Janssen *et al*., 2004; Zamzow *et al*., 2008). *ENTPD1* mRNA is transcribed into ectonucleoside triphosphate diphosphohydrolase 3

(*ENTPD3*), which has been reported as the main ectonucleotidase of microglia although we have not been able to confirm this unique localization. Finally, *NT5E* mRNA is transcribed into ecto-5-prime-nucleotidase, which we found was particularly expressed in microglia and vascular cells consistent with pericytes.

4.3 Purine metabolism in Parkinson's and Alzheimer's disease

Taken together, mRNA and protein studies help to shed light on possible implications of modified expression in different cell types. As *ENTPD3*, *NME1, NME7*, *AK1*, and *PNP1* are mainly expressed in neurons, down-regulation of these genes in the SN may be interpreted as a consequence of dopaminergic cell death. Expression of *NME1, NME7*, *AK1*, and *PNP1* in reactive astrocytes in the SN probably does not compensate for decreased mRNA expression due to the loss of neurons. On the contrary, gene up-regulation in the FC area 8 suggests a primary alteration or a compensation for altered purine metabolism in this region affected at advanced stages of PD. As only a few enzymes involved in purine metabolism have been analysed in the present study, altered expression of other factors cannot be excluded. The expression of the same series of genes was analysed in the entorhinal cortex and frontal cortex in sporadic AD at different Braak and Braak stages (Ansoleaga *et al*., 2015) (Annex). *APRT*, *DGUOK*, *POLR3B*, *ENTPD3*, *AK5*, *NME1*, *NME3*, *NME5,* and *NME7 were* down-regulated, while *ENTPD2* mRNAs were up-regulated in the entorhinal cortex of AD stages V-VI; no modifications in the mRNA expression levels of these genes were noted in the entorhinal cortex at stages III-IV. In contrast to AD entorhinal cortex, *ENTPD2*, *NME3*, *PNP,* and *PRUNE* mRNAs were significantly up-regulated in the AD FC at stages III-IV, whereas *AK5* mRNA was down-regulated and *PRUNE* mRNA up-regulated in the FC at AD stages V-VI (Ansoleaga *et al*., 2015). Differences in gene expression between AD and PD represent disease-dependent alterations of genes encoding purine metabolism-related enzymes.

These results show that (i) genes involved in purine metabolism are differentially regulated in PD and sAD at least in the FC, the only region analysed in both PD and AD brains; (ii) alteration in the expression of purine-related genes increases with disease progression; (iii) reduced gene expression occurs in the SN in PD and in the entorhinal cortex in AD, which are the most vulnerable analysed regions in these diseases, respectively; and (iv) in contrast, the expression of genes involved in purine metabolism is increased in the FC although with a different pattern in PD and sAD. As PD cases analysed in the present series have concomitant sAD-related pathology up to stage IIB of Braak and Braak, changes in the FC in PD cannot be attributed to concomitant sAD-related pathology. As a corollary, purine metabolism changes appear to be a late effect in both diseases as they appear in the FC only after significant infiltration of pathologies in these regions. Yet mRNA targets related to purine metabolism differ in PD and AD.

Present observations show for the first time modifications in the expression of genes involved in purine metabolism in a selected series of cases with PD-related pathology and minimal or absent concomitant neurological pathologies. No comparative studies between regions in the same individuals have been possible because of the very limited numbers of cases in which the three regions analysed were available. However, comparative analysis between controls and diseased brains has shown regional differences in gene expression among the SN, PUT, and FC area 8 in PD. Furthermore, changes are modified with disease progression, at least in FC, the only region with sufficient numbers of samples for study. When comparing these data with those obtained in AD, the affected genes differed from one disease to another, but gene down-regulation occurred in the substantia nigra and entorhinal cortex, two regions with significant cell loss, respectively in PD and AD, whereas gene up-regulation occurred in the frontal cortex in both conditions. As proteins encoded by affected genes are expressed in neurons or in glial cells or in both neurons and astrocytes, the present findings further

support a complex scenario of specific altered regulation of purine metabolism in PD.

5. New signalling pathways: olfactory and taste receptors in PD

The study of olfactory receptors (ORs), taste receptors (TRs), obligate downstream components of OR function, transporters, and receptor transporters provided valuable information regarding the human brain. For starters, this was the first identification of the presence of all of these elements widely distributed throughout the CNS including among other regions the cerebral cortex, the thalamus, selected nuclei of the brainstem, and the Purkinje cells of the cerebellum, demonstrating that the receptor machinery was present and able to function. In addition, the possibility of talking about a new signalling pathway in the human brain was opened. We should stress the fact that an interesting down-regulation of smell receptors was found especially in males compared to females, showing gender differences that have already been widely studied and recognized in relation to olfactory signalling. Furthermore, these results lend support to the hypothesis that alteration of cortical ORs could have implications in the pathogenesis of Parkinson's or Alzheimer's disease, and these could be considered as future disease biomarkers.

5.1 Wide distribution of OR, TRs, down-stream obligate mediators and receptor transporters in the human brain

The present study reveals that ORs are widely distributed in the human CNS, including cerebral cortex, thalamus, selected nuclei of the brainstem, and Purkinje cells of the cerebellum. Interestingly, certain nuclei of the brainstem such as the motor nuclei of the third cranial nerve, substantia nigra, and pontine nuclei, together with Purkinje cells, showed strong OR expression at least for the ORs examined (OR2H2, OR2A4, and OR6K3); within the cerebral cortex, the greatest expression occurred not only in the CA1 area and hilus of the hippocampus but also in the entorhinal cortex and neocortical areas examined. Central OR neuronal expression is accompanied by the obligate functional molecules of the

OR pathway in sensory olfactory neurons, AC3, and $G_{\alpha olf}$, indicating that the OR machinery is also able to operate, at least, in selected regions of the CNS and, particularly, in the cerebral cortex. Olfactory receptor and associated molecules were overwhelmingly localized in neurons; their presence in a few glial cells was unusual. It is noteworthy that the cytoplasmic localization obtained by cell subfractionation of the various components (OR2H2 and AC3) of study was consistent with the idea that cortical receptors required specific mechanisms to facilitate the translocation of the ORs from the cytosol to the membranes. This localization is consistent with the idea that cortical ORs may require specific mechanisms to facilitate OR translocation from the cytosol to the membranes (Saito *et al*., 2004; Behrens *et al*., 2006). According to this rationale, cofactors of G protein-coupled receptors bind to specific domains of receptors, thus facilitating or inhibiting cell surface expression (Duvernay *et al*., 2009). Receptor transporters RTP1 and RTP2, and REEP1, facilitate OR recruitment to lipid rafts (Saito *et al*., 2004). The present observations show RTP1, RTP2, and REEP1 in the cytoplasm of the vast majority of, if not all, neurons in parallel with the expression of ORs in different regions, thereby revealing that molecules specifically expressed in sensory olfactory neurons are also present in cerebral cortex and other regions of the human brain, enabling the traffic of ORs from the cytosol to the cellular membranes. UDP-glucuronosyltransferases are enzymes localized in the endoplasmic reticulum and nuclear membranes involved in the detoxification of various xenobiotic and endobiotic compounds through glucuronidation of substrates, thereby facilitating their excretion (Tukey and Strassburg, 2000; Mackenzie *et al*., 2005). Several UGTs, including UGT1A6, have been reported in the olfactory epithelium and olfactory bulb (Leclerc *et al*., 2002) and, more recently, in the olfactory cortex of rodents, where they appear to participate in both detoxification and olfactory signal termination (Heydel *et al*., 2010). Here, we show that UGT1A6 is expressed in neurons of the human cerebral cortex and may be

considered as a candidate to assist in the detoxification of chemicals triggering cortical OR activation.

5.2 Olfactory receptors function

As of now, we have no precise idea about the role of ORs in the human cerebral cortex and what might be the nature of the putative ligands of cortical ORs or the routes that ligands may use to contact the corresponding ORs in the cerebral cortex. Several natural ligands have been identified in mammals (Robertson *et al*., 1993; Glasgow *et al*., 1995; Marchese *et al*., 1998; Scaloni *et al*., 2011; Briand *et al*., 2004; Sanz *et al*., 2005; Le Danvic *et al*., 2009). These are small molecules of approximately 200 to 400 molecular weight related to steroids, fatty acid derivatives, and various terpenoid compounds (Le Danvic *et al*., 2009; Hsu et al., 2008). However, it is not known whether central ORs are functionally related to their putative counterparts within the sensory olfactory neurons. It is possible that putative exogenous ligands to ORs in the human brain are transported through the blood vessels and the extracellular space. Alternatively, brain ORs might be stimulated by local chemicals from neighboring cells, or even from the same cells, thus having a self-stimulating mechanism regulating internal trafficking. In line with these hypotheses, ORs are involved in functions other than olfactory signal recognition, including axonal guidance (Feinstein *et al*., 2004; Barnea *et al*., 2004), cell assembly functions in the embryonic period (Dreyer, 1998), muscle regeneration, and regulation of cell adhesion and migration (Griffin *et al*., 2009; Kang and Noo, 2012). Olfactory receptor expression in the testes has been implicated in sperm chemotaxis (Spehr *et al*.,2004; Fukuda *et al*., 2004). Regarding the nervous system, expression of ORs in ganglia of the autonomic nervous system has lent support to the idea that they serve functions other than odorant detection (Weber *et al*., 2002), including detection of endogenously produced chemicals in their vicinity (Otaki *et al*., 2003). $G_{\alpha olf}$ is expressed in different brain regions in the murine brain (Belluscio *et al*., 1998; Hervé *et*

al., 1995). It is worth noting that surviving homozygous mice with null mutation in Gαolf exhibit, in addition to reduced olfaction, hyperactive behaviors. These behavioral phenotypes suggest that Gαolf may also function as an essential signaling molecule more centrally in the brain (Belluscio *et al*., 1998).

5.3 ORs and TRs mRNA expression in the human brain

Results from mRNA arrays and TaqMan PCR analysis have relevant implications. First, dysregulation of ORs in FC, selected because of the amount of newly identified molecular alterations in this area in PD and related α-synucleinopathies (Ferrer *et al*., 2012; Ferrer *et al*., 2011), appears at relatively early stages of the neurodegenerative processes, along with other molecular changes (Ferrer, 2009; Ferrer *et al*., 2011), and precedes the presence of α-synuclein aggregates. Second, OR dysregulation is not related to medication because it is found in patients who have not received drug therapy. Third, there is a predominant OR down-regulation in PD, which seems not to be a consequence of a more general down-regulation of other central chemoreceptors, because certain TASRs appear to be upregulated in the same region at the same stages of the process in the same cases. Fourth, down-regulation of certain ORs in PD is probably sex-dependent, because a significant decrease in the expression of at least some genes is found mostly in men but not in women. Unfortunately, lack of appropriate antibodies against the products of dysregulated genes did not permit the validation of mRNA dysregulation at the protein level. Sex differences have been largely recognized in relation to olfactory signaling (Stowers and Logan, 2010). These are partly caused by a sexually dimorphic olfactory response despite similar olfactory circuits, suggesting that sex-specific initial signals prompt divergent behaviors (Haga *et al*., 2010). This may be caused by specific sex responses to sexually dimorphic specific ligands (Holy *et al*., 2000; Ben-Shaul *et al*., 2010) and to the imprinting of sex-dimorphic sensory olfactory modulation by hormones (Stowers and

Logan, 2010). The present findings indicate sex-specific vulnerability of certain ORs in PD, thus suggesting that at least a subpopulation of cortical ORs is modulated by steroid hormones or their derivatives. The fact that both ORs and TRs are deregulated in the cerebral cortex and in the SN suggests that the olfactory and taste impairment in PD could be the result of more widespread disorder. These changes are already seen in the early stages of neurodegeneration.

These findings lead the idea that the ORs and TRs might well be destined for physiological roles, suggesting a new scenario in the as yet little known chemical signalling in the brain. Finally, further collaborations have demonstrated expression of ORs, and the response to odorant molecules in midbrain DA neurons as well as substantial changes in AD and Creutzfeldt-Jakob disease (CJD) are increasingly opening the doors of knowledge to this new signalling pathway (Annex).

6. Molecular pathology in DLB and rpDLB

The present study was undertaken to identify alterations of several metabolic pathways which may participate in the pathogenesis of Dementia with Lewy bodies (DLB) and rapidly progressive Dementia with Lewy bodies (rpDLB) including mitochondrial function and energy metabolism, purine metabolism, protein synthesis machinery, neuroinflammation, and certain recently discovered ectopic olfactory and taste receptors expressed in brain. The focus of the study was to learn about biochemical alterations beyond the well-known modifications of target proteins in DLB such as α-synuclein and β-amyloid. The results obtained in both DLB and rpDLB showed for the first time a wide range of different metabolic pathways linked with both pathologies. These affected metabolic pathways include differences at the mRNA and especially at the protein level in the mitochondria and energy metabolism followed by variations in the protein synthesis machinery. Importantly, altered expression of proteins involved in transcription in the ribosome was found more severe in rpDLB than in DLB. On the other hand, differences in the

fold change obtained in mRNA and protein of TNF-α in the study of the inflammatory responses showed that the up-regulation obtained in rpDLB when compared to DLB might have functional implications due to the involvement of TNF-α in several metabolic pathways. Significant alterations shown by RT-qPCR in purine metabolism suggested the influence of this pathway in the variegated functions including modulation of synaptic transmission, ATP-mediated propagation of calcium waves in glial cells, neurogenesis, microglial function, and blood flow. Regarding olfactory and taste brain receptors, an increase in DLB and in rpDLB, more marked in the rapid form of the disease, was revealed to be an interesting point for further investigation of the role of these receptors and their obligate mediators in the brain. Finally, increased levels in rpDLB when compared to middle-aged (MA) and DLB cases were obtained in soluble Aβ40 and Aβ42, while membrane associated β-amyloid and α-synuclein oligomers showed an important rise in both DLB and rpDLB when compared to MA. Therefore, rpDLB has similar biochemical profiles regarding the several pathways analysed in comparison with DLB. However, two traits are substantially different in rpDLB when compared with DLB. The first is that the important levels of soluble Aβ40 and Aβ42 can precipitate disease progression in rpDLB due to that these oligomeric species can be toxic to nerve cells. The second differential aspect is that the elevated levels of TNF-α in rpDLB could be a potential biomarker, so the use of inhibitors might be considered a putative instrument to mitigate TNF-α associated harmful effects in rpDLB.

6.1. Mitochondria and energy metabolism alterations

Mitochondrial alterations in the frontal cortex area 8 (FC) are prominent in DLB. *ATP5G2* and *ATP5H* expression is decreased in DLB when compared with MA individuals; protein expression of NDUFA7, NDUFA10, NDUFB8, SDHB, UQCRC2, MTCO1, ATP5A, and ATP50 is reduced; and rpDLB shows distinct profiles as *NDUFS8* and *UQCRB*

mRNAS are up-regulated in rpDLB. NDUFA7 protein levels are significantly reduced in rpDLB, while significant differences between DLB and rpDLB are restricted to NDUFB8, SDHB, ATP5A, and ATP50. It is worth stressing that these alterations were not the mere consequence of mitochondrial loss in DLB. Expression levels of voltage-dependent anion channel (VDAC) are decreased in DLB but the expression levels of the mentioned subunits are reduced even considering VDAC for normalization of protein levels. It can be argued that the rapid course of the disease results in a reduced harmful impact on mitochondria in rpDLB when compared with DLB. Despite differences in gene and protein expression, mitochondrial enzymatic activity of complexes I, II, III, and IV is significantly decreased in FC area 8 in DLB and rpDLB. Therefore, the present observations point to altered mitochondrial function in FC as a major factor in the pathogenesis of DLB and rpDLB. Regarding energy metabolism, only *ATP4A* and *SLC6A6* are up-regulated in DLB, while *ATP6V0B* is down-regulated in DLB and rpDLB. *ATP4A* encodes a membrane-bound P-type ATPase, which permits ion transport through cell membranes (Singh *et al*., 2013). *SLC6A6* encodes a taurine transporter (SLc6a6/TauT) involved in the uptake of gamma-aminobutyric acid (GABA) (Tomi *et al*., 2008). Whether these changes have implications in GABA metabolism in DLB is not known although GABA levels in the cerebrospinal fluid (CSF) in DLB are not altered when compared with normal individuals (Molina *et al*., 2005). *ATP6V0B* encodes ATPase H+ transporting V0 subunit b, which is involved in protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation (Lu *et al*., 2007). Together, these results suggest energy metabolism impairment in frontal cortex in DLB and rpDLB.

6.2. Purine metabolism

Several genes encoding enzymes linked to purine metabolism are up-regulated in DLB and rpDB although significant values are only obtained

when comparing rpDLB with MA cases. These include *ADA*, *AK1*, *AK2*, *AK4*, *ENTPD1*, *NME1*, *NME4*, *NME6*, *NT5E*, and *PRUNE*. However, only two genes, *DGUOK* and *NME1*, are significantly up-regulated in rpDLB when compared with DLB. *ENTPD2* is down-regulated in DLB but not in rpDLB. Purines and pyrimidines are the core of DNA, RNA, nucleosides, and nucleotides. Nucleotides are involved in cell signalling and energy metabolism, and purine bases are also cofactors of several enzymatic reactions (Ribeiro *et al*., 2003; Boison, 2008; Burnstock *et al*., 2011; Ipata *et al*., 2011). Adenylate kinases (AKs) participate in the phosphorylation of AMP to ADP and dAMP to dATP (Noma 2005; Dzeja and Terzic 2009). The NME gene family encodes nucleotide diphosphate kinases which are involved in the phosphorylation of nucleotide diphosphates to form nucleotide triphosphates. These enzymes are enriched in synapses. Increased NME mRNA expression in DLB contrasts with its decrease in AD (Kim *et al*., 2000; Ansoleaga *et al*., 2015), and suggests a compensatory role of NME in response to reduced synapses in DLB. The product of PRUNE participates in the metabolism of guanosine pentaphosphate and tetraphosphate and is linked to NME in memory conservation (Li *et al*., 2012). Deoxyguanosine kinase (encoded by *DGUOK*) phosphorylates purine deoxyribonucleosides in the mitochondrial matrix (Jüllig and Eriksson 2000; Johansson *et al*., 2001; Desvignes *et al*., 2009). Adenine phosphoribosyl-transferase is related to adenine metabolism and catalyses the phosphorylation reaction, whereas ADA de-aminates adenosine. Further, ecto-nucleoside triphosphate diphosphohydrolases, encoded by *ENTPD* genes, hydrolyze the terminal phosphate group of nucleoside tri- and di-phosphates to form di- and mono-phosphates, thus controlling extracellular ATP concentrations (Yegutkin, 2008), adenosine-activated type I receptors, nucleotide-activated type 2 ligand-gated ion channels, and metabotropic P2Y receptors (Al-Rashida *et al*., 2014). Finally, ecto 5'-nucleotidase (*NT5E*) catalyzes the generation of adenosine from degradation of AMP in the extracellular space. In brain, ectonucleotidases are involved in variegated

functions including modulation of synaptic transmission, ATP-mediated propagation of calcium waves in glial cells, neurogenesis, microglial function, and blood flow (Zimmermann, 2006). Present findings show marked alterations in the expression of enzymes involved in purine metabolism in the frontal cortex in DLB and rpDLB.

6.3. Protein synthesis

Decreased mRNA expression of nuclear chaperones such as nucleolin (*NCL*), nucleoplasmin 3 (*NPM3*), and upstream binding transcription factor (*UBTF*) is found in the frontal cortex in DLB. Altered expression of *NCL*, *NPM3*, and *UBTF* is indicative of nucleolar stress, which may alter ribosomal biogenesis (Olson *et al*., 2004; Boulon *et al*., 2010; Baltanas *et al*., 2011; Hetman and Pietrak, 2012; Parlato and Kreiner, 2013). Alterations in the expression of genes encoding ribosomal proteins are seen in DLB and rpDLB, including up- and down-regulation of RPLs, thus suggesting impaired ribosome biogenesis (Granneman and Baserga, 2004; Klein *et al*., 2004; Kressler *et al*., 2010; Korobeinikova *et al*., 2012). This is accompanied by reduced protein expression of several initiation factors of transcription at the ribosome, which is more marked in rpDLB than in DLB. However, the expression levels of elongation factors eEF1A and eEF2 are preserved in DLB and rpDLB. Although the direct study of protein synthesis is not possible in human samples due to *post-mortem* delay between death and tissue processing, the present findings indicate that the machinery of protein synthesis is altered in DLB (Kapp and Lorsch, 2004; Jackson *et al*., 2010; Voorhees and Ramakrishnan, 2013). Importantly, altered expression of proteins involved in transcription in the ribosome is more severe in rpDLB than in DLB.

6.4. Inflammatory responses

No significant differences in gene expression of several cytokines and mediators of the inflammatory response are seen in DLB. These data are in line with the observation of no major increase in Iba1 protein levels and CD68 expression in FC in DLB when compared with MA individuals.

They are also in agreement with previous observations showing very limited activation of microglia in DLB (Shepherd *et al*., 2000; Streit and Xue, 2016). Subtle change refers to *TNF-α* gene expression in rpDLB. Whereas *TNF-α* mRNA is not altered in DLB, significant *TNF-α* mRNA up-regulation and increased TNF-α protein levels occur in the FC in rpDLB. TNF-α is involved in several metabolic pathways, particularly facilitating gene transcription, activating of the JNK pathway, and promoting apoptosis via caspase-dependent and caspase-independent signaling (Thomson and Lotze, 2003). Therefore, *TNF-α* up-regulation in rpDLB may have functional implications. Further molecular studies are needed to elucidate activated molecules of the TNF-α pathways in rpDLB.

6. 5. Olfactory and taste brain receptors

Olfactory and taste receptors (ORs and TASRs, respectively) are widely expressed in human and rodent brain including the cerebral cortex. ORs and TASRs in the brain are accompanied by all downstream molecules that permit a functional signaling pathway, and they are functional, as revealed in culture neurons under appropriate stimuli (Garcia-Esparcia *et al*., 2013; Grison *et al*., 2014). The expression of several genes encoding ORs is increased in DLB and rpDLB, and is more marked in the rapidly progressive form of the disease in which six of thirteen assessed genes are up-regulated. A similar up-regulation occurred for TASRs; five of six genes in DLB and all six genes are up-regulated in rpDLB. The function of ORs and TASRs in brain is not known although it has been postulated that they may participate in intra- and extra-cellular signaling in association, or not, with other receptors. Identification of natural ligands of brain ORs and TASRs should yield insights about the function of these ectopic receptors (Garcia-Esparcia *et al*., 2013).

6.6. Mitochondrial function, inflammation, and deregulation of olfactory and taste receptors in frontal cortex discriminate DLB and AD

Several studies have shown common pathogenic pathways in AD and DLB (Guerreiro *et al*., 2016; Sanchez-Mut *et al*., 2016). Commonly affected pathways include mitochondrial function and energy metabolism, oxidative stress damage, protein synthesis, altered responses to aggregated proteins, impaired protein degradation by the UPS and autophagy, and inflammation, among others. However, the present study reveals disease-specific alterations when comparing the present results in DLB with available data for AD in the same region, the FC, at similar stages of disease progression. Mitochondrial alterations and impaired activity of complex V are early events in the entorhinal cortex in AD (Terni *et al*., 2010). *NDUFA2*, *NDUFB3*, *UQCR11*, *COX7C*, *ATPD*, *ATP5L*, and *ATP50* gene expression is reduced in the entorhinal cortex with disease progression and this is accompanied by impaired activity of complexes I, II, and V (Armand-Ugon *et al*., 2016) (Annex). In contrast, mRNA expression levels of several subunits of complexes I-V and enzymatic activities of complex I, II, IV, and V are preserved in FC in AD even at stages V-VI of Braak (Armand-Ugon *et al*., 2016). Therefore, mitochondrial dysfunction markedly differs in AD and DLB, with detrimental effects in most mitochondrial complexes in DLB in comparison with AD. There is a large body of information demonstrating microglial responses and increased expression of inflammatory markers in the cerebral cortex in AD (Griffin *et al*., 1998; McGeer and McGeer, 2001; Streit *et al*., 2001; Akiyama *et al*., 2010; Graeber and Streit, 2010; Lee *et al*., 2010; Eikelenboom *et al*., 2010; Rubio-Perez and Morillas-Ruiz, 2011; Leung *et al*., 2011; Morimoto *et al*., 2011; McGeer and McGeer, 2013; Prokop *et al*., 2013; Heppner *et al*., 2015; Heneka *et al*., 2015; López-González *et al*., 2015; López-González *et al*., 2016). In contrast, inflammatory responses are very limited in DLB. Therefore, inflammation is characteristically an important factor in AD pathogenesis

whereas it has low impact in DLB. Regarding purine metabolism, *ENTPD2*, *NME3*, *PNP*, and *PRUNE* mRNAs are deregulated in the FC in AD cases at the same stages of AD-related pathology as those found in the present DLB series (Ansoleaga *et al*., 2015). Severe alterations of the machinery involved in protein synthesis from the nucleolus to the ribosome have been observed in the entorhinal cortex in AD (Hernández-Ortega *et al*., 2016) (Annex), but no similar data are available in AD FC at β-amyloid phases and AD Braak stages of NFT pathology corresponding to those assessed in DLB. Finally, *OR4F4* mRNA expression levels are increased in FC area 8 at stages III-IV, and *OR52L1* mRNA at stages III-IV and V-VI (Ansoleaga *et al*., 2013) (Annex). Regarding TASRs, no modification in the mRNA expression levels is observed in FC at any stage of AD (Ansoleaga *et al*., 2013). These changes are in contrast with the extensive deregulation of ORs and TASRs in the FC in DLB, thus indicating marked differences in the regulation of these still poorly understood brain receptors.

6.7. Specific traits in rpDLB compared with DLB

No neuropathological differences are seen in DLB and rpDLB, as have been reported in other studies (Gaig *et al*., 2011). rpDLB has similar biochemical profiles regarding mitochondrial gene and protein expression, purine metabolism, protein synthesis, and expression of olfactory and taste receptors. Gene and protein expression of subunits of mitochondrial respiratory complexes are more pronounced in DLB when compared with rpDLB, whereas alterations in purine metabolism and initiation of protein translation is more pronounced in rpDLB when compared with DLB. However, two traits are substantially different in rpDLB when compared with DLB. The first of these is the higher levels of soluble Aβ40 and Aβ42 in rpDLB in spite of similar plaque burden and β-amyloid associated with membranes in rpDLB and DLB. In this line, modified characteristics of Aβ42 have been described in rapidly progressive AD (rpAD) (Cohen *et al*., 2015), thus suggesting that β-

amyloid conformers pace disease progression in AD. Higher levels of soluble β-amyloid oligomeric species, known to be toxic to nerve cells (Lambert *et al*., 1998; Selkoe, 2002; Klein, 2002; Gandy *et al*., 2010; Hayden *et al*, 2013; Viola and Klein, 2015), can precipitate disease progression in rpDLB. Therefore, procedures geared to reducing soluble β-amyloid species (Klein *et al*., 2001; Hefti *et al*., 2013) seem to be appropriate methods to slow down disease progression in DLB cases with rapid course. The second differential aspect is the higher levels of TNF-α in rpDLB when compared with DLB. Whether this factor influences disease progression needs clinical confirmation. Following this rationale, the use of specific TNF-α inhibitors (Murdaca *et al*., 2009; Ozer and Ozbalkan, 2010) can be considered a putative instrument to mitigate TNF-α associated harmful effects in rpDLB that were once considered to be pre-treatment conditions and possible side-effects.

Concluding remarks

Our studies, among others, have shown that in spite of the absence or the relatively small numbers of LB and LN in the cerebral cortex in PD, DLB, and rpDLB until advanced stages of the disease, there is a plethora of molecular alterations, including: altered synaptic modulation and transmission, altered machinery of protein synthesis, mitochondrial dysfunction, oxidative stress damage, reduced energy metabolism, increased inflammatory responses, altered purine metabolism, and abnormal expression of receptors whose function is still poorly understood. These alterations are region- and stage- dependent, converge in the most vulnerable regions of the cerebral cortex, and extend to other regions with disease progression.

CONCLUSIONS

The findings revealed in this thesis allow us to draw the following conclusions:

General conclusion

1. Molecular alterations including damage to the protein synthesis machinery, mitochondria and energy metabolism, neuroinflammation and immune response system, purine metabolism, and new signalling pathways of olfactory and taste receptors are underlying functional cerebral changes in *post-mortem* human brain samples of Parkinson's disease, Dementia with Lewy bodies, and rapidly progressive Dementia with Lewy bodies.

Machinery of protein synthesis in PD

2. Altered machinery of protein synthesis is region- and stage-dependent and is associated with α-synuclein oligomers in Parkinson's disease.

3. Altered protein synthesis is targeted especially in the substantia nigra and in the cerebral cortex in Parkinson's disease at middle and advanced stages of the disease at mRNA and protein levels.

Mitochondria and energy metabolism in PD

4 Altered regulation of genes and proteins related to mitochondria and energy metabolism is an early region-dependent event in the frontal cortex and angular gyrus within the spectrum of Parkinson's disease.

5. Parkinson's disease and especially dementia in the context of Parkinson's disease are linked to region-specific deregulation of genomic genes encoding subunits of mitochondrial complexes and energy metabolism-related enzymes, as well as to changes in the activity of mitochondrial complexes I, II, III, and IV.

6. Mitochondrial function may be affected by the presence of α-synuclein oligomers, as demonstrated by mitochondrial-enriched fractions in PD.

Neuroinflammation in PD

7. Complex deregulation and expression of cytokines and mediators of the immune response is region- and stage-dependent in Parkinson's disease.

8. Localisation of intrinsic neuroinflammatory response in neurons and glial cells suggests intrinsic neuroinflammatory cross-information among neurons,

astrocytes, and microglia in PD with limited participation of the peripheral immune system.

9. Altered α-synuclein solubility and aggregation, and Lewy body formation, oxidative damage, and neuroinflammation converge in the pathogenesis of PD.

Purine metabolism in PD

10. Purine metabolism deregulation is region- and stage-dependent in Parkinson's disease.

11. Proteins encoded by affected genes are expressed in neurons or in glial cells or in both neurons and astrocytes, supporting a complex scenario of specific altered regulation of purine metabolism in Parkinson's disease.

New signalling pathways: Olfactory and taste receptors

12. Olfactory and taste receptors and obligate downstream functional mediators are present in the human brain and differentially regulated in Parkinson's disease at premotor and parkinsonian stages in frontal cortex area 8.

13. ORs and probably TASRs, widely distributed in the human brain, may play key physiologic roles, and suggest a new scenario in the chemical signalling system of the brain.

Molecular alterations in DLB and rpDLB

14. Several biochemical alterations, including mitochondria and energy metabolism abnormalities, purine metabolism, protein synthesis, inflammatory responses, and new signalling pathway modifications, converge in the pathogenesis of Dementia with Lewy bodies and its rapidly progressive form.

15. Altered mitochondrial function and energy metabolism impairment in the frontal cortex appear to be major factors in the pathogenesis of DLB and rpDLB.

16. Higher protein levels of soluble Aβ40 and Aβ42 in rpDLB, as well as higher levels of mRNA *TNF-α* when compared with DLB are two traits that distinguish rpDLB from DLB, and may have functional implications.

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ANNEX

ARTICLE

Dysregulation of brain olfactory and taste receptors in AD, PSP and CJD, and AD-related model.

Ansoleaga B, Garcia-Esparcia P, Llorens F, Moreno J, Aso E, Ferrer I.

Neuroscience 2013 Sep 17; 248:369-82.

ABSTRACT

Introduction: Recently, we have shown the expression of novel chemoreceptors corresponding to the olfactory receptor (OR) and taste receptor (TASR) families in the human brain. We have also shown dysregulation of ORs and TASRs in the cerebral cortex in Parkinson's disease. **Results:** The present study demonstrates the presence of OR mRNA and mRNA of obligated downstream components of OR signalling adenylyl cyclase 3 (ADYLC3) and olfactory G protein (Gnal) in the cerebral cortex of the mouse. Dysregulation of selected ORs and TASRs has been found in the entorhinal cortex and frontal cortex in Alzheimer's disease (AD) in a gradient compatible with Braak and Braak staging; frontal cortex in terminal stages of Progressive Supranuclear Palsy; and frontal cortex and cerebellum in Creutzfeldt-Jakob disease subtypes methionine/methionine at codon 129 of PRNP (MM1) and valine/valine at codon 129 of PRNP (VV2). Altered OR, ADYLC3 and Gnal mRNA expression with disease progression has also been found in APP/PS1 transgenic mice, used as a model of AD. **Conclusions:** The function of these orphan receptors is not known, but probably related to cell signalling pathways responding to unidentified ligands. Variability in the drift, either down- or up-regulation, of dysregulated genes, suggests that central ORs and TASRs are vulnerable to variegated neurodegenerative diseases with cortical involvement, and that altered expression of ORs and TASRs is not a mere reflection of neuronal loss but rather a modulated pathological response.

ARTICLE

PrP mRNA and protein expression in brain and PrP(c) in CSF in Creutzfeldt-Jakob disease MM1 and VV2.

Llorens F, Ansoleaga B, Garcia-Esparcia P, Zafar S, Grau-Rivera O, López-González I, Blanco R, Carmona M, Yagüe J, Nos C, Del Río JA, Gelpí E, Zerr I, Ferrer I.

Prion 2013 Sep-Oct; 7(5):383-93.

ABSTRACT

Introduction: Creutzfeldt-Jakob disease (CJD) is a heterogenic neurodegenerative disorder associated with abnormal post-translational processing of cellular prion protein (PrP(c)). CJD displays distinctive clinical and pathological features which correlate with the genotype at the codon 129 (methionine or valine: M or V respectively) in the prion protein gene and with size of the protease-resistant core of the abnormal prion protein PrP (sc) (type 1: 20/21 kDa and type 2: 19 kDa). MM1 and VV2 are the most common sporadic CJD (sCJD) subtypes. **Results and conclusions:** PrP mRNA expression levels in the frontal cortex and cerebellum are reduced in sCJD in a form subtype-dependent. Total PrP protein levels and PrP (sc) levels in the frontal cortex and cerebellum accumulate differentially in sCJD MM1 and sCJD VV2 with no relation between PrP (sc) deposition and spongiform degeneration and neuron loss, but with microgliosis, and IL6 and TNF-α response. In the CSF, reduced PrP(c), the only form present in this compartment, occurs in sCJD MM1 and VV2. PrP mRNA expression is also reduced in the frontal cortex in advanced stages of Alzheimer disease, Lewy body disease, progressive supranuclear palsy, and frontotemporal lobe degeneration, but PrP(c) levels in brain varies from one disease to another. Reduced PrP(c) levels in CSF correlate with PrP mRNA expression in brain, which in turn reflects severity of degeneration in sCJD.

ARTICLE

Mesencephalic dopaminergic neurons express a repertoire of olfactory receptors and respond to odorant-like molecules.

Grison A, Zucchelli S, Urzì A, Zamparo I, Lazarevic D, Pascarella G, Roncaglia P, Giorgetti A, Garcia-Esparcia P, Vlachouli C, Simone R, Persichetti F, Forrest AR, Hayashizaki Y, Carloni P, Ferrer I, Lodovichi C, Plessy C; FANTOM Consortium, Carninci P, Gustincich S.

BMC Genomics 2014 Aug 27; 15:729.

ABSTRACT

Introduction: The mesencephalic dopaminergic (mDA) cell system is composed of two major groups of projecting cells in the Substantia Nigra (SN) (A9 neurons) and the Ventral Tegmental Area (VTA) (A10 cells). Selective degeneration of A9 neurons occurs in Parkinson's disease (PD) while abnormal function of A10 cells has been linked to schizophrenia, attention deficit and addiction. **Results:** By taking advantage of transgenic labeling, laser capture microdissection coupled to nano Cap-Analysis of Gene Expression (nanoCAGE) technology on isolated A9 and A10 cells, we found that a subset of Olfactory Receptors (OR) is expressed in mDA neurons. Gene expression analysis was integrated with the FANTOM5 Helicos CAGE sequencing datasets, showing the presence of these ORs in selected tissues and brain areas outside of the olfactory epithelium. OR expression in the mesencephalon was validated by RT-qPCR and in situ hybridization. By screening 16 potential ligands on 5 mDA ORs recombinantly expressed in a heterologous in vitro system, we identified carvone enantiomers as agonists at Olfr287 and able to evoke an intracellular Ca2+ increase in solitary mDA neurons. ORs were found expressed in human SN and down-regulated in PD post mortem brains. **Conclusions:** Our study indicates that mDA neurons express ORs and respond to odor-like molecules providing new opportunities for pharmacological intervention in disease.

ARTICLE

Decrease in olfactory and taste receptor expression in the dorsolateral prefrontal cortex in chronic schizophrenia.

Ansoleaga B, Garcia-Esparcia P, Pinacho R, Haro JM, Ramos B, Ferrer I.

Journal Psychiatric Reserch 2015 Jan; 60:109-16.

ABSTRACT

Introduction: We have recently identified up- or down-regulation of the olfactory (OR) and taste (TASR) chemoreceptors in the human cortex in several neurodegenerative diseases, raising the possibility of a general deregulation of these genes in neuropsychiatric disorders. In this study, we explore the possible deregulation of OR and TASR gene expression in the dorsolateral prefrontal cortex in schizophrenia. We used quantitative polymerase chain reaction on extracts from postmortem dorsolateral prefrontal cortex of subjects with chronic schizophrenia (n = 15) compared to control individuals (n = 14). Negative symptoms were evaluated *premortem* by the Positive and Negative Syndrome and the Clinical Global Impression Schizophrenia Scales. **Results:** We report that ORs and TASRs are deregulated in the dorsolateral prefrontal cortex in schizophrenia. Seven out of eleven ORs and four out of six TASRs were down-regulated in schizophrenia, the most prominent changes of which were found in genes from the 11p15.4 locus. However, most ORs and all TASRs inversely associated with the daily chlorpromazine dose. **Conclusions:** This study identifies for the first time a decrease in brain ORs and TASRs in schizophrenia, a neuropsychiatric disease not linked to abnormal protein aggregates, suggesting that the deregulation of these receptors is associated with altered cognition of these disorders. In addition, the influence of antipsychotics on the expression of ORs and TASRs in schizophrenia suggests that these receptors could be involved in the mechanism of action or side effects of antipsychotics.

ARTICLE

Deregulation of purine metabolism in Alzheimer's disease.

Ansoleaga B, Jové M, Schlüter A, Garcia-Esparcia P, Moreno J, Pujol A, Pamplona R, Portero-Otín M, Ferrer I.

Neurobiology of Aging 2015 Jan; 36(1):68-80.

ABSTRACT

Introduction: The neuroprotective role of adenosine and the deregulation of adenosine receptors in Alzheimer's disease (AD) have been extensively studied in recent years. However, little is known about the involvement of purine metabolism in AD. We started by analyzing gene expression in the entorhinal cortex of human controls and AD cases with whole-transcript expression arrays. **Results:** Once we identified deregulation of the cluster purine metabolism, messenger RNA expression levels of 23 purine metabolism genes were analysed with RT-qPCR in the entorhinal cortex, frontal cortex area 8, and precuneus at stages I-II, III-IV, and V-VI of Braak and Braak and controls. APRT, DGUOK, POLR3B, ENTPD3, AK5, NME1, NME3, NME5, NME7, and ENTPD2 messenger RNAs were deregulated, with regional variations, in AD cases when compared with controls. In addition, liquid chromatography mass spectrometry based metabolomics in the entorhinal cortex identified altered levels of dGMP, glycine, xanthosine, inosine diphosphate, guanine, and deoxyguanosine, all implicated in this pathway. **Conclusions:** Our results indicate stage- and region-dependent deregulation of purine metabolism in AD.

ARTICLE

Neuroinflammatory signals in Alzheimer disease and APP/PS1 transgenic mice: correlations with plaques, tangles, and oligomeric species.

López-González I, Schlüter A, Aso E, Garcia-Esparcia P, Ansoleaga B, LLorens F, Carmona M, Moreno J, Fuso A, Portero-Otin M, Pamplona R, Pujol A, Ferrer I.

Journal Neuropathology Experimental Neurology 2015 Apr; 74(4):319-44.

ABSTRACT

Introduction: To understand neuroinflammation-related gene regulation during normal aging and in sporadic Alzheimer disease (sAD), we performed functional genomics analysis and analysed messenger RNA (mRNA) expression by RT-qPCR of 22 genes involved in neuroinflammation-like responses in the cerebral cortex of wild-type and APP/PS1 transgenic mice. For direct comparisons, mRNA expression of 18 of the same genes was then analysed in the entorhinal cortex, orbitofrontal cortex, and FC of middle-aged human subjects and in older subjects with sAD. **Results:** Modifications of cytokine and immune mediator mRNA expression were found with normal aging in wild-type mice and in middle-aged individuals and patients with early stages of sAD-related pathology. In APP/PS1 mice, inflammatory changes coincided with β-amyloid (Aβ) deposition; increased levels of soluble oligomers paralleled the modified mRNA expression of cytokines and mediators in wild-type mice. **Conclusions:** In patients with sAD, regulation was stage- and region-dependent and not merely acceleration and exacerbation of mRNA regulation with aging. Gene regulation at first stages of AD was not related to hyperphosphorylated tau deposition in neurofibrillary tangles, Aβ plaque burden, concentration of Aβ1-40 (Aβ40) and Aβ1-42 (Aβ42), or fibrillar Aβ linked to membranes but rather to increased levels of soluble oligomers.

ARTICLE

Complex Inflammation mRNA-Related Response in ALS Is Region Dependent.

Berjaoui S, Povedano M, Garcia-Esparcia P, Carmona M, Aso E, Ferrer I.

Neural Plasticity 2015; 2015:573784.

ABSTRACT

Introduction: Inflammatory changes are analysed in the anterior spinal cord and frontal cortex area 8 in typical spinal-predominant ALS cases. Increased numbers of astrocytes and activated microglia are found in the anterior horn of the spinal cord and pyramidal tracts. **Results:** Significant increased expression of TLR7, CTSS, and CTSC mRNA and a trend to increased expression of IL10RA, TGFB1, and TGFB2 are found in the anterior lumbar spinal cord in ALS cases compared to control cases, whereas C1QTNF7 and TNFRSF1A mRNA expression levels are significantly decreased. IL6 is significantly upregulated and IL1B shows a nonsignificant increased expression in frontal cortex area 8 in ALS cases. IL-6 immunoreactivity is found in scattered monocyte-derived macrophages/microglia and TNF-α in a few cells of unknown origin in ALS cases. Increased expression and abnormal distribution of IL-1β occurred in motor neurons of the lumbar spinal cord in ALS. Strong IL-10 immunoreactivity colocalizes with TDP-43-positive inclusions in motor neurons in ALS cases. **Conclusions:** The present observations show a complex participation of cytokines and mediators of the inflammatory response in ALS consistent with increased proinflammatory cytokines and sequestration of anti-inflammatory IL-10 in affected neurons.

ARTICLE

Altered machinery of protein synthesis in Alzheimer's: from the nucleolus to the ribosome

Hernández-Ortega K, Garcia-Esparcia P, Gil L, Lucas JJ, Ferrer I.

Brain Pathology 2015 Oct 29 *[Epub ahead of print]*.

ABSTRACT

Introduction: Ribosomes and protein synthesis have been reported to be altered in the cerebral cortex at advanced stages of Alzheimer's disease (AD). Modifications in the hippocampus with disease progression have not been assessed. 67 cases including middle-aged (MA) and AD stages I-VI were analysed. **Results:** Nucleolar chaperones nucleolin, nucleophosmin and nucleoplasmin 3, and upstream binding transcription factor RNA polymerase I gene (UBTF) mRNAs are abnormally regulated and their protein levels reduced in AD. Histone modifications H3K9me2 and H3K12ac are decreased in CA1. Nuclear tau declines in CA1 and DG, and practically disappears in neurons with neurofibrillary tangles. 28S rRNA expression is altered in CA1 and DG in AD. Several genes encoding ribosomal proteins are abnormally regulated and protein levels of translation initiation factors eIF2α, eIF3η and eIF5, and elongation factor eEF2, are altered in the CA1 region in AD. **Conclusions:** These findings show alterations in the protein synthesis machinery in AD involving the nucleolus, nucleus and ribosomes in the hippocampus in AD some of them starting at first stages (I-II) preceding neuron loss. These changes may lie behind reduced numbers of dendritic branches and reduced synapses of CA1 and DG neurons, which cause hippocampal atrophy.

ARTICLE

Human DNA methylomes of neurodegenerative diseases show common epigenomic patterns.

Sanchez-Mut JV, Heyn H, Vidal E, Moran S, Sayols S, Delgado-Morales R, Schultz MD, Ansoleaga B, Garcia-Esparcia P, Pons-Espinal M, de Lagran MM, Dopazo J, Rabano A, Avila J, Dierssen M, Lott I, Ferrer I, Ecker JR, Esteller M.

Translational Psychiatry 2016 Jan 19;6:e718.

ABSTRACT

Introduction: Different neurodegenerative disorders often show similar lesions, such as the presence of amyloid plaques, TAU-neurotangles and synuclein inclusions. The genetically inherited forms are rare, so we wondered whether shared epigenetic aberrations, such as those affecting DNA methylation, might also exist. **Results:** The studied samples were gray matter samples from the prefrontal cortex of control and neurodegenerative disease-associated cases. We performed the DNA methylation analyses of Alzheimer's disease, dementia with Lewy bodies, Parkinson's disease and Alzheimer-like neurodegenerative profile associated with Down's syndrome samples. The DNA methylation landscapes obtained show that neurodegenerative diseases share similar aberrant CpG methylation shifts targeting a defined gene set. **Conclusions:** Our findings suggest that neurodegenerative disorders might have similar pathogenetic mechanisms that subsequently evolve into different clinical entities. The identified aberrant DNA methylation changes can be used as biomarkers of the disorders and as potential new targets for the development of new therapies.

ARTICLE

Genetic and Transcriptomic Profiles of Inflammation in Neurodegenerative Diseases: Alzheimer, Parkinson, Creutzfeldt-Jakob and Tauopathies.

López González I, Garcia-Esparcia P, Llorens F, Ferrer I.

International Journal Molecular Sciences 2016 Feb 4; 17(2):206.

ABSTRACT

Introduction: Polymorphisms in certain inflammatory-related genes have been identified as putative differential risk factors of neurodegenerative diseases with abnormal protein aggregates, such as sporadic Alzheimer's disease (AD) and sporadic Parkinson's disease (sPD). **Results:** Gene expression studies of cytokines and mediators of the immune response have been made in *post-mortem* human brain samples in AD, sPD, sporadic Creutzfeldt-Jakob disease (sCJD) subtypes MM1 and VV2, Pick's disease (PiD), progressive supranuclear palsy (PSP) and frontotemporal lobar degeneration linked to mutation P301L in MAPT Frontotemporal lobar degeneration-tau (FTLD-tau). The studies have disclosed variable gene regulation which is: (1) disease-dependent in the frontal cortex area 8 in AD, sPD, sCJD MM1 and VV2, PiD, PSP and FTLD-tau; (2) region-dependent as seen when comparing the entorhinal cortex, orbitofrontal cortex, and frontal cortex area 8 (FC) in AD; the substantia nigra, putamen, FC, and angular gyrus in PD, as well as the FC and cerebellum in sCJD; (3) genotype-dependent as seen considering sCJD MM1 and VV2; and (4) stage-dependent as seen in AD at different stages of disease progression. **Conclusions:** These observations show that regulation of inflammation is much more complicated and diverse than currently understood, and that new therapeutic approaches must be designed in order to selectively act on specific targets in particular diseases and at different time points of disease progression.

ARTICLE

Altered Mitochondria, Protein Synthesis Machinery, and Purine Metabolism Are Molecular Contributors to the Pathogenesis of Creutzfeldt-Jakob Disease.

Ansoleaga B, Garcia-Esparcia P, Llorens F, Hernández-Ortega K, Carmona M, Antonio Del Rio J, Zerr I, Ferrer I.

Journal Neuropathology Experimental Neurology 2016 Jun 12.

ABSTRACT

Introduction: Neuron loss, synaptic decline, and spongiform change are the hallmarks of sporadic Creutzfeldt-Jakob disease (sCJD), and may be related to deficiencies in mitochondria, energy metabolism, and protein synthesis. **Results:** To investigate these relationships, we determined the expression levels of genes encoding subunits of the 5 protein complexes of the electron transport chain, proteins involved in energy metabolism, nucleolar and ribosomal proteins, and enzymes of purine metabolism in frontal cortex samples from 15 cases of sCJD MM1 and age-matched controls. We also assessed the protein expression levels of subunits of the respiratory chain, initiation and elongation translation factors of protein synthesis, and localization of selected mitochondrial components. We identified marked, generalized alterations of mRNA and protein expression of most subunits of all 5 mitochondrial respiratory chain complexes in sCJD cases. Expression of molecules involved in protein synthesis and purine metabolism were also altered in sCJD. **Conclusions:** These findings point to altered mRNA and protein expression of components of mitochondria, protein synthesis machinery, and purine metabolism as components of the pathogenesis of CJD.

ARTICLE

Olfactory Receptors in Non-Chemosensory Organs: The Nervous System in Health and Disease.

Ferrer I, Garcia-Esparcia P, Carmona M, Carro E, Aronica E, Kovacs GG, Grison A, Gustincich S.

Frontiers in Aging Neuroscience 2016 Jul 5; 8:163.

ABSTRACT

Introduction: Neuron loss, synaptic decline, and spongiform change are the hallmarks of sporadic Creutzfeldt-Jakob disease (sCJD), and may be related to deficiencies in mitochondria, energy metabolism, and protein synthesis. **Results:** To investigate these relationships, we determined the expression levels of genes encoding subunits of the 5 protein complexes of the electron transport chain, proteins involved in energy metabolism, nucleolar and ribosomal proteins, and enzymes of purine metabolism in frontal cortex samples from 15 cases of sCJD MM1 and age-matched controls. We also assessed the protein expression levels of subunits of the respiratory chain, initiation and elongation translation factors of protein synthesis, and localization of selected mitochondrial components. We identified marked, generalized alterations of mRNA and protein expression of most subunits of all 5 mitochondrial respiratory chain complexes in sCJD cases. Expression of molecules involved in protein synthesis and purine metabolism were also altered in sCJD. **Conclusions:** These findings point to altered mRNA and protein expression of components of mitochondria, protein synthesis machinery, and purine metabolism as components of the pathogenesis of CJD.