Targeting Aging and Alzheimer's disease: from GM1 ganglioside to Amyloid-β peptide

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Ever tried. Ever failed. No matter. Try Again. Fail again. Fail better.

Samuel Beckett

Ningú ens ha dit que el temps no deixa cap ferida però pels camins tranquils hi ha poca poesia.

Txarango

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Abstract

Aging is the main challenge that humanity will face on the near future. Brain aging is associated with cognitive deficiencies and is the main risk factor for Alzheimer's Disease (AD). AD is the most common neurodegenerative disease leading to dementia caused by the aggregation of amyloid-β peptide (Aβ). In this thesis, we have shown that neuronal aging leads to alteration in the ganglioside membrane content. Specifically, increased levels of GM1 ganglioside in membrane leads to a decrease in the calcium entry through NMDA receptors and a reduction of dendritic spines. Overall, pointing a role in the alterations in learning and memory associated to aging. On the other hand, we have showed the inhibitory effect of the human gamma immunoglobulins (IgG) in Aβ aggregation through the antigen-binding fragment (Fab), which binds to Aβ blocking fibrillation progression.

L'envelliment de la població és i serà un gran repte per la nostra societat en els pròxims anys. L'envelliment cerebral està associat amb deficiències cognitives i és el principal factor de risc pel desenvolupament de la malaltia d'Alzheimer. L'Alzheimer és la malaltia neurodegenerativa que més freqüentment provoca demència i és causada per l'agregació del pèptid β-amiloide (Aβ). En aquesta tesis hem demostrat que l'envelliment neuronal condueix a alteracions en el contingut de gangliòsids de les membranes. Particularment, l'increment del gangliòsid GM1 en la membrana condueix a una disminució en l'entrada de calci a través dels receptors de NMDA i a una reducció de les espines dendrítiques. En conjunt, indicant el rol de GM1 en les alteracions en l'aprenentatge i la memòria que es produeixen en l'envelliment. Per una altra banda, hem demostrat l'efecte inhibitori de gamma immunoglobulina humana (IgG) en la inhibició de l'agregació de l'Aβ a través del contacte del fragment d'unió a l'antigen (Fab). Fab s'uneix a Aβ inhibint la progressió de la fibril·lació.

Prologue

Humanity is facing an aging process of the population. Aging is caused by the accumulation of cellular damage. Brain function depends on a complex network of connections. Precise control of connections is necessary for maintaining correct brain function. Brain aging courses with loss of integrative function leading to stereotypical and neurophysiological changes and a variable cognitive decline even in absence of disease. The greatest risk factor for Alzheimer's Disease (AD) is aging itself. Physiological aging is a central process of pathological aging. Accordingly, slowing down or reversing brain aging could help fight against AD.

AD is the most common neurodegenerative disorder leading to dementia caused by the pathological aggregation of amyloid-β peptide (Aβ). Aβ is a 4 kDa peptide generated in every cell of the organism. It becomes neurotoxic due to a conformation change leading to β-sheet rich structure. Aβ can initiate a signaling cascade leading to neuronal apoptosis. Currently, there is no approved treatment able to fight Aβ aggregation in the brain. Therefore, it is urgent to identify a target to reduce Aβ aggregation and their pathological consequences. Moreover, aging has been associated with modifications in the ganglioside content of neuronal membrane. GM1 ganglioside is one of the more abundant one. Until now research has been mainly focus on GM1 ganglioside role in Aβ aggregation. Thus, further studies are needed to reveal the function of the different membrane components.

This thesis shows the role of GM1 ganglioside in neuronal aging. GM1 increase in neuronal membranes reduce calcium entry through NMDA receptor by interacting with its ligand binding domain leading to a decrease of synaptic spines. Furthermore, we have identified the human gamma immunoglobulins (IgG) inhibitory effect on Aβ aggregation process through a highly conserved structure in the antigen-binding fragment (Fab).

Abbreviations

α7nAChr	Alpha-7 nicotinic receptor
Aβ	Amyloid-β peptide
AChE	Acetylcholinesterase enzyme
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase family
AICD	Amyloid precursor protein intracellular domain
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Amyloid plaques
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ARIA	Amyloid-related imaging abnormalities
BBB	Brain-blood barrier
BACE1	Beta-site APP cleaving enzyme 1
CA	*Cornu Ammonis*
CAA	Cerebral amyloid angiopathy
CaMKII	Ca^{2+}/calmodulin- dependent kinase II
CGlcT	Ceramide glucosyltransferase
CNS	Central nervous system
CREB	cAMP response element-binding protein
CSF	Cerebrospinal fluid
CTX	Cholera toxin
DRM	Detergent resistant membrane
EOAD	Early-onset Alzheimer's Disease
EPSP	Excitatory postsynaptic potentials
ER	Endoplasmatic reticulum
Fab	Antigen-binding fragment
Fc	Constant fragment
FDA	U.S Food and Drug Administration

GABA	γ-aminobutyric acid
GalNAc	N-Acetyl-D-galactosamine
GalT I	Galactosyl transferase I
GalTII	Galactosyl transferase II
GM1	Monosialotetrahexosyl ganglioside
GPI	Glycosylphosphatidylinositol
GWAS	Genome-wide association studies
HDAC2	Histone deacetylase 2
IGF-1	Insulin-growth factor 1
IIS	Insulin/IGF-1 signaling
IgG	Immunoglobulin G
iGluR	Glutamate ionotropic receptors
ISF	Interstitial fluid
IPSP	Inhibitory postsynaptic potentials
IVIG	Intravenous immunoglobulin
KAR	Kainate receptor
LOAD	Late-onset Alzheimer's Disease
LTD	Long-term depression
LTP	Long-term potentiation
LRP	LDLR-related protein
MAMs	Mitochondrial associated membranes
MCI	Mild-cognitive impairment
mGluR	Metabotropic glutamate receptors
mtDNA	mitochondrial DNA
NAM	Negative allosteric modulator
NFT	Neurofibrillary tangles
NMDA	*N*-Methyl-D-aspartate or *N*-Methyl-D-aspartic acid
NMDAR	*N*-Methyl-D-aspartate receptor
NVC	Neuraminidase from *Vibrio Cholerae*
PD	Parkinson's disease

PET	Positron emission tomography
PKA	cAMP-dependent protein kinase A
PSD	Postsynaptic density
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RAGE	Advanced glycosylation end product-specific receptor
ROS	Reactive oxygen species
SAP	Serum amyloid P component
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SialTI	Sialyltransferase I
SialTII	Sialyltransferase II or GD3 synthase
SialTIII	Sialyltransferase III or GT3 synthase
sLRP	Soluble LDLR-related protein
SNARE	Soluble N-ethylmaleimide–sensitive factor attachment protein receptor
sRAGE	Soluble receptor for advanced glycosylation end product
TRPC5	Transient receptor potential C5
UPR	Unfolded protein response
VGCC	Voltage gated calcium channels

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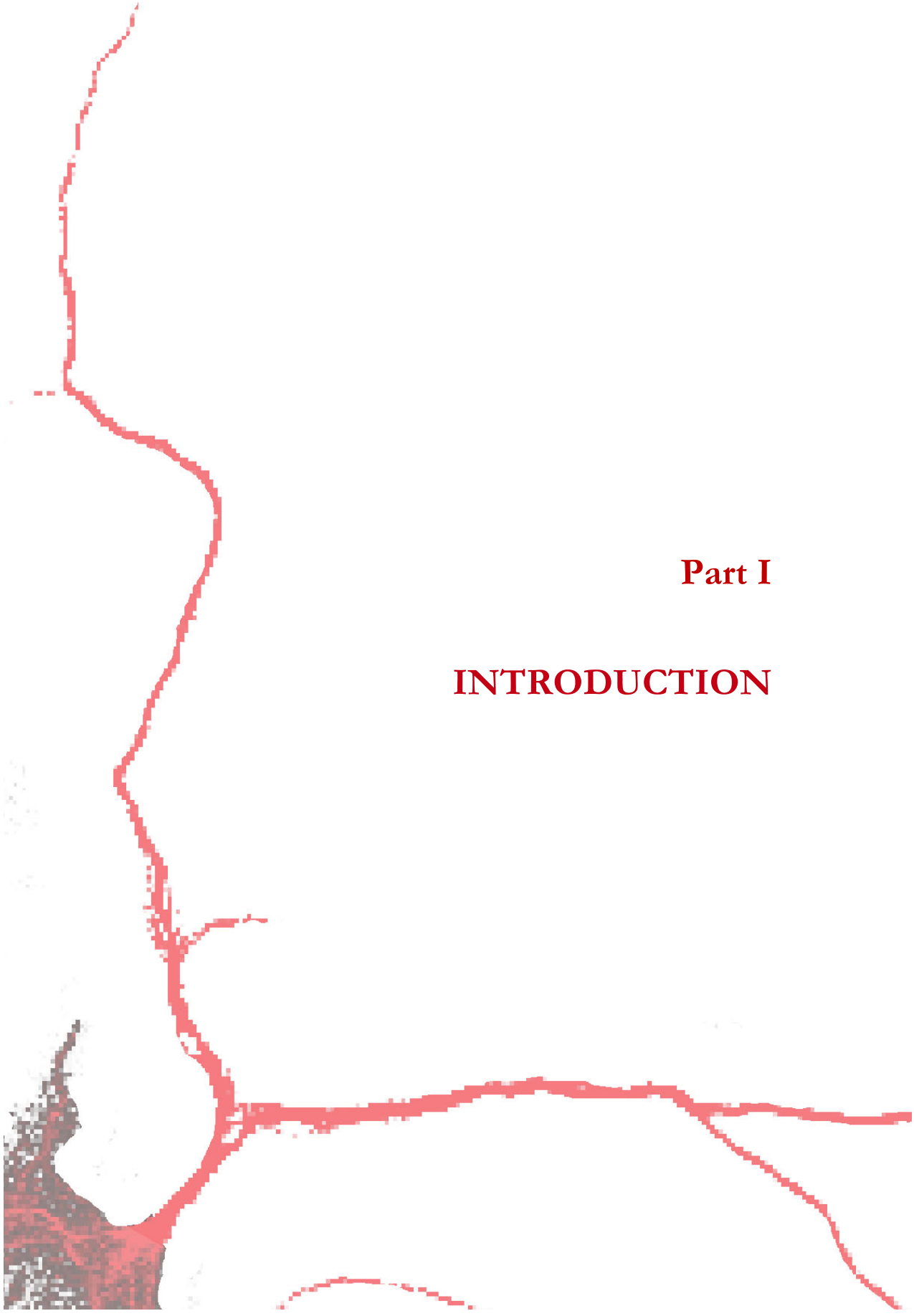


Part I

INTRODUCTION

1 Brain function and neurotransmission

Brain is a complex organ composed of more than 10^{11} neurons which transmit information through chemical and electrical synapses. Synapses are the basic and specialized junctions in the central nervous system (CNS). Electrical synapses are composed just by "connexons" in the junction of the presynaptic and postsynaptic membranes (Fig 1b). Chemical synapses are specialized communication structures that permit to control the electric messages between neurons (Fig 1a).

Each neuron receives an average of 10.000 synapses, processes signals received from other neurons and transmits information to additional neurons within the circuit. Human brain contains more than 10^{15} synapses. Precise control of synaptic connections is necessary for maintaining normal brain function [1,2].

The storage of information in the brain leads to alterations in the structure and the chemistry of synapses. Synapse are plastic and this process could induce the formation or the elimination of synapses. Synaptic plasticity of the brain is the molecular basis of learning and memory. In neurological conditions such as Alzheimer's Disease (AD) or autism spectrum disorders synaptic plasticity is greatly affected [1,3].

1.1 Chemical synapsis

Chemical synapses (Fig 1a) have an axonal ending (presynaptic terminal) that releases the synaptic vesicles containing neurotransmitters. The neurotransmitter released by the presynaptic neuron binds to the neurotransmitter receptor present in the postsynaptic terminal, which usually is a dendrite of the postsynaptic neuron. The synaptic cleft is a 20-25nm gap between the presynaptic terminal and the postsynaptic terminal, a distance maintained by transmembrane proteins. Chemical synapses are usually surrounded by glia that helps to provide a fixed structure to the synapses and, also, recaptures neurotransmitters (i.e. glutamate) [4,5].

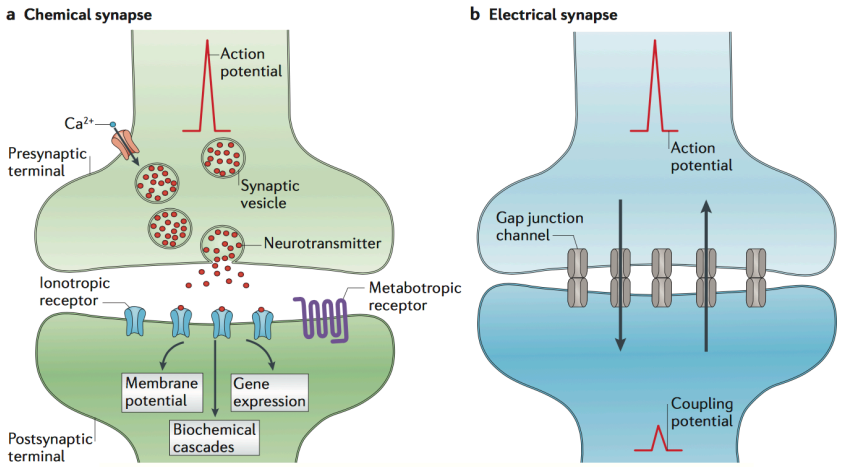


Figure 1: Main modalities of synaptic transmission. a) Chemical synapse requires presynaptic molecular machinery that regulates neurotransmitter release upon depolarization of the presynaptic terminal which leads to the activation of voltage-gated calcium channels (VGCCs). The postsynaptic terminal includes the presence of ionotropic and metabotropic receptors that are capable of detecting and translating the presynaptic message (neurotransmitters) into various postsynaptic events. b) Electrical transmission are composed by clusters of intercellular channels called gap junctions that connect the two adjacent cells, and enable the bidirectional passage of electrical currents carried by ions (arrows). From Pereda AE, 2014 [2]

1.1.1 Presynaptic terminal

Action potential produces a membrane depolarization that travels along the neuronal processes to finally reach the presynaptic terminal, where voltage-gated calcium channels (VGCC) open initiating an inward Ca^{2+} current that dissemble the specialized membrane of the active zone. It triggers the fusion of neurotransmitter-containing synaptic vesicles to the membrane forming a pore. Neurotransmitters are liberated in the active zone, a thicker region of the presynaptic membrane. It is a highly controlled and rapid mechanism termed docking that permit the progressive fusion of vesicles performing a "kiss and run" process. The docking and fusion of the vesicles is stimulated

by Ca^{2+} and controlled by the N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex [6,7].

1.1.2 Postsynaptic terminal

Neurotransmitters bind to the neurotransmitter receptor evoking an electrical response in the postsynaptic neuron. The neurotransmitter and the corresponding receptor determine if the synapse is excitatory (i.e. glutamate and kainate receptors) or inhibitory (γ-aminobutyric acid, GABAergic or glycinergic) (Fig 2). Therefore, they will produce excitatory postsynaptic potentials (EPSP) or inhibitory postsynaptic potentials (IPSP). In fact, neuronal signaling processing is mediated by the integration of several excitatory and inhibitory inputs. The final output of the postsynaptic neuron is the result of the integration of all inputs. There is a fine and precise regulation to maintain the balance between both excitatory and inhibitory inputs [7].

The postsynaptic membrane has also a highly specialized structure characterized by the presence of the postsynaptic density (PSD) located adjacent to the membrane. The PSD is an electron-dense structure with a precise three-dimensional organization containing a matrix of scaffolding proteins, receptors, ion channels, signal transduction proteins and cytoskeleton components. The PSD is localized in opposition to the active zone in the presynaptic terminal. Excitatory and inhibitory synapses can be distinguished by the composition of their PSD: excitatory synapses contain a more complex and prominent PSD than inhibitory synapses [4,5]. In excitatory PSD the key proteins are N-methyl-D-aspartate receptor (NMDAR), amino-3-hydroxy-5-methyl-4-isoazolepropionate receptor (AMPAR), PSD-95, Ca^{2+}/calmodulin- dependent kinase II (CaMKII) and Shank family proteins [8,9]. The main proteins in the inhibitory PSD are the $GABA_A$ receptors and glycine receptors. These receptors interact directly with gephyrin, a postsynaptic scaffold protein of inhibitory synapse[10] .

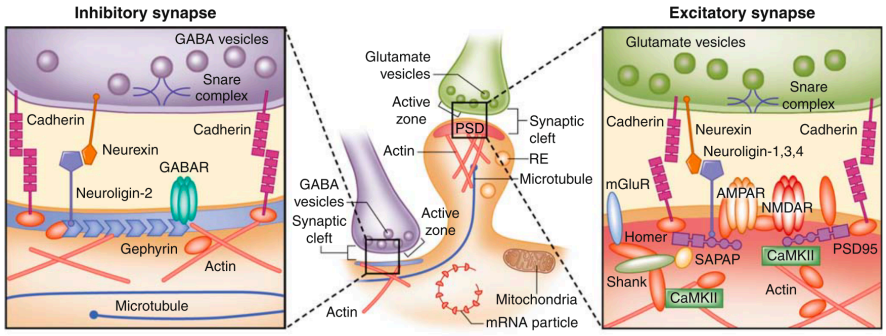


Figure 2: Molecular structure of inhibitory and excitatory synapses. Molecular structure of an inhibitory and excitatory synapse. In inhibitory synapses the presynaptic neuron (purple) contacts the postsynaptic neuron at the dendritic shaft whereas the presynaptic excitatory neuron (green) contacts the postsynaptic neuron at the dendritic spine. Both presynaptic terminals contain neurotransmitter vesicles that are released after the action potential arrives at the terminal and activates VGCCs, allowing the entry of calcium and the subsequent exocytosis of the vesicles. The postsynaptic terminal in the excitatory synapse is more complex. It contains a matrix of scaffolding proteins to concentrate and connect ion channels (NMDAR and AMPAR), receptors and signaling proteins with actin cytoskeleton. CaMKII: Ca^{2+}/calmodulin-dependent kinase II; AMPAR: amino-3-hydroxy-5-methyl-4-isoazolepropionate receptor; GABA: γ-aminobutyric acid; GABAR: GABA receptor; NMDAR: N- methyl-D-aspartate receptor; mGluR—metabotropic glutamate receptor; SAPAP—synapse-associated protein 90/PSD-95-associated protein. From Van Spronsen et al., 2010 [1]

1.1.3 Main postsynaptic channels in learning and memory

Learning is the ability to acquire new information and memory is the ability to retain this new information over time [11]. Synaptic plasticity, which is the capacity of synapse to adapt to overall neuronal activity, allows the learning process. Long-term potentiation (LTP) and long-term depression (LTD) are the two main molecular processes behind synaptic plasticity. In the hippocampus, there are several channels that have an important role such as NMDAR, AMPAR and VGCC [12,13].

1.1.3.1 Voltage gated calcium channels

VGCCs are heteromultimeric complexes composed by four or five different subunits. The α1 is the largest subunit of the channel and contains the pore, the voltage sensor, the gating apparatus and most of the regulation sites that are recognized by second messengers. The genetic diversity of this subunit permits the formation of channels with different properties that can fulfil highly specialized roles [14].

VGCCs control the depolarization-induced Ca^{2+} entry into the neurons. Ca^{2+} does not significantly modify the membrane potential but controls several physiological and pathological signaling pathways. Thereby, VGCCs have a critical function in the brain and dysfunction of these channels causes several neurological disorders such as pain, epilepsy, migraine and ataxia [14].

1.1.3.2 Neurotransmitter receptors: glutamate ionotropic receptors

There are two main types of glutamate postsynaptic receptors: ligand-gated ion channels (ionotropic receptors) and G protein-coupled (metabotropic) receptors [1].

Glutamate ionotropic receptors (iGluR) are NMDAR, AMPAR and kainate receptors (KAR)[15]. NMDAR and AMPAR play an important role for learning and memory [16,17].

NMDARs are heterotetrameric cationic channels containing two GluN1 subunits and either two GluN2 (A-D) or GluN3 (A-B) subunits (Fig 3a). GluN1 subunits binds to glycine whereas GluN2 subunits bind to glutamate (Fig 3b). The different types of subunits present in a channel confers it different biophysical and pharmacological properties. To this end, studies have focused on the inhibition or activation of NMDARs with different subunit compositions. In the adult brain, GluN2A and GluN2B subunits are mainly expressed in the neocortex and the hippocampus [16,18–21].

The activation of NMDAR requires the binding of two agonists, glycine and glutamate, together with the release of Mg^{2+}, which blocks the channel when the membrane is at resting potential (-60 to -80 mV). To remove the Mg^{2+} block the membrane must be depolarized. NMDAR activation leads to cationic influx, mainly Ca^{2+} but also Na^{+} and K^{+}. Calcium influx initiates the signalling cascade that regulates synaptic plasticity [16,18].

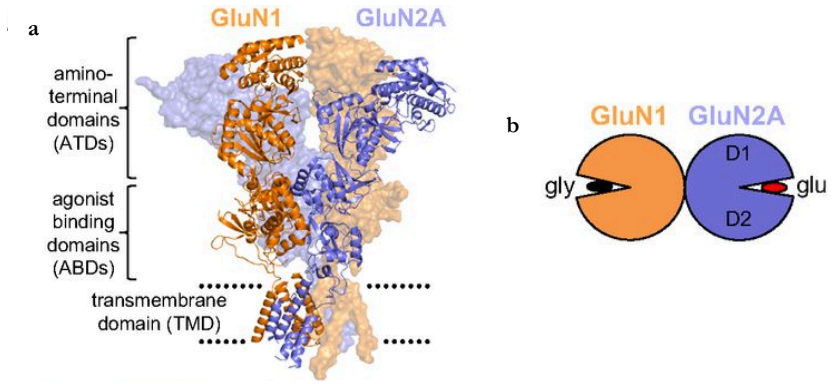


Figure 3: NMDAR structure. a) Structure of the NMDA receptor composed by two GluN1 and two GluN2 subunits (PDB ID code 4PE5) b) Cartoon illustrating the GluN1/2A heterodimer with glycine bound in GluN1 and glutamate. From Lindt et al., 2017 [19]

AMPAR is a heterotetrameric, cationic channel that is mostly permeably to Na^{+}. AMPARs mediates a faster excitatory transmission than NMDARs. Most AMPARs are expressed in the adult hippocampus and cortex and are composed of a GluA2 dimer and a dimer of GluA1, GluA3 or GluA4 subunits; GluA2-GluA1 tetramers being the most frequent. GluA2 subunits allow for the influx of Ca^{2+} [22,23].

Metabotropic glutamate receptors (mGluR) are G-protein coupled receptors that modulate synaptic transmission and plasticity. mGluR are composed by seven transmembrane domains and three extracellular and intracellular loops. mGluR can be classified in three subfamilies: group I (mGlu1 and mGlu5), group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7 and mGlu8). Group I receptors are coupled to Gq-like proteins and are mainly

post-synaptic. Group II and III receptors are coupled to Gi/Go proteins and are mainly pre-synaptic. mGluRs activation initiates complexes signaling cascades[24].

1.2 Learning and memory

1.2.1 Hippocampus

The hippocampus is part of a multimodal sensory integration system localized in the medial temporal lobe. Sensory information from cortical sensory areas travels to the hippocampus *via* the perirhinal and entorhinal cortices. The outputs from the perirhinal and entorhinal cortices project to the dentate gyrus, containing neural stem cells, and to the *Cornu Ammonis* (CA) region. The dentate gyrus and CA region are together called the hippocampal formation. The CA region consists of the CA1, CA2 and CA3, which have been widely studied (Fig 4a). CA1 is vital for long-term memory formation and has been used as a model system for understanding LTP. The CA3 is believed to be important for the rate of learning and pattern completion [11,25–27].

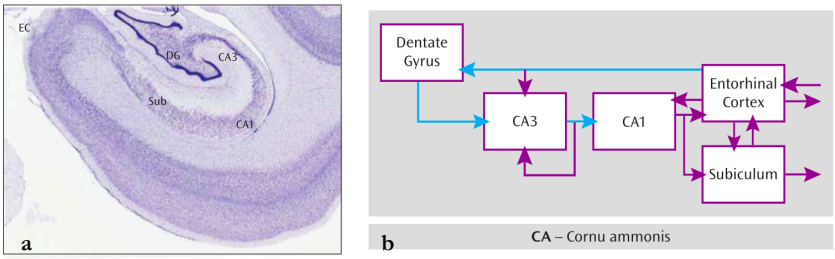


Fig 4: The hippocampus. a) Nissl staining of the human hippocampus (EC: entorhinal cortex; Sub: subiculum; DG: dentate gyrus and CA: *Cornu Ammonis)* b) Schematic draw of the connectivity within the hippocampus**.** From Tamminga et al., 2010 [27]

The primary projections from the entorhinal cortex to the hippocampus are through the perforant pathway which connects to all fields of the hippocampal formation in a unidirectional way. Furthermore, Schaffer

collaterals transfers information from CA3 to CA1. The output neurons of the hippocampus are the CA1 pyramidal neurons, which project predominantly to the entorhinal cortex and contralateral hippocampus. Secondary efferents of CA1 pyramidal neurons also project to subcortical regions (Fig 4b) [11,27].

Many studies have demonstrated an important role of the hippocampus in memory formation and consolidation, but its function is still matter of debate. Some authors [11,26,28] believe that the hippocampus does not store information for extended periods of time, but rather serves as an intermediate-duration memory buffer that is involved in maintaining memories until they are transferred for more permanent storage to the cerebral cortex (long-lasting memories). On the other hand, other scientists defend the "multiple trace theory" which states that the hippocampal memory trace is not replaced by the cortical one, but rather both memories are in continuous interplay. Thus, long-term memories could involve both the hippocampus and neocortex [29,30].

1.2.2 Synaptic plasticity: LTP and LTD

Synapses are dynamic and synaptic strength can be modified by neuronal activity. The ability of synapses to undergo activity-dependent changes is the basis for all experience-dependent plasticity such as learning and memory. Synaptic plasticity allows to the brain to adapt and respond to new environmental stimuli-leading to learning and the formation of new memories [23,31,32].

The most known, accepted and studied forms of synaptic plasticity are LTP and LTD, which occur at the excitatory synapses between the Schaffer collateral and CA1 pyramidal neurons. LTP results in a gain of synaptic strength whereas LTD leads to a decrease in synaptic strength [32,33].

Both mechanisms are NMDAR dependent, requiring an increase in the postsynaptic Ca^{2+} levels. It is known that they differ in the magnitude of the

calcium influx and waves patterns triggering different signaling cascades. LTP or LTD can be induced depending on the frequency and the duration of the stimulation. LTP is triggered by 100 Hz train of action potentials for 1s, whereas 1-3 Hz for 10 min induces LTD [32–35].

It is known that LTP is a synapse-specific event. Only stimulated synapses, but not neighbouring synapses are potentiated thanks to synaptic tagging. To achieve this specificity the entry of calcium through NMDARs is only present in potentiated synapses and absent in not stimulated ones [25,36].

The excitatory neurotransmitter glutamate is released in the synaptic cleft after depolarization of the presynaptic neuron. Glutamate binds to both AMPARs and NMDARs, however AMPARs, which are permeably to Na^+, are responsible for most of the inward current at the postsynaptic neuron because NMDARs are blocked by Mg^{2+}. When the postsynaptic membrane is simultaneously depolarized, the Mg^{2+} is released from the channel allowing the entry of Ca^{2+} and Na^+. It is thought that with repeated activation, sufficient Ca^{2+} enters the cell and induces LTP. Thereby, the calcium signal initiates signalling cascades -specifically CaMKII and the cyclic adenosine monophosphate (cAMP)-depending pathways- that lead to LTP induction and expression [17,25,36,37].

The early phase of LTP does not require protein synthesis but involves Na^+ and Ca^{2+} influx, prolonged CaMKII activity and an increase in the number of AMPAR inserted into the postsynaptic membrane [38–42]. The late phase of LTP requires de novo protein synthesis mediated by transcription factors or translation of localized mRNAs. For example, the cAMP-dependent protein kinase A (PKA) activates cAMP response element-binding protein (CREB), a transcription factor which regulates the expression of memory-associated genes [43,44].

The other synaptic mechanism is LTD. This mechanism appears to reverse LTP. Moreover, it is necessary to avoid the saturation by learning of the

synapses allowing the proper formation of memories. During LTD, the temporal pattern and the magnitude of calcium entry is different, activating different signalling cascades. Small rises in intracellular Ca^{2+} levels in postsynaptic neurons seems to facilitate the activation of calcineurin, PP1 and PP2B, phosphatases which dephosphorylate different substrates such as AMPARs, NMDARs and kinases [17,45,46].

1.2.3 Structural plasticity

Dendritic spines are small membranous protrusions arising from the dendritic shaft of postsynaptic neurons where most excitatory synapses reside. Inhibitory synapses are found directly contacting with the dendritic shaft [31,47,48]. Excitatory spines are connected to the dendritic shaft through a thin spine neck. Moreover, spines can be classified depending on their size and shape: thin, stubby, cup, mushroom and branched-shaped (Fig 5a) [4,49,50].

Spines are autonomous compartments that isolate chemical and electrical signaling and contain all the postsynaptic machinery needed to respond in a fast way including glutamate receptors, actin cytoskeleton, the PSD, endoplasmic reticulum (ER) and endosomes [31,51].

Dendritic spines are highly dynamic structures, constantly changing in number, size and shape in response to modification in the strength of synaptic connections. Both structural and morphological changes allow structural plasticity, and as consequence, learning and memory. It is not completely known how neuronal activity regulates morphology and synaptic plasticity. However, induction of LTP is associated with the enlargement of spines whereas induction of LTD is associated with shrinkage. The control of the structural and morphological changes is mainly performed by the actin filaments (f-actin), which are the principal components of the cytoskeleton in the dendritic spine (Fig 5b) [3,31,52–54].

One question is what makes a neuron decide to connect with the presynaptic neuron at the dendritic spine instead of the dendritic shaft? Each dendritic

spine contains excitatory synapses, but the dendritic shaft contains both excitatory and inhibitory synapses. Ramon y Cajal was the first to describe the dynamics of the dendritic spine as a mechanism by which dendrites increase their surface area [55]. Now it is believed that the main purpose of dendritic spines is to help to distribute the circuit connectivity matrix, to make excitatory input integration non-saturing and linear, and to make connections independently plastic. To conclude, dendritic spines create a well distributed circuit and be able to use the advantages of their design [56].

The physiological relevance of the well-functioning of spines is demonstrated when abnormal spine structures appear. These abnormal spines are associated with different neurologic disorders, such as schizophrenia [57,58], epilepsy [59–61], fragile X syndrome [62,63], Down syndrome [64], addiction [65,66] and AD [67,68].

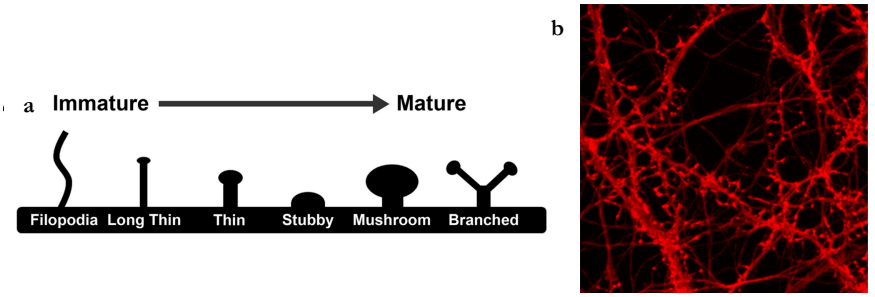


Figure 5: Dendritic spines. a) Different types of spines progressing in maturity from thin-filopodia to enlarged mushroom spines. Branched spines are occasional. From Risher et al., 2014 [50] b) Immunofluorescence staining with phalloidin of dendritic spines in hippocampal primary neurites. From Bellot et al., 2014 [49]

2 Aging

Aging is it defined as the time-dependent changes that occur in most living organisms. These changes are manifested by a decline in peak-fertility and physiological functions, such as cognitive performance [69,70].

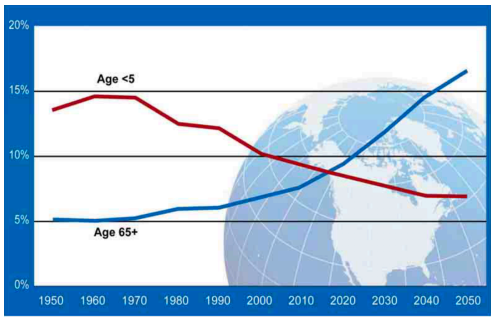


Figure 6: Demographic progression of age distribution. Graph shows the percentage of young and older people in global population (1950-2050). From Suzman et al., 2011 [70]

Humanity is facing a demographic milestone since soon people aged 65 or older will outnumber young children under 5. In 2010, an estimated 524 million people were aged 65 or older population. By 2050, this number is expected to reach 1.5 billion (Fig 6) [70].

The decreases in fertility rates and the increases in life expectancy in the last decades are the main reasons for this demographic challenge. Thanks to scientific and technological improvement, there has been a shift in the leading causes of death from infectious and parasitic diseases to non-transmissible diseases and chronic conditions (heart diseases, cancer, diabetes and neurodegenerative diseases) [70].

2.1 Cellular and molecular hallmarks

Aging is controlled by genetic and biochemical processes conserved throughout evolution. Furthermore, the main reason for aging is the accumulation of cellular damage [71,72].

Despite differences in the rate of aging, molecular and cellular damage hallmarks can be classified into three categories: primary hallmarks, antagonistic hallmarks and integrative hallmarks (Fig 7). Primary hallmarks

include clearly negative effects such as genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis. Antagonistic hallmarks consist of the cell's response to damage and they can have opposite effects- at low levels the responses are beneficial but at high levels the effects are deleterious. This category includes dysregulation of nutrient sensing, mitochondrial dysfunction and cellular senescence. Finally, the last category are integrative hallmarks that include stem cell exhaustion and alterations in cellular communication which directly affect tissue function [71].

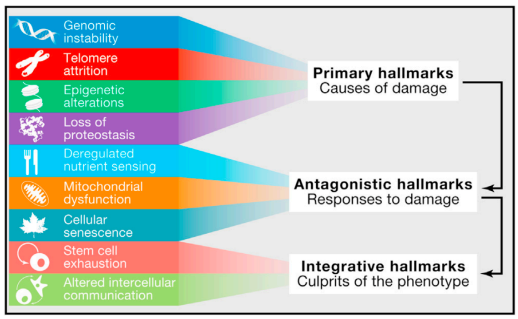


Figure 7: Hallmarks of cellular aging. Molecular and cellular hallmarks can be classified into three main categories: primary hallmarks, antagonistic hallmarks and integrative hallmarks depending on their consequences. From López-Otín et al., 2013 [71]

In order to classify an aging hallmark, each effect should be manifested during normal aging, its experimental aggravation should lead to accelerated or pathological aging, and finally, its experimental amelioration should retard normal aging and increase healthy lifespan. However, complex interactions between aging hallmarks makes it difficult to clearly demonstrate the last criteria independently of other hallmarks [71].

2.1.1 Genomic instability

Genomic stability is challenged throughout the aging process by extrinsic (exogenous physical, chemical and biological agents) and intrinsic (endogenous agents including DNA replication errors, reactive oxygen species-ROS) leading to diverse genetic mutations including point mutations, translocations, chromosomal gain and loss, telomere shortening and gene disruption caused by virus and transposons. To repair these lesions,

organisms have developed a complex DNA repair mechanism. These mechanisms allow for repair most of nuclear DNA damage, maintenance of a functional telomere length, and the integrity of mitochondrial DNA (mtDNA) [73].

The aging process unequivocally leads to the accumulation of nuclear DNA damage, even in the presence of repair mechanisms, that could affect essential genes and transcriptional pathways, creating dysfunctional cells. If not eliminated by apoptosis or senescence, these dysfunctional cells may impair tissue homeostasis. Moreover, mtDNA mutations have been showed to contribute to aging and have been considered a target due to the oxidative mitochondrial environment and the lack of histones and efficient mtDNA repair mechanisms. However, the role of mtDNA mutations on aging it is controversial due to the multiplicity of genomes [73].

Genomic instability has been also involved in brain aging. In fact, both DNA and mtDNA damage have been associated with brain aging process and age associated neurodegenerative disease [73].

2.1.2 Telomere attriation

Mammalian telomeres are particularly vulnerable to age-related deterioration due to the lack of constitutive telomerase activity (DNA polymerase that replicates completely the terminal end of linear DNA). Therefore, there is a progressive decrease in telomere length with age. A decrease in telomere length explains the limited proliferative capacity *in vitro* of some cultured cells, also called the Hayflick limit[73].

It has been shown that changes in telomeres length can modify lifespan-shortened telomeres lead to a reduced lifespan while the lengthening leads to an increase. Moreover, aging can be reversed by telomerase activation. Finally, alterations associated with brain aging such as cognitive decline and AD have been associated with modifications in telomerase expression and activity as well as shortening of telomeres [74–76].

2.1.3 Epigenetic alteration

Epigenetic modifications that can affect cells and tissues includes DNA methylation, post-translational modifications of histones and chromatin remodeling[73].

Aging has been associated with global hypomethylation. However, it has also been shown that some specific loci are hypermethylated[77]. There is controversy regarding the regulation of lifespan by modulating histone methylation. It has been showed that the demethylation of key components of insulin/insulin growth factor 1 (IGF-1) pathway can modulate lifespan[78]. However, the mechanism by which alterations of histone methylation can affect aging is not known. It could be due to direct epigenetic mechanisms, impaired DNA repair or through transcriptional alterations affecting signaling pathways outside the nucleus [71]. Aging is also associated with an increase in transcriptional noise [79] and alterations in mRNA maturation [80,81].

Epigenetic alterations are reversible and treatable with anti-aging drugs. For example, treatment with a histone deacetylase inhibitor can reverse histone 4 acetylation levels and prevent age-related memory impairment [82].

Several studies have unveiled the important role for epigenetics modifications in learning and memory, aging and neurodegenerative disease. It has been suggested that epigenetic changes during aging can cause synapse loss and structure alterations. Histone deacetylase 2 (HDAC2) for example, increases its expression and leads to a reduction in dendritic spines. Moreover, in AD patients it has been shown a reduction in general DNA methylation[83–85].

2.1.4 Loss of proteostasis

All cells have a quality control system to maintain protein homeostasis. Known as proteostasis, this process controls the proper folding of proteins, the stability and functionality of proteins and the mechanisms of protein degradation by the lysosome or the proteasome [86–88]. These mechanisms

have been shown to be altered in aging [87]. The degradation systems implicated in protein quality control are autophagy and the ubiquitin-proteasome system. Both systems are altered in aging, supporting the idea that the loss of proteostasis is an aging hallmark[89,90].

Moreover, unfolded, misfolded or aggregated proteins contribute to the development of some pathologies such as Parkinson's disease (PD) or AD, in which the main risk factor is aging.

2.1.5 Deregulated nutrient-sensing

There are several nutrient-sensing pathways, which informs the cell of the present of glucose, that have been involved in aging. One of them is the somatotrophic axis which comprises growth hormone (GH) and IGF-1 signaling. The evolutionarily conserved insulin/IGF-1 signaling (IIS) pathway carries out this function. Multiple genetic attenuations of the IIS pathway results in extended lifespan across species [91]. However, GH and IGF-1 levels are reduced in normal aging, demonstrating that decreased IIS signaling is a normal trait of aging. It could be explained if the decrease in IIS signaling reflects a defensive response by the cell to minimize metabolism and cell growth in cases of systemic damage. Models with decreased systemic IIS signaling can survive longer because they have lower rates of metabolism and cell growth, and thus, less cellular damage[92]. Other pathways involved include mTOR, AMPK and sirtuins[93–95].

In general, it is believed that anabolic pathways accelerate aging, whereas decreased nutrient sensing extends lifespan[91]. Insulin/IGF-1 pathway has been found to be altered in AD and brain aging. In AD, insulin receptors and related signaling proteins are reduced, whereas insulin levels in plasma are high and glucose disposal low [96,97]. Amyloid-β peptide (Aβ) has been shown to reduce the activation of the pathway and induce the removal of the insulin receptors from the surface [98–100]. However, there is controversy regarding the activation state of the pathway in AD brains since some

reports showed a decrease in Akt phosphorylation whereas other studies suggested an increase in Akt phosphorylation[98,101,102]. Moreover, during aging there is a reduction in insulin sensitivity. Insulin resistance is linked to accelerated aging processes[103].

2.1.6 Mitochondrial Dysfunction

A decrease in mitochondrial function is associated with aging as it decreases the efficacy of the electron transport chain, generating less ATP and more electron leakage [104,105].

Mitochondrial dysfunction can be caused by an increased level of ROS or by alterations in the integrity and biogenesis of mitochondria. In 1965, the mitochondrial free radical theory of aging was proposed stating that mitochondrial dysfunction associated with aging increases levels of ROS leading to further mitochondrial and cellular damage [106]. This theory has been evaluated since then. Now it is believed that increased production of ROS by the mitochondria is an attempt to maintain cellular survival. Low levels of ROS cause reversible post-translational modifications which can modify the activity of the target protein helping the cell to adapt to stress. However, when ROS levels reach a threshold, they aggravate the situation becoming harmful for the cell and increasing age-associated damage [107,108].

Processes that can alter mitochondrial integrity and biogenesis include the accumulation of mutations and deletions in mtDNA, oxidation of mitochondrial proteins, destabilization of electron transport chain complexes, changes in mitochondrial lipid composition, an imbalance of fission and fusion events due to altered mitochondrial dynamics, and defective mitophagy (an organelle-specific form of macroautophagy targeting defective mitochondria) [109].

Overall, mitochondrial dysfunction in mammals accelerates aging by increasing damage and decreasing turnover of mitochondria[71,110–112]. Moreover, it is known that mitochondrial dysfunction is particularly involved

in the aging process of post-mitotic cell types such as neurons and muscular cells. Particularly, neurons are highly dependent on the mitochondrial electron transport chain and the ATP generated by the mitochondria. An inefficient transport chain leads to ROS generation, overwhelming the inner antioxidant defenses and generating protein oxidation and DNA damage as found in AD [113].

2.1.7 Cellular Senescence

The stable arrest of the cell cycle coupled to stereotyped phenotypic changes is called cellular senescence [71,114–116]. It was first described by Hayflick who studied human fibroblasts passaged in culture[117]. Cellular senescence is caused by: telomere shortening, non-telomeric DNA damage and de-repression of the INK4/ARF locus[71,114,118]. Telomeres are repetitive DNA sequences that protects DNA endings from shortening and recombination. Aging process leads to their shortening [118]. Moreover, different types of DNA damage can induce cell senescence and organismal aging show higher levels of mutations. Finally, INK4/ARF locus are two critical tumor suppressor genes which expression is increased in aging [71,114].

Cellular senescence is believed to be a beneficial compensatory mechanism in response to damage that becomes deleterious and accelerates aging when tissues exhaust their regenerative capacity [118].

Cellular senescence has been studied particularly in mitotic cells. In the brain, most of studies have been done using proliferating glial cells. However, neurons with senescent hallmarks have also been found in aging brains [119].

2.1.8 Stem cell exhaustion

During chronological aging there is a decline in the regenerative potential due to a deficient proliferation of stem and progenitor cells. This is a protective process to avoid excessive proliferation that can also be deleterious by accelerating exhaustion of stem cells niches [71]. Ultimately, stem cell exhaustion is an integrative consequence of multiple damages and it

is responsible for tissue and organism aging. It has been shown that stem cell niche rejuvenation may reverse aging [120]. In fact, the dentate gyrus in the hippocampus consists of a pool of neural progenitors that are involved in learning and memory and alterations in neurogenesis are associated with AD [121].

2.1.9 Altered intercellular communication

Chronological aging occurs with changes in intercellular communication at the endocrine, neuroendocrine or neuronal level. Changes in intercellular communication affects the mechanical and functional properties of tissues[95,120,122,123].

Inflammation is one of the main causes of altered intercellular communication during aging. The chronic and low grade inflammation that occurs during aging is termed "inflammaging"[103,124]. This increase in inflammation could be explained by multiple reasons including the accumulation of pro-inflammatory tissue damage, the failure of the immune system to clear pathogens and dysfunctional host cells, the increase of pro-inflammatory cytokine secretion by senescent cells, the reduced autophagy response or an enhanced activation of the NF-KB signaling pathway [125].

Aside from the inflammation, there is evidence showing that age-related changes in one tissue can deteriorate other tissues[71]. It has been shown that senescent cells can spread senescence towards their neighbor cells through gap junctions and ROS signaling [126]. Moreover, CD4T defects have been observed in aging due to the microenvironmment, which can lead to systemic alterations [127]. Finally, modulation of life-span in one tissue can retard aging process in other tissues. For example by treating a murine progeria model with muscle-derived stem/progenitor cells improve degenerative changes and vascularization in tissues where the progenitors are not found [128].

2.2 Brain and neuronal aging

The greatest risk factor for cognitive decline and Alzheimer's Disease (AD) is aging itself. Therefore, slowing down or reversing brain aging could have a huge impact on the quality of life for the elderly and their caregivers [69,70]. To achieve that aim, more information is needed regarding the mechanisms behind the aging process, the strategies to reduce pathological symptoms and preserve cognitive functions [69–71].

Brain aging leads to stereotypical and neurophysiological changes and a variable cognitive decline even in absence of disease. The most consistently affected cognitive abilities due to aging are processing speed, working memory and episodic memory [69,113,129,130].

It has been shown that cognitive decline in normal aging arises from a global loss of integrative function. Therefore, the different brain regions that serve as higher-order structures supporting cognition show less-coordinated activation. This anatomical disconnection observed between areas could be explained by the loss of white matter and demyelination or by changes in synaptic physiology of aging neurons [69,130].

Aging hallmarks previously mentioned such as mitochondrial dysfunction, DNA damage, changes in conserved metabolic pathways (IGF/insulin) or loss of proteostasis are key factors for brain aging. However, the role of these hallmarks in the onset and progression of neurodegenerative disease are not known. More intervention for targeting those pathways in normal and pathological aging is needed to unveil the connection [69,130].

Individual vulnerability to neurodegenerative disorders during aging is determined by genetic and environmental factors that interact with molecular and cellular mechanism of aging. During pathological aging, there is an abnormal increase in normal aging molecular changes within vulnerable neuronal populations. Usually, vulnerable neurons are large and myelinated projection neurons that connect different regions of the brain. These

neurons are vulnerable because they require high levels of energy, precise axonal transport (integrity of the cytoskeleton) and are exposed to different, potentially toxic environments throughout the brain [104].

There is a debate whether a continuum exists between normal brain aging and dementia (AD). This theory is supported by the presence of AD pathology in 20-40% of older individuals cognitively normal prior death. Preservation of cognitive abilities could be due to brain reserve or due to the fact that AD pathology has not occurred yet prior death. In contrast, the occurrence of AD pathology is not always observed with age and there are differences in patterns of brain atrophy between aging and presymptomatic AD [129,131–135].

Overall, we can say that molecular alterations between normal and pathological aging are quantitative and the boundaries between them are weak. Therefore, it is known that normal aging is a central process of pathological aging even if it is not known to what extent. Accordingly, it makes sense to use models of normal aging and pathological aging to enhance our knowledge of both processes as we did in this thesis. It is expected that a modification that slows down normal aging would also help fight against neurodegenerative disease [104,129].

3 Alzheimer's disease

AD was identified for the first time by Alois Alzheimer in 1906 [136]. AD is the most common neurodegenerative disorder leading to irreversible dementia without any effective treatment.

Dementia describes a group of symptoms affecting memory, problem solving and social skills which collectively alter daily life. Today, over 46 million people suffer dementia worldwide and it is expected to increase to 74 million by 2030 due to aging of the world population [137]. Total estimated cost of dementia worldwide in 2015 was 818 million dollars. By 2030 dementia will become a 2 trillion dollar disease [137]. Dementia is and will be a major problem in the near future for high income, low or middle income countries. There are other causes of dementia such as vascular dementia, frontotemporal dementia, Lewy bodies disease and Huntington disease [137,138].

3.1 General characteristics: protein aggregation

AD is characterized by memory and cognitive decline due to neuronal death and synapse loss. AD leads to degeneration of different brain structures where protein deposition is more abundant. The process starts in the entorhinal/perirhinal cortex, then moves to the hippocampus and the association cortex and finally to the primary neocortex [134,139,140] (Fig 8A).

AD is characterized by two main histopathological hallmarks: intracellular neurofibrillary tangles (NFT) and extracellular amyloid or neuritic plaques (AP) (Fig 8B-D). NFT are intraneuronal aggregates of hyperphosphorylated tau that become extracellular when tangle-bearing neurons die. AP are extracellular deposits of aggregated Aβ classified as diffuse or dense-core base plaques, depending on their morphology and positive staining with Congo-red or Thioflavin-S [136,141–143] . Diffuse plaques are amorphous amyloid deposits, which are non-neuritic and not associated with glial response or synaptic loss. These plaques are not stained by Congo-red or Thioflavin-S. In contrast, dense-core plaques are compact, fibrillar amyloid deposits that are

surrounded by dystrophic neurites, reactive glial cells and synaptic loss. These plaques are stained with Congo-red or Thioflavin-S and are used to diagnose AD because they are correlated with cognitive impairment [135,144–149].

Eighty percent of AD patients exhibit Aß deposits in the tunica media of brain vessels in form of cerebral amyloid angiopathy (CAA) (Fig 9). Specifically, these deposits occur in posterior areas of the brain which can weaken the vessel and cause lobar hemorrhages [134,150].

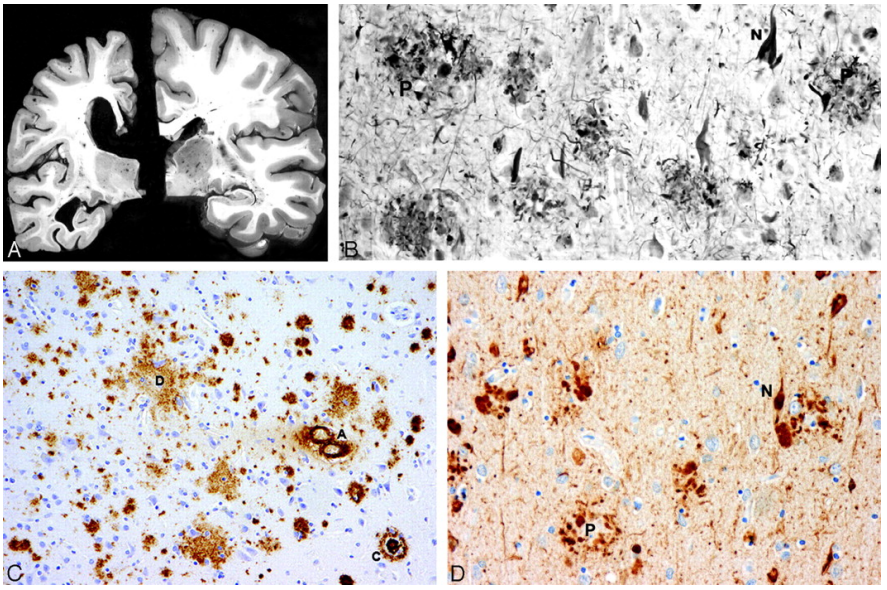


Figure 8: Histopathology of AD. A) Section of an AD (left) and a healthy (right) hemibrain. The AD brain shows marked atrophy of cerebral and entorhinal cortex and the hippocampus and enlargement of the lateral ventricle. B) Neurofibrillary tangles (N) and neuritic plaques (P) in AD hippocampus stained with modified Bielschowsky silver impregnation. C) Immunohistochemistry against β-amyloid in an AD frontal lobe. In this image it is observed a diffuse plaque (D), a cored plaque (C) and cerebral amyloid angiopathy (A). D) Phospho-Tau immunochemistry in an AD frontal lobe staining neurofibrillary tangles (N) and neuritic plaques (P). From Wippold et al., 2006[143]

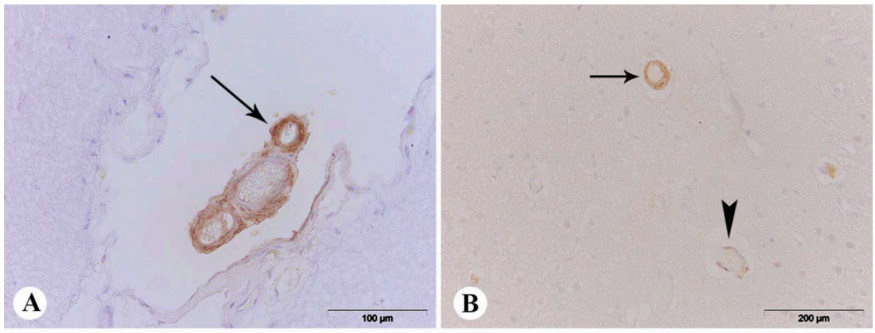


Figure 9: Histopathology of CAA. A) Immunohistochemistry against $A\beta_{1-42}$. Aß deposits in the wall of meningeal blood vessels (arrow) are stained B) Immunohistochemistry against $A\beta_{1-14}$. Aß deposits in the wall of parenchymal blood vessels (arrow) are stained. From Nešić et al., 2017 [150]

3.2 Causes and risks factors

AD incidence increases from 1% between the ages of 60-70 years old to 6-8% at 85 years or older. Moreover, AD prevalence also increases significantly with age. Therefore, the prevalence and incidence of AD suggests that aging is the most influential known risk factor [151].

AD can be classified in two subtypes based on the age of onset: early-onset Alzheimer's Disease (EOAD) and late-onset Alzheimer's Disease (LOAD).

EOAD accounts for 1-5% of all cases and includes an age of onset between 35 and 60-65 years old [151–153]. Sixty percent of EOAD cases have multiple cases of AD in their relatives and 13% of these familial EOAD, are autosomal dominant inherence with at least 3 generations affected. EOAD is a entirely genetically based disease [154–156].

Ninety-five percent of AD cases are LOAD, which is the most common form of AD and includes age of onset later than 60-65 years-old. LOAD causes are not known [151,152].

Another classification used in AD is familial versus non-familial, also called sporadic or idiopathic. However, due to the increasing knowledge regarding genetics of the disease it seems that most sporadic cases are in fact, influenced by genetic risk factors [152]. AD heritability has been estimated to be between 58-79% and genetic factors influence both men and women [157].

Early-onset disease can also occur in families with late-onset disease and both EAOD and LOAD may occur in families with positive AD histories [153,154]. Therefore, the real classification is not as easy as the theoretical one, because there is a tight correlation between all four categories. Only a few cases of autosomal dominant inheritance are single-gene disorders. Furthermore, AD is considered a complex, polygenic and multifactorial disorder in which multiple susceptibility genes and environmental factors are involved. Finally, the pattern of transmission is not consistent with Mendelian inheritance [153,158–161].

A few families are affected by several mutations (less than 5% of the cases). They are fully penetrant autosomal dominant mutations in the genes encoding for the amyloid precursor protein (*APP*), presenilin 1(*PSEN1*) or presenilin 2 (*PSEN2*) (Table 1) [152,162–165]. These mutations share a common biochemical pathway and cause AD by increasing the generation of Aß$_{42}$ and ß-amyloid deposition [135,152,160]. In fact, this discovery originally lead to the amyloid cascade hypothesis (see below).

Table 1: AD autosomal dominant mutation

Gene	Protein	Chromosome	Mutations	Variation
APP	amyloid precursor protein	21q21.2	51	Increased $A\beta_{42}$/ $A\beta_{40}$ ratio Increased Aβ production Increased Aβ aggregation
PSEN1	Presenilin 1	14q24.3	219	Increased $A\beta_{42}$/ $A\beta_{40}$ ratio
PSEN2	Presenilin 2	1q42.13	16	Increased $A\beta_{42}$/ $A\beta_{40}$ ratio

The apolipoprotein E (APOE) gene is the only risk factor that has been linked in multiple studies with LOAD, specifically the ε4 allele. The three major alleles of the APOE locus are ε2, ε3 and ε4. These variations correspond to combination of two amino acid changes at residues 112 and 158 (ε2: Cys112/ Cys158; ε3: Cys112/Arg158; ε4: Arg112/Arg158). Heterozygous ε4 carriers have a three-fold increased risk of being diagnosed with AD, whereas homozygous ε4 carriers have an eight to ten-fold increased risk. Moreover, several studies have also been showed a weak protective effect of the ε2 allele [152,153,166–168]. Although the presence of one or two APOE e4 allele is neither necessary or sufficient to cause AD [153].

Thanks to more powerful and efficient sequencing methods, more genome-wide association studies (GWAS) have been published. GWAS have allowed the characterization of several genetic risk variants associated with AD (Table 2)[152,169,170]. Three types of processes have emerged as important: cholesterol/sterol metabolism, inflammation and innate immune system and endosomal vesicle recycling [171].

Table 2: Risk genetic variants associated in AD

Gene	Protein	p value*	Function
APOE	apolipoprotein E	<1E-50	Aggregation and clearance of Ab; cholesterol metabolism
BIN1	bridging integrator 1	1.59E-26	Production and clearance of Ab. Receptor-mediated endocytosis.
CLU	clusterin	3.37E-23	Aggregation and clearance of Ab; inflammation
ABCA7	ATP binding cassette subfamily A member 7	8.17E-22	Lipid metabolism, clearance of Ab
CR1	Component complement 3b/4b receptor 1	4.72E-21	Clearance of Ab; inflammation
PICALM	Phosphatidylinositol bindind clathrin assembly protein	2.85E-20	Production and clearance of Ab. Synaptic transmission and endocytosis
MS4A6A	Membrane spanning 4-domains A6A	1.81E-11	Signal transduction
CD33	CD33 molecule	2.04E-10	Innate immune system response

* p values refer to systematic meta-analyses of the publicly available AlzGene database [169]

3.3 Amyloid-β peptide

Aβ is a 4 kDa peptide made up of 36-43 amino acids and is a major component of AP in AD [172,173]. It also plays a critical role in other pathologies such as Down's Syndrome[174] and CAA[175]. The physiological role of Aβ is not completely understood but it seems to become toxic when it is misfolded into β-sheets due to high production and low degradation and clearance rate [176–178]. Therefore, the study of Aβ production, clearance and neurotoxicity are crucial for the development of new treatments to fight AD.

3.3.1 The amyloid cascade hypothesis

The amyloid hypothesis was firstly postulated in the early 90's claiming that Aβ is the primary driver of AD pathogenesis and initiates the pathological cascade leading to AD. This hypothesis postulates that Aβ production and deposition precedes and initiates the rest of the disease process including neurofibrillary tangles formation, cell loss and vascular damage leading to dementia [173,179–183].

Several evidences support the amyloid hypothesis, some observations were originally known and others have been discovered recently (Fig 10).

1. Mutations in *APP*, *PSEN1* and *PSEN2* cause AD due to the increased production of Aβ [184,185].
2. Humans with Down Syndrome (Trisomy 21) contain three copies of *APP* and develop typical neuropathological AD. In contrast, individuals with rare mutations that leads to duplications on chromosome 21 the APP locus do not develop AD [186,187].
3. Aβ is toxic to cultured neurons and other cell types [188].
4. APOE ε4 allele is a major risk factor for developing LOAD and is associated with an increase of Aβ deposition in the brain and blood vessels in humans [189]. Using a knock-in mouse model expressing human APOE ε4 and familial AD linked-APP transgenes, it has been shown that there is decreased levels of Aβ clearance [190,191]. In contrast,

mice lacking *APOE* gene show a decrease in AP and neuritic dystrophy which is reversed when ε4 or ε3 allele are expressed [192].

5. Mutations in *APP*, specifically in the Aβ encoding region cause familial AD and CAA by increasing aggregation propensity or inhibiting the degradation of Aβ [193–195].
6. A mutation in *APP* protects against AD and cognitive decline by reducing β-secretase cleavage leading to a decrease in AP formation [196].
7. Aβ oligomers decrease synapse density impairs synaptic function and synaptic structure leading to memory impairment [197]. AD begins with a loss of hippocampal synaptic efficacy due to Aβ [67]. Moreover, synapse loss is the major correlate with cognitive decline [198,199].
8. Aβ accumulation has been shown to occur prior to neurofibrillary tangle formation. Moreover, it has been shown in familial AD stem cell-derived neurons that Aβ accumulation precedes tau alterations without tau being over-expressed [200–204]. However, it is known that Aβ neurotoxicity is partially dependent on tau presence [205–207].

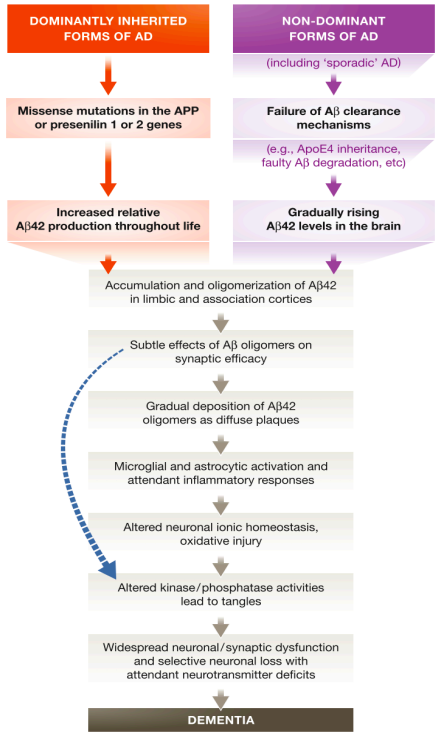


Figure 10: The amyloid cascade hypothesis. The blue arrow indicates that Aβ oligomers may directly injure the synapse and neurites in addition to activate microglia and astrocytes. From Selkoe et al., 2016 [183]

Nevertheless, there is still controversy on the matter because of several findings that appear to counter the amyloid hypothesis [208,209].

1. Correlation between AP with the degree of cognitive impairment is much less than with NT or synaptic loss. It can be explained by the deposition of oligomers which are undetectable or by an early deposition of Aβ (more than two decades before symptoms) that leads to all downstream cellular and molecular changes which cause neuronal dysfunction and cognitive decline [210]. Moreover, there is still a debate regarding the toxicity of Aβ sub-species and their detection [209].
2. Healthy, non-demented brains studied using Aβ-binding positron emission tomography (PET) ligands and post-mortem brains have been found to be rich in AP [210–212]. Some or many of these AP from non-demented patients are diffuse forms which are not linked to neuritic and glial pathology. Moreover, the differential role of AP and Aβ oligomers should be considered. The degree of cognitive decline is much more correlated with soluble forms of Aβ aggregates than AP, which are determined by histological studies [173,213–216]. In conclusion, this is consistent with the hypothesis that oligomers are the more toxic species. AP are able to sequester oligomers in a non-diffusible, less neurotoxic state until a limit is reached in which case the oligomers diffuse to surrounding synaptic membranes and other regions of the cell membrane [217].
3. Numerous clinical trials using anti-amyloid agents have not shown any clinical improvement. However, several of these trials were not designed properly in terms of the patients chosen, the choice of agent, the target or dose of the agent or the trials had to be halted due to off-target side effects. Targeting patients during the mild or very mild stages of disease have demonstrated clinical benefit [218,219]. However, it has been shown in familial AD disease model that the clearance of AP by

active and passive immunization therapies remove Aβ, improve synaptic function and cognitive performance [220].

3.3.2 Aβ production

Aβ is produced by the amyloidogenic pathway by sequential cleavage of the APP. APP is a ubiquitously expressed type-I transmembrane glycoprotein encoded by a gene located in the chromosome 21, which consists of 18 exons [221]. Human APP has three different transcripts (APP695, APP751 and APP770) generated by alternative splicing. APP695 is the predominant transcript expressed in neuronal tissue, whereas the other two isoforms are widely expressed in non-neuronal tissues [222]. APP is translocated into the ER following the secretory pathway where it undergoes post-translational modifications such as glycosylation, phosphorylation and tyrosine sulfation before arriving at the plasma membrane [223].

The physiological functions of APP are still being studied but it has been shown to be involved in cell proliferation [224], cell adhesion [225], differentiation [226] and synaptogenesis [227].

APP can be processed by three main enzymatic complexes (Fig 11): α-secretase, β-secretase beta-site APP cleaving enzyme 1 or by γ-secretase [228–230].

The non-amyloidogenic pathway involves cleavage of APP by α-secretase. This prevents Aβ formation since its cleavage site is within the Aβ domain [230]. Four enzymes with α-secretase activity have been identified within the ADAM family (A disintegrin and metalloproteinase family): ADAM9, 10,13 and 17 (tumour necrosis factor converting enzyme). ADAM 10 is the highly expressed α-secretase in the brain [231].

The amyloidogenic pathway is a sequential cleavage of APP by a group of enzymes termed β-secretase or beta-site APP cleaving enzyme 1 (BACE1) and by γ-secretase. BACE1 is type I integral membrane protein belonging to the pepsin family of aspartyl proteases localized to the intracellular

compartments of the secretory pathway, specifically the luminal site of Golgi apparatus and endosomes [232–234]. The γ-secretase is a multiprotein complex that contains five different proteins: PSEN1, PSEN2, as a catalytic core and nicastrin, anterior pharynx defective and presenilin enhancer 2 as additional indispensable subunits [235–238] .

Under physiological conditions, the non-amyloidogenic pathway is predominant [239]. APP is cleaved by the α-secretase at amino acid 83, producing a large N-terminal ectodomain called sAPPα. The α-secretase and β-secretase activities are mutually exclusive because α-secretase cleavage occurs within the Aβ region, thereby avoiding its formation [240,241].

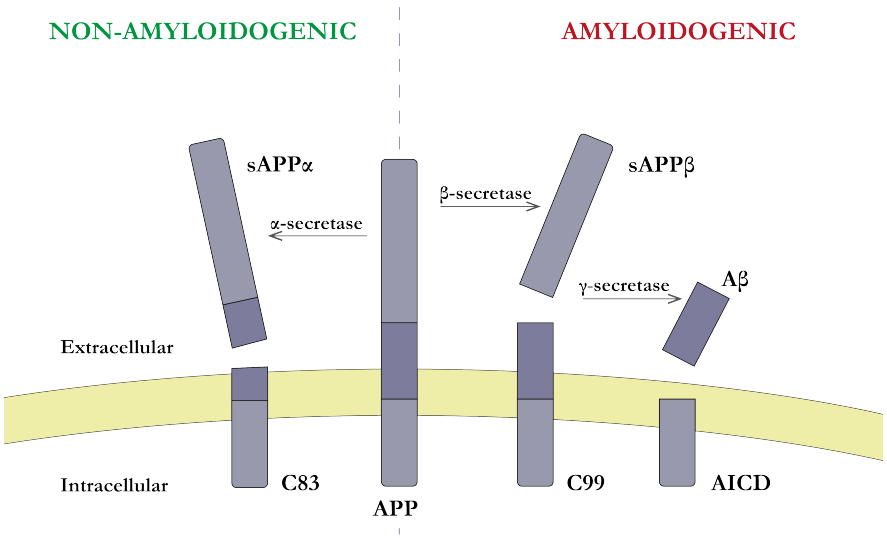


Figure 11: APP processing. APP can be processed through the non-amyloidogenic pathway (left) by α-secretase and γ-secretase generating sAPPα. Moreover, it can also be cleaved through the amyloidogenic pathway (right) by BACE1 and γ-secretase generating Aβ and AICD.

The alternative cleavage by the amyloidogenic pathway leads to Aβ generation. The first proteolytic step is mediated by BACE1 in a sequence specific manner in the early endosomes at amino acid 99 leading to release of sAPPβ fragment (N-terminal) to the lumen and C99, which remains in the membrane [242,243]. Subsequent cleavage of C99 fragment between residues 38

and 43 by the γ-secretase complex in the plasma membrane generates the Aβ peptide and the amyloid precursor protein intracellular domain (AICD) [244,245]. Depending on the γ-secretase cleavage site different Aβ species are produced. The most produced peptide of Aβ is $A\beta_{40}$ and $A\beta_{42}$ is less frequent (10%) but it is more hydrophobic and more prone to aggregate and to form fibrils [246]. The γ-secretase activity and, therefore Aβ generation is localized at the plasma membrane [247,248]. Aβ is released to the extracellular space [249] and from there can be internalized by nearby cells [250].

3.3.3 Aβ clearance

An imbalance between Aβ production and clearance leads to Aβ accumulation. In fact, Aβ clearance seems to be impaired in both EOAD and LOAD even at prodromal stages [173,251–253]. Furthermore, in AD patients, Aβ levels are increased in the brain but decreased in the cerebrospinal fluid (CSF) It has been shown that EOAD-associated presenilin mutations exhibit increased Aβ production and decreased Aβ clearance whereas patients with LOAD exhibit decreased Aβ clearance only [253–255]. Therefore, understanding Aβ clearance mechanisms and how they may become dysfunctional in AD is important to develop new therapies targeting this process to restore clearance mechanisms, eliminate Aβ deposits and delay (or even prevent) disease onset.

Aβ clearance systems in the brain interact and overlap. However, their relative contribution is still being studied. The following are the known Aβ clearance mechanisms (Fig 12):

a) Degradation clearance:
   - Intracellular Aβ is degraded by the ubiquitin- proteasome pathway [256], the endosomal-lysosomal pathway [257] or proteases such as the insulin-degrading enzyme [258].
   - Extracellular Aβ is also degraded by proteases such as neprilysin [259], glutamate carboxypeptidase II [260], metalloproteinases [261], endothelin-

converting enzyme [262], plasmin [261], tissue plasminogen activator [263], angiotensin-converting enzyme [261] and insulin-degrading enzyme [264].

- Interstitial fluid (ISF)(contains the brain extracellular residues) Aβ can also be degraded by astrocytes and microglia phagocytosis [265,266].

These degradation clearance mechanisms are impaired in aging and AD due to alterations in enzyme expression and activity, ligand affinity, and decreased initiation of intracellular degradation pathways and cellular uptake [267].

b) Blood-brain barrier (BBB) clearance: Aβ can move from the ISF through the BBB and into the blood and also, from the blood to the ISF through BBB. This mechanism allows most of the extracellular Aβ clearance. This transport is possible thanks to several receptors: low density lipoprotein family of receptors, such as LRP1 and LRP2 [268]. LRP2 is only functional in a complex with clusterin [268,269]. Another family of receptors involved in the clearance process are ATP-binding cassette transporters (ABC transporters), particularly, ABCB1(also known as MDR1) and ABCA1which mediates Aβ clearance through an APOE-dependent mechanism [270–272]. Other proteins such as α2-macroglobulin (α2M) are also involved in the clearance process [273]. Finally, free plasmatic Aβ can be transported to the ISF via the advanced glycosylation end product-specific receptor (RAGE) [269,274]. This process is prevented by sequestering agents presents in the blood which bind to plasma Aβ. The sequestering agents are the soluble form of RAGE (sRAGE), anti-Aβ immunoglobulin (IgG), serum amyloid P component (SAP) and the soluble form of LRP (sLRP)[275]. This binding allows systematic clearance of Aβ in the kidney and liver.

c) Glymphatic clearance: This is a mechanism by which the ISF is transported to CSF. The astrocytes mediate this form of Aβ transport and requires aquaporin-4 (AQP4) which is also perivascular [276–278]. Glymphatic clearance is reduced 40% in aged mice [278].

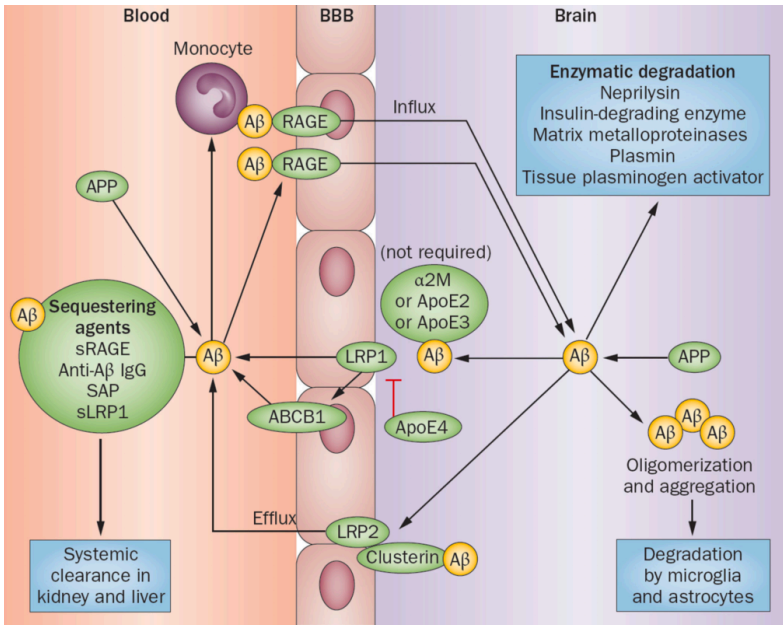


Fig 12: Aβ clearance mechanisms. APP is processed and Aβ is released in the brain and in peripheral tissues. Aβ in the brain is degraded by different specific enzymes or by microglia and astrocytes. Moreover, Aβ drainage to blood is allowed by LRP1, LRPP2/clusterin and ABCB1. Free Aβ influx to the brain is mediated by RAGE, but it is prevented by the sequestering agents present in the blood that permit the systemic clearance in the liver and the kidney. Abbreviations: α2M, α2-macroglobulin; Aβ, amyloid-β; ABCB1, multidrug resistance protein 1 (also known as P-glycoprotein 1); ApoE, apolipoprotein E; BBB, blood–brain barrier; LRP, LDL receptor-related protein; RAGE, advanced glycosylation end product- specific receptor; SAP, serum amyloid P; sLRP1, soluble LRP1; sRAGE, soluble form of RAGE. From Tarasoff-Conway et al., 2015 [267]

3.3.4 Aβ aggregation

Aβ is released from the membrane as a soluble monomer to the extracellular space. Soluble Aβ has a disorganize structure organized in random coil and α-helicoidal structures. Modulation of the *in vitro* conditions such as salt content, temperature, pH and agitation may facilitate the switch of Aβ secondary structure to β-sheet folding [279–281]. In fact, the β-sheet conformation switch and Aβ aggregation process is pH-dependent. This process is particularly promoted at pH 4-7, and the isoelectric point of the peptide is 5.5, at which Aβ aggregation and precipitation propensity are maximal [279,282].

Aβ aggregation process follows a sigmoidal growth curve (Fig 13) with three phases: lag/nucleation, growth and saturation. The lag/nucleation phase is a dynamic flux controlled by stochastic interactions between monomers, dimers and trimers until a stable misfolded nucleus is formed (seed oligomer). Once a critical aggregate size is achieved, the growth or elongation phase begins where these aggregates then interact with monomers, exponentially increasing the rates of aggregation and forming high molecular weight protofibrils. Finally, during the saturation phase, insoluble and mature fibrils are formed. The limiting factor in Aβ aggregation process is the formation of misfolded seeds that promote aggregation. Therefore, the process can be accelerated with the addition of preformed seed oligomers or truncated fibrils [279,283]. The aggregation process in the cell it is also modified by the interaction with lipid bilayers, particularly with several components of lipid rafts such as cholesterol and monosialotetrahexosyl ganglioside (GM1 ganglioside) [239].

Several AD-mutations in the APP region of Aβ sequence alter the aggregation process and propensity to aggregate (Fig 14). Leuven [284] and Flemish [285] mutations have been shown to increase Aβ production and secretion, respectively. A2V[286], Taiwanese [287] and K16N [288] mutations affect

aggregation propensity and the quantity of peptide generated. English [289], Tottori [290], Italian [291], Artic [292], Osaka [293], Dutch [294] and Iowa [295] mutations increase Aβ aggregation propensity.

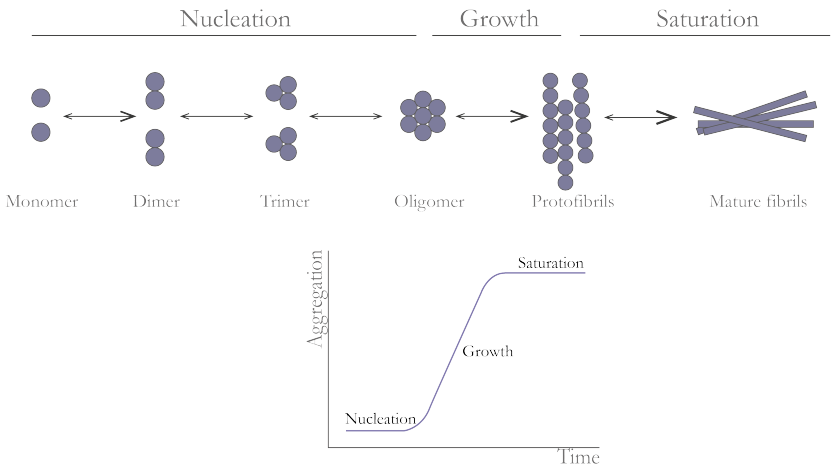


Figure 13: Aβ aggregation process. The aggregation process has three phases: nucleation, growth and saturation. The nucleation phase is a dynamic flux controlled by stochastic interaction between monomers, dimers and trimers until a stable misfolded nucleus is formed (seed oligomer). Once a critical aggregate size is achieved starts the growth or elongation phase, where these aggregates interact with monomers exponentially increasing the rates of aggregation forming high molecular weight protofibrils. Finally, insoluble and mature fibrils are formed in the saturation phase. The limiting factor in Aβ aggregation process is the formation of misfolded seeds that promote aggregation. Therefore, the process can be accelerated with the addition of preformed seed oligomers or truncated fibrils.

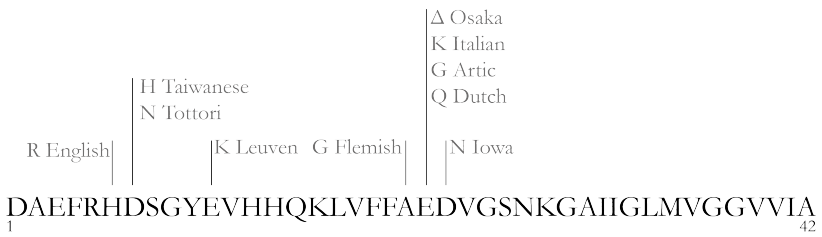


Figure 14: Mutations in Aβ region of APP. Different AD mutations have been found at position 2,6,7,11,16,21,22,23,34 and 42 yielding to early onset of AD.

Besides the mutations listed above Aβ peptide length modifies its aggregation properties. Under physiological conditions $A\beta_{40}$ is produced ten time more than $A\beta_{42}$ [296]. However, the two extra residues of $A\beta_{42}$ confer more hydrophobic properties and a lower energy barrier in all the steps of the oligomerization process, thus making $A\beta_{42}$ more aggregation prone than $A\beta_{40}$. $A\beta_{42}$ has a higher aggregation rate than $A\beta_{40}$ [246,283]. Moreover, it is believed that $A\beta_{42}$ aggregation precedes $A\beta_{40}$. In fact, $A\beta_{42}$ is the major component of AP in AD and $A\beta_{40}$ is only present in a subset of plaques [297]. It is also known, that an increased ratio of $A\beta_{42}/A\beta_{40}$ increases neurotoxicity and this is observed in patients with presenilin-mutations carriers [298,299].

Aβ oligomerization can also be modified by post-translational modifications such as oxidation, phosphorylation, nitration, racemization, isomerization, pyroglutamylation, and glycosylation. These modifications lead to the generation of peptides with altered pathological and more aggregation-prone properties [300,301].

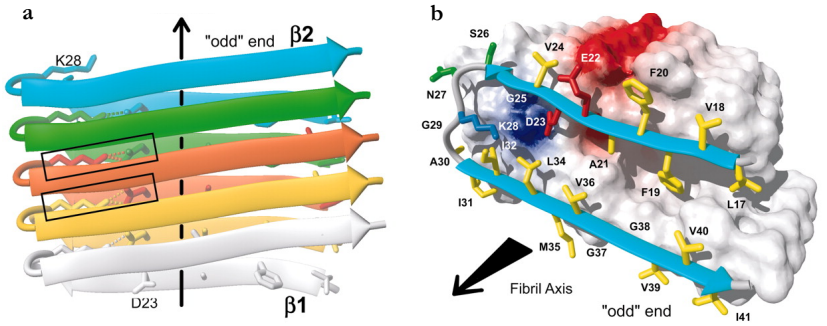


Figure 15: 3D structure of Aβ fibrils. Ribbon diagrams of the core structure of residues 17–42 showing the interactions of two antiparallel β-sheets (β1 and β2) a) Illustrate the molecular nature of intrafibrillar interactions and the supramolecular structure of the fibril. b) Represent fibril cross-sections. Black arrows represent the fibril axis. Modified from Lührs et al., 2005 [302]

The 3D structure of fibrils was resolved in 2005 (PDB ID: 2BEG). Each protofibril is formed by two antiparallel β-sheets comprising the residues L17-S26 (β1) and I31-I41 (β2). This structure is likely to be stabilize by

hydrophobic interactions, backbone hydrogen bonds and intermolecular salt bridges between D23 and K28 [302]. Interactions between protofibrilles are necessary to achieve the supramolecular structure of the fibril [303–305] (Fig 15).

3.3.5 Aβ toxicity

Aβ oligomers are the main toxic species responsible for synaptic dysfunction and neurite injury [173,306,307].

Extracellular Aβ oligomers have been reported to directly bind receptors such as the NMDAR, the AMPAR and the alpha-7 nicotinic receptor (α7nAChr), impairing glutamatergic and cholinergic transmission [308–310]. Aβ binding to NMDAR modifies channel activity and calcium influx leading to increased cytotoxicity and impaired LTP [239,311–313]. Aβ binding to AMPAR alters its internalization and dephosphorylation [310]. Moreover, α7nAChr binding to Aβ allows for peptide internalization. Other receptors have been shown to bind to Aβ such as nerve growth factor receptor [314], insulin receptor [314], frizzled receptor [315] and the cellular prion protein [316].

Intracellular direct Aβ binding to ryanodine receptors (RyR) and inositol 1,4,5-triphosphate receptors (IP_3R) modifies calcium levels in the ER and the mitochondria [317–322].

Another effect of Aβ oligomers on the plasma and lysosomal membrane is the increase of its permeability [323]. In lysosomes, increased permeability leads to the leakage of lysosomal hydrolases and Aβ to the cytosol [324].

Aβ in the mitochondria inhibits the respiratory chain complexes III and IV [325]. This could account for the abnormal distribution and morphology of the organelle and an increased mitochondrial fission observed in AD [326,327]. Moreover, Aβ oligomerization produces oxidative and nitrative stress in neurons [328]. The aggregation process produce H_2O_2 and $OH^{\cdot-}$ on its own [329–331] or through the activation of the microglia which releases more free radicals [332]. Free radicals cause further impairment of mitochondria, the

respiratory chain, lipid peroxidation, protein oxidation and nitrotyrosination [328,333]. Furthermore, it increases ROS production increasing oxidative stress leading to apoptosis [334].

Intracellular Aβ also impairs the ubiquitin-proteasome degradation pathway by inhibiting deubiquitinating enzymes and proteasome activity [335–337] and the autophagy degradation system [338,339]. Notably, Aβ is degraded by both degradation systems [267], so the presence of Aβ oligomers inhibits its own degradation, leading to a further increase in Aβ levels.

3.4 Therapeutics

Currently there is no effective treatment for curing or slowing down the progression of AD. However, five pharmacological treatment are approved by the U.S Food and Drug Administration (FDA) to treat symptoms (Table 3). These treatments are focus on two main strategies [340]:

1) Acetylcholinesterase enzyme (AChE) inhibitors: this group prevents the degradation of acetylcholine (Ach), which is significantly decreased in AD patients. AChE inhibitors treat memory, language and other cognitive deficits [340].
2) NMDAR antagonists prevent dysfunctional glutamatergic transmission and excitotoxicity. They are used to improve memory, attention, reason, language and the ability to perform simple tasks [340].

Table 3: FDA approved treatments for AD

Generic name	Name	Type	Approved for	Year
Donezepil	Aricept	AChE inhibitor	All stages	1996
Galantamine	Razadyne	AChE inhibitor	Mild to moderate	2001
Rivastigmine	Exelon	AChE inhibitor	Mild to moderate	2000
Memantine	Namenda	NMDAR antagonist	Moderate to severe	2003
Memantine + donezepil	Namzaric	Mixed	Moderate to severe	2014

3.4.1 Aβ therapeutics

The amyloid hypothesis suggests that Aβ accumulation is the primary cause that initiates the pathological cascade in AD [173,179–182]. Therefore, different approaches aiming to reduce Aβ accumulation through modulation, clearance or inhibition of Aβ aggregation are under study for AD treatment [341–343]. In the recent years these therapies have dominated AD clinical trials [344].

The main two strategies that are being studied regarding the reduction of brain Aβ are 1) the inhibition of BACE1 or γ-secretase enzymes to reduce Aβ production and 2) immunotherapy [345].

3.4.1.1 BACE1 and γ-secretase inhibitors

Both BACE1 and γ-secretase have other roles and other substrates in the cell besides APP.

BACE1 has over 100 substrates and it is known to be involved in axon myelination [346]. BACE1 inhibitors are usually small molecules with oral bioavaibility and a good penetration through the BBB. MK-8931 is the most promising inhibitor. A phase Ib clinical trial has been completed showing safety and efficacy [347] and phase II/III clinical trials finished last year in prodromal and mild to moderate AD patients (NCT01739348/NCT01953601). Another inhibitor AZD 3293 is also in phase II/III and still under study (NCT02245737) [348]. Recently, many BACE1 substrates have been discovered, increasing the fear of potential off-target effects [347,349]. In fact, the main concerns come from insufficient knowledge of the effects from BACE1 inhibition [196,350].

γ-secretase is involved in the proteolysis of more than 90 other proteins. A particularly important substrate is Notch, which has influenced the generation of second-generation inhibitors which specifically aim to avoid side effects caused by Notch proteolysis inhibition [351,352]. However, the

results from clinical trials are not promising. BMS-708163 was the first second-generation molecule tested but was discontinued due to side effects such as stomach reactions and skin problems. In addition, no cognitive improvement was observed. New molecules such as NIC5-15 have shown security and safety and CHF-5074 has also shown clinical safety [348,353].

3.4.1.2 Immunotherapy

Immunotherapy is one of the most attractive and promising areas of research for reducing cerebral Aβ levels [354]. This group includes three different approaches: passive immunization, active immunization or intravenous immunoglobulin (IVIG). It has been shown that immunoglobulins are able cross the BBB and act in the brain [355].

Passive immunization consists of an injection of exogenous prepared antibodies. The antibodies are usually humanized murine monoclonal antibodies or donor-derived human polyclonal. It is the most developed type of approach in clinical trials. This approach has several advantages such as the control of the final amount of the injected antibody in case of side effects and need of clearance and the possibility to be directed to several targets: APP, monomers, soluble oligomers or fibrils, as well as other Aβ carrier proteins. On the other hand, this therapy requires repeated infusions in order to be able to select the proper target and develop an immune reaction against the exogenous antibodies [356,357]. Moreover, it has shown side effects such as vascular edema and intracerebral microhemorrages called amyloid-related imaging abnormalities (ARIA) [358].

Several groups have shown the mechanism by which antibodies targeting the N-terminus region of Aβ inhibit Aβ aggregation [359–361]. Moreover, the peripheral administration of anti-Aβ antibodies in AD mouse models can reduce AP [355]. Furthermore, the first humanized monoclonal antibody was bapineuzumab targeting the N-terminus region which strongly binds to AP and less to soluble monomers. Bapineuzumab showed safety and tolerance,

however did not demonstrate any therapeutic effects. Furthermore, it showed relevant side effects such as ARIA [341,362,363]. More examples include solanezumab, which is specific to the middle domain of Aβ and binds to monomeric soluble forms, and gantenerumab, which is a conformational antibody and binds to aggregated Aβ. The first showed a reduction of cognitive decline in mild to moderate patients, suggesting the importance of early stage treatment [364]. The latter, gantenerumab in phase I showed reduction of AP. Both have a clinical trial ongoing with prodromal patients [348,363]. Finally, aducanumab reacts against soluble oligomers and insoluble fibrils, showing a reduction of Aβ in a dose and time-dependent manner. Phase III clinical trials are underway [348,365].

Active immunization leads to the stimulation of the immune system to produce their own antibodies. The advantages of this approach are the length of the antibody response and a fewer number of vaccinations needed to generate polyclonal antibodies with multiple specificities. On the other hand, the longer the antibody response, the longer the side effects occur (even lifelong). Moreover, the variability in the response depends on the patient and the age, where the immune system in an older individual may not be as effective [345]. AN-1792 was the first, containing a full-length pre-aggregated Aβ and an adjuvant. However, this vaccine showed severe side effects such as aseptic meningoencephalitis and no beneficial effects [354,363,366]. Another vaccine was CAD106 which contains $A\beta_{1-6}$ fragment with an adjuvant. This vaccine did not show adverse effects but the clinical efficacy was not probed [357,367,368].

Intravenous Ig (IVIG) is an alternative approach to passive immunotherapy which consists of the administration of antibodies present in a mixture of polyclonal antibodies prepared from blood plasma from more than thousand young and healthy humans [357]. This treatment has been already used for other diseases such as immunodeficiency and inflammatory syndromes or cancer. IVIG showed high affinity for oligomers and low affinity for

monomers. Furthermore, IVIG treatment was reported to be safe and to cross the BBB. However, this treatment was not able to overcome phase III due to the lack of positive results. Although it showed positive results among APOE-e4 carriers [369–371]. At the end of 2017 the AMBAR clinical trial (NCT01561053) including IVIG was completed [348] but the results are not available.

3.4.2 Difficulties for new therapies

Since 2003 no new treatments have been approved by the FDA for AD. Moreover, there is a high attrition rate and a low success rate for advancing from one phase to another and also for regulatory review, which is the lowest rate in any therapeutic area. [344].

The main two problems faced during this year in the generation of new therapies for AD, particularly the ones targeting Aβ, are: 1) difficulties in crossing the BBB [372] and 2) the selection of the patients that should be included in clinical trials. AD symptoms begin 10-20 years later than molecular pathological changes. As consequence, when the treatment is applied, it is too late because there is too much damage that is irreversible. Therefore, clinical trials should focus on prodromal or mild to moderate patients [373]. Patients carrying genetic-causing mutations which will develop the disease early are being targeted [374,375]. In this direction, it is important to find an earlier marker to be able to diagnose AD earlier and to start treatment earlier [376–379].

4. Plasma membrane and gangliosides

4.1 Plasma membrane composition; lipid rafts

Singer and Nicholson were the first to propose the fluid-mosaic model for biomembrane organization in 1972 [380]. Differences in lipid composition creates different compartments depending on the lipid organization with specific functions such as signal transduction, membrane trafficking or plasticity. Moreover, lipid composition diverges across cell types, tissues and organelles[381–383].

Lipids rafts are present in both the inner and the outer leaflets and coupled across leaflets. Moreover, they are evolutionary conserved structures present in prokaryiotic and eukaryotic organisms [382,384–387]. The membrane laterally segregates its constituents to be able to coordinate all functions. Different investigations showed lateral segregation heterogeneity [388,389] suggesting that the lateral heterogeneities in the membrane form lipid rafts. This hypothesis was made based on the broad concept of lateral heterogeneity, which is caused by differential interaction between cholesterol, relatively saturated lipids and glycosylated lipids. Their differential interaction leads to the formation of relatively ordered, tightly packed and functionally active domains able to recruit proteins and lipids [390,391] (Fig 16).

Finally, in 2006 a new consensus and operational definition was made by specialists on the field. Lipid rafts were defined as small (10–200 nm) heterogeneous, highly dynamic (in terms of both lateral mobility and association-dissociation), cholesterol and sphingolipid-enriched nano-domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger microscopic domains (>300 nm) upon clustering through protein-protein and protein-lipid interactions[382,384–387].

Originally, it was though that lipid raft formation was based on preferential interactions between sphingolipids and cholesterol [390]. Sphingomyelin has also been identified as a main component of lipids rafts due to its strong

interaction with cholesterol [392–394]. On the other hand, cholesterol has been found in both raft and non-raft domains at high levels, hence, with an enrichment in raft domains [391,395]. Gangliosides, particularly GM1, have also been found to interact with cholesterol in ordered (raft-like) domains [396,397]. Finally, saturated hydrocarbon phospholipids have also been associated with raft-like domains [391].

Lipid rafts are functional signaling platforms that regulate cellular functions. Lipid rafts localize, in proximity, membrane components to determine cell function and specificity. Moreover, they physically separate proteins present in rafts from non-rafts proteins. Protein content in raft-like and non-raft domains are not as well studied as lipid content. It is known that the proteins interacting via lipid anchors also follow the rules set by lipids: saturated lipid

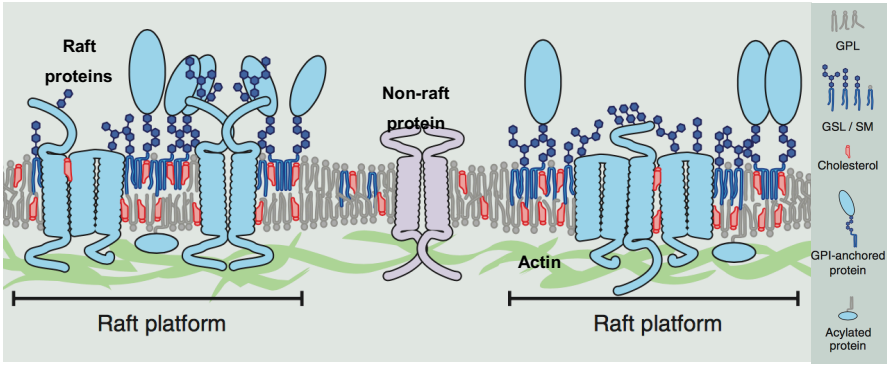


Figure 16: Lipid raft composition and heterogeneity in cell membranes. Lipid rafts are small, heterogeneous and highly dynamic nano-domains that compartimentalize cellular membranes. Lipid rafts are platforms of signaling events. The main components are saturated phospho, sphingo- and glycolipids, cholesterol, lipidated proteins and GPI-anchored proteins. Differential interaction between cholesterol, saturated lipids and glycosylated lipids determine their lateral composition. This assemblage can be accessed and/or modulated by GPI-anchored proteins, certain transmembrane proteins, acylated cytosolic effectors and cortical actin. Gray proteins do not possess the specificity to associate with this membrane and are considered non-raft. GPL, glycerophospholipid; SM, sphingomyelin. Modified from Lingwood et al., 2010 [381]

anchors (such as glycosylphosphatidylinositol (GPI)-anchored proteins) tend to be found in ordered domains. Lipid rafts proteomics have shown the presence of receptors and channels, recognition molecules, coupling factors and enzymes (Table 4). All these proteins are able to facilitate interaction and support signaling [382,386,391,398].

Table 4: Main proteins associated to lipids rafts

List of proteins
Caveolin-1
α-catenin
β-catenin
Fas/CD95
β-Actin
Ras
Integrin β-1
CD44

The main problem for lipid raft research has been the lack of accurate technical methods. First, lipid rafts were defined biochemically as detergent resistant membranes (DRMs) as they are resistant to non-ionic detergents (at low temperatures). Later, it was discovered that DRMs were not identical to lipid rafts. Moreover, new techniques have been found. However, the field is still looking for techniques that allow direct microscopic analysis of lipid rafts in living cells. As a consequence, the exact nature of rafts in live cells remains to be elucidated [386,390,391].

Therefore, since the formulation of lipid rafts hypotheses they have been implicated in several physiological and pathological processes in neurons. Currently they are reported to be highly relevant in axonal growth [399–401], synapse stabilization [402] or conversion of prion proteins [403,404].

4.2 Gangliosides

Gangliosides are a group of sialic acid-containing glycosphingolipids present in cell membranes, which contain a sialylated glycan attached to a ceramide core (Fig 17). Gangliosides can be found in all mammalian tissues. However, the brain, and particularly neurons, are enriched in gangliosides but they are still minority in neuronal membranes compared with other lipids (Fig 18). The 5 major gangliosides in the human brain are: GM1, GD1a, GD1b, GT1a, and GT1b [405–408]. It has been shown that rat cerebellar granule neurons contains 50 fmol/cell of glycerophospholipids, 5 fmol/cell of

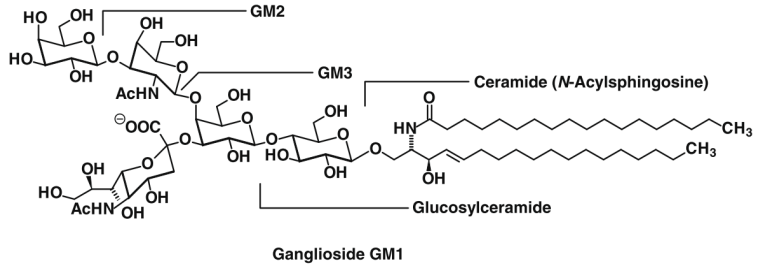


Figure 17: GM1, GM2 and GM3 ganglioside structure. Gangliosides contain a ceramide core attached to a glycan structure. The glycan structure contains a sialic acid. From Kolter et al., 2010 [408]

cholesterol, and 2 fmol/cell of sphingolipids (1 fmol/cell of gangliosides and 1fmol/cell of sphingomyelin) [409].

The sialyl glycan is composed by 3-8 saccharides with one or more sialic acid. There are four sialoglycan sequences in the brain. The major sialic acid in the brain is N-acetylneuraminic acid (Neu5Ac). On the other hand, the ceramide core of the ganglioside is composed by a single fatty acid (C18:0, stearic acid) attached to one of two sphingosines, 2-amino-4-octadecene-1,3-diol (d18:1) and 2-amino-4-dodecene-1,3-diol (d20:1). It generates a ceramide which contains highly saturated carbon chains that preferentially associate with each other and other saturated lipid chains. Therefore, gangliosides enhance the lateral self-association of lipids in the membrane. Gangliosides nomenclature

contains information about the series (G= ganglio) and the number of sialic acids (A= 0, M=1, D=2, T=3) [405–407,410].

Gangliosides are diverse, and therefore they play a wide variety of roles in the brain. They are involved in cell adhesion, cell-cell recognition, intracellular trafficking, cell proliferation and differentiation, modulation of ion channels, calcium homeostasis, signal transduction and neurotransmitter release [405,411]. The main mechanism of action is by controlling protein activity and signaling. Gangliosides can modulate membrane composition allowing for the inclusion or exclusion of proteins in the lipid raft which ultimately, affects receptor trafficking and protein interaction. Moreover, gangliosides can work as co-receptors that aid in proper positioning of the ligand to maximally activate the main receptor. Finally, membrane gangliosides can interact directly or indirectly, with receptors and modulate their function [405,411].

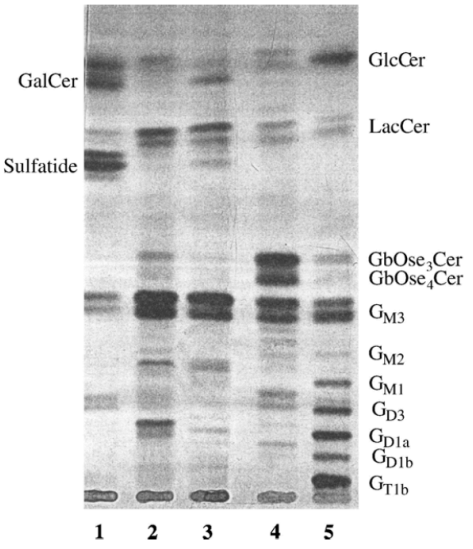


Figure 18: Gangliosides expression in different cell types. Fluorography from different cells incubated for 48h in the presence of [^{14}C] galactose. Lane 1, primary culture of oligodendrocytes; lane 2, primary culture of astrocytes; lane 3, neuroblastoma cells (B104); lane 4, fibroblasts; lane 5, primary cerebellar culture. The mobility of the lipid standards is indicated in the right. From Van Echten-Deckert et al., 2006 [407]

4.2.1 Synthesis and degradation pathway

Ganglioside synthesis is a sequential pathway in which monosaccharide and sialic acid residues are added to the lipid moiety. This process is mainly catalyzed by glycosyltransferases present on the luminal leaflet of the Golgi membrane. However, ceramide synthesis and its first glycosylation by glucosylceramide synthase (ceramide glucosyltransferase-CGlcT) occur at the cytosolic side of the ER leading to the formation of glucosylceramides [405,407]. The first glycosylation step in the Golgi is performed by galactosyl transferase I or lactosylceramide synthase (GalT I gene *B4GALT5*) to obtain lactosylceramides. Next, sialyltransferase I (SialTI gene *ST3GALT5*) also called GM3 synthase, adds a sialic acid to the chain to obtain GM3. GM3 is the simplest and the precursor of all types of complex gangliosides [412]. Sialyltransferase II (SialTII or GD3 synthase) and Sialyltransferase III (SialTIII or GT3 synthase) sequentially add sialic acids leading to GD3 and GT3 formation. Until now, we have already obtained different precursors of ganglioside series. These series are characterized by the presence of 0 (0-series), 1 (a-series), 2 (b-series) or 3 (c-series) sialic acids linked to the position 3 of the inner galactosyl residue [413]. Usually, sialic acid is bonded to the galactisyl residue through a α-2,3-linkage and to other sialic acid through α-2,8-linkage. GM3, GD3 and GT3 are the starting point of each series of gangliosides and seem to determine the relative amount of each series in the membrane [413]. Sequential action of GalNAc (N-Acetyl-D-galactosamine) transferase, also called GM2/GD2 synthase (gene *B4GALNT1*) and galactosyl transferase II (GalTII) also called, GM1a/GD1b synthase (gene *B3GALT4*) leads to the formation of the rest of the gangliosides of the series (Fig 19)[405,407,413,414].

Gangliosides are degraded on the surface of endosome and lysosome membranes after endocytosis. The degradation process is also a sequential process that functions to regulate gangliosides levels. Enzymes of the degradation pathway are soluble glycosidases that cleave monosaccharide

units in a step-wise manner from the end of the oligosaccharide chain. For the degradation of gangliosides with less than four sugars chains, due to their solubility the enzymes need assistance of glycoprotein cofactors called saposins A to D (SAP A-D), sphingolipid activator proteins and GM2 activator protein (GM2AP). Enzymes without these cofactors are not able to degrade their membrane bound-substrates. The degradation enzyme of the pathway is β-galactosidase and requires the cofactors SAP-B and GM2AP to cleave the galactose of GM1 and GA1. In addition β-hexamidase requires the GM2AP to cleave the GalNAc from the end of GM2 and GA1. Finally, the sialidase requires SAP-B to cleave the sialic acid to GM3 (Fig. 5.4) [405,407,408,415].

To study the consequences of dysregulating each step of this sequential pathway, several knock-out mice have been generated. Brains of mice deficient in GM2/GD2 synthase contain large amount of GM3 and GD3 that compensate for the lack of complex gangliosides [416]. They have a normal lifespan but they show abnormal myelination, optic nerve degeneration and altered motor function [417,418]. Moreover, knock out mice lacking GD3 synthase completely lack b-series gangliosides. However, they have high levels of a-series gangliosides and exhibit almost a normal phenotype [419]. In mice lacking sialyltransferase I, formation of 0-series gangliosides are still permitted and neurological abnormalities are absent [420]. Inactivation of both sialyltransferase I and GM2 synthase prevent ganglioside formation. Mice are viable but they suffer severe neurodegeneration [421]. In conclusion, it seems that complex gangliosides are required for the proper functioning of molecular pathways, but higher levels of 0 and a-series gangliosides are sufficient to maintain normal brain morphology [417].

Defects in the degradation pathway result in the accumulation of gangliosides in the lysosomes and other compartments in the brain and other organs. Two examples are the GM1 gangliosidosis (β-galactosidase deficiency) and GM2 gangliosidosis (Tay-Sachs disease or β-hexamidase

deficiency)[405]. Congenital deficiencies in β-galactosidase and β-hexamidase lead to GM1 and GM2 accumulation, respectively, and subsequent neurodegeneration [422,423].

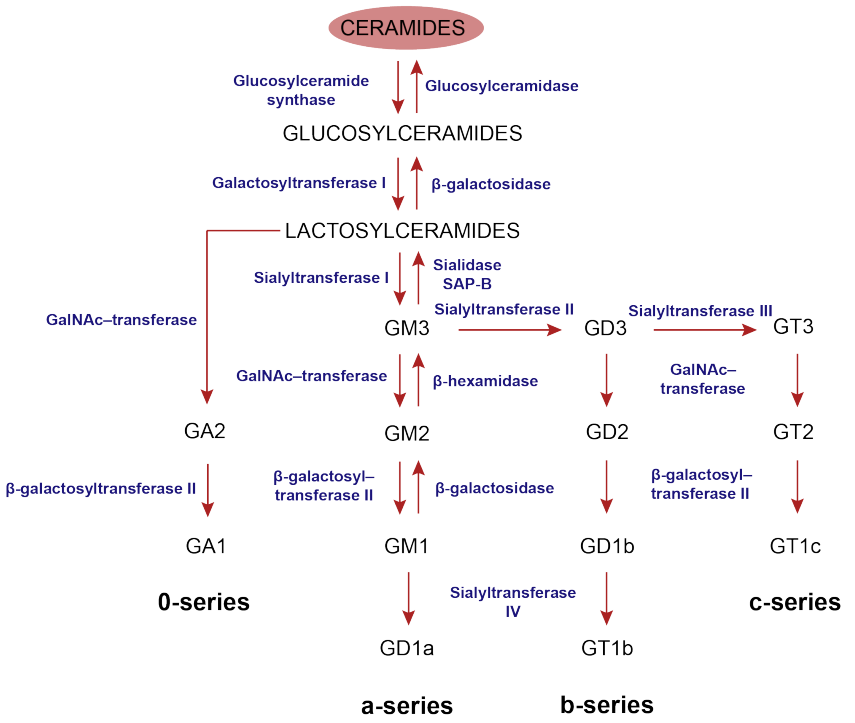


Figure 19: Synthesis and degradation pathways of gangliosides. The synthesis pathway is catalyzed by glycosyltransferases. Until the formation of the glucosylceramides the transmembrane enzymes are localized in the ER. Then, glucosylceramides are transported to the Golgi to continue the process. Degradation takes place in the endosomes or lysosomes.

4.2.2 GM1 ganglioside

GM1 represents 10-20% of total brain content and is a marker for lipids rafts due to its enrichment in these regions. GM1 ganglioside is expressed in different brain cell types, but it is mostly expressed in neurons (Fig 18) [407]. It is easy to detect using cholera toxin subunit B (CTX) [406,424,425]. Moreover, it is also the most studied ganglioside due to its involvement in several signaling processes through different mechanisms of action. GM1 contains a sialic acid (Fig 17) attached to the galactosyl residue (position II of the ganglioside) which gives a negative charge at physiological conditions (for free acid sialic pKa value is 2.6) [405,426]. Due to its particular α-2,3-linkage to the sialic acid, GM1 (and also GM2) sialic acid are resistant to hydrolytic removal by endogenous sialidases and also, by bacterial and viral sialidases [424,427].

GM1 is localized in the plasma membrane, particularly enriched in the pre and post-synaptic membranes[428], in the nuclear envelope [429], in the mitochondrial associated membranes (MAMs) [430] and the endosomes [425,431]

GM1 has been widely studied and has been involved in several cell roles such as the modulation of neuronal differentiation and neuritogenesis, modulation of ion channels and receptors, mirroring neurotrophins effect and pathological functions in AD or PD [424,425,432].

GM1 is important for neuronal development and maturation. Different studies have shown low expression levels during early phases of neuron migration and a rapid upregulation of GM1 (and other gangliosides) during out-growth and synaptogenesis under physiological conditions [433,434]. In pathological conditions such as GM1 gangliosidosis models there are ectopic neurites specifically in neurites with high levels of GM1 [435]. Supporting its role in neuritogenenesis, the treatment with GM1 has been shown to increase neurite out-growth in neuroblastoma [436] and primary neurons [437].

GM1 has also been shown to modulate sodium and calcium transport through the membrane. Studies in B4galnt1$^{-/-}$ mice show that GM1 controls

Na_v channel function through the modulation of lipid membrane integrity [438]. Moreover, GM1 also regulates calcium homeostasis. GM1 potentiates the activity of a nuclear envelope Na^+/Ca^{2+} exchanger [439], indirectly activates calcium entry to the cell through transient receptor potential C5 (TRPC5) channel by cross-linking with α5β1 integrin [440] and inhibits $Ca2^+$ uptake via the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)[441]. Finally, GM1 accumulation leads to activation of the unfolded protein response (UPR) through depletion of ER calcium stores [442]. Besides the UPR harmful effects, Ca^{2+} is uptaken by the mitochondria, that overload of Ca^{2+} , which can result in apoptosis [430].

Several studies have shown that GM1 exhibits neuroprotective effects. Trk receptors co-localize and interact tightly with GM1 in lipid rafts [443–447]. Interestingly, exogenous administration of GM1 and semi-synthetic derivatives (LIGA20) have been shown to activate TrkB receptor [448]. GM1 modulation of TrkB activity it is dependent of the glycosylation of the receptor [425]. Moreover, TrkA receptor trafficking its dependent of GM1 [449]. Finally, mice B4galnt1$^{-/-}$ or $^{+/-}$ showing no or low levels of GM1 have GDNF signaling impaired [450].

In the substantia nigra of PD patients, the levels of GM1 are decreased [451]. Moreover, B4galnt1$^{-/-}$ mice showed altered movements and neuropathological symptoms common to PD [452]. Finally, clinical trials using exogenous administration of GM1 have showed partial restoration of striatal dopamine levels and nigrostriatal neuron recovery in a mouse model of PD (MPTP-treated) [453,454] and in primates [455]. Finally, GM1's role in Alzheimer's disease will be discuss on the following section.

4.2.2.1 GM1, Aβ and Alzheimer Disease

In 1995, it was shown for the first time that Aβ binds to GM1 in the neuronal membrane. Moreover, the Aβ present in GM1-Aβ complexes is reported to have higher immunoreactivity than Aβ present in amyloid

plaques, a unique molecular characteristic responsible for the initial deposition of Aβ [456]. Later on, Choo-Smith *et al.* provided more information asserting that Aβ binds to membranes specifically containing GM1 and upon the binding Aβ undergoes a conformational transition to a rich β-sheet conformation [457]. Interestingly, an increase in GM1 levels leads to clustering that serve as a conformational catalyst to generate Aβ with a seeding ability [458]. Aβ oligomers strongly binds to GM1 which leads to a faster sequestration by membranes than monomers. Sialic acid has been shown to be required for this binding [457,459] . In fact, blocking the sialic acid in GM1 decreases the oligomer-mediated LTP impairment [217].

Conversely, the treatment with exogenous GM1 has been shown to regulate APP proteolysis. GM1 modulates γ-secretase activity leading to an increase in Aβ production [460]. Moreover, GM1 also binds to N-terminal domain of APP preventing the processing by the α-secretase [461]. Finally, there are evidences linking GM1 levels to AD. High levels of GM1 have been found in the CSF and in lipid rafts of neuronal membranes of AD patients which correlates with AP formation with and early AD outbreak [456,462]. Moreover, GM1-bound Aβ was found in CSF, showing a positive correlation with $A\beta_{42}$ levels [217].

4.2.2.2 GM1 and neurotransmission receptors

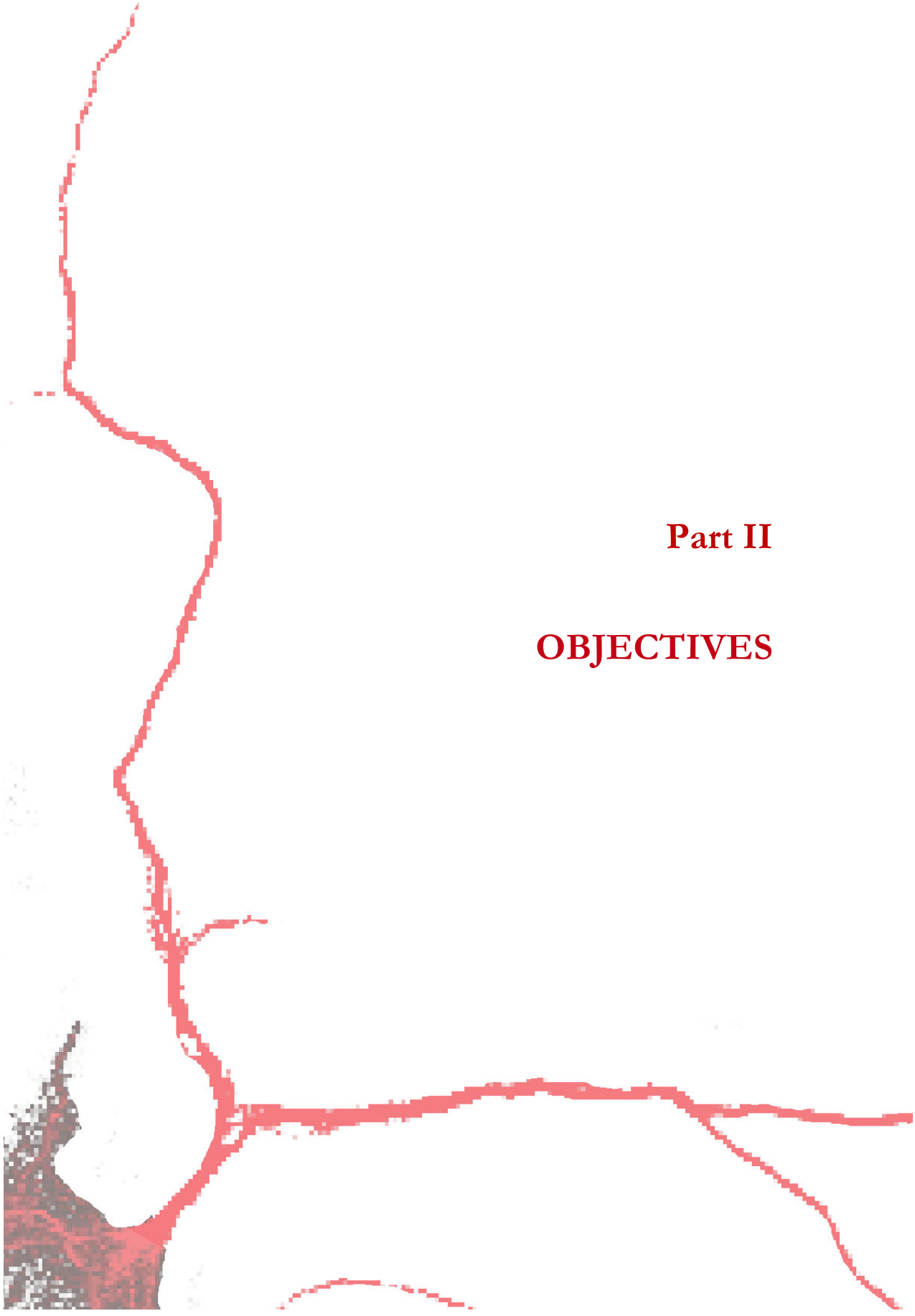
GM1 is enriched in lipids rafts, structures present in the active zone of the postsynaptic ending closely related to the PSD and where AMPARs and NMDARs are localized [463,464]. In fact, it is known that 60% of NMDAR are found in the lipid rafts [465]. Precise localization of both receptors is important for their function.

Gangliosides, particularly GM1, can affect receptor trafficking. For example, AMPAR subunit GluR2 binds selectively to GM1 whereas the proteins involved in its internalization such as thorase, NSF and nicalin bind selectively to GT1b. As a consequence, alteration of any of these

gangliosides in the membrane results in an altered distribution of AMPARs containing GluR2 [466]. The relationship between gangliosides and the major excitatory neurotransmission receptors needs to be further studied, but it could explain why congenital deficits in ganglioside biosynthesis pathway results in intellectual deficits and seizures [406].

Regarding cholesterol and NMDARs is it known that the removal of cholesterol from the cell and disrupting lipid rafts leads to a reduction of calcium entry through NMDARs, protecting the cell from excitotoxicity [467]. AMPAR basal internalization has been shown to be reduced after cholesterol depletion [464]. More research on the field is needed to understand how lipid raft content regulates trafficking and synaptic plasticity.

Finally, GM1 increase in a membrane model is able to induce tighter lipid packing due to GM1-GM1 interactions, altering membrane curvature and forming larger ordered clusters [468]. More studies are needed to determine if changes in GM1 or other gangliosides concentrations modify NMDAR and AMPAR function and activity. These modifications in membrane conditions could directly or indirectly affect receptors through indirect contact with channel-modulating proteins.



Part II

OBJECTIVES

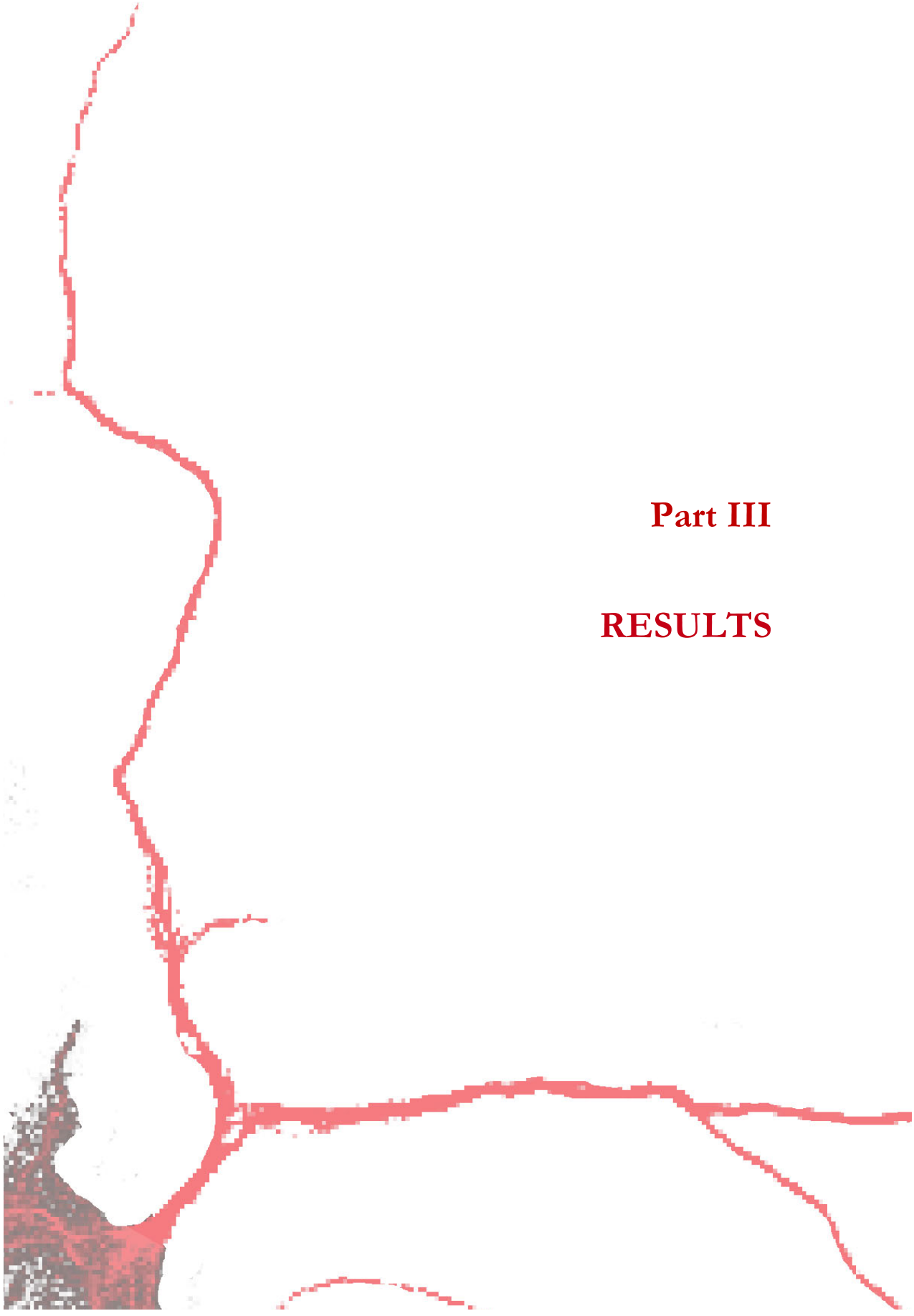
The main goals of the present thesis are to study ganglioside/glycosphingolipid alterations due to aging and its consequences on neuronal function, particularly on NMDAR and to study mechanisms to decrease Aβ oligomerization and neurotoxicity.

It is known that the aging process leads to neuronal molecular modifications. Several authors have linked GM1 ganglioside to AD by increasing Aβ aggregation. However, there are controversial results reported on GM1 content in aging, which is the main risk factor for AD. It explains our attempt to study ganglioside/glycosphingolipid modification during aging. Furthermore, both non-pathological aging and AD are associated with cognitive deficits at different degrees. For this reason, we have addressed the study of NMDAR function in aging. Overall, we propose that glycosphingolipid alteration, particularly GM1 increase, could be able to modify NMDAR activity.

Aβ aggregation turns amyloid into neurotoxic species. Therefore, the inhibition of Aβ aggregation is one of the main research targets in AD. Several studies have shown the potential effectivity of immunotherapy in AD. We attempt to study the molecular mechanism by which IgG treatment could affect Aβ aggregation and neurotoxicity. Moreover, we aim to determine the region of the immunoglobulin able to bind Aβ peptide.

The specific aims of the present thesis are:

- The study of the molecular processing pathway of membrane glycosphingolipid alterations in neuronal aging.
- The study of the consequences of these alterations in glycosphyingolipid processing pathway in neuronal function, particularly on NMDAR activity.
- The study of the effect of human IgG on Aβ aggregation and neurotoxicity.
- The identification of the IgG region important for its interaction with Aβ.



Part III

RESULTS

Chapter 1

The increase in GM1 ganglioside during aging reduces hippocampal NMDA receptor function and spine density

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The increase in GM1 ganglioside during aging reduces hippocampal NMDA receptor function and spine density

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Summary

Brain aging involves neurophysiological changes leading to cognitive decline even in the absence of pathology. GM1 is a ganglioside highly present in neurons, which is enriched in the postsynaptic terminal where NMDA receptors (NMDAR) are highly expressed. In the present study, we have analysed the glycosphingolipid/ganglioside content in murine hippocampus and primary neuronal cultures showing a consistent increase in GM1 and GM2 gangliosides in aging. Furthermore, we have studied the consequences of GM1 increase in synaptic function. Our dendritic spine study has shown a reduced spine density in GM1-enriched neurons, mimicking the effect observed in aged neurons. Moreover, in GM-enriched neurons, and not in GM2-enriched neurons, there is decrease in calcium entry after NMDA stimulation, mimicking the results we obtained in aged neurons. GM1 acts as a negative allosteric modulator of NMDAR, and our molecular dynamics simulation have shown that is partially through direct interactions with the ligand binding domain of these receptor. In conclusion, we have showed for the first time the consequences of the GM1 increase on NMDA receptor function, linking the increased levels of GM1 to the cognitive defects associated to brain aging.

1. Introduction

Nowadays, humanity is facing a demographic challenge. In 2010, an estimated 524 million people were aged 65 or older population. By 2050, this number is expected to triple, about 1.5 billion (Suzman & Beard 2011). Furthermore, the main risk factor for cognitive decline and neurodegenerative disease such as Alzheimer's Disease (AD) is aging (Bishop et al. 2010; Suzman & Beard 2011).

Brain aging leads to stereotypical and neurophysiological changes and a variable cognitive decline even in absence of pathology. This cognitive impairment is caused by the loss of coordination between high-order brain cognition areas (Bishop et al. 2010; Yankner et al. 2008; Jagust 2013; Andrews-Hanna et al. 2007).

Synapses depend on the properties of their pre- and postsynaptic membranes to allow neurotransmission. Synapses are particularly rich in lipid rafts, which have a high concentration of cholesterol and sphingolipids (Simons & Ikonen 1997; Wang & Silvius 2003; Sodt et al. 2015; Ramstedt & Slotte 1999; Levental et al. 2010), providing the required fluidity. Gangliosides are a group glycosphingolipids, which contain a sialylated glycan attached to a ceramide core (Supplementary figure 1a). The five major gangliosides in the human brain are GM1, GD1a, GD1b, GT1a, and GT1b (Ryan et al. 2013; Schnaar 2016; van Echten-Deckert & Herget 2006). GM1 ganglioside has special relevance for neurons (van Echten-Deckert & Herget 2006) and represents 10-20% of total lipid brain content. It is mostly expressed in neurons and it is found in the plasma membrane, where is enriched in pre- and post-synaptic membranes (Ledeen 1978). Interestingly, GM1 is particularly abundant in lipid rafts (Lozano et al. 2013; Harder et al. 1998) where 60% of NMDAR are found (Wheeler et al. 2009; Suzuki et al. 2011; Hering et al. 2003). Precise localization of the receptor is important for its function. Therefore, gangliosides and particularly GM1 membrane levels are important to keep its correct function. GM1 has been demonstrated to induce tighter lipid packing due to GM1-GM1 interactions, modifying membrane curvature, forming larger ordered clusters and facilitating segregation (Patel et al. 2016).

Here, we have studied the modifications in neuronal and brain gangliosides during the aging process. Particularly, we showed an increase in GM1 and GM2 in the hippocampus in vivo and in primary neurons. Finally, we studied the consequences of GM1 and GM2 increase in NMDA receptors activity, showing a reduction in the activity of these channels when GM1 is increased which leads to a reduction in the dendritic spine density.

2. Results

2.1 GM1, GM2 and lactosylceramides levels are increased in the hippocampus from aged mouse.

There is controversy regarding ganglioside expression, particularly GM1, during aging. GM1 has been postulated to be a leading cause of β-amyloid peptide (Aβ) aggregation (Choo-Smith & Surewicz 1997; Ariga et al. 2001) and therefore, AD. Here, we quantified the glycosphingolipid and ganglioside levels by mass spectrometry in hippocampi from 2 (young), 7 (adult) and 21 (aged) month-old mice (Figure 1). We observed an increase in the levels of glucosylceramides, lactosylceramides, GM3, GM2 and GM1 (Figure 1b-f) during hippocampal maduration (young to adult). Furthermore, we found an increase in lactosylceramides, GM2 and GM1 during the aging process in the hippocampus (adult to aged, Figure 1c,e and f). Figure 1h shows the relative increase of each glycosphingolipid compared with the levels in young hippocampus showing an increase localized in the A-series of the ganglioside pathway (Supplementary figure 1b).

Next, we determined the levels of the ganglioside synthesis pathway enzymes (Supplementary figure 1b). We analyzed by western blot B4GALT5, STGAL5, B4GALNT1 and B3GALT4 protein levels in the hippocampus without observing statistical differences between adult and aged (Supplementary figure 2). Supplementary figure 2f shows a diagram of the results obtained in this section.

2.2 GM1 and GM2 levels are increased in aged hippocampal primary neurons

To be able to directly study neuronal cells, we cultured primary hippocampal neurons for 14 days in vitro (DIV), as mature neurons, and 24 DIV, for the study of aged neurons (Sodero et al. 2011). We quantified the glycosphingolipid and ganglioside levels by mass spectrometry at 14 and 24 DIV (Figure 2), observing an increase in GM1 and GM2 gangliosides during aging (Figure 2e and f). Figure 2h shows the relative increase of each glycosphingolipid compared with the levels in control cells (14DIV) showing a specific increase centered of the A-series of the ganglioside pathway.

We determined by western blot the levels of the synthesis enzymes in neurons. We found an increase in the protein levels of B4GALT5, STGAL5, B4GALNT1, but no significant changes in B3GALT4 protein expression (Supplementary figure 3). Supplementary figure 3f shows a diagram of the results obtained in this section.

2.3 GM1 increase in primary neurons decreases dendritic spine density

Aging process has been linked to a decline in learning and memory. GM1, is present in the entire neuronal membrane surface, but with a higher concentration in the synaptic terminal (Aureli et al. 2016). Therefore, we hypothesized that GM1 could have a role in

the impairment of synaptic plasticity during aging. For this reason, we treated 13 DIV hippocampal neurons with GM1 ganglioside (500 nM) and neuraminidase from *Vibrio Cholerae* (NVC-0.03 U/mL) for 24h without causing neurotoxicity (Supplementary figure 4a). Exogenous GM1 can be integrated in the membrane and enter to the natural glycosphingolipid pathway. However, only GM1 monomers enter the plasma membranes, whereas GM1-micelles bind to cell surface interacting with proteins (Aureli et al. 2016; Saqr et al. 1993; Simons et al. 1999; Leskawa et al. 1989). Moreover, GM1 and GM2 particular α-2,3-linkage to the sialic acid are resistant to hydrolytic removal by bacterial neuraminidases (Ledeen & Wu 2015; Monti & Miyagi 2012) whereas the sialic acid linkages to complex gangliosides (GTs and GDs) are hydrolyzed becoming GMs during NVC treatment. Therefore, neurons treated with GM1 and NVC will have increased levels of these gangliosides in the membrane. However, our quantification by western blot (Supplementary figure 4b-c) only showed a significant increase in GM1 in neurons treated with NVC. We also analyzed GM1 levels by immunofluorescence (Supplementary figure 4d) observing an increase in GM1 after both treatments. We next analyzed the spine density and the percentage of the spine density in neurons with GM1-enriched membranes and aged neurons (Figure 3). Our findings indicates that GM1 increase reduce the density of dendritic spines (Figure 3a,b,e). Moreover, in aged neurons we also observed a reduction in dendritic spine density (Figure 3c,d). Probably, there are other factors involved in the reduction of dendritic spines during aging but our results indicate that GM1 would be playing a key role in this process.

2.4 GM1 levels in the membrane modify calcium response to NMDA

Considering that GM1 has been linked to calcium homeostasis (Wu et al. 2009; Wu et al. 2007; Ginzburg et al. 2007) and that is highly concentrated in the synaptic terminal, our next aim was to study the consequences of the increase of GM1 in neurons in the response to NMDA stimulation using calcium imaging. NMDA response is linked to synaptic plasticity and, therefore, to aging. NMDARs are heterotetrameric cationic channels that allow Ca^{2+} influx. In the adult brain, NMDARs in the hippocampus contain two GluN1 subunits and either two GluN2A or GluN2B subunits (Wang et al. 2014; Mayer et al. 1984; Lind et al. 2017; Vicini et al. 1998; Forsythe & Westbrook 1988). We increased GM1 levels in the membrane of hippocampal neurons treating them with GM1 ganglioside (500nM) and NVC (0.03 U/mL) for 24h. Treated and control neurons were loaded with the cytosolic calcium dye fura-2 AM. Stimulation with the NMDAR synthetic agonist NMDA generated a decreased calcium entry to the cytosol in neurons with high levels of GM1 (Figure 4a–c). Nevertheless, the opposite effect was observed when cells were stimulated with a physiological stimulus such as high potassium. Upon application

of a high potassium solution, which depolarizes cell membrane and activates voltage-gated calcium channels, we observed an increase in calcium entry to the cytosol in neurons with high levels of GM1 (Figure 4d–f). Thus, it appears that this effect is specific, although perhaps not exclusive, to NMDAR.

We also explored the effect of NMDAR stimulation in aged neurons. Besides having higher levels of GM1, aged neurons also suffer other alterations. Interestingly, we also observed a decrease in the entry of calcium to the cytosol in response to NMDA (Figure 4g–i). Moreover, upon the application of high potassium solution, we observed an increase in calcium entry (Figure 4j–l).

In addition, we tested the levels of the different NMDAR subunits after the treatments or in aged neurons by western blot. We did not observe differences in the levels of the different subunits in any condition (Supplementary figure 5a-h).

Finally, we tested the specificity of GM1 ganglioside in modifying NMDAR function by treating hippocampal primary neurons with GM2 for 24h without causing neurotoxicity (Supplementary figure 4a) and then, stimulating them with NMDA. We did not observe differences in the calcium response (Supplementary figure 6a-c). Neither, we observed difference after the application of high potassium solution (Supplementary figure 6d-f). Thus, differences in calcium entry of aged neurons appear to be a GM1 specific effect.

2.5 GM1 interacts with the ligand binding domain of NMDAR

Our previous results provide evidence that GM1 acts as a negative allosteric modulator (NAM) of the NMDAR. There are two possible scenarios for the NAM action of GM1: (i) GM1 could regulate the NMDAR function by direct specific contacts with the protein surface of the channel and/or (ii) GM1 could alter NMDAR function by modulating membrane properties such as fluidity, condensation and curvature. In this respect immunoprecipitation experiments (Supplementary figure 5i) suggests that GM1 is tightly bound to the NMDAR, thus indicating that part of its NAM action is mediated via direct contacts. In order to understand better the underlying molecular mechanism, we carried out short molecular dynamics simulation of the NMDAR embedded into a GM1-enriched membrane (Figure 5). GM1 consists of a glycosylated ceramide with the ceramide being inside the membrane and the oligosaccharide chain exposed to the extracellular region. Not surprisingly, we find that the ceramide fragment of the GM1 interacts with the transmembrane domain of the NMDAR in the upper leaflet of the membrane (Figure 5b). However, in contrast to non-glycosylated ceramides or phospholipids (e.g. 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)), we observe that the oligosaccharide chain is able to interact with the ligand binding domain (LBD) of the NMDAR - an important region where agonists bind for channel activation (see white arrows, Figure 5). The detected

GM1-LDB interaction could be critical for reducing the effect of ligands (i.e. NAM action) and thus NMDAR activity.

2.6 Glycosphingolipids and ganglioside levels in AD patients are not altered.

Genetic and environmental factors determine the individual vulnerability to physiological or pathological aging. It is unclear if there is a continuum between both types of aging because hallmarks of pathological aging such as Aβ in AD are present in 20-40% of old individuals cognitively normal (Jagust 2013; Imhof et al. 2007; Armstrong 2012; Mann & Jones 1990; Serrano-Pozo et al. 2011; Selkoe 2001). However, it is believed that boundaries in between normal and pathological aging are weak and aging is a core process on neurodegenerative diseases (Mattson & Magnus 2006; Jagust 2013).

Accordingly to previous studies, GM1 has been involved in Aβ aggregation process and AD (Choo-Smith & Surewicz 1997; Ariga et al. 2001). Moreover, aging is the main factor for AD. Therefore, we decided to quantify glycosphingolipids and gangliosides in hippocampal samples (Supplementary figure 7) and cerebrospinal fluid (CSF) (Table 1) from AD patients and non-demented controls by mass spectrometry. We did not observe any differences in the ganglioside and glycosphingolipid quantification between AD and control patients in the hippocampus. Furthermore, we were not able to detect gangliosides in CSF. We were only able to detected ceramides, sphingomyelin, glucosylceramides and dihydrosphingomyelin in a common pool of 5 non-demented controls and 5 AD. Higher levels of all of them were detected in the pool of AD patients CSF compared to the pool of controls.

3. Discussion

Aging is associated to cognitive decline even in absence of pathology. It is believed that in pathological aging there are increased molecular alterations compared to physiological aging. Accordingly, new studies are needed to unveil the mechanisms behind the aging process, in order to define new strategies to reduce pathological symptoms and to preserve cognitive functions in physiological and pathological aging.

Previous studies have been controversial about glycosphingolipid/ganglioside alterations in aging (Kracun et al. 1991; Sasaki et al. 2015; Ohsawa 1989). Our results demonstrate an increase of GM1 and GM2 in the cell membrane *in vivo* and *in vitro,* while glucosylceramides are increased only *in vitro,* during the aging process. Interestingly, we have found that high levels of GM1 in the neuronal membrane leads to a reduction in the calcium entry to the cytosol through NMDARs. Moreover, GM1 increase can mimic the decrease in calcium entry observed in aged neurons. This effect is specific for GM1 and NMDAR, because of (i) an increase of GM2 does not cause any

modification in NMDAR function and (ii) it is observed an increase in calcium entry in aged neurons after the stimulation with high potassium. Finally, we have showed that GM1 increase leads to a reduction in synaptic spine density, therefore reducing the number of excitatory synapses.

To further study the causes of the modifications in the glycosphingolipid/ganglioside membrane levels due to aging, we have analyzed the ganglioside synthesis pathway. Activity of the degradation pathway has already been shown to be increased in AD (Magini et al. 2015) and in aging (Dong et al. 2011). We have not been able to detect enzyme expression differences in the synthesis pathway between aged and adult *in vivo*. However, in hippocampal primary neurons, we have observed and increased expression of B4GALT5 (lactosylceramide synthase), ST3GALT5 (GM3 synthase), B4GALNT1 (GM2 synthase) whereas no significant difference in B3GALT4 (GM1 synthase) expression was observed. Our data is consistent with previous studies in endothelial cells showing an increase in GM1 levels and alterations in the synthesis pathway in senescence and senescence-induced cells (Sasaki et al. 2015).

Our results showed that dendritic spine density is reduced in aged neurons and in GM1-enriched neurons. We have increased GM1 levels by using two different treatments to prove our results and discard unspecific effects. Previous studies showed that exogenous GM1 increase neuritogenesis (Purpura & Baker 1978; Facci et al. 1984; Skaper et al. 1985). However, these studies were performed in other cellular models and focusing on neuronal maturation process. Therefore, we have showed for the first time the deleterious effect of GM1 increase in dendritic spine density in mature neurons.

In addition, NMDARs function is reduced due to the specific increase of GM1 in the membrane. Both treatments to increase GM in the neuronal membrane, exogenous GM1 and NVC, have shown a reduction in calcium entry to the cytosol after the stimulation with NMDA, mimicking the pattern observed in aged neurons. GM1 acts as a negative allosteric modulator of NMDAR, partially through direct interactions with the ligand binding domain of the receptor as our molecular dynamics simulation have shown. Moreover, any of the treatments reduced the total expression of NMDAR subunits, but we cannot discard that the increase in GM1 in the membrane lead to an alteration of the distribution or trafficking of some subunits modifying channel's activity. Interestingly, GM2 increase in the membrane did not modify NMDARs activity and calcium entry to the cytosol.

On the other hand, GM1 increase leads to an enhanced calcium entry through voltage gated calcium channels (VGCC) after depolarization with high potassium solution, mimicking the results obtained in aged cells. There are no previous studies in neurons

regarding VGCC and GM1 ganglioside, so further studies are needed to determine GM1 role on this channel function.

Our study in human hippocampus did not show differences between non-demented controls and AD groups. Since GM1 is highly expressed in neurons, our main hypothesis is that the quantification in post-mortem and advanced AD stage patients with high levels of neurodegeneration is not the optimal for GM1 differences detection. More studies in previous stages of AD samples should be done to determine if GM1 could be involved in the transition from aging to AD.

In conclusion, our finding demonstrates a key role of GM1 in neuronal synaptic function. Further studies are needed to determine GM1 physiological and pathological roles since GM1 is highly expressed in lipids rafts (Aureli et al. 2016), where it contacts with a wide number of proteins and lipids. Therefore, besides its ability to initiate and accelerate Aβ aggregation cascade (Ariga et al. 2008; Choo-Smith & Surewicz 1997; Ariga et al. 2001) GM1 would be also playing a role in the synaptic deregulation, which could be important for both pathological aging and AD progression.

4. Experimental Procedures

Mice

Six young (2 month), six adults (7 months) and six old (21 months) male C57BL6 mice were used for protein and ganglioside quantification. Hippocampi were dissected at indicated ages, and homogenized on ice in lysis buffer for WB detection or in PBS for lipidomics as indicated in the procedures below. The procedure was approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra (EC-IMIM-UPF).

Hippocampal primary cultures

Mouse cortical neurons were isolated from 18-day-old CD1 embryos. The procedure was approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra (EC-IMIM-UPF). Hippocampi were aseptically dissected in ice-cold HBSS (Life Technologies) supplemented with 4.5 g/L glucose (Sigma-Aldrich) and trypsinized for 17 min at 37ºC. After 3 washes in HBSS+glucose and mechanical dissociation, cells were seeded on DMEM (Life Technologies) containing 10% horse serum (Life Technologies) onto 1% poly-D-Lysine (Sigma-Aldrich, USA) coated plates. Cells were placed into an humidified incubator containing 95% air and 5% of CO_2. After 2 h, medium was removed and Neurobasal medium (Life Technologies) was added supplemented with 2% B27 supplement (Gibco BRL), 1% GlutaMAX (Life Technologies) and 1% Penicillin/Streptomycin. On day 3 of culture (DIV), cells were treated with 2 μM

1-β-D-arabinofuranosylcytosine (AraC; Sigma) for 24 h to eliminate proliferating non-neuronal cells. For the experiments, primary neurons were kept in culture for 14 (fully mature) and 24 (aged) days in vitro (DIV). At 14 DIV, neurons are fully mature and polarized, expressing all the functional receptors required for their proper function and we can study the structure and morphology of the dendrites. At 24 DIV neurons are used for aging studies. Aged cultures have been widely used as a model of aging by several authors because they recapitulate the aging process (Sodero et al. 2011).

Cell culture treatments

Neurons were treated with 500 nM GM1 ganglioside (Sigma), 0.03 U/mL neuraminidase from *Vibrio Cholerae* (NVC) (Sigma) or 500 nM GM2 ganglioside (Sigma) at 13 DIV.

Ganglioside quantification

Neurons were seeded in a 6 cm dish (750.000 cells/dish) and grown for 14 and 24 DIV. Next, they were washed twice with 1× PBS and centrifuged at 1800 rpm for 5 min at 4 °C. The pellet was frozen in liquid nitrogen for ganglioside and glycosphingolipid quantification. Briefly, lipid extraction and processing were performed as reported previously (Canals et al. 2009; Munoz-Olaya et al. 2008) Lipid analysis was carried out by ultraperformance liquid chromatography coupled to time-of-flight (TOF) mass spectrometry in positive electrospray ionization mode. Instrument conditions were set as in previous studies (Canals et al. 2009; Munoz-Olaya et al. 2008).

Dendritic spines quantification

Neurons were seeded in a 24-well plate (80.000 cells/well). At 12 and 22 DIV neurons neurons were transfected using Magnetofection technology (OZ BIOSCIENCES, Marseille cedex, FRANCE) and the reagent Neuromag specific for neurons with a GFP expression plasmid (pEGFP). At 13 and 23 DIV neurons were treated with GM1 and NVC during 24h. Cells were washed twice in PBS, fixed with 4% paraformaldehyde-4% sucrose in PBS for 10 minutes. For the quantification of dendritic spines, we used TRITC–phalloidin (ECM Biosciences, Versailles, KY, USA) to label actin, which allowed us to quantify dendritic length and the total number of spines. The cells were mounted in mounting medium and visualised using a Zeiss LSM 5 Pascal confocal microscope (NA 1.4) for the acquisition of sequential images of each fluorophore at maximum resolution (102461024 pixels). The images were processed using ImageJ (NIH, Bethesda, MA, USA). The dendritic length and the total number of spines values were normalized to the number of spines per 10 μm in each condition.

Immunofluorescence

Neurons plated into coverslip in 24 well plate (75.000 cells/well) were treated with GM1 (500nM) and NVC (0.03U/mL) for 24h at 13 DIV. At 14 DIV neurons were incubated with cholera toxin subunit B alexa 488-fluor conjugate (Thermo-Fisher Scientific) for 20 min at room temperature (RT). Following, cells were washed twice in PBS, fixed with 4% paraformaldehyde-4% sucrose in PBS for 10 minutes. The cells were mounted in mounting medium and images were taken with Leica TCS SP5 confocal microscope (63x objective) and analyzed with Leica confocal software.

Cell viability

Neurons were seeded in a 96-well plate ($1x10^4$ cells/well /100 µL/well). Treatments were added to Neurobasal without phenol red supplemented with 1% GlutaMAX (Life Technologies) and cells were treated for 24 h at 37ºC. Cell viability was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Briefly, 11 µL of MTT stock solution (Sigma Aldrich; 5 mg/mL) were added; after 2 h the media was replaced with 100 µL of DMSO. MTT absorbance was determined in an Infinite 200 multiplate reader (Tecan) at A540 nm and corrected by A650 nm. Untreated cells were taken as 100%.

Calcium analysis

Neurons were seeded into coverslip in a 24 well plate (75.000 cells/well). Cytosolic Ca^{2+} signal was determined at RT in cells loaded for 45 min with 4.5 µM FURA 2-AM (Life Technologies) and 0.02% pluronic and washed with isotonic solution containing 2.5 mM KCl, 140 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (305 mOsm, pH 7.4). Experiments were performed at RT using a custom-made recording chamber on a NIKON inverted microscope (×20 objective). Excitation at 340 and 380 nm was supplied by a xenon arc lamp with an optical filter changer. Emitted fluorescence at 510 nm images were acquired every 5 s with a digital camera (Hamamatsu Photonics), controlled and analysed with the AquaCosmos software. Cytosolic calcium levels were calculated as the ratio of emitted fluorescence at 510 nm following excitation at 340 and 380 nm (340/380 ratio) and are corrected for the basal individual fluorescence measured prior to cell stimulation (F/Fo). During all the experiment cells were bathed in the isotonic solution previously described. Cells were stimulated with 20 µM NMDA (Tocris) for 5 min or high potassium solution containing 52,5 mM KCl, 90 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (305 mOsm, pH 7.4) for 3 min.

Protein levels detection and quantification by Western Blot

Hippocampal neurons and hippocampi were lysed on ice with a solution containing 1x PBS, 0,1% triton, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Protein concentration was determined by Pierce® BCA protein assay kit (Thermoscientific). Protein samples were electrophoretically resolved using Nu-PAGE 4-12% Bis-Tris Protein Gels (Life Technologies) with MOPS running buffer. Gels were transferred to nitrocellulose membranes using a dry blotting system (Life Technologies). After blocking the membranes for 1h with 5% non-fat milk 0.05% Tween-TBS or 5% BSA-TBS, they were probed overnight with the indicated antibodies at 4ºC (Table 2). HRP-linked goat anti-mouse and anti-rabbit secondary antibody (1:2500, GE Healthcare UK limited) was used at RT for 1 h and membranes were developed using the ECL Substrate (Thermo Scientific) in the ChemiDoc MP imaging system (Bio-Rad). Bands were quantified with ChemiDoc MP.

Immunoprecipitation of NMDAR

Cells were washed three times in PBS and lysed on ice with a solution containing 1x PBS, 0,1% triton, protease inhibitor cocktail (Roche). GluN1 was immunoprecipitated with the mouse monoclonal anti-NR1 antibody (Thermo Fisher) using magnetic beads (Life Technologies), using a modified protocol of the manufacturer's instructions. Briefly, 2 µg of antibody were bound to 1.5 mg Dynabeads ProteinG in 200 µL of Tween-20 PBS for 1h at RT. The beads were washed and incubated with 500 µL of lysate o/n at 4ºC. The supernatant was removed, the beads were washed thoroughly and the antigen was eluted with a 67:30:3 mixture of 50 mM Glycine pH 2.8, LDS Sample Buffer (Life Technologies) and Reducing Agent (Life Technologies) and incubated at 70ºC for 10 min before running the samples in a protein gel.

Structural model of the NMDA receptor embedded in a GM1-enriched POPC membrane

The model was generated using the crystal structure of the GluN1/GluN2B NMDA receptor (PDB code: 4TLL (Lee et al. 2014)). In a first step, the GluN1/GluN2B NMDA receptor was curated (e.g. addition of missing atoms, assignment of protonation states, etc.) using the structure preparation tool of the MOE package (www.chemcomp.com). Afterwards, the curated NMDA model was embedded into a hydrated and ionized membrane composed of POPC and GM1 using the CHARMM-GUI web-based Graphical User Interface (Jo et al. 2008) yielding a system of approximately 380000 atoms. The entire protein-membrane system was equilibrated using NAMD (Phillips et al. 2005) and the standard protocol provided by CHARMM-GUI. Finally, short NVT productions runs were carried out using the ACEMD simulation engine (Harvey et al. 2009) and the

CHARMM36m force field to confirm structural stability of the obtained protein-membrane system.

Statistical analysis

Data are expressed as mean ± SEM of *n* independent experiments as indicated in the corresponding figures. Statistical analyses were performed by one-way ANOVA followed by Bonferroni's or Newman-Keuls post hoc analysis for more than two sets of data or Student unpaired t test for two sets of data using GraphPad software.

5. Tables

Table 1

CSF pool	CT	AD
Ceramides	28	56
Sphingomyelin	2262	2532
Glucosylceramides	190	258
Dihydrosphingomyelin	2316	2587

TABLE 1 Sphingolipids and glycosphingolipids quantification from CSF from non-demented humans and AD patients. Quantification by TOF mass spectrometry of a CSF pool of 5 controls and 5 AD patients. Expressed as pmol/mL.

Table 2

Name	Reference	Brand	Dilution
NMDAR1	32-0500	Thermo Fisher	1/500
NMDAR 2A	ab1555P	Abcam	1/500
NMDAR 2B clone N59/36	73-101	NeuroMab Antibodies Inc.	1/200
α-tubulin	T6074	Sigma	1/10.000
B3GALT4	ab169759	Abcam	1/1000
B4GALNT1	ab173966	Abcam	1/500
B4GALT5	ab155905	Abcam	1/500
ST3GAL5	ab155671	Abcam	1/1000

TABLE 2 List of antibodies used for WB quantification.

6. Figures

Figure 1

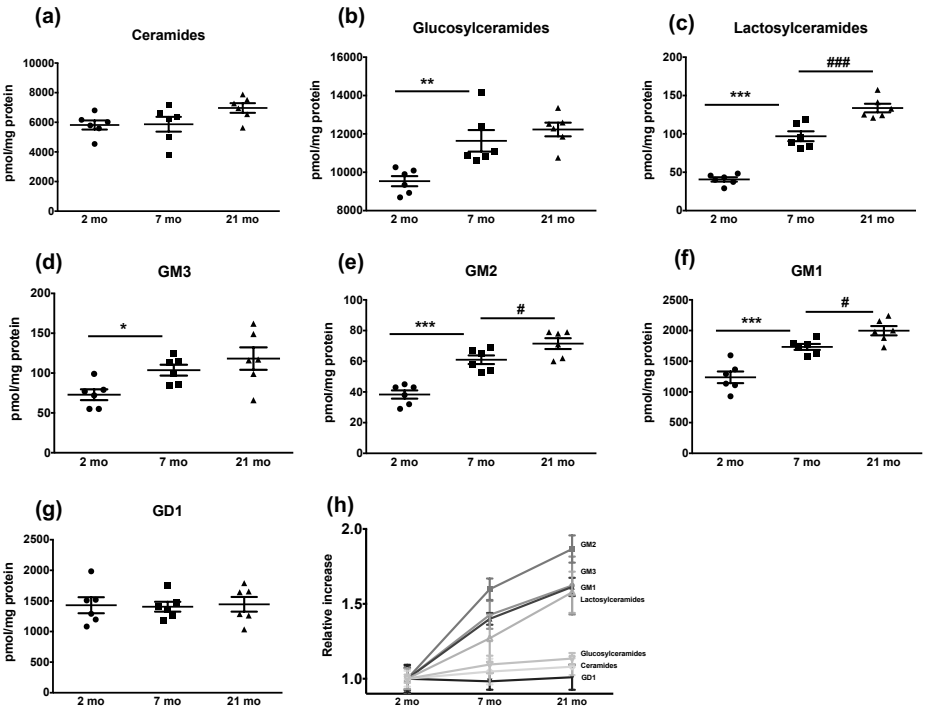


FIGURE 1 Lactosylceramides, GM1 and GM2 levels increase during aging in mice hippocampus**.** Ceramides (a), glucosylceramides (b), lactosylceramides (c), GM3 (d), GM2(e), GM1(f) and GD1 (g) quantification of mouse hippocampi from young (2 months), adult (7 months) and aged (21 months) by TOF mass spectrometry. H) Relative increase between adult and aged compared to young of the previously mentioned glycosphingolipids and gangliosides. Data are mean ± SEM of 6 independent animals/group. Statistical analysis was performed by one-way ANOVA using Newman-Keuls post test *p<0.05 compared with young : **p<0.01 compared with young; ***p<0.001 compared with young; #p<0.05 compared with adult; ### p<0.001 compared with adult by one-way ANOVA. mo: months

Figure 2

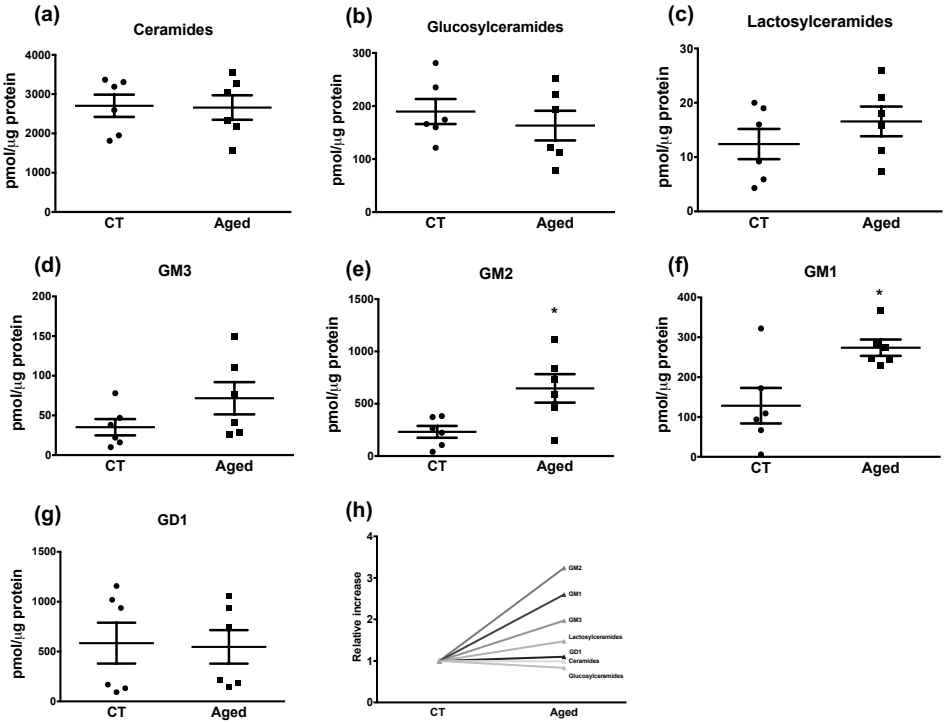


FIGURE 2 GM2 and GM1 levels increase during aging in cultured hippocampal neurons. Ceramides (a), glucosylceramides (b), lactosylceramides (c), GM3 (d), GM2 (e), GM1 (f) and GD1 (g) quantification of control (14 DIV) and aging (24 DIV) neurons by TOF mass spectrometry. Data are mean ± SEM 6 independent experiments. (h) Relative increase between aging and control cells of the previously obtained quantification of glycosphingolipids and gangliosides. Statistical analysis was performed by t-test *p<0.05.

Figure 3

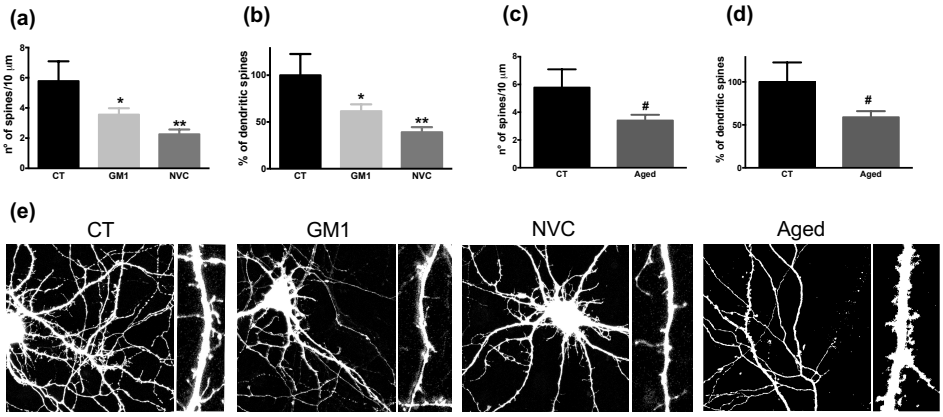


FIGURE 3 Dendritic spines density is reduced in neurons with high levels of GM1 and aging neurons. GM1 levels are increased in cultured primary neurons due to the treatment with GM1 (500 nM) and NVC (0.03 U/mL) for 24h leading to a reduction in spine density (a,b). Aged neurons also showed a reduced spine density (c,d). (e) Representative images taken by confocal microscopy of each condition labelling EGFP. Data are mean ± SEM of 2 independent experiments. Statistical analysis was performed by one-way ANOVA using Bonferroni post test or t-test *p<0.05; **p<0.01; # p<0.05.

Figure 4

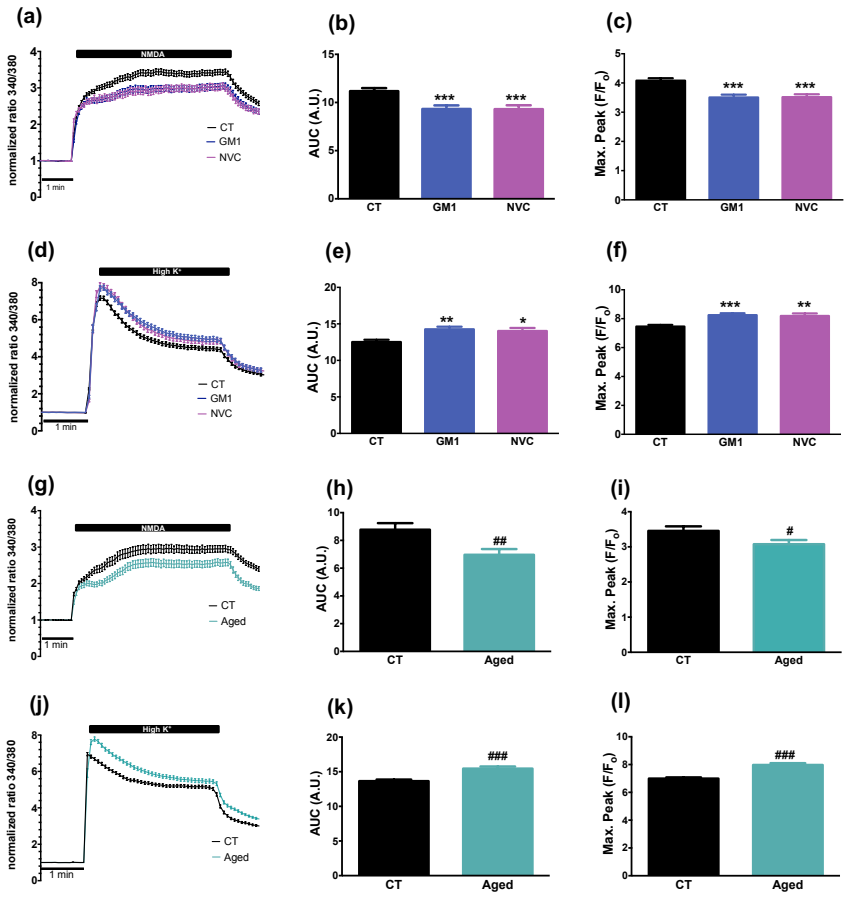


FIGURE 4 High GM1 levels and aging decrease calcium entrance in response to NMDA in cultured hippocampal neurons. Neurons were treated with GM1 (500 nM) and NVC (0.03 U/mL) for 24h, and were stimulated with 20 μM NMDA (a) or 52.5 mM KCl (d). Data are mean ± SEM between 115 and 212 cells for (a) and between 119 and 164 (d). The area under the curve (AUC) (b,e) and the maximum response peak (c,f) were calculated for each cell response. Aged and control neurons were stimulated with 20 μM NMDA (g) or 52.5 mM KCl (j). Data are mean ± SEM 114–119 cells for (g) and 100–106 for (j). The area under the curve (AUC) (h,i) and the maximum response peak (k, l) were calculated for each cell response. Statistical analysis was performed by one-way ANOVA Bonferroni post test and t-test (for two groups) *p<0.05; **p<0.01; ***p<0.001; #p<0.05; ##p<0.01, ###p<0.001

Figure 5

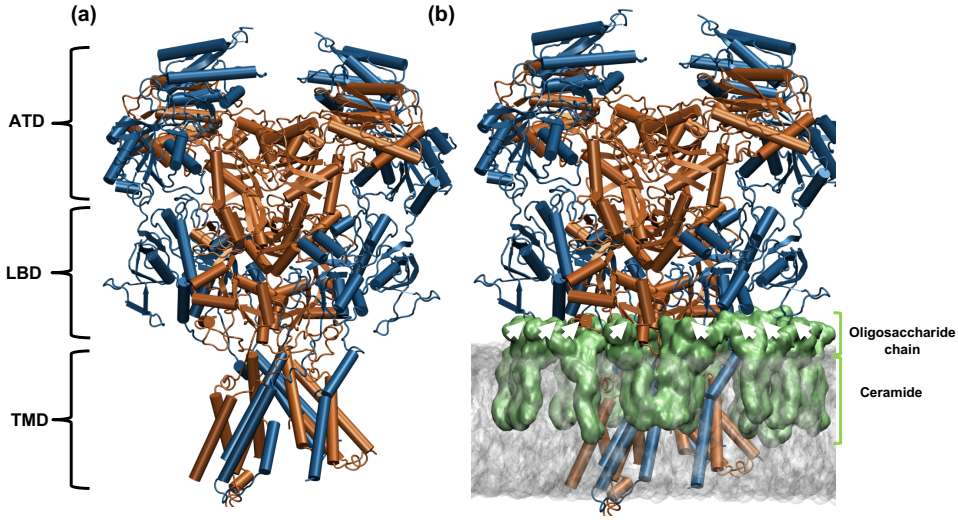
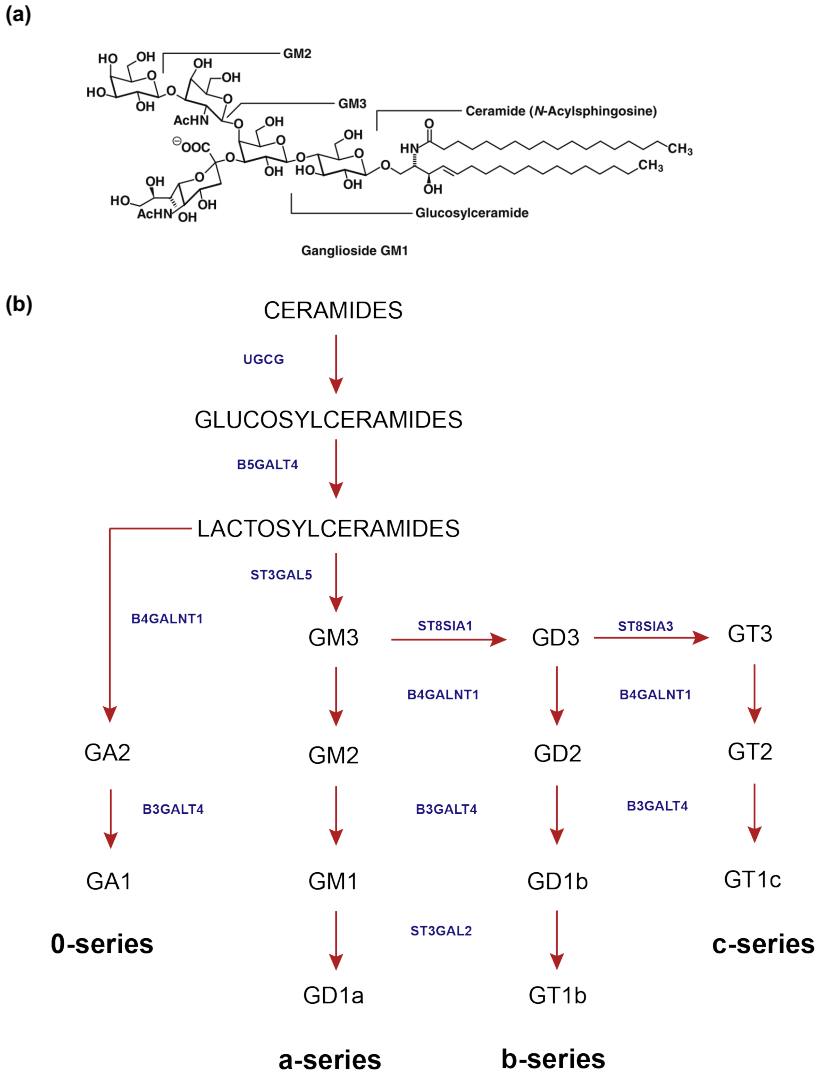


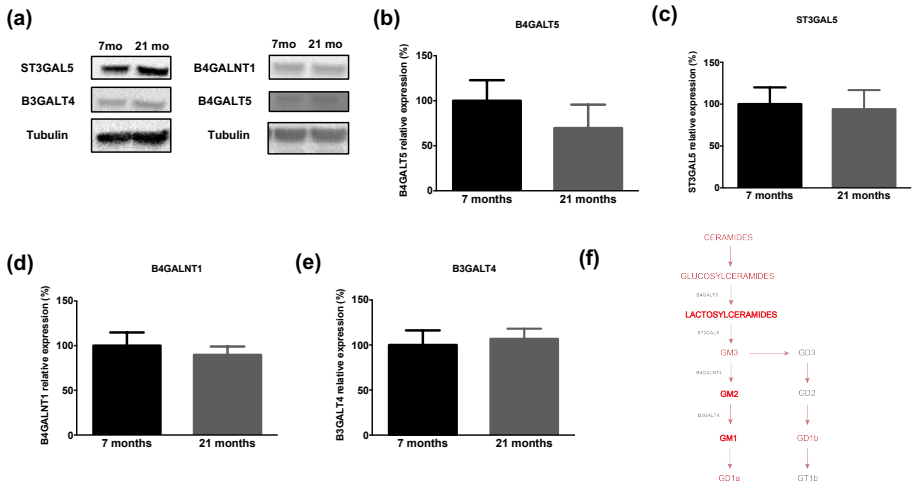
FIGURE 5 NMDA receptor structure simulation with GM1-enriched membranes showing the interaction between GM1 and NMDAR**.** (a) Crystal structure of the GluN1/GluN2B NMDA receptor (PDB ID: 4TLL). GluN1 subunits are shown in blue and GluN2B subunits in orange. (b) Structural model of the GluN1/GluN2B NMDA receptor embedded in a POPC/GM1 membrane. GM1 is composed by a ceramide core and a oligosaccharide chain and it is localize in the upper leaflet of the membrane. GM1 is showed in green. White arrows show the interaction points between GM1 and the LBD. POPC bilayer is showed in grey. ATD: amino-terminal domain; LBD: ligand-binding domain; TMD: transmembrane domain

Supplementary figure 1



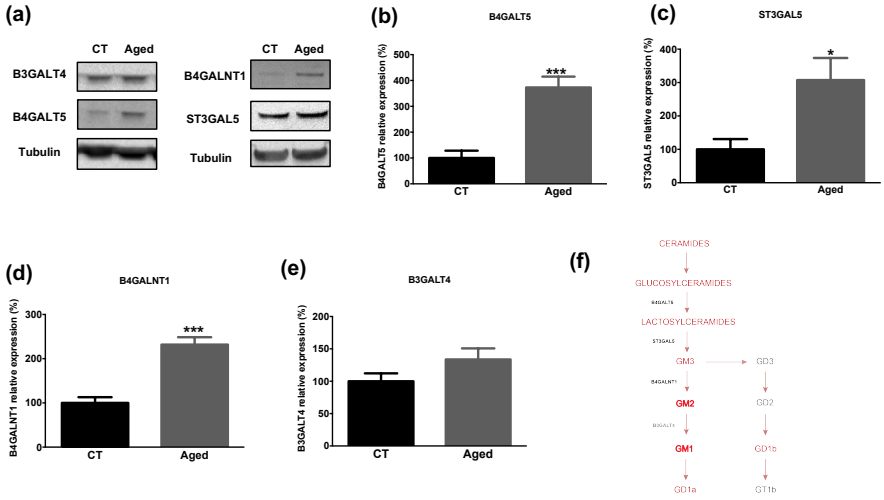
SUPPLEMENTARY FIGURE 1 Ganglioside structure and synthesis pathway. (a) GM1, GM2 and GM3 structure. From Kolter et al., 2010 (b) Ganglioside synthesis pathway. Enzymes of the synthesis pathway are named by the gene which express the protein.

Supplementary figure 2



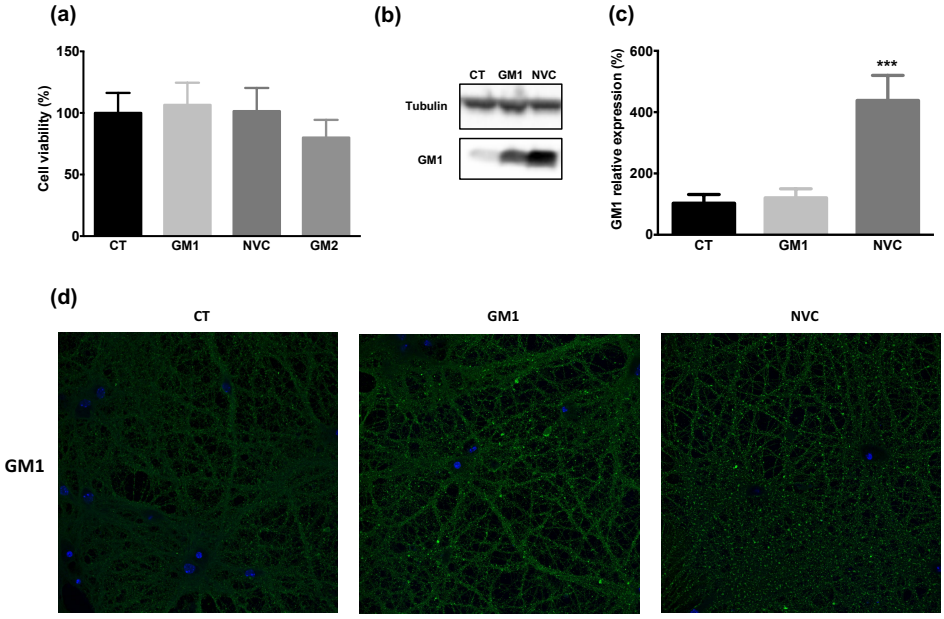
SUPPLEMENTARY FIGURE 2 Ganglioside synthesis pathway enzyme expression levels during aging in mice hippocampus are not altered. (a) Representative western blot on adult (7 months) and aged (21 months) hippocampi. Quantification from (a) of the levels of B4GALT5, ST3GALT5, B4GALNT1 and B3GALT4. (b,c,d and e). Data are mean ± SEM 6 independent experiments. Statistical analysis was performed by t-test f) Diagram of the pathway. Red: glycosphingolipids analyzed; Bold red: increased levels in aging; Grey: not modified expression enzymes. mo: months

Supplementary figure 3



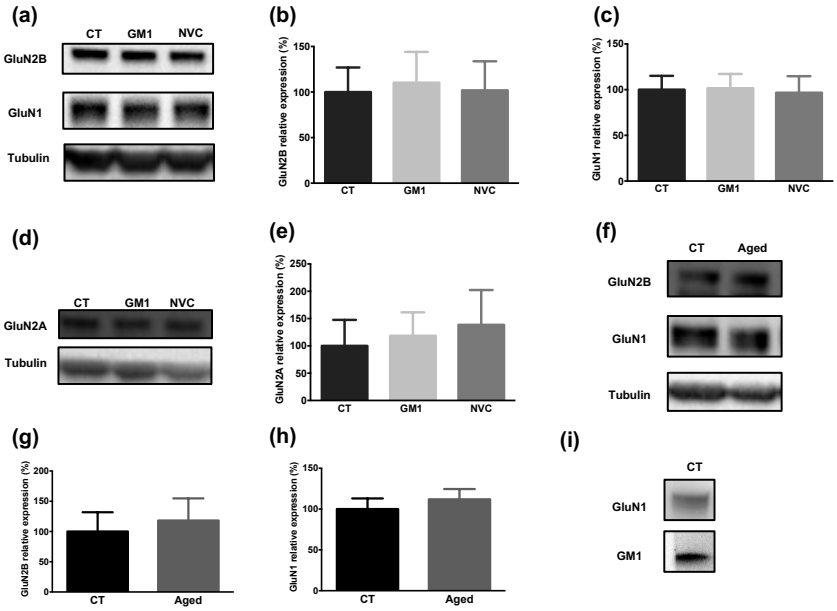
SUPPLEMENTARY FIGURE 3 Ganglioside synthesis pathway enzyme expression levels are increased during aging in cultured hippocampal neurons (a) Representative western blot from control (14 DIV) and aged cells (24 DIV). Quantification from (a) of the levels of B4GALT5, ST3GALT5, B4GALNT1 and B3GALT4. (b,c,d and e). Data are mean ± SEM 6 experiments. Statistical analysis was performed by t-test. *p<0.05; ***p<0.001 (f) Diagram of the pathway. Red: glycosphingolipids analyzed; Bold red: increased levels in aging; Grey: not modified expression enzymes; Bold black: increased levels in aging.

Supplementary figure 4



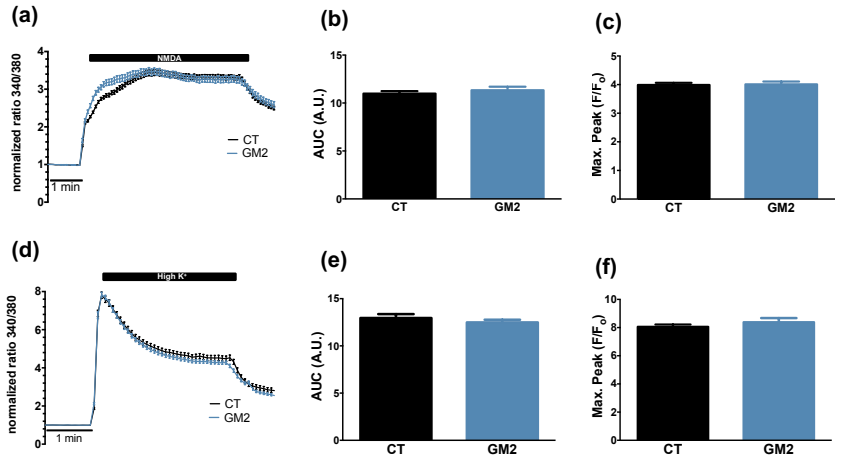
SUPPLEMENTARY FIGURE 4 Treatment with GM1, GM2 and neuraminidase do not affect cell viability**.** (a) After the treatment with GM1 (500 nM), NVC (0.03 U/mL) and GM2 (500 nM) for 24h cell viability was assessed by MTT reduction. (b) Representative Western blot from control, GM1 and NVC treated neurons using cholera toxin subunit B (CTB)–peroxidase. (c) Quantification of (b). (d) Representative images of an immunofluorescence staining of GM1 using CTB-alexa 488 in cells treated with GM1 and NVC for 24h. Data are mean ± SEM 7-8 experiments. Statistical analysis was performed by one-way ANOVA Bonferroni post test ***p<0.001.

Supplementary figure 5



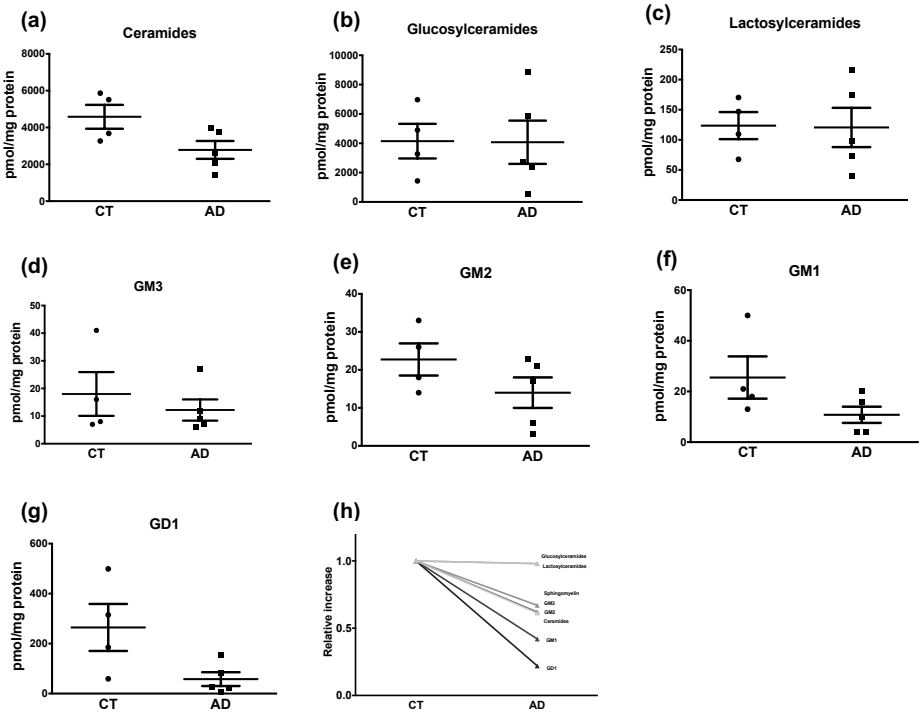
SUPPLEMENTARY FIGURE 5 GM1-enriched and aged hippocampal neurons do not present alterations in NMDAR subunit expression. (a,d) Representative western blot from control and GM1 (500 nM) and neuraminidase (NVC 0.03 U/mL) treated cells for 24h. (b,c and e) Quantification of (a) and (d). (f) Representative western blot from control (14 DIV) and aged cells (24 DIV). (g and h) Quantification of (f). (i) Representative western blot of GM1 co-immunoprecipitation with the NMDAR subunit GluN1 from control neurons. Data are mean ± SEM 6 experiments. Statistical analysis was performed by one-way ANOVA and t-test (for two groups). Non significant differences were obtained.

Supplementary figure 6



SUPPLEMENTARY FIGURE 6 GM2 increase has no effect on calcium response to NMDA and high K^+. Neurons were treated with GM2 (500 nM) for 24h and, were stimulated with 20 µM NMDA (a) or 52.5 mM KCl (d). Data are mean ± SEM between 140 and 149 cells for (a) and between 106 and 151 for (d). The area under the curve (AUC) (b, e) and the maximum response peak (c,f) were calculated for each cell response. Statistical analysis was performed by t-test. Non significant differences were obtained.

Supplementary figure 7



SUPPLEMENTARY FIGURE 7 Glycosphingolipid and ganglioside quantification in hippocampi from non-demented controls and AD patients do not show differences. Ceramides (a), glucosylceramides (b), lactosylceramides (c), GM3 (d), GM2(e), GM1(f) and GD1 (g) quantification of control (75 yo) and AD (73 yo - stage VI) by TOF mass spectrometry. Data are mean ± SEM 5-6 samples/group. Statistical analysis was performed by t-test. Non significant differences were obtained. H) Relative increase between AD and non-demented control patients of the previously obtained quantification of glycosphingolipids and gangliosides.

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Chapter 2

The antigen-binding fragment of human gamma immunoglobulin prevents amyloid β-peptide folding into β-sheet to form oligomers

Victòria Valls-Comamala, Biuse Guivernau, Jaume Bonet, Marta Puig, Alex Perálvarez-Marín, Ernest Palomer, Xavier Fernàndez-Busquets, Xavier Altafaj, Marta Tajes, Albert Puig-Pijoan, Ruben Vicente, Baldo Oliva, Francisco J. Muñoz. Oncotarget. 2017 Jun 20;8(25):41154-41165.

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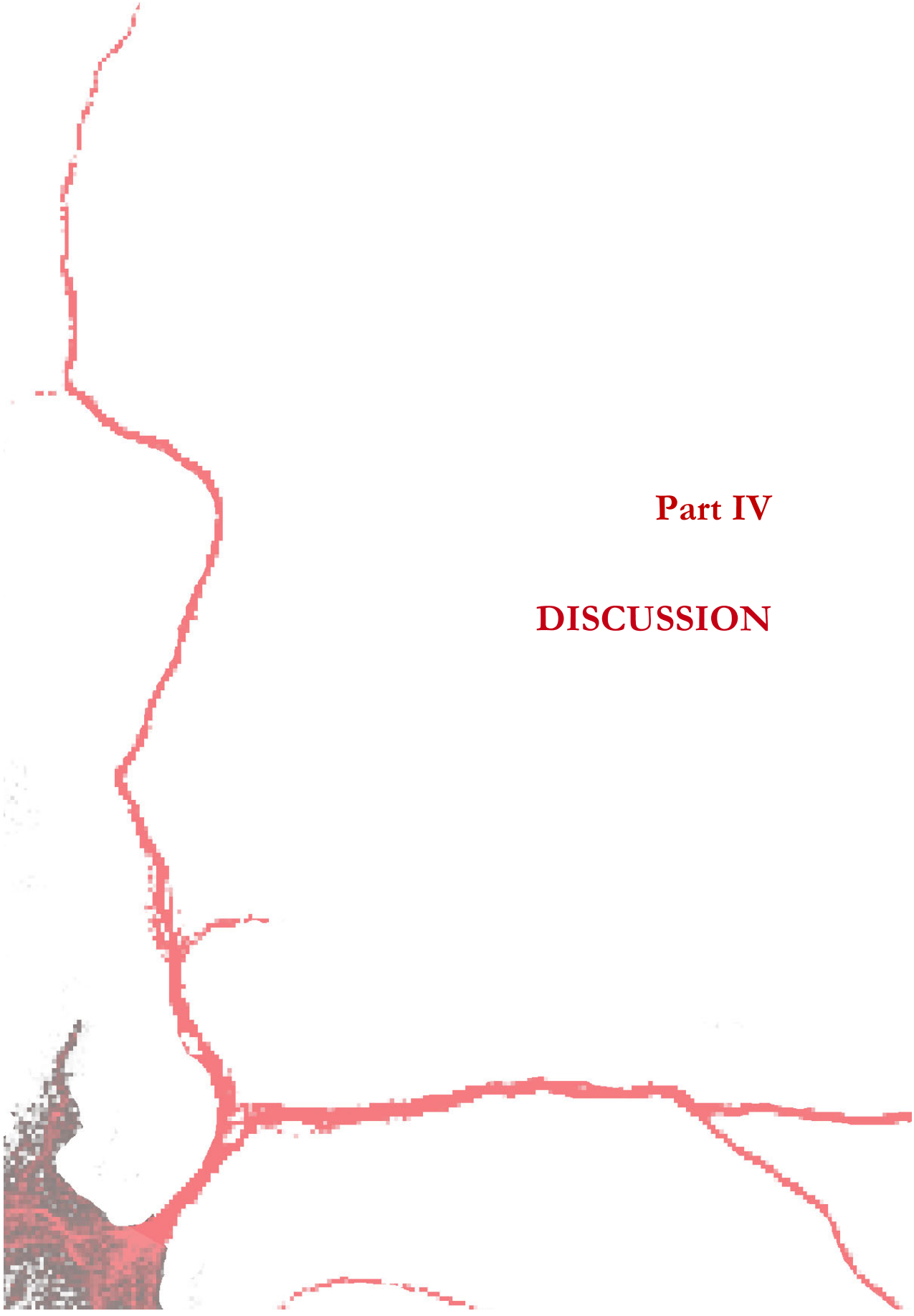
Valls-Comamala V, Guivernau B, Bonet J, Puig M, Peralvarez-Marín A, Palomer E, Fernàndez-Busquets X, Altafaj X, Tajes M, Puig-Pijoan A, Vicente R, Oliva B,Muñoz FJ. **The antigen-binding fragment of human gamma immunoglobulin prevents amyloid β-peptide folding into β-sheet to form oligomers.** Oncotarget. 2017;8(25):41154–65. DOI: 10.18632/oncotarget.17074



Part IV

DISCUSSION

Both non-pathological and pathological aging progress with cognitive decline. In pathological aging, there is an increase in molecular alterations, particularly in vulnerable neurons such as dopaminergic neurons of the brain steam that lead to PD or hippocampal glutamatergic neurons that lead to AD. In fact, some studies describe that there is a continuum between normal brain aging and AD being the bboundaries between pathological and non-pathological aging not defined. This theory is based on the presence of Aβ aggregates in samples from old cognitively normal individuals. Moreover, the main risk factor for AD is aging itself. Most of AD patients are sporadic cases without a known genetic factor but more research is needed to unlock the keys of the relation between physiological aging and AD. Since the prevalence of AD in the population older than 85 years old is up to 32% [469], it is not risky to propose that AD is the final destiny of the brain. Therefore, it is important to study in depth both of these diseases, because improving or slowing down one of them would help on the research on the other.

In the first chapter of this thesis, we have addressed a study to modify the membrane glycosphingolipid content during the neuronal aging process. We have shown an increase in several gangliosides in aged hippocampus and in primary hippocampal neurons. Specifically, our results showed an increase in GM1. GM1 is a ganglioside highly localized in the synaptic terminal, which has been already involved in the maintenance of membrane physical conditions [468] and in the acceleration of Aβ aggregation [457–459]. On this basis, we have studied the effect of GM1 increase on NMDAR activity and synaptic plasticity. NMDAR is a key receptor for LTP and, consequently, for learning and memory [33,470]. We have observed a reduction in dendritic spines density and a decrease on Ca^{2+} entry to the cytosol in both aged and GM1 increased levels neurons. On the other hand, this decrease in the NMDAR activity is not observed when we increase GM2 levels, a ganglioside that is also increased during aging. Overall, these results show the relevance of GM1, besides of accelerating Aβ aggregation, is able to decrease NMDAR

activity during aging and could be a link between non-pathological aging and sporadic AD.

In the second chapter of this thesis we have focused on the study of the effects of IgG treatment on Aβ aggregation. We have shown *in vitro* that the presence of IgG impairs Aβ oligomerization process in a dose-dependent manner and β-sheet structure is not formed, decreasing the neurotoxicity of the peptide. Moreover, we have observed that the specificity of the antibody is not important since similar results were obtained using Palivizumab, a human anti-syncytial virus antibody. Finally, we have shown that the antigen-binding fragment (Fab) is involved in the inhibitory effect over Aβ aggregation.

1 Aging modifies the glycosphingolipid content of the membranes in the brain

Different studies have been published [471–473] showing controversial results on the existence or not of changes in the glycosphingolipid levels, particularly gangliosides, during aging. Since GM1 plays a key role in the maintenance of the synaptic structure we have studied glycosphingolipid levels in aging in chapter I of this thesis.

Our findings in mouse hippocampus and hippocampal primary neurons showed modifications in the synthesis pathway leading to changes in the levels of glycosphingolipids (Fig 20). In hippocampal primary neurons, we showed an increase of GM1 and GM2 ganglioside levels due to aging (Fig 21a). Interestingly, the expression of some synthesis enzymes is increased in the early steps of the pathway including B4GALT5 (lactosylceramide synthase), ST3GALT5 (GM3 synthase) and B4GALNT1 (GM2 synthase). Surprisingly, B3GALT4 (GM1 synthase) did not show a different expression pattern (Fig 20a). Our results suggest that there is an alteration in the previous steps of the synthesis pathway, even though we are not able to detect differences in the levels of the first glycosphingolipids of the pathway.

It is worth mentioning that there is a tendency to an increase in aging in the levels of the first glycosphingolipids of the pathway compared with control neurons starting in lactosylceramides (Fig 21a) although this difference is not significant.

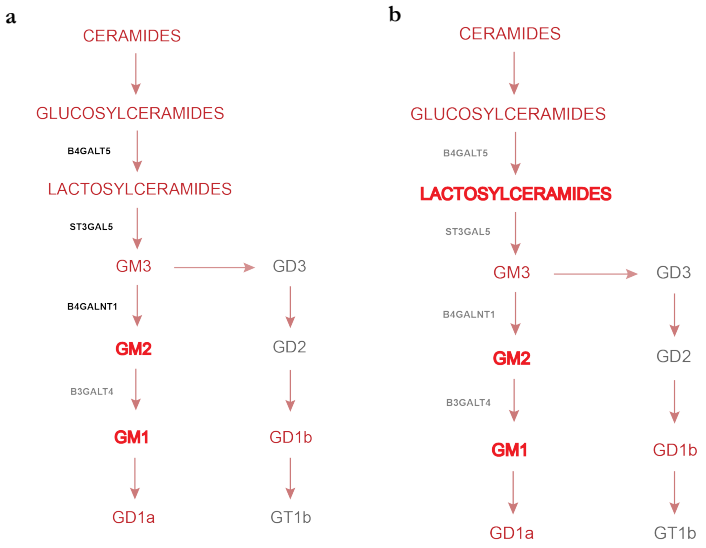


Figure 20: Synthesis pathway changes in primary neurons (a) and mouse hippocampus (b) in aging. Red: glycosphingolipids analyzed; Bold red: increased levels in aging; Grey: not modified expression enzymes. Bold black: increased levels of enzyme expression in aging.

In mice hippocampal samples we detected increased levels of lactosylceramides, GM2 and GM1 in aging (Fig 21b). GM3 levels were not significantly increased. In contrast with the study in primary cultured neurons, the synthesis pathway did not show any differences in the expression of the enzymes (B4GALT5, ST3GALT5, B4GALNT1 and B3GALT4) (Fig 20b). These results could be related with the fact that we analyzed the whole hippocampus, which contains different cell types. However, GM1 is mostly expressed in neurons and it has a low expression pattern in glial cells [407]. Therefore, we could detect the differences in ganglioside levels in the hippocampus but not in the levels of expression of

the enzyme because they are present in both neurons and glial cells. Gangliosides require specific techniques to be detected. We have used TOF mass spectrometry to study these samples but we cannot know the specific contribution of each cell type. This is the reason why a simpler model such as primary hippocampal neurons allows us to better determine the specific alterations in neurons and their molecular and functional consequences. We have planned to perform further complementary studies (out of the temporal scope of this thesis) to identify the contribution of neurons and glia to the previously mentioned results.

It is also important to mention that we have studied the expression of the synthesis enzymes but we have not studied the activity of these enzymes, which may give us further information regarding our results and the changes in the pathway. Moreover, we do not know the causes that produce the modification in the expression of the enzymes of the pathway. However, previously published data showed an increase of GM1 and the expression of these enzymes due to the exposure to oxidative stress [472]. Low and sub-toxic doses of H_2O_2, used as an oxidative stress inducer, lead to cell aging and senescence which was measured by an increase in β-galactosidase activity [474]. It would be interesting to perform further experiments to elucidate how oxidative stress, so tightly bound to aging, induces these changes on ganglioside synthesis, providing specific targets for future treatments. Furthermore, we have focused on the synthesis pathway because GM1 degradation pathway has been shown to be increased in AD [475] and aging [476].

We have studied samples from mouse hippocampi at different ages including two months (young), seven months (adult) and twenty-one months (old or aged). The increase in GM1 levels between two and seven months confirmed the previously reported role of gangliosides in neurodevelopment and maturation [414]. Young animals have not completed the brain maturation process, so we have focused in the results obtained at 7 and 21 months because our aim was to study the aging process. The aging increase in

glycosphingolipids is not as high as during maturation (Fig 21b) but, particularly in the case of GM1, this increase could be pathologic. This affirmation is based in the knowledge that small increases in GM1 levels leads to clusterization and changes in the membrane function during aging [468].

To conclude this section, we showed an increase in GM1 and GM2 in mice hippocampus and hippocampal neurons due to aging, probably as a consequence of an increase in the activity of B4GALT5, ST3GALT5 and B4GALNT1 enzymes.

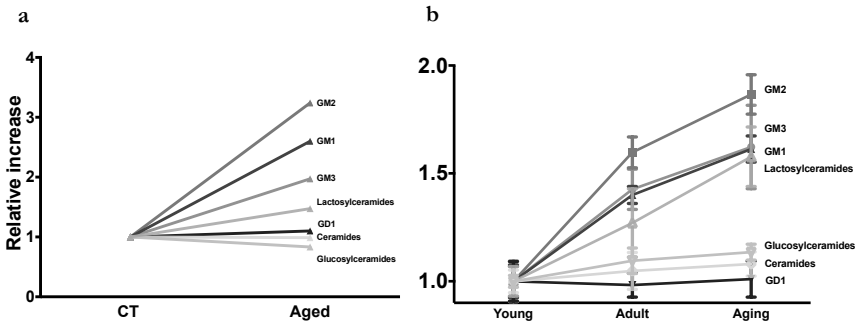


Figure 21: Relative increase of glycosphingolipids in cultured hippocampal neurons (a) **and mouse hippocampus** (b) **in aging** compared with control neurons or young mice. Data were obtained by quantifying glycosphingolipids by TOF mass spectrometry from cultured neurons or mice hippocampi (n=6 per group).

2 Glycosphingolipids in AD and aging human samples

GM1 has been broadly studied due to its effect on Aβ aggregation [457–459]. The levels of GM1 and other gangliosides have been previously determined but the published results are controversial. In frontal and temporal cortices GM1 has been found to decrease, whereas GM2 and GM3 have been found to be increased in the parietal cortex [471]. In contrast, other studies showed differences in the localization of the different GM1 molecular species but no differences in total amount of GM1 in the dentate gyrus [477–479]. GM1 is composed by a ceramide core, which can vary the length of the carbon chain

forming the different molecular species of GM1. Each GM1 molecular specie correlates with a specific localization. Our results in human hippocampi from non-demented controls and AD patients did not show differences. However, we observed a tendency in all gangliosides to decrease their expression (Fig 22). Overall, we can hypothesize that the tendency of GM1 to decrease in AD brain is due to the neurodegeneration already present in the AD brain samples, where neurons have died and they are the GM1 richest cell type [407].

In hippocampi from AD patients we were not able to observe the pattern obtained in aged mice and neurons. However, human AD brain samples are analyzed post-mortem in advanced stages of the disease. A published study in aged brain showed an increase of GM1 in the frontal cortex and a moderate decrease in the hippocampus [471]. Our results showing a decrease in the hippocampal GM1 should be due to neuronal death dramatically affecting hippocampus in AD patients.

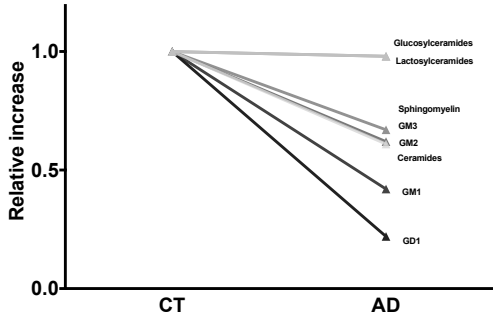


Figure 22: Relative decrease of glycosphingolipids in hippocampi from AD patients compared with non-demented humans. Samples were processed as indicated and quantified by TOF mass spectrometry (n= 4-5 per group).

CSF offers a window to analyze brain's metabolism and pathology through the detection of biomarkers [480]. Previous study analyzing CSF from control non-demented humans, mild cognition impairment (MCI) patients and AD patients showed that there is an increase of GM1 in CSF from patients with AD compared to controls, but not in MCI compared to controls [481]. The

authors conclude that the presence of GM1 could be a marker of neurodegeneration. For this reason, we also analyzed CSF (Table 5). However, we were not able to detect gangliosides since they were present in very low concentration. We only detected ceramides, sphingomyelin, glucosylceramides and dihydrosphingomielin in a common pool of 5 non-demented controls and 5 AD. Higher levels of all detected species were observed in the pool of AD compared to the pool of controls.

To conclude, we did not observe any difference in glycosphingolipids levels in hippocampi from AD patients probably due to the neurodegeneration caused by AD.

Table 5: Glycosphingolipids quantification from CSF from non-demented humans and AD patients. Quantification by TOF mass spectrometry of a CSF pool of 5 control and 5 AD patients Expressed as pmol/mL

CSF pool	CT	AD
Ceramides	28	56
Sphingomyelin	2262	2532
Glucosylceramides	190	258
Dihydrosphingomyelin	2316	2587

3 GM1 and aging reduce neuronal dendritic spine content

GM1 is highly concentrated in the membranes of the synaptic terminal [425]. Accordingly, we hypothesize that GM1 could play an important role on structural plasticity and dendritic spine density during aging in hippocampal neurons. Dendritic spines contain most of the excitatory synapses [31,47,48].

We performed two different treatments to study the effects of GM1 increase. First, we treated with exogenous GM1 ganglioside for 24h to increase its concentration in the membrane. It is known that exogenous GM1 can be integrated in the membrane and enter to the natural glycosphingolipid pathway. Only GM1 monomers enter the plasma

membranes, whereas micelles bind to cell surface interacting with proteins [425,482–484]. The unspecific binding to proteins can generate changes that do not occur in physiological conditions. However, it is known that physiological mechanisms are more often observed using low concentrations of GM1, as the ones we used (nM), compared to higher concentrations (μM and mM) [424]. Second, we increased endogenous GM1 levels using neuraminidase from *V.cholerae* (NVC), which removes sialic acid from complex gangliosides to form GM1. GM1 and GM2 sialic acid bound are resistant to hydrolytic removal by NVC [424,427]. After NVC treatment GM1 becomes the 95% of the ganglioside content in the brain [425].

Our results showed that the increase in GM1 levels and aging reduce the dendritic spine density. Preliminary studies showed that the increase in GM1 may be able to reduce the number of all types of spines (mushroom, stubby, thin and branched) except the immature spines (filipodia), pointing to its possible role in the maturation of the spines. Controversially, it has been reported that GM1 increases neuritogenesis [435–437]. However, these studies were performed in other cellular models and addressing the study of neuronal maturation. They were focused only in neurogenesis and did not study the specific formation of dendritic spines.

We can conclude that an increase in GM1 ganglioside reduces dendritic spines density, decreasing the number of synapses and modifying the synaptic network function.

4 GM1, and not GM2, modifies calcium entry through NMDAR and VGCC

To elucidate the effects of GM1 and GM2 increase in the membrane we carried out the specific experiments increasing each ganglioside. Here, we reported for the first time the role of GM1 on NMDAR activity. The increase of GM1 in the membrane leads to a decrease in Ca^{2+} entry to the cytosol through NMDAR (Fig 23). Both treatments used to increase GM1

levels in the membrane produced the same effect: a decrease in the NMDAR activity. However, when we treated the neurons with GM2 we did not observe any effect on NMDAR function.

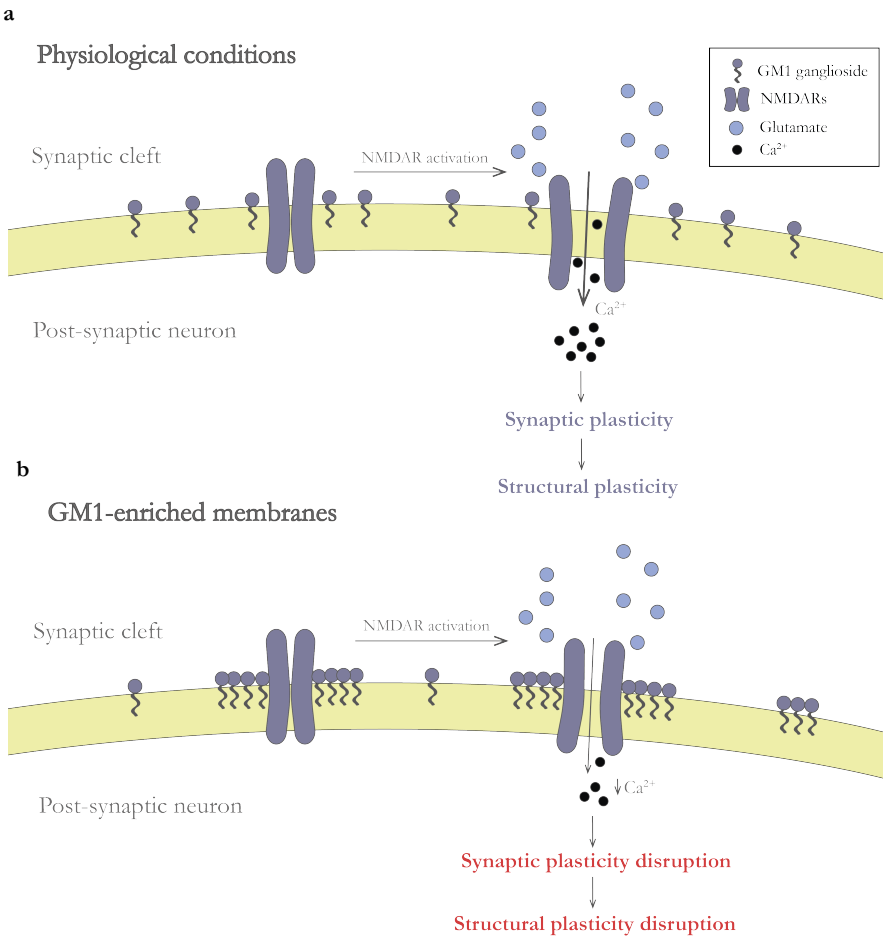


Figure 23: NMDAR function in physiological and GM1-enriched membranes. a) In physiological conditions NMDAR activation and calcium entry through the channel to the cytosol occur normally. Therefore, the downstream molecular pathways do not suffer any alteration and do not affect synaptic plasticity and dendritic spine density (structural plasticity) b) In GM1-enriched membranes, calcium entry through NMDAR is reduced. Therefore, the downstream molecular pathway is modified, leading to a reduction of synaptic plasticity and structural plasticity, measured by dendritic spine density.

We studied the possible changes on expression of NMDAR subunits (GluR1, GluR2A and GluR2B) but there were no differences in total expression between control and the different treatments in any of them. Moreover, to further study GM1 role in NMDAR function, it would be important to know more about the localization of the subunits, to reveal if GM1 can modulate its localization.

GM1 acts as negative allosteric modulator (NAM) of NMDAR through two possible scenarios: 1) GM1 increase in the membrane could have consequences on its physical properties and/or 2) GM1 could regulate NMDAR function by direct specific contacts with the surface of the receptor. Regarding membrane physical properties it is worth mentioning that NMDAR trafficking process requires focal changes in the membrane, receptor internalization needs focal invagination, while the traffic to the surface require fusion of receptor loaded vesicles to cell membrane. The plasma membrane should be able to adapt and change conditions fast to allow the proper function of the receptor [465]. On the other hand, our immunoprecipitation experiments suggested that GM1 is bound to the NMDAR, thus indicating that part of its NAM action is mediated via direct contacts. We carry out a molecular dynamics simulation to understand better the underlying mechanism. This experiment pointed out that the oligosaccharide chain of GM1 interacts with the ligand binding domain of the NMDAR, an important region where agonists bind for channel activation. Therefore, this interaction could be critical for reducing the effect of ligands (i.e. NAM action) and thus NMDAR activity.

Regarding VGCC function, we observed an increase in calcium entry after high K^+ stimulus. There are no previous studies regarding GM1 increase effects on the activity of these channels in neurons. Clearly, further studies are required to determine the specific effect of GM1 in VGCC.

In this section, we can conclude that GM1 directly interacts with NMDAR through its ligand binding domain decreasing the entry of Ca^{2+} and, therefore, reducing channel activation.

5 Aging modifies calcium entry through NMDAR and VGCC

Our results showed a similar pattern of glycosphingolipid modifications, particularly GM1 and GM2 in aged neurons and mice hippocampus. Aged cultures have been widely used as a model of aging by several authors because they recapitulate the aging process [485]. Therefore, we decided to use aged neurons to perform molecular and functional studies.

Our results in aged neurons showed a decrease in Ca^{2+} entry to the cytosol through NMDAR. It confirmed previous results that showed a decrease in NMDAR activity [486,487]. A decrease in the different subunits, particularly in GluR2B [488], has been described in aging. Surprisingly, we did not observe any differences in total expression of NMDAR receptors subunits GluR1 and GluR2B. Here, it is important to mention that we have studied the total levels of each subunit, but we do not know the exact localization of the subunits. It is known that, in aging, GluR2B levels can decrease in the synapse [489], whereas increasing its extra-synaptic localization [490], which decreases its activity.

VGCC response to high K^+ has been shown to be increased [491]. Our results showed an increase confirming what was previously shown. High K^+ is a physiological stimulus that can confirm that not all membrane receptors activity is modified in the same direction.

Studying the calcium response to high K^+ or NMDA in an aging model do not allow us to conclude that the only important factors are the increase in GM1 and GM2**. However, we showed that NMDAR and VGCC activity are altered following the same pattern than when we only modify GM1 levels in the membrane.**

6 IgG decreases Aβ neurotoxicity and Aβ oligomerization through Fab

Currently, there is no treatment able to stop or slow down AD. All the FDA-approved drugs are just symptomatic. Therefore, no approved drug can modulate Aβ aggregation [340,353].

We have shown *in vitro* that IgG at 7 mg/mL inhibits Aβ aggregation and reduces its neurotoxicity. The presence of IgGs during the aggregation process leads to formation of a globular conformation without β-sheet folding (Fig 24). Our simulation showed that IgG binds to Aβ like a monomer blocking the progression of the fibrillation. It also explains the effects of IgG in a concentration-dependent manner, which has already been observed in previous clinical trials [370,492], because a higher concentration of IgG will block all points of fibrillation. Each fibril contains two sites of growth.

In addition, we also showed that the specificity of the IgG *in vitro* is not necessary. However, in a systemic treatment it may help to keep the immunoglobulins close to the Aβ. It is important to mention that the pool of human IgG (IVIG) contains all the antibodies present in a young individual, including antibodies against Aβ [493]. Importantly, several studies have shown the presence of predominantly conformation-selective antibodies against oligomeric and fibrillary Aβ [494,495] in IVIG. Therefore, the treatment with IVIG also has some Aβ specificity that facilitates the contact between IgG and several forms of aggregated Aβ.

Another advantage of IVIG treatment compared with other immunotherapies is that this treatment has been used for a long time to treat other diseases [496]. Therefore, the safety and the tolerance of the treatment is already demonstrated, which is very important for new studies. Compared with other therapies, which can cause ARIA [497], IVIG may increase the risk of vascular events such as thrombosis and benign side effects such as fever or anemia [370].

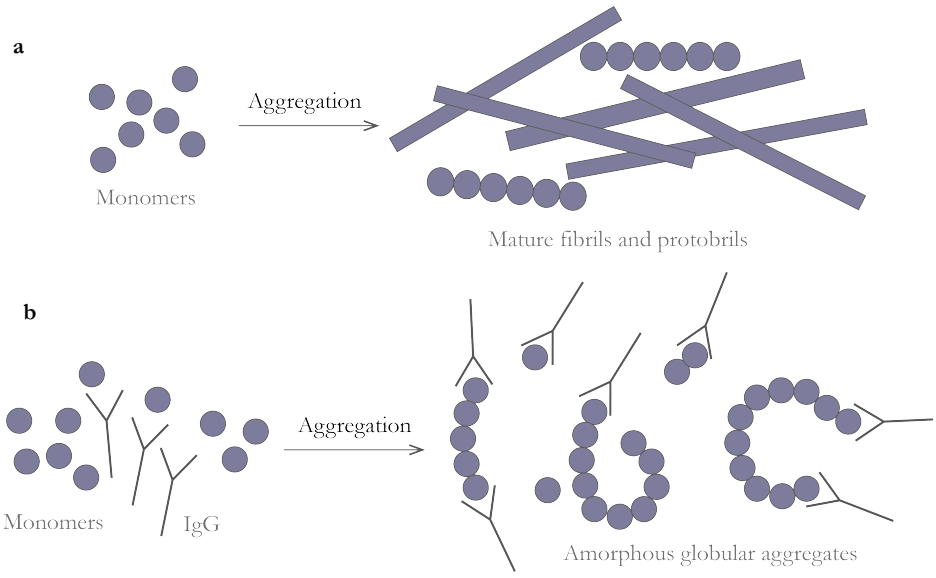


Figure 24: Model explaining the effects of IgG in Aβ aggregation process. a) Aβ aggregation results in the formation of oligomers, protofibrils and mature fibrils. b) The presence of IgGs during Aβ aggregation process leads to the formation of amorphous globular aggregates. IgG binds to Aβ blocking the progression of the fibrillation.

In this chapter, we also showed that the Fab of the IgG bind the Aβ. Considering that the side effects of the immunotherapies such as hemorrhage [498] and vascular edema [358] are related to inflammation [499,500] the creation of new peptides without the constant fragment (Fc) and only containing the Fab could be an interesting option. It is known that the inflammatory response is involved in Aβ clearance. However there is data

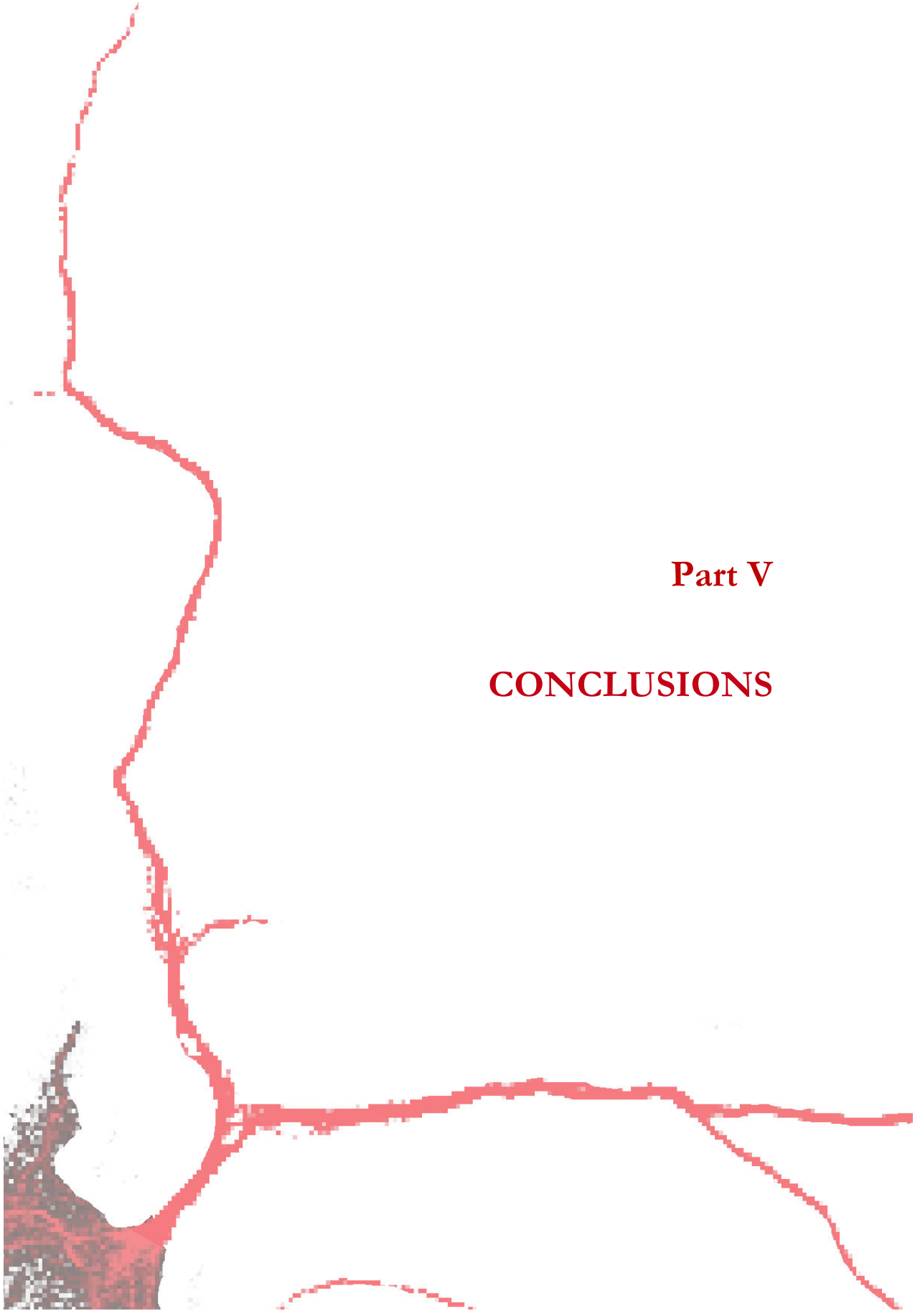
suggesting that antibodies can function without the Fc [501–503]. In conclusion, since Fc is the fragment responsible for triggering the immune response, administration of IgG without this fragment could be a useful method to overcome inflammatory problems. Moreover, it could be a manner to synthetize a new peptide with a reduced size and an enhanced capacity to cross through the BBB. IVIG crosses the BBB [370] but the facilitation of this process could be helpful.

With regard to the role of inflammation in AD, more studies using IVIG could help determine the role in the pathogenesis of naturally occurring alterations in antibody production due to age or Fc signaling. In fact, IVIG basically adds antibodies of young individuals to the old immune system.

Finally, our results, together with previous clinical trials using IVIG, support that patient selection for new clinical trials should be different. As previously mentioned, IVIG clinical trials in AD patients have not shown efficacy. In contrast, in some groups of patients, such as APOE-ε4 carriers, IVIG showed positive results [369–371]. Therefore, new clinical trials should target prodromal AD patients. It would be particularly interesting to target AD-mutation carriers to study the effect of very early treatment. It is a problem that most clinical trials in AD drugs are facing, together with the previous selection of AD patients who suffer from co-occurrence of cerebrovascular disease or other diseases. In conclusion, the combination of facing the disease at a late stage, together with the presence of other health problems complicates a clear observation of the potential efficacy of the treatments.

In conclusion, our results showed that Aβ aggregation in presence of IgGs leads to the formation of less neurotoxic amorphous globular aggregates. IgGs block the progression of Aβ fibrillation.

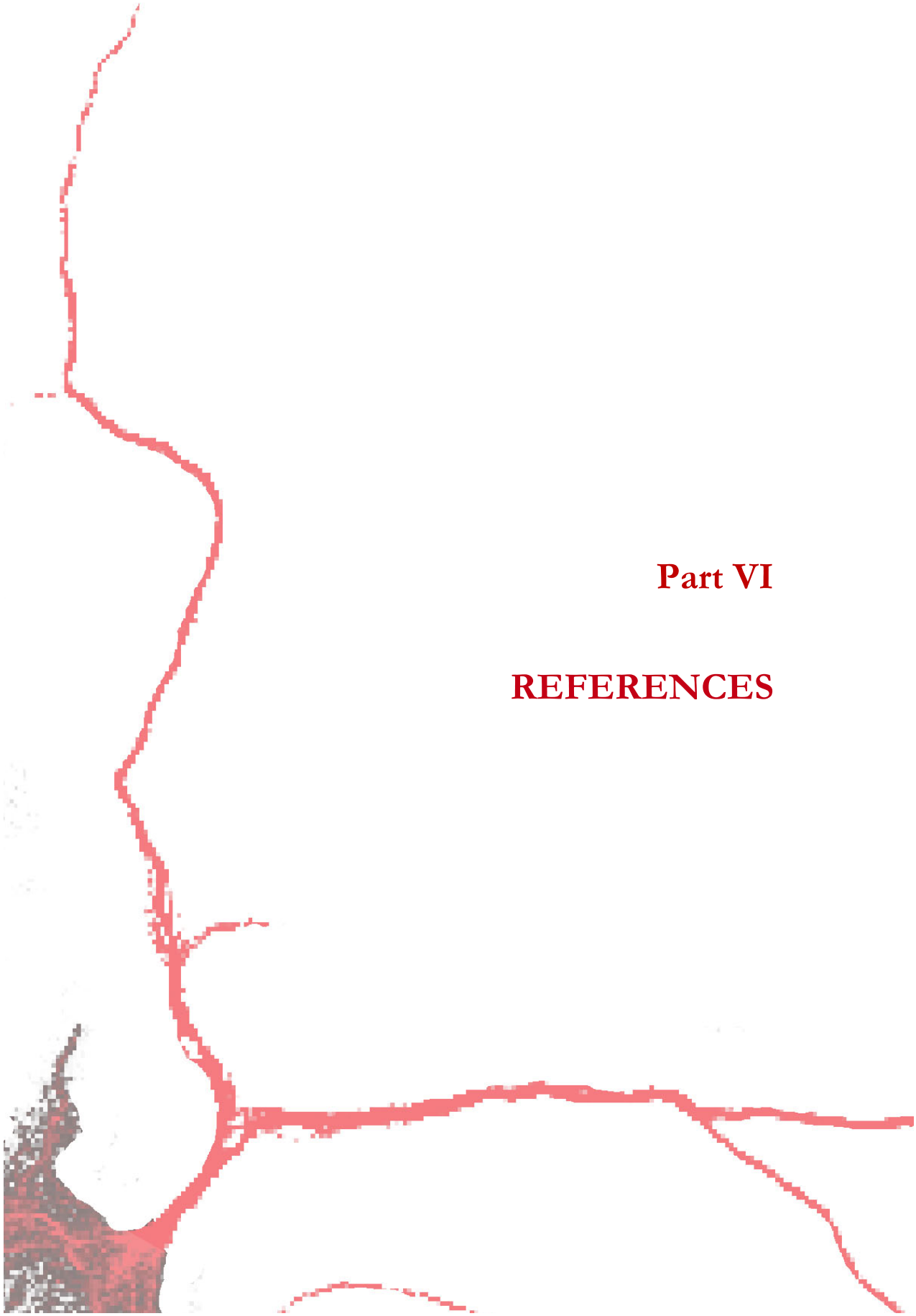
Aging is the main risk factor for AD. Boundaries between aging and AD are still not clearly defined and AD is believed to be an accelerated aging process. Several molecular factors have been involved in both processes; lipid membrane content, post-translational modifications of proteins, loss of proteostasis, epigenetic alterations, mitochondrial dysfunction, apoptosis among others. In conclusion, individual vulnerabilities to these factors may explain the final fate of each person. Moreover, the increase of altered factors during the aging process may explain the increase in the incidence of AD pathology in older people. In this thesis, we have linked for the first time the increase in GM1 levels to the cognitive defects associated to brain aging. Furthermore, we have shown that IgGs reduce Aβ neurotoxicity and aggregation encouraging for further studies regarding this treatment.



Part V

CONCLUSIONS

1. GM1 and GM2 gangliosides are increased in the hippocampus in aging due to the increase of expression of B4GALT5, ST3GALT5 and B4GALNT1.
2. GM1 acts as a negative allosteric modulator of NMDAR. High GM1 levels in the membrane reduce calcium entry through NMDAR partially through direct contact with the ligand binding domain of the receptor.
3. GM1 specific increase in the membrane increases calcium entry through VGCC.
4. Aged neurons, containing high levels of GM1, show modified activity of NMDAR and VGCC following the same pattern that neurons with high levels of GM1.
5. GM2 increase does not modify NMDAR and VGCC activity.
6. Aβ aggregation is inhibited in concentration dependent manner by IgG
7. Aggregation of Aβ in the presence of IgG generates a less neurotoxic globular structure without β-sheet folding.
8. IgG binds to new Aβ protofibrils in formation blocking the fibrillation.
9. IgG-Aβ specificity is not necessary for the inhibition of aggregation since Palivizumab, a human anti-syntytial virus antibody, can inhibit Aβ aggregation
10. Fab fragment of the IgG is responsible for the inhibition of Aβ aggregation through a highly conserved structure able to bind Aβ.



Part VI

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