Departamento de Farmacología y Toxicología
Facultad de Farmacia y Nutrición
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Adrenomedulin, a new therapeutic target for the treatment of Alzheimer's disease

Hilda Ferrero Hidalgo

Pamplona, 2017

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TESIS DOCTORAL

Adrenomedulin, a new therapeutic target for the treatment of Alzheimer's disease

Trabajo presentado por Hilda Ferrero Hidalgo para obtener el Grado de Doctor

Fdo. Hilda Ferrero Hidalgo
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A mi abuela y a mi tía

“It is the brain, the little gray cells on which one must rely ".

— Agatha Christie

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Summary

One of the consequences of the ageing world population is the increase in neurodegenerative diseases such as Alzheimer's or frontotemporal dementia.

Neurodegenerative diseases are pathologies characterized by a gradual and irreversible deterioration of neurons and presenting with different neurological syndromes depending on the affected brain area. Dementias are the most common neurodegenerative disorders, for which therapeutic options are very limited and merely symptomatic, rather than neuroprotective or neuroregenerative.

Furthermore, the most common dementia disorders, Alzheimer's disease or frontotemporal dementia, affect over 7 million people in Europe, and this figure is expected to double every 20 years as the population ages. The estimated care costs for dementia in Europe are approximately €130 billion per year, which cause a big concern on public health systems. Together with this, there is also an enormous social and human burden on patients as well as caregivers.

For this reasons, one of the major challenges faced by neuroscience research field is to bring more insights into the molecular pathomechanisms that underlie the neurodegenerative processes in dementia disorders in order to design novel pharmacological approaches and develop successful therapies for preventing or treating dementia.

The cytoskeleton plays an essential role on many fundamental neuronal processes, such as neuronal migration, cargo transport, polarity, and differentiation. Perturbations in the architecture of cytoskeleton can result in the loss of neuronal functions leading to neurodegeneration. This thesis aims to study the role of alterations in the cytoskeleton in the pathogenesis of neurodegenerative diseases. In particular, the thesis will focus on the involvement of adrenomedullin in alterations of the cytoskeleton and its relationship with two of the most common dementias, Alzheimer's disease and frontotemporal dementia.

First, an overview of the current status of adrenomedullin, with special focus on its interaction with the cytoskeleton, along with a summary of the most relevant neurodegenerative diseases and the different possible pathways by which adrenomedullin might mediate its actions will be provided in the *Chapter I, Introduction*.

Chapter II describes the hypothesis and objectives of the present thesis.

Chapter III presents a study on the involvement of adrenomedullin in Alzheimer's disease and the purported mechanism of action by which adrenomedullin exert its effects in the course of the disease.

Chapter IV describes changes in adrenomedullin expression in the ageing brain. In addition, it is described in this chapter the effects on cognition of deleting adrenomedullin gene in the CNS (AMKO mouse model) and the effects of ageing in this model.

Chapter V presents a brief study on the involvement of adrenomedullin alterations on frontotemporal dementia and the possible influence on cytoskeleton.

Finally, *Chapter VI, General Discussion*, integrates and highlights the most relevant aspects of the previous chapters, to end with the *Chapter VII, Conclusions*, summarising the main findings of the present thesis.

RESUMEN

Una de las consecuencias del envejecimiento de la población mundial, es el aumento de enfermedades neurodegenerativas como la enfermedad de Alzheimer o la demencia frontotemporal.

Las enfermedades neurodegenerativas son patologías caracterizadas por un deterioro gradual e irreversible de las neuronas y que se presentan con diferentes síndromes neurológicos dependiendo del área afectada. La demencia es el trastorno neurodegenerativo más común, para el cual las opciones terapéuticas son muy limitadas y meramente sintomáticas, en lugar de neuroprotectoras o neuroregenerativas.

Además, los trastornos de demencia más comunes, la enfermedad de Alzheimer y la demencia frontotemporal, afectan a 7 millones de personas en Europa. Se espera que se duplique cada 20 años conforme la población envejece. Los costes asociados al cuidado de personas con demencia se estiman en alrededor de 130 € billones por año en Europa. A esto se añade la carga social y humana que soportan tanto los pacientes como los cuidadores.

Por esta razón, uno de los mayores retos a los que se enfrenta el campo de la Neurociencia es ahondar en el conocimiento de los mecanismos moleculares que subyacen en estas patologías neurodegenerativas para diseñar nuevas aproximaciones farmacológicas y desarrollar terapias exitosas para prevenir o tratar la demencia.

El citoesqueleto juega un papel fundamental en muchos procesos neuronales esenciales, como la migración neuronal, el trasporte de carga, polaridad y diferenciación. Alteraciones en la citoarquitectura del citoesqueleto pueden resultar en la pérdida de funciones neuronales dando lugar a la neurodegeneración. Esta tesis trata de realizar una contribución al estudio de las enfermedades neurodegenerativas e intenta explicar el papel de adrenomedulina en su fisiopatología relacionada con el citoesqueleto.

Primero, en el *Capítulo I, Introducción* se realiza una descripción general del conocimiento actual acerca de adrenomedulina, con especial atención a su interacción con el citoesqueleto, junto con un resumen de su posible implicación en los mecanismos etiopatogénicos las enfermedades neurodegenerativas más relevantes.

El *Capítulo II*, detalla la hipótesis y objetivos de la presente tesis.

El *Capítulo III* presenta un estudio del posible papel de adrenomedulina en la enfermedad de Alzheimer y el potencial mecanismo de acción mediante el cual puede influir en el curso de la enfermedad.

El *Capítulo IV* describe cambios en la expresión de adrenomedulina en el cerebro envejecido. Además, en este capítulo se describen los efectos en la cognición tras la eliminación del gen de adrenomedulina en el SNC (modelo de ratón AMKO) y los efectos del envejecimiento en este modelo.

El *capítulo V* presenta un estudio de la implicación de alteraciones en adrenomedulina en la demencia frontotemporal y su posible influencia en el citoesqueleto.

Finalmente, el *Capítulo VI, Discusión General*, integra y resalta los aspectos más relevantes de los capítulos anteriores, finalizando con el *Capítulo VII, Conclusiones*, resumiendo los principales hallazgos de la presente tesis.

ABBREVIATIONS

AD	Alzheimer's disease
AM /ADM	adrenomedullin
AMBP-1	adrenomedullin binding protein-1
AMKO	adrenomedullin knock-out
APP	amyloid precursor protein
Aβ	amyloid-β
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
CGPR	calcitonin gene-related peptide
CNS	central nervous system
CRLR	calcitonin receptor-like receptor
FTD	frontotemporal dementia
FTLD	frontotemporal lobar degeneration
HD	Huntington's disease
IF	intermediate filament
KIF2	kinesin heavy chain member 2
KO	knock-out
LB	Lewy bodies
MAP	microtubule associated protein
MAPK	mitogen-activated protein kinase
MAPT	microtubule-associated protein tau gene
MCI	mild cognitive impairment
MF	microfilament
MMSE	mini mental state examination
MT	microtubule
NCAM	neural cell adhesion molecule
NF	neurofilament
NFT	neurofibrillary tangles
NORT	novel object recognition test
PAMP	proadrenomedullin N-terminal 20-peptide
PD	Parkinson's disease
PHF	paired helical filaments
PiD	Pick's disease
proAM	proadrenomedullin
pTau	phosphorylated Tau
RAMP	receptor activity-modifying protein
SNP	single nucleotide polymorphism
TNF-α	tumour necrosis factor-α
VaD	vascular dementia
WML	white matter lesions
WT	wild type

Chapter I
Introduction

Neurodegenerative diseases represent a heterogeneous group of disorders whose common characteristic is the progressive degeneration of neuronal structure and function. Although much knowledge has been accumulated on the pathophysiology of neurodegenerative diseases over the years, more efforts are needed to understand the processes that underlie these diseases and hence to propose new treatments.

Adrenomedullin (AM) is a multifunctional peptide involved in vasodilation, hormone secretion, antimicrobial defense, cellular growth, and angiogenesis. In neurons, AM and related peptides are associated with some structural and functional cytoskeletal proteins that interfere with microtubule dynamics. Furthermore, AM may intervene in neuronal dysfunction through other mechanisms such as immune and inflammatory response, apoptosis, or calcium dyshomeostasis. Alterations in AM expression have been described in neurodegenerative processes such as Alzheimer's disease. This introduction addresses the current state of knowledge on AM and its possible implication in neurodegenerative diseases.

1. ADRENOMEDULLIN

Adrenomedullin (AM) is a biologically active peptide that was first isolated from human pheochromocytoma and identified by its ability to stimulate cAMP production in platelets (Kitamura et al. 1993). At first, AM was characterized by its potent hypotensive effect. However, since its discovery, AM has been found to be expressed ubiquitously and participate in a variety of physiological functions including vasodilation, bronchodilatation, antimicrobial defense, growth and hormone regulation, angiogenesis, and apoptosis (Hinson et al. 2000; López and Martínez 2002). AM is involved in several pathophysiological processes such as hypertension, renal failure, septic shock, retinopathy or tumorigenesis (Kohno et al. 1996; Cheung and Leung 1997; Larráyoz et al. 2014; Iesato et al. 2016; Gillmann et al. 2017; Simon et al. 2017).

1.1 STRUCTURE

AM is a 52 amino acid peptide with an internal ring of 6 amino acids formed by a single intramolecular disulfide bond between residues 16 and 21. Another characteristic of AM is the presence of an amidated carboxyl end. Both the disulphide bond and the terminal amide are essential structural properties needed for receptor binding and for its biological activities (Kitamura et al. 1993; Eguchi et al. 1994). The structure of AM is similar to

calcitonin/calcitonin gene-related peptide (CGRP) and amylin, therefore AM has been classified as a member of the CGRP family.

Adrenomedullin human gene, *ADM*, is located in a single locus on chromosome 11 formed by four exons and three introns (Ishimitsu et al. 1994). The primary structure of the *ADM* gene is well preserved among different species (Sakata et al. 1994). *ADM* codes for a large precursor protein of 185 amino acids, termed preproadrenomedullin (preproAM), which in turn, contains a second precursor, proadrenomedullin (proAM) (**Figure 1**). Through post-translational modifications, proAM generates two active peptides, AM and proadrenomedullin N-terminal 20-peptide (PAMP).

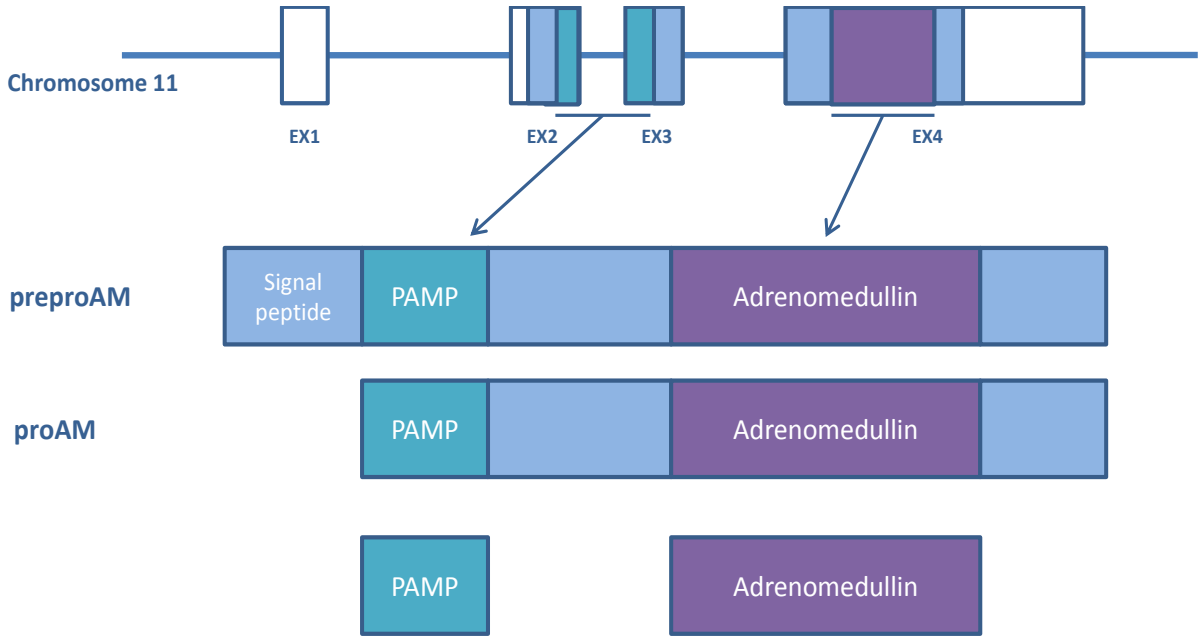

Figure 1. Schematic representation of the *Adrenomedullin* gene and the post-translational processing leading from preproadrenomedullin (preproAM) to mature adrenomedullin (AM) and proadrenomedullin N-terminal 20-peptide (PAMP). EX: exon.

Furthermore, PAMP has also its own biological activities, including hypotensive activity (Kitamura et al. 1994), antimicrobial functions (Martínez et al. 2006), and proangiogenic capabilities (Martínez et al. 2004c), among others. Factors including oxidative stress, inflammatory cytokines, circulating hormones and hypoxia promote *ADM* gene transcription (Minamino et al. 1995; Sugo et al. 1995a, b; Garayoa et al. 2000).

1.2 DISTRIBUTION, SYNTHESIS, RELEASE, AND METABOLISM.

AM was first found in pheochromocytoma (Kitamura et al. 1993) but is widely distributed in human organs and tissues including adrenal cortex, lung, heart, kidney, pancreas, spleen, small intestine, adipose tissue, and central nervous system (Ichiki et al. 1994; Sakata et al. 1994; Ueta et al. 1995; Washimine et al. 1995; Satoh et al. 1996; Montuenga et al. 1997; Serrano et al. 2000; Kitamura et al. 2002; Kato and Kitamura 2015). AM mRNA is highly expressed in a variety of cells such as myocardial, vascular endothelial, vascular smooth muscle, pulmonary or pancreatic cells, as well as human tumours or embryonic tissue (Eto et al. 1999). This peptide has been also detected in the cultured media of cardiovascular, glial tumour and neurons, which implies that AM is synthesized and secreted by them (Takahashi et al. 1997; Tsuruda et al. 1998; Tixier et al. 2008).

Initially, adrenal medulla was proposed as the major source of AM and hence its name, however, the most abundant source in the human body is vascular walls (Sugo et al. 1994). This could be due to the fact that shear stress also stimulates the production of AM on endothelial and vascular smooth muscle cells. Furthermore, AM can be measured in the blood, urine, cerebrospinal fluid, amniotic fluid, saliva, sweat and milk (López and Martínez 2002). In humans, plasma concentration of AM is around 2-10 pM, although most of blood AM is specifically bound to AM binding protein-1 (AMBP-1), which was later identified as complement factor H (Pío et al. 2001). Therefore, total plasma levels of AM could be higher (Pío et al. 2001; Dupuis et al. 2005). Circulating AM is degraded sequentially by metalloproteases followed by aminopeptidases (Martínez et al. 2004b).

1.3 RECEPTORS AND SIGNAL TRANSDUCTION

AM presents many specific binding sites in several cells and tissues such as the heart, lung, spleen, liver, kidney, and skeletal muscle (López and Martínez 2002). This variety of binding sites may be related to the several biological actions that AM plays. Furthermore, binding sites for AM have been reported in different brain regions, which provides an anatomical basis to involve AM in the physiology and neuropathology of the central nervous system (CNS) (Juaneda et al. 2003).

AM receptor is a heterodimer formed by calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein (RAMP) (Hay and Smith 2001; Muff et al. 2001). CLR presents seven transmembrane domains while RAMP only possesses a single transmembrane

domain and has three different subtypes RAMP1, RAMP2, and RAMP3. RAMPs are indispensable to transport CLR from the endoplasmic reticulum to the plasma membrane. Since AM is a member of the CGRP family, it is no surprising that CGRP can also bind to CLR (Poyner 1997). The specificity of the receptor for AM or CGRP depends on the interaction with different RAMPs: CLR/RAMP1 is the CGRP receptor while the combinations CLR/RAMP2 and CLR/RAMP3 present specificity for AM, therefore they were termed as AM_1 and AM_2 receptors, respectively (Foord et al. 1998) (**Figure 2**). It has been hypothesized that eight N-terminal residues that are conserved in RAMP2 and RAMP3 but differ in RAMP1, confer such specificity for AM (Qi et al. 2008).

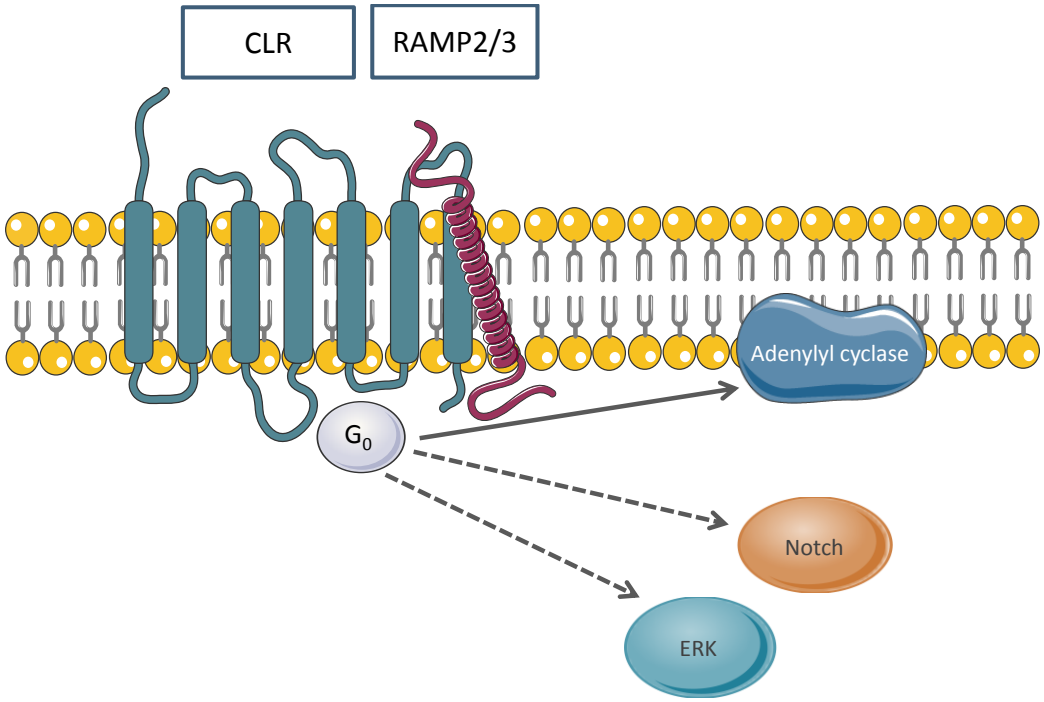

Figure 2. AM receptors. The calcitonin receptor-like receptor (CLR) component is a G protein-coupled 7-transmembrane receptor. Both receptor activity-modifying proteins (RAMPs) are single transmembrane domain proteins. The active receptor is a functional heterodimer complex of CLR with a RAMP, at the cell membrane. From the interaction of RAMP2 with CLR results the AM_1 receptor, whereas the complex CLR/RAMP3 originates the AM_2 receptor. The major signal transduction pathway activated by AM receptors, the adenylyl cyclase/cAMP system, is also represented.

AM signal transduction pathways vary between species, tissues, organs, and cell types. The major signal transduction pathway activated by AM is the adenylyl cyclase/cAMP system (Hirata et al. 1995) (**Figure 2**). In addition, AM-activated pathways include, among others, Akt and mitogen-activated protein kinase (MAPK) extracellular signal-regulated protein kinase (ERK) or Notch pathways (Nishimatsu et al. 2001; Yurugi-Kobayashi et al. 2006; Fritz-Six et al. 2008). It has been also described that AM partially mediates its action by NMDA-dependent mechanism as a neuromodulator (Xu and Krukoff 2004).

2. NEURONAL CYTOSKELETON AND ADRENOMEDULLIN

2.1 NEURONAL CYTOSKELETON

The cytoskeleton was described as the major intracellular structure whose function determines the morphology of the cells. This simple definition underestimates the chief role of cytoskeleton in cell functioning, which goes beyond the mere view of cytoskeleton as rigid structure with isolated functions. It is instead a dynamic and adaptive structure whose different components and regulatory proteins are in constant movement and reorganization. Actually, cytoskeleton exerts major functions in the correct stasis and function of cellular organelles, cell-to-cell and cell-matrix connections, and nucleocytoplasmic signalling. To ensure these labours, the cytoskeleton utilizes several specialized structures (Fletcher and Mullins 2010). In neurons, a well-organized cytoskeleton is fundamental since it divides the cell into two different functional compartments, the axonal and the somatodendritic (Craig and Banker 1994). Supported by this morphology, neurons can achieve their function of processing and transmitting information. Thereby, electrical synaptic inputs from thousands of neurons are sent to the dendrites of a single neuron, which receives and integrates this signal. Meanwhile, the axon is responsible for transmitting this integrated information as an action potential to downstream neurons. For this reason, maintaining the polarity and the integrity of the neuron requires a detailed control of the three principal cytoskeletal elements: actin filaments, intermediate filaments (IF or neurofilaments, NF), and microtubules (MT).

Actin filaments, also known as microfilaments (MF), are flexible filamentous structures polymerized from actin subunits. MFs consist of two actin protofilament polymers associated in a thin double-helical structure of approximately 7 nm in diameter (Spudich et al. 1972). These filaments present polarity with plus and minus ends, since actin monomers are oriented in the same direction. As the main component of the membrane cytoskeleton, MFs form more complex structures such as bundles or 3-dimensional networks which regulate the shape and movements of neurons (Luo 2002). Both in axon and dendrites, actin filament bundles form a meshwork. In addition, actin bundles are accumulated at the beginning of the axon so it has been proposed that they can limit the axonal transport load and regulate its progress into the axon (Watanabe et al. 2012).

Intermediate filaments include the most extended family of cytoskeletal proteins and can polymerize into filaments of 10 nm in diameter. In adult neurons, five types of IFs have been described: three different neurofilament proteins, peripherin, and α-internexin (Cairns et

al. 2004). All IF proteins have three subdomains: a helical central "rod" domain flanked on either side by non-helical "head" and "tail" domains. IFs provide strength and stability to the cytoskeleton but, in contrast with MFs, they are not polarized structures. Phosphorylation regulates the polymerization and depolymerization process of IFs. Besides, IFs´ phosphorylated tails are basic for the union between IFs and their interaction with microtubules (Eira et al. 2016). Neurofilaments are highly enriched in the axon, and they intervene in different processes such as neuronal differentiation, axonal outgrowth, and regeneration (Zhu et al. 1997).

In the neuronal cytoskeleton, microtubules play a key role since they provide the main route for axonal transport, contribute to the structural integrity of neurons (Prokop 2013), and are involved in neuronal plasticity (Conde and Cáceres 2009). Recently, it has been reported that dendritic spines, in addition to containing actin filaments, also have MTs that impact on spine development, maintenance, and function (Jaworski et al. 2009). MTs are polarized structures formed by α- and β- tubulin heterodimer subunits that constitute linear protofilaments. The helical lateral assembly of 10-15 protofilaments forming a helical tube of 24 nm in diameter results in a microtubule (Downing and Nogales 1998). The assembly of tubulin subunits makes up polarized MTs with plus and minus ends. The engine that drives microtubule dynamics results from GTP hydrolysis. Tubulin has intrinsic GTPase activity that is activated by polymerization (Erickson and O'Brien 1992). Despite its GTPase activity, tubulin polymerizes in the presence of non-hydrolysable GTP to form stable microtubules (Hyman et al. 1992). After polymerization, GTP is hydrolyzed and affinity for the adjacent tubulin units decays, thus triggering depolymerization (Mitchison and Kirschner 1984). MTs grow through their plus end and shrink through their minus end (Walker et al. 1988). In neurons, MT structure is polarized with the minus ends pointing to the cell body and plus ends towards the axon terminal. Furthermore, at the end of the MTs, there may be a stabilizing cap of GTP tubulin, whose loss leads to a catastrophic disassembly, resulting in MT shrinkage (Desai and Mitchison 1997).

MT stability is regulated by different post-translational modifications of tubulin units: tyrosination/detyrosination, acetylation, and polyglutamylation (Eira et al. 2016). Initially, α-tubulin is expressed in its tyrosinated form so the post-translational modification is detyrosination. As the number of detyrosinated tubulins increases, MTs are considered more stable. Otherwise, acetylation of α-tubulin, which occurs in the MT lumen, has been used as a marker of stable MTs (Soppina et al. 2012). Acetylation has been found mainly on stable

microtubules resistant to depolymerisation (Portran et al. 2017). Another post-translational modification is polyglutamination, which may alter MTs length (Lacroix et al. 2010).

The MT cytoskeleton is essential for molecular and cargo transport in most cells, but especially in neurons. Kinesin and dynein are the two motor protein families responsible for transporting cargoes through a polarized railway formed by the MTs in neuronal axons. Vesicles, organelles, proteins and mRNA are moved in different directions by these motors: kinesins directed towards the plus end (anterograde transport) and dyneins move towards the minus end (Hirokawa et al. 2010). Some kinesin motor proteins preferably bind to stable microtubules marked by acetylation and detyrosination (Cai et al. 2009). MTs are involved in dendritic spine morphology and synaptic plasticity (Jaworski et al. 2009). Thus, impairment of MT dynamics may trigger loss of dendritic spines, leading to synaptic dysfunction and neurodegeneration.

Several MT-associated proteins (MAPs) specifically bind tubulin subunits to regulate MT stabilization, among which Tau is key in determination the structure of neuronal axons and MAP2 in dendrites. Other members of MAPs such as MAP1A and MAP1B are found in axons and dendrites (Takemura et al. 1992), and are largely implicated in dendritic differentiation and maintenance (Conde and Cáceres 2009).

2.2 ADRENOMEDULLIN AND THE CYTOSKELETON

The distribution of proAM products in the cytoplasm and MT-enriched areas in neurons suggest a possible interaction of these peptides with cytoskeletal elements. Actually, the vast majority of AM immunoreactivities within neurons are located on the cytoskeleton or on the cytoplasmic side of mitochondrial and nuclear membranes, although never inside the synaptic vesicles (Serrano et al. 2000).

AM and PAMP peptides have been closely related to the cytoskeleton (Sackett et al. 2008; Larráyoz et al. 2013). A yeast-2 hybrid experiment identified interactions of PAMP and AM with several cytoskeletal elements, either MAPs or direct components of the MTs (Sackett et al. 2008). AM interacts directly with MAP1A and cytoskeleton associated protein 1 (CKAP1). MAP1A is critical for dendritic branching and dendritic arbour stabilization in cultured neuron (Szebenyi et al. 2005). Similar to AM, MAP1A is mainly distributed in the soma and dendritic spines of neurons, suggesting a function for MAP1A in the proper organization of somatodendritic MTs (Liu et al. 2015). On the contrary, CKAP1 is a tubulin

folding protein that plays a role in microtubule dynamics and might be crucial at some developmental or cell specialization processes, for instance during neurogenesis where tubulin turnover might be required (Kortazar et al. 2007). In contrast, PAMP interacts with tubulin β-IIa and kinesin heavy chain member 2 (KIF2). β-tubulin has six isoforms although little is known about them. One of this isoforms is tubulin β-II, mainly present in the axon and with a possible role in axonal growth (Hoffman et al. 1992). KIF2 is a member of the kinesin family and regulates the dynamics of MTs by inducing their depolymerization. As MT destabilizer, KIF2 plays a significant role in the suppression of collateral branch extension and in expansion at the growth cone of the neuronal axon (Li et al. 2006). Overall, PAMP is likely associated to tubulin depolymerisation and velocity of cargo transport by kinesin over MTs (Larráyoz and Martínez 2012), whereas AM is likely involved in tubulin destabilization by interacting with MAPs (Larráyoz et al. 2013).

Immunohistochemistry studies revealed a complete colocalization of AM and PAMP with MTs in cultured neurons. When these cultures were treated with MT-destabilizing agents (nocodazol or low temperature), the cellular distribution of AM and PAMP were closely related to the altered MTs (Sackett et al. 2008). Furthermore, downregulation of *ADM* gene through siRNA technology produces MT hyperpolymerization, leading to reduced cell motility and partial arrest at the G2 phase of the cell cycle (Sackett et al. 2008). Thus, alterations of AM and/or PAMP may induce morphological changes in the cytoskeleton and affect associated cellular functions (Fernández et al. 2008; Sackett et al. 2008).

In addition, the absence of *ADM* changed the ratio among the three possible lineages of neural stem cells: neurons, astrocytes, and oligodendrocytes. Lack of *ADM* promoted oligodendrocyte formation whereas it interfered with the production of neurons and astrocytes. The changed ratio was partially recovered for each lineage in the presence of externally added AM. In contrast, the morphological and proliferative changes appeared in cells in the absence of *ADM* could not be rescued supplementing the media with AM. This suggests the existence of distinct mechanisms by which AM regulates both differentiation and morphology in stem cells. The development of a stem cell in its progeny may be mediated through AM receptors, whereas morphological features might be dependent on an intracellular pool of the peptides acting through their direct binding to the cytoskeleton (Sackett et al. 2008; Vergaño-Vera et al. 2010; Larrayoz et al. 2012). The ability to interact and regulate cytoskeleton may be mediating the observed protective effects of AM and

PAMP against heart remodelling, hypertrophy, and other cardiac dysfunctions (Niu et al. 2004).

Taking all together AM and PAMP could be tightly involved in the regulation of cytoskeleton, including MT dynamics and hence axonal transport and synaptic plasticity.

3. ADRENOMEDULLIN AND NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are a heterogeneous group of progressive disorders characterized by neuronal function impairment. Life expectancy has raised significantly over the years, and the prevalence of neurodegenerative disorders increases dramatically with the ageing population. Interestingly, clinical symptoms appear before neuronal loss is extensive. Improper accumulation of certain proteins may compromise neuronal activity and activate a cascade of adaptive responses in neurotransmission and signalling pathways that might consequently cause synaptic deficits and ultimately the collapse of neuronal networks and neurological symptoms. So far the efforts of the scientific community have failed to discover an effective treatment to prevent or slow the progress of any of these diseases. Therefore, any progress achieved in this field will reduce the human, social and economic costs associated with these neurodegenerative disorders.

3.1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common form of dementia and neurodegenerative disease. AD is a progressive and irreversible neurodegenerative pathology produced by the gradual loss of brain neurons. Clinically, AD is characterized by severe cognitive impairment that presents with loss of memory and behavioural impairment. AD is classified by the ethiological origin (sporadic vs familial) or by the age at disease´s onset (late-onset vs early-onset). The majority of AD cases are sporadic, and their incidence increases with aging, affecting one in four people aged 85 and over (Alzheimer's Disease International. World Alzheimer Report 2016). In addition to aging, hypertension, dyslipidemia, and diabetes are potential risk factors for sporadic AD (Reitz and Mayeux 2014).

Two main pathological features distinguishes AD brains: senile plaques and neurofibrillary tangles (NFT), mainly composed of extracellular deposits of amyloid-β (Aβ) peptides and hyperphosphorylated Tau protein, respectively (Shoghi-Jadid et al. 2002; Selkoe and Hardy 2016). Neuronal dysfunction is an early event in AD that ranges from synaptic pathology to altered neuronal connectivity. Together with senile plaques and neurofibrillary

tangles, neuronal loss is the third hallmark of AD. This neuronal loss is mainly established in the cerebral cortex and the hippocampus, both areas involved in memory and learning, and occurs before senile plaques formation (Selkoe 2002). Familial AD represents less than 5% of AD cases, which is linked to hereditary autosomal dominant genetic mutations in three genes, and usually presents an early onset debut. Genes associated with familial AD codify for amyloid precursor protein (APP) and presenilins 1 and 2 (PS1 and PS2), which are responsible for amyloid metabolism. APP is the precursor of Aβ peptides and it can be processes through two different pathways: the amyloidogenic and the non-amyloidogenic routes. Amyloidogenic processing occurs predominantly in pathological conditions, as the result of a sequential cleavage carried out by two secretases, β- secretase and γ- secretase. Aβ is a physiological protein that spontaneously self-aggregates into multiple isoforms. The two main isoforms are: the soluble form, $A\beta_{40}$, representing 80-90% of the total, and the insoluble form, $A\beta_{42}$, which constitutes 5-10% approximately (Haass et al. 2012). Familial AD mutations affect amyloidogenic APP processing, leading to rapid and aberrant cleavage with overproduction of Aβ, mostly $A\beta_{42,}$ the most neurotoxic form of Aβ (O'Brien and Wong 2011). The major genetic factor that influences the risk for late-onset sporadic AD is the allele APOE4, which also accelerates Aβ deposition (Querfurth and LaFerla 2010). These mutations are the basis for "the amyloid hypothesis" that suggests an imbalance between Aβ peptide production and clearance resulting in aggregation, accumulation, and Aβ oligomerization into insoluble fibrils and subsequent Aβ deposition (Hardy and Selkoe 2002). Aβ oligomers and fibrils are considered neurotoxic and their burden correlates with the severity of cognitive deficits. The cellular changes triggered by Aβ oligomers lead to synaptic dysfunction, loss of dendritic spines, impairment on energy metabolism, increase oxidative stress, mitochondrial dysfunction, and disruption of calcium homeostasis (Haass et al. 2012; Mucke and Selkoe 2012). Taken together, these events precipitate a downstream cascade of molecular alterations that provoke neuronal loss and activate the immune response that may perpetuate a neuroinflammatory response. In addition, it has been proposed that Aβ neurotoxicity in AD is mediated by the hyperphosphorylation, disorganization and deposition of Tau in the neocortex (Iqbal et al. 2015; DeVos et al. 2017).

Tau protein is a natively unfolded MAP encoded by a single gene, *MAPT*, on human chromosome 17. In humans, Tau is predominantly expressed in neurons. As a result of alternative splicing, Tau presents six molecular isoforms and also may contain three (3R) or four (4R) MT domain repeats (Iqbal et al. 2015). The 4R Tau protein facilitates better than

3R Tau the assembly of MTs. In a healthy brain, equal amounts of 3R and 4R Tau are, as well as the six different isoforms of Tau, in different brain regions (Spillantini and Goedert 2013). The best established function of Tau is to promote assembly of the MTs through its direct union to tubulin subunits thus stabilizing their structure. Furthermore, a gradient concentration of Tau exists along the axon, with an increase at the synapse where Tau may halt the motor proteins and facilitate the local release of their cargo (Medina et al. 2016). Therefore, Tau promotes correct axonal transport and the formation and maintenance of both dendrites and axons thus contributing to neuronal integrity. The regulation of Tau function is achieved by different degrees of posttranslational modifications, mainly phosphorylation. In AD, Tau is hyperphosphorylated, this characteristic appears to confer resistance to proteolysis and therefore increases its presence in neurons (Shimura et al. 2004). This state of Tau is the result of the sum of the activities of several kinases and phosphatases such as glycogen synthase kinase 3β (GSK3β) and protein phosphatase 2A (PP2A). In its hyperphosphorylated form, Tau loses its physiological function and reduces its affinity for MTs, affecting also MAP1 and MAP2 function and causing MT disassembly (Alonso et al. 1997). In this way, the disintegration of the neuronal cytoskeleton causes the axonal transport system to collapse. Following this event, synaptic and axonal dysfunction can be initiated leading to neuronal death. In this sense, the "Tau hypothesis" suggests that hyperphosphorylated Tau is a key element responsible for triggering the cascade of molecular phenomena, which leads to cognitive impairment and memory loss. According to this model, hyperphosphorylated Tau is capable to self-associate through its MT-binding regions to form helicoidally paired filaments which finally generate intracellular NFTs (Morishima-Kawashima et al. 1995). Although Tau is mainly found in the axon, abnormal accumulation of hyperphosphorylated Tau also occur in the somatodendritic compartment in AD, which may alter the localization of dendritic spines and thereby lead to synaptic dysfunction (Hoover et al. 2010). Moreover, similar to what happens with Aβ oligomers, intermediate aggregates of abnormal Tau are insoluble and have deleterious effects, producing neurotoxicity and cognitive impairment (Querfurth and LaFerla 2010). It has been proposed that helical paired filaments may sequester toxic intermediate Tau species, as a protection mechanism (Lee et al. 2005). As the pathology of AD progresses, neuronal death leads to intracellular Tau being released into the extracellular space which may generate a detrimental environment for neighbouring neurons. Extracellular Tau increases intracellular concentrations of calcium that disrupt neuronal function (Gómez-Ramos et al. 2006). In addition, Tau aggregates can also induce damage in proteasome function, interfering with the

clearance of damaged proteins (Myeku et al. 2015). In AD, Tau pathology propagates following an anatomical pattern which correlates with the clinical cognitive status (Braak and Braak 1991a). Neurofibrillary lesions and spreading of Tau toxicity may occur through exocytosis and endocytosis of Tau species by naïve neurons nearby affected neurons by synaptic transmission and/or by synaptic dysfunction (de Calignon et al. 2012; Liu et al. 2012).

As Tau, AM might play a role in the pathophysiology of AD or might potentiate the action of pTau. AM together with its receptors are widely expressed throughout the CNS, providing an anatomical basis for its function. Recent studies have shown that AM is increased in transgenic mouse models of AD (Fernandez et al. 2016). Based on these data and with the previous notion that AM interacts directly with cytoskeletal proteins, it could be hypothesized that patients suffering from AD may have neuronal cytoskeletal disorders caused by increased levels of AM and PAMP. As a consequence of the microtubule instability generated by increasing *ADM* gene products, AM and PAMP might drive the collapse of the transport system, resulting in axon retraction and loss of synaptic connections (**Figure 3**).

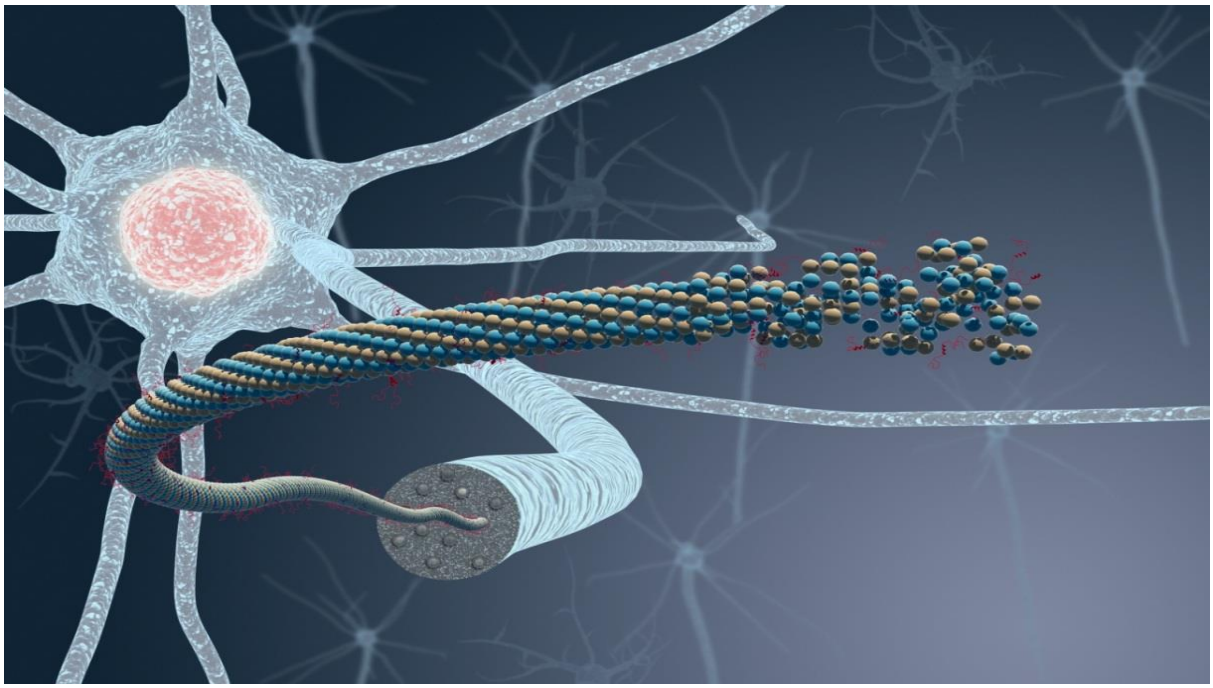

Figure 3. A neuron and its microtubule axonal transport disrupted by *ADM* derived peptides. AM and PAMP direct interaction with the tubulin subunits of the cytoskeleton may interfere with microtubule dynamics. Increased levels of AM and PAMP in AD brains may cause the disassembly of neurocytoskeleton, which may lead to the collapse of the axonal transport system and ultimately result in axon retraction and loss of synaptic connections.

AD brain presents evidence of a persistent widespread inflammatory response, which is sustained by activating glial cells, microglia and astrocytes, facilitating the neurodegenerative progression and contributing to neuronal dysfunction and death (Agostinho et al. 2010). Moreover, Aβ deposition alone might be sufficient to induce an inflammatory reaction which might perturb the CNS microenvironment that keeps immune response under tight control and leads to microglia and astrocyte activation. Both senile plaques and NFTs contain a number of these immune cells clustered around the deposits. Inflammatory and glial responses seem to interact closely with AM on brain damage (Jahnke et al. 2001). Additionally, AM is known to regulate cytokine secretion of microglia and macrophages (Wong et al. 2005; Consonni et al. 2011), thus it cannot be excluded that under pathological conditions AM may alter the phenotype of immune cells. Likewise, in the search for new AD plasma biomarkers, mid regional (MR)-proAM has been proposed as a progression marker from predementia into full fledged AD (Ewers et al. 2010; Buerger et al. 2011a; Verweij et al. 2013; Henriksen et al. 2014). Changes in mid-regional proAM parallel those in mature AM and PAMP. In addition, AM peptide has been determined as biomarker in patients with ischemic infarct, which increase in risk of dementia (Zhang et al. 2014). This latter study showed that plasma AM concentration predicted 3-month mortality and unfavourable outcome.

3.2 VASCULAR DEMENTIA

Vascular dementia (VaD) is the second most common cause of dementia in the elderly after AD (O'Brien and Thomas 2015). VaD defines a syndrome of cognitive impairment that primarily results from vascular disease in the absence of other pathologies. The predominant cognitive symptom of VaD is the dysfunction in executive function that interferes with social or occupational functioning (Román 2004). The risk of VaD increases exponentially with aging. In addition, vascular risk factors such as obesity, diabetes, hypercholesterolemia, and hypertension predispose to suffer VaD. Genetic VaD occurs mainly on rare familial syndromes such as Cadasil or cerebral autosomal dominant arteriopathy, and cerebral amyloidosis-Dutch type (HCHWA-D). Cadasil is a small-vessel disease related to a mutation in the Nocht 3 gene on chromosome 19 (Chabriat et al. 2009). HCHWA-D is caused by a mutation on the APP gene that causes abnormal deposition of amyloid peptide in vascular walls causing cerebral amyloid angiopathy. Because the pathogenesis, symptoms and risk factors of VaD are similar to those of AD, there is a frequent overlap between both diseases. In fact, postmortem studies reveal that dementia patients with significant vascular pathology

vary between 15-30%, either alone or in combination with AD (Leblanc et al. 2006). In addition, vascular risk has also emerged as a major risk factor for AD, as increased cerebrovascular damage exacerbates cognitive impairment in this dementia (Deramecourt et al. 2012). As expected from its heterogeneous etiology, neuropathological lesions of VaD are also diverse, including infarcts, white matter lesions (WMLs), microbleeds, dilated perivascular spaces, and brain atrophy. WMLs have been associated with age and considered a hallmark of VaD, but they are also present in AD and Lewy body dementias (Englund 1998; Ihara et al. 2010). On the other hand, lacunar infarcts have been described as the most common features in more than 50% of elderly VaD patients (Vinters et al. 2000). Both characteristics are the product of progressive degeneration of the vascular wall, which includes thickening and loss of vascular cells. As a consequence, vascular dysfunction produces a cerebral blood supply deficit that may lead to ischemia and neuronal death with glial proliferation in the area (Iemolo et al. 2009). As in AD, the immune and inflammatory responses contribute to neuronal dysfunction and death in VaD. Furthermore, endothelial dysfunction leads to a breakdown of the blood-brain barrier (BBB) and chronic leakage of fluid and macromolecules into the white matter (Bronge and Wahlund 2000). BBB dysfunction may contribute to the pathological processes in vascular dementia (VaD) and possibly also in AD (Farrall and Wardlaw 2009). In this context, AM may play a key role as endogenous regulator of BBB permeability and cerebral blood flow (Kis et al. 2001a, b; Bełtowski and Jamroz 2004). In fact, plasma levels of AM are almost 50% higher in brain vasculature than in peripheral vasculature (Kis et al. 2001a). AM is well known by its effects on the vascular structure, including its development, remodelling, and regeneration. Accordingly, AM may have a neuroprotective role in VaD, whereas in AD it may mediate neurodegeneration. Several studies support the neuroprotective role of AM since increased levels of this peptide reduces cerebral ischemic damage in different animal models (Dogan et al. 1997; Watanabe et al. 2001; Pickering et al. 2002; Xia et al. 2004, 2006; Maki et al. 2011). In additon, expression of AM dramatically increase after inflammatory or ischemic events, suggesting that this response may compensate brain damage by remodelling and repairing vascular tissue (Serrano et al. 2002; Miyamoto et al. 2009; Liverani and Paul 2013). Neuroprotective effects of vascular AM could be mediated by an upregulation of vascular endothelial growth factor and basic fibroblast growth factor but also by an increase in endothelial NO production (Fernandez et al. 2011; Maki et al. 2011). Conversely, AM is a potent vasodilator of the cerebral vasculature, and it may be implicated in the pathological mechanism that originates and aggravates both VaD and AD (Fernandez et al. 2016). Besides,

AM can increase cerebral microvascular perfusion, which together with endothelial damage, might advance the neuropathological state (Kis et al. 2006). In this context, AM has been evaluated as a possible biomarker for VaD caused by microvascular endothelial dysfunction, although no significant results have been found (Holm et al. 2017). In addition, AM is an autocrine regulator of BBB function, which contributes to homeostasis and neuroprotection, although increased vasodilator peptide levels and decreased vasoconstrictors, such as AM and ET-1, might lead to maintained vasodilation and thereby worsening dementia (Kis et al. 2001b, 2006; Dohgu et al. 2010). Moreover, immune response carried out by astrocytes may intervene in the production of AM and its effects may alter the BBB. Similarly to AD, MR-proAM has been proposed as a new predictor factor for VaD, since increased MR-proAM levels have been directly related to WMLs progression and inversely correlated with the degree of cognitive function (Kuriyama et al. 2017). All together, this observactions supports a role of AM in VaD pathophysiology, however, whether this involvement has positive or negative outcomes on disease development and progression needs to be elucidated.

3.3 OTHER NEURODEGENERATIVE DISEASES

Parkinson's disease (PD) is clinically characterized by progressive rigidity, postural instability, bradykinesia, and rest tremor. This disorder is caused by the loss of dopaminergic neurons in the substantia nigra. Cytoplasmic inclusions of α-synuclein protein, the Lewy bodies (LB), are the major pathological hallmark of PD (Lees et al. 2009). It is to note that on the top of the presence of senile plaques and NTFs, more than half of AD patients present LBs. In addition, although PD is a disease that manifests principally as a movement disorder, PD is commonly accompanied by dementia. When dementia precedes Parkinsonism and abundant cortical LBs are present, the disorder is designated dementia with LBs (Shaw et al. 2007). Mutations in the α-synuclein gene cause an autosomal dominant familial PD. Abnormal α-synuclein progressively forms more insoluble oligomers and fibrillar aggregates. The second most common mutation in familial PD is the parkin gene (Dawson and Dawson 2010). Parkin is involved in protein degradation and has been reported to interact with α and β tubulin subunits to stabilizes MTs in a likely ubiquitin-dependent manner (Yang et al. 2005).

Huntington’s disease (HD) patients present involuntary movements such as chorea, psychiatric disturbances, and dementia caused by a degeneration of long projection neurons in the cortex and striatum. HD is inherited in an autosomal dominant manner with a mutation

in the huntingtin gene that causes an expanded polyglutamine tract at the amino terminus of the protein (Ross and Tabrizi 2011). This polyglutamine tract can vary in length and causes a toxic gain-of-function in huntingtin. Several studies evidence the toxicity of the expanded polyglutamine containing proteins as they interfere with essential functions of the cell. Recently, it has been reported that the cytoskeleton is also affected by this neurodegenerative disease (Fernández-Nogales et al. 2016).

Frontotemporal dementia (FTD) is the second more common neurodegenerative dementia with early-onset. This neurodegenerative disorder is associated with atrophy of the frontal and temporal lobe which tends to be asymmetric between hemispheres. Clinically, a variety of overlapping clinical syndromes appear related with the frontotemporal lobar degeneration, among which the behavioral variant is the most common. Familial FTD cases are associated with mutations on different genes, although the most commonly mutated genes are progranulin (*GRN*) and Tau (*MAPT*) (Rohrer 2012). One of the FTD variants is accompanied by parkinsonism symptoms and named frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). In addition to motor symptoms, this neurodegenerative disorder also presents behavioral and personality changes and cognitive impairment. FTDP-17 is an autosomal dominant disorder that is caused by mutations in the *MAPT* gene, affecting Tau protein. FTDP-17 phenotype can vary not only between families carrying different mutations but also between and within families carrying the same mutations. Similarly to AD, the mechanisms underlying FTDP-17 are thought to be related to altered patterns in the distribution of Tau isoforms or with the loss of Tau function and its ability to bind MTs and to promote MT assembly (Wszolek et al. 2006). At this point, combined AM and Tau alterations could be deleterious and alter the state of the cytoskeleton.

The involvement of AM in the etiology and/or progression of neurodegenerative diseases has been studied at a preclinical and clinical level in AD and VaD**.** AM may exert a negative influx aggravating the MT instability on these neurodegenerative diseases, collapsing the cytoskeleton, and leading to axonal transport impairment (**Figure 3**).

For this reason, the inhibition of AM could be a novel target and therapeutic approach for these neurodegenerative diseases. Indeed, small molecules have been synthesized and characterized as antagonists of AM, and these molecules have already been tested in the treatment of osteoporosis, tumours, and retinopathies (Larráyoz et al. 2014; Martínez-Herrero et al. 2016).

4. REFERENCES

Agostinho, P., Cunha, R.A., and Oliveira, C. (2010). Neuroinflammation, oxidative stress and the pathogenesis of Alzheimer's disease. Curr. Pharm. Des. 16, 2766–2778.

Alonso, A.D., Grundke-Iqbal, I., Barra, H.S., and Iqbal, K. (1997). Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. Proc. Natl. Acad. Sci. U. S. A. 94, 298–303.

Bełtowski, J., and Jamroz, A. (2004). Adrenomedullin--what do we know 10 years since its discovery? Pol. J. Pharmacol. 56, 5–27.

Braak, H., and Braak, E. (1991). Demonstration of amyloid deposits and neurofibrillary changes in whole brain sections. Brain Pathol. 1, 213–216.

Bronge, L., and Wahlund, L.O. (2000). White matter lesions in dementia: an MRI study on blood-brain barrier dysfunction. Dement. Geriatr. Cogn. Disord. 11, 263–267.

Buerger, K., Uspenskaya, O., Hartmann, O., Hansson, O., Minthon, L., Blennow, K., Moeller, H.-J., Teipel, S.J., Ernst, A., Bergmann, A., et al. (2011). Prediction of Alzheimer's Disease Using Midregional Proadrenomedullin and Midregional Proatrial Natriuretic Peptide. J. Clin. Psychiatry 72, 556–563.

Cai, D., McEwen, D.P., Martens, J.R., Meyhofer, E., and Verhey, K.J. (2009). Single molecule imaging reveals differences in microtubule track selection between Kinesin motors. PLoS Biol. 7, e1000216.

Cairns, N.J., Lee, V.M.-Y., and Trojanowski, J.Q. (2004). The cytoskeleton in neurodegenerative diseases. J. Pathol. 204, 438–449.

Chabriat, H., Joutel, A., Dichgans, M., Tournier-Lasserve, E., and Bousser, M.-G. (2009). CADASIL. Lancet Neurol. 8, 643–653.

Conde, C., and Cáceres, A. (2009). Microtubule assembly, organization and dynamics in axons and dendrites. Nat. Rev. Neurosci. 10, 319–332.

Consonni, A., Morara, S., Codazzi, F., Grohovaz, F., and Zacchetti, D. (2011). Inhibition of lipopolysaccharide-induced microglia activation by calcitonin gene related peptide and adrenomedullin. Mol. Cell. Neurosci. 48, 151–160.

Craig, A.M., and Banker, G. (1994). Neuronal Polarity. Annu. Rev. Neurosci. 17, 267–310.

Dawson, T.M., and Dawson, V.L. (2010). The role of parkin in familial and sporadic Parkinson's disease. Mov. Disord. 25, S32–S39.

de Calignon, A., Polydoro, M., Suárez-Calvet, M., William, C., Adamowicz, D.H., Kopeikina, K.J., Pitstick, R., Sahara, N., Ashe, K.H., Carlson, G.A., et al. (2012). Propagation of Tau Pathology in a Model of Early Alzheimer's Disease. Neuron 73, 685–697.

Deramecourt, V., Slade, J.Y., Oakley, A.E., Perry, R.H., Ince, P.G., Maurage, C.-A., and Kalaria, R.N. (2012). Staging and natural history of cerebrovascular pathology in dementia. Neurology 78, 1043–1050.

Desai, A., and Mitchison, T.J. (1997). Microtubule Polymerization Dynamics. Annu. Rev.

Cell Dev. Biol. 13, 83–117.

DeVos, S.L., Miller, R.L., Schoch, K.M., Holmes, B.B., Kebodeaux, C.S., Wegener, A.J., Chen, G., Shen, T., Tran, H., Nichols, B., et al. (2017). Tau reduction prevents neuronal loss and reverses pathological tau deposition and seeding in mice with tauopathy. Sci. Transl. Med. 9, eaag0481.

Dogan, A., Suzuki, Y., Koketsu, N., Osuka, K., Saito, K., Takayasu, M., Shibuya, M., and Yoshida, J. (1997). Intravenous Infusion of Adrenomedullin and Increase in Regional Cerebral Blood Flow and Prevention of Ischemic Brain Injury after Middle Cerebral Artery Occlusion in Rats. J. Cereb. Blood Flow Metab. 17, 19–25.

Dohgu, S., Sumi, N., Nishioku, T., Takata, F., Watanabe, T., Naito, M., Shuto, H., Yamauchi, A., and Kataoka, Y. (2010). Cyclosporin A induces hyperpermeability of the blood–brain barrier by inhibiting autocrine adrenomedullin-mediated up-regulation of endothelial barrier function. Eur. J. Pharmacol. 644, 5–9.

Downing, K.H., and Nogales, E. (1998). Tubulin and microtubule structure. Curr. Opin. Cell Biol. 10, 16–22.

Dupuis, J., Caron, A., and Ruël, N. (2005). Biodistribution, plasma kinetics and quantification of single-pass pulmonary clearance of adrenomedullin. Clin. Sci. (Lond). 109, 97–102.

Eguchi, S., Hirata, Y., Kano, H., Sato, K., Watanabe, Y., Watanabe, T.X., Nakajima, K., Sakakibara, S., and Marumo, F. (1994). Specific receptors for adrenomedullin in cultured rat vascular smooth muscle cells. FEBS Lett. 340, 226–230.

Eira, J., Silva, C.S., Sousa, M.M., and Liz, M.A. (2016). The cytoskeleton as a novel therapeutic target for old neurodegenerative disorders. Prog. Neurobiol. 141, 61–82.

Englund, E. (1998). Neuropathology of white matter changes in Alzheimer's disease and vascular dementia. Dement. Geriatr. Cogn. Disord. 9 Suppl 1, 6–12.

Erickson, H.P., and O'Brien, E.T. (1992). Microtubule Dynamic Instability and GTP Hydrolysis. Annu. Rev. Biophys. Biomol. Struct. 21, 145–166.

Eto, T., Kitamura, K., and Kato, J. (1999). Biological and clinical roles of adrenomedullin in circulation control and cardiovascular diseases. Clin. Exp. Pharmacol. Physiol. 26, 371–380.

Ewers, M., Mielke, M.M., and Hampel, H. (2010). Blood-based biomarkers of microvascular pathology in Alzheimer's disease. Exp. Gerontol. 45, 75–79.

Farrall, A.J., and Wardlaw, J.M. (2009). Blood–brain barrier: Ageing and microvascular disease – systematic review and meta-analysis. Neurobiol. Aging 30, 337–352.

Fernandez, A.P., Serrano, J., Amorim, M.A., Pozo-Rodrigalvarez, A., and Martinez-Murillo, R. (2011). Adrenomedullin and nitric oxide: implications for the etiology and treatment of primary brain tumors. CNS Neurol. Disord. Drug Targets 10, 820–833.

Fernandez, A.P., Masa, J.S., Guedan, M.A., Futch, H.S., and Martínez-Murillo, R. (2016). Adrenomedullin Expression in Alzheimer's Brain. Curr. Alzheimer Res. 13, 428–438.

Fernández, A.P., Serrano, J., Tessarollo, L., Cuttitta, F., Martínez, A., Fernandez, A.P., Serrano, J., Tessarollo, L., Cuttitta, F., and Martinez, A. (2008). Lack of adrenomedullin in the mouse brain results in behavioral changes, anxiety, and lower survival under stress conditions. Proc. Natl. Acad. Sci. U. S. A. 105, 12581–12586.

Fernández-Nogales, M., Santos-Galindo, M., Hernández, I.H., Cabrera, J.R., and Lucas, J.J. (2016). Faulty splicing and cytoskeleton abnormalities in Huntington's disease. Brain Pathol. 26, 772–778.

Fletcher, D.A., and Mullins, R.D. (2010). Cell mechanics and the cytoskeleton. Nature 463, 485–492.

Foord, S.M., McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., and Lee, M.G. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393, 333–339.

Fritz-Six, K.L., Dunworth, W.P., Li, M., and Caron, K.M. (2008). Adrenomedullin signaling is necessary for murine lymphatic vascular development. J. Clin. Invest. 118, 40–50.

Garayoa, M., Martínez, A., Lee, S., Pío, R., An, W.G., Neckers, L., Trepel, J., Montuenga, L.M., Ryan, H., Johnson, R., et al. (2000). Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. Mol. Endocrinol. 14, 848–862.

Gómez-Ramos, A., Díaz-Hernández, M., Cuadros, R., Hernández, F., and Avila, J. (2006). Extracellular tau is toxic to neuronal cells. FEBS Lett. 580, 4842–4850.

Haass, C., Kaether, C., Thinakaran, G., and Sisodia, S. (2012). Trafficking and proteolytic processing of APP. Cold Spring Harb. Perspect. Med. 2, a006270.

Hardy, J., and Selkoe, D.J. (2002). The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. Science. 297, 353–356.

Hay, D.L., and Smith, D.M. (2001). Adrenomedullin receptors: molecular identity and function. Peptides 22, 1753–1763.

Henriksen, K., O'Bryant, S.E., Hampel, H., Trojanowski, J.Q., Montine, T.J., Jeromin, A., Blennow, K., Lönneborg, A., Wyss-Coray, T., Soares, H., et al. (2014). The future of blood-based biomarkers for Alzheimer's disease. Alzheimer's Dement. 10, 115–131.

Hirata, Y., Hayakawa, H., Suzuki, Y., Suzuki, E., Ikenouchi, H., Kohmoto, O., Kimura, K., Kitamura, K., Eto, T., and Kangawa, K. (1995). Mechanisms of adrenomedullin-induced vasodilation in the rat kidney. Hypertens. 25, 790–795.

Hirokawa, N., Niwa, S., and Tanaka, Y. (2010). Molecular Motors in Neurons: Transport Mechanisms and Roles in Brain Function, Development, and Disease. Neuron 68, 610–638.

Hoffman, P.N., Lopata, M.A., Watson, D.F., and Luduena, R.F. (1992). Axonal transport of class II and III beta-tubulin: evidence that the slow component wave represents the movement of only a small fraction of the tubulin in mature motor axons. J Cell Biol. 119, 595–604.

Holm, H., Nägga, K., Nilsson, E.D., Ricci, F., Melander, O., Hansson, O., Bachus, E., Magnusson, M., and Fedorowski, A. (2017). Biomarkers of microvascular endothelial dysfunction predict incident dementia: a population-based prospective study. J. Intern. Med. 282, 94–101.

Hoover, B.R., Reed, M.N., Su, J., Penrod, R.D., Kotilinek, L.A., Grant, M.K., Pitstick, R., Carlson, G.A., Lanier, L.M., Yuan, L.-L., et al. (2010). Tau Mislocalization to Dendritic Spines Mediates Synaptic Dysfunction Independently of Neurodegeneration. Neuron 68, 1067–1081.

Hyman, A.A., Salser, S., Drechsel, D.N., Unwin, N., and Mitchison, T.J. (1992). Role of GTP hydrolysis in microtubule dynamics: information from a slowly hydrolyzable analogue, GMPCPP. Mol. Biol. Cell 3, 1155–1167.

Ichiki, Y., Kitamura, K., Kangawa, K., Kawamoto, M., Matsuo, H., and Eto, T. (1994). Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. FEBS Lett. 338, 6–10.

Iemolo, F., Duro, G., Rizzo, C., Castiglia, L., Hachinski, V., and Caruso, C. (2009). Pathophysiology of vascular dementia. Immun. Ageing 6, 13.

Ihara, M., Polvikoski, T.M., Hall, R., Slade, J.Y., Perry, R.H., Oakley, A.E., Englund, E., O'Brien, J.T., Ince, P.G., and Kalaria, R.N. (2010). Quantification of myelin loss in frontal lobe white matter in vascular dementia, Alzheimer's disease, and dementia with Lewy bodies. Acta Neuropathol. 119, 579–589.

Iqbal, K., Liu, F., and Gong, C.-X. (2015). Tau and neurodegenerative disease: the story so far. Nat. Rev. Neurol. 12, 15–27.

Ishimitsu, T., Kojima, M., Kangawa, K., Hino, J., Matsuoka, H., Kitamura, K., Eto, T., and Matsuo, H. (1994). Genomic Structure of Human Adrenomedullin Gene. Biochem. Biophys. Res. Commun. 203, 631–639.

Jahnke, G.D., Brunssen, S., Maier, W.E., and Harry, G.J. (2001). Neurotoxicant-induced elevation of adrenomedullin expression in hippocampus and glia cultures. J. Neurosci. Res. 66, 464–474.

Jaworski, J., Kapitein, L.C., Gouveia, S.M., Dortland, B.R., Wulf, P.S., Grigoriev, I., Camera, P., Spangler, S.A., Di Stefano, P., Demmers, J., et al. (2009). Dynamic Microtubules Regulate Dendritic Spine Morphology and Synaptic Plasticity. Neuron 61, 85–100.

Juaneda, C., Dumont, Y., Chabot, J.G., Fournier, A., and Quirion, R. (2003). Adrenomedullin receptor binding sites in rat brain and peripheral tissues. Eur. J. Pharmacol. 474, 165–174.

Kato, J., and Kitamura, K. (2015). Bench-to-bedside pharmacology of adrenomedullin. Eur. J. Pharmacol. 764, 140–148.

Kis, B., Deli, M.A., Kobayashi, H., Abrahám, C.S., Yanagita, T., Kaiya, H., Isse, T., Nishi, R., Gotoh, S., Kangawa, K., et al. (2001a). Adrenomedullin regulates blood-brain barrier functions in vitro. Neuroreport 12, 4139–4142.

Kis, B., Abrahám, C.S., Deli, M.A., Kobayashi, H., Wada, A., Niwa, M., Yamashita, H., and Ueta, Y. (2001b). Adrenomedullin in the cerebral circulation. Peptides 22, 1825–1834.

Kis, B., Chen, L., Ueta, Y., and Busija, D.W. (2006). Autocrine peptide mediators of cerebral endothelial cells and their role in the regulation of blood-brain barrier. Peptides 27, 211–222.

Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H., and Eto, T. (1993). Adrenomedullin: A Novel Hypotensive Peptide Isolated from Human Pheochromocytoma. Biochem. Biophys. Res. Commun. 192, 553–560.

Kitamura, K., Kangawa, K., Ishiyama, Y., Washimine, H., Ichiki, Y., Kawamoto, M., Minamino, N., Matsuo, H., and Eto, T. (1994). Identification and hypotensive activity of proadrenomedullin N-terminal 20 peptide (PAMP). FEBS Lett. 351, 35–37.

Kitamura, K., Kangawa, K., and Eto, T. (2002). Adrenomedullin and PAMP: Discovery, structures, and cardiovascular functions. Microsc. Res. Tech. 57, 3–13.

Kortazar, D., Fanarraga, M.L., Carranza, G., Bellido, J., Villegas, J.C., Avila, J., and Zabala, J.C. (2007). Role of cofactors B (TBCB) and E (TBCE) in tubulin heterodimer dissociation. Exp. Cell Res. 313, 425–436.

Kuriyama, N., Ihara, M., Mizuno, T., Ozaki, E., Matsui, D., Watanabe, I., Koyama, T., Kondo, M., Tokuda, T., Tamura, A., et al. (2017). Association between Mid-Regional Proadrenomedullin Levels and Progression of Deep White Matter Lesions in the Brain Accompanying Cognitive Decline. J. Alzheimer's Dis. 56, 1253–1262.

Lacroix, B., van Dijk, J., Gold, N.D., Guizetti, J., Aldrian-Herrada, G., Rogowski, K., Gerlich, D.W., and Janke, C. (2010). Tubulin polyglutamylation stimulates spastin-mediated microtubule severing. J. Cell Biol. 189, 945–954.

Larrayoz, I.M., Ochoa-Callejero, L., García-Sanmartín, J., Vicario-Abejón, C., and Martínez, A. (2012). Role of Adrenomedullin in the Growth and Differentiation of Stem and Progenitor Cells. Int Rev Cell Mol Biol. 297, 175–234.

Larráyoz, I.M., and Martínez, A. (2012). Proadrenomedullin N-terminal 20 peptide increases kinesin's velocity both in vitro and in vivo. Endocrinology 153, 1734–1742.

Larráyoz, I.M., Martínez-herrero, S., Ochoa-callejero, L., García-sanmartín, J., and Martínez, A. (2013). Is the Cytoskeleton an Intracellular Receptor for Adrenomedullin and PAMP ? Curr Protein Pept Sci.14, 429–443.

Larráyoz, I.M., Martínez-Herrero, S., García-Sanmartín, J., Ochoa-Callejero, L., and Martínez, A. (2014). Adrenomedullin and tumour microenvironment. J. Transl. Med. 12, 339.

Leblanc, G.G., Meschia, J.F., Stuss, D.T., and Hachinski, V. (2006). Genetics of Vascular Cognitive Impairment: The Opportunity and the Challenges. Stroke 37, 248–255.

Lee, H., Perry, G., Moreira, P.I., Garrett, M.R., Liu, Q., Zhu, X., Takeda, A., Nunomura, A., and Smith, M.A. (2005). Tau phosphorylation in Alzheimer's disease: pathogen or protector? Trends Mol. Med. 11, 164–169.

Lees, A.J., Hardy, J., and Revesz, T. (2009). Parkinson's disease. Lancet 373, 2055–2066.

Li, C., Zheng, Y., Qin, W., Tao, R., Pan, Y., Xu, Y., Li, X., Gu, N., Feng, G., and He, L. (2006). A family-based association study of kinesin heavy chain member 2 gene (KIF2)

and schizophrenia. Neurosci. Lett. 407, 151–155.

Liu, L., Drouet, V., Wu, J.W., Witter, M.P., Small, S.A., Clelland, C., and Duff, K. (2012). Trans-Synaptic Spread of Tau Pathology In Vivo. PLoS One 7, e31302.

Liu, Y., Lee, J.W., and Ackerman, S.L. (2015). Mutations in the Microtubule-Associated Protein 1A (Map1a) Gene Cause Purkinje Cell Degeneration. J. Neurosci. 35, 4587–4598.

Liverani, E., and Paul, C. (2013). Glucocorticoids alter adrenomedullin receptor expression and secretion in endothelial-like cells and astrocytes. Int. J. Biochem. Cell Biol. 45, 2715–2723.

López, J., and Martínez, A. (2002). Cell and molecular biology of the multifunctional peptide, adrenomedullin. Int. Rev. Cytol. 221, 1–92.

Luo, L. (2002). Actin Cytoskeleton Regulation in Neuronal Morphogenesis and Structural Plasticity. Annu. Rev. Cell Dev. Biol. 18, 601–635.

Maki, T., Ihara, M., Fujita, Y., Nambu, T., Miyashita, K., Yamada, M., Washida, K., Nishio, K., Ito, H., Harada, H., et al. (2011). Angiogenic and Vasoprotective Effects of Adrenomedullin on Prevention of Cognitive Decline After Chronic Cerebral Hypoperfusion in Mice. Stroke 42, 1122–1128.

Martínez, A., Zudaire, E., Portal-Núñez, S., Guédez, L., Libutti, S.K., Stetler-Stevenson, W.G., and Cuttitta, F. (2004a). Proadrenomedullin NH2-Terminal 20 Peptide Is a Potent Angiogenic Factor, and Its Inhibition Results in Reduction of Tumor Growth. Cancer Res. 64, 6489–6494.

Martínez, A., Oh, H.-R., Unsworth, E.J., Bregonzio, C., Saavedra, J.M., Stetler-Stevenson, W.G., and Cuttitta, F. (2004b). Matrix metalloproteinase-2 cleavage of adrenomedullin produces a vasoconstrictor out of a vasodilator. Biochem. J. 383, 413–418.

Martínez, A., Bengoechea, J.A., and Cuttitta, F. (2006). Molecular Evolution of Proadrenomedullin N-Terminal 20 Peptide (PAMP): Evidence for Gene Co-Option. Endocrinology 147, 3457–3461.

Martínez-Herrero, S., Larrayoz, I.M., Ochoa-Callejero, L., Fernández, L.J., Allueva, A., Ochoa, I., and Martínez, A. (2016). Prevention of Bone Loss in a Model of Postmenopausal Osteoporosis through Adrenomedullin Inhibition. Front. Physiol. 7, 280.

Medina, M., Hernández, F., and Avila, J. (2016). New Features about Tau Function and Dysfunction. Biomolecules 6, 21.

Minamino, N., Shoji, H., Sugo, S., Kangawa, K., and Matsuo, H. (1995). Adrenocortical steroids, thyroid hormones and retinoic acid augment the production of adrenomedullin in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 211, 686–693.

Mitchison, T., and Kirschner, M. (1984). Dynamic instability of microtubule growth. Nature 312, 237–242.

Miyamoto, N., Tanaka, R., Shimosawa, T., Yatomi, Y., Fujita, T., Hattori, N., and Urabe, T. (2009). Protein Kinase A-Dependent Suppression of Reactive Oxygen Species in Transient Focal Ischemia in Adrenomedullin-Deficient Mice. J. Cereb. Blood Flow

Metab. 29, 1769–1779.

Montuenga, L.M., Martínez, A., Miller, M.J., Unsworth, E.J., and Cuttitta, F. (1997). Expression of Adrenomedullin and Its Receptor during Embryogenesis Suggests Autocrine or Paracrine Modes of Action. Endocrinology 138, 440–451.

Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K., and Ihara, Y. (1995). Proline-directed and non-proline-directed phosphorylation of PHF-tau. J. Biol. Chem. 270, 823–829.

Mucke, L., and Selkoe, D.J. (2012). Neurotoxicity of Amyloid -Protein: Synaptic and Network Dysfunction. Cold Spring Harb. Perspect. Med. 2, a006338–a006338.

Muff, R., Born, W., and Fischer, J.A. (2001). Adrenomedullin and related peptides: receptors and accessory proteins. Peptides 22, 1765–1772.

Myeku, N., Clelland, C.L., Emrani, S., Kukushkin, N. V, Yu, W.H., Goldberg, A.L., and Duff, K.E. (2015). Tau-driven 26S proteasome impairment and cognitive dysfunction can be prevented early in disease by activating cAMP-PKA signaling. Nat. Med. 22, 46–53.

Nishimatsu, H., Suzuki, E., Nagata, D., Moriyama, N., Satonaka, H., Walsh, K., Sata, M., Kangawa, K., Matsuo, H., Goto, A., et al. (2001). Adrenomedullin induces endothelium-dependent vasorelaxation via the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. Circ. Res. 89, 63–70.

Niu, P., Shindo, T., Iwata, H., Iimuro, S., Takeda, N., Zhang, Y., Ebihara, A., Suematsu, Y., Kangawa, K., Hirata, Y., et al. (2004). Protective Effects of Endogenous Adrenomedullin on Cardiac Hypertrophy, Fibrosis, and Renal Damage. Circulation 109, 1789–1794.

O'Brien, J.T., and Thomas, A. (2015). Vascular dementia. Lancet 386, 1698–1706.

O'Brien, R.J., and Wong, P.C. (2011). Amyloid precursor protein processing and Alzheimer's disease. Annu. Rev. Neurosci. 34, 185–204.

Pickering, M.C., Cook, H.T., Warren, J., Bygrave, A.E., Moss, J., Walport, M.J., and Botto, M. (2002). Uncontrolled C3 activation causes membranoproliferative glomerulonephritis in mice deficient in complement factor H. Nat. Genet. 31, 424–428.

Pío, R., Martínez, A., Unsworth, E.J., Kowalak, J.A., Bengoechea, J.A., Zipfel, P.F., Elsasser, T.H., Cuttitta, F., Pio, R., Martinez, A., et al. (2001). Complement Factor H Is a Serum-binding Protein for Adrenomedullin, and the Resulting Complex Modulates the Bioactivities of Both Partners. J. Biol. Chem. 276, 12292–12300.

Portran, D., Schaedel, L., Xu, Z., Théry, M., and Nachury, M.V. (2017). Tubulin acetylation protects long-lived microtubules against mechanical ageing. Nat. Cell Biol. 19, 391–398.

Poyner, D.R. (1997). Molecular pharmacology of receptors for calcitonin-gene-related peptide, amylin and adrenomedullin. Biochem. Soc. Trans. 25, 1032–1036.

Prokop, A. (2013). The intricate relationship between microtubules and their associated motor proteins during axon growth and maintenance. Neural Dev. 8, 17.

Qi, T., Christopoulos, G., Bailey, R.J., Christopoulos, A., Sexton, P.M., and Hay, D.L. (2008). Identification of N-terminal receptor activity-modifying protein residues important for calcitonin gene-related peptide, adrenomedullin, and amylin receptor function. Mol. Pharmacol. 74, 1059–1071.

Querfurth, H.W., and LaFerla, F.M. (2010). Alzheimer's Disease. N. Engl. J. Med. 362, 329–344.

Reitz, C., and Mayeux, R. (2014). Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers. Biochem. Pharmacol. 88, 640–651.

Rohrer, J.D. (2012). Structural brain imaging in frontotemporal dementia. Biochim. Biophys. Acta - Mol. Basis Dis. 1822, 325–332.

Román, G.C. (2004). Facts, myths, and controversies in vascular dementia. J. Neurol. Sci. 226, 49–52.

Ross, C.A., and Tabrizi, S.J. (2011). Huntington's disease: from molecular pathogenesis to clinical treatment. Lancet Neurol. 10, 83–98.

Sackett, D.L., Ozbun, L., Zudaire, E., Wessner, L., Chirgwin, J.M., Cuttitta, F., and Martínez, A. (2008). Intracellular proadrenomedullin-derived peptides decorate the microtubules and contribute to cytoskeleton function. Endocrinology 149, 2888–2898.

Sakata, J., Shimokubo, T., Kitamura, K., Nishizono, M., Iehiki, Y., Kangawa, K., Matsuo, H., and Eto, T. (1994). Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. FEBS Lett. 352, 105–108.

Satoh, F., Takahashi, K., Murakami, O., Totsune, K., Sone, M., Ohneda, M., Sasano, H., and Mouri, T. (1996). Immunocytochemical localization of adrenomedullin-like immunoreactivity in the human hypothalamus and the adrenal gland. Neurosci. Lett. 203, 207–210.

Selkoe, D.J. (2002). Alzheimer's Disease Is a Synaptic Failure. Science. 298, 789–791.

Selkoe, D.J., and Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol. Med. 8, 595–608.

Serrano, J., Uttenthal, L.O., Martínez, A., Fernández, A.P., Martínez de Velasco, J., Alonso, D., Bentura, M.L., Santacana, M., Gallardo, J.R., Martínez-Murillo, R., et al. (2000). Distribution of adrenomedullin-like immunoreactivity in the rat central nervous system by light and electron microscopy. Brain Res. 853, 245–268.

Serrano, J., Alonso, D., Fernández, A.P., Encinas, J.M., López, J.C., Castro-Blanco, S., Fernández-Vizarra, P., Richart, A., Santacana, M., Uttenthal, L.O., et al. (2002). Adrenomedullin in the central nervous system. Microsc. Res. Tech. 57, 76–90.

Shaw, L.M., Korecka, M., Clark, C.M., Lee, V.M.-Y., and Trojanowski, J.Q. (2007). Biomarkers of neurodegeneration for diagnosis and monitoring therapeutics. Nat. Rev. Drug Discov. 6, 295–303.

Shimura, H., Schwartz, D., Gygi, S.P., and Kosik, K.S. (2004). CHIP-Hsc70 Complex Ubiquitinates Phosphorylated Tau and Enhances Cell Survival. J. Biol. Chem. 279, 4869–4876.

Shoghi-Jadid, K., Small, G.W., Agdeppa, E.D., Kepe, V., Ercoli, L.M., Siddarth, P., Read, S., Satyamurthy, N., Petric, A., Huang, S.-C., et al. (2002). Localization of neurofibrillary tangles and beta-amyloid plaques in the brains of living patients with Alzheimer disease. Am. J. Geriatr. Psychiatry 10, 24–35.

Soppina, V., Herbstman, J.F., Skiniotis, G., and Verhey, K.J. (2012). Luminal localization of α-tubulin K40 acetylation by cryo-EM analysis of fab-labeled microtubules. PLoS One 7, e48204.

Spillantini, M.G., and Goedert, M. (2013). Tau pathology and neurodegeneration. Lancet Neurol. 12, 609–622.

Spudich, J.A., Huxley, H.E., and Finch, J.T. (1972). Regulation of skeletal muscle contraction. II. Structural studies of the interaction of the tropomyosin-troponin complex with actin. J. Mol. Biol. 72, 619–632.

Sugo, S., Minamino, N., Kangawa, K., Miyamoto, K., Kitamura, K., Sakata, J., Eto, T., and Matsuo, H. (1994). Endothelial Cells Actively Synthesize and Secrete Adrenomedullin. Biochem. Biophys. Res. Commun. 201, 1160–1166.

Sugo, S., Minamino, N., Shoji, H., Kangawa, K., Kitamura, K., Eto, T., and Matsuo, H. (1995a). Interleukin-1, Tumor Necrosis Factor and Lipopolysaccharide Additively Stimulate Production of Adrenomedullin in Vascular Smooth Muscle Cells. Biochem. Biophys. Res. Commun. 207, 25–32.

Sugo, S., Minamino, N., Shoji, H., Kangawa, K., and Matsuo, H. (1995b). Effects of vasoactive substances and cAMP related compounds on adrenomedullin production in cultured vascular smooth muscle cells. FEBS Lett. 369, 311–314.

Szebenyi, G., Bollati, F., Bisbal, M., Sheridan, S., Faas, L., Wray, R., Haferkamp, S., Nguyen, S., Caceres, A., and Brady, S.T. (2005). Activity-Driven Dendritic Remodeling Requires Microtubule-Associated Protein 1A. Curr. Biol. 15, 1820–1826.

Takahashi, K., Satoh, F., Hara, E., Sone, M., Murakami, O., Kayama, T., Yoshimoto, T., and Shibahara, S. (1997). Production and secretion of adrenomedullin from glial cell tumors and its effects on cAMP production. Peptides 18, 1117–1124.

Takemura, R., Okabe, S., Umeyama, T., Kanai, Y., Cowan, N.J., and Hirokawa, N. (1992). Increased microtubule stability and alpha tubulin acetylation in cells transfected with microtubule-associated proteins MAP1B, MAP2 or tau. J. Cell Sci. 103 (Pt 4), 953–964.

Tixier, E., Leconte, C., Touzani, O., Roussel, S., Petit, E., and Bernaudin, M. (2008). Adrenomedullin protects neurons against oxygen glucose deprivation stress in an autocrine and paracrine manner. J. Neurochem. 106, 1388–1403.

Tsuruda, T., Kato, J., Kitamura, K., Kuwasako, K., Imamura, T., Koiwaya, Y., Tsuji, T., Kangawa, K., and Eto, T. (1998). Adrenomedullin: a possible autocrine or paracrine inhibitor of hypertrophy of cardiomyocytes. Hypertens. 31, 505–510.

Ueta, Y., Kitamura, K., Isse, T., Shibuya, I., Kabashima, N., Yamamoto, S., Kangawa, K., Matsuo, H., Eto, T., and Yamashita, H. (1995). Adrenomedullin-immunoreactive neurons in the paraventricular and supraoptic nuclei of the rat. Neurosci. Lett. 202, 37–40.

Vergaño-Vera, E., Fernández, A.P., Hurtado-Chong, A., Vicario-Abejón, C., and Martínez, A. (2010). Lack of adrenomedullin affects growth and differentiation of adult neural stem/progenitor cells. Cell Tissue Res. 340, 1–11.

Verweij, N., Mahmud, H., Leach, I.M., de Boer, R.A., Brouwers, F.P., Yu, H., Asselbergs, F.W., Struck, J., Bakker, S.J.L., Gansevoort, R.T., et al. (2013). Genome-Wide Association Study on Plasma Levels of Midregional-Proadrenomedullin and C-Terminal-Pro-Endothelin-1. Hypertension 61, 602–608.

Vinters, H. V, Ellis, W.G., Zarow, C., Zaias, B.W., Jagust, W.J., Mack, W.J., and Chui, H.C. (2000). Neuropathologic substrates of ischemic vascular dementia. J. Neuropathol. Exp. Neurol. 59, 931–945.

Walker, R.A., O'Brien, E.T., Pryer, N.K., Soboeiro, M.F., Voter, W.A., Erickson, H.P., and Salmon, E.D. (1988). Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies. J. Cell Biol. 107, 1437–1448.

Washimine, H., Asada, Y., Kitamura, K., Ichiki, Y., Hara, S., Yamamoto, Y., Kangawa, K., Sumiyoshi, A., and Eto, T. (1995). Immunohistochemical identification of adrenomedullin in human, rat, and porcine tissue. Histochem. Cell Biol. 103, 251–254.

Watanabe, K., Takayasu, M., Takagi, T., Suzuki, Y., Noda, A., Hara, M., and Yoshia, J. (2001). Adrenomedullin Reduces Ischemic Brain Injury after Transient Middle Cerebral Artery Occlusion in Rats. Acta Neurochir. (Wien). 143, 1157–1161.

Watanabe, K., Al-Bassam, S., Miyazaki, Y., Wandless, T., Webster, P., and Arnold, D. (2012). Networks of Polarized Actin Filaments in the Axon Initial Segment Provide a Mechanism for Sorting Axonal and Dendritic Proteins. Cell Rep. 2, 1546–1553.

Wong, L.Y.F., Cheung, B.M.Y., Li, Y.-Y., and Tang, F. (2005). Adrenomedullin Is Both Proinflammatory and Antiinflammatory: Its Effects on Gene Expression and Secretion of Cytokines and Macrophage Migration Inhibitory Factor in NR8383 Macrophage Cell Line. Endocrinology 146, 1321–1327.

Wszolek, Z.K., Tsuboi, Y., Ghetti, B., Pickering-Brown, S., Baba, Y., and Cheshire, W.P. (2006). Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). Orphanet J. Rare Dis. 1, 30.

Xia, C.-F., Yin, H., Borlongan, C. V., Chao, J., and Chao, L. (2004). Adrenomedullin gene delivery protects against cerebral ischemic injury by promoting astrocyte migration and survival. Hum. Gene Ther. 15, 1243–1254.

Xia, C.-F., Smith, R.S., Shen, B., Yang, Z.-R., Borlongan, C. V., Chao, L., and Chao, J. (2006). Postischemic Brain Injury Is Exacerbated in Mice Lacking the Kinin B2 Receptor. Hypertension 47, 752–761.

Xu, Y., and Krukoff, T.L. (2004). Adrenomedullin in the rostral ventrolateral medulla increases arterial pressure and heart rate: roles of glutamate and nitric oxide. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287, R729-34.

Yang, F., Jiang, Q., Zhao, J., Ren, Y., Sutton, M.D., and Feng, J. (2005). Parkin Stabilizes Microtubules through Strong Binding Mediated by Three Independent Domains. J. Biol. Chem. 280, 17154–17162.

Yurugi-Kobayashi, T., Itoh, H., Schroeder, T., Nakano, A., Narazaki, G., Kita, F., Yanagi, K., Hiraoka-Kanie, M., Inoue, E., Ara, T., et al. (2006). Adrenomedullin/cyclic AMP pathway induces Notch activation and differentiation of arterial endothelial cells from vascular progenitors. Arterioscler. Thromb. Vasc. Biol. 26, 1977–1984.

Zhang, H., Tang, B., Yin, C.-G., Chen, Y., Meng, Q.-L., Jiang, L., Wang, W.-P., and Niu, G.-Z. (2014). Plasma adrenomedullin levels are associated with long-term outcomes of acute ischemic stroke. Peptides 52, 44–48.

Zhu, Q., Couillard-Després, S., and Julien, J.-P. (1997). Delayed Maturation of Regenerating Myelinated Axons in Mice Lacking Neurofilaments. Exp. Neurol. 148, 299–316.

Chapter II
Hypothesis and Objectives

HYPOTHESIS

The central role of the cytoskeleton is to maintain the highly asymmetrical shape and structural polarity of neurons that are essential for neuronal physiology. Microtubules are one of the major cytoskeletal components of neurons that play important roles in many aspects of neuronal biology that include establishing and conserving neuronal polarity, transporting cargo, maintaining neuronal morphology and modulating signaling events. Perturbations in the architecture of this main element of cytoskeleton can result in neurodegeneration.

The hypothesis of this work states that elevated levels of adrenomedullin gene products, AM and PAMP might compromise the proper organization of neuronal cytoarchitecture by directly interacting and destabilizing microtubules. Microtubule disassembly may thereby lead to impaired axonal transport and defects in neurotransmission and synaptic plasticity, which could be the cause, or at least contribute to neurodegeneration.

OBJECTIVES

The major goal of the present work is to study the role of adrenomedullin in the in the pathophysiology of neurodegenerative dementias.

To accomplish this goal, specific objectives are formulated as follows:

1. To study the involment of adrenomedullin in the neuropathology of Alzheimer's disease. The main findings of this study are shown in *Chapter III: Increased levels of adrenomedullin in the neuropathology of Alzheimer's disease.*

   1.1. To measure adrenomedullin gene products in Alzheimer's disease brains.

   1.2. To determine the realtionship of adrenomedullin with the microtubules, key elements involved in the organization and structure of neuronal cytoskeleton in Alzheimer's disease brains.

   1.3. To evaluate the implication of adrenomedullin on the neurodegenerative process including synaptic and neurotransmission function.

2. To characterize the effects of ageing in adrenomedullin expression. The main findings of this study are shown in *Chapter IV: Adrenomedullin contributes to age-related memory loss in mice and is elevated in aging human brains*.

2.1. To perform a cognitive and biochemical characterization of a conditional knock-out mouse model for adrenomedullin gene in the central nervous system.

2.2. To determine the pathological crosstalk between Tau and adrenomedullin on cognitive function in the aged knock-out mouse model.

2.3. To measure the effects of ageing on adrenomedullin expression in human post-mortem samples.

3. To check the role of adrenomedullin in frontotemporal lobar degeneration. The main findings of this study are shown in *Chapter V: Reduced adrenomedullin parallels microtubule dismantlement in frontotemporal lobar degeneration.*

3.1. To characterize the status of adrenomedullin in brains of frontotemporal lobar dementia, a neurodegenerative dementia with primary tauopathy.

3.2. To study the association of adrenomedullin with other markers of neuronal cytoskeleton dismantlement in frontotemporal lobar degeneration.

Chapter III
Increased levels of Brain Adrenomedullin in the Neuropathology of Alzheimer's disease

SUMMARY

Alzheimer´s disease (AD) is characterized by the loss of synaptic contacts caused in part by cytoskeleton disruption. In neurons, derived peptides of adrenomedullin (*ADM)* are associated with some structural and functional cytoskeletal proteins, causing microtubule destabilization. Here we describe the relationships between *ADM* and other signs of AD in clinical specimens. Frontal cortex from AD patients and controls were studied for *ADM*, acetylated tubulin, NCAM, Ox-42 and neurotransmitters. *ADM* was increased in AD compared with controls, while levels of acetylated tubulin, NCAM and neurotransmitters were decreased. Interestingly, increases in *ADM* statistically correlated with the decrease in these markers. Furthermore, Ox42 overexpression in AD correlated with levels of *ADM*. It is proposed that AD patients may have neural cytoskeleton failure associated with increased of *ADM* levels, resulting in axon transport collapse and synaptic loss. These observations suggest that reducing *ADM* expression may constitute a new avenue to prevent/treat AD.

1. INTRODUCTION

Alzheimer´s disease (AD) is an irreversible degenerative pathology of the brain characterized by progressive deterioration of cognitive functions, affecting mainly neurons in the hippocampus and the cerebral cortex. Histological features of AD are senile plaques, made up of accumulations of β-amyloid (Aβ) peptide, and neurofibrillary tangles (NFT), which are fibrillar deposits of hyperphosphorylated Tau protein (pTau) (Hernandez et al. 2013).

There is overwhelming evidence that Aβ accumulation is central to the pathogenesis of AD. One of the consequences of Aβ accumulation is the hyperphosphorylation of a microtubule-associated protein (MAP) known as Tau. Pathological hyperphosphorylation of Tau leads to the destabilization of axonal cytoskeleton and loss of neural connections (synapses), chief markers of clinical manifestation which stem from the inability of Tau to regulate neural microtubule dynamics (Querfurth and LaFerla 2010).

In the search for blood biomarkers of AD, some studies have found that mid-regional proadrenomedullin is elevated in AD patients and that the concentration of this protein could have prognostic value in the progression from predementia to clinical AD (Buerger et al. 2011b; Henriksen et al. 2014). The proadrenomedullin gene, *ADM*, codes for a 185 amino acid preprohormone which, after post-translational modifications, generates 2 biologically active peptides: prodrenomedullin N-terminal 20 peptide (PAMP) and adrenomedullin (AM). Both peptides are amidated at their carboxy terminus and their 3-dimensional structure is based on a central α-helix (Pérez-Castells et al. 2012). These peptides are ubiquitously expressed and perform several functions, including vasodilatation, bronchodilatation, angiogenesis, hormone secretion regulation, growth modulation, and antimicrobial activities, among others (López and Martínez 2002). In the central nervous system (CNS), adrenomedullin is expressed throughout the whole brain and spinal cord (Serrano et al. 2000) and acts as neuromodulator partially through NMDA-dependent mechanisms (Xu and Krukoff 2004b).

An intriguing finding showed that both AM and PAMP decorate the microtubules in a variety of cell types, including neurons (Sackett et al. 2008). Yeast-2-hybrid analysis demonstrated that AM binds to several MAPs, whereas PAMP binds directly to tubulin and kinesin. Cell physiology studies point to a direct involvement of PAMP in regulating

microtubule dynamics and kinesin speed (Larráyoz and Martínez 2012). Downregulation of *ADM* expression, through either gene knock-down or targeted knock-out, results in a massive hyperpolymerization of the tubulin cytoskeleton, an increase on Glu- and acetylated-tubulin, a reduction of kinesin velocity, and the apparition of actin filopodia in CNS stem/progenitor cells (Vergaño-Vera et al. 2010). In addition, *ADM* immunoreactivity increases in the brain of mouse models of AD and seems to be associated with activated astrocytes in the vicinity of amyloid plaques (Fernandez et al. 2016).

Taking all this into consideration the aim of the present work was to investigate the potential association of *ADM* derived peptides with the pathological mechanisms of AD in patient samples.

2. MATERIAL AND METHODS

2.1 HUMAN BRAIN TISSUE

Brain tissues were obtained from the Oxford Project to Investigate Memory and Ageing (OPTIMA, see www.medsci.ox.ac.uk/optima). Subjects for this study constituted a randomly selected subset of the participants, now part of the Thomas Willis Oxford Brain Collection within the Brains for Dementia Research Initiative (BDR). Informed consent was obtained from the patients' next-of-kin before collection of brains and the study was approved by the UK National Research Ethics Service. All subjects (n=19, 7 were used for immunohistochemical studies, and 12 were used for western blotting studies) fulfilled Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria for the neuropathological diagnosis of

AD and were staged at Braak V/VI. Memory status in the AD group in the last pre-mortem examination indicated a mini-mental state examination (MMSE) score = 5.8 ± 0.8. Age-matched and sex-matched controls (n=19, 7 were used for immunohistochemical studies, and 12 were used for western blotting studies) did not have dementia or other neurological diseases, did not meet CERAD criteria for AD diagnosis, and were staged at Braak 0-II. Frontal [Brodmann area (BA)10 or BA9] cortex were dissected free of meninges. To partially mitigate the possible effects of cause of death on neurochemical determinations, brain pH was measured in homogenates of frontal cortex in deionised water as an index of acidosis associated with terminal coma. Brain pH is used as tissue quality indication in post-mortem research, with pH > 6.1 considered acceptable (Kirvell et al. 2006). All subsequent analyses

were performed blind to clinical information. All biochemical studies (western blot, NCAM and neurochemical measurements) were performed in the same tissues to allow correlation studies.

2.2 IMMUNOHISTOCHEMISTRY

Formalin fixed, wax-embedded blocks (n=7 in each group), cut into 7 μm sections and mounted onto slides, were used for immunohistochemistry. Briefly, the sections were dewaxed and rehydrated using Histoclear and alcohol dilutions. Antigen retrieval was carried out by microwaving the sections for ten minutes in citrate buffer pH 6.0. Following blocking of endogenous peroxidases (0.3% H_2O_2 in PBS for 30 minutes), sections were incubated overnight with a polyclonal antibody for ADM (1:200, produced in house), previously characterized (Serrano et al. 2000; Fernandez et al. 2016). Development of the sections was performed using biotinylated antirabbit secondary antibody, ABC reagents and a DAB kit (all Vector Laboratories, Peterborough, UK). Sections were dehydrated and mounted with DPX and coverslipped. For control experiments, the secondary biotinylated antibody was omitted. All tissues were processed, reacted and developed at the same time. Images were captured on a Leica DMRB microscope equipped with DC420 digital camera.

2.3 WESTERN BLOTTING

Samples (12 controls and 12 AD patients) were homogenized in RIPA buffer (Thermo Scientific) containing protease (EDTA-free complete, Roche, Basilea, Switzerland) and phosphatase (PhosStop, Roche) inhibitors. Homogenates were centrifuged for 30 minutes at 15,000 x g and the supernatants collected. Protein concentration was determined by the BCA kit (Pierce, Rockford, IL), with bovine serum albumin as standard, using a spectrophotometer (POLARstar Omega, BMG Labtech, Ortenberg, Germany). Then, 25 μg of protein from each sample were mixed with 4x sample buffer (Invitrogen) and heated for 10 minutes at 70°C. Samples were run on 4-12% SDS–polyacrylamide gels. Seeblue plus 2 Prestained Standards (Invitrogen) were used as molecular weight markers. Proteins were transferred onto 0.2 μm nitrocellulose membranes (Amersham GE HealthCare). Membranes were incubated overnight at 4°C with primary antibodies followed by peroxidase- labeled secondary antibodies.

The primary antibodies used were: ADM antibody (1:200, produced in house), acetylated tubulin antibody (mouse monoclonal, 1:15000, Sigma), and Ox-42 antibody

(rabbit polyclonal, 1:500, Thermo Scientific). Immunoreactive bands were visualized using fluorescence and quantified by an image analyzer (Image Studio Lite, LI-COR Biosciences, Lincoln NE, USA). β-Actin (mouse monoclonal, 1:10000, Sigma-Aldrich) was used as an internal loading control. Results were calculated as the percentage of optical density values of normal controls.

2.4 QUANTIFICATION OF TOTAL LEVELS OF NCAM

To obtain crude synaptosomal pellets, frontal cortical tissue was homogenized in 10 volumes of ice-cold sucrose (0.32 M) and HEPES (5 mM) buffer that contained a cocktail of protease inhibitors (Complete TM, Boehringer Mannheim, UK) and centrifuged at 1,000g for 5 min. The supernatant was then centrifuged again at 15,000g for 15 min, and the pellet resuspended in Krebs buffer.

NCAM levels were quantified according to a previously described protocol (Aisa et al. 2010). Flat bottom 96 well microplates were allowed to adsorb a coating solution (Na_2CO_3 0.1 M/ $NaHCO_3$, 0.1 M) for 2 h at room temperature. The solution was removed and 50 µl of pellet samples added at a concentration of 10 mg/ml to each well of polystyrene flat-bottom ELISA plates. Plates were incubated overnight at 4ºC and then washed three times with 1 M phosphate buffered saline (PBS) containing 0.05% Tween 20, pH 7.4. Additional binding sites were blocked with bovine serum albumin (BSA) (3%) for 2 h at room temperature. Wells were incubated with 50 µl aliquots of primary antibody Ab5032 (1:15,000 TBST; Chemicon) for 20–24 h at 4ºC and subsequently, 50 µl aliquots of antirabbit IgG peroxidase conjugate antibody (1:500; Sigma, UK) were added for a 2 h incubation period. TMB (Promega) was used as a chromogenic substrate. The reaction was terminated by the addition of 1 N hydrochloric acid. Absorbancies were measured at 450 nm using an automatic ELISA microplate reader. Results were expressed as absolute absorbance values.

2.5 NEUROCHEMICAL MEASUREMENTS

As previously described (Aisa et al. 2010), concentrations of different neurotransmitters were determined by high performance liquid chromatography (HPLC) with electrochemical detection (Waters Spheribor® S10 0DS2 4,6x150 mm), including precolumn derivatization with o-phthaldehyde and β-mercapthoethanol for GABA and glutamate determinations. The limit of detection was 1 pg/10 µl for 5-HT and DA, 20 pg/10 µl for glutamate, and 50 pg/10 µl for GABA content.

Cholinergic activity was measured in terms of cholinacetyltransferase (ChAT) activity. This assay was performed as described (Garcia-Alloza et al. 2005). Results were expressed as percentage of control values.

2.6 STATISTICAL ANALYSIS

Data were analyzed by SPSS for Windows, release 15.0. Normality was checked by Shapiro-Wilks's test ($p > 0.05$). Student's t-test was used in comparisons between controls and AD samples. Correlation studies between biochemical variables were performed by Pearson's or Spearman's correlation coefficients, according to the normality of variables.

3. RESULTS

There were no significant differences in age, post-mortem delay, or brain pH between the control and AD groups. As shown in **Figure 1**, the *ADM* antibody stained several structures, including neurons, in cortical BA9 area in both AD and control human tissue. *ADM* immunoreactivity was particularly prominent in pyramidal cells of cortical layers IV–V, where either nuclei and/or cytosolic compartments were immunostained.

A detailed analysis revealed stronger *ADM* labelling in apical dendrites and axons from AD pyramidal neurons (**Figure 1B**) when compared to normal controls (**Figure 1A**).

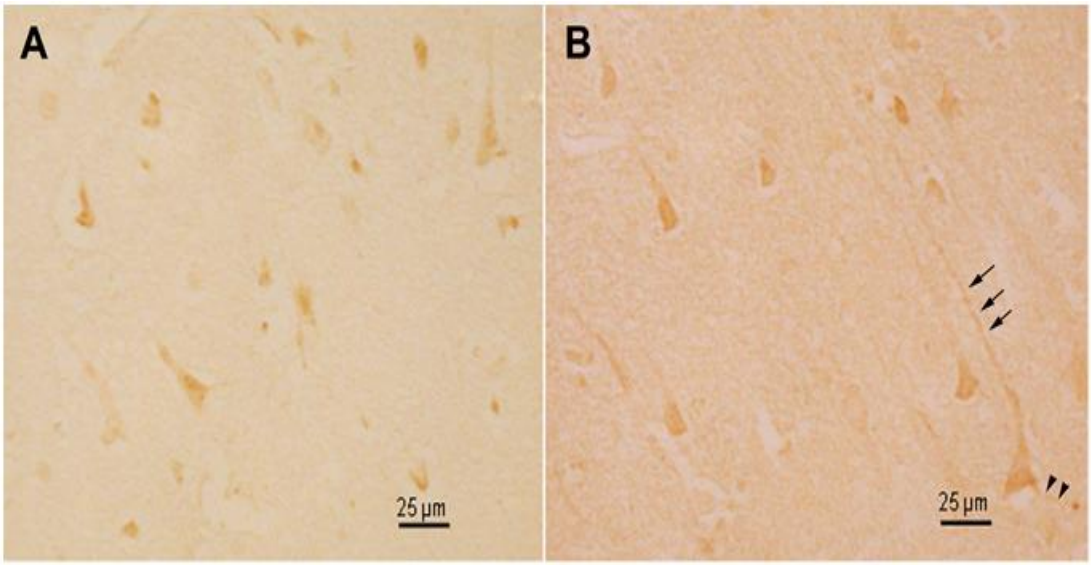

Figure 1. Immunostaining for *ADM* in the frontal cortex of a control subject (A) and an Alzheimer´s disease patient (B). *ADM* staining is more intense in the apical dendrites (arrows) and axons (arrowheads) of pyramidal neurons in AD. Scale bar = 25 µm.

Western blot experiments revealed two different bands of about 14 and 55 kDa using the *ADM* antibody (**Figure 2**). 14 kDa band corresponds to the proadrenomedullin (*ADM*) protein which includes PAMP and AM moieties. Second band at around 55 kDa purportedly shows the association between *ADM* gene products and tubulin as previously described (Sackett et al. 2008). Significant increases of both *ADM* bands were found in AD compared with controls (**Figure 2**).

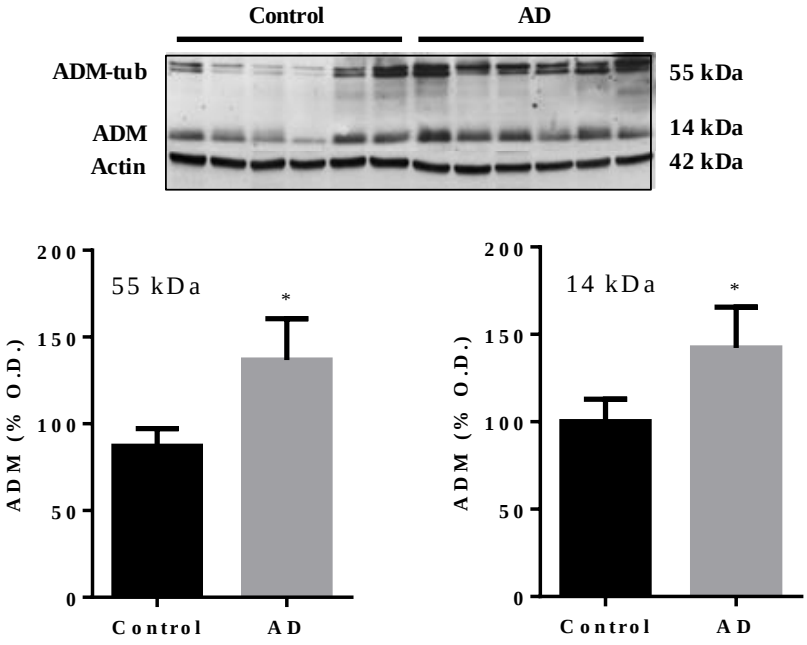

Figure 2. Changes of *ADM* expression in Alzheimer's disease (AD) and control patients. Both *ADM* (14 kDa) and *ADM* associated to tubulin (AM-tub, 55 kDa) show significantly increased levels in AD. Panels show percentage of optical density (O.D.) values of control and representative pictures of the blotting. β-Actin is used as internal loading control. *p<0.05, Student´s t-test.

Given the demonstrated relationship among AM, PAMP, and tubulin polymerization (Sackett et al. 2008), we studied the expression of acetylated tubulin and found that it was significantly decreased in AD samples (**Figure 3A**). These data suggest that the higher levels of AM and/or PAMP could be associated with cytoskeleton destabilization in AD. Supporting this idea, there was a strong trend when correlating products of *ADM* gene (55 kDa) and acetylated tubulin expression ($r = -0.540$; $p=0.07$, Pearson´s correlation coefficient).

The expression of another synaptic plasticity protein, the neural cell adhesion molecule (NCAM) was significantly lower in AD frontal cortex compared to controls (Student´s t-test; $p<0.05$, **Figure 3B**). Decreases in NCAM expression were significantly correlated to increases in *ADM* (14 kDa) levels ($r = -0.641$; $p<0.05$, Pearson´s correlation coefficient).

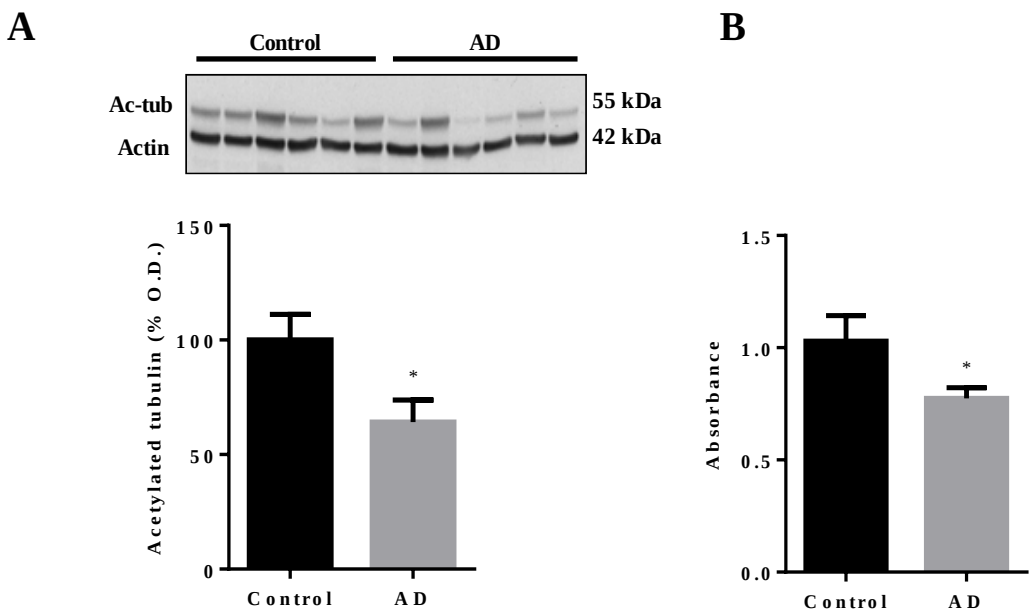

Figure 3. Decreased levels of acetylated tubulin (Ac-tub, panel A) and NCAM (panel B) were found in the frontal cortex (BA10) of Alzheimer´s disease (AD) patients. Panel A show percentage of optical density (O.D.) values of control and representative pictures of the blotting. β-Actin is used as internal loading control. NCAM levels are expressed as absolute absorbance value. *p<0.05, Student´s t-test.

The expression of Ox-42, a microglia activation marker, was significantly enhanced in AD over controls (Student´s t-test; p<0.05, **Figure 4**). *ADM* (14 kDa) and Ox-42 expression were significantly and positively intercorrelated in AD (r = 0.702; p<0.05, Pearson´s correlation coefficient).

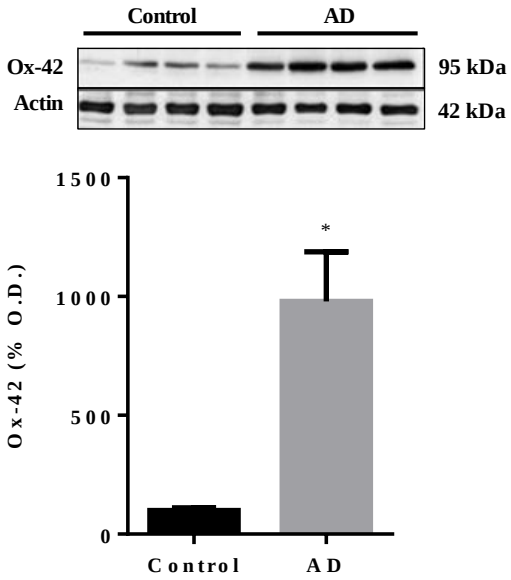

Figure 4. Increased expression of Ox-42, as marker of activated microglia, in the frontal (BA10) cortex of Alzheimer´s disease (AD) patients. Panel shows percentage of optical density (O.D.) values of control and representative pictures of the blotting. β-Actin is used as internal loading control *p<0.05, Student´s t-test.

Levels of different neurotransmitters were also measured in cortical samples of AD patients and controls. Significant decreases in ChAT activity and 5-HT, DA, and GABA levels were observed in AD patients (Student´s t-test; $p<0.01$, **Table 1**). A significant correlation between the reduced levels of neurotransmitters and increased levels of *ADM* (14 kDa) was found for GABA ($r = -0.709$; $p<0.05$, Spearman´s correlation coefficient) and glutamate ($r = -0.718$; $p<0.05$, Spearman´s correlation coefficient).

Table 1. Neurochemical measurements in the frontal (BA10) cortex of controls and Alzheimer´s disease (AD) patients.

	Control	AD
ChAT	100.32±8.94	**31.68±3.53 ***
5-HT	48.66±5.90	**26.09±3.58 ***
DA	91.44±19.39	**38.56±6.52 ***
GABA	752.39±99.41	**569.33±45.97 ***
Glutamate	6639.55±625.98	5450.75±561.90

Values are mean±S.E.M from control (n=12) and Alzheimer´s disease patients (n=12). Choline acetyltransferase (ChAT) activity is expressed as percentage of activity relative to control. Serotonine (5-HT), dopamine (DA), gaba gamma aminobutyric acid (GABA) and glutamate levels are expressed as pg/mg of tissue. * Significantly lower than Control, Student t-test, $p<0.01$.

4. DISCUSSION

In this study we have shown that human brains from AD patients have a higher expression of *ADM* derived peptides than age-matched normal controls. This increased *ADM* correlated with lower levels of acetylated tubulin, NCAM, and neurotransmitters, and a higher level of activated microglia.

Previous reports have indicated that mid-regional proadrenomedullin levels may constitute an early prognostic marker of AD (Buerger et al. 2011b; Henriksen et al. 2014). This intermediate peptide does not have a known function but is used as a surrogate of adrenomedullin expression since it has a longer half-life than the mature peptide (Morgenthaler et al. 2005). Currently, our study seems to be the first report on the increased expression of *ADM* peptides in the brain of AD patients, although a similar observation was

made in mouse models of AD (Fernandez et al. 2016). The specific localization of *ADM*-immunoreactivity in the soma and apical dendrites of cortical neurons coincides with original observations in rat brains (Serrano et al. 2000). Electron microscopy (Serrano et al. 2000) and immunofluorescence (Sackett et al. 2008) studies have shown that intracellular *ADM* is associated to the neuronal microtubules. The fact that the increase on *ADM* immunoreactivity correlates with a reduction in acetylated tubulin levels suggests a role for the *ADM* derived peptides in neuronal cytoskeleton maintenance. In the case of microtubule dynamics, the contribution of PAMP peptide is likely to exert a more specific contribution to microtubule dynamics than AM, since previous studies have shown the profound impact of PAMP on microtubule fluidity (Sackett et al. 2008).

Tubulin acetylation is a post-translational modification of α-tubulin that sustains microtubule stability (Strzyz 2016). The reduced levels of acetylated tubulin levels in AD patients when compared to non-demented controls suggests the presence of less rigid microtubules in these patients, which might contribute and precipitate synaptic disconnections. We had to consider that our AD patients had a MMSE score of 5.8 ± 0.8, which is considered a telltale for severe cognitive impairment (Mungas 1991). Other α-tubulin modifications have been also reported to increase in neurodegenerative diseases, including AD (Vu et al. 2017).

NCAM is part of a family of cell-surface glycoproteins that plays key roles in normal brain development, including axonal/dendritic growth and branching, and synaptic plasticity (Rønn et al. 2000; Kiss and Muller 2001; Kleene and Schachner 2004; Walmod et al. 2004). NCAMs have been implicated as critical components in the induction of long-term potentiation (LTP) and in memory formation (Rønn et al. 2000; Kiss and Muller 2001). NCAMs have been shown to play critical roles in ontogenetic development and are thus surrogate markers of age-related pathology, particularly in AD (Montag-Sallaz et al. 2002; Aisa et al. 2009).

Synaptic loss is the major neurobiological substrate of cognitive dysfunction in AD. Synaptic failure is an early disease event that can be already detectable in patients with mild cognitive impairment, a prodromal state of AD [reviewed by (Arendt 2009)]. This pathological feature progresses during the course of AD though in most early stages involves mechanisms of compensation (synaptic remodeling or synaptogenesis) before reaching a stage of decompensated function (degeneration) (Mikkonen et al. 1999, 2001).

There are previously published studies that have reported either no changes (Gillian et al. 1994) or increased (Mikkonen et al. 1999; Jin et al. 2004) levels of NCAM in the hippocampus of AD patients. The latter observations have been related to enhanced neurogenesis and may be indicative of adaptive brain mechanisms to compensate for the structural and functional damage caused by the disease. Soluble forms of NCAM are increased in the cerebrospinal fluid of both AD and Parkinson disease patients, but these increases seem to be related to ageing and neurodegeneration and not to dementia as such (Strekalova et al. 2006). In the serum samples, only levels of low molecular weight forms of NCAM correlated to severity of dementia (Todaro et al. 2004). In agreement with our observations, decreased levels of NCAM have been found in the frontal cortex of AD patients (Yew et al. 1999), which might stem from the synaptic loss associated to the course of disease.

The correlation between Ox-42 and *ADM* expression suggests that increased *ADM* products expression is somewhat related to the activation of microglia, the resident immune cells of CNS, therefore indicating that increases in brain *ADM* peptides expression could be contributing to cerebral amyloid-associated inflammation in AD. Inflammatory responses in the brain, which can be measured by changes in markers for microglia activation, such as Ox-42, are a common feature of human neurodegenerative diseases. A crucial role has been described for neuroinflammation in AD pathogenesis and emerging evidence suggests that neuroinflammation could be both a cause and a consequence of AD (Calsolaro and Edison 2016).

Neuron and synapse loss, together with neurotransmitter dysfunction, Aβ deposition, and NFTs, are recognized hallmarks of AD (Francis 2003). Furthermore, clinical and preclinical studies point to neuronal loss and associated neurochemical alterations of several transmitter systems as a main factor underlying both cognitive and neuropsychiatric symptoms (Francis et al. 2010). The neurodegenerative process, as indicated by NFT pathology, proceeds from the hippocampus and entorhinal cortex to involve increasingly the glutamatergic neurons of the cortical association areas in a topographical progression (Braak and Braak 1991b). Other studies have reported dysfunction in the major cortical inhibitory GABAergic system in AD which may in part be dependent on disease severity and behavioral symptoms of dementia, such as depression (Garcia-Alloza et al. 2006).

Clinicopathological correlation studies have been crucial to generate hypotheses about the pathophysiology of the disease and potential targets of intervention. From our present observations in AD postmortem tissue it might be inferred that therapeutical approaches aimed at reducing AM/PAMP levels may constitute a novel path to prevent/delay AD neurodegeneration. Few years ago, a particular single nucleotide polymorphism (SNP) in the proximity of the *ADM* gene was found to be associated with reduced circulating levels of AM peptide (Cheung et al. 2011) and lower risk of developing cancer (Martínez-Herrero and Martínez 2013). Therefore, it would be interesting to perform follow-up studies on these carriers in order to evaluate whether this particular haplotype prevents from susceptibility of predeveloping AD. In addition, several physiological inhibitors of AM peptide have been proposed for clinical development, including a monoclonal antibody (Martínez et al. 1996), the peptide fragment AM22-52 (Ishikawa et al. 2003), and a number of small molecules that target either AM or PAMP (Martínez et al. 2004a; Roldós et al. 2012). Therefore some of these inhibitors might be developed for the pharmacological prevention of AD in individuals at high risk (Hickman et al. 2016) or in patients suffering moderate AD in high risk of rapid cognitive decline (Barbe et al. 2016).

Overall, increased *ADM* peptides levels are found in brains of AD patients correlating negatively with markers of cytoskeleton stability and cell-to-cell interactions. Therefore, *ADM* gene products might represent novel pathological features contributing to perturbations of neuronal maintenance and synaptic function in AD, and their pharmacological inhibition may constitute a novel approach to the treatment of AD.

5. REFERENCES

Aisa, B., Elizalde, N., Tordera, R., Lasheras, B., Del Río, J., and Ramírez, M.J. (2009). Effects of neonatal stress on markers of synaptic plasticity in the hippocampus: implications for spatial memory. Hippocampus 19, 1222–1231.

Aisa, B., Gil-Bea, F.J., Solas, M., García-Alloza, M., Chen, C.P., Lai, M.K., Francis, P.T., and Ramírez, M.J. (2010). Altered NCAM expression associated with the cholinergic system in Alzheimer's disease. J. Alzheimers. Dis. 20, 659–668.

Arendt, T. (2009). Synaptic degeneration in Alzheimer's disease. Acta Neuropathol. 118, 167–179.

Barbe, C., Morrone, I., Novella, J.L., Dramé, M., Wolak-Thierry, A., Aquino, J.-P., Ankri, J., Jolly, D., and Mahmoudi, R. (2016). Predictive Factors of Rapid Cognitive Decline in Patients with Alzheimer Disease. Dement. Geriatr. Cogn. Dis. Extra 6, 549–558.

Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 82, 239–259.

Buerger, K., Uspenskaya, O., Hartmann, O., Hansson, O., Minthon, L., Blennow, K., Moeller, H.-J., Teipel, S.J., Ernst, A., Bergmann, A., et al. (2011). Prediction of Alzheimer's disease using midregional proadrenomedullin and midregional proatrial natriuretic peptide: a retrospective analysis of 134 patients with mild cognitive impairment. J. Clin. Psychiatry 72, 556–563.

Calsolaro, V., and Edison, P. (2016). Neuroinflammation in Alzheimer's disease: Current evidence and future directions. Alzheimer's Dement. 12, 719–732.

Cheung, B.M.Y., Ong, K.L., Tso, A.W.K., Leung, R.Y.H., Cherny, S.S., Sham, P.C., Lam, T.H., Lam, K.S.L., and Investigators of Hong Kong Cardiovascular Risk Factor Prevalence Study (2011). Plasma adrenomedullin level is related to a single nucleotide polymorphism in the adrenomedullin gene. Eur. J. Endocrinol. 165, 571–577.

Fernandez, A.P., Masa, J.S., Guedan, M.A., Futch, H.S., and Martínez-Murillo, R. (2016). Adrenomedullin Expression in Alzheimer's Brain. Curr. Alzheimer Res. 13, 428–438.

Francis, P.T. (2003). Glutamatergic systems in Alzheimer's disease. Int. J. Geriatr. Psychiatry 18, S15–S21.

Francis, P.T., Ramírez, M.J., and Lai, M.K. (2010). Neurochemical basis for symptomatic treatment of Alzheimer's disease. Neuropharmacology 59, 221–229.

Garcia-Alloza, M., Gil-Bea, F.J., Diez-Ariza, M., Chen, C.P.L.-H., Francis, P.T., Lasheras, B., and Ramirez, M.J. (2005). Cholinergic-serotonergic imbalance contributes to cognitive and behavioral symptoms in Alzheimer's disease. Neuropsychologia 43, 442–449.

Garcia-Alloza, M., Tsang, S.W., Gil-Bea, F.J., Francis, P.T., Lai, M.K., Marcos, B., Chen, C.P., and Ramirez, M.J. (2006). Involvement of the GABAergic system in depressive symptoms of Alzheimer's disease. Neurobiol. Aging 27, 1110–1117.

Gillian, A.M., Brion, J.P., and Breen, K.C. (1994). Expression of the neural cell adhesion molecule (NCAM) in Alzheimer's disease. Neurodegeneration 3, 283–291.

Henriksen, K., O'Bryant, S.E., Hampel, H., Trojanowski, J.Q., Montine, T.J., Jeromin, A., Blennow, K., Lönneborg, A., Wyss-Coray, T., Soares, H., et al. (2014). The future of blood-based biomarkers for Alzheimer's disease. Alzheimer's Dement. 10, 115–131.

Hernandez, F., Lucas, J.J., and Avila, J. (2013). GSK3 and tau: two convergence points in Alzheimer's disease. J. Alzheimers. Dis. 33 Suppl 1, S141-4.

Hickman, R.A., Faustin, A., and Wisniewski, T. (2016). Alzheimer Disease and Its Growing Epidemic: Risk Factors, Biomarkers, and the Urgent Need for Therapeutics. Neurol. Clin. 34, 941–953.

Ishikawa, T., Chen, J., Wang, J., Okada, F., Sugiyama, T., Kobayashi, T., Shindo, M., Higashino, F., Katoh, H., Asaka, M., et al. (2003). Adrenomedullin antagonist suppresses in vivo growth of human pancreatic cancer cells in SCID mice by suppressing angiogenesis. Oncogene 22, 1238–1242.

Jin, K., Peel, A.L., Mao, X.O., Xie, L., Cottrell, B.A., Henshall, D.C., and Greenberg, D.A. (2004). Increased hippocampal neurogenesis in Alzheimer's disease. Proc. Natl. Acad. Sci. 101, 343–347.

Kirvell, S.L., Esiri, M., and Francis, P.T. (2006). Down-regulation of vesicular glutamate transporters precedes cell loss and pathology in Alzheimer's disease. J. Neurochem. 98, 939–950.

Kiss, J.Z., and Muller, D. (2001). Contribution of the neural cell adhesion molecule to neuronal and synaptic plasticity. Rev. Neurosci. 12, 297–310.

Kleene, R., and Schachner, M. (2004). Glycans and neural cell interactions. Nat. Rev. Neurosci. 5, 195–208.

Larráyoz, I.M., and Martínez, A. (2012). Proadrenomedullin N-terminal 20 peptide increases kinesin's velocity both in vitro and in vivo. Endocrinology 153, 1734–1742.

López, J., and Martínez, A. (2002). Cell and molecular biology of the multifunctional peptide, adrenomedullin. Int. Rev. Cytol. 221, 1–92.

Martínez, A., Weaver, C., López, J., Bhathena, S.J., Elsasser, T.H., Miller, M.J., Moody, T.W., Unsworth, E.J., and Cuttitta, F. (1996). Regulation of insulin secretion and blood glucose metabolism by adrenomedullin. Endocrinology 137, 2626–2632.

Martínez, A., Julián, M., Bregonzio, C., Notari, L., Moody, T.W., and Cuttitta, F. (2004). Identification of vasoactive nonpeptidic positive and negative modulators of adrenomedullin using a neutralizing antibody-based screening strategy. Endocrinology 145, 3858–3865.

Martínez-Herrero, S., and Martínez, A. (2013). Cancer protection elicited by a single nucleotide polymorphism close to the adrenomedullin gene. J. Clin. Endocrinol. Metab. 98, E807-10.

Mikkonen, M., Soininen, H., Tapiola, T., Alafuzoff, I., and Miettinen, R. (1999). Hippocampal plasticity in Alzheimer's disease: changes in highly polysialylated NCAM immunoreactivity in the hippocampal formation. Eur. J. Neurosci. 11, 1754–1764.

Mikkonen, M., Soininen, H., Alafuzof, I., and Miettinen, R. (2001). Hippocampal plasticity

in Alzheimer's disease. Rev. Neurosci. 12, 311–325.

Montag-Sallaz, M., Schachner, M., and Montag, D. (2002). Misguided axonal projections, neural cell adhesion molecule 180 mRNA upregulation, and altered behavior in mice deficient for the close homolog of L1. Mol. Cell. Biol. 22, 7967–7981.

Morgenthaler, N.G., Struck, J., Alonso, C., and Bergmann, A. (2005). Measurement of midregional proadrenomedullin in plasma with an immunoluminometric assay. Clin. Chem. 51, 1823–1829.

Mungas, D. (1991). In-office mental status testing: a practical guide. Geriatrics 46, 54–58, 63, 66.

Pérez-Castells, J., Martín-Santamaría, S., Nieto, L., Ramos, A., Martínez, A., Pascual-Teresa, B. de, and Jiménez-Barbero, J. (2012). Structure of micelle-bound adrenomedullin: a first step toward the analysis of its interactions with receptors and small molecules. Biopolymers 97, 45–53.

Querfurth, H.W., and LaFerla, F.M. (2010). Alzheimer's Disease. N. Engl. J. Med. 362, 329–344.

Roldós, V., Carbajo, R.J., Schott, A.-K., Pineda-Lucena, A., Ochoa-Callejero, L., Martínez, A., Ramos, A., and de Pascual-Teresa, B. (2012). Identification of first proadrenomedullin N-terminal 20 peptide (PAMP) modulator by means of virtual screening and NMR interaction experiments. Eur. J. Med. Chem. 55, 262–272.

Rønn, L.C., Berezin, V., and Bock, E. (2000). The neural cell adhesion molecule in synaptic plasticity and ageing. Int. J. Dev. Neurosci. 18.

Sackett, D.L., Ozbun, L., Zudaire, E., Wessner, L., Chirgwin, J.M., Cuttitta, F., Martínez, A., and Mart??nez, A. (2008). Intracellular proadrenomedullin-derived peptides decorate the microtubules and contribute to cytoskeleton function. Endocrinology 149, 2888–2898.

Serrano, J., Uttenthal, L.O., Martínez, A., Fernández, A.P., Martínez de Velasco, J., Alonso, D., Bentura, M.L., Santacana, M., Gallardo, J.R., Martínez-Murillo, R., et al. (2000). Distribution of adrenomedullin-like immunoreactivity in the rat central nervous system by light and electron microscopy. Brain Res. 853, 245–268.

Strekalova, H., Buhmann, C., Kleene, R., Eggers, C., Saffell, J., Hemperly, J., Weiller, C., Müller-Thomsen, T., and Schachner, M. (2006). Elevated levels of neural recognition molecule L1 in the cerebrospinal fluid of patients with Alzheimer disease and other dementia syndromes. Neurobiol. Aging 27, 1–9.

Strzyz, P. (2016). Post-translational modifications: Extension of the tubulin code. Nat. Rev. Mol. Cell Biol. 17, 609–609.

Todaro, L., Puricelli, L., Gioseffi, H., Guadalupe Pallotta, M., Lastiri, J., Balde de Kier Joffé, E., Varela, M., and Sacerdote de Lustig, E. (2004). Neural cell adhesion molecule in human serum. Increased levels in dementia of the Alzheimer type. Neurobiol. Dis. 15, 387–393.

Vergaño-Vera, E., Fernández, A.P., Hurtado-Chong, A., Vicario-Abejón, C., and Martínez, A. (2010). Lack of adrenomedullin affects growth and differentiation of adult neural stem/progenitor cells. Cell Tissue Res. 340, 1–11.

Vu, H.T., Akatsu, H., Hashizume, Y., Setou, M., and Ikegami, K. (2017). Increase in α-tubulin modifications in the neuronal processes of hippocampal neurons in both kainic acid-induced epileptic seizure and Alzheimer's disease. Sci. Rep. 7, 40205.

Walmod, P.S., Kolkova, K., Berezin, V., and Bock, E. (2004). Zippers make signals: NCAM-mediated molecular interactions and signal transduction. Neurochem. Res. 29, 2015–2035.

Xu, Y., and Krukoff, T.L. (2004). Adrenomedullin in the rostral ventrolateral medulla increases arterial pressure and heart rate: roles of glutamate and nitric oxide. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287, R729-34.

Yew, D.T., Li, W.P., Webb, S.E., Lai, H.W., and Zhang, L. (1999). Neurotransmitters, peptides, and neural cell adhesion molecules in the cortices of normal elderly humans and Alzheimer patients: a comparison. Exp. Gerontol. 34, 117–133.

Chapter IV
Adrenomedullin contributes to Age-related Memory loss in Mice and is elevated in Aging human brains

SUMMARY

Memory decline is common in elderly individuals and is the hallmark of Alzheimer´s disease (AD). Memory failure follows the loss of synaptic contacts in the cerebral cortex and hippocampus, caused in part by cytoskeleton disruption.The expression of adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP), has been identified as a potential biomarker for predicting progression from predementia to clinical AD. Here we analyze the connection between AM levels and memory preservation. Mice lacking neuronal AM and PAMP (knock-out) and their wild type littermates were subjected, at different ages, to the novel object recognition test and the contextual fear conditioned test. Aged KO mice have significantly better retention memory than their WT counterparts. This feature was more prominent in females than in males. Prefrontal cortex and hippocampus samples from these animals were subjected to Western blotting for phosphorylated Tau and acetylated tubulin. Aged female KO mice had significantly less accumulation of phosphorylated Tau than their WT littermates. In addition, protein extracts from the frontal cortex of non-demented mature (65.10 ± 3.86 years) and aged (77.14 ± 2.77 years) human donors were analyzed by Western blotting. Aged human brains had significantly higher levels of AM and lower levels of acetylated tubulin than younger donors. These observations suggest that drugs or interventions that reduce AM/PAMP expression may constitute a new avenue to prevent memory decline during normal aging and in patients suffering moderate AD in high risk of rapid cognitive decline.

1. INTRODUCTION

Memory loss is a common characteristic of normal aging (Leal et al. 2016) which gets greatly accelerated in some neurodegenerative diseases. Alzheimer´s disease (AD) is the most frequent cause of memory loss and other dementia symptoms among elderly patients (Hickman et al. 2016). AD is an irreversible degenerative pathology of the brain characterized by progressive deterioration of cognitive functions, affecting mainly neurons in the hippocampus and the cerebral cortex. The histological hallmarks of AD include senile plaques, made up by accumulations of β-amyloid (Aβ) peptide, and neurofibrillary tangles, which are deposits of Tau, a microtubule associated protein (MAP), which gets abnormally phosphorylated (pTau) (Hernandez et al. 2013). Pathological accumulation of pTau, which compromises synaptic transmission and neuronal viability by contributing to cytoskeleton collapse, correlates better with the severity of cognitive decline in AD than senile plaques (Nelson et al. 2012).

The causes of memory loss during normal aging are not completely understood. Atrophy of some brain areas has been shown in normal aging (Pini et al. 2016) and changes in intrinsic neural electrical excitability associated with oxidative stress have been hypothesized as potential causes (Hermann et al. 2014). Subtle perturbations in stabilization of neuronal cytoskeleton, reminiscent of those occurring during AD neurodegeneration, may also be an important underlying cause of age-associated neuronal dysfunction and cognitive decline (Savva et al. 2009). In this line, modifications on pTau expression and status (tauopathies) are also typical of normal aging (Delacourte et al. 2002) and their distribution pattern correlates with memory capabilities (Guillozet et al. 2003).

Some interventions have been proposed to delay aging-related cognitive deficits and thus improve the quality of life in older people. Some of these interventions include mental stimulation and physical activity (Collette and Salmon 2014) but a better knowledge of the mechanisms underlying the loss of memory may help in devising novel pharmaceutical approaches.

In the search for predictive blood biomarkers of AD cognitive decline, some studies have found that mid-regional proadrenomedullin is elevated in the plasma of AD patients and that the concentration of this peptide could have predictive value in the progression from predementia to clinical AD (Buerger et al. 2011; Henriksen et al. 2014), although a recent

study on a Swedish population found no correlation (Holm et al. 2017). The proadrenomedullin gene, *ADM*, codes for a preprohormone which, after post-translational modifications, generates 2 biologically active peptides: proadrenomedullin N-terminal 20 peptide (PAMP) and adrenomedullin (AM). Both peptides are amidated at their carboxy terminus and their 3-dimensional structure is based on a central α-helix (Perez-Castells et al. 2012). Expression of these peptides is widespread and several functions have been ascribed to them, including vasodilatation, bronchodilatation, angiogenesis, hormone secretion regulation, growth modulation, and antimicrobial activities, among others (Lopez and Martinez 2002).

In the central nervous system (CNS), AM is expressed throughout the whole brain and spinal cord (Serrano et al. 2000) where it acts as a neuromodulator through mechanisms dependent and independent of NMDA receptors (Xu and Krukoff 2004). It has been shown that plasma levels of AM increase with normal aging (Kato et al. 2002).

Knock-out studies have shown that total abrogation of *adm* results in embryo lethality (Caron and Smithies 2001). To circumvent this problem, we generated a conditional knock-out model where *adm* was eliminated just from neurons by using Cre/loxP technology, and the physiological consequences of such manipulation have been published (Fernandez et al. 2008;Fernandez et al. 2010; Hurtado et al. 2010).

An intriguing finding showed that both AM and PAMP decorate the microtubules in a variety of cell types, including neurons (Sackett et al. 2008). Yeast-2-hybrid analysis demonstrated that AM binds to several MAPs, whereas PAMP binds directly to tubulin and kinesin. Cell physiology studies point to a direct involvement of PAMP in regulating microtubule dynamics and kinesin speed (Larrayoz and Martinez 2012). Downregulation of *adm* expression, through either gene knock-down or targeted knock-out, results in a massive hyperpolymerization of the tubulin cytoskeleton, an increase on Glu- and acetylated-tubulin, a reduction of kinesin velocity, and the apparition of actin filopodia in CNS stem/progenitor cells (Sackett et al. 2008;Vergano-Vera et al. 2010). In addition, *ADM* related peptides immunoreactivity increases in the brain of AD patients (CHAPTER III) and in mouse models of AD where it seems to be associated with activated astrocytes in the vicinity of amyloid plaques (Fernandez et al. 2016).

Taking all this into consideration we decided to investigate the potential connection between *adm* gene products and normal aging memory loss in a mouse model and the expression of AM in human brains.

2. MATERIAL AND METHODS

2.1 MICE LACKING NEURONAL AM

Conditional knock-out mice where *adm* gene was eliminated (AMKO mice) from neurons have been previously described (Fernandez et al. 2008). KO and wild type (WT) littermates of both sexes were allowed free access to food and water under standard laboratory conditions, with light/dark cycles of 12/12 h, and a constant temperature of 24ºC. All procedures were carried out in accordance with the European Communities Council Directive (86/609/CEE) on animal experiments and with approval from the ethical committees on animal welfare of our institutions (OEBA-CIBIR and University of Navarra protocol 054-12).

2.2 BEHAVIORAL TESTS

2.2.1 NOVEL OBJECT RECOGNITION TEST (NORT)

Test was performed as previously described (Gerenu et al. 2013) in young (3 months old) and old (18 months old) mice. In short, animals (n=8 per group) were first familiarized with the arena (35 x 35 x 45 cm) for 30 min and after one day mice were allowed to explore two identical objects during 5 min (training). Retention was assessed 24 h post-training, when one object was replaced by a novel one. Retention score is expressed as discrimination index (percentage of time exploring the novel object to the total time of object exploration).

2.2.2 CONTEXTUAL FEAR CONDITIONED TEST

After 24 h of rest, all mice were subjected to a fear conditioned test. The behavioral procedure involved three phases: habituation, training, and testing. Mice were habituated for 5 min to the context, which consisted in a soundproof box with white walls, light, and a background noise produced by a fan. The training phase was conducted 24 h later, where mice were placed in the same context and allowed to explore for 2 min prior to a 2 s footshock (0.3 mA) stimulus. After 30 s mice were returned to their home cage. 24 h later mice were placed back in the conditioning box and allowed to explore the context for 2 min, during which freezing time was recorded (contextual long-term memory). Freezing behavior

was defined as an absence of cage displacement. Freezing scores were expressed as percentages of total freezing time. The conditioning procedure was carried out in a StartFear system (Panlab S.L., Barcelona, Spain) that allows movement recording by a high-sensitivity Weight Transducer system and data analysis by the built-in FREEZING and STARTLE software.

2.3 HUMAN BRAIN TISSUE

Brain tissues were obtained from the Oxford Project to Investigate Memory and Ageing (OPTIMA, see www.medsci.ox.ac.uk/optima). Subjects for this study constituted a randomly selected subset of the participants, now part of the Thomas Willis Oxford Brain Collection within the Brains for Dementia Research Initiative (BDR). At death, informed consent had been obtained from the patients' next-of-kin before collection of brains and the study was approved by the UK National Research Ethics Service.

All cases were selected based on clinic-pathological consensus diagnoses. A total of 12 individuals were included in the study. They included 6 mature donors (age at death = 65.10 ± 3.86 years; post mortem delay = 34.10 ± 4.91 h) and 6 older donors (age at death = 77.14 ± 2.77 years; post mortem delay = 49.42 ± 6.82 h). These participants were classified as normal controls, did not have dementia or other neurological diseases, did not meet CERAD criteria for AD diagnosis, and were staged at Braak 0-II. Frontal (Brodmann Area, BA10) cortices were dissected free of meninges.

To partially mitigate the possible effects of cause of death on neurochemical determinations, brain pH was measured as an index of acidosis associated with terminal coma. Brain pH is used as an indication of tissue quality in post-mortem research, with pH>6.1 considered acceptable (Bahn et al. 2001; Lewis 2002). All the tissue used fulfilled this condition.

2.4 WESTERN BLOTTING

Two days after completing behavioral testing, all mice were euthanized and prefrontal cortex and hippocampus were dissected out. Mouse and human samples were homogenized in RIPA buffer (Thermo Scientific, Rockford, IL) containing protease (EDTA-free complete, Roche, Basilea, Switzerland) and phosphatase (PhosStop, Roche) inhibitors. Homogenates were centrifuged for 30 min at 15,000 x g and the supernatants collected.

Protein concentration was determined by the BCA kit (Pierce, Rockford, IL), with bovine serum albumin as standard, using a spectrophotometer (POLARstar Omega, BMG Labtech, Ortenberg, Germany). Then, 25 µg of protein from each sample were mixed with 4x sample buffer (Invitrogen, Carlsbad, CA) and heated for 10 min at 70°C.

Samples were run on 4-12% SDS–polyacrylamide gels. Seeblue plus 2 Prestained Standards (Invitrogen) were used as molecular weight markers. Proteins were transferred onto 0.2 µm nitrocellulose membranes (Amersham GE HealthCare, Pittsburgh, PA).

Membranes were incubated overnight at 4°C with primary antibodies followed by fluorescence secondary antibodies (**Table 1**). Immunoreactive bands were visualized using fluorescence and quantified by an image analyzer (Image Studio Lite, LI-COR Biosciences, Lincoln NE, USA). Membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Scientific). β-Actin was used as an internal loading control. Results were calculated as the percentage of optical density values of WT.

Table 1. Antibodies and conditions used in this study.

Target	Species	Dilution	Reference
Adrenomedullin	Rabbit polyclonal	1:200	Novus NBP1-19731
AT8	Mouse monoclonal	1:1,000	Thermo Scientific MN1020
PHF1	Mouse monoclonal	1:1,000	Gift from Peter Davies
TAU	Mouse monoclonal	1:1,000	Cell Signaling 4019
Acetylated tubulin	Mouse monoclonal	1:15,000	Sigma T7451
β-Actin	Mouse monoclonal	1:10,000	Sigma AC74

2.5 STATISTICAL ANALYSIS

Data were analyzed by SPSS for Windows, release 15.0. Normalcy and homoscedasticity were checked by Shapiro–Wilks's and Levene´s tests, respectively. Normally distributed data were analyzed by Student´s *t*-test or two-way ANOVA (genotype x age) followed by Tukey´s post hoc test. P values lower than 0.05 were considered statistically significant.

3. RESULTS

3.1 MOUSE EXPERIMENTS

The conditional *adm* KO animals and their WT littermates were tested in two different cognitive paradigms: the novel object recognition test (NORT) and the fear conditioning test. Animals included were either young (3 months) or aged (18 months) mice. Each age group was composed by KO (n=8) and WT (n=8) littermates.

First of all, to ensure that WT and KO mice had no differences in general locomotor activity or other psychological/physiological traits that may interfere with memory determination, their investigative activity was recorded on the first and second day of the experiment.

On the first day, in the first 30 min, WT mice travelled 18,871 ± 2,362 cm whereas KO mice walked for 16,486 ± 1,666 cm (*t* test, $p>0.05$). On the second day (training), when mice were presented with two identical objects, all groups of mice had a ratio close to 50% and there were no significant differences among them (*t* test, $p>0.05$; **Figure 1A**, males; **1B,** females). These results show that genotype does not influence mice ability to move freely or to show curiosity when presented with different objects.

When presented with the novel object, young animals had no significant differences in recognition memory between those carrying *adm* and the knock-outs (**Figure 1C**, males; **1D** females). Recognition memory was significantly facilitated in aged AMKO animals, both in males (**Figure 1C**, two way ANOVA, significant interaction $F=12.44$, $p<0.05$, Tukey's multiple comparisons test, $p<0.05$) and females (**Figure 1D**, two way ANOVA, significant interaction $F=11.11$, $p<0.05$, Tukey's multiple comparisons test, $p<0.05$).

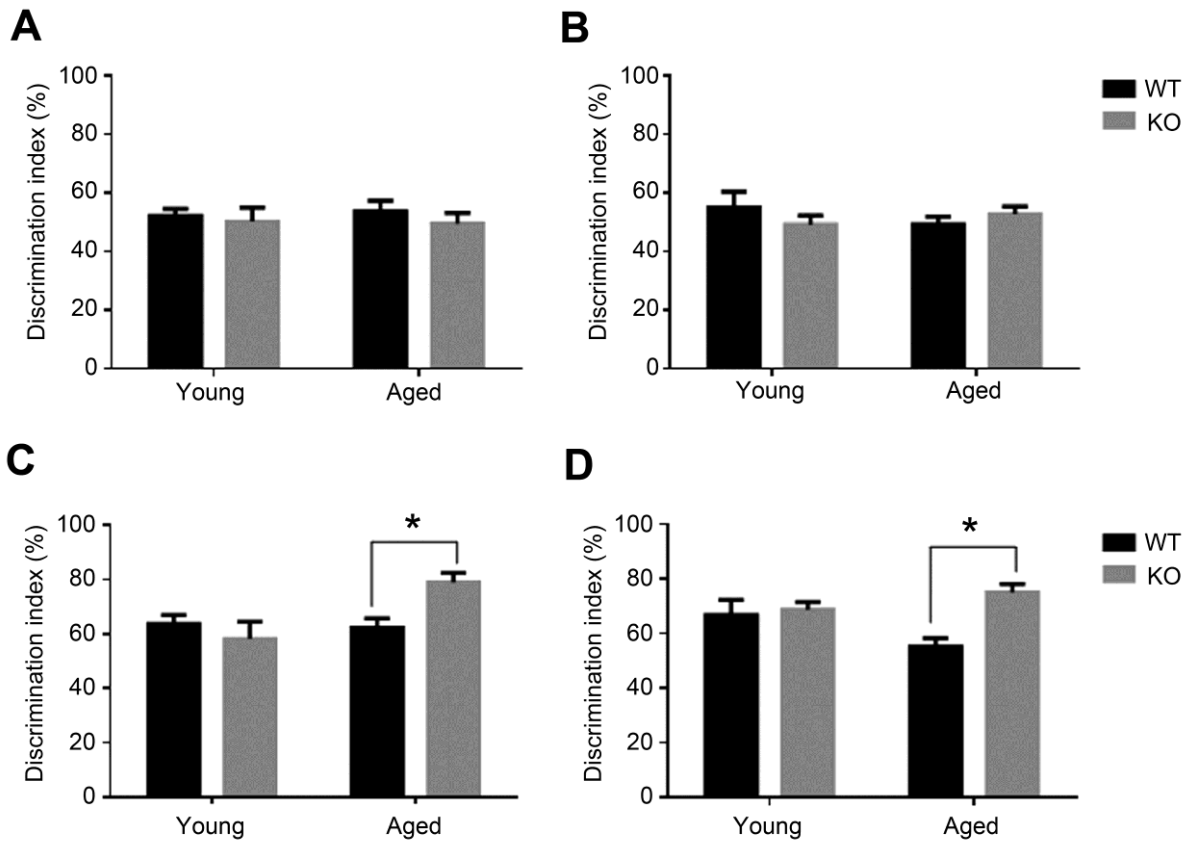

Figure 1. Cognitive phenotype in animals lacking neuronal adm has tested in the novel object recognition test (NORT, A-D). Behavioral NORT data are shown as percentage of discrimination index (time exploring new object/total time of exploration x 100) in male (A, C) and female (B, D), young (3 months), and aged (18 months) mice. Discrimination index was measured with two identical objects on day 2 (A, B) and after introducing the novel object on day 3 (C, D). Two-way ANOVA (genotype x age), *p < 0.05 interaction. WT: wild type; KO: adm knock-out.

In the fear conditioning test (contextual learning), first we measured the freezing levels during training and found they were around 40% for both genotypes (*t* test, p>0.05) during the first 2.5 min of their stay in the chamber. We also tested foot shock sensitivity by measuring the freezing levels immediately following the foot shock. These levels were close to 80% for both genotypes (*t* test, p>0.05) for the 30 s following the shock, indicating that the genotype has no influence in regular freezing behaviour or in foot pain sensitivity.

During the memory test period, male mice showed no statistically significant differences in freezing behaviour irrespective of age or genotype (**Figure 2**).

In contrast, aged female WT mice presented an age-related memory loss as demonstrated by a statistically significant reduction in freezing time when compared with younger females of the same genotype (**Figure 2B**, two way ANOVA, significant interaction $F=9.02$, $p<0.05$, Tukey's multiple comparisons test, $p<0.05$). Interestingly, aged female mice lacking neuronal *adm* showed better memory than their WT littermates (Tukey's multiple comparisons test $p<0.05$).

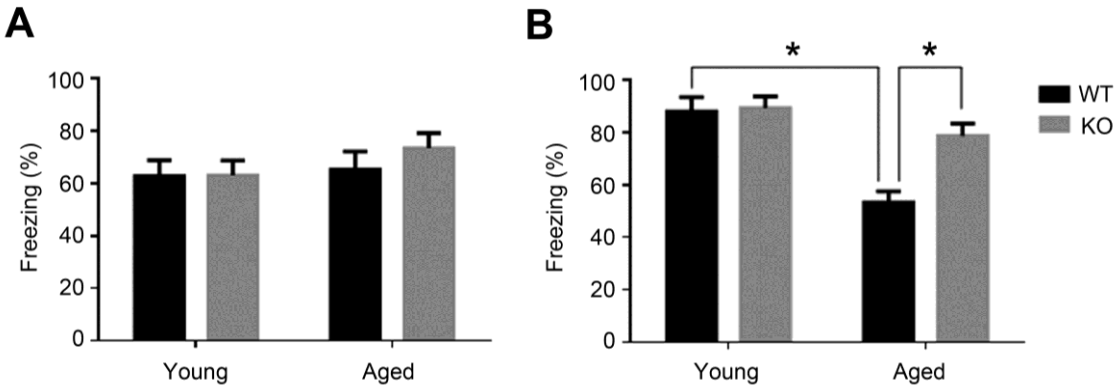

Figure 2. Cognitive phenotype in animals lacking neuronal *adm* has tested in the fear conditioning (A, B). Fear conditioning data are shown as percentage of freezing over the 2 min test in male (A) and female (B) young and aged mice. Two-way ANOVA (genotype x age), *$p < 0.05$ interaction. WT: wild type; KO: *adm* knock-out.

Altogether, both cognitive tests point to a memory protection phenotype in animals lacking neuronal *adm*.

To investigate the possible mechanism underlying these behavioral observations, changes in pTau expression were checked in the mouse prefrontal cortex and hippocampus using two different antibodies: AT8 (**Figure 3**) and PHF1 (**Figure 4**).

In male mice, there was an increased pTau/Tau ratio associated to aging that was not significantly affected by deleting the *adm* gene (two way ANOVA, main effect of age, $F=21.50$, $p<0.01$, **Figure 3A, C**). However, in female mice, a significant interaction between genotype and age was found (**Figure 3B, D**), and the increased expression of pTau associated to aging was counteracted in mice lacking the *adm* gene (two way ANOVA, significant interaction $F=16.81$, $p<0.05$, Tukey's multiple comparisons test, $p<0.05$).

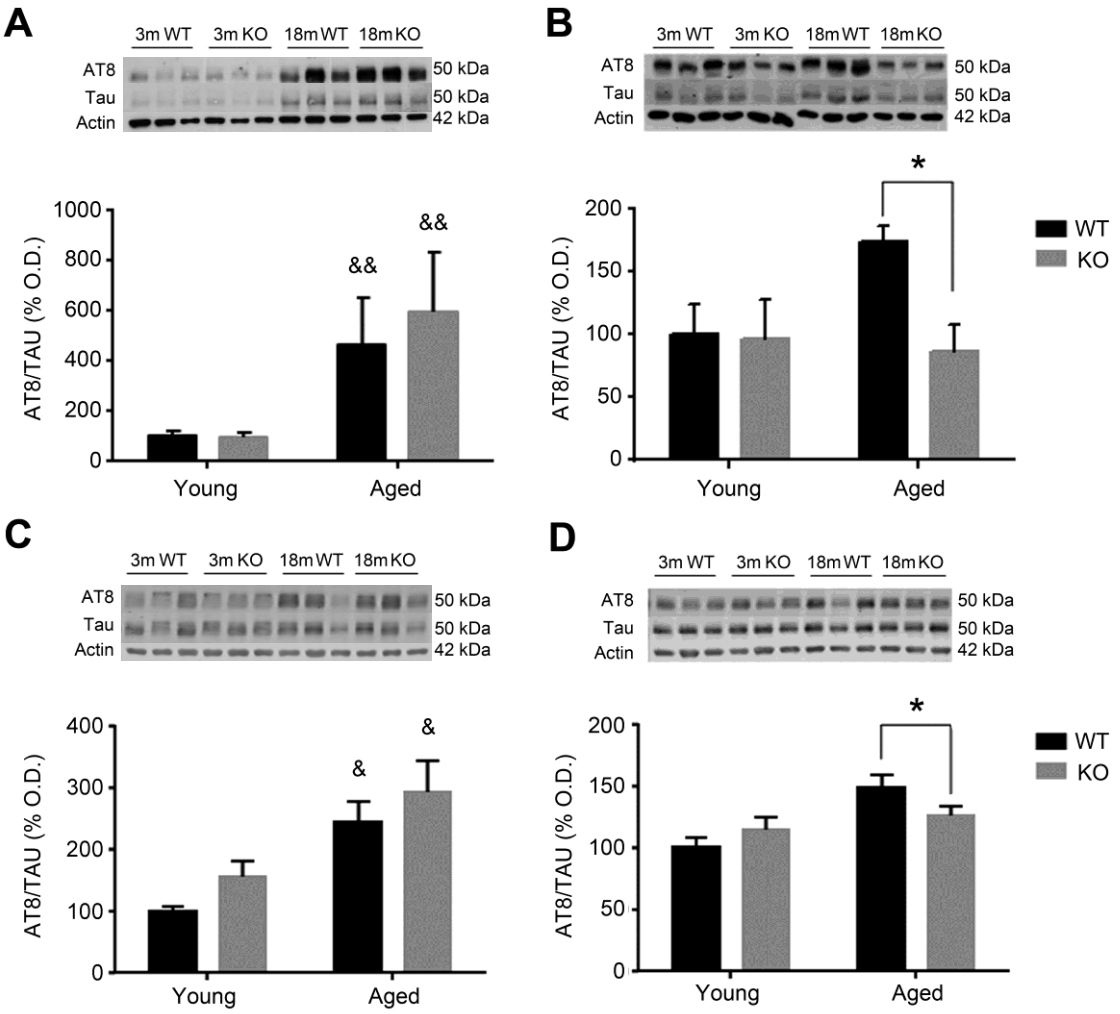

Figure 3. Expression of pTau (using the AT8 antibody) in the frontal cortex (A, B) and the hippocampus (C, D) of male (A, C) and female (B, D), young (3 months, 3 m) and aged (18 months, 18 m), control (wild type, WT) and *adm* knock-out (KO) mice. Two-way ANOVA: *p < 0.05 interaction, && p< 0.001 main effect of age. Panels show percentage of optical density (OD) values of control and representative pictures of the blotting. β-Actin is used as internal loading control.

For the other antibody, PHF1 (**Figure 4**), results were perfectly parallel to those observed with AT8. There was an increased pTau/Tau ratio associated to aging in males that was not significantly affected by deleting the *adm* gene (two way ANOVA, main effect of age, F=28.81, p<0.05, **Figure 4A, C**). In females, a significant interaction between genotype and age was found (**Figure 4B, D**), and the increased expression of pTau associated to aging was counteracted in mice lacking the *adm* gene (two way ANOVA, significant interaction F=18.24, p<0.05 Tukey's multiple comparisons test, p<0.05).

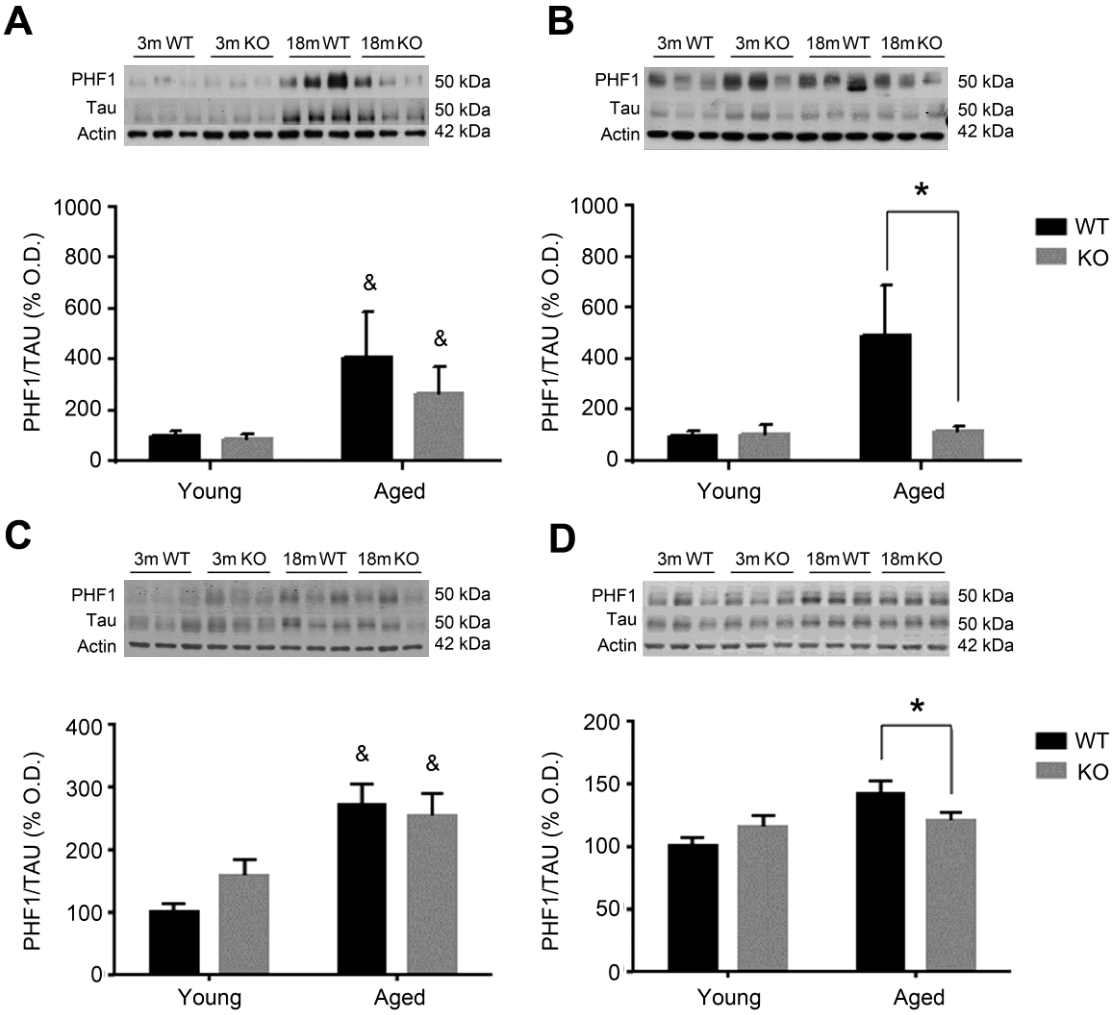

Figure 4. Expression of pTau (using the PHF1 antibody) in the frontal cortex (A, B) and the hippocampus (C, D) of male (A, C) and female (B, D), young (3 months, 3 m) and aged (18 months, 18 m), control (wild type, WT) and *adm* knock-out (KO) mice. Two-way ANOVA: *p < 0.05 interaction, & p< 0.05 main effect of age. Panels show percentage of optical density (OD) values of control and representative pictures of the blotting. β-Actin is used as internal loading control. β-Actin and total Tau are the same as in Figure 3 and are repeated here to allow a direct comparison with the total protein and loading controls.

To further study cytoskeleton stability, tubulin acetylation was checked in the frontal cortex and hippocampus of these animals. Aged male mice showed a decrease in the expression of acetylated tubulin that was independent of the genotype (two way ANOVA, main effect of age, F=39.23, p<0.01, **Figure 5A, C**). No effect associated to either age or genotype was found in female mice (**Figure 5B, D**).

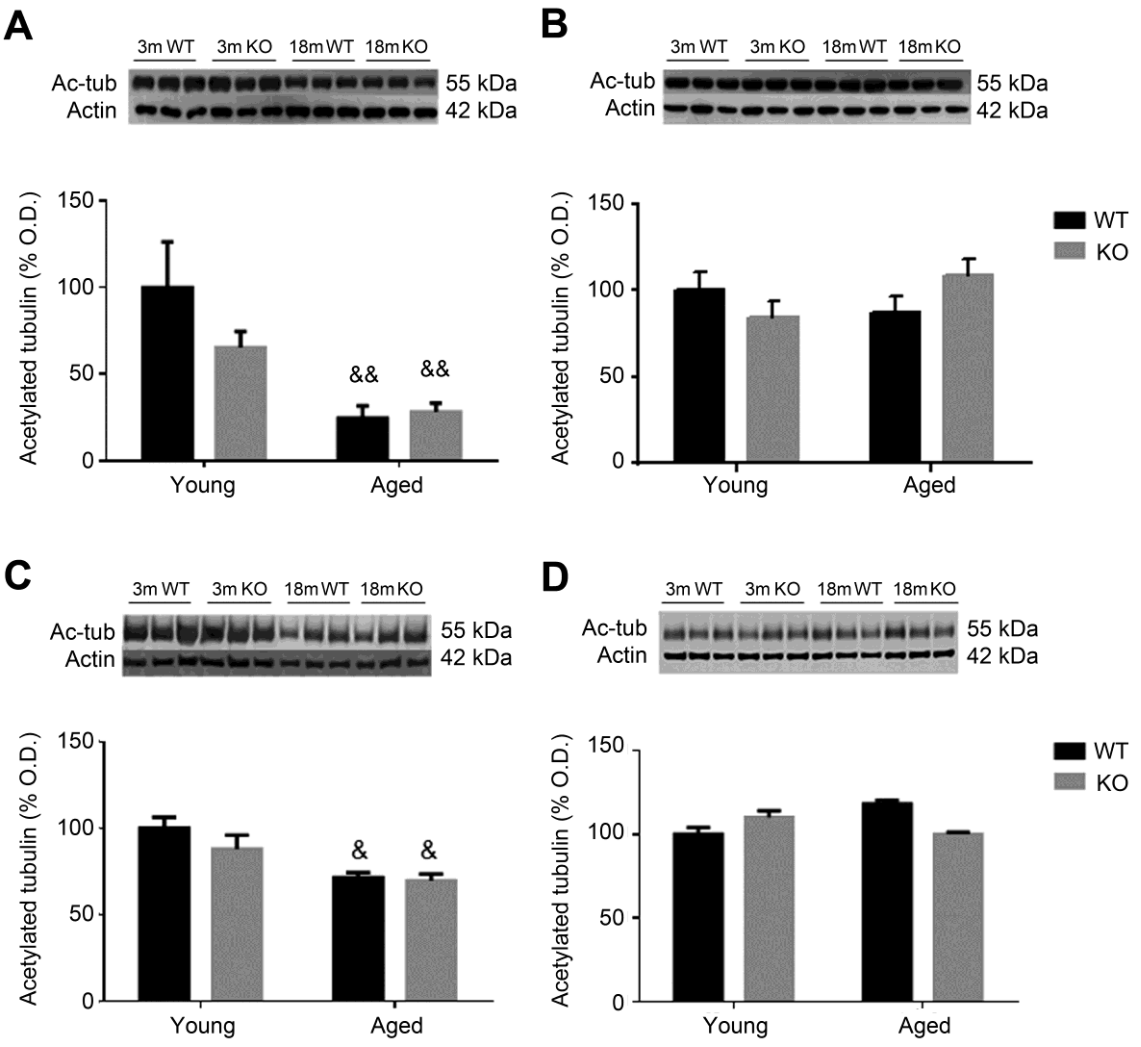

Figure 5. Expression of acetylated tubulin (Ac-tub) in the frontal cortex (A, B) and hippocampus (C, D) of male (A, C) and female (B, D), young (3 months, 3 m) and aged (18 months, 18 m), control (wild type, WT) and *adm* knock-out (KO) mice. Two-way ANOVA: & $p < 0.05$ main effect of age, and && $p < 0.001$ main effect of age. Panels show percentage of optical density (OD) values of control and representative pictures of the blotting. β-Actin is used as internal loading control.

3.2. HUMAN SPECIMENS

To test whether similar changes are present in humans, frontal cortex protein extracts were obtained from mature and aged human donors. Western blots for AM showed a significant ($p<0.05$) increase in the relative levels of this peptide in aged brains, which was more than double the levels observed in younger samples (**Figure 6A**). In contrast, the relative levels of acetylated tubulin were significantly ($p<0.05$) reduced by aging (**Figure 6B**).

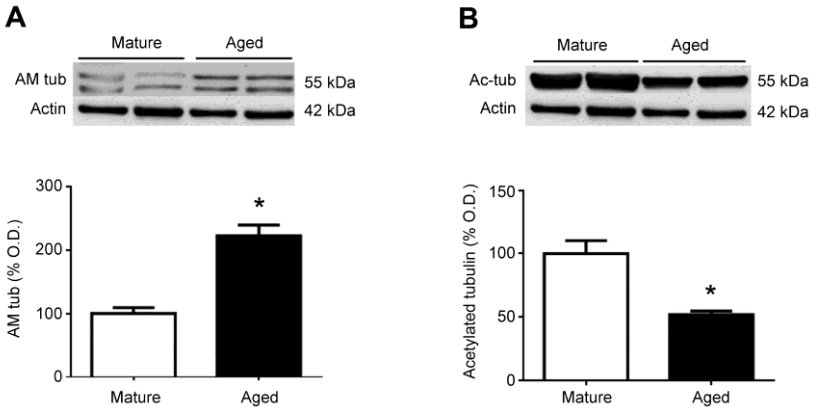

Figure 6. Expression of AM (A) and acetylated tubulin (B) in the frontal cortex of mature and aged human donors. The band of immunoreactive tubulin-associated AM (~55 kDa) is more intense in the aged brain whereas acetylated tubulin is significantly lower in aged individuals. Student´s t test, *$p < 0.05$. β-Actin is used as internal loading control. β-Actin blot in panel B is the same as in panel A and is repeated here to allow a direct comparison with the loading controls.

4. DISCUSSION

In this study we have shown that aged mice that lack neuronal AM have better contextual and recognition memory than their WT littermates. In parallel, the brain cortex and hippocampus of these mice have a lower accumulation of pTau, suggesting that pTau may be the link between lack of AM and memory preservation, although we cannot rule out other alternative molecular pathways. In addition, we also showed that older human individuals present higher levels of AM and lower levels of acetylated tubulin in their brains than younger controls.

Previous studies had found that plasma AM increases with age (Kato et al. 2002), but a description of the levels of AM in the aging brain was lacking. Here we have demonstrated that normal aging is accompanied by an increase of AM protein expression, at least in the frontal cortex. Elevated ADM related peptides levels have been shown to occur in the brain of AD patients (CHAPTER III) and that plasma levels of the related peptide, mid-regional proadrenomedullin, may constitute an early marker for progression to AD (Buerger et al. 2011; Henriksen et al. 2014). Obviously, patients in their path to develop AD should have a larger AM increase than normally aging individuals. These correlation studies provide important information by identifying potential markers to detect early cases of rapidly declining individuals, but they do not help in understanding the underlying biochemical

mechanisms. Our mouse study provides a first insight into the potential mechanisms linking AM levels to contextual memory loss, through the regulation of pTau, especially among females. Although a protein-protein contact between Tau and AM was not detected by yeast-2-hybrid experiments (Sackett et al. 2008), these two proteins are MAPs and are located in close proximity on the microtubule surface, so a direct physical interaction cannot be excluded. Alternatively, AM could modulate any of the kinases that phosphorylate Tau (Tell et al. 2016). The exact biochemical mechanism linking AM to Tau phosphorylation should be addressed in future studies.

Two different antibodies were used in this study to asses Tau phosphorylation. AT8 is a phospho-specific antibody that recognizes phosphorylation in Ser202 and Thr205 (Goedert et al. 1995), whereas the PHF1 antibody recognizes phosphorylation on Ser396 and Ser404 of the Tau protein (Otvos, et al. 1994). All these phosphorylation sites have been involved in pathological findings (Mondragon-Rodriguez et al. 2014). PHF1 is generally considered a later marker in the dynamic sequence of Tau phosphorylation events during the evolution of neurofilaments in AD, when compared to AT8 (Oh et al. 2010). Having very similar results with both antibodies indicates that we are detecting highly phosphorylated forms of Tau, which are the hallmarks of AD and other tauopathies related to memory loss (Tepper et al. 2014).

The levels of acetylated tubulin, a post-translational modification of α-tubulin which results in microtubule stability (Strzyz 2016), were also studied as another potential mechanism of AM-mediated memory preservation. Aged human donors and male mice had lower levels than their younger counterparts, although this did not happen with female mice. In these animals we did not see any difference between genotypes, although we expected them based on previous studies (Sackett et al. 2008). Perhaps this loss of hyperpolymerized microtubules in the neurons occurs late in the aging process, resulting in synaptic disconnection, and is not so prevalent in females, where Tau phosphorylation may be more relevant. Of course, another possibility is that female hormones may have a neuroprotective effect in this context. Other α-tubulin modifications have been also reported to increase in neurodegenerative diseases, including AD (Vu et al. 2017), suggesting an important contribution of the cytoskeleton to the pathogenesis of memory loss.

An intriguing feature is the clear sexual dimorphism we observed in the behavioral and biochemical studies. Our fear conditioning results suggest that aged female mice are more

prone to contextual memory loss than their male counterparts, a condition that was compensated by the lack of AM expression in neurons. Albeit using different memory tests, these sexual differences have been also reported in humans where women have an increased risk for memory disorders relative to men later in life (Jacobs et al. 2016; Reed et al. 2017). It seems that this risk depends on sex steroids, whose decline after menopause correlates with more pronounced alterations in hippocampal connectivity (Jacobs et al. 2016). In addition, the physiological effects of AM have shown to be also sex-dependent with females being more susceptible to changes in the levels of AM expression (Martinez-Herrero et al. 2016).

We have previously found that lack of AM has an impact on locomotor activity (Fernandez et al. 2008) and pain perception (Fernandez et al. 2010) in young mice. These characteristics may have an influence in the way the KO mice respond to the memory tests. To address these issues we performed an open field test in old mice before the NORT and found no differences in locomotor activity, as previously shown for mice of this age (Fernandez et al. 2008). Since we do not observe any memory differences in younger mice and the older mice do not have locomotor activity differences, we can rule out the influence of this feature on the results of the NORT. Also, to ensure that the different sensitivity to pain did not influence the fear conditioning results, we measured the freezing behavior of mice of either genotype before and after the foot shock. In both cases, the differences were not statistically significant, indicating that this is not an issue for this specific test.

Previous studies have shown that neuronal AM may have a neuroprotective effect, especially in the context of stroke (Hurtado et al. 2010) and exposure to hypobaric environments (Fernandez et al. 2008). Nevertheless, we cannot generalize and say that AM is always neuroprotective. For instance, high levels of circulating AM correlate with increased neurological severity in stroke patients (Serrano-Ponz et al. 2016) and endothelial AM seems to have the reverse effect on stroke and brain damage than neuronal AM (Ochoa-Callejero et al. 2016). Until we acquire a more complete understanding on the effects of AM in the CNS, our present data need to be interpreted on their own, indicating that lower levels of AM are beneficial for memory preservation, especially in females.

AM has been shown to bind to complement factor H (CFH) in the blood stream, and each molecule influences the physiological activities of the other (Pio et al. 2001). CFH is also present at high levels in the CNS (Serrano et al. 2003). Although there are no data on the evolution of brain CFH with age, some studies point to a correlation between lower levels of

circulating CFH and more severe cognitive impairment during the development of AD, both in patients (Gezen-Ak et al. 2013) and in mouse models (Wang et al. 2016). It would be interesting to investigate the potential impact of CFH on AM-mediated memory loss during normal aging.

Our data suggest that reducing AM/PAMP levels may constitute a novel path to preventing/delaying memory loss. Few years ago, a particular single nucleotide polymorphism (SNP) close to the *ADM* gene was found to be responsible for a natural reduction in the circulating levels of AM (Cheung et al. 2011) and to correlate with cancer susceptibility (Martinez-Herrero and Martinez 2013). Therefore, it would be interesting to test whether carriers of this SNP are more protected from developing memory impairment. Also, several physiological inhibitors of AM have been proposed for clinical development, including a monoclonal antibody (Martinez et al. 1996), the peptide fragment AM_{22-52} (Ishikawa et al. 2003), and a number of small molecules that target either AM or PAMP (Martinez et al. 2004; Roldos et al. 2012). Therefore some of these inhibitors may be used for the pharmacological prevention of age-related memory loss.

In conclusion, normal aging results in higher expression of AM in the brain and AM ablation prevents Tau phosphorylation in female mice and favors memory preservation in advanced age. These observations suggest that a pharmacological inhibition of *adm* gene products may constitute a novel approach to preventing memory loss in normal aging and in patients suffering moderate AD in high risk of rapid cognitive decline.

5. REFERENCES

Bahn, S., Augood, S.J., Ryan, M., Standaert, D.G., Starkey, M., and Emson, P.C. (2001). Gene expression profiling in the post-mortem human brain--no cause for dismay. J. Chem. Neuroanat. 22, 79–94.

Buerger, K., Uspenskaya, O., Hartmann, O., Hansson, O., Minthon, L., Blennow, K., Moeller, H.-J., Teipel, S.J., Ernst, A., Bergmann, A., et al. (2011). Prediction of Alzheimer's disease using midregional proadrenomedullin and midregional proatrial natriuretic peptide: a retrospective analysis of 134 patients with mild cognitive impairment. J. Clin. Psychiatry. 72, 556–563.

Caron, K.M., and Smithies, O. (2001). Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. Proc. Natl. Acad. Sci. 98, 615–619.

Cheung, B.M.Y., Ong, K.L., Tso, A.W.K., Leung, R.Y.H., Cherny, S.S., Sham, P.C., Lam, T.H., Lam, K.S.L., and Investigators of Hong Kong Cardiovascular Risk Factor Prevalence Study (2011). Plasma adrenomedullin level is related to a single nucleotide polymorphism in the adrenomedullin gene. Eur. J. Endocrinol. 165, 571–577.

Collette, F., and Salmon, E. (2014). The effect of normal and pathological aging on cognition. Rev. Med. Liege 69, 265–269.

Delacourte, A., Sergeant, N., Wattez, A., Maurage, C.-A., Lebert, F., Pasquier, F., and David, J.-P. (2002). Tau aggregation in the hippocampal formation: an ageing or a pathological process? Exp. Gerontol. 37, 1291–1296.

Fernandez, A.P., Masa, J.S., Guedan, M.A., Futch, H.S., and Martínez-Murillo, R. (2016). Adrenomedullin Expression in Alzheimer's Brain. Curr. Alzheimer Res. 13, 428–438.

Fernández, A.P., Serrano, J., Tessarollo, L., Cuttitta, F., Martínez, A., Fernandez, A.P., Serrano, J., Tessarollo, L., Cuttitta, F., and Martinez, A. (2008). Lack of adrenomedullin in the mouse brain results in behavioral changes, anxiety, and lower survival under stress conditions. Proc. Natl. Acad. Sci. U. S. A. 105, 12581–12586.

Fernández, A.P., Serrano, J., Martínez-Murillo, R., and Martínez, A. (2010). Lack of Adrenomedullin in the Central Nervous System Results in Apparently Paradoxical Alterations on Pain Sensitivity. Endocrinology. 151, 4908–4915.

Gerenu, G., Dobarro, M., Ramirez, M.J., and Gil-Bea, F.J. (2013). Early cognitive stimulation compensates for memory and pathological changes in Tg2576 mice. Biochim. Biophys. Acta - Mol. Basis Dis. 1832, 837–847.

Gezen-Ak, D., Dursun, E., Hanağası, H., Bilgiç, B., Lohman, E., Araz, Ö.S., Atasoy, I.L., Alaylıoğlu, M., Önal, B., Gürvit, H., et al. (2013). BDNF, TNFα, HSP90, CFH, and IL-10 serum levels in patients with early or late onset Alzheimer's disease or mild cognitive impairment. J. Alzheimers. Dis. 37, 185–195.

Goedert, M., Jakes, R., and Vanmechelen, E. (1995). Monoclonal antibody AT8 recognises tau protein phosphorylated at both serine 202 and threonine 205. Neurosci. Lett. 189, 167–169.

Guillozet, A.L., Weintraub, S., Mash, D.C., and Mesulam, M.M. (2003). Neurofibrillary

Tangles, Amyloid, and Memory in Aging and Mild Cognitive Impairment. Arch. Neurol. 60, 729.

Henriksen, K., O'Bryant, S.E., Hampel, H., Trojanowski, J.Q., Montine, T.J., Jeromin, A., Blennow, K., Lönneborg, A., Wyss-Coray, T., Soares, H., et al. (2014). The future of blood-based biomarkers for Alzheimer's disease. Alzheimer's Dement. 10, 115–131.

Hermann, P.M., Watson, S.N., and Wildering, W.C. (2014). Phospholipase A2 - nexus of aging, oxidative stress, neuronal excitability, and functional decline of the aging nervous system? Insights from a snail model system of neuronal aging and age-associated memory impairment. Front. Genet. 5, 419.

Hernandez, F., Lucas, J.J., and Avila, J. (2013). GSK3 and tau: two convergence points in Alzheimer's disease. J. Alzheimers. Dis. 33 Suppl 1, S141-4.

Hickman, R.A., Faustin, A., and Wisniewski, T. (2016). Alzheimer Disease and Its Growing Epidemic: Risk Factors, Biomarkers, and the Urgent Need for Therapeutics. Neurol. Clin. 34, 941–953.

Holm, H., Nägga, K., Nilsson, E.D., Ricci, F., Melander, O., Hansson, O., Bachus, E., Magnusson, M., and Fedorowski, A. (2017). Biomarkers of microvascular endothelial dysfunction predict incident dementia: a population-based prospective study. J. Intern. Med. 282, 94–101.

Hurtado, O., Serrano, J., Sobrado, M., Fernández, A.P., Lizasoain, I., Martínez-Murillo, R., Moro, M.A., and Martínez, A. (2010). Lack of adrenomedullin, but not complement factor H, results in larger infarct size and more extensive brain damage in a focal ischemia model. Neuroscience 171, 885–892.

Ishikawa, T., Chen, J., Wang, J., Okada, F., Sugiyama, T., Kobayashi, T., Shindo, M., Higashino, F., Katoh, H., Asaka, M., et al. (2003). Adrenomedullin antagonist suppresses in vivo growth of human pancreatic cancer cells in SCID mice by suppressing angiogenesis. Oncogene 22, 1238–1242.

Jacobs, E.G., Weiss, B.K., Makris, N., Whitfield-Gabrieli, S., Buka, S.L., Klibanski, A., and Goldstein, J.M. (2016). Impact of Sex and Menopausal Status on Episodic Memory Circuitry in Early Midlife. J. Neurosci. 36, 10163–10173.

Kato, J., Kitamura, K., Uemura, T., Kuwasako, K., Kita, T., Kangawa, K., and Eto, T. (2002). Plasma levels of adrenomedullin and atrial and brain natriuretic peptides in the general population: their relations to age and pulse pressure. Hypertens. Res. 25, 887–892.

Larráyoz, I.M., and Martínez, A. (2012). Proadrenomedullin N-terminal 20 peptide increases kinesin's velocity both in vitro and in vivo. Endocrinology 153, 1734–1742.

Leal, S.L., Noche, J.A., Murray, E.A., and Yassa, M.A. (2017). Age-related individual variability in memory performance is associated with amygdala-hippocampal circuit function and emotional pattern separation. Neurobiol. Aging 49, 9–19.

Lewis, D. (2002). The Human Brain Revisited Opportunities and Challenges in Postmortem Studies of Psychiatric Disorders. Neuropsychopharmacology 26, 143–154.

López, J., and Martínez, A. (2002). Cell and molecular biology of the multifunctional peptide, adrenomedullin. Int. Rev. Cytol. 221, 1–92.

Martínez, A., Weaver, C., López, J., Bhathena, S.J., Elsasser, T.H., Miller, M.J., Moody, T.W., Unsworth, E.J., and Cuttitta, F. (1996). Regulation of insulin secretion and blood glucose metabolism by adrenomedullin. Endocrinology 137, 2626–2632.

Martínez, A., Julián, M., Bregonzio, C., Notari, L., Moody, T.W., and Cuttitta, F. (2004). Identification of vasoactive nonpeptidic positive and negative modulators of adrenomedullin using a neutralizing antibody-based screening strategy. Endocrinology 145, 3858–3865.

Martínez-Herrero, S., and Martínez, A. (2013). Cancer protection elicited by a single nucleotide polymorphism close to the adrenomedullin gene. J. Clin. Endocrinol. Metab. 98, E807-10.

Martínez-Herrero, S., Larrayoz, I.M., Narro-Íñiguez, J., Villanueva-Millán, M.J., Recio-Fernández, E., Pérez-Matute, P., Oteo, J.A., and Martínez, A. (2016). Lack of Adrenomedullin Results in Microbiota Changes and Aggravates Azoxymethane and Dextran Sulfate Sodium-Induced Colitis in Mice. Front. Physiol. 7, 595.

Mondragón-Rodríguez, S., Perry, G., Luna-Muñoz, J., Acevedo-Aquino, M.C., and Williams, S. (2014). Phosphorylation of tau protein at sites Ser 396-404 is one of the earliest events in Alzheimer's disease and Down syndrome. Neuropathol. Appl. Neurobiol. 40, 121–135.

Nelson, P.T., Alafuzoff, I., Bigio, E.H., Bouras, C., Braak, H., Cairns, N.J., Castellani, R.J., Crain, B.J., Davies, P., Del Tredici, K., et al. (2012). Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. J. Neuropathol. Exp. Neurol. 71, 362–381.

Ochoa-Callejero, L., Pozo-Rodrigálvarez, A., Martínez-Murillo, R., Martínez, A., Lopez, J., Martinez, A., Ichiki, Y., Shindo, T., Fritz-Six, K.L., Dunworth, W.P., et al. (2016). Lack of adrenomedullin in mouse endothelial cells results in defective angiogenesis, enhanced vascular permeability, less metastasis, and more brain damage. Sci. Rep. 6, 33495.

Oh, K.-J., Perez, S.E., Lagalwar, S., Vana, L., Binder, L., and Mufson, E.J. (2010). Staging of Alzheimer's pathology in triple transgenic mice: a light and electron microscopic analysis. Int. J. Alzheimers. Dis. 2010.

Otvos, L., Feiner, L., Lang, E., Szendrei, G.I., Goedert, M., and Lee, V.M.-Y. (1994). Monoclonal antibody PHF-1 recognizes tau protein phosphorylated at serine residues 396 and 404. J. Neurosci. Res. 39, 669–673.

Pérez-Castells, J., Martín-Santamaría, S., Nieto, L., Ramos, A., Martínez, A., Pascual-Teresa, B. de, and Jiménez-Barbero, J. (2012). Structure of micelle-bound adrenomedullin: a first step toward the analysis of its interactions with receptors and small molecules. Biopolymers 97, 45–53.

Pini, L., Pievani, M., Bocchetta, M., Altomare, D., Bosco, P., Cavedo, E., Galluzzi, S., Marizzoni, M., and Frisoni, G.B. (2016). Brain atrophy in Alzheimer's Disease and aging. Ageing Res. Rev. 30, 25–48.

Pío, R., Martínez, A., Unsworth, E.J., Kowalak, J.A., Bengoechea, J.A., Zipfel, P.F., Elsasser, T.H., Cuttitta, F., Pio, R., Martinez, A., et al. (2001). Complement Factor H Is a Serum-binding Protein for Adrenomedullin, and the Resulting Complex Modulates the

Bioactivities of Both Partners. J. Biol. Chem. 276, 12292–12300.

Reed, J.L., Gallagher, N.M., Sullivan, M., Callicott, J.H., and Green, A.E. (2017). Sex differences in verbal working memory performance emerge at very high loads of common neuroimaging tasks. Brain Cogn. 113, 56–64.

Roldós, V., Carbajo, R.J., Schott, A.-K., Pineda-Lucena, A., Ochoa-Callejero, L., Martínez, A., Ramos, A., and de Pascual-Teresa, B. (2012). Identification of first proadrenomedullin N-terminal 20 peptide (PAMP) modulator by means of virtual screening and NMR interaction experiments. Eur. J. Med. Chem. 55, 262–272.

Sackett, D.L., Ozbun, L., Zudaire, E., Wessner, L., Chirgwin, J.M., Cuttitta, F., and Martínez, A. (2008). Intracellular proadrenomedullin-derived peptides decorate the microtubules and contribute to cytoskeleton function. Endocrinology 149, 2888–2898.

Savva, G.M., Wharton, S.B., Ince, P.G., Forster, G., Matthews, F.E., Brayne, C., and Medical Research Council Cognitive Function and Ageing Study (2009). Age, Neuropathology, and Dementia. N. Engl. J. Med. 360, 2302–2309.

Serrano, J., Uttenthal, L.O., Martínez, A., Fernández, A.P., Martínez de Velasco, J., Alonso, D., Bentura, M.L., Santacana, M., Gallardo, J.R., Martínez-Murillo, R., et al. (2000). Distribution of adrenomedullin-like immunoreactivity in the rat central nervous system by light and electron microscopy. Brain Res. 853, 245–268.

Serrano, J., Encinas, J.M., Fernández, A.P., Castro-Blanco, S., Alonso, D., Fernández-Vizarra, P., Richart, A., Bentura, M.L., Santacana, M., Cuttitta, F., et al. (2003). Distribution of immunoreactivity for the adrenomedullin binding protein, complement factor H, in the rat brain. Neuroscience 116, 947–962.

Serrano-Ponz, M., Rodrigo-Gasqué, C., Siles, E., Martínez-Lara, E., Ochoa-Callejero, L., and Martínez, A. (2016). Temporal profiles of blood pressure, circulating nitric oxide, and adrenomedullin as predictors of clinical outcome in acute ischemic stroke patients. Mol. Med. Rep. 13, 3724–3734.

Strzyz, P. (2016). Post-translational modifications: Extension of the tubulin code. Nat. Rev. Mol. Cell Biol. 17, 609–609.

Tell, V., Hilbrich, I., Holzer, M., Totzke, F., Schachtele, C., Slynko, I., Sippl, W., and Hilgeroth, A. (2016). Drug Development of Small-Molecule Inhibitors of AD-Relevant Kinases as Novel Perspective Multitargeted Approach. Curr. Alzheimer Res. 13, 1330–1336.

Tepper, K., Biernat, J., Kumar, S., Wegmann, S., Timm, T., Hübschmann, S., Redecke, L., Mandelkow, E.-M., Müller, D.J., and Mandelkow, E. (2014). Oligomer Formation of Tau Protein Hyperphosphorylated in Cells. J. Biol. Chem. 289, 34389–34407.

Vergaño-Vera, E., Fernández, A.P., Hurtado-Chong, A., Vicario-Abejón, C., and Martínez, A. (2010). Lack of adrenomedullin affects growth and differentiation of adult neural stem/progenitor cells. Cell Tissue Res. 340, 1–11.

Vu, H.T., Akatsu, H., Hashizume, Y., Setou, M., and Ikegami, K. (2017). Increase in α-tubulin modifications in the neuronal processes of hippocampal neurons in both kainic acid-induced epileptic seizure and Alzheimer's disease. Sci. Rep. 7, 40205.

Wang, D., Di, X., Fu, L., Li, Y., Han, X., Wu, H., Cai, L., Meng, X., Jiang, C., Kong, W., et al. (2016). Analysis of serum β-amyloid peptides, α2-macroglobulin, complement factor H, and clusterin levels in APP/PS1 transgenic mice during progression of Alzheimer's disease. Neuroreport. 27, 1114–1119.

Xu, Y., and Krukoff, T.L. (2004). Adrenomedullin in the rostral ventrolateral medulla increases arterial pressure and heart rate: roles of glutamate and nitric oxide. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287, R729-34. Bahn, S., Augood, S.J., Ryan, M., Standaert, D.G., Starkey, M., and Emson, P.C. (2001). Gene expression profiling in the post-mortem human brain--no cause for dismay. J. Chem. Neuroanat. 22, 79–94.

Chapter V
Reduced Adrenomedullin parallels Microtubule Dismantlement in Frontotemporal lobar degeneration

SUMMARY

In the present study we explore the involvement of *ADM* in the neuropathology of frontotemporal lobar degeneration with primary tauopathy (FTLD-Tau). Proteins from frontal cortex of FTLD-Tau patients and age- and sex-matched non-demented controls were analyzed with antibodies against different microtubule components, including adrenomedullin, and synaptic markers. FTLD-Tau frontal cortices under study showed marked Tau pathology. Levels of total βIII-tubulin as well as acetylated- and detyrosinated-tubulin, two markers of stabilized and aged microtubules, were significantly reduced and directly correlated with levels of PSD95 and proBDNF in FTLD-Tau patients when compared to non-demented controls. In contrast, no change in actin cytoskeleton was found. Interestingly, these changes in microtubule elements, which likely indicate axonal loss, were accompanied by decreased levels of free adrenomedullin but no change in tubulin-bound adrenomedullin. The distribution of adrenomedullin in FTLD-Tau brains failed to display any association with reduced levels of microtubule components.

1. INTRODUCTION

Frontotemporal lobar degeneration (FTLD) is a pathological condition that predominantly presents with frontotemporal dementia (FTD) and results from the selective and progressive deterioration of the frontal and temporal lobes of the brain. Depending on the affected regions, patients with FTLD can display progressive changes in behavior, executive dysfunction and/or language abnormalities, giving rise to distinct clinical symptoms: behavioural variant of frontotemporal dementia (bvFTD), semantic variant primary progressive aphasia (svPPA), and progressive non-fluent aphasia (PNFA). The neuropathology of FTLD is also heterogeneous, and therefore FTLD has been classified into broad categories depending on the intracellular abnormal accumulation of disease-specific proteins: Tau, transactive response DNA-binding protein 43 (TDP-43) or fused-in-sarcoma (FUS), which may each be positive or negative for ubiquitin protein (Mann and Snowden 2017).

Primary tauopathies are defined by the presence of insoluble and hyperphosphorylated Tau proteins in neurons and glial cells. These disorders fall into the clinical spectrum of FTLD (hereinafter referred as pathological subtype FTLD-Tau), predominantly presenting with bvFTD, svPPA and PNFA but also with atypical parkinsonism syndromes such as progressive supranuclear palsy and corticobasal degeneration (Revesz and Holton 2003; Kovacs 2015). The rest of FTLD cases can be assigned to one of the two other major pathological subtypes: FTLD-FUS and FTLD-TDP (Mackenzie et al. 2010). FTLD is highly linked to family history (up to 50% of cases), and mutations in microtubule-associated protein Tau (*MAPT*) gene are frequent in patients with FTLD-Tau, causing a subtype of frontotemporal dementia with parkinsonism linked to chromosome 17-Tau (FTDP-17T) (Rademakers et al. 2004). Tau is a microtubule-associated protein with a chief role in microtubule stabilization by promoting their polymerization and suppressing their dynamics when assembled. Phosphorylation of Tau is important for microtubule dynamics in physiological conditions. However, when Tau is abnormally hyperphosphorylated in response to a number of stressors or mutations, Tau dissociates from the microtubule cytoskeleton leading to its instability and causing axonal degeneration (Iqbal et al. 2009). Unbound hyperphosphorylated Tau is prone to aggregate and assemble into intracellular fibrillar deposits. In FTLD-Tau, fibrils vary in their composition, which usually is enriched in one of the six Tau isoforms, and thus define different subtypes of neuropathological

phenotypes, in contrast to Alzheimer's disease (AD) where Tau deposits are made up of all isoforms (Goedert et al. 1992; Rossi and Tagliavini 2015). However all these pathological phenotypes share a common feature: abnormal hyperphosphorylation of Tau is primary and central to the disease by leading to microtubule disruption and axonal degeneration.

Microtubules are composed of globular tubulin proteins. Post-translational modifications (eg. acetylation, detyrosination) of tubulins and their binding to other cellular proteins orchestrate the dynamics of microtubule polymerization and depolymerization, which are necessary for proper axonal and dendrite organization in neurons. Previous studies identified novel microtubule interactors with prominent role in tubulin dynamics (Sackett et al. 2008). In particular, two adrenomedullin (*ADM*) gene products, proadrenomedullin N-terminal peptide (PAMP) and adrenomedullin (AM), can bind directly to tubulin, kinesin and to other microtubule associated proteins, and participate in specific functions such as destabilization of microtubule polymerization and increased transport velocity through microtubules (Sackett et al. 2008; Larráyoz and Martínez 2012). Further, increased *ADM* immunoreactivities were found in apical dendrites and axons in brains from AD patients and mouse models (Fernandez et al. 2016; CHAPTER III), and the fraction of *ADM* peptides bound to tubulin was reported to be increased in aged (submitted work) and AD brains (CHAPTER III). It is interesting to note that conditional deletion of *ADM* in rodent brains is able to revert aged-related memory impairment and abnormal Tau phosphorylation (submitted work). Taking all these data into account, we decided to explore the status of *ADM* products and its relationship to microtubule dismantlement in a neurodegenerative condition where tauopathy is a primary pathological event, such as FTLD-Tau.

2. MATERIAL AND METHODS

2.1 HUMAN BRAIN TISSUE

To this purpose, brain tissues from FTLD patients were obtained from the Brains for Dementia Research Initiative (BDR, UK). Informed consent was obtained from the patients' next-of-kin before collection of brains, and the study was approved by the UK National Research Ethics Service. Demented subjects (n = 10) fulfilled criteria for the clinical and neuropathological diagnosis of FTLD-Tau. Controls (n = 10) matched for age (62.5 ± 2.2 vs 61.9 ± 3.3, controls and FTLD respectively), and post-mortem delay (34.1 ± 4.9 vs 31.0 ± 4.3 h) did not have dementia or any other neurological diseases, and were staged at Braak 0-II. Small frozen and meninge-free pieces of frontal cortices from Brodmann area (BA)10 were

used for subsequent analysis, which were performed blind to clinical information. Brain pH measurements were determined for each frontal cortex in deionized water as an index of acidosis associated with terminal coma, and cases were subsequently excluded if the pH was found to be below 6.1.

2.2 WESTERN BLOTTING

Proteins from BA10 homogenates were isolated in RIPA buffer, resolved by SDS-glycine gel electrophoresis (as described in CHAPTER III) and subsequently immunoblotted with different antibodies against cytoskeletal components, including adrenomedullin, and synaptic markers (**Table 1**).

Table 1. Antibodies and conditions use in this study.

Target	Species	Dilution	Source
Adrenomedullin	Rabbit polyclonal	1:200	In house
Acetylated tubulin	Mouse monoclonal	1:15,000	Sigma T7451
Glu-tubulin	Rabbit polyclonal	1:1,000	Millipore AB3201
βIII-tubulin	Rabbit polyclonal	1:1,000	Sigma T2200
β -Actin	Mouse monoclonal	1:10,000	Sigma AC74
GAPDH	Mouse monoclonal	1:10,000	Sigma G8795
Tau, pSer202/pThr205 (AT8)	Mouse monoclonal	1:1,000	Fisher NM1020
Tau, pSer396/pSer404 (PHF1)	Mouse monoclonal	1:1,000	Gift from Peter Davies
Tau, total (Tau12)	Mouse monoclonal	1:1,000	Biolegend SIG-39416
Pro-BDNF	Rabbit monoclonal	1:1,000	Abcam 72439
PSD95	Rabbit polyclonal	1:1,000	Cell signaling 2507

3. RESULTS

As shown in **Figure 1**, the antibody Tau12 showed no particular changes in the amount of total Tau in BA10 frontal cortex between FTLD-Tau patients and control individuals, but the antibody PHF1 that is specific for Ser396/Ser404 detected variable but marked pattern of Tau phosphorylation in FTLD-Tau brains. Similarly, the antibody AT8 that recognizes Ser202/Thr205, showed a relative increase in Tau phosphorylation.

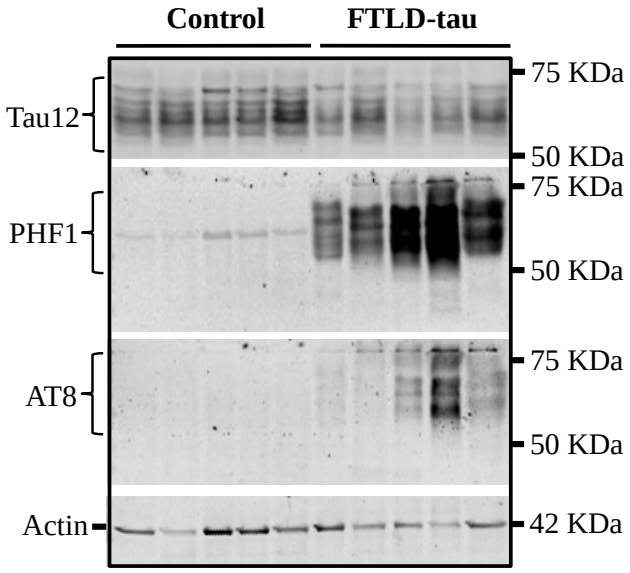

Figure 1. Representative western blot images showing Tau pathology in BA10 frontal cortex of FTLD-Tau patients and matched non-demented controls. Antibody Tau12 does not detect significant changes in total levels of Tau protein in FTLD-Tau frontal cortices. Antibody PHF1 that recognizes Ser396/Ser404 shows a more prominent pattern of Tau phosphorylation than antibody AT8 that is specific for Ser202/Thr205. β-actin is used as internal loading control.

Neuron-specific βIII-tubulin was significantly reduced in FTLD-Tau when compared to controls (Student's t test; $t = 2.12$, $P<0.05$; **Figure 2B**). Post-translational modifications of tubulin, in particular acetylated-tubulin and detyrosinated tubulin (glu-tub), were also significantly decreased in FTLD-Tau brains ($t = 1.95$, $p<0.05$; $t = 1.91$, $p<0.05$, respectively; **Figure 2B**). However, actin cytoskeleton remained unchanged, as shown by similar levels of β-actin between controls and FTLD-Tau groups ($t = -0.23$, $p= 0.41$; **Figure 2**).

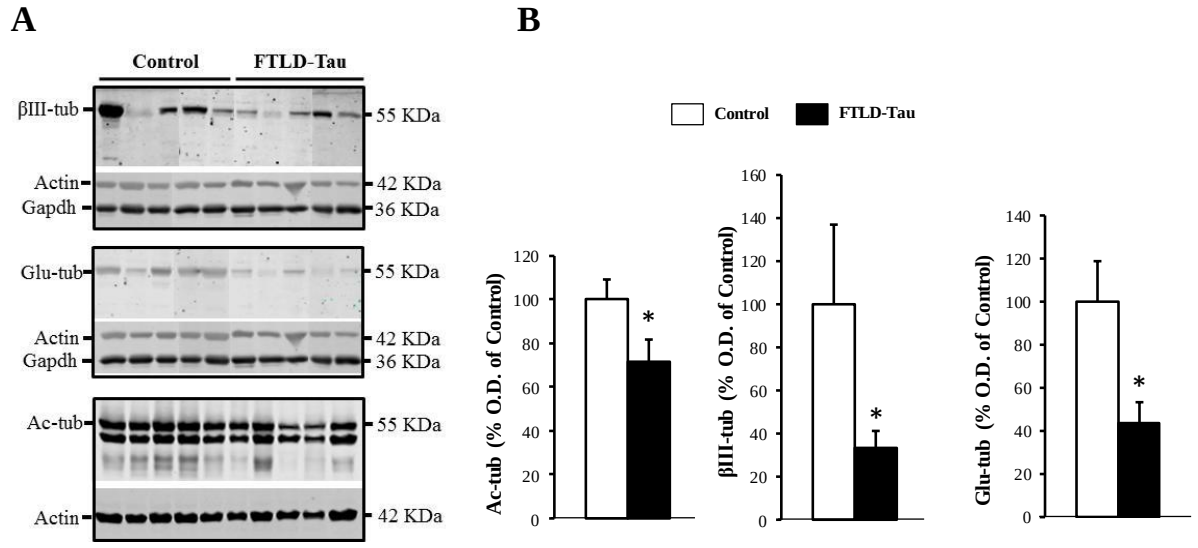

Figure 2. Changes of various microtubule elements in frontotemporal lobar degeneration with primary tauopathy (FTLD-Tau) compared to non-demented patients. Panel B shows percentage of optical density (O.D.) values relativized to the control group, as mean±SEM. βIII-tubulin (βIII-tub), detyronisated-tubulin (glu-tub), acetylated-tubulin (Ac-tub). *p < 0.05, Student's t test. Gapdh is used as internal loading control.

PSD95 and proBDNF proteins, both of which are important for synaptic function and predominantly recruited at post-synaptic sites, were found significantly decreased in FTLD-Tau (54.04 ± 11.21% of control for PSD95, t = 3.89, p<0.01; 69.28 ± 9.58% of control for proBDNF, t = 2.99, p<0.01) (**Figure 3A, 3B**). However, PSD95 was not correlated with either βIII-tubulin (Spearman's rho= 0.301, p= 0.39, n=10), acetylated-tubulin (Spearman's rho= 0.539, p= 0.11, n= 10) or detyrosinated-tubulin (Spearman's rho= 0.03, p= 0.93, n= 10) in FTLD-Tau brains.

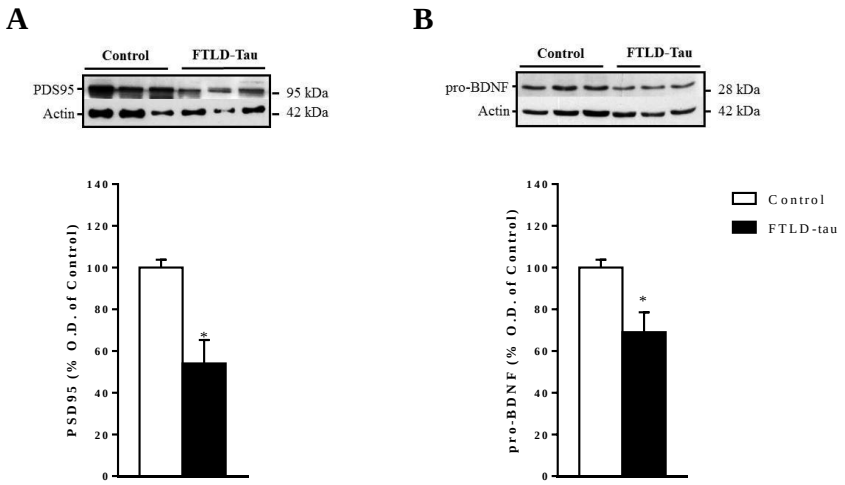

Figure 3. Decrease of PSD95 (A) and pro-BDNF (B) levels in frontotemporal lobar degeneration with primary tauopathy (FTLD-Tau). Panels show percentage of optical density (O.D.) values relativized to the control group, as mean±SEM. PSD95 and pro-BDNF are found significantly decreased in FTLD-Tau BA10 frontal cortex when compared to non-demented controls. *p < 0.05, Student's t test. β-actin is used as internal loading control.

As previously reported, the anti-AM antibody showed two different bands in the brain at around 14 and 55 kDa (**Figure 4A**) (CHAPTER III). The lower band is assigned to proAM protein containing PAMP and AM moieties, while the upper band is purportedly assigned to tubulin-bound ADM peptides (Sackett et al. 2008). As seen in **Figure 4**, significant decreases of ADM peptides (Student's t test; $t = 2.72$, $p<0.05$) parallels those of microtubule components observed in FTLD-Tau, although no changes in tubulin-bound adrenomedullin band were found ($t = 0.46$, $p = 0.33$).

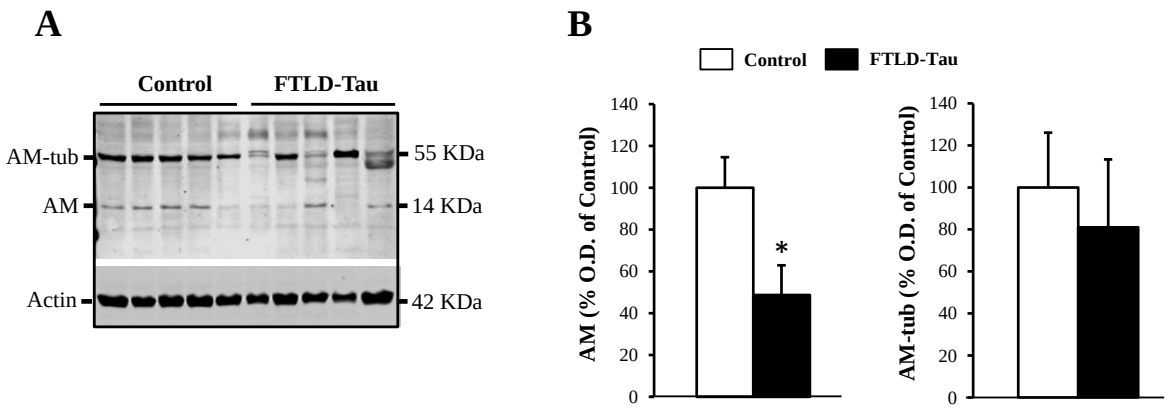

Figure 4. Changes of *ADM* related peptides in frontotemporal lobar degeneration with primary tauopathy (FTLD-Tau) when compared to non-demented control patients. Panel A shows representative pictures of blotting images. Panel B shows percentage of optical density (O.D.) values relativized to the control group, as mean±SEM. ProAM peptides (14 kDa) are significantly decreased while tubulin-associated ADM products (AM-tub, 55 kDa) remain unchanged in FTLD-Tau BA10 frontal cortex. *$p < 0.05$, Student's t test. β-actin is used as internal loading control.

4. DISCUSSION

Changes found in FTLD-postmortem human samples using AT8 and PHF1 antibodies confirm the expected Tau pathology in frontal cortex of FTLD-Tau brains, although the explanations for the intrapatient variability and the distinct intensities of Tau phosphorylation between antibodies are unclear. Perhaps regional differences may account for the distinct intensity signals given by AT8 and PHF1 antibodies, since the former preferentially recognizes the white matter pathology while the latter has preference for gray matter in AD brains (Forman et al. 2002).

On the other hand, the cytoskeleton study reveal a reduction on βIII-tubulin, a main element of microtubules, and two post-translational modifications of tubulin, acetylated-tubulin and detyrosinated tubulin, both of which associated with microtubule stabilization, in

FTLD-Tau. Such decreases in microtubule components undoubtedly represent a disassembly of microtubule cytoskeleton in FTLD-Tau pathology and confirm the known effects of tauopathy on microtubule stability and axonal degeneration (Iqbal et al. 2009). Marked decreases of βIII-tubulin and other elements of the neurocytoskeleton, such as neurofilament proteins, with no changes in microfilament β-actin protein have been already reported in frontal cortex of Pick disease patients (Pollak et al. 2003), a dementia syndrome that belongs to the subtbype FTLD-Tau. Notwithstanding, reductions in acetylated- and detyrosinated-tubulin in brains of FTLD-Tau patients are reported here for the first time to our knowledge, supporting previous observations on both decreased tubulin acetylation and detyrosination in neurofibrillary tangle-bearing neurons of AD brains and in Tau-depleted neurons (Hempen and Brion 1996; Rapoport et al. 2002; Ma et al. 2014).

The decreased of synaptic markers, PSD95 and proBDNF, represents the expected disruption of postsynaptic density that takes place in neurodegenerative dementias (Yuesong Gong and Lippa 2010). Loss of synapses may account for the observed reductions of microtubule immunoreactivities; hence stronger synaptic disruption should be accompanied by lower levels of tubulins. However, these data suggest that loss of microtubule components is not merely related to synaptic loss but instead may be specific to Tau pathology. The lack of changes in other cytoskeletal proteins further confirms the specificity of Tau pathology on microtubule cytoskeleton (Rossi and Tagliavini 2015; Guo et al. 2017).

Since *ADM* peptides have been shown to decorate microtubules (Sackett et al. 2008), the observed reduction of AM/PAMP levels might be part of the general disassembly of microtubules in FTLD-Tau brains. However, no associations between microtubule components and *ADM* related peptides or tubulin-bound *ADM* have been found in FTLD-Tau brains (data not shown). In contrast to previous studies that have reported increased *ADM* in AD brains as a seemingly primary event to microtubule disruption and axonal degeneration (CHAPTER III), the *ADM* changes observed in FTLD-Tau brains might not be primary to the neurophatology of these dementia disorders. Instead, as *ADM* downregulation results in microtubule stabilization and increased posttranslational modifications of tubulin such as acetylation and detyrosination in vitro (Sackett et al. 2008), it is more likely that proAM reductions in FTLD-Tau brain are the result of an adaptive but barely successful response of neurons to counteract microtubule destabilization. Hence, it will be challenging to explore whether pharmacological manipulation of *ADM* peptides by small molecules (as those

reported in (Ishikawa et al. 2003; Robinson et al. 2009)) will boost microtubule stabilization under these neurodegenerative conditions. Several microtubule-stabilizing drugs, commonly used in the treatment of cancer, have been tested in animal models of neurodegeneration and they provided particular benefit to tauopathies (Brunden et al. 2014).

In view of the present observations and in line with previous research work, small molecules that specifically target and inhibit *ADM* peptides should be considered candidates for microtubule-stabilizing therapies to treat FTLD-Tau in early stages before massive microtubule disassembly takes place.

5. REFERENCES

Brunden, K.R., Trojanowski, J.Q., Smith, A.B., Lee, V.M.-Y., and Ballatore, C. (2014). Microtubule-stabilizing agents as potential therapeutics for neurodegenerative disease. Bioorg. Med. Chem. 22, 5040–5049.

Fernandez, A.P., Masa, J.S., Guedan, M.A., Futch, H.S., and Martínez-Murillo, R. (2016). Adrenomedullin Expression in Alzheimer's Brain. Curr. Alzheimer Res. 13, 428–438.

Forman, M.S., Zhukareva, V., Bergeron, C., Chin, S.S.-M., Grossman, M., Clark, C., Lee, V.M.-Y., and Trojanowski, J.Q. (2002). Signature tau neuropathology in gray and white matter of corticobasal degeneration. Am. J. Pathol. 160, 2045–2053.

Goedert, M., Spillantini, M.G., Cairns, N.J., and Crowther, R.A. (1992). Tau proteins of Alzheimer paired helical filaments: abnormal phosphorylation of all six brain isoforms. Neuron 8, 159–168.

Guo, T., Noble, W., and Hanger, D.P. (2017). Roles of tau protein in health and disease. Acta Neuropathol. 133, 665–704.

Hempen, B., and Brion, J.P. (1996). Reduction of acetylated alpha-tubulin immunoreactivity in neurofibrillary tangle-bearing neurons in Alzheimer's disease. J. Neuropathol. Exp. Neurol. 55, 964–972.

Iqbal, K., Liu, F., Gong, C.-X., Alonso, A. del C., and Grundke-Iqbal, I. (2009). Mechanisms of tau-induced neurodegeneration. Acta Neuropathol. 118, 53–69.

Ishikawa, T., Chen, J., Wang, J., Okada, F., Sugiyama, T., Kobayashi, T., Shindo, M., Higashino, F., Katoh, H., Asaka, M., et al. (2003). Adrenomedullin antagonist suppresses in vivo growth of human pancreatic cancer cells in SCID mice by suppressing angiogenesis. Oncogene 22, 1238–1242.

Kovacs, G.G. (2015). Invited review: Neuropathology of tauopathies: principles and practice. Neuropathol. Appl. Neurobiol. 41, 3–23.

Larráyoz, I.M., and Martínez, A. (2012). Proadrenomedullin N-terminal 20 peptide increases kinesin's velocity both in vitro and in vivo. Endocrinology 153, 1734–1742.

Ma, Q.-L., Zuo, X., Yang, F., Ubeda, O.J., Gant, D.J., Alaverdyan, M., Kiosea, N.C., Nazari, S., Chen, P.P., Nothias, F., et al. (2014). Loss of MAP Function Leads to Hippocampal Synapse Loss and Deficits in the Morris Water Maze with Aging. J. Neurosci. 34, 7124–7136.

Mackenzie, I.R.A., Neumann, M., Bigio, E.H., Cairns, N.J., Alafuzoff, I., Kril, J., Kovacs, G.G., Ghetti, B., Halliday, G., Holm, I.E., et al. (2010). Nomenclature and nosology for neuropathologic subtypes of frontotemporal lobar degeneration: an update. Acta Neuropathol. 119, 1–4.

Mann, D.M.A., and Snowden, J.S. (2017). Frontotemporal lobar degeneration: Pathogenesis, pathology and pathways to phenotype. Brain Pathol. 27, 723–736.

Pollak, D., Cairns, N., and Lubec, G. (2003). Cytoskeleton derangement in brain of

patients with Down syndrome, Alzheimer's disease and Pick's disease. J. Neural Transm. Suppl. 149–158.

Rademakers, R., Cruts, M., and van Broeckhoven, C. (2004). The role of tau (MAPT) in frontotemporal dementia and related tauopathies. Hum. Mutat. 24, 277–295.

Rapoport, M., Dawson, H.N., Binder, L.I., Vitek, M.P., and Ferreira, A. (2002). Tau is essential to beta -amyloid-induced neurotoxicity. Proc. Natl. Acad. Sci. U. S. A. 99, 6364–6369.

Revesz, T., and Holton, J.L. (2003). Anatamopathological spectrum of tauopathies. Mov. Disord. 18, 13–20.

Robinson, S.D., Aitken, J.F., Bailey, R.J., Poyner, D.R., and Hay, D.L. (2009). Novel Peptide Antagonists of Adrenomedullin and Calcitonin Gene-Related Peptide Receptors: Identification, Pharmacological Characterization, and Interactions with Position 74 in Receptor Activity-Modifying Protein 1/3. J. Pharmacol. Exp. Ther. 331, 513–521.

Rossi, G., and Tagliavini, F. (2015). Frontotemporal lobar degeneration: old knowledge and new insight into the pathogenetic mechanisms of tau mutations. Front. Aging Neurosci. 7, 192.

Sackett, D.L., Ozbun, L., Zudaire, E., Wessner, L., Chirgwin, J.M., Cuttitta, F., and Martínez, A. (2008). Intracellular proadrenomedullin-derived peptides decorate the microtubules and contribute to cytoskeleton function. Endocrinology 149, 2888–2898.

Yuesong Gong, Y., and Lippa, C.F. (2010). Review: Disruption of the Postsynaptic Density in Alzheimer's Disease and Other Neurodegenerative Dementias. Am. J. Alzheimer's Dis. Other Dementiasr 25, 547–555

Chapter VI
General discussion

Since the discovery of *ADM* gene, the number of studies that have focused on the biology of the peptides derived from this gene has grown exponentially, and up to date new physiological and pathological functions are still being discovered. In the present work, it was proposed that elevated levels of *ADM* peptides may be deleterious to neurons by affecting the dynamics of microtubules. Based on this assumption, it was studied the role of *ADM* in the pathophysiology of neurodegenerative dementias.

Firstly, our study has evaluated the state of *ADM* on AD, the main current cause of dementia, and has shown that the expression of *ADM* is increased in the prefrontal cortex of AD patients, one of the areas most affected by the disease (CHAPTER III). Various biomarker studies have indeed reported increased levels of *ADM* in plasma samples from AD patients when compared with age-matched non-demented individuals. Two of these studies found *ADM* correlated with disease progression (Buerger et al. 2011b; Henriksen et al. 2014) and a third one reported altered *ADM* together with other blood markers of endothelial vasodilatory function in the prodromal stage of mild cognitive impairment (MCI) (Ewers et al. 2010). These data provided evidence on the potential sensitivity of *ADM* as biomarker for early detection and prognosis of AD.

Regarding the results collected in the present thesis, increased levels of *ADM* in frontal cortex of AD patients were particularly localized within the soma and apical dendrites of pyramidal neurons. Previous studies have shown that *ADM* derived peptides are able to interact with tubulin subunits to regulate the dynamics of microtubules (Sackett et al. 2008), therefore the increased levels of *ADM* in such subcellular compartments might be interfering with the proper maintenance of the neuronal cytoskeleton in AD. Microtubules are key components of neuronal cytoskeleton since they provide the main way for axonal transport, contribute to the integrity of the complex dendritic arborization (Prokop 2013), and are involved in synaptic plasticity (Jaworski et al. 2009; Conde and Cáceres 2009). Microtubule stability is commonly associated to the levels of α-tubulin subunits with different post-translational modifications, among which are detyrosination and acetylation (Eira et al. 2016). Indeed, acetylation of α-tubulin is a valuable marker of microtubule stability (Portran et al. 2017) and therefore it has been utilized along the present studies to evaluate the presumable involvement of *ADM* in neuronal microtubule destabilization. In fact, decreases of acetylated tubulin have been presently found, that inversely

correlate with increased *ADM* in AD brains, suggesting that altered expression of *ADM* is linked to vulnerable microtubules and might be likely contributing to the collapse of neuronal cytoskeleton, axonal loss and synaptic dysfunction in these patients.

To check synaptic function, NCAM levels were measured in the prefrontal cortex of AD patients, described as critical components in the induction of long-term potentiation (LTP) and in memory formation (Kiss and Muller, 2001; Rønn et al., 2000). In agreement with our observations, decreased levels of NCAM have been described in the frontal cortex of AD patients (Yew et al. 1999; Aisa et al. 2010), which might stem from the synaptic loss associated to the course of disease. Neuronal degeneration leads to neurochemical dysfunction of several transmitter systems, a fact that is considered a main factor underlying both cognitive and neuropsychiatric symptoms (Francis et al. 2010). Consequently, our study has shown neurochemicals alterations in cholinergic, serotonergic, dopaminergic and gabaergic neurotransmitters, although not at the glutamatergic level.

On the other hand, the inflammatory response has been considered both a cause and consequence of AD (Calsolaro and Edison 2016). Specifically, the ability of microglia and astrocytes to mediate neuroinflammation in AD has been implicated as a significant contributor to the pathogenesis of the disease. Microglia plays an important role in regulating synaptic plasticity, i.e M1 microglia can remove damaged cells as well as dysfunctional synapses (Kettenmann et al. 2013). However, exacerbated microglial responses in AD brains can potentiate the production of toxic pro-inflammatory mediators, thereby contributing to neuronal loss (Solito and Sastre 2012). Ox-42, a marker of active microglial response, was found dramatically increased in AD frontal cortex and positively correlated with *ADM* expression. This observation suggests that increased *ADM* expression may be related to neuroinflammation in AD. As shown in CHAPTER I, AM peptide plays a key role on inflammation and immune response. Previously, it has been reported that in vitro AM inhibits microglia activation and release of proinflammatory compounds (Consonni et al. 2011). In contraposition, AM peptide can activate microglia and astrocytes in the CNS and the blockade of AM receptor abolishes glial activation (Wong et al. 2005; Zeng et al. 2014). Furthermore, AM has been reported to promote astrocyte survival and migration (Xia et al. 2004). However, AM role in inflammation is still controversial (Sardi et al. 2014). Several studies suggest that AM exerts anti-inflammatory activity by downregulating the

production of a wide panel of inflammatory mediators (Gonzalez-Rey et al. 2007; Miksa et al. 2007; Consonni et al. 2011; Pedreño et al. 2014). Meanwhile, it has also been reported that AM stimulates the increases cytokines such as interleukin IL-1β, IL-6, and TNF-α, acting as proinflammatory mediator (Wong et al. 2005; Ma et al. 2006; Zeng et al. 2014; Li et al. 2015). Perhaps AM and its related pepetide PAMP might be involved in a feed-forward mechanisms that exacerbate neuroinflammatory processes under pathological conditions.

Overall, increased *ADM* levels have been found in brains of AD patients correlating negatively with markers of cytoskeleton stability and cell-to-cell interactions. Taking all this data into account, *ADM* gene peptides might be a primary event to microtubule disruption and axonal degeneration. However, given the many functions that are attributed to these peptides, such as their role in inflammation or immune response, they may be participating in the disease by other mechanisms that will have to be evaluated in further studies. Although the present clinicopathological study could contribute to generate hypotheses about the potential involvement of *ADM* in the neuropathology of AD, further experimental work is needed to elucidate whether altered *ADM* in AD brains is crucial in the pathological cascade of cognitive decline or is a mere disease bystander.

Aging is one of the key risk factors contributing to AD development. In agreement with previous studies that have reported increased plasma levels of AM over aging (Kato et al. 2002). The present thesis has shown that normal aging is accompanied also by an increase of AM peptide in the prefrontal cortex of the brain (CHAPTER IV). For this reason, it has been investigated the role of *ADM* gene products in aged-related memory loss using a mouse model with conditional KO of brain *adm* (AMKO mouse) (CHAPTER IV).

A battery of cognitive tests was applied to AMKO mice to assess whether *adm* is primarily involved in memory function. The novel object recognition test (NORT) measures working memory and the fear conditioning test evaluates contextual fear memory. In NORT, aged AMKO mice showed significant cognitive improvement, with no significant differences regarding the sex. However, in the fear conditioning test the memory-enhancement effect of *adm* deletion was only present in aged females. Contextual fear memory is a type of learning with high reliance on the amygdala and

the medial prefrontal cortex (Gilmartin et al. 2014). It seems paradoxical that aged male AMKO mice do not shown any improvement in freezing behavior but presents unaffected discrimination to explore the novel object in the NORT when compared to age-matched WT littermates. This is likely to be explained by the sexual divergence that exists between the different areas involved in the fear circuitry. Supporting this fact, fear conditioning studies performed in men and women have shown sex differences that have been explained by the differences in several brain regions during the test (Lebron-Milad et al. 2012; Merz et al. 2013). Indeed, important sex differences in prefrontal cortex function has been reported (Baran et al. 2010).

Following the main hypothesis of this thesis about the potential implication of alterations in the neuronal cytoskeleton underlying the effects of *adm* in cognitive function, it was determined the state of microtubules in the AMKO mouse by analyzing the levels of Tau phosphorylation and tubulin acetylation. Tau is a MAP with a chief role in microtubule stabilization. Although phosphorylation of Tau is crucial for microtubule dynamics in physiological conditions, when Tau is abnormally hyperphosphorylated it dissociates from microtubule cytoskeleton leading to its instability and utimately axonal degeneration (Iqbal et al. 2009). In this study, it has been found that male aged mice from both genotypes present an increase of Tau phosphorylation. However a significant decrease of AT8 (Ser202/Thr205) and PHF1 (Ser396/Ser404) phosphorylation has been found in prefrontal cortex from aged females with *adm* deletion. Hence, the reduced Tau phosphorylation load, which is commonly associated with less neuronal damage and better cognitive outcomes, may presumably account for the memory improvement observed in aged female AMKO mice. However, to our knowledge no study has intended to analyse differential sex effects on Tau pathology in either aging or neurodegenerative conditions.

Epidemiological studies have widely shown that women not only have a higher prevalence of AD and memory disorders, but also showed significantly age-related faster decline and greater deterioration of cognition than elderly male (Li and Singh 2014; Jacobs et al. 2016; Reed et al. 2017). It seems that this risk depends on sex steroids, which exert neuroprotective effect on pre-menopause although a decline in ther levels after menopause correlates with alterations in hippocampal connectivity (Jacobs et al. 2016). In addition, the physiological effects of *ADM* have shown to be also sex-

dependent with females being more susceptible to changes in the levels of AM expression (Martínez-Herrero et al. 2016).

Regarding cytoskeleton stability, it has been found that aging, both in humans and male mice (but not female), is associated with lower levels of acetylated tubulin in brain. Indeed, there is a trend towards an increase of acetylated tubulin and a decrease of phosphorylated Tau in aged female AMKO mice. Previous studies shown changes on microtubules stability in AMKO mice (Sackett et al. 2008), however it has not been found any differences between genotypes in the present work.

The study on AMKO mice has provided for the first time evidence into the involvement of *adm* in the cellular mechanisms regulating the cognitive function. The fact that the lack of brain *adm* induces improved memory retention in aged mice certainly provides *adm* with a role in the age-associated cognitive decline. Although the mechanisms that link *adm* with cognitive decline are still unclear, the present work has posed the alteration in Tau phosphorylation and the disorganization of microtubules as candidate mechanisms. The direct contact between Tau and *ADM* was not detected in previous experiments using the model yeast-2-hybrid (Sackett et al. 2008); however these two proteins are MAPs located on the microtubule surface and therefore transient physical interactions between them cannot be excluded. In this sense, *ADM* could be allied with the aging process in a vicious cycle that will precipitate microtubule dismantlement by destabilizing Tau-microtubule interactions and therefore making Tau more prone to aging-related phosphorylation events. It is also plausible to speculate that *ADM* might interfere in the phosphorylation cascade of Tau, through its described action on PI3K/AKT pathway (Nishimatsu et al. 2001) or other kinase activities, such as PKA or MEK-ERK, that are equally associated with Tau phosphorylation. In this regard, further studies are needed to elucidate the mechanism of action by which *ADM* could alter the phosphorylated state of Tau, either by direct contact or by its involvement in the phosphorylation cascade.

In view of these findings, it was next investigated the status of *ADM* in frontotemporal lobar dementia, a neurodegenerative disease which primordial cause is tauopathy (FTLD-Tau) (CHAPTER IV). Firstly, we confirmed the presence of extensive tauopathy in the prefrontal cortex FTLD-Tau patients. Similar to what it has just been described in the study using the AMKO model, two different epitopes of phosphorylated

Tau have been analysed. Both of them, AT8 and PHF1, were found increased in FTLD-Tau. However AT8 and PHF1 immunoreactivities presented huge inter-patient variability, being PHF1 immunoreactivity much more intense than the one of AT8. These results may be explained by the preferential distribution of each epitope throughout the brain regions; AT8 more prone to detect white matter pathology whereas PHF1 recognizes gray matter alterations (Forman et al. 2004).

Cytoskeleton analysis of FTLD-Tau has shown a decrease in βIII-tubulin, a fundamental component of microtubules, while actin cytoskeleton remained unchanged. These results agree with previous studies of Pick disease patients, a subtype of FTLD-Tau (Pollak et al. 2003). Moreover, we have reported for the first time that FTLD-Tau is accompanied with lower levels of acetylated-tubulin and detyrosinated tubulin (glu-tub), both markers of microtubule stability. Undoubtedly, such decreases in the components of microtubules represent a large depolymerization of the microtubules in the FTLD-Tau pathology. Thus, it was confirmed the already described effects of tauopathy on the stability of microtubules, which ultimatelly leads to axonal degeneration (Iqbal et al. 2009). For this reason, synaptic function was next evaluated, where a significant reduction on PSD95 and proBDNF levels in FTLD-Tau has been found, probably reflecting the expected disruption of postsynaptic density that takes place in neurodegenerative dementias (Yuesong Gong and Lippa 2010). The anterorograde/retrograde movement of cargoes, such as mRNA, signaling proteins or synaptic vesicle precursors and organelles along microtubules is crucial for the remodeling and plasticity of synaptic contacts (Verstraelen et al. 2017; Dent 2017), and therefore any alteration in the numbers of microtubule components or in the stability of microtubules will inevitably lead to the loss of synapses. However, none of the microtubules components assessed in this study has been correlated with postsynaptic markers in FTLD-Tau brains. Consequently, these data suggests that loss of microtubule components is not a mere consequence of decreased synapse and their cytoskeletal load, but instead might be specific to Tau pathology. The lack of changes in other cytoskeletal proteins (i.e. β-actin) further supports the specificity of Tau pathology in the microtubule cytoskeleton (Rossi and Tagliavini 2015; Guo et al. 2017).

On the other hand, contrary to the observed results in AD, where the levels of *ADM* peptides were found increased with respect to non-demented controls, FTLD-Tau brains showed decreased levels of *ADM*. Although no statistically significant due to

inter-patient variability, a trend towards reduced tubulin-bound *ADM* (50 kDa) can be observed in FTLD-Tau. This variability could be explained by the heterogeneous etiology of FTLD-Tau subtypes. Therefore, since *ADM* peptides decorate microtubules (Sackett et al. 2008), the observed reduction of *ADM* peptides in FTLD-Tau brains might be part of the general disassembly of microtubules. Furthermore, no correlation between the components of the microtubules and *ADM* levels were found, perhaps due to the deterioration of the cytoskeleton. This suggests that *ADM* changes observed in FTLD-Tau brains might not be primary in the neuropathology of this neurodegenerative disease. As the lack of *ADM* in vitro results in microtubule stabilization (Sackett et al. 2008), it is plausible to speculate that the reduction in *ADM* levels observed in FTLD-Tau brains may be instead an adaptive compensatory response, although barely successful, of neurons to counteract the extent of microtubule destabilization that challenges cellular function and viability. As in AD, further studies on different stages of FTLD-Tau should be done to specify the potential gradual impact of *ADM* on the cytoskeleton.

Concluding remarks

As the population ages, the number of people suffering memory disorders will increase and neurodegenerative diseases will represent a challenge for global public health and economy. It is urgent to clarify the mechanism of each neurodegenerative disease both at the molecular and clinical level and thus propose new targets and therapies that could be studied and validated appropriately in clinical trials. On the other hand, since the discovery of *ADM* gene considerable advances have been made in understanding the physiological and pathological role of AM and PAMP in several processes including the regulation of BBB, inflammation, immunity, ion homeostasis, and apoptosis. All of these effects could be part of the development and progression of neurodegenerative diseases. For this purpose, this study has tried to contribute to the present knowledge of the role of *ADM* in neurodegenerative diseases, such as AD and FTLD-Tau, and its implication on memory processes in aging. However, several limitations of this study should be pointed out. The inability to discriminate using the available antibodies between levels of PAMP and AM peptides, which are likely to play different roles in microtubule dynamics. On top of this fact, another possible limitation is the heterogeneity of *ADM* peptides in terms of distribution, functions and

pathological different variations. In addition, given the results achieved in AMKO study, it could be desirable to study whether the involvement of *ADM* in AD neuropathology is affected by sex.

The increase of *ADM* peptides may interfere by its direct interaction with microtubules and its effects on the cytoskeleton on AD and perhaps FTLD-Tau on early stages. Common alterations in neuronal cytoskeleton occur in several neurodegenerative disorders and the equilibrium of microtubules dynamics is critical to maintaining neuronal integrity. Accordingly, the significant increase of *ADM* levels may play a pivotal role in microtubules dynamics in the course of neurodegenerative diseases, leading to cytoskeleton disruption and therefore axonal transport collapse, and neuronal loss. On this basis, *ADM* derived peptides may be a possible novel target and the pharmacological inhibition of this peptide could be a promising therapeutic strategy for neurodegenerative disorders. However, further research is needed in order to clarify the implication of *ADM* in neurodegenerative diseases and its role as a therapeutic target

REFERENCES

Aisa, B., Gil-Bea, F.J., Solas, M., García-Alloza, M., Chen, C.P., Lai, M.K., Francis, P.T., and Ramírez, M.J. (2010). Altered NCAM Expression Associated with the Cholinergic System in Alzheimer's Disease. J. Alzheimer's Dis. 20, 659–668.

Baran, S.E., Armstrong, C.E., Niren, D.C., and Conrad, C.D. (2010). Prefrontal cortex lesions and sex differences in fear extinction and perseveration. Learn. Mem. 17, 267–278.

Buerger, K., Uspenskaya, O., Hartmann, O., Hansson, O., Minthon, L., Blennow, K., Moeller, H.-J., Teipel, S.J., Ernst, A., Bergmann, A., et al. (2011). Prediction of Alzheimer's disease using midregional proadrenomedullin and midregional proatrial natriuretic peptide: a retrospective analysis of 134 patients with mild cognitive impairment. J. Clin. Psychiatry 72, 556–563.

Calsolaro, V., and Edison, P. (2016). Neuroinflammation in Alzheimer's disease: Current evidence and future directions. Alzheimer's Dement. 12, 719–732.

Conde, C., and Cáceres, A. (2009). Microtubule assembly, organization and dynamics in axons and dendrites. Nat. Rev. Neurosci. 10, 319–332.

Consonni, A., Morara, S., Codazzi, F., Grohovaz, F., and Zacchetti, D. (2011). Inhibition of lipopolysaccharide-induced microglia activation by calcitonin gene related peptide and adrenomedullin. Mol. Cell. Neurosci. 48, 151–160.

Dent, E.W. (2017). Of microtubules and memory: implications for microtubule dynamics in dendrites and spines. Mol. Biol. Cell 28, 1–8.

Eira, J., Silva, C.S., Sousa, M.M., and Liz, M.A. (2016). The cytoskeleton as a novel therapeutic target for old neurodegenerative disorders. Prog. Neurobiol. 141, 61–82.

Ewers, M., Mielke, M.M., and Hampel, H. (2010). Blood-based biomarkers of microvascular pathology in Alzheimer's disease. Exp. Gerontol. 45, 75–79.

Forman, M.S., Trojanowski, J.Q., and Lee, V.M.-Y. (2004). Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. Nat. Med. 10, 1055–1063.

Francis, P.T., Ramírez, M.J., and Lai, M.K. (2010). Neurochemical basis for symptomatic treatment of Alzheimer's disease. Neuropharmacology 59, 221–229.

Gilmartin, M.R., Balderston, N.L., and Helmstetter, F.J. (2014). Prefrontal cortical regulation of fear learning. Trends Neurosci. 37, 455–464.

Gonzalez-Rey, E., Chorny, A., O'Valle, F., and Delgado, M. (2007). Adrenomedullin protects from experimental arthritis by down-regulating inflammation and Th1 response and inducing regulatory T cells. Am. J. Pathol. 170, 263–271.

Guo, T., Noble, W., and Hanger, D.P. (2017). Roles of tau protein in health and disease. Acta Neuropathol. 133, 665–704.

Henriksen, K., O'Bryant, S.E., Hampel, H., Trojanowski, J.Q., Montine, T.J., Jeromin, A., Blennow, K., Lönneborg, A., Wyss-Coray, T., Soares, H., et al. (2014). The

future of blood-based biomarkers for Alzheimer's disease. Alzheimer's Dement. 10, 115–131.

Iqbal, K., Liu, F., Gong, C.-X., Alonso, A. del C., and Grundke-Iqbal, I. (2009). Mechanisms of tau-induced neurodegeneration. Acta Neuropathol. 118, 53–69.

Jacobs, E.G., Weiss, B.K., Makris, N., Whitfield-Gabrieli, S., Buka, S.L., Klibanski, A., and Goldstein, J.M. (2016). Impact of Sex and Menopausal Status on Episodic Memory Circuitry in Early Midlife. J. Neurosci. 36, 10163–10173.

Jaworski, J., Kapitein, L.C., Gouveia, S.M., Dortland, B.R., Wulf, P.S., Grigoriev, I., Camera, P., Spangler, S.A., Di Stefano, P., Demmers, J., et al. (2009). Dynamic Microtubules Regulate Dendritic Spine Morphology and Synaptic Plasticity. Neuron 61, 85–100.

Kato, J., Kitamura, K., Uemura, T., Kuwasako, K., Kita, T., Kangawa, K., and Eto, T. (2002). Plasma levels of adrenomedullin and atrial and brain natriuretic peptides in the general population: their relations to age and pulse pressure. Hypertens. Res. 25, 887–892.

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: New Roles for the Synaptic Stripper. Neuron 77, 10–18.

Kiss, J.Z., and Muller, D. (2001). Contribution of the neural cell adhesion molecule to neuronal and synaptic plasticity. Rev. Neurosci. 12, 297–310.

Lebron-Milad, K., Abbs, B., Milad, M.R., Linnman, C., Rougemount-Bücking, A., Zeidan, M.A., Holt, D.J., and Goldstein, J.M. (2012). Sex differences in the neurobiology of fear conditioning and extinction: a preliminary fMRI study of shared sex differences with stress-arousal circuitry. Biol. Mood Anxiety Disord. 2, 7.

Li, R., and Singh, M. (2014). Sex differences in cognitive impairment and Alzheimer's disease. Front. Neuroendocrinol. 35, 385–403.

Li, H.W.R., Liao, S.-B., Chiu, P.C.N., Yeung, W.S.B., Ng, E.H.Y., Cheung, A.N.Y., Tang, F., and O, W.S. (2015). Effects of adrenomedullin on the expression of inflammatory cytokines and chemokines in oviducts from women with tubal ectopic pregnancy: an in-vitro experimental study. Reprod. Biol. Endocrinol. 13, 120.

Ma, W., Chabot, J.-G., and Quirion, R. (2006). A role for adrenomedullin as a pain-related peptide in the rat. Proc. Natl. Acad. Sci. 103, 16027–16032.

Martínez-Herrero, S., Larrayoz, I.M., Ochoa-Callejero, L., Fernández, L.J., Allueva, A., Ochoa, I., and Martínez, A. (2016). Prevention of Bone Loss in a Model of Postmenopausal Osteoporosis through Adrenomedullin Inhibition. Front. Physiol. 7, 280.

Merz, C.J., Wolf, O.T., Schweckendiek, J., Klucken, T., Vaitl, D., and Stark, R. (2013). Stress differentially affects fear conditioning in men and women. Psychoneuroendocrinology 38, 2529–2541.

Miksa, M., Wu, R., Cui, X., Dong, W., Das, P., Simms, H.H., Ravikumar, T.S., and

Wang, P. (2007). Vasoactive hormone adrenomedullin and its binding protein: anti-inflammatory effects by up-regulating peroxisome proliferator-activated receptor-gamma. J. Immunol. 179, 6263–6272.

Nishimatsu, H., Suzuki, E., Nagata, D., Moriyama, N., Satonaka, H., Walsh, K., Sata, M., Kangawa, K., Matsuo, H., Goto, A., et al. (2001). Adrenomedullin induces endothelium-dependent vasorelaxation via the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. Circ. Res. 89, 63–70.

Pedreño, M., Morell, M., Robledo, G., Souza-Moreira, L., Forte-Lago, I., Caro, M., O'Valle, F., Ganea, D., and Gonzalez-Rey, E. (2014). Adrenomedullin protects from experimental autoimmune encephalomyelitis at multiple levels. Brain. Behav. Immun. 37, 152–163.

Pollak, D., Cairns, N., and Lubec, G. (2003). Cytoskeleton derangement in brain of patients with Down syndrome, Alzheimer's disease and Pick's disease. J. Neural Transm. Suppl. 149–158.

Portran, D., Schaedel, L., Xu, Z., Théry, M., and Nachury, M.V. (2017). Tubulin acetylation protects long-lived microtubules against mechanical ageing. Nat. Cell Biol. 19, 391–398.

Prokop, A. (2013). The intricate relationship between microtubules and their associated motor proteins during axon growth and maintenance. Neural Dev. 8, 17.

Reed, J.L., Gallagher, N.M., Sullivan, M., Callicott, J.H., and Green, A.E. (2017). Sex differences in verbal working memory performance emerge at very high loads of common neuroimaging tasks. Brain Cogn. 113, 56–64.

Rønn, L.C., Berezin, V., and Bock, E. (2000). The neural cell adhesion molecule in synaptic plasticity and ageing. Int. J. Dev. Neurosci. 18.

Rossi, G., and Tagliavini, F. (2015). Frontotemporal lobar degeneration: old knowledge and new insight into the pathogenetic mechanisms of tau mutations. Front. Aging Neurosci. 7, 192.

Sackett, D.L., Ozbun, L., Zudaire, E., Wessner, L., Chirgwin, J.M., Cuttitta, F., and Martínez, A. (2008). Intracellular proadrenomedullin-derived peptides decorate the microtubules and contribute to cytoskeleton function. Endocrinology 149, 2888–2898.

Sardi, C., Zambusi, L., Finardi, A., Ruffini, F., Tolun, A.A., Dickerson, I.M., Righi, M., Zacchetti, D., Grohovaz, F., Provini, L., et al. (2014). Involvement of calcitonin gene-related peptide and receptor component protein in experimental autoimmune encephalomyelitis. J. Neuroimmunol. 271, 18–29.

Solito, E., and Sastre, M. (2012). Microglia function in Alzheimer's disease. Front. Pharmacol. 3, 14.

Verstraelen, P., Detrez, J.R., Verschuuren, M., Kuijlaars, J., Nuydens, R., Timmermans, J.-P., and De Vos, W.H. (2017). Dysregulation of Microtubule Stability Impairs Morphofunctional Connectivity in Primary Neuronal Networks. Front. Cell. Neurosci. 11, 173.

Wong, L.Y.F., Cheung, B.M.Y., Li, Y.-Y., and Tang, F. (2005). Adrenomedullin Is Both Proinflammatory and Antiinflammatory: Its Effects on Gene Expression and Secretion of Cytokines and Macrophage Migration Inhibitory Factor in NR8383 Macrophage Cell Line. Endocrinology 146, 1321–1327.

Xia, C.-F., Yin, H., Borlongan, C. V., Chao, J., and Chao, L. (2004). Adrenomedullin gene delivery protects against cerebral ischemic injury by promoting astrocyte migration and survival. Hum. Gene Ther. 15, 1243–1254.

Yew, D.T., Li, W.P., Webb, S.E., Lai, H.W., and Zhang, L. (1999). Neurotransmitters, peptides, and neural cell adhesion molecules in the cortices of normal elderly humans and Alzheimer patients: a comparison. Exp. Gerontol. 34, 117–133.

Yuesong Gong, Y., and Lippa, C.F. (2010). Review: Disruption of the Postsynaptic Density in Alzheimer's Disease and Other Neurodegenerative Dementias. Am. J. Alzheimer's Dis. Other Demen. 25, 547–555.

Zeng, X., Lin, M.Y., Wang, D., Zhang, Y., and Hong, Y. (2014). Involvement of adrenomedullin in spinal glial activation following chronic administration of morphine in rats. Eur. J. Pain 18, 1323–1332.

Chapter VII
Conclusions

From the results obtained in the present Doctoral Thesis it can be concluded that:

1. Levels of *ADM* peptides are increased in prefrontal cortices from Alzheimer's disease (AD) patients, particularly in apical dendrites and axons of pyramidal neurons and increases in *ADM* peptides statistically correlate with the decrease in acetylated tubulin and NCAM. These observations suggest that altered *ADM* is associated with impaired microtubule function in AD patients, which might contribute to synaptic disconnections.
2. Decreased levels of several neurochemical alterations of several neurotransmitter (cholinergic, serotoninergic, dopaminergic and gabaergic) systems have been found in AD brains that may be associated to the alterations in *ADM* peptides.
3. The positive correlation between *ADM* gene products and activated microglia (Ox-42) in AD brains suggest that *ADM* may contribute to the neuroinflammatory processes involved in disease progression.
4. Conditional deletion of brain *adm* improves retention of contextual and recognition memories and reduces levels of Tau phosphorylation in aged female mice. In addition, human elders show higher levels of AM peptide and lower levels of acetylated tubulin in brains compared to mature individuals. Altogether, *ADM* may play a role in age-associated cognitive decline by affecting Tau phosphorylation and microtubule dynamics.
5. Levels of microtubule subunits βIII-tubulin acetylated- and detyrosinated-tubulin, but not levels of other cytoskeletal components, are significantly reduced in frontal cortex from frontotemporal lobar dementia (FTLD) patients that present with primary tauopathy. These observations suggest that primary Tau pathology specifically affects microtubule cytoskeleton.
6. Decreased levels of free *ADM* peptides but no change in tubulin-bound *ADM* have been found in frontal cortex from FTLD-Tau patients. Since the reduction of *ADM* peptides is not associated to reduced levels of microtubule components it is suggested that reduced levels of *ADM* might not be directly linked to microtubule dismantle in FTLD-Tau but they may rather stem from a likely adaptive response of neurons to mitigate microtubule disruption.
7. Finally, all these results provide new insight into the role of *ADM* peptides in neurodegenerative diseases. Although further studies are needed, AM/PAMP

somehow intervene in these diseases, and therefore the modulation of *ADM* peptides should be considered as a new pharmacological approach for neurodegenerative dementias

De los resultados obtenidos en ka oresente Tesis Doctoral se puede concluir que:

1. Los niveles de los péptidos de *ADM* están aumentados en la corteza frontal de pacientes con enfermedad de Alzheimer (EA), particularmente en dendritas apicales y axones de neuronas piramidales y correlacionan con niveles disminuidos de tubulina acetilada y NCAM. Estas observaciones sugieren que la alteración de *ADM* está asociada a la función alterada de los microtúbulos en pacientes EA, lo cual podría contribuir a la desconexión sináptica.
2. Alteraciones neuroquímicas de varios sistemas neurotransisores (colinérgico, serotonérgico, dopaminérgico y gabaérgico) en cerebros con EA podrían asociarse a las alteraciones de los péptidos *ADM*.
3. La correlación positiva entre los productos del gen *ADM* y la microglia activada (Ox-42) en cerebros EA sugiere que *ADM* podría contribuir a los procesos de neuroinflamación involucrados en la progresión de la enfermedad.
4. La supresión condicional de *adm* en el cerebro mejora la retención de recuerdos contextuales y de reconocimiento y reduce los niveles de fosforilación de Tau en ratones hembras envejecidas. Además, personas envejecidas muestran niveles más elevados del péptido AM y niveles reducidos de tubulina acetilada que en persona de mediana edad. Con todo ello, *ADM* podría jugar un papel en declive cognitivo asociado a la edad al afectar a la phosphorilación de Tau y a la dinámica de microtúbulos.
5. Los niveles βIII-tubulina junto con la acetilación y detyrosinación de tubulina, se encuentran disminuidos significativamente en la corteza frontal de pacientes con demencia lobar frontotemporal (DLTF) que presentan tautopatía primaria, sin embargo no ocurre así con otros componentes del citoesqueleto. Estas observaciones sugieren que la patología Tau primaria afecta específicamente a los microtúbulos del citoesqueleto.
6. Se han encontrado niveles reducidos de peptidos de *ADM* libre pero no en los niveles de *ADM* unida a tubulina en la corteza de pacientes DLTF-Tau. Como la reducción de los péptidos *ADM* no está asociada a niveles

reducidos de componentes de microtúbulos, se sugiere que los niveles reducidos de ADM podrían no estar directamente relacionados con el desemsamblaje de los microtúbulos en DLTF-Tau, sino que podrían derivarse de una posible respuesta adaptativa de las neuronas para mitigar la ruptura de los microtúbulos.

7. Finalmente, todos estos resultados proporcionan una nueva visión del papel de los péptidos *ADM* en enfermedades neurodegenerativas. Aunque se necesitan más estudios, los péptidos *ADM* de alguna manera intervienen en estas enfermedades, por lo que su modulación debe considerarse como un nuevo enfoque farmacológico para las demencias neurodegenerativas.