Instituto de Neurociencias
Consejo Superior de Investigaciones Científicas
Universidad Miguel Hernández

Cross-talk between Acetylcholinesterase and Presenilin 1, implications for Alzheimer's disease

Doctoral Thesis presented by:

Maria-Letizia Campanari

Directors:

Dr. Javier Sáez Valero

Dra. María Salud García Ayllón

Sant Joan d' Alacant, 2014

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Prof. Juan Lerma
DIRECTOR

Prof. **Juan Lerma Gómez**, Director del Instituto de Neurociencias, centro mixto del Consejo Superior Investigaciones Científicas, CSIC y la Universidad Miguel Hernández, UMH

CERTIFICA

Que la Tesis Doctoral "**Cross-talk between Acetylcholinesterase and Presenilin 1, implications for Alzheimer's disease**", ha sido realizada por D[a]. Maria-Letizia Campanari, Licenciada en Neurociencias, bajo la dirección del Dr. Javier Sáez Valero y la Dra. Maria Salud García Ayllón y da su conformidad para que se presente el borrador de tesis a la Comisión de Doctorado de la Universidad Miguel Hernández.

Para que así conste, a los efectos oportunos, firma el presente certificado en San Juan de Alicante a 20 de Marzo de 2014.

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Fdo.: Juan Lerma Gómez

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El Dr. **Javier Sáez Valero**, Profesor Titular del Departamento de Bioquímica y Biología Molecular de la Universidad Miguel Hernández (UMH) e Investigador adscrito al Instituto de Neurociencias de Alicante, centro mixto UMH-Consejo Superior de Investigaciones Científicas (CSIC), y la Dra. **María Salud García Ayllón**, Investigadora Miguel Servet de la Fundacion para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana en el Hospital General Universitario de Elche.

CERTIFICAN

Que Dª. Maria-Letizia Campanari, Licenciada en Neurociencias, ha realizado bajo su dirección el trabajo experimental que recoge en su Tesis Doctoral "**Cross-talk between Acetylcholinesterase and Presenilin 1, implications for Alzheimer's disease**".

Que han revisado los contenidos científicos y los aspectos formales del trabajo y dan su conformidad para que se presente el borrador de tesis a la Comisión de Doctorado de la Universidad Miguel Hernández.

Para que así conste, y a los efectos oportunos, expiden y firman el presente Certificado en San Juan de Alicante a 20 de Marzo de 2014.

Fdo.: Javier Sáez Valero

Fdo. : María Salud García Ayllón

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A Federico, Valentina, Pierrick e Neve

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En primer lugar me gustaría agradecer a mis jefes, Javier y Salu. Lo que habeis hecho para mí durante este doctorado tiene un valor enorme. Gracias por haberme apoyado en mis inicios en la ciencia. Gracias por vuestra paciencia con mis errores. Nunca olvidaré (¡espero!) vuestros consejos, vuestras atenciones y todas las tonterías que hacen de vosotros los mejores jefes del mundo.

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(^{a}): amphiphilic

(^{na}): non-amphiphilic soluble

A: asymmetric form of AChE

ACh: acetylcholine

AChE: acetylcholinesterase

AChE-I: acetylcholinesterase inhibitors

AChE-H: hydrophobic AChE

AChE-R: readthrough AChE

AChE-T: tailed AChE

AD: Alzheimer Disease

ADAM: a disintegrin and metalloprotease family

AICD: amyloid intracellular domain

Aph-1: anterior pharynx-defective 1

ApoE: apolipoprotein E

APP: amyloid β-protein precursor

Asp: aspartate

Aβ: amyloid-β peptide

Aβ40 and Aβ42: soluble amyloid beta peptide 1-40 and 1-42

BACE or Asp2: Aβ-site APP cleaving enzyme

BuChE: butyrylcholinesterase

ChAT: choline acetyl transferase

ChEs: cholinesterases

CSF: cerebrospinal fluid

CTF: C-terminus fragment

ER: endoplasmic reticulum

FAD: familiar AD

FHB: four-helix bundle motive

G: globular form of AChE

MAPs: microtubule-associated proteins

NCT: nicastrin

NFT: neurofibrillary tangles

NGF: nerve growth factor

GPI: glycosyl phosphatidyl inositol

GSK3β: glycogen synthase kinase 3 β

HMW: high-molecular weight

M: muscarinic ACh receptor

MAPs: microtubule-associated proteins

NCT: nicastrin

NFT: neurofibrillary tangles

NGF: nerve growth factor

NMDAR: N-methyl-d-aspartate receptor

NMJ: neuromuscular junction

NO: nitric oxide

NTF: N-terminus fragment

P-tau: phospho-tau

PAS: peripheral binding anionic site

Pen-2: presenilin enhancer 2

PHF: paired helical filaments

PNS: peripheral nervous system

PRAD: proline rich anchor domain sequence

PRiMA: proline rich membrane anchor

PS1 and PS2: presenilin 1 and presenilin 2

TACE or ADAM-17: tumour necrosis factor-K (TNFK)-converting enzyme

TGN: trans-golgi network

TMD: transmembrane domain

T-tau: total amount of tau

WAT: tryptophan amphiphilic tetramerization motive

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of memory and cognition, wherein a deterioration of cholinergic synapses occurs in hippocampus and neocortex. Decreased concentration of the neurotransmitter acetylcholine (ACh) appears to be a contributing factor in the development of the dementia. In this regard, current therapeutic approach to treat AD symptoms is based in the inhibition of acetylcholinesterase (AChE), the enzyme responsible for ACh degradation. The AD brain is characterized by co-existence of amyloid plaques, extracellular protein deposits where the major component is the β-amyloid peptide (Aβ), and of neurofibrillary tangles (NFT), composed of paired helical filaments of the microtubule-associated protein tau abnormally hyperphosphorylated (P-tau). The Aβ peptide is a small polypeptide generated by processing of a much larger transmembrane protein, the β-amyloid precursor protein (APP) through the successive action of two proteolytic enzymes, β-secretase and γ–secretase. γ-Secretase is an intramembranous multi-protein complex that cleaves many type-I proteins with critical roles in neuronal function. Presenilin-1 (PS1) is the catalytic component of the γ-secretase complex. Our group has previously demonstrated by reciprocal co-immunoprecipitation that AChE can interact with PS1. Moreover, AChE can modulate PS1 levels. In cellular models AChE over-expression increases PS1 levels, while AChE knock-down with siRNA leads decrease level of PS1 protein in transfected cells.

Our present study addresses how AChE influences PS1 expression by examining changes in PS1, at both protein and transcriptional levels, in several conditions where distinct AChE variant and molecular forms have been modulated. We demonstrate a modulation of PS1 by AChE variants via non-cholinergic mechanisms,

and by a mechanism independent of its catalytic activity. Our data also suggest that AChE may function as an inhibitor of γ-secretase activity, and provide evidence that γ-secretase inhibition could result in PS1 up-regulation.

In this regard, we have re-analyzed the expression levels of AChE in the brain of AD subjects. We demonstrated by Western blotting and immunohistochemistry that a prominent pool of enzymatically inactive AChE protein existed in the AD brain. The potential significance of these unexpected levels of inactive AChE protein in the AD brain may is of relevance in the context of protein-protein interactions with PS1.

Conversely, we have also studied the influence of PS1/ γ-secretase activity on AChE. The major AChE variant expressed in the brain is a tetramer (G4) of four catalytic subunits attached to the plasma membrane by a proline-rich membrane anchor subunit (PRiMA). We demonstrate that PS1 participates in AChE processing, cleaving PRiMA with the consequent release of a C-terminal PRiMA fragment. We are also able to localize PRiMA immunoreactivity in the nucleus, which suggest the possibility that the PRiMA segment participates in the regulation of gene transcription, a possibility that deserves thoroughly investigation.

Understanding the relationship between PS1 and AChE may be useful not only for the physiopathology of the disease, but also to develop more effective Alzheimer's therapies.

La enfermedad de Alzheimer (EA) es una enfermedad neurodegenerativa caracterizada por la pérdida progresiva de memoria y capacidades cognitivas, siendo especialmente afectadas las áreas y conexiones colinérgicas del hipocampo y neocortex. Así, la depleción en los niveles del neurotransmisor acetilcolina (ACh) aparece como un factor de importancia en el desarrollo y progresión de la demencia. En este contexto, las actuales terapias paliativas con las que se trata la EA se basan en mantener niveles altos de ACh, mediante la inhibición de la enzima que hidroliza al neurotransmisor, la acetilcolinestarase (AChE). En el cerebro de sujetos con EA co-existen dos entidades neuropatológicas que la caracterizan, los depósitos proteinaceos extracelulares, cuyo componente mayoritario es el péptido β-amiloide (Aβ), y los ovillos neurofibrilares intraneuronales, un conglomerado anormal de proteínas compuesto por pequeñas fibrillas entrelazadas de la proteína citoesquelética tau anormalmente hiperfosforilada (P-tau).

El Aβ es un pequeño polipeptido, de 40 a 42 aminoácidos de longitud en sus isoformas mayoritarias, generado tras el procesamiento de una proteína de transmembrana tipo I denominada precursor de la proteína amiloide o APP. El Aβ se genera tras el procesamiento secuencial del APP por las enzimas proteolíticas β-secretasa y γ–secretasa. La actividad γ-secretasa es llevada a cabo por un complejo proteico que procesa un gran número de proteínas transmembrana tipo I con un corto domino intracelular, muchas de ellas de conocida importancia en la función neuronal. La enzima presenilina-1 (PS1) es el componente catalítico del complejo γ–secretasa. Nuestro grupo ha demostrado previamente, mediante experimentos de co-inmunoprecipitación recíproca, que AChE puede interaccionar con PS1. Además, AChE aparece como una proteína

moduladora de los niveles de PS1. En modelos celulares, la sobre-expresión de AChE promueve aumentos de PS1, mientras que el silenciamiento de la expresión de la colinesterasa causa disminución de PS1 en células transfectadas con siRNA de AChE.

El estudio recogido en esta memoria se adentra en el mecanismo de dicha interacción, analizando la influencia de las diferentes variantes y formas moleculares de AChE en los niveles de expresión de PS1. Demostramos que la modulación de PS1 por AChE es llevada a cabo por mecanismo no colinérgicos, y en concreto independientes de la capacidad catalítica de AChE, dado que mutantes inactivos de la proteína mantienen la capacidad de influenciar los niveles de PS1. Nuestros datos indican que AChE puede funcionar como un inhibidor de la γ-secretasa, y, lo que es más importante, sugieren que la inhibición de la actividad γ-secretasa puede promover, mediante un mecanismo compensatorio, el aumento de expresión de su componente activo, la PS1.

En nuestros estudios también hemos re-analizado los niveles de expresión de AChE en extractos cerebrales de sujetos con EA; hasta ahora la inmensa mayoría de los estudios clásicos habían abordado tan solo los niveles de actividad enzimática. Nuestro análisis mediante técnicas de Western blotting e inmunohistoquímica indican que mientras los niveles de actividad están disminuidos, los de proteína AChE aparecen preservados, lo que demuestra la existencia de un importante reservorio de proteína AChE no activa en el cerebro afectado por la patología. El potencial papel y significado fisiopatológico de esta AChE inactiva en el cerebro de enfermos de EA requiere ser analizado, con mayor interés si cabe a la vista de la capacidad de AChE de influir sobre PS1 mediante interacciones proteína-proteína independientes de su actividad catalítica.

Finalmente, hemos estudiado la influencia recíproca de PS1/ γ-secretasa sobre AChE, y en concreto la posibilidad de que la actividad secretasa participe del procesamiento de AChE. La variante de AChE mayormente expresada en el cerebro humano es un tetrámero de subunidades catalíticas de AChE (G4) anclada a la membrana mediante una subunidad estructural rica en prolina y denominada PRiMA, una proteína transmembrana de tipo I. Hemos demostrado que PS1 participa en el procesamiento de AChE, actuando sobre PRiMA y liberando un pequeño fragmento intracelular de la misma. Caracterizamos y localizamos dicho fragmento de PRiMA en el núcleo, lo que sugiere su potencial participación en mecanismo de regulación transcripcional, una posibilidad que abre una nueva vía de estudio que puede resultar de importancia tanto en condiciones fisiológicas como patológicas.

Una mayor comprensión de las complejas inter-relaciones de PS1 y AChE puede ser de importancia no sólo para avanzar en el conocimiento de los mecanismos fisiopatológicos afectados en la demencia, sino también para el desarrollo de nuevas y mejoradas estrategias terapéuticas para el tratamiento de la EA.

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CHAPTER 1: INTRODUCTION

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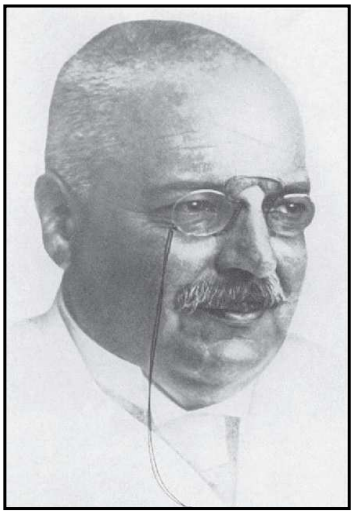
1. Alzheimer's Disease

In 1907, in the article "UbereineeigenartigeErlranliung der Hirnrinde", the Bavarian psychiatrist Alois Alzheimer described for the first time the special disease of a patient called Auguste D, a 51-year-old woman from Frankfurt who had shown progressive cognitive impairment. From 1901, Alzheimer followed Auguste D's case until her death in 1906. After that, using the newly developed Bielschowsky's silver staining method, Alzheimer observed and described the anatomical features of a new disease, different from all the others known at this time. It was named after his name in 1910 by Emil Kraepelin (Alois Alzheimer's superior) (Ramirez-Bermudez 2012). For this reason, he is considered to be the founding father of the neuropathology.



Alois Alzheimer

He reported:

…She is entirely disoriented to time and place. Once in a while she makes comments that she does not understand anything going on; or has lost track of things…

…A single one or a few fibrils come to prominence on the inside of an otherwise still "normal" appearing cell. Then, during further progression, many such fibrils running next to each other show changes in the same way. They subsequently fold together into dense bundles and move towards the cell surface. Eventually the nucleus and the cell disintegrate, and only a tangled bundle of fibrils indicates the place which had formerly been occupied by a ganglion cell. Since these fibrils are stainable with other dyes than normal neurofibrils, a chemical transformation of the fibril substance must have taken place; which appears to be the cause

for the fibrils' persistence after demise of the cell. The transformation of the fibrils seems to go hand in hand with the deposition of a not yet more closely examined pathological metabolic product into the ganglion cell… (Alzheimer et al 1995).

1.1 Social Impact

There are 7.7 million new cases of dementia each year, implying that there is a new case of dementia somewhere in the world every four seconds. Alzheimer's disease (AD) is responsible for 60-80% of all these dementia cases, which represented in 2010 an estimated 35.6 million people suffering of this senile dementia worldwide (from WORLD ALZHEIMER REPORT 2013). Nowadays, this disease is considered as a major public health problem, affecting 30% of aged people in the Western world.

Clinically, AD is an irreversible, progressive brain disease that slowly impairs memory and cognitive skills. Symptoms of AD usually develop slowly and gradually worsen over time, progressing from mild forgetfulness to widespread brain impairment. The earliest changes that are part of the pathology of AD usually occur in medial temporal lobe structures (e.g., hippocampus, entorhinal cortex; [see (Braak & Braak 1991)], interrupting the neural network critical for episodic memory function. Thus, the ability to learn and remember new information (i.e., anterograde amnesia) is the clinical hallmark of AD pathology. However, many changes occur in cortical areas (i.e., posterior cingulate, inferior parietal lobule, lateral temporal neocortex, ventromedial and dorsomedial prefrontal cortex) that project heavily to medial temporal lobe structures (Buckner et al 2008). All these chemical and structural changes in the brain slowly destroy the ability to create, remember, learn, reason, and relate to others. Drastic

personality loss occurs and the body systems fail (Weintraub et al 2012).

1.2. Genetics

From a genetic point of view, AD is divided in two forms: the genetic or familiar AD (FAD) and the sporadic AD (referred hereafter as AD).

1.2.1 Familiar AD

Familiar AD (FAD) represents less than 1-5% of all AD cases. It is also known as early-onset AD as it can begin in the second decade of life, however not all the early-onset AD cases are responding to genetic predispositions. The first candidate gene for FAD to be discovered was the gene encoding the amyloid β-protein precursor (APP). Since that, 30 AD-causing mutations on APP gene have been reported, but they all together explain only one-tenth of all FAD cases (Cruts & Van Broeckhoven 1998). Interestingly, most of the mutations in APP gene occur around the putative γ-secretase cleaving site, which generates the amyloid-β peptide (Aβ), suggesting the critical implication of this proteolytic enzyme in FAD (Goate et al 1991). Indeed, most of the mutations linked to FAD are identified in the Presenilin-1 (PS1) gene, that encodes the catalytic subunit of the γ-secretase complex; also mutations in its close homolog Presenilin-2 (PS2) gene, which protein can substitute PS1 as the catalytic part of the γ-secretase complex, has been identified (Lleo et al 2002, Thinakaran & Parent 2004). All the mutations identified in FAD share a common feature, all increase the generation of the amyloidogenic isoforms of Aβ (see section 3.2.1).

The discovery of these genetic mutations also has allowed researchers to create transgenic animal models that display some

important aspects of the disease and serve as a basis of AD research.

1.2.2 Sporadic AD

Sporadic AD is associated to aging and is usually diagnosed after age 65. The sporadic is the major form of the disease and is not caused by a mutation in a single protein. In fact, multiple genetic and environmental risk factors have been related with the progression of this disease.

The strongest genetic risk factor associated to AD is the ε4 allele of the apolipoprotein E (*APOE*) gene on chromosome 19. ApoE regulates lipid homeostasis by mediating lipid transport from one tissue or cell type to another. In the brain, ApoE is mainly produced by astrocytes and transports cholesterol to neurons via ApoE receptors, which are members of the low-density lipoprotein receptor (LDLR) family [for a review see (Liu et al 2013)]. The human *APOE* gene exists as three polymorphic alleles, ε2, ε3 and ε4, which have a frequency of 8.4%, 77.9% and 13.7%, respectively. However, the frequency of the ε4 allele is dramatically increased to ~40% in patients with AD. A meta-analysis of clinical and autopsy-based studies demonstrated that, compared with individuals with an ε3/ε3 genotype, risk of AD was increased in individuals with one copy of the ε4 allele (3-4 times) or two copies (~15 times) among Caucasian subjects (Farrer et al 1997). The biochemical consequences of APOEε4 in AD pathogenesis are not yet fully understood. Current hypotheses propose that APOEε4 predisposes patients to increased plasma cholesterol levels and has direct toxic effects on the cerebrovascular system. High plasma cholesterol, in turn, has been correlated with increased Aβ deposition and stabilization in the AD brain (Zlokovic 2013). Interestingly, in APOEε4 homozygous patients, the pathological process differed from that typically seen in AD. These patients showed a heavy burden of

perivascular tau-immunopositive cell processes associated with severe amyloid beta protein angiopathy, neurofibrillary tangles, some cortical Lewy bodies and an absence of neuritic plaques (Vidal et al 2000).

Other genetic risk factors have been described, moreover in the last 10 years but their contribution is probably minor (Tanzi 2013). In parallel, sedentary and smoking behavior, obesity and head trauma are usually accepted as common risk factors of sporadic AD (Borenstein et al 2006).

1.3. Pathogenesis

At the microscopic level, the characteristics lesions described by Alzheimer are now defined as extracellular *senile or neuritic plaques*, deposits of the β-amyloid protein (Aβ), and intracellular *neurofibrillary tangles* (NFTs), composed of paired helical filaments of the microtubule-associated protein tau abnormally hyperphosphorylated (P-tau).

1.3.1 Senile Plaques

The amyloid beta (Aβ) peptide, at sufficiently high concentration, form a beta sheet-rich tertiary structure that aggregates to form amyloid fibrils that deposit outside neurons in dense formations known as *senile plaques* or neuritic plaques and in less dense aggregates called *diffuse plaques*. In some cases, Aβ can aggregate inside the walls of small blood vessels in the brain in a process called *amyloid angiopathy*. Senile plaques, together with the amyloid angiopathy and the abundance of microglia and astrocytes, are typical degenerative structures of AD. Diffuse plaques are more considered a byproduct of senescence or biological aging.

Large numbers of senile plaques is characteristic features of AD (Figure 1).

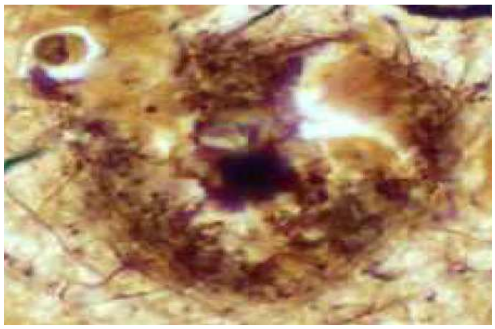


Figure 1. Human neuritic plaque. Large extracellular plaque with amyloid core from a patient who died of AD. The histologic section of neocortex has been stained with a modified Bielschowsky method (Schellenberg & Montine 2012).

As mentioned above, Aβ represents the core of the amyloid plaques in AD and, depending by the different sequential proteolysis of the largest β-amyloid precursor protein (APP), we can distinguish different forms of different lengths. Aβ40 and Aβ42 (with 40 and 42 amino acids of lenght) are the two major forms in AD. An immunohistochemical study revealed that the longer (Aβ40 and Aβ42) and shorter (Aβ17) Aβ peptides are differently distributed along the various types of amyloid deposits in AD. In fact, while the amyloid angiopathy and senile plaques are constituted of both longer and shorter Aβ peptides, the diffuse plaques have Aβ17 peptides as its principal component (Rabano et al 2005).

APP is a single pass transmembrane protein, with a large external N-terminal and a short cytosolic C-terminal, thus, classified as a Type-I protein. It is characterized by the presence of a β-domain of 39/43 amino acids partly located at the ectodomain and mainly within the transmembrane domain (TMD) (Thinakaran & Koo 2008). Three

proteases, called α-, β- and γ-secretase, are involved in the APP specific processing. The name "secretases" refers to the secretion of the proteolytically cleaved substrates. The activities of the three proteases are described below.

In the APP processing, we discriminate two principal pathways: the *amyloidogenic pathway*, which leads to Aβ generation; and the *non-amylodoigenic pathway*, which prevents Aβ generation (Figure 2).

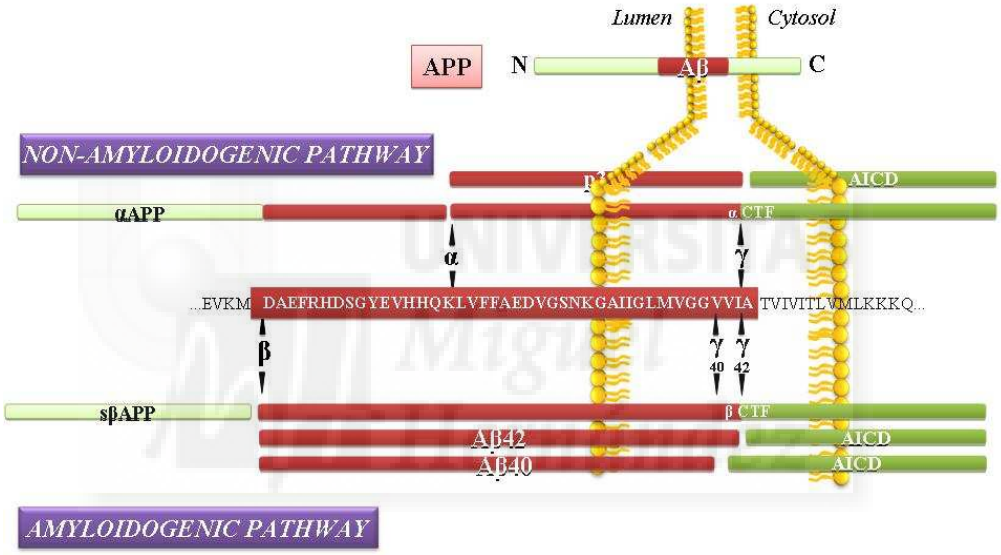


Figure 2. Proteolytic processing of APP. A schematic structure of APP is shown with the Aβ domain in red. In the non-amyloidogenic processing of APP the α-secretase cleaves within the Aβ domain, thus precluding the generation of an intact Aβ peptide. The amyloidogenic processing of APP is carried out by sequential action of membrane β- and γ-secretase. sAPPβ: soluble ectodomain APPβ; sAPPα: soluble ectodomain APPα; CTF: C-terminal fragment; AICD: Amyloid intracellular domain.

In normal conditions, most of the APP molecules are cleaved by the α-secretase, leading the non-amylodoigenic pathway. Several zinc metalloproteinases, members of the "A Disintegrin And Metalloprotease" (ADAM) family, such as ADAM9, ADAM10,

TACE/ADAM17 and ADAM19, can function as α-secretases (Allinson et al 2003). Recent evidence suggests that, at least in neurons, the principal constitutive α-secretase activity is exerted by ADAM10 (Kuhn et al 2010). α-Secretase cleaves APP extracellulary, within the β domain inducing the shedding of nearly the entire ectodomain. This cut generates an α- C-terminal fragment (α-CTF) bound to the membrane. Subsequently, γ-secretase cut inside the transmembrane domain of the α-CTF and generates a cytoplasmic polypeptide termed AICD and a non-fibrilar 3 kDa peptide that is released in the medium (p3).

In the alternative amylodogenic pathway, APP is first cleaved by β-secretase. β-secretase mediates the initial and the rate-limiting processing step during Aβ generation. β-secretase activity has been identified in a unique enzyme which originally was referred with different names, such as memapsin, aspartyl protease 2, or BACE1 (β-site APP cleaving enzyme-1); BACE1 is now the generally accepted term for the enzyme harboring the β-secretase activity [for a review see: (Wang et al 2013a)]. β-Secretase also cleaves APP at the ectodomain, generating the N-terminal of the Aβ fragment (see Figure 2). The resulting membrane-bound C-terminal fragment, as in the non-amylodoigenic pathway, is a γ-secretase substrate, releasing the cytoplasmic polypeptide AICD and the Aβ peptides. γ-secretase cleaves within the membrane-spanning domain of APP at multiple potential cleavage sites, thus generating peptide isoforms of 36–43 amino acids. The Aβ40 is the most common specie, but the Aβ42 variant is the most amyloidogenic form of the peptide and more intimately associated to AD progression [for a review see: (Steiner et al 2008)].

γ-Secretase is an intramembranous protease complex, composed of four components: presenilin-1 (PS1), nicastrin (NCT), presenilin enhancer 2 (Pen-2) and anterior pharynx-defective 1 (Aph). PS1 constitutes the catalytic domain of γ-secretase (for further details see

section 3.1). The APP protein processing, to produce Aβ peptide, has been described *in vitro* in several cellular membranes, including the endoplasmic reticulum (ER), the trans-golgi network (TGN), the early and late endosomes, recycling endosomes, and in lysosomes (Choy et al 2012). Despite large localization of PS1 and BACE1 within the endoplasmic reticulum and golgi, it is assumed that APP cleavage occurs on the surface and in endosomes/lysosomes compartments, where the proteolytically active PS1/γ-secretase is principally localized [for a review see: (Haass et al 2012)]. Moreover, it has been suggested that the amyloidogenic processing occurs in the cholesterol- and sphingolipid-enriched membrane raft microdomains of intracellular organelles and cell surface (Vetrivel et al 2004).

Under normal conditions, both, amyloidogenic and non-amyloidogenic pathways co-exist and Aβ is found in appreciable amounts in the non-pathological human brain. The Aβ peptide can be degraded in the brain by several peptidases including the insulin-degrading enzyme, neprilysin, and the endothelin-converting enzyme (Finder 2010, Miners et al 2011). Moreover, Aβ is also cleared from the brain in a process balanced by the efflux and the influx across the blood–brain barrier. It is a matter of controversy whether disturbance in Aβ clearing mechanism contributed to AD. Indeed, it has been proposed that Aβ accumulation in the brain, but not necessarily his production, is the event leading to neuronal degeneration and dementia. The so-called *Amyloid Cascade hypothesis* was proposed in 1991 by John Hardy and David Allsop (Hardy & Allsop 1991, Hardy & Higgins 1992) and reformulated during the last decade to focus on oligomeric aggregates of Aβ as the prime toxic species causing AD (Hardy & Selkoe 2002). The Amyloid Cascade hypothesis is supported by the findings that the unique mutations identified in FAD are present in the genes that encodes both the substrate (APP) and the proteolytic

enzymes (presenilins) responsible of Aβ generation [for a review see: (Karran et al 2011)].

To date, it is accepted that the initiation of the neurotoxic downstream changes in AD is due to the increase of Aβ diffusion and assembly, to form, from monomers of 4 kDa, Aβ oligomers of 8, 12 kDa and larger size aggregates (Lue et al 1999). The dynamic and the mechanism (or mechanisms) of toxicity of these soluble Aβ species are still unclear. Moreover, Aβ peptides can also operate inside the cells. They accumulate in distal neurites and synaptic compartment compromising the synaptic activity. Part of this pool can be subsequently secreted or released as a consequence of degenerating neuronal processes (Gouras et al 2005). Recently, it has been shown in SH-SY5Y neuroblastoma cells that the intracellular Aβ is not preferentially localized to any particular organelle and, to a large extent, is secreted from the cells (Zheng et al 2013). Soluble Aβ monomers and oligomers finally diffuse and associate to gradually form the extracellular senile plaques that may further disrupt neuronal circuits.

Several studies tried to correlate the progressive cognitive impairment and the morphological alterations in human autopsied brain. Surprising, is not the increase of insoluble Aβ aggregation to correlate with the severity of the disease, or the numbers of amyloid plaques, but the smaller soluble pool, that can move and interact with many other proteins, changing their functions (McLean et al 1999). Many studies in human demonstrated that the highest statistical correlation between cognitive impairment and morphological alterations, is the cortical levels of soluble Aβ and the extent of synaptic loss [for a review see: (Shankar & Walsh 2009)]. It seems in fact that the synaptic function is compromised before its physical degeneration (Alonso-Nanclares et al 2013).

1.3.2 Neurofibrillary Tangles

Tau is a soluble Microtubule-Associated Protein (MAPs) that, inside a neuron, participates in several physiological functions including microtubule assembly and stability, vesicle transport, neuronal outgrowth and neuronal polarity (Grundke-Iqbal et al 1986, Weingarten et al 1975). These functions are strictly regulated by the degree of tau phosphorylation (Figure 3).

In a pathological state, tau is abnormally hyperphosphorylated at certain residues and displays different solubility, as well tends to form clusters of paired helical filaments (PHF) (Wang et al 2013b).

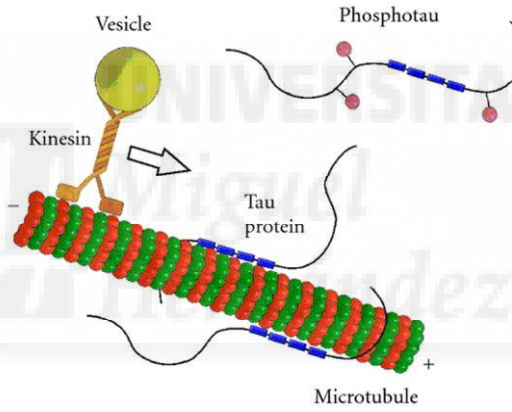


Figure 3. Normal function of tau protein. Tau protein stabilizes microtubules through the tubulin binding domains (blue boxes). The binding is maintained in equilibrium by coordinated actions of kinases and phosphatases. The phosphorylation of tau (pink balls) regulates its activity to bind to microtubules and can affect axonal transport. Tau protein may inhibit the plus-end-directed transport of vesicles along microtubules by kinesin (Kolarova et al 2012).

PHF are filamentous structures of a modified version of tau, highly stable to proteolysis, insoluble and toxic, able to aggregate to form the neurofibrillar tangles (NFTs). The core of a PHF is composed of hyperphosphorylated tau that can be truncated at the C- and N-

terminals. It has been proposed that such truncations favored tau polymerization and the subsequent NFT formation (Fasulo et al 2000, Guillozet-Bongaarts et al 2005, Wischik et al 1988a, Wischik et al 1988b). The PHF-core tau is not only unable to bind tubulin, but also binds normally-phosphorilated tau, sequestering it and blocking its physiological function (Alonso et al 1994). The final results are the disruption of microtubules, of the cellular structure and of the cargo transport. In the hippocampus, the amygdala and the cerebral cortex of AD patients, tau is mislocalized and shows a prominent immunoreactivity in the somatodendritic compartment of the neurons (Figure 4).

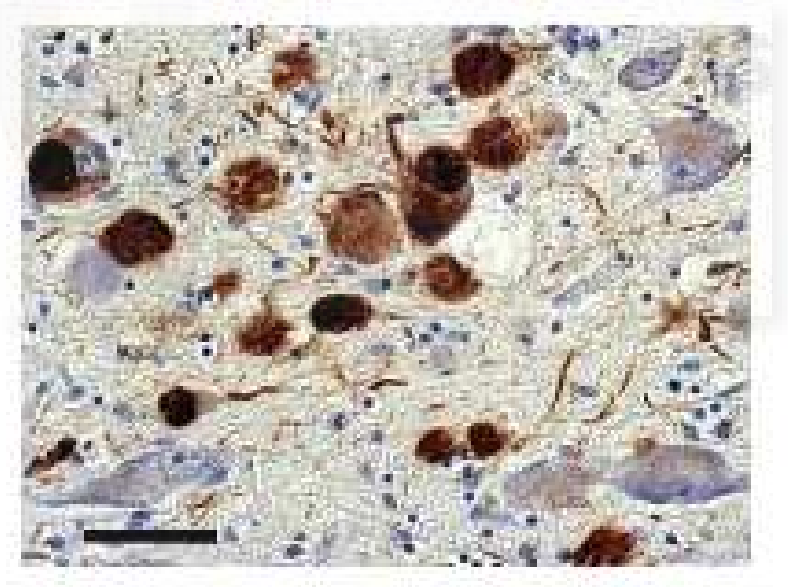


Figure 4. NFTs Immunostaining with antibody against phospho-tau protein. Cholinergic neurons from the nucleus basalis of Meynert (NbM) show NFTs mainly distributed in the perinuclear area of the neuron and in proximal processes. Midstage (AD). Scale bar = 50μM. (Nelson et al 2009).

Althought the mechanism of tangles formation is not completely understood, many researchers proposed the *Tau hypothesis* of AD, where tau protein dysfunction is the primary pathological event that

brings to neurofibrillary tangles formation, susceptibility to Aβ toxicity, degeneration and dementia (Gotz et al 2011).

Despite that in the AD field, the "Amyloid" is the dominant hypothesis and it is still a matter of controversy whether tangles are also a primary causative factor in AD, or play a more peripheral role.

1.4 Diagnosis

An early and accurate diagnosis of AD during life is essential. The diagnostic of AD requires careful evaluation of the patient medical history, mental status and physiological condition through tests (such as blood tests and brain imaging) to rule out the dementia-like symptoms. Recognize an AD case largely depend on the exclusion of other dementias and in any case cannot be diagnosed before the disease become severe and the memory impaired. Thus, potential biomarkers are of capital importance.

Both, Aβ and P-tau are key pathological effectors, but also they are recognized biomarkers that can be monitored in the cerebrospinal fluid (CSF). Numerous laboratories have reported an increase in P-tau and total tau (T-tau) levels in CSF, although tau alone lacks of specificity since it is also increased in other neurological processes (Rosen et al 2013). Abnormal metabolism of Aβ is considered a more specific phenomenon related to AD. The increasing deposition of the Aβ peptide, especially of the Aβ42 form, determines that its level in CSF is decreased while pathological Aβ42 species are increased in the AD brain (Blennow et al 2010).

The combination of Aβ42 and P-tau/T-tau leads to high (~80%) levels of sensitivity, specificity, and diagnostic accuracy. However, there is a continuing search for new CSF (or blood) biomarkers to improve the clinical diagnosis, especially on the early stages of AD, and the clinical trials [reviewed in (Cedazo-Minguez & Winblad 2010)].

1.5 The cholinergic hypothesis of AD

For a long time AD was considered a cholinergic disease treatable. Today, AD is known to be an enormous complex of cellular and biochemical changes inside the brain, where cell death is only the late step of several dysfunction cascades.

A global vision of AD, considering senile plaques and NFT as final conditions, leads to several questions. First of all, where does AD happen and where does it start?

Comparing a normal aged brain (left) and the brain of a person with Alzheimer's (right), there is a massive neuronal atrophy in the AD brain that affects many regions like the temporal, frontal and parietal lobe of the cortex, the limbic structures, like the hippocampus and the amygdale. In addition, the ventricles, that contain CSF, are noticeably enlarged (Figure 5).

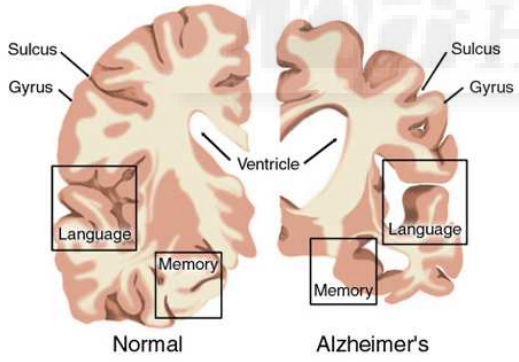


Figure 5. Healthy brain versus Alzheimer's Brain. View of the massive cell loss, that in AD change the whole brain conformation. See the text.

Cholinergic neurons, coming from the medial septum of the brain and directed to the hippocampus, neocortex and amygdale, are the first to undergo protein changes, that can be defined at the beginning of AD (Muir 1997). As consequence, all cholinergic proteins resulted affected, with decreased in the acetylcholine-synthesizing enzyme, choline acetyltransferase (ChAT), as well as the acetylcholine-hydrolyzing enzyme, acetylcholinesterase (AChE) (Atack et al 1983, Davies 1979,

Perry et al 1977). In addition, there is a reduction of the muscarinic acetylcholine (ACh) receptors (M2), most of them pre-synaptically located (Quirion 1993). Finally, the discovery of the cholinergic neurons loss coming from the nucleus basalis of Meynert, confirmed a substantial presynaptic cholinergic deficit [reviewed in (Burns et al 1997)]. Thus, the *cholinergic hypothesis* proposes that the cholinergic neurons degeneration, associated with the loss of cholinergic neurotransmission to the cerebral cortex and the subsequently reduced synthesis of the neurotransmitter ACh, contributes fundamentally to the AD cognitive function decline. This hypothesis has been supported by studies showing the emerging role of ACh in learning and memory (Drachman & Leavitt 1974). Moreover, even if the mechanisms underlying the cholinergic-induced memory formation remain unclear, it has been recently demonstrated that the cholinergic denervation of hippocampus leads to impairment of spatial memory acquisition possibly through the activation of the muscarinic receptors (Gil-Bea et al 2011).

The cholinergic hypothesis was postulated more than 30 years ago (Bartus et al 1982), and today it is well accepted that, during the early pathological period of AD, many other dysfunctions appear. For instance, the Nerve Growth Factor (NGF) metabolic pathway, fundamental for the maintenance of the biochemical and anatomical phenotype of the basal cholinergic neurons coming from the basal forebrain, is deregulated (Bruno & Cuello 2006). It was further shown that this deregulation decreases the cholinergic presynaptic buttons, inducing cholinergic atrophy and decrease in ACh release (Cuello & Bruno 2007, Cuello et al 2010). This can explain, at least in part, the susceptibility of some cholinergic neurons to develop the first symptoms of AD. In parallel, impairments of the part of the monoaminergic system coming from the serotonergic raphe nuclei, and

of a part of the noradrenergic system coming from the locus coeruleus, induce respectively a reduction of the Serotonine and Noradrenaline release (Trillo et al 2013). All together these dysfunctions may be able to explain the progression and extension of memory loss, the cognitive decline, correlating with a drastic change in behaviour including an increase of aggression, depression, fear-induced stress and wandering mood.

Anyhow, which is the cause of AD? As commented above, the first step in AD generation is still unclear but it is plausible that the abnormal Aβ metabolism together with the P-tau formation, are closely related with particular affectation of the cholinergic system.

Thus, the soluble Aβ, in the form of monomers or oligomers, might exert a pathological influence in cholinergic targets. Recently, several laboratories have reported that very low concentrations of Aβ peptides (picomolar to nanomolar) can induce cholinergic hypofunction (Kar et al 1996). Aβ peptides, under acute conditions, can decrease endogenous ACh release and the uptake of choline in slices from rat hippocampus and cortex, but exhibit no effect on ChAT activity (Kar et al 1998, Kar et al 1996). In SN 56 cells, derived from mouse basal forebrain cholinergic neurons, Aβ42 and Aβ28 reduce the ACh content accompanied by proportional decrease in ChAT activity (Pedersen et al 1996). These results were confirmed *in vivo,* since the continuous infusion of Aβ into rat cerebral ventricle impairs learning ability and decreases ChAT activity and ACh release (Itoh et al 1996).

Conversely, stimulation of muscarinic receptors with carbachol caused a time-dependent 2-fold increase in the release of soluble APP, parallel with a decrease in Aβ production into the medium (Wolf et al 1995). Thus, in pathological conditions, sub-toxic levels of Aβ may disrupt carbachol-induced muscarinic signal transduction leading to a decreased processing of APP via the α-secretase pathway, and

potentially increased formation of Aβ which, in turn, might exert additional modulation of the cholinergic system (Kelly et al 1996). Soluble Aβ peptide can also cause excitotoxicity at the pyramidal neurons inducing the over-activation of the N-methyl-d-aspartate receptor (NMDAR), the cationic channels gated by the neurotransmitter glutamate (Gotz et al 2011). This over-activation results in neuronal damage and death due to the generation of excessive nitric oxide (NO) (Law et al 2001). NO can mediate excitotoxicity by triggering down-stream protein misfolding and aggregation, as well as mitochondrial fragmentation. Moreover the majority of transduction signal systems end on the activation or inactivation of enzymes responsible of tau phosphorylation and de-phosphorylation (Billingsley & Kincaid 1997, Nuydens et al 1997, Rapoport et al 2002, Sindou et al 1992). However, the mechanism that regulates *in vivo* the activities of brain protein kinases and phosphatases on tau phosphorylation are not fully understood.

In addition, our group has recently demonstrated *in vitro* and *in vivo* that P-tau can trigger an increase in AChE expression (Silveyra et al 2012). The possibility that Aβ might influence AChE is discussed below (see section 2.6).

All these results indicate that Aβ and also P-tau may trigger cholinergic dysfunction that strength AD. Improving the understanding of the relationship between Aβ/P-tau and cholinergic enzymes will help to identify ways to prevent or stop the damage that causes the disease.

2. Deep in cholinergic dysfunction

As stated above, one of the major lesions in AD brain, together with the amyloid plaque and NTF deposition, is the substantial loss of cholinergic innervation of the cerebral cortex. This loss is predominant

in the temporal lobe, the entorhinal cortex, the amygdala and the hippocampus, where up to 80% of cholinergic axons are depleted (Geula and Mesulam 1996). Furthermore, the fact that the cholinergic lesion is part of AD degeneration is corroborating by the evidence that pharmacological therapy with cholinesterase inhibitors improved mild dementia (Cummings 2004, Giacobini 2003, Lleo 2007). Intriguingly, despite the overall decrease of AChE activity in AD brain, AChE levels are increased around the amyloid plaques and NFT (Mesulam et al 1987, Ulrich et al 1990).

2.1 Cholinesterase

Cholinesterases (ChEs) are crucial enzymes for nerve response and functions. They are acetylhydrolases degrading the neurotransmitter ACh in the synaptic cleft of peripheral (PNS) and central nervous system (CNS), producing inactive metabolites, choline and an acetate group (Figure 6).

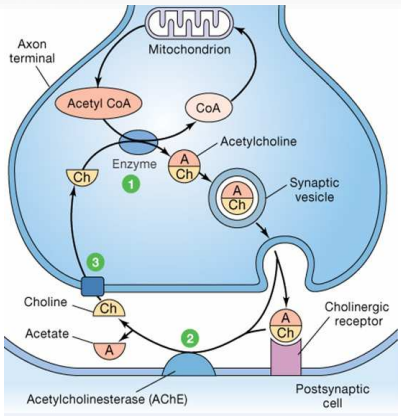


Figure 6. Biosynthetic pathway of ACh neurotransmitter. ACh is an ester of acetyl CoA and choline. ACh is synthesized in certain presynaptic neurons by the enzyme choline acetylcholine transferase (ChAT). After release in the synaptic cleft ACh is rapidly hydrolysed into the inactive metabolites by the enzyme AChE; and choline and acetate transported back to the axon terminal, where will be used to make more ACh. AChE is one of the fastest enzymes in nature.

Thus, ChEs activity serves to terminate synaptic transmission. All vertebrates possess two types of ChEs, corresponding to two distinct genes: acetylcholinesterase (*AChE*, EC 3.1.1.7) and butylcholinesterase (*BChE* or *BuChE*, EC 3.1.1.8). The two enzymes primarily differ on the basis of their substrate specificities: ACh for AChE and BuCh (butirilcholine) for BuChE, and secondly on their different sensitivity to selective inhibitors, e.g. BW284c51 for AChE and iso-OMPA for BuChE (Austin & Berry 1953).

In mammals AChE is encoded by a single *AChE* gene containing 6 exons (Figure 7).

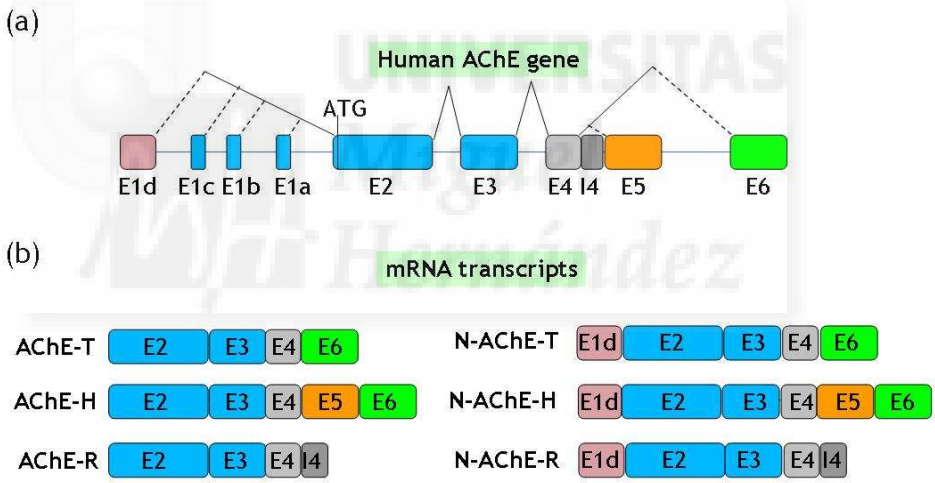


Figure 7. Schematic representation of the gene structure and transcripts of mammalian. **(a)** ACHE gene contains 6 exons that are depicted as cylinders, introns as horizontal lines. Splicing options are shown as lines above the gene. E1a–1d are alternative versions of human AChE exon 1. **(b)** Alternative transcripts of the human AChE. Exons 2, 3 and 4 encode the core of human AChE, including the catalytic domain. The alternative exons are produced by readthrough of the end of coding exon 4, or by alternative splicing to either exons 5 or 6 the text. (Meshorer et al 2004).

Diversity in the transcribed products arises from alternative mRNA splicing at the 3' and 5' termini and from post-translational

modifications (Massoulie 2002, Meshorer et al 2004, Taylor & Radic 1994). The alternative splicing at the 3' region allows the production of three distinct variants, each of them with a different C-terminal sequence.

The principle AChE mRNA transcript in brain and muscle tissues, the **AChE-T** (tail) variant, is formed by joining exon (E) 4 to E6 to give rise an amphipathic C-terminus of 40 amino acids (peptide t). This transcript is finally constituted by (E1)-E2-E3-E4-E6. The second most common transcript in the body is the **AChE-H** (hydrophobic), where the E4 links E5 and encodes a 43 amino acid C-terminal peptide (peptide H). This transcript is finally constituted by (E1)-E2-E3-E4-E5. The cleavage after amino acid 14 of the open reading frame in E5 enables linkage to glycosylphosphatidyl inositol (GPI), integration and thus anchorage to membrane surfaces, however, this transcript is insignificantly expressed in the brain. The third transcript is the **AChE-R** (readthrough) that is produced by readthrough of the end of coding exon 4. Intron 4 encodes a hydrophilic C-terminal extension of 26 amino acid residues (peptide r). This transcript is finally constituted by (E1)-E2-E3-E4-I4. The 'readthrough' AChE-R is normally expressed in low amounts in most tissues, including brain [for a review see ((Massoulie et al 2005)].

In addition to the 3' alternatively spliced species of AChE that generate proteins with distinct C termini, the 5' end is also subject to intricate regulation, as recently demonstrated by Soreq and co-workers (Meshorer et al 2004). In human at least three exons 1 (E1) at the N-terminus have been described: hE1a, hE1b, hE1c and hE1d exons. The hE1d exon encodes an additional 46 amino acids that prevent the cleavage of the human AChE signal peptide. In this AChE variants [N-AChE-T, N-AChE-H and N-AChE-R; (Figure 7)] the signal peptide sequence, that is not removed, could then serve as a transmembrane

domain, enabling N-AChE to anchor itself to the pasmatic membrane. Several N-extended AChE mRNAs have been identified in humans (Munoz-Delgado et al 2010).

In contrast, the BCHE gene produces a single type of transcript, which generates a single type of variant equivalent to AChE-T (Arpagaus et al 1990). The protein product forms G_1, G_2 and G_4 species, with hydrophilic or amphiphilic properties.

2.2 AChE structure

Knowledge of the AChE 3D structure is essential to understand its remarkable catalytic efficacy and to develop therapeutic approaches. The structure is characterized by a deep and narrow gorge leading to the active site, consisting in a Ser-200, a His-440 and a Glu-327, surrounded by a ring of 14 conserved aromatic residues (Figure 8). At the opposite side of the catalytic triad, there is the Peripheral binding Anionic Site (PAS), that, at the first step of the catalytic pathway, binds transiently ACh, like many others molecules (Johnson & Moore 2006, Mallender et al 2000).

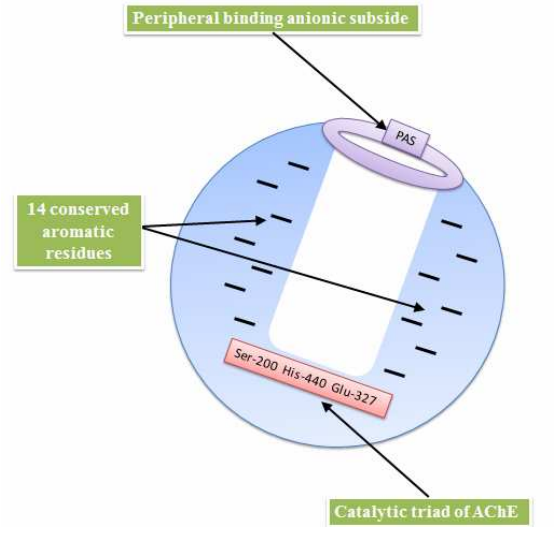


Figure 8. Schematic representation of an AChE monomer. The PAS is on the surface of AChE, approximately at 20 Å distant from the active site itself (Sussman et al 1991). The gorge has an aromatic character that might contribute to the high rate of ligand binding and, thereby, to the high catalytic activity.

PAS is an extraordinary versatile area consisting of 5 anionic residues (Tyr-70, Asp-72, Tyr-121, Trp-279 and Tyr-334) clustered around the entrance of the active site gorge (Ordentlich et al 1993). For this reason, PAS plays a significant role in the electrostatic attraction of cationic substrates and inhibitors. Moreover, it is able to mediate protein-protein interactions independently of its cholinergic functions. Some examples are the binds with the extracellular matrix molecules laminin-1 and collagen IV (Johnson & Moore 2003) and, particularly, with the Aβ peptide promoting amyloid fibril formation (De Ferrari et al 2001).

2.3 AChE molecular forms

All transcripts are able to generate AChE monomers, sharing a common catalytic domain, but distinct C-terminal domains.

The C-terminal end of the AChE-R variant end is a short sequence of 30 amino acids without cystein, thus lacking the possibility to bind others AChE subunits by disulfide bond. Therefore, AChE-R exists only as non-amphiphilic monomers (Figure 9). AChE-R transcript has been identified in human tumor cell lines with different tissue origins (Karpel et al 1994), in tissue from mice (Birikh et al 2003, Legay et al 1995) and human (Cohen et al 2003, Garcia-Ayllon et al 2012). However, AChE-R normally represents the minor brain AChE specie. Considering its low abundance *in vivo* (less than 2% of total active AChE in brain), it seems unlikely to contribute significantly to the cholinergic regulation by hydrolyzing ACh in the nervous system. It has been assumed that AChE-R is mainly expressed during brain development, and increases after stress stimulation (Kaufer et al 1998).

The AChE-H transcript encodes a subunit anchored to the membrane by glycosylphosphatidyl inositol (GPI). AChE-H can exist

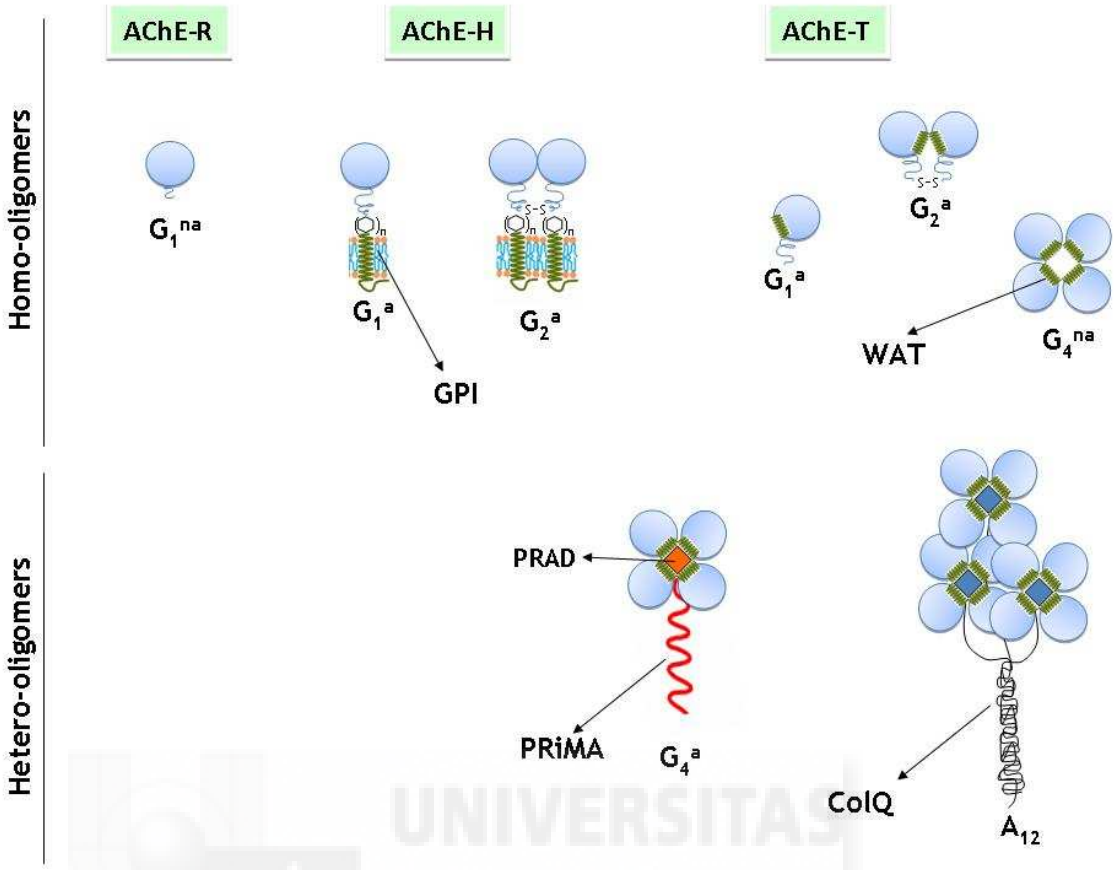


Figure 9. AChE protein polymorphism. AChE forms are classified as homo and heterooligomers depending on the presence of structural elements. Thus, the globular monomers (G1), dimers (G2) and hydrophilic tetramers (G4) which do not possess structural elements are homomeric forms, while the amphiphilic tetramers linked to the PRiMA subunit and the asymmetric forms, consisting of one (A4), two (A8), or three (A12) tetramers linked to a collagenic ColQ tail, are heteromeric species. (na) non-amphiphilic, (a) amphiphilic; WAT, Tryptophan Amphiphilic Tetramerization domain; PRAD, an extracellular domain of the mature PRiMA protein that contains a proline-rich motif (positions 56–70), which is similar to the proline-rich motif of ColQ.

like monomers or dimmers, because its C-terminal region also contains one or two cysteines, which allow dimerization by disulfide bonds. Most AChE activity of non-nervous tissues arises from the AChE-H (Montenegro et al 2013) which is not present in brain (Legay et al 1993). AChE-H is also abundant in the surface of blood cells, mostly in erythrocyte (Gomez et al 2003). The large pool of AChE in the blood

cells probably serves, together with the plasma BuChE, to hydrolyze circulating ACh and other choline esters (Mehlert et al 1993). However, AChEs may also exert other alternative functions (see below).

The AChE-T transcript exists in all vertebrate species. It is expresses in all tissues cell types except in erythrocyte, being particularly abundant in brain and muscle cells. The specific C-terminal peptide contains ~40 amino acidic sequence rich in cysteine and aromatic residues that allow AChE-T subunits to form homo-oligomers, mostly dimers and tetramers, which also can associate with non-catalytic anchoring protein subunits (Figure 9). Based on their quaternary structure and on their hydrodynamic properties, we can distinguish two classes of AChE-T: globular (G) and asymmetric (A). The globular forms consist of amphiphilic monomers (G_1^a), dimers (G_2^a), non-amphiphilic tetramers (G_4^{na}), and the membrane anchored amphiphilic tetramers (G_4^a). The amphiphilic tetrameric form is bound to the membrane through the transmembrane subunit PRiMA (Proline Rich Membrane Anchor) and it constitutes the major AChE form in the brain. The asymmetric forms contain one to three tetramers (A4, A8 or A12) and are attached to a triple helical tail of the collagenic protein ColQ, playing an essential role in the hydrolysis of ACh at the neuromuscular junctions [for a complete review see (Massoulie et al 2005)].

The C-terminal "t" peptide of the AChE-T subunit largely controls the cellular fate of AChE-T and its interactions with PRiMA and ColQ subunits, controlling the folding, oligomerization, segregation and physiological localization of the enzyme.

2.4 PRiMA protein

A small protein of ~20kDa associated to G4 forms of AChE was purified

from bovine brain in 1987 by Inestrosa (Inestrosa et al 1987).

Prof. Massoulié named this small protein the P subunit, and was the first to hypothesize its role in anchoring AChE-T in neurons, as ColQ anchors AChE in the basal lamina of neuromuscular junctions (Massoulie et al 1993). Few years later, the molecular and genetic structure of the P subunit was described in mice and humans and was re-named PRiMA (Massoulie et al 2005, Perrier et al 2002).

In 2003, a new splice variant encoding PRiMA was discovered (Perrier et al 2003). This second variant called PRiMA II contains an additional exon (exon 4b), absent in PRiMA I. The two resulting proteins differ in their C-terminal cytoplasmic domains and appear equivalent in their capacity to anchor G4 to the cell membrane (Perrier et al 2002) (Figure 10).

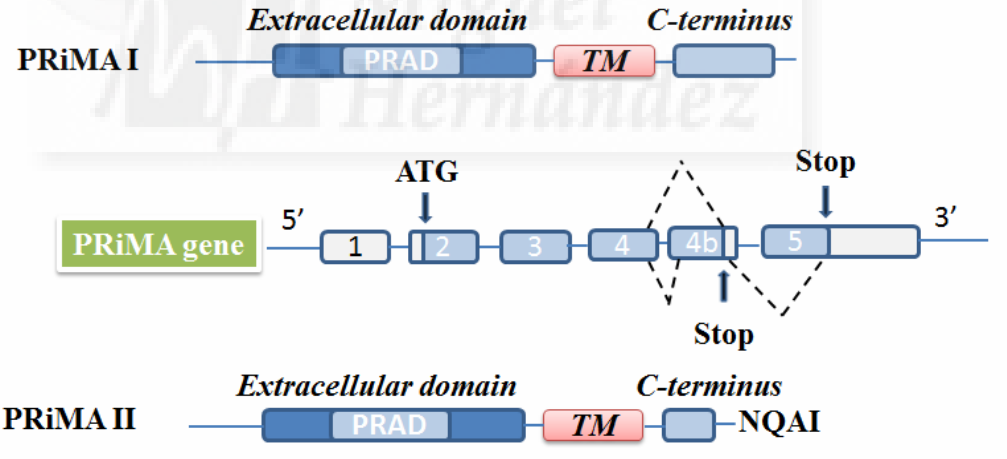


Figure 10: Schematic representation of PRiMA gene, variants transcripts and resulting proteins. PRiMA mRNA consists of a 5' non-encoding exon and four coding exons, the last of them contains the 3' STOP sequence. The starting (ATG) and the stop (STOP) codons are indicated. The two resulting PRiMA variants differ only in their C-terminal cytoplasmic domain; PRIMA II encodes a shorter protein with a smaller cytoplasmic domain. Both proteins bind G4 AChE and target it to the plasma membrane.

PRiMA I is the major form expressed in the brain, whereas PRiMA II is only detected as a minor component at the adult stage in the brain (Perrier et al 2003).

PRiMA I is a type I transmembrane protein, therefore its N-terminal corresponds to the extracellular domain containing the PRAD (Prolines-Rich Anchor Domain) motif, followed by a transmembrane domain (TMD) and a short, ~40 amino acids, cytoplasmic domain (Noureddine et al 2007) (Figure 11).

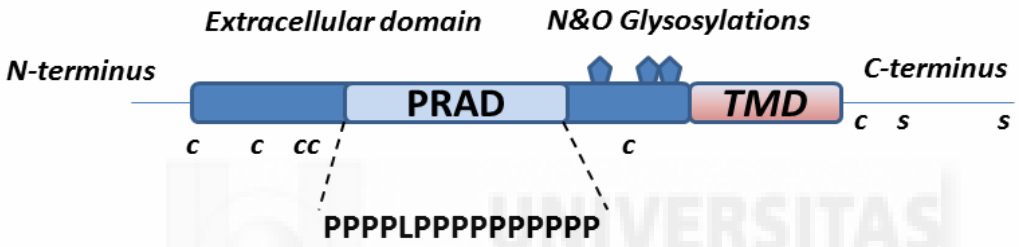


Figure 11: Schematic representation of PRiMA protein. The extracellular domain is characterized by the presence of five cysteines (c), a proline-rich sequence (PRAD) and three putative N- and O-glycosilation sites. The N-terminal is modified by fatty acylation. The cytoplasmic region contains one cystein close to the border with the membrane and putative phosphorilation sites on serines (s). The PRAD contains 14 prolines. TMD: transmembrane domain.

PRiMA is an accessory partner for the disposition of AChE at the plasma membrane (Dobbertin et al 2009), and represents a limiting factor for production of the G4 AChE (Perrier et al 2003).

2.4.1 AChE-T and PRiMA Association

PRiMA and AChE-T associate early in the endoplasmic reticulum (ER). The AChE-T "t-peptide" or "Tryptophan Amphiphilic Tetramerization domain" (WAT) can form an amphiphilic α-helix and

represents an autonomous interaction domain (Harel et al 1993, Massoulie et al 1993, Simon et al 1998). Crystallographic analyses of AChE G4 revealed that four WAT (one from each monomer) form a coiled-coil structure around the PRAD of PRiMA (Bon et al 1997, Perrier et al 2002). The complex is stabilized by the formation of disulfide bonds between the C-terminal cysteine of AChE-T subunits and the four N-terminus cysteines of PRiMA (Figure 12).

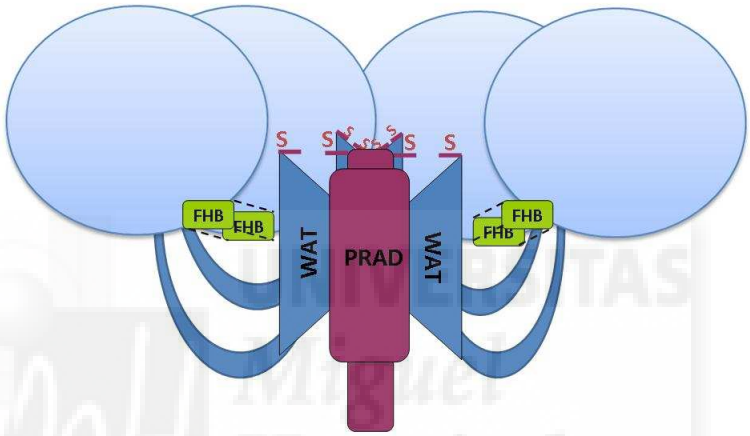


Figure 12: Oligomerization of monomers of AChE-T with PRiMA subunit. In violet is representing the PRiMA N-terminal. WAT domain from each AChE-T peptide binds the PRAD with a hydrophobic interaction. The intercatenary disulfide bonds between the t-peptide of AChE-T subunits and the N-terminal PRiMA are shown. The anchoring is also realized by a third type of hydrophobic interaction between two FHB, for the formation of a dimmer.

The catalytic domain of AChE also influences the oligomerization patterns, providing a contact zone called “four-helix bundle” (FHB). One catalytic domain formed α-helices, which contact the FHB on another AChE subunit (Chen et al 2010, Morel et al 2001, Sussman et al 1991)).

The assembly of AChE-T tetramers with PRiMA appears to proceed through a stepwise recruitment of two homo-dimers followed

by the association with PRiMA (Chen et al 2011) (Figure13).

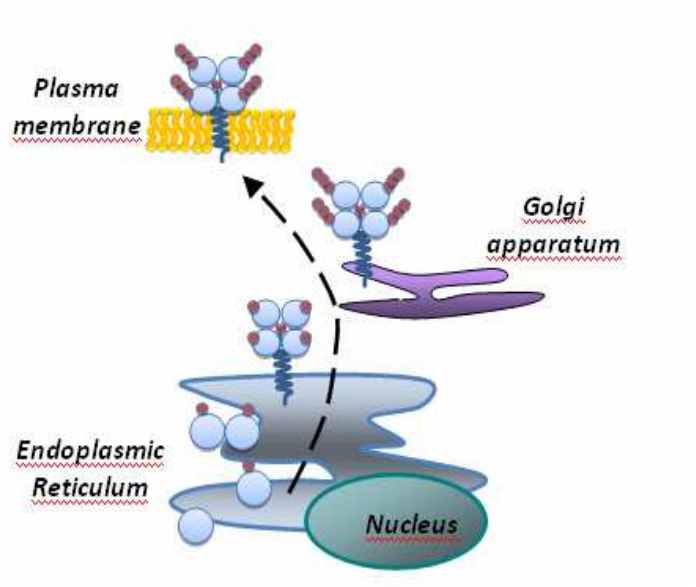


Figure 13: Assembly of AChE complexes. After transcription, in ER, AChE-T polypeptide is glycosylated and then it associates with other monomer of AChE-T to form homodimers. Then, PRiMA enters in contact with them, inducing the formation of PRiMA-linked G4 AChE. PRiMA also targets the complex to Golgi apparatus, where AChE subunits are further glycosylated. The proper glycosylation makes AChE subunits fully functional and send the complex to the plasma membrane. Without glycosylation AChE proteins are able to olygomerize, but are inactive and retained in the ER (Chen et al 2010).

Thus, PRiMA participates in the tetramerization of AChE subunits and is a necessary accessory partner for the cellular disposition of G4 AChE on the plasma membrane. PRiMA represents a limiting factor for production of the G4 AChE that is the predominant form in mammalian brain. This PRiMA-G4 AChE probably represents the cholinergic species located properly in order to hydrolyse the neurotransmitter ACh. In accordance with this, it has been recently demonstrated that PRiMA address G4 AChE in membrane rafts, a high specialized area of the plasma membrane with high content in sphingolipids, cholesterol and synaptic proteins (Xie et al 2010). These

specialized membrane microdomains compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules, influencing membrane fluidity, membrane protein trafficking and regulating neurotransmission. Rafts exist at neuron-neuron synapses and some synaptic molecules are concentrated in rafts. Thus, since PRiMA controls AChE raft localization, also limit potential interactions with others proteins (Perrier et al 2002).

2.5 AChE alternative Functions

The catalytic role of AChE at the cholinergic synapse is note like the *classical function.* However, many others activities not correlated with the cholinergic synapses and maybe not at all based on the hydrolysis of ACh have been described, and so called *non-classical functions*.

The first non-classical activity of AChE, clearly distinguished from its hydrolytic capacity, was its role in neuritogenesis. In this regard, it has been shown that AChE expression occurs largely before the onset of synaptogenesis, and in absence of noticeable ACh (Layer & Kaulich 1991, Small et al 1992, Small et al 1995). Non-catalytic morphogenic activity of AChE can be trigger by over-expression of catalytically inactive AChE and functional manipulations of splice variants (Dori et al 2005, Grifman et al 1998, Grisaru et al 2006, Sternfeld et al 1998). These results, together with the sequence homologies of AChE and several known cell-adhesion proteins, increased the evidence of a role for AChE in cell adhesion-related processes. The cholinesterase-like proteins are catalytically inactive and contain a cholinesterase-like domain that has high sequence similarity with AChE, and also acts as a protein–protein interaction domain. This protein family includes the Drosophila glutactin,

neurotactin, gliotactin and the mammalian neuroligins (de la Escalera et al 1990). The existence of these proteins provided a convincing reason to assume that AChE itself may be engaged in protein–protein interactions contributing to the formation of cellular junctions by binding to other extracellular ligands. AChE also enhances dopamine release from midbrain dopamine neurons in an autocrine form (Greenfield 1991). A soluble form of AChE is released from the dendrites of dopamine nigrostriatal neurons, independently from the cholinergic transmission. This stimulation has a "modulatory" action, enhancing the sensitivity of cells to synaptic inputs (Greenfield 1991). Other studies reported that AChE plays a role in haematopoiesis and thrombopoiesis, acting as a regulator of thrombocytic precursor proliferation (Paoletti et al 1992). AChE also seems to be implicated in synaptogenesis and stress response (Soreq & Seidman 2001).

In resume, many evidences present AChE as a multifunctional protein, with roles independent of its catalytic activity. Remain to be elucidate whether some of these roles are related with changes in AChE during neurodegeneration.

2.6 AChE dysfunction in AD

Despite the overall decrease of AChE activity in AD brain, it has been demonstrated the presence of AChE as a constituent of the amyloid plaque deposits (Ulrich et al 1990). With the progression of AD, AChE-positive neurons decrease throughout the entire neocortex and most of the cortical AChE activity is predominantly associated with the amyloid core (Gomez-Ramos et al 1992, Moran et al 1993) (Figure 14).

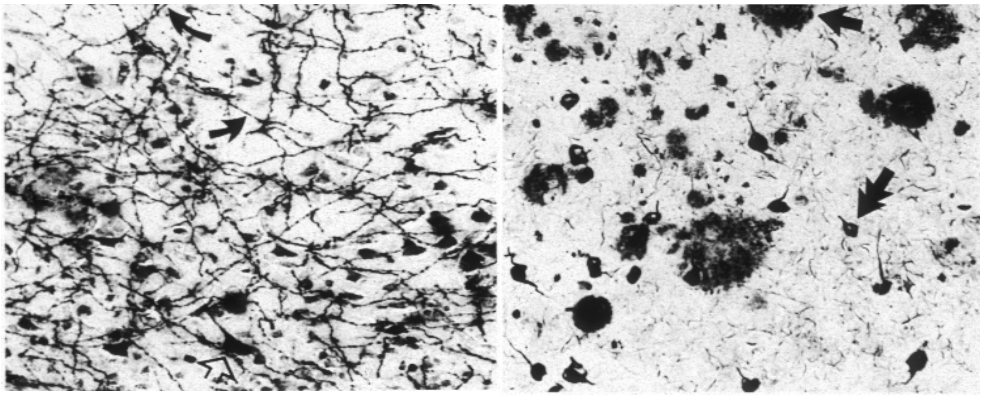


Figure14. Cholinergic expression in human brain. The neocortex has been stained with the Karnowsky method. Comparision between a non-demented case (on the left) and an AD one (on the right). In AD the cholinergic extentions decreased and AChE is predominatly associated with the amyloid core. Arrows show amyloid and tau deposits (Figure kindly concessed by MM Mesulam).

Several other proteins, such as ApoE (Namba et al 1991), α1-anti-chymotrypsin (Abraham et al 1988), and heparin sulfate proteoglycans (Snow et al 1988) have been identified in the amyloid plaque suggesting possible involvement of these in the amyloid deposition.

AChE is considered as an endogenous factor that can modulate Aβ fibrillogenesis and deposition, playing a significant role in AD pathogenesis, nucleation-dependent polymerization and plaque formation (Harper & Lansbury 1997, Inestrosa et al 1996). Indeed, inside the amyloid core, AChE may directly promote the assembly of Aβ peptide into amyloid fibrils, acting like a 'pathological chaperone' (Alvarez et al 1995). The potential domain of interaction between Aβ and AChE is the PAS, near the entrance of the catalytic gorge of AChE. Thus, the use of molecules able to bind the PAS prevents the enhancing action of AChE on Aβ fibril formation *in vitro* (Alvarez et al 1995, Inestrosa et al 1996). Moreover, biochemical studies in vitro demonstrated that the incorporation of AChE into amyloid aggregates

is an early event during the polymerization process, and that this incorporation is a thermodynamically favored process, since a small amount of AChE is required to promote aggregation (Alvarez et al 1998). The participation of AChE in the amyloid fibrillation changes the biochemical and pharmacological properties of the enzyme with respect to pH optimum, inhibitor sensitivity, and inhibition by excess substrate, and cause an increase in the neurotoxicity of the β-amyloid fibrils (Alvarez et al 1998, Geula & Mesulam 1989, Mesulam et al 1987). The pathogenic interactions between Aβ and AChE were successively described *in vivo* in a double transgenic mouse offspring of a cross between the Tg2576 line, which incorporates the human APP Swedish mutation associated with increased production of Aβ40 and Aβ42, and a hAChE line, which overexpresses human AChE in a CNS-selective pattern. In these double transgenic mice, the development of amyloid plaques is accelerated. Plaques are already mature at 6 months of age in contrast to Tg2576 line that develops plaques at 9 months, and also contain human AChE in addition to Aβ. It seems that the clusters of small plaques might be a consequence of the elevated levels of AChE expression providing multiple sites of nucleation (Rees et al 2003).

In another hand, different reports have corroborated the possibility that Aβ might also influence AChE expression, increasing its activity levels (Hu et al 2003, Sberna et al 1997). For example, transgenic mice models of AD such as APPC100 and Tg2576, which overproduce human Aβ, (Fodero et al 2002, Silveyra et al 2012) and rats treated with intracerebral Aβ (Saez-Valero et al 2002) display an increase in AChE levels, particularly of the minor G1 form.

Not all the molecular forms of AChE are equally affected in AD brain. In fact, the selective loss in the cholinergic G4 form, probably related with the cholinergic neurons loss (Fishman et al 1986), is not parallel to the lighter G1 species levels, which are preserved (Atack et

al., 1983; Fishman et al., 1986) or even increased in severe cases of AD (Arendt et al 1992, Saez-Valero et al 1999). Similarly, changes in AChE molecular forms in CSF reflect changes in the brain, with increasing amounts of G1 AChE in AD affected patients (Saez-Valero et al 1999), (Saez-Valero et al 2000a). Also, in plasma from AD subjects the light AChE species, which represent the major form of this fluid, are increased (Garcia-Ayllon et al 2010). Moreover, this monomeric form displays different glycosylation pattern (Saez-Valero et al 2000b, Saez-Valero et al 1997, Saez-Valero et al 1999).

Different cell types add different carbohydrate moieties onto AChE, so homologous AChE isoforms from different tissues and even from the same tissue differ in their glycosylation pattern (Vidal 1996). Differences in glycosylation can be detected by lectins, proteins that avidly bind to sugar moieties of glycoproteins (Lis & Sharon 1986). The ability of lectins to recognize specific carbohydrates in glycoproteins, detecting subtle differences in glycosylation patterns, makes them excellent tools to investigate glycosylation changes in pathological tissues. The pathological impairment of the glycosylation machinery could significantly compromise the processing of many glycoproteins, thereby resulting in loss of their physiological functions. Therefore, abnormal incorporation of carbohydrate moieties in AChE subunits can compromise its functional role and/or oligomerization, and also can reflect a change in AChE expression pattern.

Our group has previously characterized in *post mortem* brain and CSF of AD subjects, an increase of the monomeric form of AChE which display diminishing affinity for the lectin concanavalin A (Con A) (Saez-Valero et al 1997, Saez-Valero et al 1999). An altered AChE glycosylation patter has been also identified in *ante mortem* CSF samples from subject with probable AD (Garcia-Ayllon et al 2007, Saez-Valero et al 2000a).

Furthermore, many others proteins, such as tau and transferrin, show an altered glycosylation pattern in both extracts and CSF of AD (Fodero et al 2001, Guevara et al 1998, Kanninen et al 2004, Sihlbom et al 2008).

The expression pattern of AChE, within the embryonic development, depends on the developmental stage (Perry et al 1986, Zakut et al 1985). Indeed, in the human embryonic brain, the major form of AChE is a monomeric specie (Muller et al 1985). During this period, the monomeric AChE seems to participate in many functions such as neuronal differentiation, regulation of cell growth and cell adhesion. All these roles may depend on the protein-protein interactions rather than the catalytic activity (Small et al 1996). In particular, in the embryonic cells, AChE-R G1 appears to be the predominant form (Grisaru et al 1999). Intringly, aging involves a gradual increase of the AChE-R G1 form that attenuates the age-associated neurodeterioration (Sklan et al 2004, Sternfeld et al 2000). Moreover, it has been shown that stress, head injury and exposure to cholinesterase inhibitors can induce AChE-R mRNA accumulation (Meshorer & Soreq 2006). Reversely, other studies indicated only a minor change in AChE-R level after stress and anticholinesterase inhibitors (Perrier et al 2005, Perrier et al 2006).

Interestingly, it has been suggested that changes in AChE-R expression are able to modulate β-amyloid pathology. This AChE-R splice variant reduces Aβ fibril formation *in vitro*, inversely from what was reported for the AChE-T form (Berson et al 2008). A study of the specific changes of the AChE splice variants in AD brain is still pending.

3. γ-Secretase and Presenilin-1

The notion of a cholinergic-amyloid interrelationship is supported by the facts that cholinergic mechanisms modulate amyloid

metabolism (Nitsch et al 1992, Rossner et al 1998) as well as AChE inhibitors affect APP processing in cells, animals models and in AD-treated patients (Lahiri et al 1994, Mori et al 1995, Zimmermann et al 2005).

In this context, our group has recently demonstrated by reciprocal co-immunoprecipitation an interaction between AChE and PS1, the catalytic protein of the γ-secretase complex (Silveyra et al 2008). This interaction, that doesn't depend on the PAS domain, involves both tetrameric and monomeric forms of AChE and can be relevant in the pathological progress of AD and in the design of therapeutic strategies. Thus, it is important to explore the consequences of the AChE-PS1 interactions.

3.1 PS1 protein structure and activity

PS1 protein has been resistant to crystallographic analysis for long time, causing confusion around its topology. PS1 has ten hydrophobic regions, which, theoretically, can all form membrane-spanning domains. Various topology models have been proposed, including models suggesting 6 and 7 transmembrane domains (TMD) (Lehmann et al 1997, Nakai et al 1999), although the prevalent model that has been accepted during many years is a model with 8 TMD with the large hydrophilic loop, the N-terminal, and C-terminal domains oriented towards the cytosol (Doan et al 1996). The insertion of glycosylation sequences into potential loop regions finally demonstrates that PS1 is a 9 TMD domain protein (Laudon et al 2005). In this last model, PS1 has the N-terminus and a large hydrophilic loop in the cytosol, and the C-terminus in the lumen/extracellular space (Figure 15).

The final 3 TMD segments has been the most difficult to confirm. TMD 7 is a relatively short hydrophobic domain, that contains one of

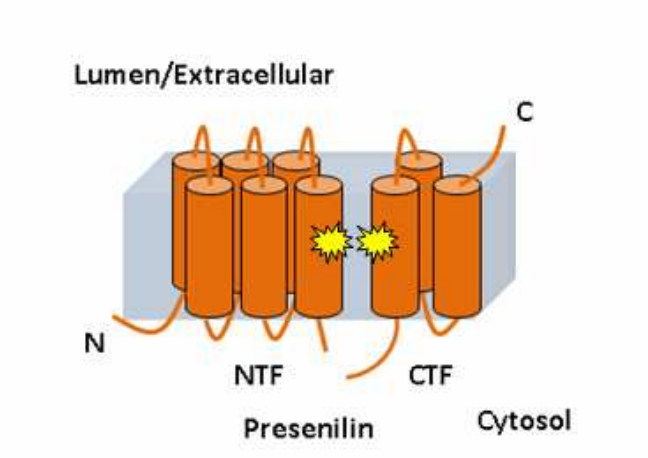


Figure 15. Membrane topology of presenilin 1. PS1 consists of 9 TMD with the N-terminus and the hydrophilic loop in the cytosol, and the C-terminus in the lumen/extracellular space. The active site is formed by 2 conserved aspartates, in the TMD 6 (Asp257) and 7 (Asp385). During the assembly with the other components of γ-secretase, PS1 undergoes endoproteolysis within the large loop region, forming a C-terminus (CTF) and a N- terminus fragment (NTF). Each subunit of PS1 provides one of the catalytic Asp to the active site (Wolfe 2013). Yellow stars symbolize the active Asp(s) involved in catalytic activity.

the conseved aspartates in the middle (Laudon et al 2005). TMD 8 and 9 are membrane spanning, but TMD 8 requires the presence of the TMD 9 to integrate into the membrane (Oh & Turner 2005). Moreover, it seems that PS1, in presence of its substrate, is finally reorganized in a ring structure (Cao & Sudhof 2001). PS1 acts as a membrane-embedded aspartyl protease, in which the catalytic activity depends of the two conserved and essential aspartates (Asp) located at the interface of the TM domain 6 of PS-NTF and domain 7 of PS1-CTF (Figure 15).

Although the PS1 holoprotein is synthesized as a polypeptide with an apparent size of 42-43 kDa, the mature and active PS1 undergoes an endoproteolysis that occurs at the aminoacids 292 and 299, and results in a ~29 kDa N-terminal fragment (NTF; containing TMD1-6) and a ~20 kDa C-terminal fragment (CTF; with TMD 7-9), which are the more abundant immunoractive bands in brain extracts.

The NTF/CTF assembly is the biologically active form of PS1 (Podlisny et al 1997, Saura et al 1999).

3.1.1 PS1 localization

PS1 is ubiquitously expressed in peripheral tissue and in the CNS. Several studies have investigated the PS1 subcellular localization in neurons using biochemical methods, immunostaining and immunoelectron microscopy. It has been reported that PS1 resides principally in the endoplasmic reticulum and trans-golgi network, but it is also present in small synaptic vesicles, synaptic plasma membranes, synaptic adhesion sites and neurite grown cone membranes (Annaert & De Strooper 1999, Georgakopoulos et al 1999). However, mature forms of PS1 have been found at the cell surface in complex with other membrane associated proteins, like nicastrin (Chyung et al 2005). Thus, despite the large proportion of PS1 localized within the endoplasmic reticulum and early Golgi, it is assumed that APP cleavage occurs on the cell surface and in endosomes/lysosomes compartments, where the proteolytically active PS1/γ-secretase is principally localized [for a review see (Haass et al 2012)].

3.2 γ-Secretase

We have previously described the γ-secretase complex formed by PS1, together with nicastrin, anterior pharynx-defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2). Diverse studies focusing on the topology of these proteins revealed that Aph-1 has 7 TMD, with an N-terminus in the lumen/extracellular space and the C-terminus in the cytosol (Fortna et al 2004) (Figure 16). Pen-2 spans the membrane twice, with the N- and C-termini facing the lumen space (Crystal et al 2003). In contrast, nicastrin has the typical topology of type I

transmembrane protein, with a single TMD and an N-terminus spanning in the lumen/extracellular space with many potential glycosylation sites (Yu et al 2000).

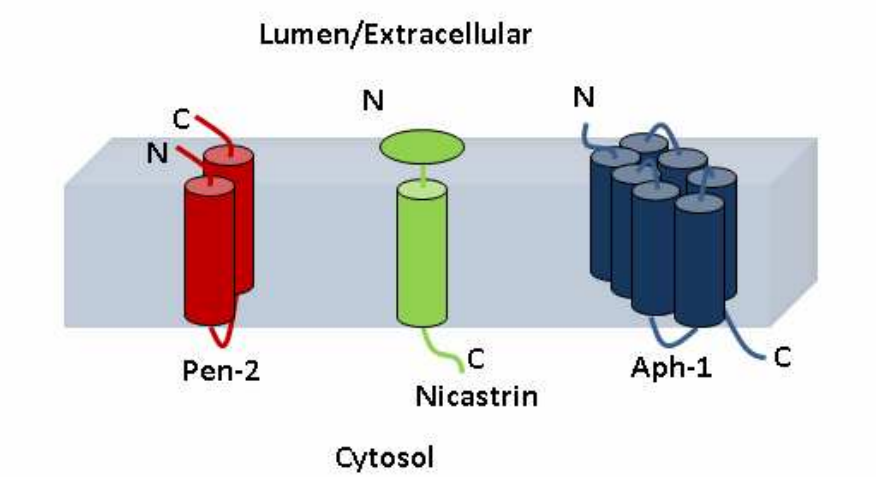


Figure 16. Membrane topology of the three components, that with PS1, form γ-secretase: nicastrin, Aph-1 and Pen-2. See the text and for a review (Wolfe 2013).

A close homologue of PS1, PS2, shares with it the catalytic activity and can be also found in the γ-secretase, forming similar but independent complexes. Similarly, in humans there are two Aph-1 homologues, Aph-1α (on chromosome 1) and APH-1β (on chromosome 15). Aph-1α has two C-terminal splicing variants: Aph-1αL (long variant) and Aph-1αS (short variant). Aph-1β, Aph-1αL and Aph-1αS are functionally redundant in terms of their ability to form active γ-secretase complexes with the other three subunits. Thus, γ-secretase complexes composed by 4 subunits yield six possible distinct γ-secretase complexes with the possible combinations of Aph-1 and PS isoforms (Shirotani et al 2007, Wakabayashi & De Strooper 2008).

The assembly of γ-secretase complex begins in the ER soon after translation and membrane insertion. Several studies indicate that Aph-1 interacts with the immature, hypoglycosylated form of nicastrin in an early stage (Gu et al 2003), forming a low-molecular weight sub-

complex before the incorporation of the other components (LaVoie et al 2003).

At this point, the C-terminus of the nascent PS1 holoprotein binds Aph-1 and nicastrin, forming a high molecular weight inactive complex where the proteins are stabilized. In this context, PS1 acts as a chaperone protein and facilitates nicastrin maturation inducing the transport of the complex to the medial Golgi compartments, where nicastrin is N-glycosylated (Fraering et al 2004, Kaether et al 2002, LaVoie et al 2003). Subsequently, nicastrin undergoes a major conformational change that involves the entire ectodomain and becomes it selectively resistant to trypsin. This structural conformational change doesn't occur in absence of PS1, and is required for the γ-secretase assembly and activity (Shirotani et al 2003). The last step consists in the incorporation of Pen-2 into the PS1-nicastrin-Aph-1 trimeric intermediate. Indeed, it seems that Pen-2 binds to the TMD 4 of PS1 and provokes its endoproteolysis into NTF-CTF heterodimers confering the proteolytic activity (Fraering et al 2004, Watanabe et al 2005) (Figura 17). The assembled γ-secretase complex is transported to the post-Golgi compartments including the plasma membrane where can be found in lipid rafts.

Although the constituents of the γ-secretase complex were identified several years ago, there has been controversy concerning the stoichiometry of the γ-secretase complex. Sizes of 100-150 kDa up to 2 MDa have been reported (Capell et al 1998, Edbauer et al 2002, Evin et al 2005, Yu et al 1998) even if the expected molecular weight based on the protein sequence, assuming that the γ-secretase complex is formed of one copy of each subunit, is of about 200-250 kDa. Purified γ-secretase runs on some Blue Native gels at around 500 kDa, implying that γ-secretase might be a dimeric complex; however, molecular sizing based on gel electrophoresis can be unreliable (Sato et al 2007).

Finally, it has been accepted a stoichiometry of 1:1:1:1 for the active complex of γ-secretase, even if it is plausible the existence of larger aggregates. Consistent with this stoichiometry, the absolute mass of the purified γ-secretase measured by scanning transmission electron microscopy (STEM) is ~230 kDa (Osenkowski et al 2009).

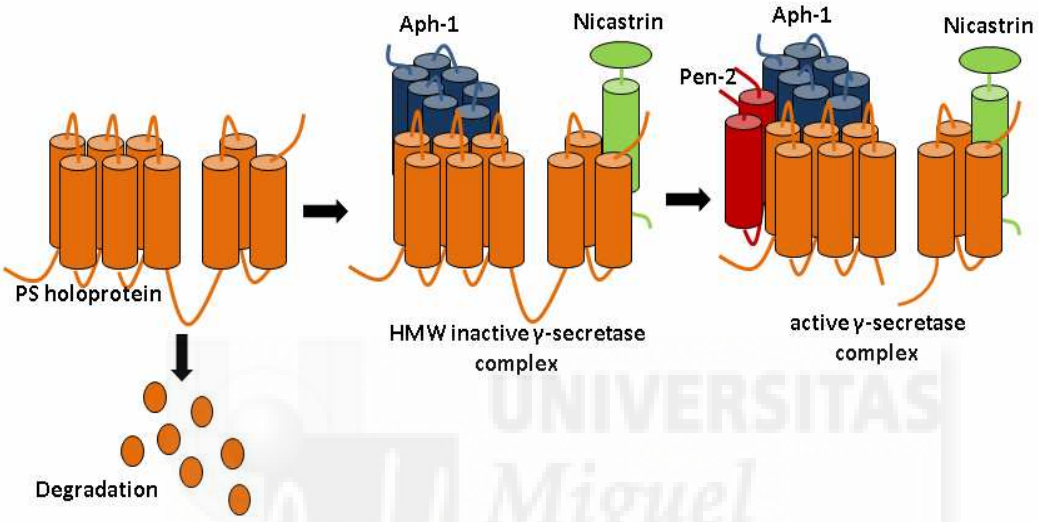


Figure 17. Schematic representation of the γ-secretase complex formation. The nascent PS1 holoprotein is stabilised by the binding to Aph-1-nicastrin complex. When this binding doesn't take place, is rapidly degraded. In the complex Aph-1 and nicastrin are bind to the PS-CTF fragment while Pen-2 binds the PS-NTF fragment. The coloured tubes represent the transmembrane domains of each protein.

3.2.1 Proteolytic functions of γ-secretase, the case of APP

γ-Secretase belongs to a diverse family of Intramembrane-Cleaving Proteases (I-CLiPs). To date, γ-secretase is also the unique intramembrane protease identified that functions as a multi-subunit protein complex; all the other I-CLiPs, in fact, are single-protein enzymes.

The notion that PS1 bears the γ-secretase active site was strongly supported by the observation that mutations of either of the

two conserved aspartate residues substantially reduced Aβ production, with a concomitant accumulation of APP βCTF (Kimberly et al 2000, Steiner et al 1999, Wolfe et al 1999b) and by the fact that the γ-secretase activity was inhibited by aspartyl protease substrate-based peptidomimetic inhibitors (Esler et al 2000, Wolfe et al 1999a). As commented above, in the active γ-secretase complex, PS1 is cleaved between residues Asn-292 and Val-293 resulting a NTF and a CTF, which remain bound together (Thinakaran et al 1996). This endoproteolysis appears to be an intramolecular autocatalytic event that is carried out by the same γ-secretase activity (Brunkan et al 2005, Wolfe et al 1999b).

In addition of APP and Notch more than 90 other transmembrane protein substrates have been described for PS1-mediated γ-secretase cleavage (Beel & Sanders 2008, Hemming et al 2008, Lleo & Saura 2011, Wakabayashi & De Strooper 2008)

In general, the requirements for being a γ-secretase substrate are broad: a type I transmembrane helix with a small ectodomain (<300 amino acids), usually resulting from a prior shedding by a metalloprotease-like protease (Lleo & Saura 2011). The previous shedding of the extracellular domain is usually mediated by specific proteases, α- or β-secretases (Brou et al 2000). However, the γ-secretase cleavage doesn't depend critically on a specific amino acid sequence or on endocytosis (Struhl & Adachi 2000). After that, the resulting C-terminal fragment is cleaved inside its TMD by the γ-secretase complex that executes an endopeptidase-like cleavage, followed by carboxypeptidase-like processive/successive cleavage. The transmembrane substrate is first proteolyzed at the border between the cytosol and membrane, which is called the ε-site (Kimberly et al 2003, Lichtenthaler et al 1999). This ε-cleavage allows the liberation of the intracellular domains (ICDs) of the substrates from the membrane.

Some ICDs have been identified as signaling mediators in several pathways, including Notch signaling. The remaining hydrophobic sequence of the substrate is processed by the γ-secretase carboxypeptidase activity, shedding shorter fragments (Qi-Takahara et al 2005, Takami et al 2009).

In the case of APP, the progressive cleavage model of γ-secretase is consistent with the detection of several Aβ peptides with lengths intermediate between Aβ49 (ε-cleavage) and Aβ42 (γ-cleavage) (Figure 18).

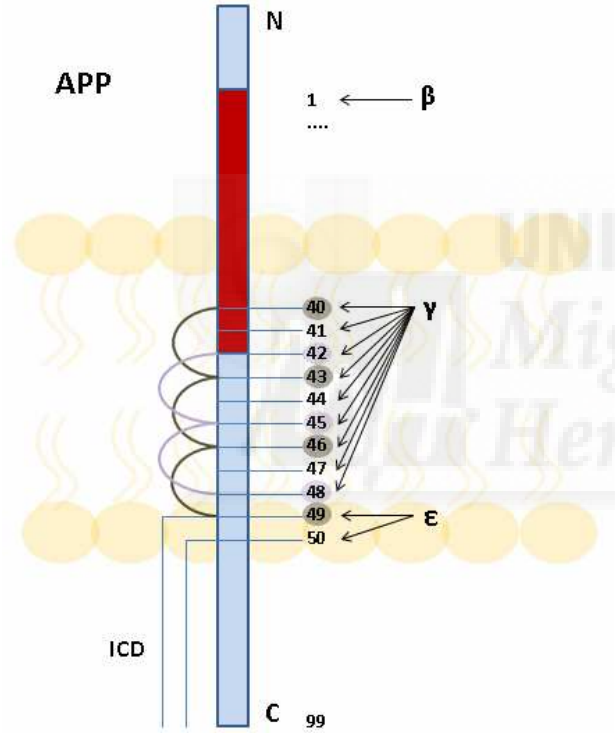


Figure18. APP processing by γ-secretase. B-cleavage of the substrate is followed by γ-secretase cleavage.
γ-Secretase processes APP at several γ sites producing soluble Aβ peptides with different C-terminal ends. Processing probably occurs by progressive cleavage, acting first at ε sites, close to the membrane-cytoplasm boundary, seeding the soluble ICD.

In theory, the ε-cleavage yields the formation of an Aβ peptides of 49 amino acids, but in practice, Aβ49 is extremely rare to find due to the progressive cleavages of PS1. In APP the recognition sequence for γ-secretase consists in 11 amino acids (Thr639-Lys649) inside the TMD at the C-terminal end, downstream the γ40 cleavage site (Cao & Sudhof 2001). One time recognized, APP is presented to the catalytic

domain of γ-secretase, which in turn recognizes many hydrophobic residues where it can acts (Barthet et al 2012, Tischer & Cordell 1996).

Interestingly, it seems that the whole known FAD mutations, that shift the γ-secretase cleavage toward Aβ42 production, are within the small binding site region, probably affecting the presentation of APP to γ-secretase (Selkoe 1998).

At the end, the cytoplasmic APP-ICD (or AICD) binds directly the molecular adaptor Fe65 protein, which promotes its entry in the nucleus, where it can regulate gene transcription (Baek et al 2002, Cao & Sudhof 2001, Wiley et al 2007).

The large number of γ-secretase substrates that have been identified, plus the multiple cellular localizations of this complex, suggest that PS1/γ-secretase participates in many biological processes including cell adhesion, lateral inhibition, neurotrophin signaling, cell differentiation, ligand-receptor binding, calcium influx, NMDA receptor activation, substrate recruitment and enzyme trafficking (Thinakaran & Parent 2004). The use of PS1 knock-out mice also showed that PS1 is not only important in the adult stage of the brain, but is fundamental in somitogenesis, axial skeleton formation and neuronal population stabilization during development (Shen et al 1997).

On the other hand, it has been proposed that PS1 is involved in the regulation of protein functions independently of the γ-secretase activity, playing a critical role in many events during development and aging (Parks & Curtis 2007). In this regard, many reports showed, in neurons, a large pool of resident PS1 in the early compartments of the biosynthetic pathway (Culvenor et al 1997, Huynh et al 1997). It has also been suggested that the over-expression of either the wild type or mutant PS1 disturbs glycoprotein processing within the golgi (Farquhar et al 2003). Indeed, it has been demonstrated that PS1 regulates the glycosylation and the intracellular trafficking of APP and

selected membrane proteins (Leem et al 2002), possibly including AChE (Sylveyra et al 2008). Recently, it has been also described the implication of PS1 protein in the regulation of neurotransmitter release during synaptic transmission. In fact, the presynaptic inactivation of PS1 decreases the probability of glutamate release (Zhang et al 2009), probably due to its role in modulation of calcium release from intracellular stores. Interestingly, it was also proposed that PS1 could form calcium leak channels in the ER, independently from its activity in the γ-secretase complex (Tu et al 2006). Moreover, PS1/γ-secretase can regulate ACh muscarinic receptor-mediated signal transduction [to review see (Cowburn et al 2007)].

However, the participation of PS1 in those biological processes, independently from its γ-secretase activity, is not clearly defined.

3.3 Presenilin dysfunction in AD

To date, more than 150 mutations have been identified in PS1 and PS2, harbour approximately 90% of FAD (De Strooper 2007). These mutations alter APP proteolysis, with an increase of Aβ production, especially of the large and amyloidogenic Aβ42 (Duff et al 1996). Reversely, in PS1 knockout mice, the Aβ42 production is reduced (Naruse et al 1998, Qian et al 1998).

In sporadic AD, it is still unclear if the γ-secretase activity is altered. Reported levels of PS1 in AD brains have been contradictory. Some of them displayed an increase (Borghi et al 2010, Kakuda et al 2012), unchanged (Mathews et al 2000) or even a decrease level (Davidsson et al 2001, Verdile et al 2004) compared to the one of non-demented brains. At the transcriptional level, early reports indicate no differences between PS1 mRNA levels in AD brain compared to controls (Johnston et al 1996). However, subsequent researches

suggested that PS1 mRNA levels, in human AD brains, are significantly higher than in those with no dementia (Ikeda et al 2000).

The progressive memory loss and cognitive decline characteristic of AD are strictly regulated by the synaptic spines morphology, composition, and stability (Rao & Craig 2000). The discovery that PS1 is directly associated with actin filament, modulating the synaptic structure, has caught much attention because a PS1-mediated synaptic alteration will provide rationales for the neuronal defects associated with AD (Sych et al 2000). This discovery is also supported by genetic studies in adult mice where loss of PS1 function results in progressive synaptic and memory impairments prior to age-dependent neurodegeneration (Saura et al 2004, Selkoe 2002, Zhang et al 2009). For instance, in a triple transgenic mice model over-expressing mutant PS1, APP and tau, in an early stage where the amyloid and tau pathologies are absent the first alterations are associated with the ACh and NMDA receptor components (Wang et al 2009). This phenomenon may be related with the adverse effects of mutant PS1 on synaptic plasticity and proteins trafficking. Similar effects have been observed in PS1 conditional knock-out mice, where the loss of PS1 causes a reduction in NMDA-receptors mediated responses and LTP deficits, which may be due to a defect in intracellular trafficking and synaptic delivery (Saura et al 2004).

Recently, our group has characterized an impairment of AChE maturation and glycosylation in mice expressing a PS1-FAD mutation (Silveyra et al 2008). In these mice AChE activity is decreased and its glycosylation altered. An inactive, delocalized AChE may have physiological consequences such as the loss of cholinergic receptors regulation, which can be related with AD impairments.

In this Thesis we aim to further explore the complex cholinergic-amyloid relationship, through the study of the PS1 and AChE cross-talk. A better understanding of their relationship, will be relevant to understand the pathological processes related with AD progression, in order to design possible therapeutic strategies.

CHAPTER 2: OBJECTIVES

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The ultimate goal of this Thesis is to explore and determine the interrelationship between PS1 and AChE and its implication in Alzheimer's disease. Specifically, the main objectives of our study are:

I. To investigate the consequences of AChE-PS1 interactions, mainly to examine the mechanism exerted by AChE for modulating PS1 levels, playing special attention to AChE variants, molecular form and enzymatic activity. Finally to study whether altered levels of PS1, triggered by AChE, induce changes in γ-secretase activity

II. To analyse whether the expression of AChE protein is altered in the AD brain, investigating AChE catalytic activity levels, but also levels of protein and mRNA of AChE in hipoccampus and cerebral cortex of AD.

III. To investigate whether PS1 participates in the processing of the cholinergic AChE specie, via cleavage of the PRiMA subunit; and whether resulting PRiMA fragments could translocate to the nucleus. To examine also in a PS1 conditional knockout mice whether PS1 influences the localization of AChE in brain lipid rafts.

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CHAPTER 3: RESULTS

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ARTICLE I:

Acetylcholinesterase modulates Presenilin-1 levels and γ-secretase activity

Our group has previously identified presenilin-1 (PS1), as an interacting protein of acetylcholinesterase (AChE) and has also demonstrated that genetic modulation of AChE expression influences PS1 levels. In this Thesis we further explore the consequences of AChE-PS1 interactions. We have found that PS1 is able to co-immunoprecipitate with all the AChE variants (AChE-R and AChE-T) and molecular forms (tetramers and light subunits) present in the human brain. Then, we have demonstrated that the overexpression of AChE-T or AChE-R in CHO cells lead to a significant increase in PS1 levels, compared to the untransfected cells. This influence of AChE in PS1 levels is exerted by a mechanism independent of its catalytic activity since over-expression of inactive mutants of AChE-T and AChE-R, in which the catalytic serine200 was replaced with a valine, also result in increases in PS1 levels. This modulatory capacity of AChE depends on its subcellular localization, because the increase in PS1 levels is further augmented in cells over-expressing AChE with the membrane anchoring subunit proline-rich membrane anchor (PRiMA), which restricts the localization of the resulting AChE tetramer to the outer plasma. Thus, the most significant variable that determines how AChE influences PS1 levels is their co-localization outside the plasma membrane. The incubation of untransfected cells with pure soluble AChE (from Electrophorus electricus, eel-AChE) also triggers an increase in endogenous PS1, at both protein and transcript levels. However, we have found that AChE-PS1 up-regulation may be linked to an inhibitory effect of AChE on γ-secretase activity. The incubation of CHO-PS70 cells, which stably overexpress wild-type human PS1 and

wild-type APP, with eel-AChE results in a decrease on γ-secretase activity, monitored by measuring the accumulation of the APP-CTF levels (C-terminus of the amyloid β-protein precursor). This inhibitory effect of AChE on γ-secretase activity was also observed by directly assessing accumulation of CTF-APP in cell-free membrane preparations incubated with eel-AChE. Our data suggest that inhibition of PS1 by AChE may initiate a feedback process that leads to up-regulation of PS1.

These results were published in the manuscript entitled: "Acetylcholinesterase modulates Presenilin-1 levels and γ-secretase activity" *Journal of Alzheimer's Disease* **(In press).**

Saez Valero, Javier

De: javila@cbm.uam.es
Enviado el: domingo, 09 de marzo de 2014 10:32
Para: Saez Valero, Javier
Asunto: Manuscript 14-0426 Decision

Dear Dr. Sáez-Valero:

Your manuscript (14-0426) has now been accepted in the Journal of Alzheimer's Disease. In keeping with the policy of JAD, you are cordially invited to become an Associate Editor of the journal. More information about the role of an Associate Editor is available at our website (http://www.j-alz.com/editorrole); our structure is quite unique and centered on the open dissemination of novel ideas. If you choose to accept, your one-year term would begin January 1, 2015 and is renewable by future submissions or handling of manuscripts.

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Acetylcholinesterase modulates Presenilin-1 levels and γ-secretase activity

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Abstract

The cholinergic enzyme acetylcholinesterase (AChE) and the catalytic component of the γ-secretase complex, presenilin-1 (PS1), are known to interact. In this study, we investigate the consequences of AChE-PS1 interactions, particularly the influence of AChE in PS1 levels and γ-secretase activity. PS1 is able to co-immunoprecipitate all AChE variants (AChE-R and AChE-T) and molecular forms (tetramers and light subunits) present in the human brain. Over-expression of AChE-R or AChE-T, or their respective inactive mutants, all trigger an increase in PS1 protein levels. The AChE specie capable of triggering the biggest increase in PS1 levels is a complex of AChE with the membrane anchoring subunit proline-rich membrane anchor (PRiMA), which restricts the localization of the resulting AChE tetramer to the outer plasma membrane. Incubation of cultured cells with soluble AChE

demonstrates that AChE is able to increase PS1 at both the protein and transcript levels. However, the increase of PS1 caused by soluble AChE is accompanied by a decrease in γ-secretase activity as shown by the reduction of the processing of the β-amyloid precursor protein. This inhibitory effect of AChE on γ-secretase activity was also demonstrated by directly assessing accumulation of CTF-APP in cell-free membrane preparations incubated with AChE. Our data suggest that AChE may function as an inhibitor of γ-secretase activity.

Introduction

Acetylcholinesterase (AChE) is a key enzyme in the cholinergic nervous system. Due to its physiological role of hydrolyzing acetylcholine and supporting neurotransmission, this enzyme has been extensively investigated and targeted for pharmacological intervention. In Alzheimer's disease (AD), the loss of forebrain cholinergic neurons is accompanied by a progressive decline in acetylcholine [1,2]. Deficits in cholinergic function most likely contribute to AD symptoms, affecting cognition, behaviour and daily living activities. Although changes in other elements of the cholinergic system [3,4] are also involved in AD, current AD therapy is mostly focused on inhibitors of AChE [5,6]. Thus, randomized clinical trials have demonstrated the efficacy of AChE inhibitors across a wide range of AD severity [7].

Many studies suggest that AChE could have alternative functions unrelated to cholinergic neurotransmission [8-12], or its catalytic activity [13-15]. AChE exists as different variants derived from alternative RNA splicing, generating different polypeptide encoding transcripts with the same catalytic domain but distinct C-terminal peptides, which determine the ability of the molecule to form oligomers [16]. These different transcripts may also influence protein-protein interactions. In the brain, the major T-transcript encodes subunits

which produce monomeric (G1) and tetrameric (G4, the cholinergic species) AChE forms; while the R-transcript, that is normally present at low levels, encodes monomeric soluble subunits [17]. The particular subcellular distribution of each AChE species allows for its interaction with specific proteins.

Brain accumulation of the β-amyloid peptide (Aβ) is a critical feature of AD pathogenesis. Aβ is the main component of extracellular amyloid plaques and is generated by processing of the larger transmembrane β-amyloid precursor protein (APP) [18,19], by the successive action of two proteolytic enzymes, β-secretase and γ-secretase [20]. We have previously identified presenilin-1 (PS1), the active component of the γ-secretase complex [21], as an interacting protein of AChE [22]. We have also shown that genetic modulation of AChE expression influences PS1 levels [23].

In this study, we further explore the consequences of AChE-PS1 interactions. We investigate which AChE variant and molecular form influences PS1 levels and if the AChE enzymatic activity is responsible for modulating PS1 expression. Finally we address whether altered levels of PS1, triggered by AChE, induce changes in γ-secretase activity.

Material and methods

Cell Cultures

Chinese Hamster Ovary (CHO) cells were grown in D-MEM+GlutaMAX™-I (Dulbecco's Modified Eagle medium; Gibco®, Life technologies Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin solution (P/S; 100 U/mL) (Gibco). Cells were seeded at a density of 8×105 cells on 35 mm tissue culture dishes and were transfected the following day with plasmid cDNA using Lipofectamine™ 2000 (Invitrogen™, Life technologies

Paisley, UK) according to the manufacturer's instructions. The plasmids employed encoded either human AChE-T (4μg) or AChE-R (1μg) under the cytomegalovirus (CMV) promoter-enhancer (a generous gift from Dr. H. Soreq, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel). The PCI "empty" vector (Promega, Madison, USA) served as negative control. After 48 hours of transfection, cells were washed with phosphate-saline buffer (PBS) and resuspended in 120 μL ice-cold extraction buffer: 50 mM Tris-HCl, pH 7.4 / 150 mM NaCl / 5 mM EDTA / 1% (w/v) Nonidet P-40 / 0.5% (w/v) Triton X-100 supplemented with a cocktail of protease inhibitors. Cell lysates were then sonicated and centrifuged at 70,000×*g* at 4 ºC for 1 hour. The supernatants were collected and frozen at -80ºC until biochemical analysis. Alternatively, AChE-T and AChE-R were over-expressed in SH-SY5Y neuroblastoma cells, grown as described elsewhere [23].

To determine if localization of AChE in the plasma membrane influences PS1 levels, CHO cells were seeded at a density of 6×10^5 cells on 35 mm tissue culture dishes and transfected with 2μg of AChE-T cDNA, with or without 2μg of PRiMA plasmid cDNA using Lipofectamine™ 2000. The cDNA encoding the mouse PRiMA isoform I tagged with an HA epitope (YPYDVPDYA) inserted before the stop codon at the C-terminus [24], was a generous gift from Dr. K.W.K. Tsim (The Hong Kong University of Science and Technology, Hong Kong, China). The cells were collected for analysis 48 hours after the transfection.

To estimate the AChE activity at the plasma membrane, CHO cells previously transfected with AChE-T cDNA (2μg) with or without 2μg of PRiMA plasmid cDNA, were treated with the AChE inhibitor tacrine, 10μM (Sigma-Aldrich, St. Louis, MO, USA). Forty-eight hours after transfection, cells were washed with PBS and intact cultured cells were

measured for AChE activity using a modified microassay version of the colorimetric Ellman's method [25].

CHO cells stably overexpressing wild-type human PS1 and wild-type APP (CHO-PS70, a generous gift from Dr. D. Selkoe, Brigham and Women's Hospital, Boston; see ref. 26), were grown in Opti-MEM® (Gibco) containing 10% FBS, 1% P/S and additionally supplemented with 200 μg/ml G418 and 2.5 μg/ml Puromycin (Sigma-Aldrich). These cells were treated with soluble AChE from *Electrophorus electricus* (*eel*-AChE; Sigma-Aldrich) or vehicle (PBS) for 18 hours, solubilized, and C-terminal fragments of APP (CTF-APP) quantified by Western blot, and PS1 transcript levels by quantitative RT-PCR (*q*RT-PCR).

Generation of inactive catalytic mutants of AChE

Catalytically inactive species of AChE-R and AChE-T were generated by site-directed mutagenesis using the QuickChangeTM site directed mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. AChE activity was removed in the plasmid cDNA of both active AChE-R and AChE-T by replacing the centre active serine200 with valine [27].

Inactive mutants (*im*AChE-T or *im*AChE-R; 3μg of the cDNAs) were overexpressed in CHO cells using the Lipofectamine™ 2000 protocol. Cells were harvested and solubilized after 48 hours. Protein AChE over-expression was assessed by Western blot, while the inactive character of the mutants was determined by measuring AChE activity levels.

Human brain samples

Samples of adult brain prefrontal cortex from non-demented subjects (three cases, 2 females and 1 male, 58 ±3 years) were obtained from the Banco de Tejidos, Fundación CIEN (Madrid, Spain). Tissues stored

at -80°C were thawed gradually at 4°C and small pieces of prefrontal cortex were homogenized (10% w/v) in ice-cold 50 mM Tris-HCl (pH 7.4)-500 mM NaCl-5 mM EDTA-1% (w/v) Nonidet P-40-0.5% (w/v) Triton X-100 supplemented with a cocktail of protease inhibitors. The homogenates were sonicated and centrifuged at 70,000×*g* at 4°C for 1 hour; the supernatant was collected, aliquoted and frozen at -80°C until use. This study was approved by the local ethics committees and carried out in accordance with the Declaration of Helsinki.

AChE enzyme assay and protein determination

A modified microassay version of the colorimetric Ellman's method was used to measure AChE [25]. One mU of AChE activity was defined as the number of nmoles of acetylthiocholine hydrolyzed per minute at 22°C. Total protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Analysis of AChE molecular forms

Molecular forms of AChE were separated according to their sedimentation coefficients by ultracentrifugation on continuous 5% to 20% (w/v) sucrose gradients containing 0.5% (w/v) Triton X 100, as previously described [25,28]. Enzymes of known sedimentation coefficient, bovine liver catalase (11.4S) and calf intestinal alkaline phosphatase (6.1S) were used in the gradients to identify individual AChE forms (G_4 = tetramers; G_2 =dimers; G_1 =monomers).

Preparation of membrane fractions and γ-secretase activity assay.

Alternatively, for analysis of γ-secretase activity cell membrane preparations were used [29]. CHO-PS70 cells were washed in PBS, harvested and homogenized using a mechanical pestle homogenizer in buffer containing 10 mM KCl and 10 mM HEPES, pH 7.0, supplemented

with a protease inhibitor cocktail. The cell homogenates were centrifuged at 1,000×*g* for 10 min, and the post-nuclear supernatant was obtained after centrifugation at 100,000×*g* for 1 hour. Membrane fractions were resuspended in buffer containing 20 mM Hepes pH 7.0, 150 mM NaCl, 5 mM EDTA and a protease inhibitor cocktail. Protein concentration was measured by the BCA Protein Assay Kit (Thermo Scientific) and maintained at 3–5 mg/ml. The samples were incubated in the absence or presence of *eel*-AChE (Sigma-Aldrich) or the γ-secretase inhibitor DAPT, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (Calbiochem), at 37ºC for 16 hours. γ-Secretase activity was assessed by measuring the levels of CTF-APP and the CTF of another γ-secretase substrate, the apolipoprotein E receptor 2 or ApoER2 [30] by Western blotting.

Western blot

Samples from cell lysates or brain extracts (30 to 50 µg of protein, equal amount in each lane) were resolved by electrophoresis on 10% SDS-polyacrylamide slab gels (SDS-PAGE) under fully reducing conditions. Samples were denatured at 50°C for 15 minutes (PS1) or 98°C for 7 minutes (all the other proteins). For blue-native gel electrophoresis, samples were analyzed as previously described [31], and NativeMark™ Unstained Protein Standards (Life Technologies) were used as molecular weight markers. Following electrophoresis, proteins were blotted onto nitrocellulose membranes (Schleider & Schuell Bioscience GmbH, Dassel, Germany), and membranes were blocked with 5% nonfat milk. The membranes were probed with the following primary antibodies: anti-CTF-APP (Sigma-Aldrich), anti-CTF apolipoprotein E receptor 2 (ApoER2; Abcam), anti-N-terminal PS1 (Calbiochem®, Merck KGaA, Darmstadt, Germany), anti- PEN2

(presenilin enhancer 2; from Sigma), anti-AChE antibody N-19 (Santa Cruz Biotech), an anti-AChE antibody raised to the unique C-terminus of human AChE-R (also a generous gift from Dr. H. Soreq), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, UK). Western blots for different antibodies were performed individually, to avoid re-using blots. The blots were then incubated with the corresponding secondary antibody conjugated to horseradish peroxidase and the signal was detected using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) in a Luminescent Image Analyzer LAS-1000 Plus (Fujifilm, Tokyo, Japan). For semi-quantitative analysis, the intensity of bands was measured by densitometry with the Science Lab Image Gauge v4.0 software provided by Fujifilm. Protein levels were normalized to GAPDH.

PS1 immunoprecipitation

Brain extracts were pre-cleared by incubation with protein A-Sepharose (Sigma-Aldrich) for 2 hours at 4°C. Immunoprecipitations were performed at 4°C by first incubating 800 µg of protein overnight with the N-terminal PS1 antibody 98/1 (a generous gift form J. Culvenor, Department of Pathology, The University of Melbourne, Australia) previously coupled to protein A-Sepharose by dimethyl pimelimidate dihydrochloride (Sigma-Aldrich). Precipitated proteins were washed with PBS and eluted with 0.1M glycine buffer at pH 2.5. After pH neutralization, supernatants were denatured in Laemmli sample buffer at 97ºC for 7 min and subjected to SDS-PAGE/Western blotting. Blots were incubated with the anti-AChE antibodies Ab31276 and anti-AChE-R.

RNA isolation and analysis of transcripts by *q*RT-PCR

Total RNA was isolated from control CHO-PS70 cells or cells treated with ***eel***-AChE using TRIzol Reagent in the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen) according to the

manufacturer's protocol. First-strand cDNAs were obtained by reverse transcription of 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; life technologies Paisley, UK), according to the manufacturer's instructions. Quantitative PCR amplification was performed in a StepOne™ Real-Time PCR System (Applied Biosystems) with TaqMan GenExpression Assays (Hs00997789 for PS1 and Hs03929097 for GAPDH) and TaqMan PCR Master Mix. Transcript levels for PS1 were calculated using the relative standard curve method normalized to GADPH.

Co-localization of AChE and PS1

CHO cells were transiently co-transfected with either 500 ng each of PS1-GFP (kindly provided by Dr. O. Berezovska; Massachusetts General Hospital, MA, USA) and AChE-T plasmids or 300 ng of each of PS1-GFP, AChE-T and PRiMA plasmids. Cells were fixed with 4% paraformaldehyde after 24 hours and immunostained for AChE using anti-AChE followed by an Alexa647-tagged secondary antibody (Molecular Probes, Inc, USA). Confocal images were taken with a SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) using a 63× objective (4× zoom). Laser power was kept low to avoid crossover between the two channels and to avoid pixel saturation. Confocal images were taken in multiple z planes (1micron apart). Analysis was performed using ImageJ software (v1.46g) [32]. Briefly, channels were thresholded to create a binary image and Manders' co-efficients [33] were calculated using the JACoP JaCoP ImageJ plugin [34]. The Manders' co-efficient corresponds to the fraction of AChE-positive pixels that are also positive for PS1. Images showing the pixels where the two channels co-localize were generated for the binary thresholded images using the Co-localization highlighter ImageJ plugin.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Data were analyzed using SigmaStat (Version 2.0; SPSS Inc.) by Student's t-test (two tailed) or by one-way analysis of variance (ANOVA), followed by Tukey test for pair-wise comparisons. Statistical significance was designated as $\boldsymbol{p} < 0.05$.

Results

Several AChE variants and isoforms interact with PS1

We first investigated whether PS1 antibodies were able to co-precipitate the R and T variants, and G_4 and G_1 AChE-T species (Fig. 1). Human brain cortex samples were immunoprecipitated using an anti-PS1 antibody, and the bound fraction was analysed by Western blotting using different anti-AChE antibodies raised against different C-terminal peptides of R and T AChE variants. Western blot analysis of the immunoprecipitates demonstrated that both AChE subunits, T and R, are potential PS1-interacting proteins (Fig. 1A). In agreement with our previous study (Silveyra et al., 2008), ultracentrifugation in sucrose density gradients confirmed that both peaks corresponding to the major AChE G_4 (tetramers of T subunits) and to the minor light forms (monomers of T, and potentially of R subunits) were decreased after immunoprecipitation with PS1 antibodies (Fig. 1B).

We next examined whether these AChE species influence PS1 levels (Fig. 2A). Over-expression of AChE-T and AChE-R in CHO cells, as monomeric forms, leads to a statistically significant increase in PS1 levels, compared to untransfected cells (Fig. 2A). The differences between AChE-R (67 ±19%) and AChE-T increase (36 ±5%) on PS1 levels is not statistically significant (p= 0.18). Over-expression of AChE-T and AChE-R in the neuroblastoma cell line SH-SY5Y yield similar increases in PS1 levels (Supplementary Fig. 1).

Influence of AChE in PS1 levels is not dependent on its catalytic activity

All the molecular forms and variants of AChE have been demonstrated to be virtually equivalent in their catalytic activity [35,36]. We next examined whether the suppression of AChE catalytic activity affects its ability to modulate PS1 levels. As it has been previously shown that mutation of serine200 to valine abolishes detectable AChE activity [27], we over-expressed site-directed mutants at serine200 for both AChE-T and AChE-R. Over-expression of the inactive mutants, ***im***AChE-T and ***im***AChE-R resulted in an increase in AChE protein levels, as assessed by Western blotting, with no substantial increase in specific activity (Fig. 2B). However, both inactive mutants were able to induce an increase in PS1 levels (Fig. 2B), indicating that the modulatory capacity of AChE is exerted by a mechanism independent of its catalytic activity.

Influence of AChE in PS1 levels is dependent on its subcellular localization

The proline-rich membrane anchor (PRiMA) subunit is a small transmembrane protein that represents a limiting factor for the restricted localization of AChE into the plasma membrane. It transforms monomeric AChE-T into a tetrameric AChE (G4)-PRiMA complex which anchors to the outer cell surface [37-39]. We examined if co-expression of the PRiMA subunit with AChE-T further affects PS1 levels. A CHO cell line over-expressing AChE-T was co-transfected with the PRiMA subunit. As expected, cells over-expressing AChE and PRiMA produced significant amounts of G4 AChE in comparison with those over-expressing AChE only (Fig. 3A). Greater AChE activity was detected on the outer cell surface of intact (non-permeabilized) cultured cells over-expressing AChE and PRiMA compared to cells

transfected with AChE in the absence of PRiMA (Fig. 3B). Immunocytochemistry was also used to compare the distribution of PS1 and AChE, expressed as a monomer or as a tetrameric PRiMA-linked AChE. Immunofluorescence labelling of cells confirmed localization of PS1 to both the cytoplasmic region and the periphery (plasma membrane) (Fig. 3C, D), a finding consistent with previous reports by us and others [22, 40-42]. In the absence of PRiMA, AChE co-localized with PS1 mainly within the cytoplasmic region (80 ±7% of AChE pixels were also positive for PS1; Fig. 3C). In contrast, in the presence of PRiMA, AChE was, as expected, targeted to the plasma membrane with staining predominantly localized to the cell periphery, with minor cytoplasmic co-localization with PS1 (only 50 ±6% of AChE pixels were also positive for PS1; p= 0.03 versus AChE without PRiMA; Fig. 3D). The levels of PS1 in cells over-expressing AChE with PRiMA is higher than in cells over-expressing intracellular AChE alone, while over-expression of PRiMA alone fails to trigger noticeable change in PS1 levels (Fig. 3E). The PRiMA subunit is an accessory partner for the cellular disposition of AChE [39], at the plasma membrane always in the presence of AChE. In conclusion, the AChE induced increase in the levels of PS1 is further augmented by the presence of PRiMA at the plasma membrane.

AChE increases PS1 protein and mRNA levels

Our results indicate that the ability of AChE to induce an increase in PS1 levels is not dependent on its C-terminal (variant), oligomerization status (molecular form) or enzymatic activity. The most significant variable which determines how AChE influences PS1 levels is co-localization outside the plasma membrane. We therefore assessed if soluble AChE (a G4 species from *Electrophorus electricus*, *eel*-AChE) is able to modulate endogenous PS1 levels in untransfected CHO cells.

After an 18 hour treatment with soluble *eel*-AChE, the levels of PS1 were significantly increased from 0.5±1 to 34±1 mU/mL (Fig. 4A). We next determined whether AChE influences the *PS1* expression by measuring *PS1* mRNA levels by *q*RT-PCR. Levels of the *PS1* transcripts were significantly increased (44 ±1%, $p< 0.001$) in *eel*-AChE treated cells compared to vehicle control cells (Fig. 4B).

AChE inhibits γ-secretase activity

Up-regulation of protein levels as a reaction to inhibition is a recognized phenomenon documented for several proteins [43,44], including AChE [17, 46,47]. To assess if AChE-mediated PS1 up-regulation is linked to an inhibitory effect of AChE on γ-secretase activity, we treated with *eel*-AChE CHO-PS70 cells, which stably overexpress wild-type human PS1 and wild-type APP and exhibit elevated γ-secretase activity [26]. The potential inhibitory effect of AChE on γ-secretase activity was monitored by measuring the accumulation of APP-CTF levels. Cells were treated for 18 hours with increasing amounts of *eel*-AChE, and levels of APP-CTF were determined in cellular extracts by Western blotting using an antibody raised against the APP C-terminal. A dose-dependent effect of AChE on γ-secretase activity was observed, with increased amount of APP-CTF in treated cells (Fig. 5A). The inhibitory effect of AChE on γ-secretase activity was then determined in membrane preparations isolated from CHO-PS70 cells obtained as described elsewhere [29]. The presence of the γ-secretase complex in these membrane preparations was first confirmed by blue native-PAGE (Fig. 5B). A predominant PS1 immunoreactive band, with a molecular mass of ~450 kDa (closed arrowhead), was detected together with other high molecular mass bands, corresponding to large γ-secretase complexes [48,49]. These

bands were also immunoreactive for the γ-secretase component PEN2 (presenilin enhancer 2) [50]. To determine the effect of inhibition of γ-secretase activity on γ-secretase cleavage of APP, cell membranes were incubated at 37ºC for 16 hours in the absence or presence of N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT), a well-known γ-secretase inhibitor that targets PS1 [51]. The efficiency of 5 μM DAPT to inhibit γ-secretase activity was monitored by measuring the accumulation of APP-CTF in membrane preparations (Fig. 5C). Accumulation of APP-CTF was also observed in membrane preparations incubated with ~34±1 mU/mL of *eel*-AChE (Fig. 5C). The increased levels in the membrane preparation treated with *eel*-AChE of the CTF of ApoER2, another γ-secretase substrate [30], which was not over-expressed in CHO-PS70 cells, served to confirm the decrease in γ-secretase in presence of AChE (Fig. 5C). These results suggest that AChE may act as an inhibitor of γ-secretase activity.

Discussion

The cholinergic system has been shown to modulate APP metabolism [52,53] and AChE inhibitors affect amyloid production [54-56]. In turn, different reports have supported the possibility that Aβ may up-regulate AChE [57-61]. While characterization of the functional cross-talk between AChE/cholinergic neurotransmission and APP processing is of major interest, there is currently no consensus on the mechanisms which regulate these reciprocal interactions. Recent evidence demonstrates that cholinergic AChE can be down-regulated in neuronal cell lines by APP independently of secretase activity [62]. However, other studies have reported modulatory effects of AChE inhibitors on α-secretase [63,64] and β-secretase [65-67]. Our previous studies have also described that AChE inhibitors are able to modulate PS1 levels [23].

We have previously explored some of the potential consequences of the interaction between AChE and PS1, and demonstrated that AChE knockdown with siRNA, as well as AChE inhibition, decreased cellular PS1 levels; whereas AChE over-expression exerted an opposing effect [23]. Our previous data also suggested that AChE does not exert its modulatory action on PS1 via a cholinergic mechanism, as the cholinergic agonist carbachol had no effect on PS1 [23]. Hence, the mechanisms employed by AChE to influence APP processing remained unclear. Our present study addresses how AChE influences PS1 expression by examining changes in PS1, at both protein and transcriptional levels, in several conditions where distinct AChE variant and molecular forms have been modulated. We first confirmed that AChE does not exert its modulatory action on PS1 via a cholinergic mechanism since mutant inactive variants also influence PS1 levels.

Although all the AChE variants (R and T) and molecular forms (monomers and tetramers) tested can influence PS1 levels, the AChE species that triggered the major increase in PS1 levels was the PRiMA-linked AChE form. The PRiMA subunit restricts localization of cholinergic tetrameric AChE to the outer plasma membrane. PS1 and AChE are located in the same intracellular compartments, including perinuclear compartments, but interestingly PRiMA has been shown to restrict AChE localization to the membrane of synapses [68-70]. Similarly, PS1 is targeted to the cell surface as an active γ-secretase complex [71]. However, the subcellular localization of biologically active γ-secretase is still a matter of controversy. Our studies demonstrate that AChE inhibits APP processing catalyzed by γ-secretase in both cells and membrane preparations. The possibility that AChE inhibits cleavage of APP by γ-secretase has been recently suggested [72]. Therefore, we postulated that, under non-pathological

conditions, it is the cholinergic species of AChE which likely interacts with PS1, within the active γ-secretase complex, but by a mechanism independent of its catalytic activity.

The mechanisms employed by AChE to influence APP processing remain unclear. Besides the involvement of the catalytic activity of AChE, a direct effect based on protein-protein interaction also seems plausible. Indeed, AChE is much more than a cholinergic enzyme with distinct biological functions than merely hydrolysis of acetylcholine. In this context, excess of enzymatically inactivated brain AChE by transgenic over-expression have demonstrated different biological functions [13-15]. Native AChE is also present in non-cholinergic tissues and shares high sequence similarity with several neural cell adhesion proteins [73]. The presence of a cholinesterase-like domain in non-catalytic proteins structurally related to AChE may reflect its capacity for protein-protein interactions. This cholinesterase-like domain may have adhesive properties [74]. Therefore, AChE may inhibit APP processing by blocking access of γ-secretases to APP. We have recently demonstrated that γ-secretase is involved in the cleavage of PRiMA [75]. Neuroligin-1, a postsynaptic adhesion molecule whose extracellular domain is homologous to AChE, is also cleaved by γ-secretase [76]. In general, the specific requirements for a γ-secretase substrate are vague, and do not depend on a specific amino acid sequence or on endocytosis [77]. More than 90 type-I integral membrane proteins are known to be potentially cleaved by γ-secretase [78], but which of those are "common" substrates for γ-secretase in physiological conditions remains unclear. We favor the hypothesis that AChE acts as an inhibitor of γ-secretase activity by interacting with PS1. Nonetheless, we can speculate that some potential substrates of γ-secretase, such as PRiMA from the AChE cholinergic complex, are not "common" substrates and only interact under specific physiological

conditions, but which results in low catalytic efficiency. Likewise binding of AChE subunits to PS1 may restrict γ-secretase activity, similar to a negative feedback by end-product inhibition. Further extensive research is needed to determine how AChE blocks or interferes with PS1 and γ-secretase activity and which pool of AChE is involved in the process.

In this study we report that an increase in AChE blocks γ-secretase activity. Up-regulation in reaction to inhibition is a recognized phenomenon documented for several proteins [43,44], including AChE [17,45]. Our data suggest that inhibition of PS1 by AChE may initiate a feedback process that leads to up-regulation of PS1. Regarding the pathological condition, AChE activity (particularly the cholinergic specie) is decreased in the AD brain [28, 79-81], therefore impeding its ability to modulate γ-secretase activity. Interestingly, therapy with inhibitors of AChE demonstrated weak disease-modifying effects in AD-treated patients, including modulation of APP expression and metabolism [63, 82-85]. As previously mentioned, the mechanisms employed by AChE inhibitors to influence APP processing remain unclear but may involve multiple mechanisms that vary according to the type of AChE inhibition. Specifically, how AChE inhibitors trigger a decrease in PS1 levels is unclear. However, it is important to note that the positive modulation of AChE inhibitors on APP failed to have a long-term effect in patients [83]. We propose that a limited response to AChE inhibitors may be associated with AChE up-regulation in reaction to chronic inhibition, a feedback process that leads to accumulation of AChE in parallel with the lack of effect on PS1 levels [23]. This phenomenon of AChE up-regulation, as a response to anti-AChE therapy, has been confirmed in patients under AChE inhibitor therapy [46,47,86]. Nonetheless, the subcellular localization of this new pool of

AChE, and therefore the potential to interact with PS1, merits further investigation.

In addition, under non-disease conditions AChE occurs as both active and inactive subunits [87,88], and the existence of inactive AChE has been demonstrated in brain [89]. We have recently shown by Western blotting and immunohistochemistry that a prominent pool of enzymatically inactive AChE protein existed in the AD brain [90]. The physiological significance of non-catalytic AChE in brain and how it is affected during pathology and treatment remain unexplored.

In conclusion, our data concur with other reports suggesting the regulation of APP processing by AChE. This modulatory effect may involve cholinergic and non-cholinergic mechanisms, independent of the catalytic activity of AChE. We demonstrate a modulation of PS1 by the AChE species via non-cholinergic mechanisms. We also provide evidence that γ-secretase inhibition could result in PS1 up-regulation which is of particular importance for AD therapy [91-93]. Elucidation of the mechanisms involved in the PS1-AChE interaction and reciprocal regulation are important for the optimization of current therapies based on AChE pharmacological interventions.

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Disclosure: None of the authors have any actual or potential financial conflicts or conflict of interest related with this study.

Figures

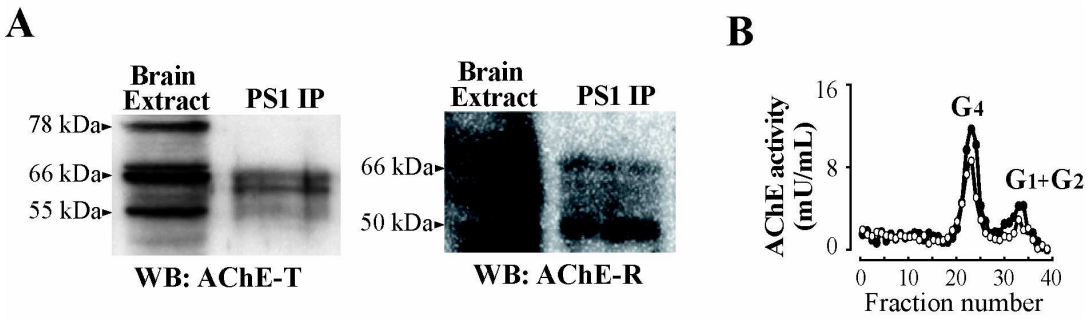


***Figure 1*. PS1 interacts with AChE-T and AChE-R variants**. (**A**) Co-precipitation of PS1 and AChE. Human brain extracts (frontal cortex from three non-demented subjects, mean age 58 ±3 years; one example is shown) were immunoprecipitated with anti-PS1 antibody 98/1. PS1-immunoprecipitated proteins (PS1 IP) were immunoblotted with the indicated anti-AChE antibody specific for particular AChE variants (T and R). Extracts incubated with protein A-Sepharose, without antibody, were analyzed in parallel as negative controls (not shown). (**B**) The non-immunoprecipitated fraction was analyzed for molecular forms of AChE by sucrose gradient ultracentrifugation. Approximately 40 fractions were collected from the bottom of each tube and assayed for AChE activity. Representative profiles of AChE molecular forms (tetramers: G4; and light dimers and monomers: G1+G2) prior (●) and after (□) immunoprecipitation are shown. Experiments were performed in triplicate.

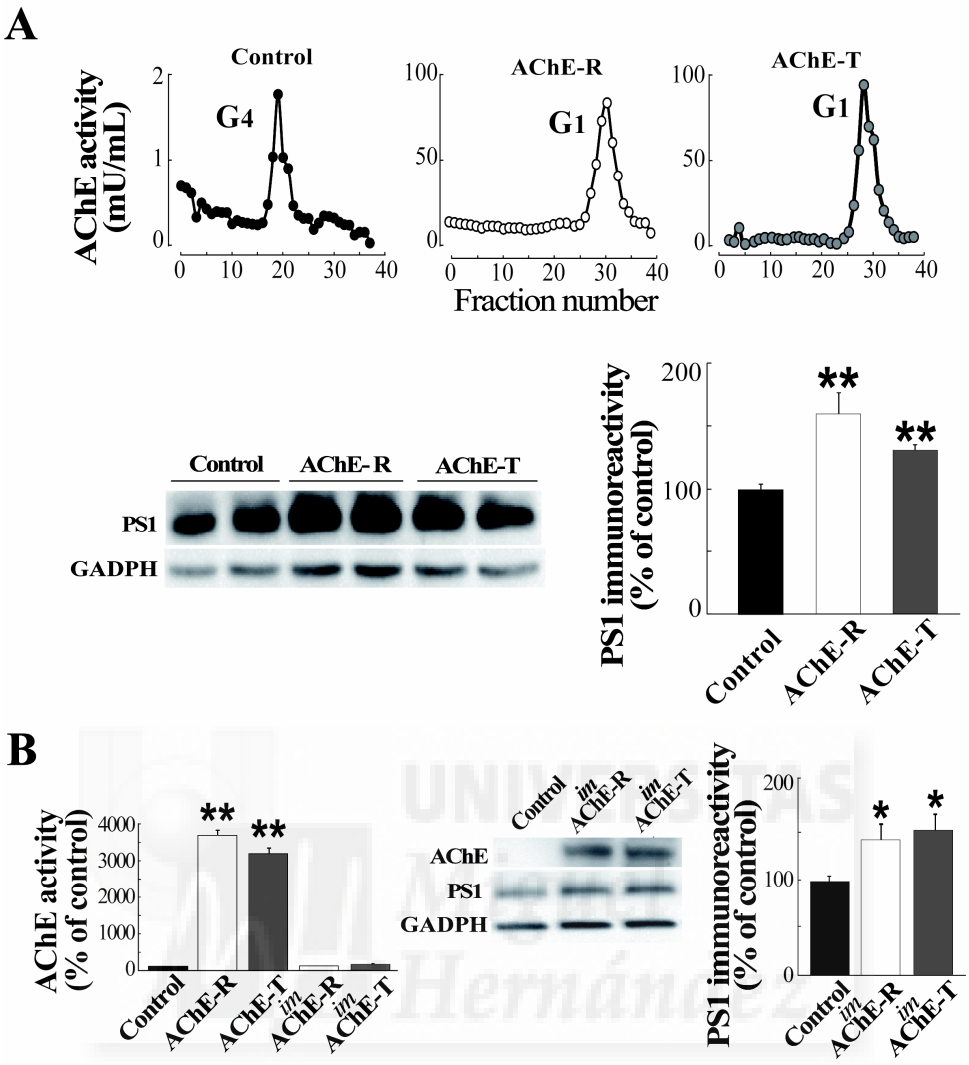


Figure 2. **PS1 levels are regulated by AChE independent of its enzymatic activity.** (**A**) Representative molecular profiles of AChE and immunodetection of PS1-NTF in CHO cells stably transfected with constructs carrying either the AChE-R or AChE-T cDNA. CHO cells stably transfected with a PCI vector served fas controls (Control). The results were confirmed in three independent experiments. The densitometric quantification of PS1-NTF immunoreactivity is represented. Protein levels were normalized to glyceraldehyde 3-phosphate deydrogenase (GADPH). (**B**) PS1 immunodetection and densitometric quantification in cells transfected with the inactive form of AChE-R (***im***AChE-R) and AChE-T (***im***AChE-T). Immunoblots with the anti-AChE antibody N19 antibody confirmed the expression of equal amounts of ***im***AChE-R and ***im***AChE-T in transfected cells. Columns represent mean ± SEM from three different experiments (n= 12 for each condition). Representative immunoblots are shown. *****p*** < 0.01 and ****p***< 0.05, significant difference from the control group.

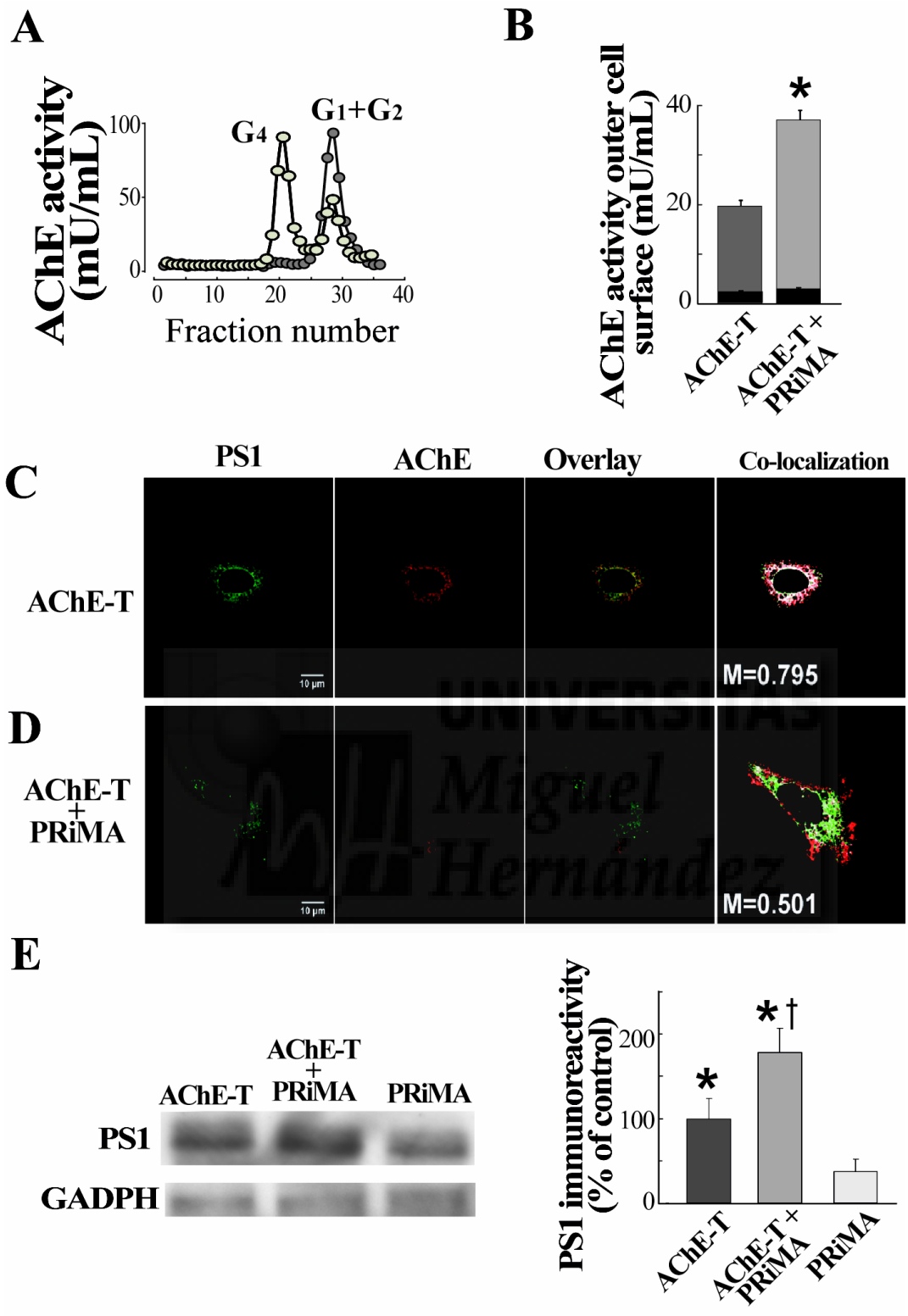


***Figure 3*. Regulation of PS1 levels by tetrameric PRiMA-linked AChE located in the plasma membrane**. (**A**) Representative profiles of AChE in CHO cells stably transfected with AChE-T cDNA without (●; AChE-T) and with PRiMA co-expression (●; AChE-T+PRiMA) (G4=tetramers; G1+G2=monomers and dimers). The results were

confirmed in four independent determinations. (**B**) AChE activity was also assayed directly in plasma membranes from cultured cells transfected with AChE-T in presence or absence of PRiMA cDNA. The inner dark columns represent AChE activity levels after treatment with the AChE inhibitor tacrine (10μM). (**C, D**) AChE-T is transported from the cytoplasm to the cell periphery in the presence of PRiMA. Representative images of CHO cells transiently co-expressing PS1 and AChE-T (C), or PS1, AChE-T and PRiMA (D). PS1; PS1-GFP (488 nm) channel, AChE; Anti-AChE N19 (647 nm) channel. Overlay; overlay of 488 nm and 647 nm channels. Co-localization; channel overlay with pixels positive for both PS1 and AChE, marked in white. M; Mander's co-localization co-efficient. Localization of PS1 and AChE-T in the absence of PRiMA is observed mainly in the cytoplasmic region (**C**). The mean number of AChE-T pixels co-localizing with PS1 was 79.5% (n=3). Cells expressing AChE-T+PRiMA and PS1 show localization of AChE at the cell periphery with only 50.1% of AChE pixels co-localized with PS1 (n=3). (**E**) Immunodection and densitometric quantification of PS1-NTF (normalized to GADPH) in CHO cells transfected with AChE-T, AChE-T+PRiMA or PRiMA alone. Columns represent mean ± SEM from two different experiments (n= 10 for each condition). Significantly different ($\boldsymbol{p} < 0.05$) from cells over-expressing PRiMA alone (*), or from the AChE-T cells (†).

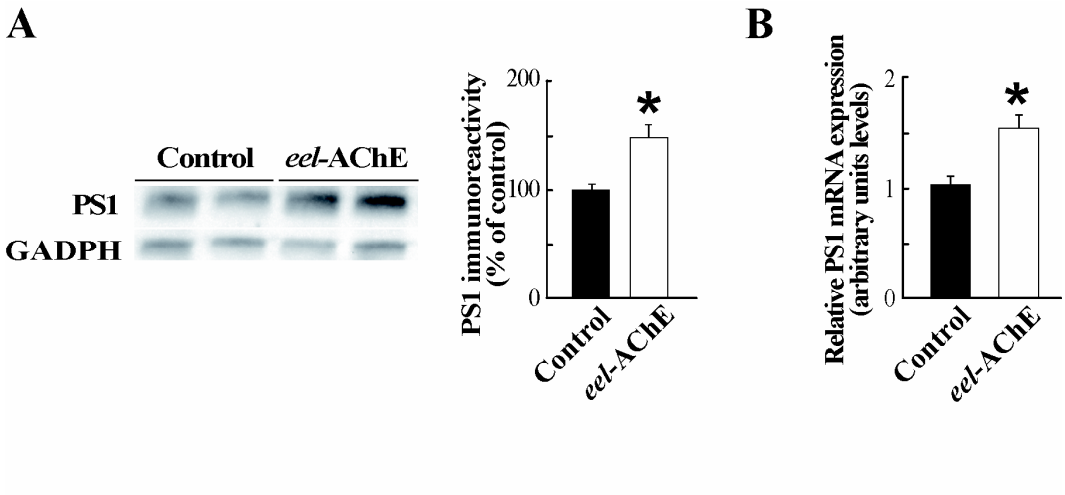


Figure 4*. The presence of exogenous AChE increases PS1 expression.** CHO cells were treated for 18 hours with AChE from ***Electrophorus electricus (***eel***-AChE; at ~34±1 mU/mL of enzymatic activity) or saline (Control). (**A**) Cell extracts were analyzed by Western blot with an anti-N-terminal PS1 antibody. Equivalent amounts of protein were loaded in each lane and GAPDH was used as a loading control. An increase in PS1 immunoreactivity was observed in cells treated with ***eel***-AChE (**B**) Messenger RNA levels of the PS1 transcript were measured by ***q***RT-PCR from cell extracts. Values were calculated using relative standard curves and normalized to GAPDH obtained from the same cDNA preparations. mRNA levels were significantly increased in cells treated with ***eel***-AChE. Data represent mean ± SEM from a minimum of 15 independent determinations from three independent experiments. *$\boldsymbol{p} < 0.001$.

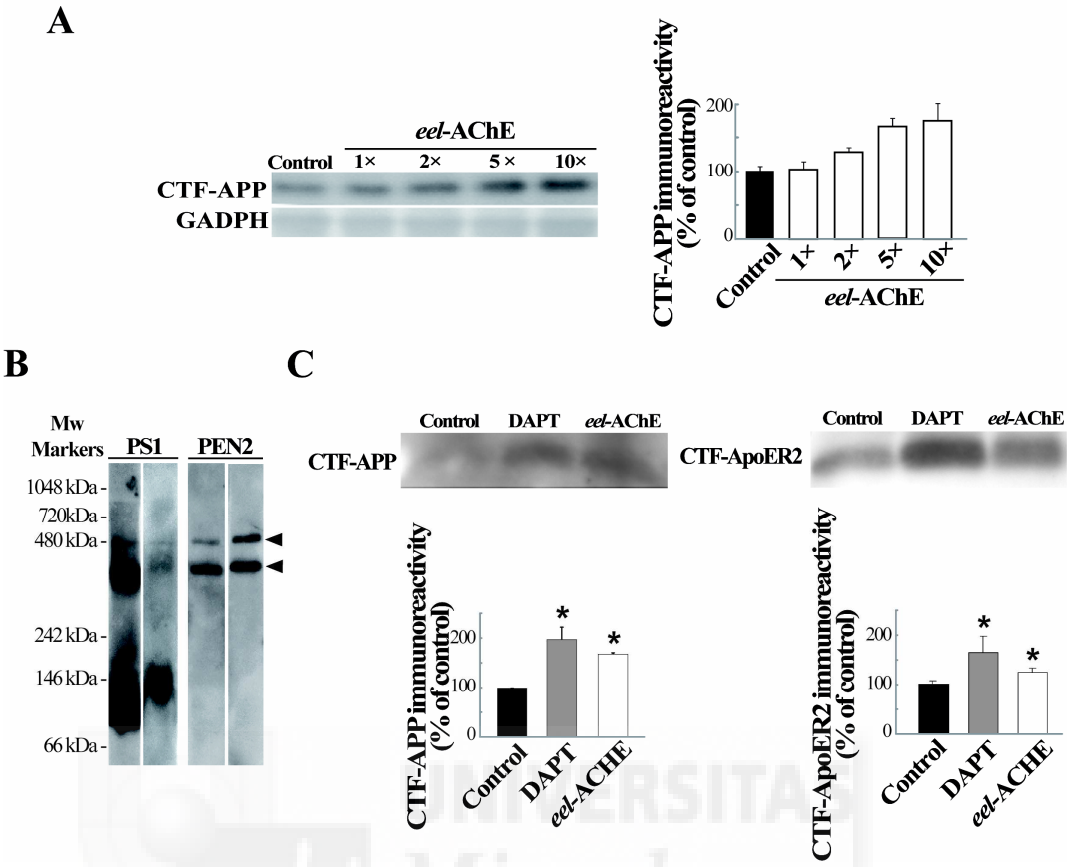
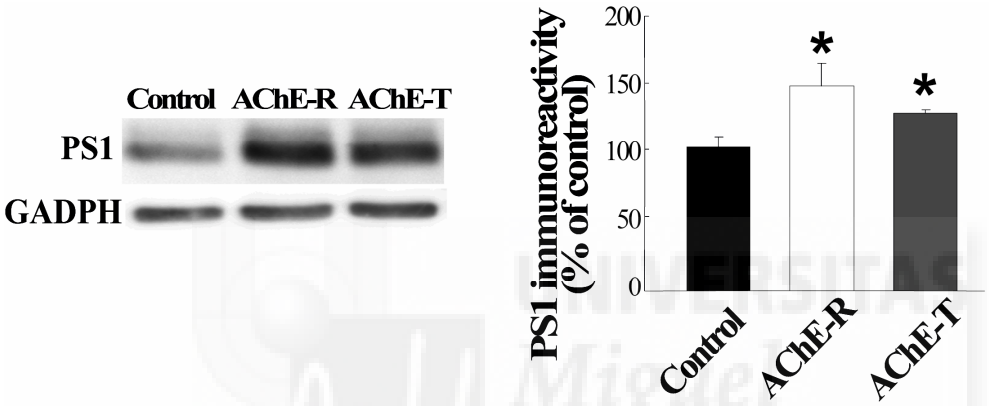


Figure 5*. Inhibition of PS1/γ-secretase processing of C-terminal fragments of APP by AChE.** (**A**) Dose-dependent effect of soluble AChE from ***Electrophorus electricus (***eel***-AChE) on APP processing. CHO cells were treated with 0 (saline; Control), ~9±1 mU/mL (1×), ~17±1 mU/mL (2×), ~34±1 mU/mL (5×) or ~70±1 mU/mL (10×) of active ***eel***-AChE. Cell extracts blotted with a C-terminal anti-APP antibody demonstrated APP CTF accumulation in treated cells as a result of the inhibition of γ-secretase processing. (**B**) CHO cells over-expressing PS1 were homogenized and membranes isolated by sequential centrifugation (see Material & Methods). γ-Secretase complexes were characterized by blue native-PAGE using an anti-PS1 antibody. Complexes of different molecular mass were detected. Similar immunoreactive bands (arrowheads) were detected for PEN2, a subunit of the γ-secretase complex. (**C**) γ-Secretase cleavage of endogenous APP in membrane preparations of CHO cells was assessed in the presence of ~34±1 mU/mL of ***eel***-AChE. γ-secretase activity was inhibited in cells treated with 5 μM of the γ-secretase inhibitor DAPT. Data represent the percentage relative to control cells. The results were confirmed in two independent experiments (n= 8 determinations). *$\boldsymbol{p} < 0.05$.

SUPPLEMENTARY DATA

To confirm our hypothesis we over-expressed AChE-T and AChE-R in neuroblastoma cell line SH-SY5Y. Again, AChE increases PS1 levels.



Supplementary Figure. Effect of the AChE over-expression on PS1 levels in SH-SY5Y cells. Immunodetection and densitometric quantification of PS1-NTF for AChE-R or AChE-T transfected, and control cells transfected with a PCI vector. Protein levels were normalized to glyceraldehyde 3-phosphate deydrogenase (GADPH). Data represent percentage relative to control cells, expressed as means ± SEM of 10 independent determinations from at two different experiments. *$p < 0.05$.

References

1. Davies P, Maloney AJ (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet* 2, 1403.
2. Perry EK, Perry RH, Blessed G, Tomlinson BE (1978) Changes in brain cholinesterases in senile dementia of Alzheimer type.Neuropathol Appl Neurobiol 4, 273-277.
3. Kása P, Rakonczay Z, Gulya K (1997) The cholinergic system in Alzheimer's disease. *Prog Neurobiol* 52, 511-535.
4. Schliebs R, Arendt T (2011) The cholinergic system in aging and neuronal degeneration. *Behav Brain Res* 221, 555-563.
5. Giacobini E (2003) Cholinergic function and Alzheimer's disease. *Int J Geriatr Psychiatry* 18, S1-5.
6. Lleó A, Greenberg SM, Growdon JH (2006) Current pharmacotherapy for Alzheimer's disease. *Annu Rev Med* 57, 513-533.
7. Di Santo SG, Prinelli F, Adorni F, Caltagirone C, Musicco M (2013) A meta-analysis of the efficacy of donepezil, rivastigmine, galantamine, and memantine in relation to severity of Alzheimer's disease. *J Alzheimers Dis* 35, 349-361.
8. Massoulié J, Sussman J, Bon S, Silman I (1993) Structure and functions of acetylcholinesterase and butyrylcholinesterase. *Prog Brain Res* 98, 139-146.
9. Layer PG (1995) Nonclassical roles of cholinesterases in the embryonic brain and possible links to Alzheimer disease. *Alzheimer Dis Assoc Disord* 9, 29-36.
10. Small DH, Michaelson S, Sberna G (1996) Non-classical actions of cholinesterases: role in cellular differentiation, tumorigenesis and Alzheimer's disease. *Neurochem* 28, 453-483.
11. Soreq H, Seidman S (2001) Acetylcholinesterase-new roles for an old actor. *Nat Rev Neurosci* 2, 294-302.
12. Silman I, Sussman JL (2005) Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. *Curr Opin Pharmacol* 5, 293-302.
13. Sternfeld M, Ming G, Song H, Sela K, Timberg R, Poo M, Soreq H (1998) Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. *J Neurosci* 18, 1240-1249.
14. Dori A, Cohen J, Silverman WF, Pollack Y, Soreq H (2005) Functional manipulations of acetylcholinesterase splice variants highlight alternative splicing contributions to murine neocortical development. *Cereb Cortex* 15, 419-430.
15. Grisaru D, Pick M, Perry C, Sklan EH, Almog R, Goldberg I, Naparstek E, Lessing JB, Soreq H, Deutsch V (2006) Hydrolytic and non enzymatic functions of acetylcholinesterase comodulate hemopoietic stress responses. *J Immunol* 176, 27-35.
16. Massoulié J (2002) The origin of the molecular diversity and functional anchoring of cholinesterases. *Neurosignals* 11, 130-143.
17. Kaufer D, Friedman A, Seidman S, Soreq H (1998) Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature* 393, 373-377.
18. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer's disease and Down syndrome. *Proc Natl Acad Sci USA* 82, 4245-4249.

19. Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Müller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325, 733-736.

20. Thinakaran G, Koo EH (2008) Amyloid precursor protein trafficking, processing, and function. *J Biol Chem* 283, 29615-29619.

21. De Strooper B, Iwatsubo T, Wolfe MS (2012) Presenilins and γ-secretase: structure, function, and role in Alzheimer Disease. *Cold Spring Harb Perspect Med* 2, a006304.

22. Silveyra MX, Evin G, Montenegro MF, Vidal CJ, Martínez S, Culvenor JG, Sáez-Valero J (2008) Presenilin 1 interacts with acetylcholinesterase and alters its enzymatic activity and glycosylation. *Mol Cell Biol* 28, 2908-2919.

23. Silveyra MX, García-Ayllón MS, Serra-Basante C, Mazzoni V, García-Gutierrez MS, Manzanares J, Culvenor JG, Sáez-Valero J (2012) Changes in acetylcholinesterase expression are associated with altered presenilin-1 levels. *Neurobiol Aging* 33, 627.e27-37.

24. Chen VP, Choi RC, Chan WK, Leung KW, Guo AJ, Chan GK, Luk WK, Tsim KW (2011) The Assembly of Proline-rich Membrane Anchor (PRiMA)-linked Acetylcholinesterase Enzyme. Glycosylation is required for enzymatic activity but not for oligomerization. *J Biol Chem* 286, 32948–32961.

25. Sáez-Valero J, Tornel PL, Muñoz-Delgado E, Vidal CJ (1993) Amphiphilic and hydrophilic forms of acetyl- and butyrylcholinesterase in human brain. *J Neurosci Res* 35, 678–689.

26. Xia W, Zhang J, Kholodenko D, Citron M, Podlisny MB, Teplow DB, Haass C, Seubert P, Koo EH, Selkoe DJ (1997) Enhanced production and oligomerization of the 42-residue amyloid beta-protein by Chinese hamster ovary cells stably expressing mutant presenilins. *J Biol Chem* 272, 7977-7982.

27. Gibney G, Camp S, Dionne M, MacPhee-Quigley K, Taylor P (1990) Mutagenesis of essential functional residues in acetylcholinesterase. *Proc Natl Acad Sci USA* 87, 7546-7550.

28. Sáez-Valero J, Sberna G, McLean CA, Small DH (1999) Molecular isoform distribution and glycosylation of acetylcholinesterase are altered in brain and cerebrospinal fluid of patients with Alzheimer's disease. *J Neurochem* 72, 1600-1608.

29. Frånberg J, Karlström H, Winblad B, Tjernberg LO, Frykman S (2010) γ-Secretase dependent production of intracellular domains is reduced in adult compared to embryonic rat brain membranes. *PLoS One* 5, e9772.

30. Balmaceda V, Cuchillo-Ibáñez I, Pujadas L, García-Ayllón MS, Saura CA, Nimpf J, Soriano E, Sáez-Valero J (2013) ApoER2 processing by presenilin-1 modulates reelin expression. *FASEB J* In press;doi: 10.1096/fj.13-239350.

31. Schägger H, Von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal Biochem* 199, 223–231.

32. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9, 671-675.

33. Manders EMM, Verbeek FJ, Aten JA (1993) Measurement of co-localization of objects in dual-colour confocal images. *J Microsc* 169, 375-382.

34. Bolte S, Cordelieres FP (2006) A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* 224, 213-232.

35. Vigny M, Bon S, Massoulié J, Leterrier F (1978) Active-site catalytic efficiency of acetylcholinesterase molecular forms in Electrophorus, torpedo, rat and chicken. *Eur J Biochem* 85, 317-323.

36. Soreq H, Gnatt A, Loewenstein Y, Neville LF (1992) Excavations into the active-site gorge of cholinesterases. *Trends Biochem Sci* 17, 353-358.

37. Perrier AL, Massoulié J, Krejci E (2002) PRiMA: the membrane anchor of acetylcholinesterase in the brain. *Neuron* 33, 275-285.

38. Perrier NA, Khérif S, Perrier AL, Dumas S, Mallet J, Massoulié J (2003) Expression of PRiMA in the mouse brain: membrane anchoring and accumulation of 'tailed' acetylcholinesterase. *Eur J Neurosci* 18, 1837-1847.

39. Dobbertin A, Hrabovska A, Dembele K, Camp S, Taylor P, Krejci E, Bernard V (2009) Targeting of acetylcholinesterase in neurons in vivo: a dual processing function for the proline-rich membrane anchor subunit and the attachment domain on the catalytic subunit. *J Neurosci* 29, 4519-4530.

40. Kovacs DM, Fausett HJ, Page KJ, Kim TW, Moir RD, Merriam DE, Hollister RD, Hallmark OG, Mancini R, Felsenstein KM, Hyman BT, Tanzi RE, Wasco W (1996) Alzheimer-associated presenilins 1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells. *Nat Med* 2, 224-229.

41. Huynh DP, Vinters HV, Ho DH, Ho VV, Pulst SM (1997) Neuronal expression and intracellular localization of presenilins in normal and Alzheimer disease brains. *J Neuropathol Exp Neurol* 56, 1009-1017.

42. Herl L, Lleo A, Thomas AV, Nyborg AC, Jansen K, Golde TE, Hyman BT, Berezovska O (2006) Detection of presenilin-1 homodimer formation in intact cells using fluorescent lifetime imaging microscopy. *Biochem Biophys Res Commun* 340, 668-674.

43. Xu L, Kappler CS, Mani SK, Shepherd NR, Renaud L, Snider P, Conway SJ, Menick DR (2009) Chronic administration of KB-R7943 induces up-regulation of cardiac NCX1. *J Biol Chem* 284, 27265-27272.

44. Serfőző Z, Lontay B, Kukor Z, Erdődi F (2012) Chronic inhibition of nitric oxide synthase activity by N(G)-nitro-L-arginine induces nitric oxide synthase expression in the developing rat cerebellum. *Neurochem Int* 60, 605-615.

45. Chiappa S, Padilla S, Koenigsberger C, Moser V, Brimijoin S (1995) Slow accumulation of acetylcholinesterase in rat brain during enzyme inhibition by repeated dosing with chlorpyrifos. *Biochem Pharmacol* 49, 955-963.

46. Darreh-Shori T, Almkvist O, Guan ZZ, Garlind A, Strandberg B, Svensson AL, Soreq H, Hellström-Lindahl E, Nordberg A (2002) Sustained cholinesterase inhibition in AD patients receiving rivastigmine for 12 months. *Neurology* 59, 563-572.

47. García-Ayllón MS, Silveyra MX, Andreasen N, Brimijoin S, Blennow K, Sáez-Valero J (2007) Cerebrospinal fluid acetylcholinesterase changes after treatment with donepezil in patients with Alzheimer's disease. *J Neurochem* 101, 1701-1711.

48. Edbauer D, Winkler E, Haass C, Steiner H (2002) Presenilin and nicastrin regulate each other and determine amyloid beta-peptide production via complex formation. *Proc Natl Acad Sci USA* 99, 8666-8671.

49. Kimberly WT, LaVoie MJ, Ostaszewski BL, Ye W, Wolfe MS, Selkoe DJ (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. *Proc Natl Acad Sci USA* 100, 6382-6387.

50. Kaether C, Haass C, Steiner H (2006) Assembly, trafficking and function of gamma-secretase. *Neurodegener Dis* 3, 275-283.

51. Morohashi Y, Kan T, Tominari Y, Fuwa H, Okamura Y, Watanabe N, Sato C, Natsugari H, Fukuyama T, Iwatsubo T, Tomita T (2006) C-terminal fragment of presenilin is the molecular target of a dipeptidic gamma-secretase-specific inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester). *J Biol Chem* 281, 14670-14676.

52. Nitsch RM, Slack BE, Wurtman RJ, Growdon JH (1992) Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. *Science* 258, 304-307.

53. Rossner S, Ueberham U, Schliebs R, Pérez-Polo JR, Bigl V (1998) The regulation of amyloid precursor protein metabolism by cholinergic mechanisms and neurotrophin receptor signaling. *Prog Neurobiol* 56, 541-569.

54. Mori F, Lai CC, Fusi F,Giacobini E (1995) Cholinesterase inhibitors increase secretion of APPs in rat brain cortex. *Neuroreport* 6, 633-636.

55. Lahiri DK, Farlow MR, Nurnberger JI Jr, Greig NH (1997) Effects of cholinesterase inhibitors on the secretion of beta-amyloid precursor protein in cell cultures. *Ann N Y Acad Sci* 826, 416-421.

56. Zimmermann M, Gardoni F, Marcello E, Colciaghi F, Borroni B, Padovani A, Cattabeni F, Di Luca M (2004) Acetylcholinesterase inhibitors increase ADAM10 activity by promoting its trafficking in neuroblastoma cell lines. *J Neurochem* 90, 1489-1499.

57. Sberna G, Sáez-Valero J, Beyreuther K, Masters CL, Small DH (1997) The amyloid beta-protein of Alzheimer's disease increases acetylcholinesterase expression by increasing intracellular calcium in embryonal carcinoma P19 cells. *J Neurochem* 69, 1177-1184.

58. Sberna G, Sáez-Valero J, Li QX, Czech C, Beyreuther K, Masters CL, McLean CA, Small DH (1998) Acetylcholinesterase is increased in the brains of transgenic mice expressing the C-terminal fragment (CT100) of the beta-amyloid protein precursor of Alzheimer's disease. *J Neurochem* 71, 723-731.

59. Hu W, Gray NW, Brimijoin S (2003) Amyloid-beta increases acetylcholinesterase expression in neuroblastoma cells by reducing enzyme degradation. *J Neurochem* 86, 470-478.

60. Melo JB, Agostinho P, Oliveira CR (2003) Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. *Neurosci Res* 45, 117-127.

61. Li G, Klein J, Zimmermann M (2013) Pathophysiological amyloid concentrations induce sustained upregulation of readthroughacetylcholinesterase mediating anti-apoptotic effects. *Neuroscience* 240, 349-360.

62. Hicks DA, Makova NZ, Gough M, Parkin ET, Nalivaeva NN, Turner AJ (2013) The amyloid precursor protein represses expression of acetylcholinesterase in neuronal cell lines. *J Biol Chem* 288, 26039-26051.

63. Zimmermann M, Borroni B, Cattabeni F, Padovani A, Di Luca M (2005) Cholinesterase inhibitors influence APP metabolism in Alzheimer disease patients. *Neurobiol Dis* 19, 237-242.

64. Peng Y, Jiang L, Lee DY, Schachter SC, Ma Z, Lemere CA (2006) Effects of huperzine A on amyloid precursor protein processing and beta-amyloid generation in human embryonic kidney 293 APP Swedish mutant cells. *J Neurosci Res* 84, 903-911.

65. Lahiri DK, Chen D, Maloney B, Holloway HW, Yu QS, Utsuki T, Giordano T, Sambamurti K, Greig NH (2007) The experimental Alzheimer's disease drug posiphen [(+)-phenserine] lowers amyloid-beta peptide levels in cell culture and mice. J. Pharmacol. *Exp Ther* 320, 386-396.

66. Fu H, Li W, Luo J, Lee NT, Li M, Tsim KW, Pang Y, Youdim MB, Han Y (2008) Promising anti-Alzheimer's dimer bis(7)-tacrine reduces beta-amyloid generation by directly inhibiting BACE-1 activity. *Biochem Biophys Res Commun* 366, 631-636.

67. Li Q, Wu D, Zhang L, Zhang Y (2010) Effects of galantamine on β-amyloid release and beta-site cleaving enzyme 1 expression in differentiated human neuroblastoma SH-SY5Y cells. *Exp Gerontol* 45, 842-847.

68. Xie HQ, Liang D, Leung KW, Chen VP, Zhu KY, Chan WK, Choi RC, Massoulié J, Tsim KW (2010a) Targeting acetylcholinesterase to membrane rafts: a function mediated by the proline-rich membrane anchor (PRiMA) in neurons. *J Biol Chem* 285, 11537-11546.

69. Xie HQ, Leung KW, Chen VP, Chan GK, Xu SL, Guo AJ, Zhu KY, Zheng KY, Bi CW, Zhan JY, Chan WK, Choi RC, Tsim KW (2010b) PRiMA directs a restricted localization of tetrameric AChE at synapses. *Chem Biol Interact* 187, 78-83.

70. Henderson Z, Matto N, John D, Nalivaeva NN, Turner AJ (2010) Co-localization of PRiMA with acetylcholinesterase in cholinergic neurons of rat brain: an immunocytochemical study. *Brain Res* 1344, 34-42.

71. Kaether C, Lammich S, Edbauer D, Ertl M, Rietdorf J, Capell A, Steiner H, Haass C (2002) Presenilin-1 affects trafficking and processing of beta APP and is targeted in a complex with nicastrin to the plasma membrane. *J Cell Biol* 158, 551-561.

72. Niu X, Zhang X, Xie J, Zhang X (2012) Acetylcholinesterase blocks cleavage of APP by γ-secretase in 293 cells and mouse brain. *Mol Neurodegener* 7, S11.

73. Krejci E, Duval N, Chatonnet A, Vincens P, Massoulié J (1991) Cholinesterase-like domains in enzymes and structural proteins: functional and evolutionary relationships and identification of a catalytically essential aspartic acid. *Proc Natl Acad Sci USA* 88, 6647-6651.

74. Darboux I, Barthalay Y, Piovant M, Hipeau-Jacquotte R (1996) The structure-function relationships in Drosophila neurotactin show that cholinesterasic domains may have adhesive properties. *EMBO J* 15, 4835-4843.

75. García-Ayllón MS, Campanari ML, Montenegro MF, Cuchillo-Ibáñez I, Belbín O, Lleó A, Saura CA, Tsim KM, Vidal CJ and Sáez-Valero J (2014) Presenilin-1 influences processing of the acetylcholinesterase membrane-binding tail PRiMA. *Neurobiol Aging* in press. doi: 10.1016/j.neurobiolaging.2014.01.147.

76. Suzuki K, Hayashi Y, Nakahara S, Kumazaki H, Prox J, Horiuchi K, Zeng M, Tanimura S, Nishiyama Y, Osawa S, Sehara-Fujisawa A, Saftig P, Yokoshima S, Fukuyama T, Matsuki N, Koyama R, Tomita T, Iwatsubo T (2012) Activity-dependent proteolytic cleavage of neuroligin-1. *Neuron* 76, 410-422.

77. Struhl G, Adachi A (2000) Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. *Mol Cell* 6, 625-636.

78. Lleó A, Saura CA (2011) γ-secretase substrates and their implications for drug development in Alzheimer's disease. *Curr Top Med Chem* 11, 1513-1527.

79. Atack JR, Perry EK, Bonham JR, Perry RH, Tomlinson BE, Blessed G, Fairbairn A (1983) Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type: selective loss of the intermediate (10S) form. *Neurosci Lett* 40, 199-204.

80. Fishman EB, Siek GC, MacCallum RD, Bird ED, Volicer L, Marquis JK (1986) Distribution of the molecular forms of acetylcholinesterase in human brain, alterations in dementia of the Alzheimer type. *Ann Neurol* 19, 246-252.

81. Schegg KM, Harrington LS, Neilsen S, Zweig RM, Peacock JH (1992) Soluble and membrane-bound forms of brain acetylcholinesterase in Alzheimer's disease. *Neurobiol Aging* 13, 697-704.

82. Clarke NA, Soininen H, Gustafson L, Minthon L, Alhainen K, Francis PT (2001) Tacrine may alter APP-like protein levels in the lumbar CSF of Alzheimer patients. *Int J Geriatr Psychiatry* 16, 1104-1106.

83. Borroni B, Colciaghi F, Pastorino L, Pettenati C, Cottini E, Rozzini L, Monastero R, Lenzi GL, Cattabeni F, Di Luca M, Padovani A (2001) Amyloid precursor protein in platelets of patients with Alzheimer disease: effect of acetylcholinesterase inhibitor treatment. *Arch Neurol* 58, 442-446.

84. Basun H, Nilsberth C, Eckman C, Lannfelt L, Younkin S (2002) Plasma levels of Abeta42 and Abeta40 in Alzheimer patients during treatment with the acetylcholinesterase inhibitor tacrine. *Dement Geriatr Cogn Disord* 14, 156-160.

85. Pakaski M, Kasa P (2003) Role of acetylcholinesterase inhibitors in the metabolism of amyloid precursor protein. *Curr Drug Targets CNS Neurol Disord* 2, 163-171.

86. Sobow T, KloszewskaI (2005) Short-term treatment with rivastigmine and plasma levels of Abeta peptides in Alzheimer's disease. *Folia Neuropathol* 43, 340-344.

87. Darreh-Shori T, Hellström-Lindahl E, Flores-Flores C, Guan ZZ, Soreq H, Nordberg A (2004) Long-lasting acetylcholinesterase splice variations in anticholinesterase-treated Alzheimer's disease patients. *J Neurochem* 88, 1102-1113.

88. Stieger S, Brodbeck U, Witzemann V (1987) Inactive monomeric acetylcholinesterase in the low-salt-soluble extract of the electric organ from Torpedo marmorata. *J Neurochem* 49, 460-467.

89. Rotundo RL (1988) Biogenesis of acetylcholinesterase molecular forms in muscle.Evidence for a rapidly turning over, catalytically inactive precursor pool.*J Biol Chem* 263, 19398-19406.

90. Chatel JM, Grassi J, Frobert Y, Massoulié J, Vallette FM (1993) Existence of an inactive pool of acetylcholinesterase in chicken brain. *Proc Natl Acad Sci USA* 90, 2476-2480.

91. Campanari ML, García-Ayllón MS, Blazquez-Llorca L, Luk WK, Tsim K, Sáez-Valero J (2013) Acetylcholinesterase Protein Level Is Preserved in the Alzheimer's Brain. *J Mol Neurosci* In press;doi: 10.1007/s12031-013-0183-5.

92. Wolfe MS (2012) γ-Secretase as a target for Alzheimer's disease. *Adv Pharmacol* 64, 127-153.

93. Wagner SL, Tanzi RE, Mobley WC, Galasko D (2012) Potential use of γ-secretase modulators in the treatment of Alzheimer disease *Arch Neurol* 69, 1255-1258.

94. Gandy S, DeKosky ST (2013) Toward the treatment and prevention of Alzheimer's disease: rational strategies and recent progress. *Annu Rev Med* 64, 367-383

ARTICLE II:

Acetylcholinesterase protein level is preserved in the Alzheimer's brain

The majority of investigations about AChE on AD progression and therapy have been based in the determination of its enzymatic activity level, which is depleted in the AD brain. An inactive pool of AChE has been demonstrated in brain and CSF and alternative functions unrelated with the hydrolysis of acetylcholine are suspected for AChE. However, AChE protein levels have not been examined in the brain of subjects affected by AD. We have measured AChE protein levels amount in brain cortex of AD and non-demented controls by Western blotting using an anti-AChE antibody (N19) raised against a peptide that maps the N-terminus of human AChE, common to all variants. We have found that the levels of immunoreactive AChE bands were not significantly different in AD samples in comparison with non-demented controls. However, AChE enzymatic activity was decreased in AD samples. Therefore, the decrease in AChE enzyme activity was not paralleled by changes in AChE protein immunoreactivity.

Immunohistochemical examination of AD brain yielded similar results. The AChE protein-staining pattern was studied in hippocampus. Sections from AD and control brain showed similar immunoreactivity to the anti-AChE antibody, whereas histochemical staining for AChE confirmed the depletion in activity. Moreover, AChE-positive fibers stained with the N19 antibody seem to be denser around the amyloid plaques in AD patients when compared with other regions far from plaque or the control case. This lack of correlation between catalytic activity and immunostaining may be attributable, at least in part, to the presence of a large amount of inactive AChE subunits that

may exert a variety of unpredictable effects and induce vulnerability in the pathological brain. This result is of potential relevance in the context of protein-protein interactions, between AChE and PS1 (presenilin-1).

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Acetylcholinesterase Protein Level Is Preserved in the Alzheimer's Brain

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Abstract Acetylcholinesterase (AChE) is a key enzyme in the cholinergic nervous system and is one of the most studied proteins in the field of Alzheimer's disease (AD). Moreover, alternative functions of AChE unrelated with the hydrolysis of acetylcholine are suspected. Until now, the majority of investigations on AChE in AD pathology have been focused on the determination of its enzymatic activity level, which is depleted in the AD brain. Despite this overall decrease, AChE activity increases at the vicinity of the two hallmarks of AD, the amyloid plaques and the neurofibrillary tangles (NFT). In fact, AChE may directly interact with Aβ in a manner that increases the deposition of Aβ to form plaques. In the context of protein–protein interactions, we have recently reported that AChE can interact with presenilin-1, the catalytic component of γ-secretase, influencing its expression level and also its activity. However, the alteration of AChE protein in the AD brain has not been determined. Here, we demonstrated by Western blotting and immunohistochemistry that a prominent pool of enzymatically inactive AChE protein existed in the AD brain. The potential significance of these unexpected levels of inactive AChE protein in the AD brain was discussed, especially in the context of protein–protein interactions with β-amyloid and presenilin-1.

Keywords Alzheimer's disease · Acetylcholinesterase · β-Amyloid · Presenilin-1

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive loss of memory and cognition. Cholinergic depletion is a classical feature in the AD brain, associated with amyloid plaques and neurofibrillary tangles (NFT) (Davies and Maloney 1976; Perry et al. 1977). A decrease in acetylcholinesterase (AChE), the enzyme that rapidly degrades the neurotransmitter acetylcholine at cholinergic synapses, has been reported in a large number of studies (for a review, see García-Ayllón et al. 2011). Nonetheless, because of the substantial reduction in acetylcholine levels, a molecule with a critical role in cognitive function, the therapies designed to reverse the cholinergic deficit characteristics of AD are based on inhibitors of AChE (AChE-I) (Giacobini 2003; Lleó et al. 2006). Furthermore, evidence indicates that

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cholinergic mechanisms can modulate amyloid metabolism (Nitsch et al. 1992; Rossner et al. 1998) and AChE-I affect the amyloid expression (Mori et al. 1995; Zimmermann et al. 2005; Lahiri et al. 1997), although the therapeutic effect of present AChE-I drugs used in AD therapy is rather modest and has not met expectations (Small 2005).

The majority of investigations about AChE on AD progression and therapy have been based on the determination of its enzymatic activity level. However, AChE is much more than a cholinergic enzyme, and indeed other biological functions unrelated with its catalytic activity have been reported (Sternfeld et al. 1998; Dori et al. 2005; Grisaru et al. 2006). These alternative functions are probably related with the rich polymorphism of AChE that exists in different variants by alternative splicing, as well as the existence of different molecular forms (for a review, see Massoulié 2002). Moreover, AChE is present in noncholinergic tissues and shares with several neural cell adhesion proteins high sequence similarity (Krejci et al. 1991). The presence of a cholinesterase-like domain in those adhesion proteins may reflect the capacity of AChE in acting in protein–protein interactions.

We have recently demonstrated an interaction between AChE and presenilin-1 (PS1), the catalytic component of the γ-secretase complex (Silveyra et al. 2008, 2012a). γ-Secretase is a proteolytic enzymatic complex that participates in the processing of the amyloid precursor protein (APP), generating the β-amyloid peptide or Aβ (Kaether et al. 2006), the major constituent of the amyloid plaques (Masters et al. 1985; Kang et al. 1987). Moreover, extensive studies by Inestrosa and co-workers showed that AChE directly interacted with Aβ (Alvarez et al. 1995; Inestrosa et al. 1996a, b). Indeed, despite the overall decrease in the AD brain, AChE is increased at the vicinity of amyloid plaques and NFT (Ulrich et al. 1990; Gomez-Ramos et al. 1992; Wright et al. 1993). The pathological implications of these and other AChE-amyloid interrelationships have remained elusive, but it is evident that AChE can influence amyloid processing and fibrillation via protein–protein interactions and not only through its cholinergic activity. Accordingly, new dual binding AChE-I, able to inhibit the AChE-induced Aβ aggregation, are in development (García-Palomero et al. 2008; Belluti et al. 2011; Viayna et al. 2013). In this context, it has been recently demonstrated that a novel AChE-I, NP-9 (9-anthraldehyde pyrazoline derivative), which is able to interfere Aβ AChE interaction, attenuates Aβ aggregation and amyloid-induced toxicity in Alzheimer animal models (Mishra et al. 2013).

Surprisingly, the vast majority of AChE studies in the AD brain, including histological studies, are focused on the AChE enzymatic level and not the protein level. Here, we present evidence that, in the AD brain, the level of AChE protein does not accord with the decrease in enzymatic activity level. We also discuss the potential physiopathological consequences of the presence of this unexpected large pool of nonenzymatically active AChE in the pathological brain.

Material and Methods

Collection of Human Brain Samples

This study was approved by the Ethics Committee of the Miguel Hernandez University and was carried out in accordance with the Declaration of Helsinki. Frozen brain samples [five AD cases, aged 78–91 years (mean 83 years) and five control cases, aged 60–73 years (mean 66 years)] were obtained from the UIPA Neurological Tissue Bank (Unidad de Investigación Proyecto Alzheimer; Madrid, Spain). Fixed human brain tissues [four AD cases, aged 75–82 years (mean 80) and one control case, aged 69 years] were obtained from the UIPA Neurological Tissue Bank, the Instituto de Neuropatología (IDIBELL-Hospital Universitario de Bellvitge, Barcelona, Spain), and from the Servicio de Patología Forense (Instituto Vasco de Medicina Legal, Bilbao, Spain). After neuropathological examination, sporadic AD cases were categorized as stages V–VI of Braak and Braak (1991). Samples from nondisease individuals correspond to cases with no clinical dementia and no evidence of brain pathology. The mean postmortem interval of the tissue was between 1.5 and 6 h, with no significant difference between each group of samples.

Preparation of Human Brain Samples for Biochemical Analysis

Samples (~0.1 g) of human frontal cortex, stored at −80 °C, were thawed slowly at 4 °C and homogenized (10 %*v*/*v*) in 50 mM Tris–HCl, pH 7.5/150 mM NaCl/5 mM EDTA/1 % (*w*/*v*) Nonidet P-40/0.5 % (*w*/*v*) Triton X-100 and supplemented with a cocktail of protease inhibitors (Sáez-Valero et al. 1993). The homogenates were sonicated and centrifuged at 70,000×*g* at 4 °C for 1 h, and the supernatants were collected and frozen at −80 °C until assay.

AChE Enzymatic Assay and Protein Determination

A modified microassay version of the colorimetric Ellman's method was used to measure AChE (Sáez-Valero et al. 1993), using 50 μM tetraisopropyl pyrophosphoramide (iso-OMPA) as specific inhibitor for the structurally related butyrylcholinesterase, an enzyme that coexists with AChE in the human brain. One milliunit (mU) of AChE activity was defined as the number of nanomoles of acetylthiocholine hydrolyzed per minute at 22 °C. Total protein concentrations were determined using the bicinchoninic acid method (Pierce). AChE-specific

activity is expressed as the activity of the enzyme in milliunits per milligram (mg) of total protein (mU/mg).

Western Blotting Assays

Fifty micrograms of protein from brain extracts (equal amount of protein in each lane) was separated by SDS–PAGE electrophoresis. Samples were denatured at 98 °C for 7 min. The separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience GmbH) and probed with the goat anti-AChE antibody N19 (Santa Cruz Biotech) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam). The blots were incubated with the corresponding secondary antibody conjugated to horseradish peroxidase, and the signal was detected using ECL Plus detection reagent according to the manufacturer's instructions (GE Healthcare) in a Luminescent Image Analyzer LAS-1000 Plus (FUJIFILM). For semiquantitative analysis, protein levels were normalized to GAPDH, and the intensity of the bands was measured by densitometry with the Science Lab Image Gauge v4.0 software provided by FUJIFILM.

Preparation of Human Brain Samples for Immunohistochemistry and Histochemistry

Upon removal, the brain tissue was immediately fixed in cold 4 % paraformaldehyde in phosphate buffer (PB 0.1 M, pH 7.4), and after 2 h, the tissue was cut into small blocks and post-fixed in the same fixative for 24–48 h at 4 °C. After fixation, all the specimens were immersed in graded sucrose solutions, and they were stored in a cryoprotectant solution at −20 °C. Serial vibratome sections (50 μm) of cortical tissue were obtained, and the sections from each region and case were batch-processed for immunocytochemical staining. The sections immediately adjacent to those stained immunocytochemically were Nissl-stained so as to identify the cortical areas to which they pertained and the laminar boundaries. The same AD and ND cases were extensively studied by scores for the amyloid and neurofibrillary pathologies, as in a previous study (Blazquez-Llorca et al. 2011).

Immunohistochemistry and Staining Procedure

Free-floating hippocampal sections were pretreated in 1 % H_2O_2 for 30 min to remove endogenous peroxidase activity, and subsequently, they were blocked for 1 h in PB with 0.25 % Triton-X and 3 % normal rabbit serum (1:200, Vector Laboratories Inc.). The sections were incubated overnight at 4 °C with the N19 (1:500) antibody, and the following day, the sections were rinsed and incubated for 2 h with biotinylated rabbit anti-goat IgG (1:200, BA-5000, Vector Laboratories). The sections were then incubated for 1 h in an avidin–biotin peroxidase complex (Vectastain ABC Elite PK6100, Vector) and finally with the 3,3′-diaminobenzidine tetrahydrochloride chromogen (DAB, Sigma-Aldrich). After staining, the sections were dehydrated, cleared with xylene, and coverslipped. Control sections were processed as above but without the primary antibodies, and no significant staining was seen under these conditions.

The AChE enzymatic reaction was performed according to the method of Karnovsky and Roots (1964). Slices were incubated for 2 h in the medium containing the following: acetylthiocholine iodide (0.05 %), 5 mM $K_3Fe(CN)_4$ (0.164 %), 30 mM $CuSO_4$ (0.75 %), and 0.1 mM sodium citrate (2.94 %) in 0.2 M acetate buffer (pH 6.0) (1.64 %). The reaction was stopped with washes in phosphate-buffered saline. Then, the sections were dehydrated, cleared with xylene, and coverslipped.

Statistical Analysis

All data were analyzed using SigmaStat (Version 2.0; SPSS Inc.) by Student's t test (two tailed). Results are presented as means ± SEM. p values<0.05 were considered significant.

Results

The levels of AChE in frontal cortex extracts from AD and ND subjects were screened for enzymatic activity. As expected, the AChE assay revealed that AD samples had lower enzymatic activity (40 % decrease; $p=0.003$), in comparison to age- and gender-matched ND controls (Fig. 1a).

Sample aliquots were analyzed by SDS–PAGE under fully reducing conditions, followed by Western blotting using anti-AChE antibody N19, raised against a peptide that maps the N-terminus of human AChE, common to all variants. N19 detected AChE bands of ~75 and ~65 kDa and a doublet of bands at ~55 and ~50 kDa in brain samples from both AD and nondisease (ND) control subjects (Fig. 1b). Although the predicted molecular weight of the AChE subunits is ~70 kDa in size, our as well as other studies strongly suggest the identity of lower AChE molecular weight bands (García-Ayllón et al. 2006, 2007, 2010, 2012; Santos et al. 2007; Sternfeld et al. 2000; Xie et al. 2011). The lower molecular weight bands might originate by posttranslation modification and/or resulted from the reducing conditions used during electrophoresis. We have previously confirmed the specificity of the AChE bands, including the lighter subunits, by AChE immunoprecipitation and probing blots with alternative anti-human AChE antibodies (García-Ayllón et al. 2007, 2010). Interestingly, the decrease in AChE enzyme activity was not paralleled by changes in AChE protein immunoreactivity. The levels of immunoreactive AChE bands were not significantly different in AD samples in comparison with ND controls

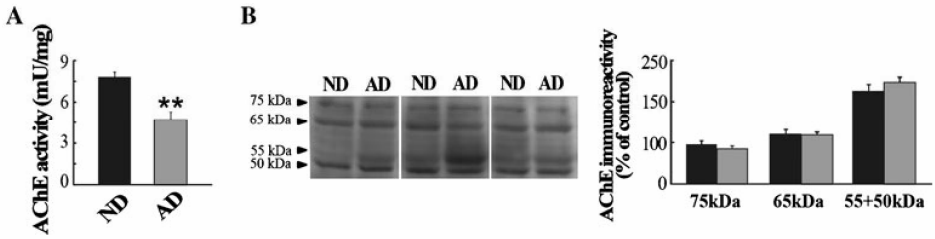

Fig. 1 Decrease in AChE activity does not parallel changes in AChE protein in the Alzheimer's brain. **a** AChE activity levels in frontal cortex extracts from nondemented (ND) controls ($n=5$) and patients with Alzheimer's disease (AD) ($n=5$). **b** Aliquots of the same brain extracts were immunoblotted with the anti-AChE antibody N19, which recognizes all variants. Representative immunoblot (*left panel*) and densitometric quantification (*right panel*) of the AChE-immunoreactive bands, expressed in arbitrary units (a.u.), are shown and expressed as percentage relative to immunoreactivity of the 75-kDa band from the ND group. For the semiquantitative analysis, protein levels were normalized to GAPDH. The results were confirmed in two independent determinations with equivalent amounts of protein loaded in each lane. Columns represent means ± SEM. *$p<0.05$, significantly different from the ND group

(Fig. 1b). Immunohistochemical examination of AD brain yielded similar results (Fig. 2).

The AChE protein staining pattern was studied in hippocampus from three AD patients and one ND control case. Sections from AD and control brain showed similar immunoreactivity to the anti-AChE antibody N19. Moreover, AChE-positive fibers stained with the N19 antibody seemed to be denser around amyloid plaques in AD patients (Fig. 2b), when compared with other regions far from the plaque or the control case. Similar to previous reports (Ulrich et al. 1990; Gomez-Ramos et al. 1992; Wright et al. 1993), when the AChE enzymatic reaction was performed by the Karnovsky and Roots method (Karnovsky and Roots 1964), no fibers were observed, but the amyloid plaques and NFT were stained (Fig. 2b, inset).

Discussion

Here we report that AChE protein level appears unexpectedly preserved in the AD brain, with respect to the decline in activity level. Frontal cortex samples, assayed by immunoblotting, and hippocampus sections, resolved by immunohistochemistry, demonstrate that AChE protein levels are comparable between AD and ND subjects. Despite the limitation of the low number of samples analyzed, our data signify discrepancies between the levels of AChE activity and AChE protein immunoreactivity, which may be attributable in part to the inactive or subnormal active character of AChE subunits present in the pathological tissues. Moreover, AChE banding pattern obtained by electrophoretic analysis/Western blotting has no simple relationship to specific molecular forms or enzymatic activity (García-Ayllón et al. 2006, 2007). For this reason, the detriment in the major tetrameric form of AChE is not patent in immunoreactivity changes of particular bands. Further analyses with antibodies for specific variants are pending.

There is an extensive number of investigations being done to identify alterations in AChE level as a consequence of AD processes. Most of them are based on biochemical determination of enzymatic activity, probably because of the facility in measuring the activity of the enzyme and also because of the interest derived from the use of AChE-I in therapy. Today, it is well established that a substantial loss of AChE activity is due to loss of a tetrameric form linked to the plasma membrane (Atack et al. 1983; Sáez-Valero et al. 1999). Enzymatic histochemical

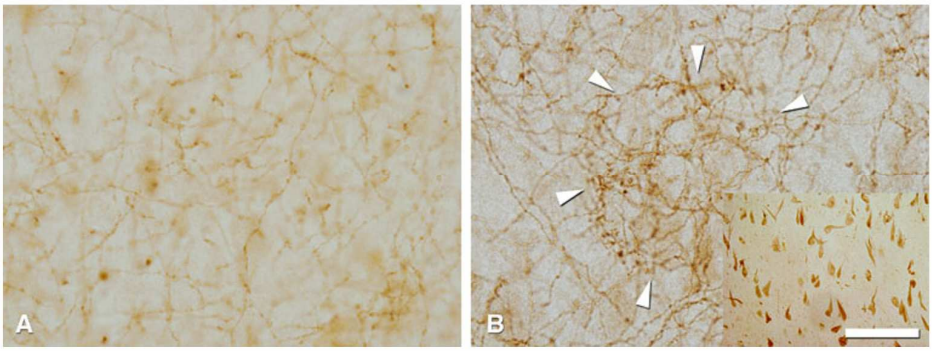

Fig. 2 Immunohistochemical detection of AChE in human hippocampus. Immunostaining with the anti-AChE antibody N19, in the subiculum from a control case (**a**) and an AD patient (**b**). *Inset* in **b** corresponds to AChE enzymatic reaction in the subiculum from the same AD subject. AChE-positive fibers stained with the N19 antibody seem to be denser around amyloid plaques (surrounded by *arrowheads*). When the AChE enzymatic reaction was performed, no fibers were observed, but the amyloid plaques and NFT (*inset* in **b**) were stained. *Scale bar* = 35 μm in **a**, **b**; 80 μm in *inset* in **b**

examination of the AD brain yielded similar results than biochemical determination with an overall depletion of AChE activity (Ulrich et al. 1990; Gomez-Ramos et al. 1992; Wright et al. 1993). In vivo studies by positron emission tomography, based on the assessment of enzymatic activity using radiolabeled analogs of acetylcholine, also suggested that AChE activity could be reduced in the AD brain (Iyo et al. 1997; Herholz 2008; Mori et al. 2012).

Several laboratories attempted to develop an enzyme-linked immunosorbent assay (ELISA) for AChE (Rasmussen et al. 1989; Blennow et al. 2007; Darreh-Shori et al. 2008), but very few develop a two-site immunoassay (Brimijoin et al. 1987; Weikert et al. 1994; Konings et al. 1995). Hammond and Brimijoin (1988) reported a parallel variation in AChE enzyme activity and AChE immunoreactivity, assessed by a two-site ELISA in the AD brain, but this ELISA probably assessed only protein levels for the major tetrameric form. Previously, we had noted that a large amount of AChE subunits found in AD samples and identified by Western blotting with several polyclonal anti-AChE antibodies, displayed low affinity for certain monoclonal antibodies, which recognized native AChE (Sáez-Valero et al. 2000; García-Ayllón et al. 2007, 2010). As commented above, the lack of correlation between catalytic activity and immunostaining may be attributable, at least in part, to the presence of a large amount of inactive AChE subunits.

The existence of inactive AChE has been demonstrated in the brain (Chatel et al. 1993). These inactive AChE species are probably monomers, which could serve as a reservoir for the active enzyme (Stieger et al. 1987; Rotundo 1988). Anyhow, the presence of a large amount of inactive monomers in fluids, such as cerebrospinal fluid (CSF) and plasma (Darreh-Shori et al. 2004; García-Ayllón et al. 2007, 2010), suggests that these light species might have more than a simple role as precursors of enzymatically active oligomers. Despite several evidences strongly supporting the possibility that AChE can act independently of acetylcholine hydrolysis, the physiological significance of the nonenzymatic AChE in the brain is still controversial, as catalytic and noncatalytic effects cannot be easily distinguished (reviewed in Massoulié et al. 1993; Balasubramanian and Bhanumathy 1993; Small et al. 1996; Layer 1995; Soreq and Seidman 2001; Greenfield et al. 2008). Indeed, transgenic overexpression of enzymatically inactivated AChE (Sternfeld et al. 1998; Dori et al. 2005; Grisaru et al. 2006) or heat-inactivated AChE (Klegeris et al. 1994) has demonstrated to exert distinct biological functions. Possible biological functions for the pool of AChE nonenzymatically active in the normal brain, and particularly in the AD brain, therefore need to be elucidated.

The occurrence of AChE subnormal active in the pathological tissue cannot be discarded. In this context, AChE activity present in the AD brain associated with plaques and NFT displays particular enzymatic properties and sensitivity to inhibitors (Geula and Mesulam 1989; Wright et al. 1993). However, it is not clear whether the atypical histochemical behavior of AChE observed in AD is due to alterations in kinetic properties of these enzymes (Ciro et al. 2012), or due to interactions with other molecules within these lesions (Darvesh et al. 2010). In the context of the presence of AChE within plaques and NFT, it is important to note that both AD effectors, Aβ and abnormally hyperphosphorylated tau, can influence AChE levels in vitro (Sberna et al. 1997; Sáez-Valero et al. 2003; Hu et al. 2003; Melo et al. 2003) and in rodent models (Sberna et al. 1998; Sáez-Valero et al. 2002; Silveyra et al. 2012b; Kreutz et al. 2013). We have also demonstrated that Aβ alters AChE glycosylation in vivo (Sberna et al. 1998; Fodero et al. 2002), and this change is similar to the change in the glycosylation pattern of AChE in AD brain (Sáez-Valero et al. 1997, 1999, 2000). Correct glycosylation determines the adequate biological function by influencing trafficking, folding, assembly, and final localization of glycoproteins. Changes in glycosylation could compromise enzymatic capacities. The glycosylation pattern of the inactive AChE pool in Alzheimer's condition has not been studied.

Noncatalytic AChE is not the unique AChE pool that remains unaltered, or even increase, in the AD brain. The significance of different variants and forms of AChE is intriguing, as their specific subcellular distribution is thought to reflect specific physiological functions. Despite the loss of the major tetrameric and cholinergic AChE, the minor monomeric form increases in respect to the normal levels in nonpathological brain (Atack et al. 1983; Arendt et al. 1992; Sáez-Valero et al. 1999). So far, the significance of an increase in minor AChE species is controversial, as the relative increase is compared in absolute terms with the major cholinergic form. However, a variation in a particular AChE form (with little impact on cholinergic equilibrium) could have an impact at the functional level with biological consequences, since particular forms or variants may have specific functions. Indeed, recent evidences indicate distinct and, in certain cases, inverse cell fate outcome under enforced expression of the human N- and C-terminally modified AChE variants, all of which have similar enzymatic activities (Berson et al. 2008; Toiber et al. 2008; Greenberg et al. 2010). Brain AChE splice variants show indistinguishable enzymatic activity, but may present particular banding pattern by Western blotting (Meshorer and Soreq 2006). Additional studies regarding the specific roles of particular AChE species are mandatory in order to develop more effective therapies. In this regard, targeting a specific AChE variant (e.g., by antisense or siRNA agents) could hence offer specific advantages. Indeed, the AChE-targeted microRNA-132 has been recently reported to be down-regulated during the progression of AD (Lau et al. 2013), and the possibility to intercept microRNA-132-mediated damages by modulating AChE expression has been demonstrated viable (Shaltiel et al. 2013).

The interaction of AChE with the catalytic subunit of the γ-secretase complex, PS1, is also of interest. We have demonstrated the reciprocal co-immunoprecipitation of AChE with PS1 (Silveyra et al. 2008) and the modulation of PS1 levels by AChE-I (Silveyra et al. 2012a). More interestingly, we have recently seen changes in PS1 levels under the influence of AChE genetic expression (Silveyra et al. 2012a), even using of inactive catalytic mutants of the enzyme (unpublished). The modulatory effect on PS1 levels was not sustained when AChE-I promotes feedback AChE upregulation (Silveyra et al. 2012b). All together these results suggest that excess of inactive or inactivated brain AChE can exert a variety of unpredictable effects and may induce vulnerability in the pathological brain. In conclusion, a better understanding of the alternative functions of AChE and estimation of its protein levels could be relevant for deciphering their implication in the progression of Alzheimer's pathology and the development of new therapy.

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References

Alvarez A, Bronfman F, Pérez CA, Vicente M, Garrido J, Inestrosa NC (1995) Acetylcholinesterase, a senile plaque component, affects the fibrillogenesis of amyloid-beta-peptides. Neurosci Lett 201:49–52

Arendt T, Brückner MK, Lange M, Bigl V (1992) Changes in acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease resemble embryonic development—a study of molecular forms. Neurochem Int 21:381–396

Atack JR, Perry EK, Bonham JR et al (1983) Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type: selective loss of the intermediate (10S) form. Neurosci Lett 30:199–204

Balasubramanian AS, Bhanumathy CD (1993) Noncholinergic functions of cholinesterases. FASEB J 7:1354–1358

Belluti F, Bartolini M, Bottegoni G et al (2011) Benzophenone-based derivatives: a novel series of potent and selective dual inhibitors of acetylcholinesterase and acetylcholinesterase-induced beta-amyloid aggregation. Eur J Med Chem 46:1682–1693

Berson A, Knobloch M, Hanan M et al (2008) Changes in readthrough acetylcholinesterase expression modulate amyloid-beta pathology. Brain 131:109–119

Blazquez-Llorca L, Garcia-Marin V, Merino-Serrais P, Avila J, DeFelipe J (2011) Abnormal tau phosphorylation in the thorny excrescences of CA3 hippocampal neurons in patients with Alzheimer's disease. J Alzheimers Dis 26:683–698

Blennow K, Zetterberg H, Minthon L et al (2007) Longitudinal stability of CSF biomarkers in Alzheimer's disease. Neurosci Lett 419:18–22

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

Brimijoin S, Hammond P, Rakonczay Z (1987) Two-site immunoassay for acetylcholinesterase in brain, nerve, and muscle. J Neurochem 49:555–562

Chatel JM, Grassi J, Frober Y, Massoulié J, Vallette FM (1993) Existence of an inactive pool of acetylcholinesterase in chicken brain. Proc Natl Acad Sci U S A 90:2476–2480

Ciro A, Park J, Burkhard G, Yan N, Geula C (2012) Biochemical differentiation of cholinesterases from normal and Alzheimer's disease cortex. Curr Alzheimer Res 9:138–143

Darreh-Shori T, Hellström-Lindahl E, Flores-Flores C et al (2004) Long-lasting acetylcholinesterase splice variations in anticholinesterase-treated Alzheimer's disease patients. J Neurochem 88:1102–1113

Darreh-Shori T, Kadir A, Almkvist O et al (2008) Inhibition of acetylcholinesterase in CSF versus brain assessed by 11C-PMP PET in AD patients treated with galantamine. Neurobiol Aging 29:168–184

Darvesh S, Reid GA, Martin E (2010) Biochemical and histochemical comparison of cholinesterases in normal and Alzheimer brain tissues. Curr Alzheimer Res 7:386–400

Davies P, Maloney AJF (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 2:1403

Dori A, Cohen J, Silverman WF, Pollack Y, Soreq H (2005) Functional manipulations of acetylcholinesterase splice variants highlight alternative splicing contributions to murine neocortical development. Cereb Cortex 15:419–430

Fodero LR, Sáez-Valero J, McLean CA et al (2002) Altered glycosylation of acetylcholinesterase in APP (SW) Tg2576 transgenic mice occurs prior to amyloid plaque deposition. J Neurochem 81:441–448

García-Ayllón MS, Silveyra MX, Candela A et al (2006) Changes in liver and plasma acetylcholinesterase in rats with cirrhosis induced by bile duct ligation. Hepatology 43:444–453

García-Ayllón MS, Silveyra MX, Andreasen N, Brimijoin S, Blennow K, Sáez-Valero J (2007) Cerebrospinal fluid acetylcholinesterase changes after treatment with donepezil in patients with Alzheimer's disease. J Neurochem 10:1701–1711

García-Ayllón MS, Riba-Llena I, Serra-Basante C, Alom J, Boopathy R, Sáez-Valero J (2010) Altered levels of acetylcholinesterase in Alzheimer plasma. PLoS One 5:e8701

García-Ayllón MS, Small DH, Avila J, Sáez-Valero J (2011) Revisiting the role of acetylcholinesterase in Alzheimer's disease: cross-talk with P-tau and β-amyloid. Front Mol Neurosci 4:22

García-Ayllón MS, Millán C, Serra-Basante C, Bataller R, Sáez-Valero J (2012) Readthrough acetylcholinesterase is increased in human liver cirrhosis. PLoS One 7:e44598

García-Palomero E, Muñoz P, Usan P et al (2008) Potent beta-amyloid modulators. Neurodegener Dis 5:153–156

Geula C, Mesulam M (1989) Special properties of cholinesterases in the cerebral cortex of Alzheimer's disease. Brain Res 498:185–189

Giacobini E (2003) Cholinergic function and Alzheimer's disease. Int J Geriatr Psychiatry 18:1–5

Gomez-Ramos P, Mufson EJ, Moran MA (1992) Ultrastructural localization of acetylcholinesterase in neurofibrillary tangles, neuropil threads and senile plaques in aged and Alzheimer's brain. Brain Res 569:229–237

Greenberg DS, Toiber D, Berson A, Soreq H (2010) Acetylcholinesterase variants in Alzheimer's disease: from neuroprotection to programmed cell death. Neurodegener Dis 7:60–63

Greenfield SA, Zimmermann M, Bond CE (2008) Non-hydrolytic functions of acetylcholinesterase. The significance of C-terminal peptides. FEBS J 275:604–611

Grisaru D, Pick M, Perry C et al (2006) Hydrolytic and nonenzymatic functions of acetylcholinesterase comodulate hemopoietic stress responses. J Immunol 176:27–35

Hammond P, Brimijoin S (1988) Acetylcholinesterase in Huntington's and Alzheimer's diseases: simultaneous enzyme assay and immunoassay of multiple brain regions. J Neurochem 50:1111–1116

Herholz K (2008) Acetylcholine esterase activity in mild cognitive impairment and Alzheimer's disease. Eur J Nucl Med Mol Imaging 35: 25–29

Hu W, Gray NW, Brimijoin S (2003) Amyloid-beta increases acetylcholinesterase expression in neuroblastoma cells by reducing enzyme degradation. J Neurochem 86:470–478

Inestrosa NC, Alvarez A, Pérez CA et al (1996a) Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. Neuron 16: 881–891

Inestrosa NC, Alvarez A, Calderón F (1996b) Acetylcholinesterase is a senile plaque component that promotes assembly of amyloid beta-peptide into Alzheimer's filaments. Mol Psychiatry 1:359–361

Iyo M, Namba H, Fukushi K, Shinotoh H et al (1997) Measurement of acetylcholinesterase by positron emission tomography in the brains of healthy controls and patients with Alzheimer's disease. Lancet 349:1805–1809

Kaether C, Haass C, Steiner H (2006) Assembly, trafficking and function of gamma-secretase. Neurodegener Dis 3:275–283

Kang J, Lemaire HG, Unterbeck A et al (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325:733–736

Karnovsky MJ, Roots L (1964) A "direct-coloring" thiocholine method for cholinesterases. J Histochem Cytochem 12:219–221

Klegeris A, Budd TC, Greenfield SA (1994) Acetylcholinesterase activation of peritoneal macrophages is independent of catalytic activity. Cell Mol Neurobiol 14:89–98

Konings CH, Kuiper MA, Mulder C, Calliauw J, Wolters EC (1995) CSF acetylcholinesterase in Parkinson disease: decreased enzyme activity and immunoreactivity in demented patients. Clin Chim Acta 235: 101–105

Krejci E, Duval N, Chatonnet A, Vincens P, Massoulié J (1991) Cholinesterase-like domains in enzymes and structural proteins: functional and evolutionary relationships and identification of a catalytically essential aspartic acid. Proc Natl Acad Sci U S A 88: 6647–6651

Kreutz F, Scherer EB, Ferreira AG et al (2013) Alterations on Na+, K + −ATPase and acetylcholinesterase activities induced by amyloid-β peptide in rat brain and GM1 ganglioside neuroprotective action. Neurochem Res 38:2342–2350

Lahiri DK, Farlow MR, Nurnberger JI Jr, Greig NH (1997) Effects of cholinesterase inhibitors on the secretion of beta-amyloid precursor protein in cell cultures. Ann N Y Acad Sci 826:416–421

Lau P, Bossers K, Janky R et al (2013) Alteration of the microRNA network during the progression of Alzheimer's disease. EMBO Mol Med 5:1613–1634

Layer PG (1995) Nonclassical roles of cholinesterases in the embryonic brain and possible links to Alzheimer disease. Alzheimer Dis Assoc Disord 9:29–36

Lleó A, Greenberg SM, Growdon JH (2006) Current pharmacotherapy for Alzheimer's disease. Annu Rev Med 57:513–533

Massoulié J (2002) The origin of the molecular diversity and functional anchoring of cholinesterases. Neurosignals 11:130–143

Massoulié J, Pezzementi L, Bon S, Krejci E, Vallette FM (1993) Molecular and cellular biology of cholinesterases. Prog Neurobiol 41:31–91

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer's disease and Down syndrome. Proc Natl Acad Sci U S A 82:4245–4249

Melo JB, Agostinho P, Oliveira CR (2003) Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. Neurosci Res 45:117–127

Meshorer E, Soreq H (2006) Virtues and woes of AChE alternative splicing in stress-related neuropathologies. Trends Neurosci 29: 216–224

Mishra N, Sasmal D, Singh KK (2013) Attenuating Aβ1-42-induced toxicity by a novel acetylcholinesterase inhibitor. Neuroscience 250:309–319

Mori F, Lai CC, Fusi F, Giacobini E (1995) Cholinesterase inhibitors increase secretion of APPs in rat brain cortex. Neuroreport 6:633–636

Mori T, Maeda J, Shimada H et al (2012) Molecular imaging of dementia. Psychogeriatrics 12:106–114

Nitsch RM, Slack BE, Wurtman RJ, Growdon JH (1992) Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. Science 258:304–307

Perry EK, Perry RH, Blessed G, Tomlinson BE (1977) Necropsy evidence of central cholinergic deficits in senile dementia. Lancet 1: 189

Rasmussen AG, Arends J, Larsen SO (1989) Evaluation and quality control of a monoclonal antibody based enzyme antigen immunoassay of acetylcholinesterase in amniotic fluid. Scand J Clin Lab Invest 49:503–511

Rossner S, Ueberham U, Schliebs R, Pérez-Polo JR, Big V (1998) The regulation of amyloid precursor protein metabolism by cholinergic mechanisms and neurotrophin receptor signaling. Prog Neurobiol 56:541–569

Rotundo RL (1988) Biogenesis of acetylcholinesterase molecular forms in muscle. Evidence for a rapidly turning over, catalytically inactive precursor pool. J Biol Chem 263:19398–19406

Sáez-Valero J, Tornel PL, Muñoz-Delgado E, Vidal CJ (1993) Amphiphilic and hydrophilic forms of acetyl- and butyrylcholinesterase in human brain. J Neurosci Res 35:678–689

Sáez-Valero J, Sberna G, McLean CA, Masters CL, Small DH (1997) Glycosylation of acetylcholinesterase as diagnostic marker for Alzheimer's disease. Lancet 350:929

Sáez-Valero J, Sberna G, McLean CA, Small DH (1999) Molecular isoform distribution and glycosylation of acetylcholinesterase are altered in brain and cerebrospinal fluid of patients with Alzheimer's disease. J Neurochem 72:1600–1608

Sáez-Valero J, Mok SS, Small DH (2000) An unusually glycosylated form of acetylcholinesterase is a CSF biomarker for Alzheimer's disease. Acta Neurol Scand 176:49–52

Sáez-Valero J, de Ceballos ML, Small DH, de Felipe C (2002) Changes in molecular isoform distribution of acetylcholinesterase in rat cortex and cerebrospinal fluid after intracerebroventricular administration of amyloid beta-peptide. Neurosci Lett 325:199–202

Sáez-Valero J, Fodero LR, White AR, Barrow CJ, Small DH (2003) Acetylcholinesterase is increased in mouse neuronal and astrocyte cultures after treatment with beta-amyloid peptides. Brain Res 965: 283–286

Santos SC, Vala I, Miguel C et al (2007) Expression and subcellular localization of a novel nuclear acetylcholinesterase protein. J Biol Chem 282:25597–25603

Sberna G, Sáez-Valero J, Beyreuther K, Masters CL, Small DH (1997) The amyloid b-protein of Alzheimer's disease increases acetylcholinesterase expression by increasing intracellular calcium in embryonal carcinoma P19 cells. J Neurochem 69:1177–1184

Sberna G, Sáez-Valero J, Li QX et al (1998) Acetylcholinesterase is increased in the brains of transgenic mice expressing the C-terminal fragment (CT100) of the beta-amyloid protein precursor of Alzheimer's disease. J Neurochem 71:723–731

Shaltiel G, Hanan M, Wolf Y et al (2013) Hippocampal microRNA-132 mediates stress-inducible cognitive deficits through its acetylcholinesterase target. Brain Struct Funct 218:59–72

Silveyra MX, Evin G, Montenegro MF et al (2008) Presenilin 1 interacts with acetylcholinesterase and alters its enzymatic activity and glycosylation. Mol Cell Biol 28:2908–2919

Silveyra MX, García-Ayllón MS, Serra-Basante C et al (2012a) Changes in acetylcholinesterase expression are associated with altered presenilin-1 levels. Neurobiol Aging 33:627.e27-37

Silveyra MX, García-Ayllón MS, De Barreda EG et al (2012) Altered expression of brain acetylcholinesterase in FTDP-17 human tau transgenic mice. Neurobiol Aging 33:624.e23-34

Small DH (2005) Acetylcholinesterase inhibitors for the treatment of dementia in Alzheimer's disease: do we need new inhibitors? Expert Opin Emerg Drugs 10:817–825

Small DH, Michaelson S, Sberna G (1996) Non-classical actions of cholinesterases: role in cellular differentiation, tumorigenesis and Alzheimer's disease. Neurochem Int 28:453–483

Soreq H, Seidman S (2001) Acetylcholinesterase—new roles for an old actor. Nat Rev Neurosci 2:294–302

Sternfeld M, Ming G, Song H et al (1998) Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. J Neurosci 18:1240–1249

Sternfeld M, Shoham S, Klein O, Flores-Flores C, Evron T, Idelson GH et al (2000) Excess "read-through" acetylcholinesterase attenuates but the "synaptic" variant intensifies neurodeterioration correlates. Proc Natl Acad Sci U S A 97:8647–8652

Stieger S, Brodbeck U, Witzemann V (1987) Inactive monomeric acetylcholinesterase in the low-salt-soluble extract of the electric organ from *Torpedo marmorata*. J Neurochem 49:460–467

Toiber D, Berson A, Greenberg D, Melamed-Book N, Diamant S, Soreq H (2008) N-acetylcholinesterase-induced apoptosis in Alzheimer's disease. PLoS One 3:e3108

Ulrich J, Meier-Ruge W, Probst A, Meier E, Ipsen S (1990) Senile plaques: staining for acetylcholinesterase and A4 protein: a comparative study in the hippocampus and entorhinal cortex. Acta Neuropathol 80:624–628

Viayna E, Sabate R, Muñoz-Torrero D (2013) Dual inhibitors of β-amyloid aggregation and acetylcholinesterase as multitarget anti-Alzheimer drug candidates. Curr Top Med Chem 13: 1820–1842

Weikert T, Rathjen FG, Layer PG (1994) Use of ELISA to G4 antigen to quantitate neurite outgrowth in the chick both in vivo and in vitro. J Neurochem 62:1570–1577

Wright CI, Geula C, Mesulam MM (1993) Neurological cholinesterases in the normal brain and in Alzheimer's disease: relationship to plaques, tangles, and patterns of selective vulnerability. Ann Neurol 34:373–384

Xie J, Jiang H, Wan YH et al (2011) Induction of a 55 kDa acetylcholinesterase protein during apoptosis and its negative regulation by the Akt pathway. J Mol Cell Biol 3:250–259

Zimmermann M, Borroni B, Cattabeni F, Padovani A, Di Luca M (2005) Cholinesterase inhibitors influence APP metabolism in Alzheimer disease patients. Neurobiol Dis 19:237–242

ARTICLE III:

Presenilin-1 influences processing of the acetylcholinesterase membrane anchor PRiMA

γ-Secretase processes, in addition to APP and Notch, more than 90 type I integral membrane proteins after a prior shedding by α-secretase or β-secretase. The major acetylcholinesterase (AChE) form present in the brain is a tetramer of active monomers anchored to the plasma membrane through a non-catalytic subunit, a type I transmembrane protein called PRiMA (proline-rich membrane anchor). The PRiMA subunit is an accessory partner for the cellular disposition of the cholinergic AChE and represents a limiting factor for production of the active AChE-PRiMA complex. In the context of a PS1-AChE interaction it is interesting to know if γ-secretase is involved in the processing of the cholinergic AChE via cleavage of its PRiMA anchor. First, we have proved an interaction between PS1 and the AChE forms containing the PRiMA subunit. In extracts from CHO cells stably over-expressing the AChE-T variant and co-transfected with PRiMA (CHO-AChE/PRiMA), PS1 co-immunoprecipitates PRiMA, resolved as bands of ~22 and ~20 kDa, which probably correspond to the mature (fully glycosylated) and immature PRiMA. We have also evidenced that through its γ-secretase activity, PS1 participates in the processing of PRiMA-linked AChE. We showed that CHO-AChE/PRiMA treated with DAPT, a well characterized γ-secretase inhibitor, resulted in an increased levels of the tetrameric AChE (the form linked to PRiMA), with concomitant increases in PRiMA content, suggesting that PS1 can cleave PRiMA at the membrane spanning domain. The cleavage of type I transmembrane proteins by γ-secretase, releases C-terminal intracellular domains, with possible signaling properties. We have

identified, by immunoprecipitation and immunofluorescence labelling, a C-terminal PRiMA fragment of ~14 KDa in the nucleus. This finding suggests that PRiMA fragment participates in modulation of gene transcription, a possibility that deserves more investigation. Furthermore, our analysis on the raft composition, in a PS1 conditional knockout mouse, revealed that that this is the subcellular location where PRiMA processing by γ-secretase could take place. The physiological relevance of the proteolytic events described in this study is still not well understood but reveals that PS1 may participate in AChE processing and subsequent signaling events.

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Presenilin-1 influences processing of the acetylcholinesterase membrane anchor PRiMA

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ABSTRACT

Presenilin-1 (PS1) is the catalytic component of the γ-secretase complex. In this study, we explore if PS1 participates in the processing of the cholinergic acetylcholinesterase (AChE). The major AChE variant expressed in the brain is a tetramer (G_4) bound to a proline-rich membrane anchor (PRiMA). Overexpression of the transmembrane PRiMA protein in Chinese hamster ovary cells expressing AChE and treated with the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester have enabled us to study whether, through its γ-secretase activity, PS1 participates in the processing of PRiMA-linked AChE. γ-Secretase inhibition led to a notable increase in the level of PRiMA-linked AChE, suggesting that γ-secretase is involved in the cleavage of PRiMA. We demonstrate that cleavage of PRiMA by γ-secretase results in a C-terminal PRiMA fragment. Immunofluorescence labeling allowed us to identify this PRiMA fragment in the nucleus. Moreover, we have determined changes in the proportion of the raft-residing AChE-PRiMA in a PS1 conditional knockout mouse. Our results are of interest as both enzymes have therapeutic relevance for Alzheimer's disease.

1. Introduction

The accumulation of the amyloid-β (Aβ) peptide in the brain together with the abnormal phosphorylation of the tau protein are significant features for Alzheimer's disease (AD) pathogenesis (Blennow et al., 2006). The Aβ polypeptide arises from the proteolytic processing of a larger transmembrane protein, the amyloid precursor protein (APP), through the successive cleavage by enzymes called secretases. Sequential cleavage by β-secretase cleaving enzyme (BACE) and γ-secretase produces the Aβ peptide fragment. Alternative processing occurs if APP is first processed by a metalloproteinase, α-secretase, instead of BACE, resulting in a membrane-bound C-terminal fragment (CTF) that is also a γ-secretase substrate, but generating nonamyloidogenic products (for a review, see Thinakaran and Koo, 2008). γ-Secretase is a protein complex consisting of presenilin (PS), nicastrin, anterior pharynx-defective 1 (APH-1), and PS enhancer 2 (PEN-2) (Kaether et al., 2006). PS1, an aspartyl protease that cleaves substrates within the cell membrane, is the catalytic subunit of the γ-secretase complex.

Many other type I integral membrane proteins are also known to be cleaved by γ-secretase, after a previous shedding by α- or β-secretase (for a review, see Lleó and Saura, 2011). We have recently demonstrated an interaction between the acetylcholine-hydrolyzing enzyme, acetylcholinesterase (AChE), and the catalytic γ-secretase component PS1 (Silveyra et al., 2008, 2012). The brain cholinergic variant of AChE (a tetramer of active subunits) is anchored to the plasma membrane through a noncatalytic subunit, a type 1 transmembrane protein called proline-rich membrane anchor (PRiMA) (Chen et al., 2011; Perrier et al., 2002). Thus, the PRiMA subunit is an accessory partner for the

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cellular disposition of AChE (Dobbertin et al., 2009) and represents a limiting factor for the production of the tetrameric AChE (G_4)-PRiMA complex (Perrier et al., 2003). PRiMA expression is predominantly associated to the cholinergic system and strongly colocalizes with AChE within the neurons and particularly at the plasma membrane (Henderson et al., 2012). PRiMA consists of a signal peptide, an extracellular domain that contains a proline-rich motif, a transmembrane domain, and a cytoplasmic domain (Noureddine et al., 2007). Indeed, during the progression of AD, AChE activity decreases in the cerebral cortex and other affected areas, and a major tetrameric form containing the PRiMA subunit is markedly altered, whereas monomeric forms of AChE are relatively preserved (Atack et al., 1983; Fishman et al., 1986; Sáez-Valero et al., 1999).

The possibility of spontaneous and evoked release of a soluble form of AChE within different brain areas has been demonstrated in vivo in several rodent species (Appleyard and Smith, 1987; Appleyard et al., 1988; Greenfield and Shaw, 1982), a process that is likely mediated by secretion (Anglade et al., 1999). Moreover, recently the participation of a metalloproteinase activity, likely α-secretase, has been proposed in the shedding process of plasma membrane-anchored AChE (Hicks et al., 2011, 2013). Subsequent processing of the membrane anchor subunit PRiMA and the potential physiological consequences have been not investigated to date.

In this study, we investigate whether PS1 participates in the processing of the cholinergic AChE form via cleavage of the PRiMA subunit and whether PRiMA fragments could translocate to the nucleus. Using PS1 conditional knockout mice (*PS1* cKO, Yu et al., 2001), we have also examined whether PS1 influences the localization of PRiMA-linked G_4 AChE in brain lipid rafts in vivo.

2. Material and methods

2.1. Cell culture and treatments

Chinese hamster ovary (CHO) cells stably overexpressing the human AChE-T variant (a generous gift from H. Soreq, The Institute of Life Sciences, The Hebrew University of Jerusalem) were cultured in Dulbecco Modified Eagle medium + GlutaMAX-I (Gibco Invitrogen Corporation) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and G-410 (200 μg/mL) and maintained at 37 °C in saturated humidity containing 95% air and 5% CO_2. Complete depletion of AChE from FBS was achieved by passing the serum through edrophonium-sepharose (Flores-Flores et al., 1996). Cells were seeded at a density of 8×10^5 cells on 35 mm tissue culture dishes. CHO cells were transfected with 4 μg of PRiMA plasmid complementary DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The complementary DNA encoding the full-length mouse PRiMA isoform I was tagged with an hemagglutinin (HA) epitope (YPYDVP DYA) inserted before the stop codon at the C-terminus. A pCI "empty" vector (Promega, Madison, WI, USA) served as the negative control. After 48 hours of the transfection, the cells were washed 2 times with phosphate-buffered saline (PBS) and resuspended in 120 μL ice-cold extraction buffer: 50 mM Tris-HCl, pH 7.4/150 mM NaCl/5 mM EDTA/1% (w/v) Nonidet P-40/0.5% (w/v) Triton X-100 supplemented with a cocktail of protease inhibitors. The cell lysates were sonicated and centrifuged at 70,000× *g* at 4 °C for 1 hour, and the supernatants were collected and frozen at −80 °C before assaying for AChE and PRiMA.

CHO cells stably overexpressing wild-type human PS1 and wild-type APP (PS70, a generous gift from Dr Selkoe; see Xia et al., 1997) were cultured in Opti-MEM supplemented with 10% FBS, G-418 (200 μg/mL), and puromycin (2.5 μg/mL). These cells were transfected with 2 μg of PRiMA DNA and 2 μg of plasmid DNA encoding the human AChE-T under the cytomegalovirus promoter-enhancer (Soreq and Seidman, 2001). Cells were collected for analysis 48 hours after the transfection.

Transfected cells were treated with 0.1, 0.5, 1, 5, or 10 μM of the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT; Calbiochem) or vehicle (dimethyl sulfoxide; Sigma-Aldrich Co). Alternatively, the α-secretase inhibitor batimastat (25 μM) or the BACE inhibitor 2-β-secretase inhibitor III, GL189 (H-EVNstatineVAEF-NH_2, 5 μM), both from Calbiochem, Merck Chemicals, were used. After 24 hours of treatment, cells were collected for analysis.

For some experiments, cells overexpressing AChE and PRiMA were treated for 24 hours with the neurotoxin from snake venom fasciculin-2 (2 nM; Abcam), which binds AChE with high affinity (Bourne et al., 2003) or with the monoclonal anti-AChE antibody HR2 (dilution 1:1000; ABR-Affinity BioReagents).

Cell viability after each treatment (as previously described) was tested in cells cultured in 96-well plates using using the MTS tetrazolium assay [MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; CellTiter 96 AQueous Assay; Promega], according to the manufacturer's instructions.

2.2. PS1 conditional knockout mice and tissue preparation

All animal procedures were approved by the Animal Care and Use Committee of Universidad Miguel Hernandez. Brain samples were collected from ten 2.5- to 3.5-month-old *PS1* cKO, (see Yu et al., 2001) and 10 age-matched littermates in a C57BL6/129 hybrid background (all generously provided by C.A. Saura, Institut de Neurociències, Universitat Autònoma de Barcelona, Spain). PS1 expression has been selectively eliminated in the postnatal forebrain of these cKO mice beginning at P18. No PS1 immunoreactivity is detected in the brains of these mice at 2–3 months of age. In addition, no significant alterations in normal brain cytoarchitecture, neuronal number or morphology, general behavior, motor coordination, or exploratory anxiety were observed in these mice (Saura et al., 2004; Yu et al., 2001).

Brain cortices were homogenized (10% w/v) in extraction buffer: 50 mM Tris-HCl, pH 7.4/500 mM NaCl/5 mM EDTA/1% (w/v) Nonidet P-40/0.5% (w/v) Triton X-100 supplemented with a cocktail of protease inhibitors (Sáez-Valero et al., 1993). The homogenates were sonicated and centrifuged as described previously, and the supernatants were collected and frozen at −80 °C until assay.

Raft membrane subdomains were isolated at 4 °C, as described previously but with minor modifications (Xie et al., 2010a). Briefly, brain cortices (1 g/10 mL) were homogenized at 9500 rpm (IKA, Staufen, Germany), 6 times (10 seconds each time) in buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM benzamidine HCl, 10 μg/mL aprotinin, 10 μg/mL leupeptin). Homogenized tissues were then sonicated 3 times at low intensity (0.5 seconds periods, with 30 seconds intervals to avoid overheating). The sonicated preparation was centrifuged at 500× *g* for 5 minutes to remove cell debris and nuclei, and the postnuclear fraction was centrifuged at 35,000 rpm for 30 minutes in a Sorvall TST 60.4 rotor. The resulting pellet was washed and resuspended by sonication in 600 μL of buffer A containing 5% glycerol. This pellet was used as the total membrane fraction. For analysis of membrane rafts, a 500-μL sample of the total membrane fraction was incubated with 0.1% Triton X-100 on ice for 1 hour and applied to a discontinuous flotation gradient. This fraction was then mixed with an equal volume of 80% sucrose in buffer A, placed at the bottom of a 4-mL ultracentrifugation tube and overlaid with 2.4 mL of buffer A containing 30% sucrose followed by 0.6 mL of buffer A containing 5% sucrose. The resulting discontinuous gradient was spun at

50,000 rpm in a Sorvall TST 60.4 rotor for 18 hours at 4 °C. Fourteen fractions of ~250 μL were collected from the top of the tube and used for various assays.

2.3. Nuclear protein fractionation

CHO PS70 cells, hereafter referred to as CHO-PS1, were cultured in T25 flasks (8×10^5 cells per plate). Two days after transfection with PRiMA and AChE-T, cells were washed twice with cold PBS and gently scraped off the plates with 10 mL of cold PBS. Cell debris was discarded by centrifugation for 5 minutes at 500× *g*, 4 °C. Cell lysis and isolation of cellular nuclei were performed using the QProteome Nuclear Protein kit (Qiagen), according to the manufacturer's instructions. Cytosolic and nuclear protein fractions were assayed by Western blotting using α-tubulin and acetylated histone H2B as internal markers.

2.4. Immunoprecipitation of PRiMA-HA

Immunoprecipitations were carried out at 4 °C by incubating samples overnight with the antibodies coupled to protein A-sepharose using dimethyl pimelimidate dihydrochloride (Sigma-Aldrich Co). Immune complexes were eluted with glycine. For PS1 immunoprecipitation, extracts from CHO cells stably overexpressing the AChE-T variant (CHO-AChE) transfected with PRiMA were incubated with the 98/1 anti-PS1 antibody (Evin et al., 2001) and analyzed by Western blotting using an anti-HA antibody. For PRiMA-HA immunoprecipitation extracts from CHO cells stably transfected with PS1 (CHO-PS1), cells transfected with PRiMA and AChE-T were incubated with the anti-HA antibody (Sigma-Aldrich Co) and analyzed by Western blotting using an anti-PRiMA antibody. Extracts incubated with protein A-sepharose coupled to a nonspecific rabbit IgG (Vector) were analyzed as negative controls.

2.5. AChE enzymatic assay and protein determination

A modified microassay version of the colorimetric Ellman method was used to measure AChE (Sáez-Valero et al., 1993), using 50 μM tetraisopropyl pyrophosphoramide (*iso*-OMPA) as a specific inhibitor for the structurally related butyrylcholinesterase, an enzyme that coexists with AChE. Total protein concentrations were determined using the bicinchoninic acid method (Pierce).

2.6. Sedimentation analysis

Molecular forms of AChE were separated according to their sedimentation coefficients by centrifugation on 5%–20% (w/v) sucrose gradients containing 0.5% (w/v) Triton X-100. Ultracentrifugation was performed at 150,000× *g* in an SW 41Ti Beckman rotor for 18 hours, at 4 °C. Approximately 40 fractions were collected from the bottom of each tube and assayed for AChE activity to identify individual AChE forms (G_4 = tetramers and G_1 = monomers) by comparison with the position of molecular weight markers, catalase (11.4S), and alkaline phosphatase (6.1S). We defined the ratio of AChE forms G_4/G_1 as the proportion of G_4 molecules versus the light form, G_1.

2.7. Western blotting, measurement of PRiMA by enzyme-linked immunosorbent and alkaline phosphatase assays

Protein of 20–50 μg from cell lysates (equal amount of protein in each lane) or 40 μL of each lipid raft fraction was separated by sodium dodecyl–polyacrylamide gel electrophoresis. Samples were denatured at 50 °C for 15 minutes (PS1) or 98 °C for 7 minutes (all other proteins). The separated proteins were transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience GmbH) and probed with the following primary antibodies: anti-AChE antibody E-19 (Santa Cruz Biotech), anti-N-terminal PS1 (Calbiochem), anti-HA (Sigma-Aldrich), and anti-C-terminal PRiMA (Leung et al., 2009). An anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Abcam) was used as a loading control. Antibodies against flotillin 2 (BD Biosciences) were used as a lipid raft marker, whereas antibodies against calnexin (Sigma-Aldrich Co) and anti-β-cop (Thermo Scientific Pierce) were used as nonraft markers. An antibody against acetylated histone H2B (generous gift of Dr A. Barco) was used to distinguish the nuclear fraction, and an α-tubulin (Sigma-Aldrich Co) antibody was used to distinguish the cytosolic fraction. Western blots for different antibodies were performed separately to avoid reprobing of blots. The blots were incubated with the corresponding secondary antibody conjugated to horseradish peroxidase, and the signal was detected using the ECL Plus detection reagent according to the manufacturer's instructions (GE Healthcare) in a luminescent image analyzer LAS-1000 plus (Fujifilm). For semiquantitative analysis, protein levels were normalized to glyceraldehyde-3-phosphate dehydrogenase and the intensity of bands was measured by densitometry with the Science Lab Image Gauge version 4.0 software provided by Fujifilm.

PRiMA levels in cortical extracts from *PS1* cKO mice were determined using specific enzyme-linked immunosorbent assay (Cusabio Biotech Co, Hubei, China) according to the manufacturer's instructions.

Alkaline phosphatase (AP) was measured using 0.75 mM *p*-nitrophenylphosphate in 0.1 mM diethanolamine buffer, pH 9.8. One unit (U) of AP activity is equal to 1 nmol of *p*-nitrophenol formed per minute.

2.8. Confocal microscopy

CHO-PS1 were transfected with human PRiMA tagged to HA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 4 hours, the medium was replaced with fresh medium containing 5 μM DAPT or the equivalent volume of dimethyl sulfoxide as the vehicle control. After 24 hours, cells were washed with cold Hank-buffered salt solution and fixed with methanol for 10 minutes at −20 °C. Nonspecific sites were blocked with 2% (w/v) bovine serum albumin and 40 μg/mL digitonin in PBS for 30 minutes. Cells were then incubated with anti-HA antibody (rabbit; Sigma-Aldrich) for 1 hour followed by the secondary antibody (Cy5 anti-rabbit; GE-Healthcare) for 1 hour. After 2 washes with cold Hank-buffered salt solution, cells were incubated briefly with Hoechst 33,258 dye to label nuclei (Invitrogen). Pictures were acquired in a Leica upright TCL-SL confocal microscope using an HCX Plan Apochromat 63×/1.32–0.6 NA oil objective. To measure the intensity of PRiMA-HA fluorescence in the nucleus, a circle was hand drawn over the blue fluorescence of the Hoechst dye in the merge channel, and the red fluorescence from PRiMA-HA that overlapped this fluorescence was quantified. Analysis was performed with LAS AF Lite software. Nontransfected cells were used as controls, and nonspecific fluorescence was not detected.

Human H4 neuroglioma cells were seeded on glass coverslips and transiently cotransfected with 300 ng of each of the AChE-T, PRiMA, and PS1-green fluorescence protein constructs in serum-free media using the XtremeGene9 DNA transfection reagent (Roche Diagnostics GmbH) according to the manufacturer's instructions. FBS (10%) was added to the media 4 hours after transfection. After 24 hours, the lipid rafts were labeled as described previously (Guardia-Laguarta et al., 2009). Briefly, cells on the glass coverslips were incubated with Cholera toxin subunit B tagged with an Alexa-694 fluorophore (Molecular Probes, Inc) followed by an anti-Cholera toxin subunit B antibody (Molecular Probes, Inc) to cross-link lipid rafts into distinct patches on the plasma membrane. Cells were washed twice with PBS, fixed with 4% paraformaldehyde, and then immunostained for

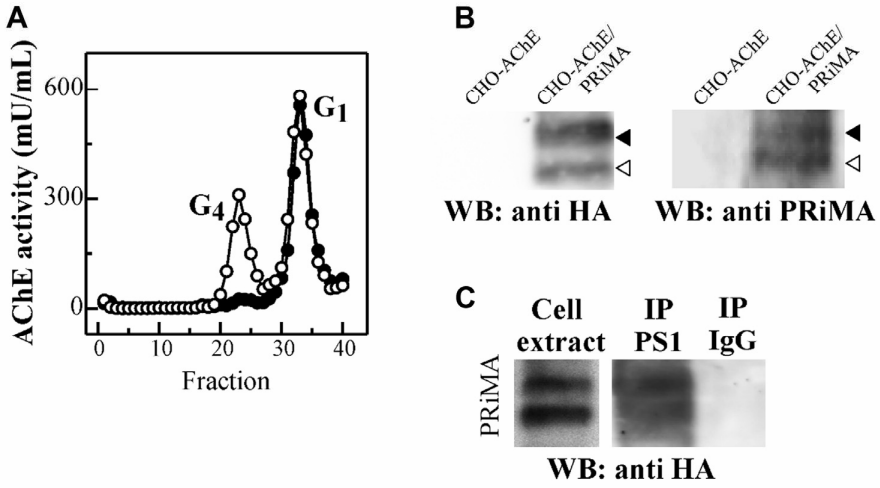

Fig. 1. Proline-rich membrane anchor (PRiMA) interacts with presenilin-1 (PS1). (A) Cellular extracts from Chinese hamster ovary cells stably transfected with the acetylcholinesterase-T variant (CHO-AChE, closed symbols) were cotransfected with the noncatalytic PRiMA subunit carrying an HA tag at its C-terminus (CHO-AChE/PRiMA, open symbols). The generation of the tetrameric G_4 AChE form in CHO-AChE/PRiMA cells was assessed by ultracentrifugation in a continuous sucrose density gradient. (B) The over-expressed PRiMA subunit was identified by blotting with an anti-HA antibody or by an anti-C-terminal PRiMA antibody. Two PRiMA bands were identified at ~22 kDa (most likely the fully glycosylated mature protein, closed arrowhead) and ~20 kDa (immature protein, open arrowhead) in CHO-AChE/PRiMA cell extracts. (C) Cellular extracts from CHO-AChE/PRiMA cells were immunoprecipitated with an anti-N-terminal PS1 antibody. Precipitated proteins were immunoblotted with an anti-HA antibody. Two PRiMA bands at ~22 and ~20 kDa were detected. Extracts incubated with protein A-sepharose coupled with a nonspecific rabbit IgG were analyzed in parallel as negative controls.

AChE with the anti-AChE N19 antibody (Santa Cruz Biotechnology) followed by an Alexa 647–tagged secondary antibody (Molecular Probes, Inc). Confocal images were taken with an SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) using a 63× objective (4× zoom) in 3 channels, 488 nm (PS1-green fluorescence protein), 561 nm (lipid rafts), and 647 nm (AChE-T). Laser power was kept at a low gain to avoid crossover between the 3 channels and to avoid pixel saturation. Confocal images were taken in multiple *z* planes (1 micron apart) to capture the entire cell. All *z* planes were used for quantification of colocalization. Only the *z* plane at the midpoint of the cell was used for the confocal images presented here. Colocalization analysis was performed using ImageJ software (version 1.46g) (Schneider et al., 2012). The 3 confocal channels (AChE, PS1, and lipid rafts) containing multiple *z* stacks across the cell were individually thresholded using the Otsu method implemented in ImageJ to create a binary image. Manders' coefficients (Manders et al., 1993) for pairwise colocalization of AChE-T and lipid rafts, AChE-T and PS1, or PS1 and lipid rafts were calculated for the binary threshold images using the JACoP ImageJ plug-in (Bolte and Cordelieres, 2006). The Manders' coefficients correspond to the fraction of pixels that are positive for the first protein that are also positive for the second, with a value of 1 representing 100% colocalization and 0 being 0% colocalization. Images showing the pixels where the 2 channels colocalize were generated for the binary threshold images using the colocalization highlighter ImageJ plug-in.

2.9. Statistical analysis

All data were analyzed using SigmaStat (version 2.0; SPSS Inc) by Student *t* test (2 tailed). Results are presented as means ± standard error of the mean. *p* Values <0.05 were considered significant.

3. Results

3.1. PRiMA interacts with PS1 and undergoes proteolytic processing

We previously demonstrated an interaction between PS1 and AChE, and the co-immunoprecipitation of G_4 AChE with PS1 antibodies (Silveyra et al., 2008). To confirm that this interaction occurs in AChE forms containing the PRiMA subunit, we used an anti-PS1 antibody to co-immunoprecipitate PRiMA in CHO-AChE and cotransfected with a C-terminal HA-tagged PRiMA subunit. Sedimentation analysis by sucrose density gradients was used to characterize AChE molecular forms and to confirm the expression of PRiMA-linked G_4 AChE in transfected CHO cells (Fig. 1A). Two PRiMA bands of ~22 and ~20 kDa were observed in all cellular extracts by Western blot analysis with an anti-HA antibody (Fig. 1B). These 2 bands likely correspond to mature (fully glycosylated) and immature PRiMA (Chan et al., 2012). The specificity of the signal was confirmed in PS1 immunoprecipitates using an anti-PRiMA antibody (Fig. 1B). The PRiMA signal was not detected in the negative immunoprecipitation controls, using a nonspecific rabbit IgG antibody (Fig. 1C).

We next examined if γ-secretase was able to mediate PRiMA processing. CHO-AChE, cotransfected with PRiMA, were treated with a well-known γ-secretase inhibitor DAPT, which has been demonstrated to reduce levels of Aβ in cell culture and in vivo (Lanz et al., 2003). The efficiency of DAPT to inhibit γ-secretase activity was monitored by measuring the accumulation of the APP-CTF in cells treated for 24 hours with 5 μM of DAPT (Fig. 2A). In cells treated with DAPT, a trend toward a decrease in soluble (secreted) AChE activity was observed, albeit that this was not statistically significant compared with controls (12% ± 5%, $p = 0.1$). Conversely, a small but statistically significant increase (16% ± 6%, $p = 0.03$) in the cellular AChE activity was observed compared with untreated cells (Fig. 2B). Specifically, the levels of PRiMA-linked G_4 AChE were increased in DAPT-treated cells, with minimal change in monomeric G_1 (the species devoid of PRiMA subunit), leading to a significant increase in G_4/G_1 ratio (Fig. 2C). The concomitant increase in PRiMA content (180% ± 40%, $p < 0.001$, Fig. 2D) in DAPT-treated cells, compared with untreated controls, suggests that PS1 can cleave PRiMA at the membrane spanning domain by preventing γ-secretase processing. A dose-dependent effect of DAPT treatment on PRiMA accumulation is observed (Supplementary Fig. S1). No significant change in cell viability was observed after 24 hours of treatment with any of the secretase inhibitors, as assessed by the MTS method.

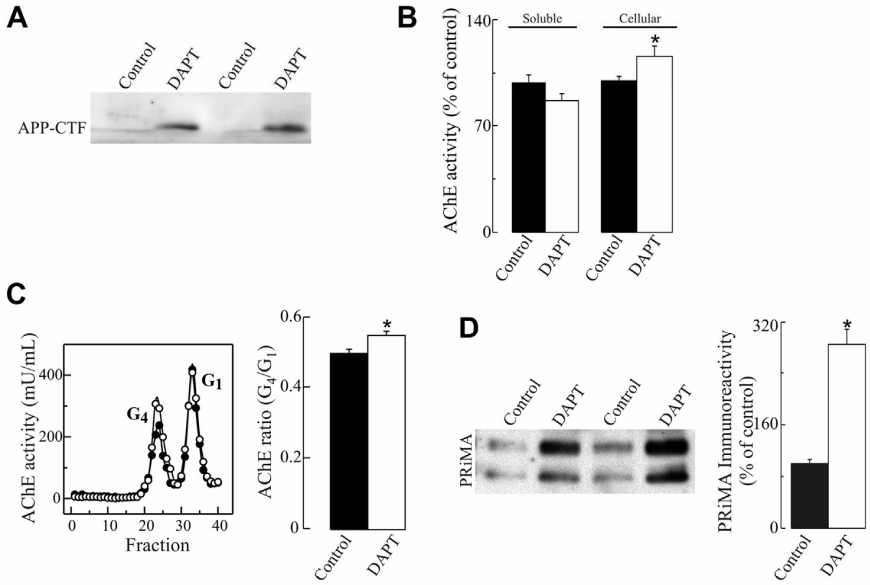

Fig. 2. Inhibition of γ-secretase increases levels of tetrameric acetylcholinesterase (AChE) and of the linked proline-rich membrane anchor (PRiMA) subunit. Chinese hamster ovary (CHO) cells stably transfected with the AChE-T variant and cotransfected with PRiMA tagged to HA were treated for 24 hours with 5 μM of *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) or vehicle control (dimethyl sulfoxide [DMSO]). (A) The presence of the C-terminal amyloid precursor protein fragment (CTF-APP) indicates inhibition of γ-secretase activity by DAPT. (B) The relative percentage of soluble (secreted) and cellular AChE activity after the addition of DAPT or vehicle is shown. (C) Fractions from the sucrose density gradient were collected and assayed for AChE activity to identify individual AChE isoforms in extracts from cells treated with vehicle (DMSO, control, closed symbols) or 5 μM DAPT (open symbols). The corresponding G_4/G_1 ratio was calculated in controls and in DAPT-treated cells. (D) Immunoreactivity of PRiMA-HA from DAPT-treated cells. Each determination was made in duplicate. Data represent means ± standard error of the mean from at least 9 independent determinations from 3 independent experiments. * $p < 0.05$.

These results suggest that PRiMA-linked G_4 AChE is a new target of γ-secretase. However, because CTFs of PRiMA (PRiMA-CTF) were not detected in this cell line, it is possible that they were present at subdetectable levels and is indicative of the inherent instability of these fragments. To investigate this further, CHO-PS1, which exhibit elevated γ-secretase activity (Xia et al., 1997), were transfected with AChE-T and PRiMA tagged to HA at the C-terminus in an attempt to identify the PRiMA-CTF. An ~14 kDa band was detected by the anti-HA antibody corresponding to the expected size of PRiMA-CTF (Fig. 3A). The specificity of the band was confirmed by immunoprecipitation with an anti-HA antibody and Western blotting using anti-PRiMA antibody (an antibody raised to the C-terminus of PRiMA, Fig. 3B).

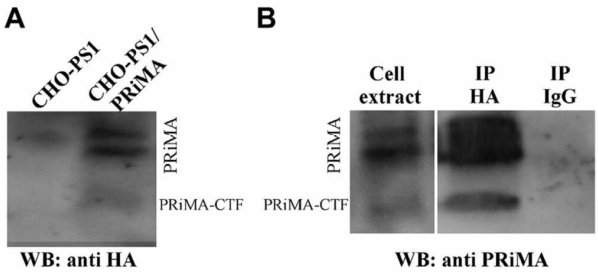

Fig. 3. Identification of a C-terminal fragment (CTF) of proline-rich membrane anchor (PRiMA) in Chinese hamster ovary cells stably transfected with PS1 (CHO-PS1). (A) CHO-PS1 were transfected with PRiMA tagged to HA at its C-terminus (CHO-PS1/PRiMA). The full-length PRiMA subunit and CTF were identified by blotting with an anti-HA antibody. (B) Cell extracts from CHO-PS1/PRiMA cells were immunoprecipitated with an anti-HA antibody, and immunoprecipitated (IP) proteins were immunoblotted with an anti-C-terminal PRiMA antibody. The anti-HA antibody was able to immunoprecipitate both full-length PRiMA (bands of ~22 and ~20 kDa) and its CTF (~14 kDa). Negative controls were extracts incubated with protein A-sepharose coupled with rabbit IgG.

Moreover, accordingly with the expected sequential actions of secretases, a first one, α- or β-secretase, is expected to cleave PRiMA-linked AChE before shedding of PRiMA anchor by γ-secretase. Thus, we also monitored, under the same conditions used for DAPT treatment, the efficiency of α- and β-secretase–specific inhibitors to target PRiMA-linked AChE by determining changes in AChE release and in the cellular levels of PRiMA. Treatment of the cells with batimastat, a matrix metalloproteinase inhibitor that has previously been shown to influence AChE release (Hicks et al., 2013) prevented AChE release and increased PRiMA levels (Fig. 4). Interestingly, treatment with GL189, a specific inhibitor commonly used to block the BACE proteolytic activity of solubilized membrane fractions (Capell et al., 2002), also led to increased levels of PRiMA, but it failed to alter the levels of secreted AChE activity (Fig. 4).

We next determined whether the addition of an AChE inhibitor or an AChE antibody to the cell system, and hence associated conformational changes in the complex, would affect the processing of PRiMA. Fasciculin-2 is a strong AChE inhibitor that binds to the peripheral anionic site rather than the active site of AChE, whereas HR2 is an antibody raised against human cerebellar AChE. Neither of the assayed compounds were able to induce changes in PRiMA levels (Supplementary Fig. S2), revealing that epitopes involved in these interaction are unrelated with secretase processing.

3.2. Localization of PRiMA immunoreactivity in the cell nucleus

Several transmembrane proteins have been demonstrated to undergo γ-secretase cleavage, releasing soluble intracellular fragments that translocate to the nucleus and act as transcriptional regulators (Rochette and Murphy, 2002). In contrast, other

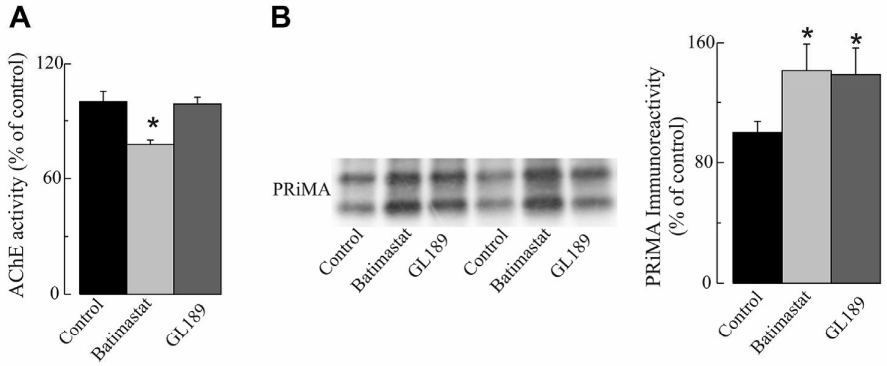

Fig. 4. Inhibition of α- and β-secretase increases levels of the proline-rich membrane anchor (PRiMA) subunit. Chinese hamster ovary cells overexpressing acetylcholinesterase-T variant (AChE-T) and PRiMA (tagged to HA) were treated for 24 hours with 25 μM of the α-secretase inhibitor Batimastat, with 5 μM of the β-secretase code named GL189, or with vehicle (control). (A) Soluble AChE activity was determined in conditioned media and expressed as percentage (%) with respect to control cells. (B) The accumulation of PRiMA was monitored with an anti-HA antibody. Data represent percentage of HA immunoreactivity relative to control cells. Data represent mean ± standard error of the mean from at least 10 independent determinations from 2 independent experiments. * $p < 0.05$.

transmembrane proteins, processed by γ-secretase, are only cleaved for degradation (for a review, see Lleó and Saura, 2011). Immunofluorescence labeling of nuclear DNA by 4′,6-diamidino-2-phenylindole allowed us to examine the presence of PRiMA-HA in the nucleus of CHO-PS1 transfected with PRiMA. Localization of PRiMA-HA immunoreactivity confirmed that PRiMA-CTF enters the nucleus. The level of the nuclear PRiMA-HA signal was decreased in transfected cells treated with DAPT compared with controls (45% ± 3% decrease, $p = 0.002$; Fig. 5A). Western blotting of HA immunoprecipitates from nuclear extracts corroborated the presence of a PRiMA-CTF in the nuclear fraction (Fig. 5B).

3.3. Colocalization of PS1 and AChE in membrane rafts

Because PS1 has been described as a lipid raft resident protein (Parkin et al., 1999; Vetrivel et al., 2004; Wada et al., 2003), and PRiMA can target G_4 AChE to membrane rafts (Xie et al., 2010a), we investigated whether both PS1 and PRiMA-AChE colocalize to lipid rafts. The distribution of PS1 and AChE was compared by immunocytochemistry in H4 cells (a cell line widely used for lipid raft studies; Asai et al., 2007; Guardia-Laguarta et al., 2009; Hinz et al., 2004) overexpressing both AChE-T and PRiMA subunits (Fig. 6). Confocal microscopy analysis demonstrated that ∼42% of total AChE and ∼10% of total PS1 colocalized with lipid rafts in H4 cells. Within the lipid rafts, ∼23% of AChE colocalized with PS1 and conversely ∼26% of PS1 colocalized with AChE.

3.4. The PRiMA-linked G_4 AChE form is increased in lipid rafts of the PS1 *cKO mouse brain*

To assess whether γ-secretase is involved in the control of PRiMA-linked G_4 AChE levels in vivo, total AChE activity, AChE isoforms, and PRiMA levels were measured in brain homogenates from 3-month-old *PS1* cKO mice (Yu et al., 2001) and littermate controls. No significant differences were detected in total AChE activity, PRiMA levels, or in the contribution of each AChE molecular form in *PS1* cKO mice compared with littermate controls (Supplementary Fig. S3). Analyses of membrane raft preparations were then conducted in the *PS1* cKO mice to examine if PS1 influences the localization (and/or processing) of AChE into brain lipid rafts in vivo. To prepare raft-enriched fractions, membrane pellets from brain cortices were treated with 0.1% Triton X-100 at 4 °C and extracts subjected to flotation in discontinuous sucrose gradients. Raft-associated proteins, flotillin-2 (Volonte et al., 1999) and alkaline phosphatase (Parkin et al., 1997), and nonraft proteins, calnexin and β-cop (García-Marcos et al., 2006), were used to characterize raft-enriched fractions (Fig. 7A). In the membrane preparations obtained from cortices of wild-type mice, the majority of the PS1 immunoreactivity was associated with raft membranes, whereas AChE was identified in both raft and nonraft fractions (Fig. 7B). The proportion of raft-localized AChE was higher in samples from *PS1* cKO mice than wild-type mice, resulting in increased values for a quotient between raft-associated AChE and nonraft AChE, enabling the distinction between *PS1* cKO mice and littermate controls (Fig. 7C). Sedimentation analysis confirmed that the PRiMA-linked G_4 form is the major, if not the only, AChE form associated with rafts in both groups of mice (Fig. 7D).

4. Discussion

The loss of cholinergic neurons in the forebrain and the associated decline of cholinergic neurotransmission are features of AD (Davies and Maloney, 1976; Perry et al., 1977). The AChE enzyme, as part of the cholinergic system, is decreased in the brain of AD subjects, where the cholinergic PRiMA-linked G_4 form is particularly affected (Atack et al., 1983; Fishman et al., 1986). Other molecular forms and variants of AChE are present in lower quantities within the brain. These include soluble G_4 isoforms and light forms, mainly monomers, of the T (tailed) variant (Sáez-Valero et al., 1993, 1999) and also monomers of the minor R (read-through) variant (Berson et al., 2008). The 3′ alternative splicing of *AChE* also generates additional N-terminally extended AChE variants (Meshorer et al., 2004). All molecular forms and variants of AChE display similar catalytic activity; however, the structural polymorphism of AChE differs within cellular compartments and between different cell types (for a review, see Massoulié, 2002). It has been proposed that different molecular forms of AChE may have different physiological functions, other than its classical cholinergic role (Grisaru et al., 1999; Small et al., 1996). These polymorphisms enable the localization of AChE into particular cellular compartments. Individual components can be sorted separately by this fine regulation of AChE function (either the classical cholinergic activity or other activities), either by the protein itself or in combination with other protein partners. Recent studies have demonstrated that a significant portion of the membrane-bound G_4 AChE is localized in membrane rafts within the brain and that PRiMA directs this restricted localization (Xie et al., 2010a, 2010b). This suggests that the targeting of PRiMA-linked AChE to rafts in the brain may

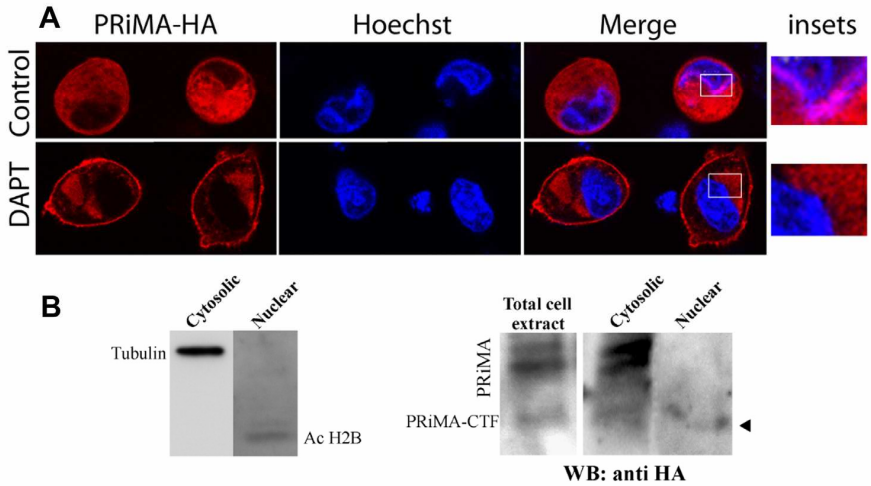

Fig. 5. Localization of proline-rich membrane anchor (PRiMA)-HA immunoreactivity in the nucleus. (A) Confocal images of Chinese hamster ovary cells stably overexpressing presenilin-1 (CHO-PS1) and cotransfected with PRiMA-HA. Cells were incubated for 24 hours with dimethyl sulfoxide (control) or with 5 μM *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) and fixed, and immunodetection was performed with an antibody against HA. Nuclei were stained with Hoechst 33,258 dye. The insets show magnification of selected areas from the merged images, highlighting the low amount of PRiMA-HA in the nucleus after DAPT incubation. (B) Nuclear and cytoplasmic fractions from CHO-PS1/PRiMA cells were analyzed by Western blotting with an anti-HA antibody. A faint band of a C-terminal PRiMA fragment (~14 kDa, arrowhead) was detected in the nuclear fraction, whereas full-length PRiMA was detected in the cytosolic fractions. Acetylated histone H2B (AcH2B) and tubulin were used as markers for nuclear and cytoplasmic fractions, respectively.

account for its synaptic localization and function. It is thus important to elucidate which particular AChE molecular forms are affected in the AD brain, and within which particular cell compartment they reside, to optimize therapies.

In this study, we show that PS1, the catalytic component of the γ-secretase complex, can interact with the PRiMA subunit. Our data demonstrate that secretases can participate in the processing of PRiMA-linked AChE and that PS1/γ-secretase cleaves PRiMA in a cellular model. We were also able to characterize a single 14-kDa PRiMA CTF and localize PRiMA immunoreactivity in the nucleus. Furthermore, the silencing of PS1 in the *PS1* cKO mouse model led to changes in the distribution of PRiMA-linked G_4 AChE forms in raft and nonraft membranes.

We have previously demonstrated that expression of the PS1-A246E pathogenic mutation in transgenic mice leads to decreased brain AChE activity (Silveyra et al., 2008). Reciprocally, AChE influences PS1 levels as AChE overexpression increases PS1 levels, whereas AChE knockdown with small interfering RNA leads to decreased PS1 in transfected cells (Silveyra et al., 2012). An interrelationship between signaling and amyloid processing has been

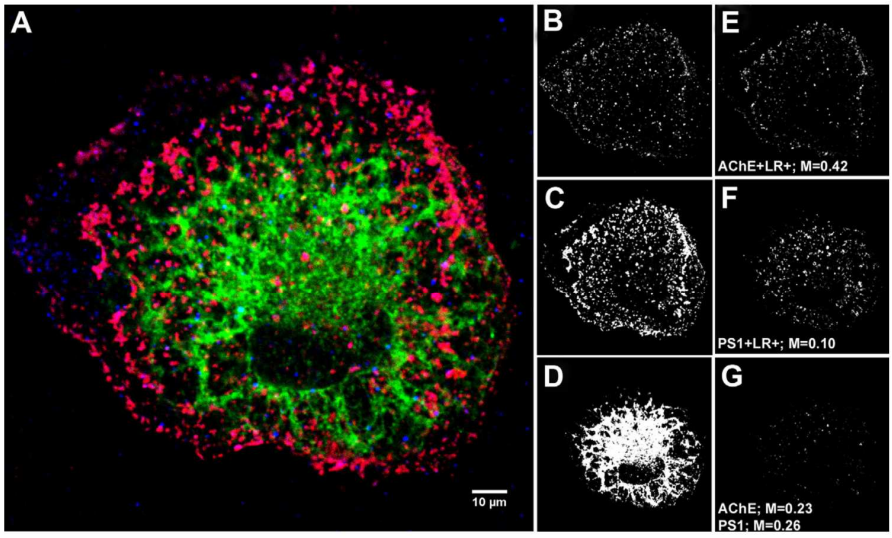

Fig. 6. Acetylcholinesterase (AChE) colocalizes with presenilin-1 (PS1) within lipid rafts. (A) H4 cell transiently expressing green fluorescence protein–tagged PS1 (green), AChE (blue), and proline-rich membrane anchor. Lipid rafts are labeled in red. Binarized images of (B) AChE, (C) lipid raft, and (D) PS1 channels were generated to quantify colocalization with lipid rafts and PS1 using the Manders' coefficient (M, estimated in $n = 7$ cells). (E) Pixels positive for both AChE and the lipid raft marker represent approximately 42% ± 3% of the total AChE-positive pixels. (F) Pixels positive for both PS1 and the lipid raft marker represent approximately 10% ± 1% of the total PS1-positive pixels. (G) Pixels positive for AChE, PS1, and lipid raft marker constitute approximately 23% ± 5% of AChE and approximately 26% ± 4% of PS1 that localize to the lipid rafts.

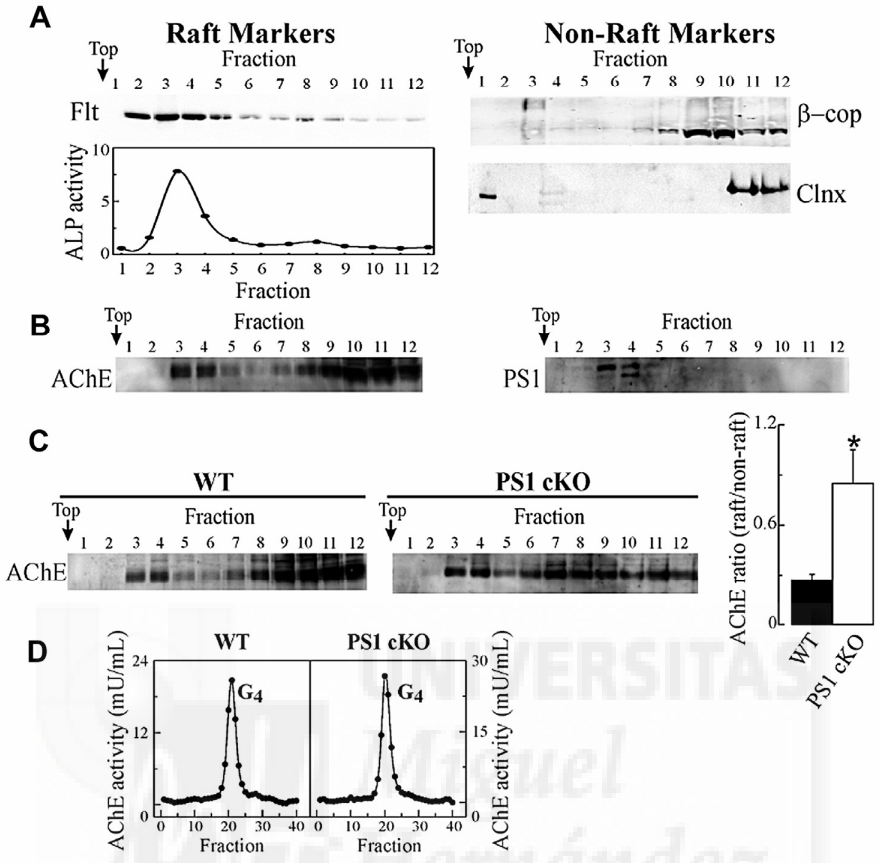

Fig. 7. G_4 acetylcholinesterase (AChE) is increased in membrane rafts isolated from the cortex of PS1 conditional knockout mice (*PS1* cKO) mice. Isolation of lipid rafts from cortices of 3-month-old *PS1* cKO mice ($n = 6$) and age-matched littermate controls ($n = 6$) was achieved by centrifugation in a discontinuous sucrose density gradient (fractions were collected from the top of the tubes). (A) Raft-enriched fractions (2–6) and nonraft fractions (8–12) were defined after determination of positive and negative lipid raft markers. Equal volumes from each fraction were analyzed by immunoblotting. Flotillin 2 (Flt) and alkaline phosphatase (ALP) enzymatic activity (expressed as arbitrary units) were used as positive lipid raft markers and calnexin (Clnx) and β-cop as non–lipid raft markers. (B) Raft localization of PS1 in control mice was verified by immunoblotting with an anti-N-terminal PS1 antibody. Aliquots of all fractions were immunodetected with an anti-N-terminal antibody E19, which recognizes all the variant subunits of AChE. AChE and PS1 were localized in lipid raft–rich fractions as determined in (A). (C) Immunoblotting demonstrated increased AChE partitioning into lipid raft fractions in *PS1* cKO versus control mice, as assessed by a ratio of the densitometric quantitation of the immunoreactivity of AChE bands identified in raft fractions 2–6 divided by the immunoreactivity of AChE bands identified in nonraft fractions 8–12. (D) Sedimentation analysis of raft-residing AChE (fractions 2–6) demonstrated a single molecular form corresponding to proline-rich membrane anchor–linked G_4 AChE in both wild-type (WT) and *PS1* cKO mice.

reported for neuroligin-1, a postsynaptic adhesion molecule whose extracellular domain is homologous to AChE and which is sequentially cleaved by α-secretase/ADAM10 and PS1/γ-secretase (Suzuki et al., 2012). This proteolytic processing of neuroligin-1 is regulated by synaptic *N*-methyl D-aspartate receptor activation or interaction with soluble neurexin ligands (Suzuki et al., 2012). Whether neuronal activity regulates PS1-mediated AChE processing and under what biological conditions remain to be determined. It has been previously suggested that AChE shedding is related with activation of the α7 nicotinic acetylcholine receptor, and also with metabolism of APP, associated with the location of AChE into lipid raft domains (Hicks et al., 2011, 2013).

APP, one of the most investigated γ-secretase substrates, is alternatively processed through the successive actions of β- and γ-secretase (amyloidogenic pathway) or by α- and γ-secretase (nonamyloidogenic pathway). More than 90 type I integral membrane proteins are known to be cleaved by γ-secretase; most of them are previously processed uniquely by α- or β-secretase, and some, similarly to APP, can be processed by both secretases (see Lleó and Saura, 2011). It is therefore important to identify the protease that cleaves PRiMA-linked G_4 before γ-secretase processing. Identification of these proteases will enable the design of strategies to interfere with its processing. We found that treatments with inhibitors of both, α- or β-secretase, resulted in accumulation of PRiMA. Cellular treatment with batimastat, a matrix metalloproteinase inhibitor with effects in several enzymes possessing α-secretase activity, led to a significant reduction in AChE activity in the conditioned media, whereas, interestingly, no effect of the β-secretase inhibitor GL189 was observed. The mechanism by which AChE is released is still unclear. Our data corroborate the possibility that a metallosecretase was involved in AChE secretion (Hicks et al., 2013), whereas processing of PRiMA-linked AChE by β- and γ-secretase may result in the degradation of the active AChE subunits. More research is needed to characterize difference between both pathways.

Also in this regard, it has been suggested that AChE may be targeted for endocytosis (Hu et al., 2009). Despite a large proportion of PS1 localized within the endoplasmic reticulum and early Golgi, it is assumed that APP cleavage occurs on the cell surface and in endosomes/lysosomes, where proteolytically active PS1/γ-secretase is principally localized (for a review, see Haass et al., 2012). A putative domain related with endocytosis has been identified in the cytoplasmic tail of PRiMA (Hu et al., 2009). As regard with the pathologic condition, this internalization pathway for AChE from

the cell surface to lysosomes appears to be influenced by extracellular Aβ (Hu et al., 2009).

The identification of PS1 (Vetrivel et al., 2004), and PRiMA-linked G_4 isoforms (Xie et al., 2010a, 2010b) in lipid raft, and our results showing that PS1 and PRiMA-AChE interact in vivo (Silveyra et al., 2008) suggest that the lipid rafts may be the location of the PS1 and PRiMA-AChE interaction. Remarkably, and in accordance with the subcellular sites of PRiMA-linked AChE processing by γ-secretase, whereas β-secretase activity is enriched within lipid rafts, alongside γ-secretase (Hattori et al., 2006; Riddell et al., 2001), α-secretase is not localized to rafts (Harris et al., 2009). Our studies using H4 cells demonstrate that PS1 colocalizes in the same raft microdomain with AChE. This is further substantiated by the observation that localization of G_4 AChE into rafts is altered in *PS1* cKO mice. The increased levels of raft-bound AChE in the *PS1* cKO mice may arise from impaired proteolytic events resulting in decreased γ-secretase activity or from the influence of PS1 itself on the localization of AChE into rafts. PS1 coprecipitates both fully glycosylated (22-kDa band) and immature PRiMA (20-kDa band). PS1 has the capacity to alter both the lipid composition (Grimm et al., 2006) and the lipid packing order of neuronal raft membranes (Eckert and Müller, 2009). Increasing evidence points to the relevance of membrane composition of both raft and nonraft domains to AD progression in different AD models and in the human disease (Williamson and Sutherland, 2011). The possibility that perturbations in lipid rafts can influence not only APP metabolism but also signaling events that may involve cholinergic neurotransmission has been suggested (Hicks et al., 2012). Our data corroborate this possibility. More interestingly, the interrelationship between neurotransmission and amyloid processing is plausible.

For the known secretase substrates, γ-secretase is always the final enzyme that cleaves the membrane-spanning domain. In general, the requirements for being a γ-secretase substrate are broad: a type I transmembrane helix with a small ectodomain (<300 amino acids), usually resulting from a previous shedding by a metalloprotease-like protease. However the requirements for γ-secretase cleavage do not depend critically on a specific amino acid sequence or on endocytosis (Struhl and Adachi, 2000). Moreover, substrates such as Notch, CD44, or the β-CTF of APP are cleaved at multiple sites by γ-secretase, resulting in various cleavage products (Lleó and Saura, 2011). In spite of the lack for specific requirements to be a potential γ-secretase substrate, it is interesting to note that the expected membrane-bound CTFs of APP and PRiMA are similar in size (~40 to 47 amino acids for the APP intracellular domain and ~40 amino acids for PRiMA). It is also worth noting that several valine residues are present at the juxtamembrane domain of PRiMA, and it is known that γ-secretase cleaves close to valines (Maruyama et al., 1996). The identification of CTFs of PRiMA is consistent with the mechanism of action of γ-secretase.

As γ-secretase cleavage of some substrates releases intracellular domains with critical signaling properties, further characterization of the generated intracellular fragment of PRiMA is required. Although PRiMA intracellular fragments may be rapidly degraded, we have been able to monitor the production of a PRiMA CTF and predict that it is translocated to the nucleus. In the absence of the PRiMA cytoplasmic tail of any of the nuclear localization signals known to date, the possibility remains that the PRiMA fragment uses an adaptor protein in its way to the nucleus. The identification of a PRiMA *trans*-acting cytoplasmic adaptor protein that participates and regulates the translocation will be valuable for understanding the role of the PRiMA fragment in the cell nucleus. Although the occurrence of AChE in the nucleus has been described in the early stages of apoptosis (Huang et al., 2005; Yang et al., 2002), there is the possibility that the PRiMA segment participates in the regulation of gene transcription, a possibility that deserves thoroughly investigation.

The physiological relevance of the proteolytic events described in this study is still not well understood. AChE inhibitors are currently at the forefront of AD therapy (Cummings, 2004; Giacobini, 2003; Lleó, 2007), and PS1 is a new emerging drug target (Wagner et al., 2012; Wolfe, 2008). Understanding the relationship between PS1 and AChE may be useful not only for the physiopathology of the disease but also to develop more effective Alzheimer therapies.

Disclosure statement

None of the authors have any actual or potential financial conflicts or conflicts of interest related with this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2014.01.147.

References

Anglade, P., Grassi, J., Motelica-Heino, I., Hashikawa, T., Tsuji, S., 1999. Ultrastructural evidence for dendritic release of acetylcholinesterase in the rat substantia nigra. Folia Histochem. Cytobiol. 37, 243–247.

Appleyard, M.E., Smith, A.D., 1987. Spontaneous and carbachol-evoked in vivo secretion of acetylcholinesterase from the hippocampus of the rat. Neurochem. Int. 11, 397–406.

Appleyard, M.E., Vercher, J.L., Greenfield, S.A., 1988. Release of acetylcholinesterase from the guinea-pig cerebellum in vivo. Neuroscience 25, 133–138.

Asai, M., Iwata, N., Yoshikawa, A., Aizaki, Y., Ishiura, S., Saido, T.C., Maruyama, K., 2007. Berberine alters the processing of Alzheimer's amyloid precursor protein to decrease Abeta secretion. Biochem. Biophys. Res. Commun. 352, 498–502.

Atack, J.R., Perry, E.K., Bonham, J.R., Perry, R.H., Tomlinson, B.E., Blessed, G., Fairbairn, A., 1983. Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type: selective loss of the intermediate (10S) form. Neurosci. Lett. 40, 199–204.

Berson, A., Knobloch, M., Hanan, M., Diamant, S., Sharoni, M., Schuppli, D., Geyer, B.C., Ravid, R., Mor, T.S., Nitsch, R.M., Soreq, H., 2008. Changes in read-through acetylcholinesterase expression modulate amyloid-beta pathology. Brain 131, 109–119.

Blennow, K., de Leon, M.J., Zetterberg, H., 2006. Alzheimer's disease. Lancet 368, 387–403.

Bolte, S., Cordelieres, F.P., 2006. A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224, 213–232.

Bourne, Y., Taylor, P., Radic, Z., Marchot, P., 2003. Structural insights into ligand interactions at the acetylcholinesterase peripheral anionic site. EMBO J. 22, 1–12.
Capell, A., Meyn, L., Fluhrer, R., Teplow, D.B., Walter, J., Haass, C., 2002. Apical sorting of beta-secretase limits amyloid beta-peptide production. J. Biol. Chem. 277, 5637–5643.
Chan, W.K., Chen, V.P., Luk, W.K., Choi, R.C., Tsim, K.W., 2012. N-linked glycosylation of proline-rich membrane anchor (PRiMA) is not required for assembly and trafficking of globular tetrameric acetylcholinesterase. Neurosci. Lett. 523, 71–75.
Chen, V.P., Choi, R.C., Chan, W.K., Leung, K.W., Guo, A.J., Chan, G.K., Luk, W.K., Tsim, K.W., 2011. The assembly of proline-rich membrane anchor (PRiMA)-linked acetylcholinesterase enzyme: glycosylation is required for enzymatic activity but not for oligomerization. J. Biol. Chem. 286, 32948–32961.
Cummings, J.L., 2004. Alzheimer's disease. N. Engl. J. Med. 351, 56–67.
Davies, P., Maloney, A.J., 1976. Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 2, 1403.
Dobbertin, A., Hrabovska, A., Dembele, K., Camp, S., Taylor, P., Krejci, E., Bernard, V., 2009. Targeting of acetylcholinesterase in neurons in vivo: a dual processing function for the proline-rich membrane anchor subunit and the attachment domain on the catalytic subunit. J. Neurosci. 29, 4519–4530.
Eckert, G.P., Müller, W.E., 2009. Presenilin 1 modifies lipid raft composition of neuronal membranes. Biochem. Biophys. Res. Commun. 382, 673–677.
Evin, G., Sharples, R.A., Weidemann, A., Reinhard, F.B., Carbone, V., Culvenor, J.G., Holsinger, R.M., Sernee, M.F., Beyreuther, K., Masters, C.L., 2001. Aspartyl protease inhibitor pepstatin binds to the presenilins of Alzheimer's disease. Biochemistry 40, 8359–8368.
Fishman, E.B., Siek, G.C., MacCallum, R.D., Bird, E.D., Volicer, L., Marquis, J.K., 1986. Distribution of the molecular forms of acetylcholinesterase in human brain: alterations in dementia of the Alzheimer type. Ann. Neurol. 19, 246–252.
Flores-Flores, C., Martinez-Martinez, A., Muñoz-Delgado, E., Vidal, C.J., 1996. Conversion of acetylcholinesterase hydrophilic tetramers into amphiphilic dimers and monomers. Biochem. Biophys. Res. Commun. 219, 53–58.
García-Marcos, M., Pochet, S., Tandel, S., Fontanils, U., Astigarraga, E., Fernández-González, J.A., Kumps, A., Marino, A., Dehaye, J.P., 2006. Characterization and comparison of raft-like membranes isolated by two different methods from rat submandibular gland cells. Biochim. Biophys. Acta 1758, 796–806.
Giacobini, E., 2003. Cholinergic function and Alzheimer's disease. Int. J. Geriatr. Psychiatry 18, S1–S5.
Greenfield, S.A., Shaw, S.G., 1982. Release of acetylcholinesterase and aminopeptidase in vivo following infusion of amphetamine into the substantia nigra. Neuroscience 7, 2883–2893.
Grimm, M.O., Tschäpe, J.A., Grimm, H.S., Zinser, E.G., Hartmann, T., 2006. Altered membrane fluidity and lipid raft composition in presenilin-deficient cells. Acta Neurol. Scand. Suppl. 185, 27–32.
Grisaru, D., Sternfeld, M., Eldor, A., Glick, D., Soreq, H., 1999. Structural roles of acetylcholinesterase variants in biology and pathology. Eur. J. Biochem. 264, 672–686.
Guardia-Laguarta, C., Pera, M., Clarimón, J., Molinuevo, J.L., Sánchez-Valle, R., Lladó, A., Coma, M., Gómez-Isla, T., Blesa, R., Ferrer, I., Lleó, A., 2009. Mild cholesterol depletion reduces amyloid-beta production by impairing APP trafficking to the cell surface. J. Neurochem. 110, 220–230.
Haass, C., Kaether, C., Thinakaran, G., Sisodia, S., 2012. Trafficking and proteolytic processing of APP. Cold Spring Harb. Perspect. Med. 2, a006270.
Harris, B., Pereira, I., Parkin, E., 2009. Targeting ADAM10 to lipid rafts in neuroblastoma SH-SY5Y cells impairs amyloidogenic processing of the amyloid precursor protein. Brain Res. 1296, 203–215.
Hattori, C., Asai, M., Onishi, H., Sasagawa, N., Hashimoto, Y., Saido, T.C., Maruyama, K., Mizutani, S., Ishiura, S., 2006. BACE1 interacts with lipid raft proteins. J. Neurosci. Res. 84, 912–917.
Henderson, Z., Matto, N., John, D., Nalivaeva, N.N., Turner, A.J., 2012. Co-localization of PRiMA with acetylcholinesterase in cholinergic neurons of rat brain: an immunocytochemical study. Brain Res. 1344, 34–42.
Hicks, D., John, D., Makova, N.Z., Henderson, Z., Nalivaeva, N.N., Turner, A.J., 2011. Membrane targeting, shedding and protein interactions of brain acetylcholinesterase. J. Neurochem. 116, 742–746.
Hicks, D.A., Makova, N.Z., Nalivaeva, N.N., Turner, A.J., 2013. Characterisation of acetylcholinesterase release from neuronal cells. Chem. Biol. Interact 203, 302–308.
Hicks, D.A., Nalivaeva, N.N., Turner, A.J., 2012. Lipid rafts and Alzheimer's disease: protein-lipid interactions and perturbation of signaling. Front. Physiol. 3, 189.
Hinz, B., Ramer, R., Eichele, K., Weinzierl, U., Brune, K., 2004. R(+)-methanandamide-induced cyclooxygenase-2 expression in H4 human neuroglioma cells: possible involvement of membrane lipid rafts. Biochem. Biophys. Res. Commun. 324, 621–626.
Hu, W., Gray, N.W., Brimijoin, S., 2009. Amyloid-beta alters trafficking of internalized acetylcholinesterase and dextran. Int. J. Physiol. Pathophysiol. Pharmacol. 1, 15–24.
Huang, X., Lee, B., Johnson, G., Naleway, J., Guzikowski, A., Dai, W., Darzynkiewicz, Z., 2005. Novel assay utilizing fluorochrome-tagged physostigmine (Ph-F) to in situ detect active acetylcholinesterase (AChE) induced during apoptosis. Cell Cycle 4, 140–147.
Kaether, C., Haass, C., Steiner, H., 2006. Assembly, trafficking and function of gamma-secretase. Neurodegener. Dis. 3, 275–283.
Lanz, T.A., Himes, C.S., Pallante, G., Adams, L., Yamazaki, S., Amore, B., Merchant, K.M., 2003. The gamma-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester reduces A beta levels in vivo in plasma and cerebrospinal fluid in young (plaque-free) and aged (plaque-bearing) Tg2576 mice. J. Pharmacol. Exp. Ther. 305, 864–871.
Leung, K.W., Xie, H.Q., Chen, V.P., Mok, M.K., Chu, G.K., Choi, R.C., Tsim, K.W., 2009. Restricted localization of proline-rich membrane anchor (PRiMA) of globular form acetylcholinesterase at the neuromuscular junctions—contribution and expression from motor neurons. FEBS J. 276, 3031–3042.
Lleó, A., 2007. Current therapeutic options for Alzheimer's disease. Curr. Genomics 8, 550–558.
Lleó, A., Saura, C.A., 2011. γ-Secretase substrates and their implications for drug development in Alzheimer's disease. Curr. Top. Med. Chem. 11, 1513–1527.
Manders, E.M., Verbeek, F.J., Aten, J.A., 1993. Measurement of co-localization of objects in dual-colour confocal images. J. Microsc. 169, 375–382.
Maruyama, K., Tomita, T., Shinozaki, K., Kume, H., Asada, H., Saido, T.C., Ishiura, S., Iwatsubo, T., Obata, K., 1996. Familial Alzheimer's disease-linked mutations at Val717 of amyloid precursor protein are specific for the increased secretion of A beta 42(43). Biochem. Biophys. Res. Commun. 227, 730–735.
Massoulié, J., 2002. The origin of the molecular diversity and functional anchoring of cholinesterases. Neurosignals 11, 130–143.
Meshorer, E., Toiber, D., Zurel, D., Sahly, I., Dori, A., Cagnano, E., Schreiber, L., Grisaru, D., Tronche, F., Soreq, H., 2004. Combinatorial complexity of 5' alternative acetylcholinesterase transcripts and protein products. J. Biol. Chem. 279, 29740–29751.
Noureddine, H., Schmitt, C., Liu, W., Garbay, C., Massoulié, J., Bon, S., 2007. Assembly of acetylcholinesterase tetramers by peptidic motifs from the proline-rich membrane anchor, PRiMA: competition between degradation and secretion pathways of heteromeric complexes. J. Biol. Chem. 282, 3487–3497.
Parkin, E.T., Hussain, I., Karran, E.H., Turner, A.J., Hooper, N.M., 1999. Characterization of detergent-insoluble complexes containing the familial Alzheimer's disease-associated presenilins. J. Neurochem. 72, 1534–1543.
Parkin, E.T., Hussain, I., Turner, A.J., Hooper, N.M., 1997. The amyloid precursor protein is not enriched in caveolae-like, detergent-insoluble membrane microdomains. J. Neurochem. 69, 2179–2188.
Perrier, A.L., Massoulié, J., Krejci, E., 2002. PRiMA: the membrane anchor of acetylcholinesterase in the brain. Neuron 33, 275–285.
Perrier, N.A., Khérif, S., Perrier, A.L., Dumas, S., Mallet, J., Massoulié, J., 2003. Expression of PRiMA in the mouse brain: membrane anchoring and accumulation of 'tailed' acetylcholinesterase. Eur. J. Neurosci. 18, 1837–1847.
Perry, E.K., Perry, R.H., Blessed, G., Tomlinson, B.E., 1977. Necropsy evidence of central cholinergic deficits in senile dementia. Lancet 1, 189.
Riddell, D.R., Christie, G., Hussain, I., Dingwall, C., 2001. Compartmentalization of beta-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts. Curr. Biol. 11, 1288–1293.
Rochette, M.J., Murphy, M.P., 2002. Gamma-secretase: substrates and inhibitors. Mol. Neurobiol. 26, 81–95.
Sáez-Valero, J., Sberna, G., McLean, C.A., Small, D.H., 1999. Molecular isoform distribution and glycosylation of acetylcholinesterase are altered in brain and cerebrospinal fluid of patients with Alzheimer's disease. J. Neurochem. 72, 1600–1608.
Sáez-Valero, J., Tornel, P.L., Muñoz-Delgado, E., Vidal, C.J., 1993. Amphiphilic and hydrophilic forms of acetyl- and butyrylcholinesterase in human brain. J. Neurosci. Res. 35, 678–689.
Saura, C.A., Choi, S.Y., Beglopoulos, V., Malkani, S., Zhang, D., Shankaranarayana Rao, B.S., Chattarji, S., Kelleher 3rd, R.J., Kandel, E.R., Duff, K., Kirkwood, A., Shen, J., 2004. Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. Neuron 42, 23–36.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.
Silveyra, M.X., Evin, G., Montenegro, M.F., Vidal, C.J., Martínez, S., Culvenor, J.G., Sáez-Valero, J., 2008. Presenilin 1 interacts with acetylcholinesterase and alters its enzymatic activity and glycosylation. Mol. Cell. Biol. 28, 2908–2919.
Silveyra, M.X., García-Ayllón, M.S., Serra-Basante, C., Mazzoni, V., García-Gutierrez, M.S., Manzanares, J., Culvenor, J.G., Sáez-Valero, J., 2012. Changes in acetylcholinesterase expression are associated with altered presenilin-1 levels. Neurobiol. Aging 33, 627.e27–627.e37.
Small, D.H., Michaelson, S., Sberna, G., 1996. Non-classical actions of cholinesterases: role in cellular differentiation, tumorigenesis and Alzheimer's disease. Neurochem. Int. 28, 453–483.
Soreq, H., Seidman, S., 2001. Acetylcholinesterase—new roles for an old actor. Nat. Rev. Neurosci. 2, 294–302.
Struhl, G., Adachi, A., 2000. Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. Mol. Cell. 6, 625–636.
Suzuki, K., Hayashi, Y., Nakahara, S., Kumazaki, H., Prox, J., Horiuchi, K., Zeng, M., Tanimura, S., Nishiyama, Y., Osawa, S., Sehara-Fujisawa, A., Saftig, P., Yokoshima, S., Fukuyama, T., Matsuki, N., Koyama, R., Tomita, T., Iwatsubo, T., 2012. Activity-dependent proteolytic cleavage of neuroligin-1. Neuron 76, 410–422.
Thinakaran, G., Koo, E.H., 2008. Amyloid precursor protein trafficking, processing, and function. J. Biol. Chem. 283, 29615–29619.
Vetrivel, K.S., Cheng, H., Lin, W., Sakurai, T., Li, T., Nukina, N., Wong, P.C., Xu, H., Thinakaran, G., 2004. Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J. Biol. Chem. 279, 44945–44954.

Volonte, D., Galbiati, F., Li, S., Nishiyama, K., Okamoto, T., Lisanti, M.P., 1999. Flotillins/cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with caveolins in vivo. Characterization and epitope-mapping of a novel flotillin-1 monoclonal antibody probe. J. Biol. Chem. 274, 12702–12709.

Wada, S., Morishima-Kawashima, M., Qi, Y., Misono, H., Shimada, Y., Ohno-Iwashita, Y., Ihara, Y., 2003. Gamma-secretase activity is present in rafts but is not cholesterol-dependent. Biochemistry 42, 13977–13986.

Wagner, S.L., Tanzi, R.E., Mobley, W.C., Galasko, D., 2012. Potential use of γ-secretase modulators in the treatment of Alzheimer disease. Arch. Neurol. 69, 1255–1258.

Williamson, R., Sutherland, C., 2011. Neuronal membranes are key to the pathogenesis of Alzheimer's disease: the role of both raft and non-raft membrane domains. Curr. Alzheimer Res. 8, 213–221.

Wolfe, M.S., 2008. Gamma-secretase inhibition and modulation for Alzheimer's disease. Curr. Alzheimer Res. 5, 158–164.

Xia, W., Zhang, J., Kholodenko, D., Citron, M., Podlisny, M.B., Teplow, D.B., Haass, C., Seubert, P., Koo, E.H., Selkoe, D.J., 1997. Enhanced production and oligomerization of the 42-residue amyloid beta-protein by Chinese hamster ovary cells stably expressing mutant presenilins. J. Biol. Chem. 272, 7977–7982.

Xie, H.Q., Liang, D., Leung, K.W., Chen, V.P., Zhu, K.Y., Chan, W.K., Choi, R.C., Massoulié, J., Tsim, K.W., 2010a. Targeting acetylcholinesterase to membrane rafts: a function mediated by the proline-rich membrane anchor (PRiMA) in neurons. J. Biol. Chem. 285, 11537–11546.

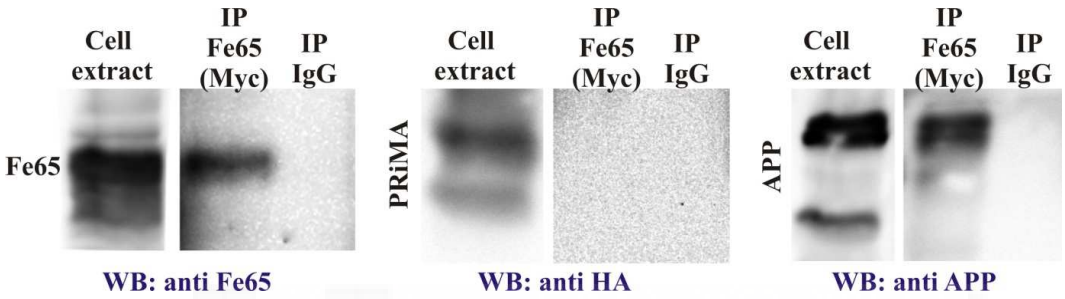
Xie, H.Q., Leung, K.W., Chen, V.P., Chan, G.K., Xu, S.L., Guo, A.J., Zhu, K.Y., Zheng, K.Y., Bi, C.W., Zhan, J.Y., Chan, W.K., Choi, R.C., Tsim, K.W., 2010b. PRiMA directs a restricted localization of tetrameric AChE at synapses. Chem. Biol. Interact 187, 78–83.

Yang, L., He, H.Y., Zhang, X.J., 2002. Increased expression of intranuclear AChE involved in apoptosis of SK-N-SH cells. Neurosci. Res. 42, 261–268.

Yu, H., Saura, C.A., Choi, S.Y., Sun, L.D., Yang, X., Handler, M., Kawarabayashi, T., Younkin, L., Fedeles, B., Wilson, M.A., Younkin, S., Kandel, E.R., Kirkwood, A., Shen, J., 2001. APP processing and synaptic plasticity in presenilin-1 conditional knockout mice. Neuron 31, 713–726.

SUPPLEMENTARY DATA

In our CHO cellular model we attempt to co-immunoprecipitate PRiMA with Fe65 in cells with and without Fe65 over-expression. However, we failed to demonstrate an interaction between Fe65 and PRiMA.



Supplementary Figure: co-immunoprecipitation experiments of Fe65, APP and HA proteins. CHO PS70 cells (over-expressing wild-type human PS1 and APP; see manuscript for details) were transfected with 2 µg of PRiMA cDNA and 2 µg of Fe65 cDNA with a C-terminal Myc tag (Lleo et al 2003). Protein cellular extracts were immunoprecipitated with an anti-Myc antibody (Abcam). Precipitated proteins [IP Fe65 (Myc)] were immunoblotted with an anti-Fe65 antibody (Millipore) to positively demonstrated immunoprecipitation, and with the anti-HA to check whether PRiMA co-immunoprecipitate. Precipitated proteins were also immunoblotted with the anti-APP antibody to positively check the efficiency of Fe65 to co-immunoprecipitate App, a well-known interacting protein (the blot shows the pull down of the full-length APP band in Fe65 immunoprecipitates). Extracts incubated with protein A-Sepharose coupled with a non-specific rabbit IgG (IP IgG), were analyzed in parallel as negative controls.

CHAPTER 4: DISCUSSION

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Although this chapter represents the general discussion of this Thesis, I would like to clarify that the intention is not to repeat the discussion of each articles presented here and published; although, obviously, the discussion of our result will result inevitably reiterative in some extension. Rather, the goal is to give a generic idea of the results, underlying the notions or hypotheses that, according to our point of view, are consider more relevant. This discussion is also an opportunity to speculate about the interpretation of our results. Even if the three different manuscripts contained in this Thesis correspond to different and independent objectives, they, altogether, try to explore the implication of AChE and PS1 cross-talk in non-disease and AD conditions.

Brains from AD patients show several distinct neuropathological features, including extracellular Aβ peptide-containing plaques, intracellular NFT of abnormally phosphorylated tau, astrocytic gliosis, reactive micoglia, inflammation, as well degeneration of cholinergic neurons of the basal forebrain. Interestingly, many results indicate a link between cholinergic mechanisms and the pathogenic events that characterize AD, notably in relation to Aβ. There are, in fact, several interactions between the cholinergic system and Aβ, not only related with the well described process of Aβ plaques depositions within the basal cholinergic forebrain nuclei (Arendt et al 1985, Arendt et al 1988).

Different studies, for instance, show that the soluble forms of Aβ, rather than the insoluble, act as negative modulators of ACh synthesis and release and interfere with normal signalling mediated by muscarinic and nicotinic receptors (Hartley et al 1999, McLean et al 1999). Indeed, interactions between nicotinic receptors (nAChR) and Aβ, where Aβ blocks the nicotinic current, have been physiologically confirmed (Pettit et al 2001). Consequently, Aβ influences

neurotransmitter release from non-cholinergic neurons that normally respond to cholinergic input. These results can in part explain the significant hypofunction of the basal forebrain cholinergic system in AD.

Reversely, ACh is known to promote the non-amyloidogenic processing of APP (Roberson & Harrell 1997, Rossner et al 1998) and reduce tau phosphorylation by reducing the activity of GSK3β (Zhang et al 2014). In normal condition most of APP is processing through the non-amyloidogenic pathway; thus generation of Aβ by brain cells of healthy people remains constitutively low.

We can presume the existence of a mechanism whereby normal cholinergic innervation participates in the regulation of the non-amyloidogenic processing of APP via the α-secretase pathway, whereas, in turn, the amyloidogenic Aβ production depress the activity of cholinergic neurons. Thus, a shift between these two activities may be a key factor in cholinergic signalling disruption and Aβ accumulation.

Also in this context, it has been shown that AChE interacts with Aβ, rendering Aβ more neurotoxic (Inestrosa et al 1996) and, moreover, that AChE it is detectable in the amyloid plaques, where its enzymatic properties are altered (Alvarez et al 1998, Geula & Mesulam 1989, Inestrosa et al 1996). This discovery, together with the evidence of an overall decrease of AChE activity in AD brain, may explain the high susceptibility of cholinergic neurons to injury by Aβ, but also indicate the participation of AChE in the development of the Alzheimer pathology by interacting with Aβ.

In this scenario, our lab, as well as many others research groups, have studied and described the alteration of AChE in AD. Alterations in the contribution of AChE molecular form levels and glycosylation have been identified in CSF and brain from AD cases, as compared with non-disease subjects (Saez-Valero et al 2000b, Saez-Valero et al 1997, Saez-

Valero et al 1999). In particular, lighter forms of AChE (G1+G2), with an abnormal glycosylation pattern, resulted increased. In the same way, treatments with Aβ peptide *in vitro* were inducing an increase of AChE (Hu et al 2003, Melo et al 2003, Saez-Valero et al 2003, Sberna et al 1997). The same results were confirmed *in vivo,* in a mouse model overexpressing Aβ; ones again the G1 and G2 forms of AChE were increased (Sberna et al 1998). Altogether these results suggested the importance to consider Aβ as a direct cause of AChE alterations in the AD brain, in terms of expression, maturation and glycosylation. In fact, glycosylation controls the correct folding and final localization of many proteins, including AChE.

With the goal to continue exploring the cholinergic-amyloid interrelationship, our group identified AChE as a PS1-interacting protein, the catalytic subunit of the γ-secretase complex responsible for Aβ production (Silveyra et al 2008). This interaction, that doesn't depend on the PAS, the AB interacting domain of AChE, take place in the adult and in the embryonic brain, as well as in the normal and AD brain.

Following this line of research, in our manuscript published in the *Journal of Alzheimer's Disease* we further analyzed the interaction between AChE and PS1.

First of all we tested if the interaction with PS1 was mediated by a specific variant or molecular form of AChE, in order to better understand the mechanism. In fact, AChE possesses a complex structural polymorphism. In the brain the major variant is the AChE-T, which is mainly presented as tetrameric forms, but also as monomers and dimmers. The minor variant AChE-R is expressed as soluble monomers. Co-immunoprecipitation, with specific antibodies against the AChE-T and –R, and ultracentrifugation analysis, revealed that all AChE species interact with PS1 independently of their state of

oligomerization. A previous work from our lab has shown that AChE exerts a modulatory effect on PS1 levels. In fact, AChE knockdown with siRNA decreased cellular PS1 levels; whereas AChE over-expression exerted an opposing effect (Silveyra et al 2012). In our study, we also demonstrated that over-expression of the different AChE variants, as monomers (–T and –R) and tetramers (T+PRiMA), are able to increase PS1 level. Interestingly, the modulation of PS1 protein seems to be stronger in presence of the subunit PRiMA. PRiMA is the protein that induce tetramerization of AChE-T subunits and, moreover, restrict its localization at the plasmatic membrane where will exert its catalytic activity. Probably PRiMA guiding AChE distribution inside the cell, increase the physical disposition of AChE to interact with PS1.

Even if the domain of interaction of AChE with PS1 is unknown, has to be excluded the intermediation of the PAS domain, the C-terminal (exclusive of each variant) and of the catalytic domain of AChE. From our results, it seems that the modulatory capacity of AChE is exerted by a mechanism independent of its enzymatic activity. In fact, the overexpression of AChE inactive mutants also resulted in a PS1 protein increase.

The possibility that AChE/γ-secretase interaction leads to the modulation of APP cleavage has been recently suggested (Niu et al 2012). In our study we confirmed this possibility. We show that AChE, by a mechanism independent of its catalytic activity, interacts with PS1, probably within the active γ-secretase complex and prevents its catalytic function. Indeed, in both cellular extracts and membrane preparations we demonstrate a decrease in the γ-secretase activity, as consequence of soluble AChE treatment. The mechanism of interaction and inhibition of AChE on PS1 activity is still unclear, but we hypothesize that this inhibitory effect could explain the AChE triggered increase in PS1 messenger and protein levels. In fact, up-regulation in

reaction to inhibition is a recognized phenomenon documented for several enzymes, as a mechanism attempting to re-stabilize the deficit of activity. An example is the accumulation of AChE in CSF of AD patient after exposure to AChE inhibitors (AChE-I) (Garcia-Ayllon et al 2007).

The role of AChE as "PS1 modulator" is a new way in the knowledge of the functional cross-talk between AChE/cholinergic system and APP processing.

Currently, the treatment of AD is largely based on AChE-Is which provide a temporary increase of ACh disposition and symptomatic relief. AChE-Is are able to modulate and decrease Aβ generation, not only interfering with PS1 (Silveyra et al 2012), but also activating the non-amyloidogenic pathway or inhibiting the β-secretase activity (Fu et al 2008, Racchi et al 2004). However, the positive effects of AChE-Is therapy on APP processing decrease after long term inhibition. Again, long-term treatment with AChE-Is results in a significant up-regulation of AChE protein levels in CSF (Darreh-Shori & Soininen 2010, Garcia-Ayllon et al 2007). This increased pool of AChE probably interacts with Aβ and increases its fibrillation and toxicity. Also, AChE up-regulation in response to inhibition is followed by PS1 increase (Silveyra et al 2012). An increase of AChE may block γ-secretase activity and an increase of PS1 levels may result in an enhancement of Aβ generation. Interestingly, AChE up-regulation occurs after days/weeks of maintain inhibition, but PS1 up-regulation appears rapidly. In this complex scenario, Aβ will also induce an increase of AChE (Sberna et al 1998), which in turn binds the amyloid core closing an aberrant loop. In this way Aβ, PS1 and AChE could establish a toxic triad.

Moreover, as explain previously in this Thesis, PS1 may have other functions inside the cell, independently of its catalytic activity. Thus, the possibility that AChE interferes with other roles of PS1 has not to be excluded. Enzymatically inactive species of AChE with

potential physiological significance have been described (Chatel et al 1993, Dori et al 2005, Grisaru et al 2006, Sternfeld et al 1998).

AChE pattern expression, as stated throughout the Thesis, seems to be highly regulated spatially and temporally (Drews 1975). Furthermore, AChE is express in cholinergic and non-cholinergic neurons; as well as in non-neuronal tissues, like meninges (Razon et al 1984), blood vessel endothelium (Kasa et al 1991) and glia (Keller et al 2001). To increase the complexity of this enzyme, a number of non-catalytic proteins share high sequence similarity with AChE. Most of these proteins, with cholinesterase-like domains, are structural proteins expressed during embryogenesis, before the formation of the cholinergic synapses and are also present in non-cholinergic tissues (Drews 1975, Krejci et al 1991). In these adhesion proteins the catalytic capacity has been lost during evolution, and the physiological functionality is carried through protein-protein interactions, exerting structural roles. The structural homologies between AChE and these adhesion proteins, suggests that AChE can act as a structural protein.

In the data contained in the manuscript published in the *Journal of Molecular Neuroscience*, we evaluated the level of expression of AChE and its activity levels, in frontal cortex extracts from AD and ND cases. Accordingly with many previous reports the AChE activity decreases. In contrast the general AChE protein content is preserved. These results confirm the presence of an inactive pool of AChE.

The existence of an inactive pool of AChE was firstly demonstrated in primary culture of chicken myotubes by Rotundo and co-workers (Rotundo et al 1989). They found that most of the AChE polypeptide newly synthesized remains catalytically inactive and is rapidly degraded in non-lysosomal compartments. A large AChE inactive pool was later proved in chicken brain *in vivo* (Chatel et al 1993). Recently, our group also reported the existence of an inactive

pool of AChE in the human CSF (Garcia-Ayllon et al 2007). Interestingly, this unexpectedly large pool of non-catalytic AChE was unresponsive to long-term treatment. Probably it represents a pool unrelated with the previously commented phenomenon of feed-back up-regulation.

To date, contrasted reports indicated changes in expression of the active species of AChE in AD. In fact, it has been demonstrated a decrease in the major form G4, while the light forms, G1 and G2, are relatively preserved or increased (Arendt et al 1992, Atack et al 1983, Fishman et al 1986, Saez-Valero et al 1999). Curiously, the G1 form of AChE, increased in AD, corresponds to the main pool of AChE normally present during development of the embryo brain. During this period, AChE acts in many others non-cholinergic functions, improving growing and cellular contacts. This non-cholinergic role could be conducted by catalytic and non-catalytic subunits.

Perhaps, the increase of AChE lighter forms in the pathogenic brain could indicate a "dedifferentiation" state, where the regression to the developmental form of AChE can exert a "neuroprotective" roles (Layer 1995).

In this context, Soreq and co-workers shown, *in vitro* and *in vivo*, that the minor AChE–R variant exerts an opposite action respect to the major AChE–T, decreasing the fibrillation and the toxicity of Aβ during plaques formation (Berson et al 2008). To complicate the interpretation of this complex scenario, AChE-I also promotes changes in the proportion of AChE variants and molecular forms. AChE-Is in fact, reverse the AD-induced decrease in AChE protein levels into an increase (Darreh-Shori et al 2002, Darreh-Shori et al 2004). In particular, using the immunoblot technique, it was found that long-term tacrine treatment induces an increase of both AChE-T and AChE-R in the CSF (Darreh-Shori et al 2004), while rivastigmine treatment caused a selective and mild up-regulation of the AChE-R variant

(Darreh-Shori et al 2002). This change in AChE-R is related with a better cognitive performance, in agreement with previous reports where this variant demonstrate to exert neuroprotective effects (Sternfeld et al 2000, Darreh-Shori et al 2006).

To date, are still unclear the levels of the different AChE variants in AD brain. For this reason additional studies are mandatory, in order to understand the specific physiological functions and to design more effective and specific therapies.

In the manuscript published in the journal *Neurobiology of Aging*, we have considered PRiMA a possible substrate for PS1/γ-secretase. We have been able to demonstrate that PS1/γ-secretase processes PRiMA after a previous cleavage of the linked-AChE subunit by α- or β-secretase. We have also characterized an intracellular PRiMA fragment (ICD) of ~14 kDa which translocates into the nucleus. We have failed to detect the putative PRiMA fragment in brain extracts, probably due to its low content and very short half-life, commonly to other ICDs generated by γ-secretase such as APP (Cupers et al 2001). At the present, the nuclear function of the PRiMA ICD has not been studied. The possibility that PRiMA fragment may trigger biological activities such as modulation of gene transcription is not excluded.

Similarly, whether neuronal activity or ligand-binding regulates PS1-mediated AChE processing, and under which biological conditions, remain to be determined. As the soluble APPα fragment, AChE release is enhanced by muscarinic receptor activation (Hicks et al 2013) and by Ca2+ influx from nicotinic receptors (Hicks et al 2011, Hicks et al 2013). The findings that Aβ disrupts the muscarinic and nicotinic receptors signaling, lead us to consider the possibility that Aβ exert a modulation on the cholinergic system changing the cell signalling and additionally AChE release. Also in this regard, it has been demonstrated that Aβ treatment increases AChE levels in the neuron-like N1E.115

neuroblastoma cell by reducing AChE degradation and surface shedding (Hu et al 2003). Furthermore, a proteolytic processing of neuroligin-1 by secretases has been described (Suzuki et al 2012). The processing of neuroligin-1, a protein with a large extracellular domain homolog to AChE, is regulated by synaptic NMDA receptor activation or interaction with soluble neurexin ligands. Thus, we can speculate a ligand-binding, or activity dependent, control of AChE processing by PS1.

Finally, the localization of AChE into membrane lipid rafts, determined by PRiMA (Xie et al 2010), results to be of particular interest taking in consideration that lipid rafts are probably the subcellular site where amyloidogenic processing predominates. In fact, APP, BACE I and γ-secretase/PS1 are present in rafts [for a revision see (Hicks et al 2012)]. We have been able to describe the co-localization of AChE with PS1 within lipid rafts. This physical co-localization, could be the reason of the favoured interaction between AChE G4/PRiMA and PS1 (demonstrated in the first article). On the other hand, PS1 co-precipitates mature PRiMA, fully glycosylated, but also immature protein, suggesting that the interaction between the two proteins, AChE/PRiMA and PS1, could be also an early event. Thus, PS1 may exert a role in AChE/PRiMA maturation such as glycosylation (Silveyra et al 2008) and influence trafficking to the plasma membrane. Therefore, the increase amount of G4 AChE/PRiMA in membrane rafts isolated from the cortex of *PS1* cKO mice probably is due to the impaired proteolytic events, and the lack of PS1 trafficking regulation.

In conclusion, our results have the intention to bring some clues in the intricate relationship between cholinergic and amyloid pathways. Even if we cannot present a more complete picture of the molecular cross-talk between AChE and PS1, we try to solve some questions and to give new inputs. The desirable outcome is the

development of new therapeutic strategies, more adapted to the complex pathology of Alzheimer.

Due to the results obtained in the presented works, in this chapter, I resume some of the mandatory experiments arising from our results.

First of all, we consider necessarry a better understanding of the complex cholinergic/amyloid crosstalk to optimize the current therapies based on AChE pharmacological interventions, as well to develop new strategy to inhibit γ-secretase activity. The possibility that inhibition of PS1 by AChE may initiate a feedback process that leads to PS1 up-regulation, should be take in consideration during therapy based on γ-secretase inhibition. Thus, it's important to clarify the mechanism that regulates γ-secretase activity block consequently the AChE binding. In this context, it is mandatory to establish which domain, inside AChE, is responsible of its interaction with PS1. To date, we only know that this domain is present in all variants of AChE and that doesn't depend on the PAS domain or the active gorge. To know that, it is also important in order to interfere in this interaction. Of course, the importance of the AChE/PS1 cross-talk has to be proved *in vivo* in order to implement this knowledge in a physiological and pathological conditions.

Also, our results show that both soluble and PRiMA-banded form of AChE interacts with PS1 and both of them are able to interfere with its activity and expression. In turn, we show that PS1 processes PRiMA after the initial cleavage mediated by α- or β-secretase. Indeed, remain to be elucidated if the soluble AChE, released by the sequential cleavages of the α- or β-secretases, blocks or interferes with PS1 and γ-secretase activity. In the same way, it's mandatory to establish the role or roles of the PRiMA ICD in the nucleus, as well as its specific cytoplasm/nucleus trans-acting adaptor protein. The possibility that the PRiMA segment, originates after processing by secretases,

participates in the regulation of gene transcription, is a possibility that deserves thoroughly investigation.

Another question that arises from our data is whether AChE/PRiMA complex is physiologically a "common" substrate for secretases, or only regulated under specific physiological conditions. In this regards, it is interesting to explore which are the stimuli that induce this processing. Therefore, more research is needed in order to better characterize the process of AChE shedding in neurons, particularly using primary neurons and animal models.

Finally, the large pool of inactive AChE present in AD brain is intriguing and little information is available on its role and distribution in normal and pathological conditions.

Currently, we are complementing our biochemical study of human brain AChE variants by blotting the samples with different AChE antibodies and studying the levels of the different AChE mRNAs. In this work we are analysing the tailed (AChE-T) and read-through (AChE-R) variants generated by alternative splicing at the 3' end and the recently characterized N-extended AChE variants generated by the 5' regulation. To date, very few studies have addressed the levels of AChE splicing variants in human brain, since the AChE-T cholinergic variant represents the major AChE specie. The significance of an increase in the minor AChE species is controversial, as the relative increase is compared in absolute terms with the major cholinergic form. However, since particular forms or variants may have specific functions, a variation in a AChE forms (with little impact on cholinergic equilibrium) could have impact at functional level causing biological consequences.

In this context, also is relevant to establish the subcellular localization of this new inactive pool of AChE in order to define the potencial interaction with PS1.

CHAPTER 5: CONCLUSIONS

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The results discussed in this Thesis are summaries in the following points:

1. The AChE protein is able to modulate levels of PS1, the catalytic component of γ-secretase, via a non-cholinergic mechanism that doesn't depend of the AChE catalytic activity. In a cellular model, the over-expression of the splicing variant AChE-R or AChE-T, or of their respective inactive mutants, triggers the increase in PS1 protein level.

2. The AChE specie capable of triggering the biggest increase in PS1 level is a complex of AChE with the membrane anchoring subunit proline-rich membrane anchor (PRiMA), which restricts the localization of the resulting AChE tetramer to the plasma membrane.

3. The possible mechanism by which AChE modulates PS1 expression level depends on its ability to decrease γ-secretase activity.

4. The decrease in AChE activity in human AD brain is not paralleled by changes in AChE protein immunoreactivity, indicating the existence of a prominent pool of enzymatically inactive AChE.

5. We demonstrate that PS1/γ-secretase can participate in the processing of the cholinergic AChE, cleaving PRiMA subunit.

6. Cleavage of PRiMA by γ-secretase results in a C-terminal PRiMA fragment, which translocates into the nucleus.

7. AChE co-localizes with PS1 within the lipid rafts. The preserved level of the raft-residing AChE-PRiMA in a PS1 conditional knockout mouse maybe is due to the impaired proteolytic events caused by PS1 decreased expression.

De los resultados presentados en la presente Memoria de Tesis se extraen las siguientes conclusiones:

1. AChE es capaz de modular los niveles de PS1, el componente catalítico del complejo γ-secretasa, mediante un mecanismo independiente de su actividad enzimática. En modelos celulares la sobre-expresión de las variantes AChE-R o AChE-T, o de sus respectivos mutantes carentes de actividad enzimática, provoca un aumento en los niveles de PS1.

2. La forma de AChE capaz de promover el mayor incremento en los niveles de PS1 es la forma colinérgica, un tetrámero de subunidades catalíticas anclado a la membrana por la subunidad estructural PRiMA (del inglés *proline-richmembrane anchor*), que determina su localización en la cara externa de la membrana plasmática.

3. El posible mecanismo por el que AChE modula los niveles de expresión de PS1 es dependiente de su capacidad para disminuir o inhibir la actividad γ-secretasa.

4. El descenso en la actividad AChE en cerebro humano de sujetos con la enfermedad de Alzheimer no se corresponde con cambios en los niveles de inmunoreactividad de la proteína AChE, lo que indica que en el cerebro patológico existe una prominente cantidad de AChE enzimáticamente inactiva.

5. Se demuestra que PS1/γ-secretasa puede participar en el procesamiento de la variante colinérgica de AChE mediante el procesamiento de la subunidad de anclaje PRiMA.

6. El corte de PRiMA por el complejo γ-secretase produce un fragmento C-terminal que se transloca al núcleo.

7. AChE colocaliza con PS1 en las regiones lipídicas de membrana denominadas lipid rafts. En ratones silentes condicionales de PS1 los niveles de AChE-PRiMA en el raft resultan más elevados que en los ratones no mutantes, debido probablemente a la afectación de los procesos proteolíticos provocados por el descenso en los niveles de PS1.

In addition to the articles presented in this Thesis, I would like to mention that I am the second and third author of two manuscripts, which have been pubblished during my PhD.

The articles are the following:

García-Ayllón MS, **Campanari ML**, Brinkmalm G, Rábano A, Alom J, Saura CA, Andreasen N, Blennow K and Sáez-Valero J. **CSF Presenilin-1 complexes are increased in Alzheimer's disease.** Acta Neuropathologica Communications 2013, 1:46.

Bi CWC, Luk WKW, **Campanari ML**, Liu YH, Xu L, Lau KM, Xu ML, Choi RCY, Sáez-Valero J, Tsim KWK. **Quantification of the Transcripts Encoding Different Forms of AChE in Various Cell Types: Real-Time PCR Coupled with Standards in Revealing the Copy Number.** J Mol Neurosci. 2014 Jan 3. 10.1007/s12031-013-0210-6.

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CHAPTER 6: BIBLIOGRAFY

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Abraham CR, Selkoe DJ, Potter H. 1988. Immunochemical identification of the serine protease inhibitor alpha 1-antichymotrypsin in the brain amyloid deposits of Alzheimer's disease. *Cell* 52: 487-501

Alonso-Nanclares L, Merino-Serrais P, Gonzalez S, DeFelipe J. 2013. Synaptic changes in the dentate gyrus of APP/PS1 transgenic mice revealed by electron microscopy. *Journal of neuropathology and experimental neurology* 72: 386-95

Alonso AC, Zaidi T, Grundke-Iqbal I, Iqbal K. 1994. Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America* 91: 5562-6

Alvarez A, Alarcon R, Opazo C, Campos EO, Munoz FJ, et al. 1998. Stable complexes involving acetylcholinesterase and amyloid-beta peptide change the biochemical properties of the enzyme and increase the neurotoxicity of Alzheimer's fibrils. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18: 3213-23

Alvarez A, Bronfman F, Perez CA, Vicente M, Garrido J, Inestrosa NC. 1995. Acetylcholinesterase, a senile plaque component, affects the fibrillogenesis of amyloid-beta-peptides. *Neuroscience letters* 201: 49-52

Alzheimer A, Stelzmann RA, Schnitzlein HN, Murtagh FR. 1995. An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". *Clin Anat* 8: 429-31

Allinson TM, Parkin ET, Turner AJ, Hooper NM. 2003. ADAMs family members as amyloid precursor protein alpha-secretases. *Journal of neuroscience research* 74: 342-52

Annaert W, De Strooper B. 1999. Presenilins: molecular switches between proteolysis and signal transduction. *Trends in neurosciences* 22: 439-43

Arendt T, Bigl V, Tennstedt A, Arendt A. 1985. Neuronal loss in different parts of the nucleus basalis is related to neuritic plaque formation in cortical target areas in Alzheimer's disease. *Neuroscience* 14: 1-14

Arendt T, Bruckner MK, Lange M, Bigl V. 1992. Changes in acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease resemble embryonic development--a study of molecular forms. *Neurochem Int* 21: 381-96

Arendt T, Taubert G, Bigl V, Arendt A. 1988. Amyloid deposition in the nucleus basalis of Meynert complex: a topographic marker for degenerating cell clusters in Alzheimer's disease. *Acta neuropathologica* 75: 226-32

Arpagaus M, Kott M, Vatsis KP, Bartels CF, La Du BN, Lockridge O. 1990. Structure of the gene for human butyrylcholinesterase. Evidence for a single copy. *Biochemistry* 29: 124-31

Atack JR, Perry EK, Bonham JR, Perry RH, Tomlinson BE, et al. 1983. Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type: selective loss of the intermediate (10S) form. *Neuroscience letters* 40: 199-204

Austin L, Berry WK. 1953. Two selective inhibitors of cholinesterase. *Biochem J* 54: 695-700

Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, Rosenfeld MG. 2002. Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. *Cell* 110: 55-67

Barthet G, Georgakopoulos A, Robakis NK. 2012. Cellular mechanisms of gamma-secretase substrate selection, processing and toxicity. *Progress in neurobiology* 98: 166-75

Bartus RT, Dean RL, 3rd, Beer B, Lippa AS. 1982. The cholinergic hypothesis of geriatric memory dysfunction. *Science (New York, N.Y.)* 217: 408-14

Beel AJ, Sanders CR. 2008. Substrate specificity of gamma-secretase and other intramembrane proteases. *Cellular and molecular life sciences : CMLS* 65: 1311-34

Berson A, Knobloch M, Hanan M, Diamant S, Sharoni M, et al. 2008. Changes in readthrough acetylcholinesterase expression modulate amyloid-beta pathology. *Brain : a journal of neurology* 131: 109-19

Billingsley ML, Kincaid RL. 1997. Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. *Biochem J* 323 (Pt 3): 577-91

Birikh KR, Sklan EH, Shoham S, Soreq H. 2003. Interaction of "readthrough" acetylcholinesterase with RACK1 and PKCbeta II correlates with intensified fear-induced conflict behavior. *Proceedings of the National Academy of Sciences of the United States of America* 100: 283-8

Blennow K, Hampel H, Weiner M, Zetterberg H. 2010. Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. *Nature reviews. Neurology* 6: 131-44

Bon S, Coussen F, Massoulie J. 1997. Quaternary associations of acetylcholinesterase. II. The polyproline attachment domain of the collagen tail. *The Journal of biological chemistry* 272: 3016-21

Boncristiano S, Calhoun ME, Kelly PH, Pfeifer M, Bondolfi L, et al. 2002. Cholinergic changes in the APP23 transgenic mouse model of cerebral amyloidosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22: 3234-43

Borenstein AR, Copenhaver CI, Mortimer JA. 2006. Early-life risk factors for Alzheimer disease. *Alzheimer disease and associated disorders* 20: 63-72

Borghi R, Piccini A, Barini E, Cirmena G, Guglielmotto M, et al. 2010. Upregulation of presenilin 1 in brains of sporadic, late-onset Alzheimer's disease. *Journal of Alzheimer's disease : JAD* 22: 771-5

Braak H, Braak E. 1991. Neuropathological stageing of Alzheimer-related changes. *Acta neuropathologica* 82: 239-59

Brou C, Logeat F, Gupta N, Bessia C, LeBail O, et al. 2000. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Molecular cell* 5: 207-16

Brunkan AL, Martinez M, Walker ES, Goate AM. 2005. Presenilin endoproteolysis is an intramolecular cleavage. *Molecular and cellular neurosciences* 29: 65-73

Bruno MA, Cuello AC. 2006. Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. *Proceedings of the National Academy of Sciences of the United States of America* 103: 6735-40

Burns A, Whitehouse P, Arendt T, Forsti H. 1997. Alzheimer's disease in senile dementia: loss of neurones in the basal forebrain. Whitehouse, P., Price, D., Struble, R., Clarke, A., Coyle, J. and Delong, M. Science (1982), 215, 1237-1239. *International journal of geriatric psychiatry* 12: 7-10

Cao X, Sudhof TC. 2001. A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science (New York, N.Y.)* 293: 115-20

Capell A, Grunberg J, Pesold B, Diehlmann A, Citron M, et al. 1998. The proteolytic fragments of the Alzheimer's disease-associated presenilin-1 form heterodimers and occur as a 100-150-kDa molecular mass complex. *The Journal of biological chemistry* 273: 3205-11

Cedazo-Minguez A, Winblad B. 2010. Biomarkers for Alzheimer's disease and other forms of dementia: clinical needs, limitations and future aspects. *Experimental gerontology* 45: 5-14

Cohen O, Reichenberg A, Perry C, Ginzberg D, Pollmacher T, et al. 2003. Endotoxin-induced changes in human working and declarative memory associate with cleavage of plasma "readthrough" acetylcholinesterase. *Journal of molecular neuroscience : MN* 21: 199-212

Cowburn RF, Popescu BO, Ankarcrona M, Dehvari N, Cedazo-Minguez A. 2007. Presenilin-mediated signal transduction. *Physiology & behavior* 92: 93-7

Cruts M, Van Broeckhoven C. 1998. Molecular genetics of Alzheimer's disease. *Ann Med* 30: 560-5

Crystal AS, Morais VA, Pierson TC, Pijak DS, Carlin D, et al. 2003. Membrane topology of gamma-secretase component PEN-2. *The Journal of biological chemistry* 278: 20117-23

Cuello AC, Bruno MA. 2007. The failure in NGF maturation and its increased degradation as the probable cause for the vulnerability of cholinergic neurons in Alzheimer's disease. *Neurochemical research* 32: 1041-5

Cuello AC, Bruno MA, Allard S, Leon W, Iulita MF. 2010. Cholinergic involvement in Alzheimer's disease. A link with NGF maturation and degradation. *Journal of molecular neuroscience : MN* 40: 230-5

Culvenor JG, Maher F, Evin G, Malchiodi-Albedi F, Cappai R, et al. 1997. Alzheimer's disease-associated presenilin 1 in neuronal cells: evidence for localization to the endoplasmic reticulum-Golgi intermediate compartment. *Journal of neuroscience research* 49: 719-31

Cummings JL. 2004. Alzheimer's disease. *The New England journal of medicine* 351: 56-67

Cupers P, Orlans I, Craessaerts K, Annaert W, De Strooper B. 2001. The amyloid precursor protein (APP)-cytoplasmic fragment generated by gamma-secretase is rapidly degraded but distributes partially in a nuclear fraction of neurones in culture. *Journal of neurochemistry* 78: 1168-78

Chatel JM, Grassi J, Frobert Y, Massoulie J, Vallette FM. 1993. Existence of an inactive pool of acetylcholinesterase in chicken brain. *Proceedings of the National Academy of Sciences of the United States of America* 90: 2476-80

Chen VP, Luk WK, Chan WK, Leung KW, Guo AJ, et al. 2011. Molecular Assembly and Biosynthesis of Acetylcholinesterase in Brain and Muscle: the Roles of t-peptide, FHB Domain, and N-linked Glycosylation. *Front Mol Neurosci* 4: 36

Chen VP, Xie HQ, Chan WK, Leung KW, Chan GK, et al. 2010. The PRiMA-linked cholinesterase tetramers are assembled from homodimers: hybrid molecules composed of acetylcholinesterase and butyrylcholinesterase dimers are up-regulated during development of chicken brain. *The Journal of biological chemistry* 285: 27265-78

Choy RW, Cheng Z, Schekman R. 2012. Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid beta (Abeta) production in the trans-Golgi network. *Proceedings of the National Academy of Sciences of the United States of America* 109: E2077-82

Chyung JH, Raper DM, Selkoe DJ. 2005. Gamma-secretase exists on the plasma membrane as an intact complex that accepts substrates and effects intramembrane cleavage. *The Journal of biological chemistry* 280: 4383-92

Darreh-Shori T, Almkvist O, Guan ZZ, Garlind A, Strandberg B, et al. 2002. Sustained cholinesterase inhibition in AD patients receiving rivastigmine for 12 months. *Neurology* 59: 563-72

Darreh-Shori T, Hellstrom-Lindahl E, Flores-Flores C, Guan ZZ, Soreq H, Nordberg A. 2004. Long-lasting acetylcholinesterase splice variations in anticholinesterase-treated Alzheimer's disease patients. *Journal of neurochemistry* 88: 1102-13

Darreh-Shori T, Meurling L, Pettersson T, Hugosson K, Hellstrom-Lindahl E, et al. 2006. Changes in the activity and protein levels of CSF acetylcholinesterases in relation to cognitive function of patients with mild Alzheimer's disease following chronic donepezil treatment. *Journal of neural transmission (Vienna, Austria : 1996)* 113: 1791-801

Darreh-Shori T, Soininen H. 2010. Effects of cholinesterase inhibitors on the activities and protein levels of cholinesterases in the cerebrospinal fluid of patients with Alzheimer's disease: a review of recent clinical studies. *Current Alzheimer research* 7: 67-73

Davidsson P, Bogdanovic N, Lannfelt L, Blennow K. 2001. Reduced expression of amyloid precursor protein, presenilin-1 and rab3a in cortical brain regions in Alzheimer's disease. *Dementia and geriatric cognitive disorders* 12: 243-50

Davies P. 1979. Neurotransmitter-related enzymes in senile dementia of the Alzheimer type. *Brain research* 171: 319-27

De Ferrari GV, Canales MA, Shin I, Weiner LM, Silman I, Inestrosa NC. 2001. A structural motif of acetylcholinesterase that promotes

amyloid beta-peptide fibril formation. *Biochemistry* 40: 10447-57

de la Escalera S, Bockamp EO, Moya F, Piovant M, Jimenez F. 1990. Characterization and gene cloning of neurotactin, a Drosophila transmembrane protein related to cholinesterases. *The EMBO journal* 9: 3593-601

De Strooper B. 2007. Loss-of-function presenilin mutations in Alzheimer disease. Talking Point on the role of presenilin mutations in Alzheimer disease. *EMBO reports* 8: 141-6

Doan A, Thinakaran G, Borchelt DR, Slunt HH, Ratovitsky T, et al. 1996. Protein topology of presenilin 1. *Neuron* 17: 1023-30

Dobbertin A, Hrabovska A, Dembele K, Camp S, Taylor P, et al. 2009. Targeting of acetylcholinesterase in neurons in vivo: a dual processing function for the proline-rich membrane anchor subunit and the attachment domain on the catalytic subunit. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 4519-30

Dori A, Cohen J, Silverman WF, Pollack Y, Soreq H. 2005. Functional manipulations of acetylcholinesterase splice variants highlight alternative splicing contributions to murine neocortical development. *Cerebral cortex (New York, N.Y. : 1991)* 15: 419-30

Drachman DA, Leavitt J. 1974. Human memory and the cholinergic system. A relationship to aging? *Archives of neurology* 30: 113-21

Drews U. 1975. Cholinesterase in embryonic development. *Progress in histochemistry and cytochemistry* 7: 1-52

Duff K, Eckman C, Zehr C, Yu X, Prada CM, et al. 1996. Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature* 383: 710-3

Edbauer D, Winkler E, Haass C, Steiner H. 2002. Presenilin and nicastrin regulate each other and determine amyloid beta-peptide production via complex formation. *Proceedings of the National Academy of Sciences of the United States of America* 99: 8666-71

Esler WP, Kimberly WT, Ostaszewski BL, Diehl TS, Moore CL, et al. 2000. Transition-state analogue inhibitors of gamma-secretase bind directly to presenilin-1. *Nature cell biology* 2: 428-34

Evin G, Canterford LD, Hoke DE, Sharples RA, Culvenor JG, Masters CL. 2005. Transition-state analogue gamma-secretase inhibitors stabilize a 900 kDa presenilin/nicastrin complex. *Biochemistry* 44: 4332-41

Farquhar MJ, Gray CW, Breen KC. 2003. The over-expression of the wild type or mutant forms of the presenilin-1 protein alters glycoprotein processing in a human neuroblastoma cell line. *Neuroscience letters* 346: 53-6

Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, et al. 1997. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *JAMA : the journal of the American Medical Association* 278: 1349-56

Fasulo L, Ugolini G, Visintin M, Bradbury A, Brancolini C, et al. 2000. The neuronal microtubule-associated protein tau is a substrate for caspase-3 and an effector of apoptosis. *Journal of neurochemistry* 75: 624-33

Finder VH. 2010. Alzheimer's disease: a general introduction and pathomechanism. *Journal of Alzheimer's disease : JAD* 22 Suppl 3: 5-19

Fishman EB, Siek GC, MacCallum RD, Bird ED, Volicer L, Marquis JK. 1986. Distribution of the molecular forms of acetylcholinesterase in human brain: alterations in dementia of the Alzheimer type. *Annals of neurology* 19: 246-52

Fodero LR, Saez-Valero J, Barquero MS, Marcos A, McLean CA, Small DH. 2001. Wheat germ agglutinin-binding glycoproteins are decreased in Alzheimer's disease cerebrospinal fluid. *Journal of neurochemistry* 79: 1022-6

Fodero LR, Saez-Valero J, McLean CA, Martins RN, Beyreuther K, et al. 2002. Altered glycosylation of acetylcholinesterase in APP (SW) Tg2576 transgenic mice occurs prior to amyloid plaque deposition. *Journal of neurochemistry* 81: 441-8

Fortna RR, Crystal AS, Morais VA, Pijak DS, Lee VM, Doms RW. 2004. Membrane topology and nicastrin-enhanced endoproteolysis of APH-1, a component of the gamma-secretase complex. *The Journal of biological chemistry* 279: 3685-93

Fraering PC, LaVoie MJ, Ye W, Ostaszewski BL, Kimberly WT, et al. 2004. Detergent-dependent dissociation of active gamma-secretase reveals an interaction between Pen-2 and PS1-NTF and offers a model for subunit organization within the complex. *Biochemistry* 43: 323-33

Fu H, Li W, Luo J, Lee NT, Li M, et al. 2008. Promising anti-Alzheimer's dimer bis(7)-tacrine reduces beta-amyloid generation by directly inhibiting BACE-1 activity. *Biochemical and biophysical research communications* 366: 631-6

Garcia-Ayllon MS, Millan C, Serra-Basante C, Bataller R, Saez-Valero J. 2012. Readthrough acetylcholinesterase is increased in human liver cirrhosis. *PloS one* 7: e44598

Garcia-Ayllon MS, Riba-Llena I, Serra-Basante C, Alom J, Boopathy R, Saez-Valero J. 2010. Altered levels of acetylcholinesterase in Alzheimer plasma. *PloS one* 5: e8701

Garcia-Ayllon MS, Silveyra MX, Andreasen N, Brimijoin S, Blennow K, Saez-Valero J. 2007. Cerebrospinal fluid acetylcholinesterase changes after treatment with donepezil in patients with Alzheimer's disease. *Journal of neurochemistry* 101: 1701-11

Georgakopoulos A, Marambaud P, Efthimiopoulos S, Shioi J, Cui W, et al. 1999. Presenilin-1 forms complexes with the cadherin/catenin cell-cell adhesion system and is recruited to intercellular and synaptic contacts. *Molecular cell* 4: 893-902

Geula C, Mesulam M. 1989. Special properties of cholinesterases in the cerebral cortex of Alzheimer's disease. *Brain research* 498: 185-9

Giacobini E. 2003. Cholinergic function and Alzheimer's disease. *International journal of geriatric psychiatry* 18: S1-5

Gil-Bea FJ, Solas M, Mateos L, Winblad B, Ramirez MJ, Cedazo-Minguez A. 2011. Cholinergic hypofunction impairs memory acquisition

possibly through hippocampal Arc and BDNF downregulation. *Hippocampus* 21: 999-1009

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, et al. 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349: 704-6

Gomez-Ramos P, Mufson EJ, Moran MA. 1992. Ultrastructural localization of acetylcholinesterase in neurofibrillary tangles, neuropil threads and senile plaques in aged and Alzheimer's brain. *Brain research* 569: 229-37

Gomez JL, Nieto-Ceron S, Campoy FJ, Munoz-Delgado E, Vidal CJ. 2003. Purification and properties of hydrophilic dimers of acetylcholinesterase from mouse erythrocytes. *The international journal of biochemistry & cell biology* 35: 1109-18

Gotz J, Eckert A, Matamales M, Ittner LM, Liu X. 2011. Modes of Abeta toxicity in Alzheimer's disease. *Cellular and molecular life sciences : CMLS* 68: 3359-75

Gouras GK, Almeida CG, Takahashi RH. 2005. Intraneuronal Abeta accumulation and origin of plaques in Alzheimer's disease. *Neurobiology of aging* 26: 1235-44

Greenfield SA. 1991. A noncholinergic action of acetylcholinesterase (AChE) in the brain: from neuronal secretion to the generation of movement. *Cell Mol Neurobiol* 11: 55-77

Grifman M, Galyam N, Seidman S, Soreq H. 1998. Functional redundancy of acetylcholinesterase and neuroligin in mammalian neuritogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 95: 13935-40

Grisaru D, Pick M, Perry C, Sklan EH, Almog R, et al. 2006. Hydrolytic and nonenzymatic functions of acetylcholinesterase comodulate hemopoietic stress responses. *Journal of immunology (Baltimore, Md. : 1950)* 176: 27-35

Grisaru D, Sternfeld M, Eldor A, Glick D, Soreq H. 1999. Structural roles of acetylcholinesterase variants in biology and pathology. *European journal of biochemistry / FEBS* 264: 672-86

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM. 1986. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *The Journal of biological chemistry* 261: 6084-9

Gu Y, Chen F, Sanjo N, Kawarai T, Hasegawa H, et al. 2003. APH-1 interacts with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin.nicastrin complexes. *The Journal of biological chemistry* 278: 7374-80

Guevara J, Espinosa B, Zenteno E, Vazguez L, Luna J, et al. 1998. Altered glycosylation pattern of proteins in Alzheimer disease. *Journal of neuropathology and experimental neurology* 57: 905-14

Guillozet-Bongaarts AL, Garcia-Sierra F, Reynolds MR, Horowitz PM, Fu Y, et al. 2005. Tau truncation during neurofibrillary tangle evolution in Alzheimer's disease. *Neurobiology of aging* 26: 1015-22

Haass C, Kaether C, Thinakaran G, Sisodia S. 2012. Trafficking and proteolytic processing of APP. *Cold Spring Harbor perspectives in medicine* 2: a006270

Hardy J, Allsop D. 1991. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends in pharmacological sciences* 12: 383-8

Hardy J, Selkoe DJ. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science (New York, N.Y.)* 297: 353-6

Hardy JA, Higgins GA. 1992. Alzheimer's disease: the amyloid cascade hypothesis. *Science (New York, N.Y.)* 256: 184-5

Harel M, Schalk I, Ehret-Sabatier L, Bouet F, Goeldner M, et al. 1993. Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. *Proceedings of the National Academy of Sciences of the United States of America* 90: 9031-5

Harper JD, Lansbury PT, Jr. 1997. Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and

physiological consequences of the time-dependent solubility of amyloid proteins. *Annual review of biochemistry* 66: 385-407

Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, et al. 1999. Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19: 8876-84

Hemming ML, Elias JE, Gygi SP, Selkoe DJ. 2008. Proteomic profiling of gamma-secretase substrates and mapping of substrate requirements. *PLoS biology* 6: e257

Hicks D, John D, Makova NZ, Henderson Z, Nalivaeva NN, Turner AJ. 2011. Membrane targeting, shedding and protein interactions of brain acetylcholinesterase. *Journal of neurochemistry* 116: 742-6

Hicks DA, Makova NZ, Nalivaeva NN, Turner AJ. 2013. Characterisation of acetylcholinesterase release from neuronal cells. *Chemico-biological interactions* 203: 302-8

Hicks DA, Nalivaeva NN, Turner AJ. 2012. Lipid rafts and Alzheimer's disease: protein-lipid interactions and perturbation of signaling. *Frontiers in physiology* 3: 189

Hu W, Gray NW, Brimijoin S. 2003. Amyloid-beta increases acetylcholinesterase expression in neuroblastoma cells by reducing enzyme degradation. *Journal of neurochemistry* 86: 470-8

Huynh DP, Vinters HV, Ho DH, Ho VV, Pulst SM. 1997. Neuronal expression and intracellular localization of presenilins in normal and Alzheimer disease brains. *Journal of neuropathology and experimental neurology* 56: 1009-17

Ikeda K, Urakami K, Arai H, Wada K, Wakutani Y, et al. 2000. The expression of presenilin 1 mRNA in skin fibroblasts and brains from sporadic Alzheimer's disease. *Dementia and geriatric cognitive disorders* 11: 245-50

Inestrosa NC, Alvarez A, Perez CA, Moreno RD, Vicente M, et al. 1996. Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron* 16: 881-91

Inestrosa NC, Roberts WL, Marshall TL, Rosenberry TL. 1987. Acetylcholinesterase from bovine caudate nucleus is attached to membranes by a novel subunit distinct from those of acetylcholinesterases in other tissues. *The Journal of biological chemistry* 262: 4441-4

Itoh A, Nitta A, Nadai M, Nishimura K, Hirose M, et al. 1996. Dysfunction of cholinergic and dopaminergic neuronal systems in beta-amyloid protein--infused rats. *Journal of neurochemistry* 66: 1113-7

Johnson G, Moore SW. 2003. Human acetylcholinesterase binds to mouse laminin-1 and human collagen IV by an electrostatic mechanism at the peripheral anionic site. *Neuroscience letters* 337: 37-40

Johnson G, Moore SW. 2006. The peripheral anionic site of acetylcholinesterase: structure, functions and potential role in rational drug design. *Curr Pharm Des* 12: 217-25

Johnston JA, Froelich S, Lannfelt L, Cowburn RF. 1996. Quantification of presenilin-1 mRNA in Alzheimer's disease brains. *FEBS letters* 394: 279-84

Kaether C, Lammich S, Edbauer D, Ertl M, Rietdorf J, et al. 2002. Presenilin-1 affects trafficking and processing of betaAPP and is targeted in a complex with nicastrin to the plasma membrane. *J Cell Biol* 158: 551-61

Kakuda N, Shoji M, Arai H, Furukawa K, Ikeuchi T, et al. 2012. Altered gamma-secretase activity in mild cognitive impairment and Alzheimer's disease. *EMBO molecular medicine* 4: 344-52

Kanninen K, Goldsteins G, Auriola S, Alafuzoff I, Koistinaho J. 2004. Glycosylation changes in Alzheimer's disease as revealed by a proteomic approach. *Neuroscience letters* 367: 235-40

Kar S, Issa AM, Seto D, Auld DS, Collier B, Quirion R. 1998. Amyloid beta-peptide inhibits high-affinity choline uptake and acetylcholine release in rat hippocampal slices. *Journal of neurochemistry* 70: 2179-87

Kar S, Seto D, Gaudreau P, Quirion R. 1996. Beta-amyloid-related peptides inhibit potassium-evoked acetylcholine release from rat hippocampal slices. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 16: 1034-40

Karpel R, Ben Aziz-Aloya R, Sternfeld M, Ehrlich G, Ginzberg D, et al. 1994. Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. *Experimental cell research* 210: 268-77

Karran E, Mercken M, De Strooper B. 2011. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov* 10: 698-712

Kasa P, Pakaski M, Joo F, Lajtha A. 1991. Endothelial cells from human fetal brain microvessels may be cholinoceptive, but do not synthesize acetylcholine. *Journal of neurochemistry* 56: 2143-6

Kaufer D, Friedman A, Seidman S, Soreq H. 1998. Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature* 393: 373-7

Keller M, Robitzki A, Layer PG. 2001. Heterologous expression of acetylcholinesterase affects proliferation and glial cytoskeleton of adherent chicken retinal cells. *Cell and tissue research* 306: 187-98

Kelly JF, Furukawa K, Barger SW, Rengen MR, Mark RJ, et al. 1996. Amyloid beta-peptide disrupts carbachol-induced muscarinic cholinergic signal transduction in cortical neurons. *Proceedings of the National Academy of Sciences of the United States of America* 93: 6753-8

Kimberly WT, Esler WP, Ye W, Ostaszewski BL, Gao J, et al. 2003. Notch and the amyloid precursor protein are cleaved by similar gamma-secretase(s). *Biochemistry* 42: 137-44

Kimberly WT, Xia W, Rahmati T, Wolfe MS, Selkoe DJ. 2000. The transmembrane aspartates in presenilin 1 and 2 are obligatory for gamma-secretase activity and amyloid beta-protein generation. *The Journal of biological chemistry* 275: 3173-8

Kolarova M, Garcia-Sierra F, Bartos A, Ricny J, Ripova D. 2012. Structure and pathology of tau protein in Alzheimer disease. *Int J Alzheimers Dis* 2012: 731526

Krejci E, Duval N, Chatonnet A, Vincens P, Massoulie J. 1991. Cholinesterase-like domains in enzymes and structural proteins: functional and evolutionary relationships and identification of a catalytically essential aspartic acid. *Proceedings of the National Academy of Sciences of the United States of America* 88: 6647-51

Kuhn PH, Wang H, Dislich B, Colombo A, Zeitschel U, et al. 2010. ADAM10 is the physiologically relevant, constitutive alpha-secretase of the amyloid precursor protein in primary neurons. *The EMBO journal* 29: 3020-32

Lahiri DK, Lewis S, Farlow MR. 1994. Tacrine alters the secretion of the beta-amyloid precursor protein in cell lines. *Journal of neuroscience research* 37: 777-87

Laudon H, Hansson EM, Melen K, Bergman A, Farmery MR, et al. 2005. A nine-transmembrane domain topology for presenilin 1. *The Journal of biological chemistry* 280: 35352-60

LaVoie MJ, Fraering PC, Ostaszewski BL, Ye W, Kimberly WT, et al. 2003. Assembly of the gamma-secretase complex involves early formation of an intermediate subcomplex of Aph-1 and nicastrin. *The Journal of biological chemistry* 278: 37213-22

Law A, Gauthier S, Quirion R. 2001. Say NO to Alzheimer's disease: the putative links between nitric oxide and dementia of the Alzheimer's type. *Brain research. Brain research reviews* 35: 73-96

Layer PG. 1995. Nonclassical roles of cholinesterases in the embryonic brain and possible links to Alzheimer disease. *Alzheimer disease and associated disorders* 9 Suppl 2: 29-36

Layer PG, Kaulich S. 1991. Cranial nerve growth in birds is preceded by cholinesterase expression during neural crest cell migration and the formation of an HNK-1 scaffold. *Cell and tissue research* 265: 393-407

Leem JY, Saura CA, Pietrzik C, Christianson J, Wanamaker C, et al. 2002. A role for presenilin 1 in regulating the delivery of amyloid precursor protein to the cell surface. *Neurobiology of disease* 11: 64-82

Legay C, Bon S, Massoulie J. 1993. Expression of a cDNA encoding the glycolipid-anchored form of rat acetylcholinesterase. *FEBS letters* 315: 163-6

Legay C, Huchet M, Massoulie J, Changeux JP. 1995. Developmental regulation of acetylcholinesterase transcripts in the mouse diaphragm: alternative splicing and focalization. *The European journal of neuroscience* 7: 1803-9

Lehmann S, Chiesa R, Harris DA. 1997. Evidence for a six-transmembrane domain structure of presenilin 1. *The Journal of biological chemistry* 272: 12047-51

Lichtenthaler SF, Wang R, Grimm H, Uljon SN, Masters CL, Beyreuther K. 1999. Mechanism of the cleavage specificity of Alzheimer's disease gamma-secretase identified by phenylalanine-scanning mutagenesis of the transmembrane domain of the amyloid precursor protein. *Proceedings of the National Academy of Sciences of the United States of America* 96: 3053-8

Lis H, Sharon N. 1986. Lectins as molecules and as tools. *Annual review of biochemistry* 55: 35-67

Liu CC, Kanekiyo T, Xu H, Bu G. 2013. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. *Nature reviews. Neurology* 9: 106-18

Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, et al. 1999. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am J Pathol* 155: 853-62

Lleo A. 2007. Current therapeutic options for Alzheimer's disease. *Current genomics* 8: 550-8

Lleo A, Berezovska O, Ramdya P, Fukumoto H, Raju S, et al. 2003. Notch1 competes with the amyloid precursor protein for gamma-secretase and down-regulates presenilin-1 gene expression. *The Journal of biological chemistry* 278: 47370-5

Lleo A, Blesa R, Queralt R, Ezquerra M, Molinuevo JL, et al. 2002. Frequency of mutations in the presenilin and amyloid precursor protein genes in early-onset Alzheimer disease in Spain. *Archives of neurology* 59: 1759-63

Lleo A, Saura CA. 2011. gamma-secretase substrates and their implications for drug development in Alzheimer's disease. *Current topics in medicinal chemistry* 11: 1513-27

Mallender WD, Szegletes T, Rosenberry TL. 2000. Acetylthiocholine binds to asp74 at the peripheral site of human acetylcholinesterase as the first step in the catalytic pathway. *Biochemistry* 39: 7753-63

Massoulie J. 2002. The origin of the molecular diversity and functional anchoring of cholinesterases. *Neurosignals* 11: 130-43

Massoulie J, Bon S, Perrier N, Falasca C. 2005. The C-terminal peptides of acetylcholinesterase: cellular trafficking, oligomerization and functional anchoring. *Chemico-biological interactions* 157-158: 3-14

Massoulie J, Pezzementi L, Bon S, Krejci E, Vallette FM. 1993. Molecular and cellular biology of cholinesterases. *Progress in neurobiology* 41: 31-91

Mathews PM, Cataldo AM, Kao BH, Rudnicki AG, Qin X, et al. 2000. Brain expression of presenilins in sporadic and early-onset, familial Alzheimer's disease. *Molecular medicine (Cambridge, Mass.)* 6: 878-91

McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, et al. 1999. Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Annals of neurology* 46: 860-6

Mehlert A, Varon L, Silman I, Homans SW, Ferguson MA. 1993. Structure of the glycosyl-phosphatidylinositol membrane anchor of acetylcholinesterase from the electric organ of the electric-fish, Torpedo californica. *Biochem J* 296 (Pt 2): 473-9

Melo JB, Agostinho P, Oliveira CR. 2003. Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. *Neuroscience research* 45: 117-27

Meshorer E, Soreq H. 2006. Virtues and woes of AChE alternative splicing in stress-related neuropathologies. *Trends Neurosci* 29: 216-24

Meshorer E, Toiber D, Zurel D, Sahly I, Dori A, et al. 2004. Combinatorial complexity of 5' alternative acetylcholinesterase transcripts and protein products. *The Journal of biological chemistry* 279: 29740-51

Mesulam MM, Geula C, Moran MA. 1987. Anatomy of cholinesterase inhibition in Alzheimer's disease: effect of physostigmine and tetrahydroaminoacridine on plaques and tangles. *Annals of neurology* 22: 683-91

Miners JS, Barua N, Kehoe PG, Gill S, Love S. 2011. Abeta-degrading enzymes: potential for treatment of Alzheimer disease. *Journal of neuropathology and experimental neurology* 70: 944-59

Montenegro MF, Nieto-Ceron S, Cabezas-Herrera J, Munoz-Delgado E, Campoy FJ, Vidal CJ. 2013. Most Acetylcholinesterase Activity of Non-Nervous Tissues and Cells Arises from the AChE-H Transcript. *Journal of molecular neuroscience : MN*

Moran MA, Mufson EJ, Gomez-Ramos P. 1993. Colocalization of cholinesterases with beta amyloid protein in aged and Alzheimer's brains. *Acta neuropathologica* 85: 362-9

Morel N, Leroy J, Ayon A, Massoulie J, Bon S. 2001. Acetylcholinesterase H and T dimers are associated through the same contact. Mutations at this interface interfere with the C-terminal T peptide, inducing degradation rather than secretion. *The Journal of biological chemistry* 276: 37379-89

Mori F, Lai CC, Fusi F, Giacobini E. 1995. Cholinesterase inhibitors increase secretion of APPs in rat brain cortex. *Neuroreport* 6: 633-6

Muir JL. 1997. Acetylcholine, aging, and Alzheimer's disease. *Pharmacol Biochem Behav* 56: 687-96

Muller F, Dumez Y, Massoulie J. 1985. Molecular forms and solubility of acetylcholinesterase during the embryonic development of rat and human brain. *Brain research* 331: 295-302

Munoz-Delgado E, Montenegro MF, Campoy FJ, Moral-Naranjo MT, Cabezas-Herrera J, et al. 2010. Expression of cholinesterases in human kidney and its variation in renal cell carcinoma types. *The FEBS journal* 277: 4519-29

Nakai T, Yamasaki A, Sakaguchi M, Kosaka K, Mihara K, et al. 1999. Membrane topology of Alzheimer's disease-related presenilin 1. Evidence for the existence of a molecular species with a seven membrane-spanning and one membrane-embedded structure. *The Journal of biological chemistry* 274: 23647-58

Namba Y, Tomonaga M, Kawasaki H, Otomo E, Ikeda K. 1991. Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain research* 541: 163-6

Naruse S, Thinakaran G, Luo JJ, Kusiak JW, Tomita T, et al. 1998. Effects of PS1 deficiency on membrane protein trafficking in neurons. *Neuron* 21: 1213-21

Nelson PT, Braak H, Markesbery WR. 2009. Neuropathology and cognitive impairment in Alzheimer disease: a complex but coherent relationship. *Journal of neuropathology and experimental neurology* 68: 1-14

Nitsch RM, Slack BE, Wurtman RJ, Growdon JH. 1992. Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. *Science (New York, N.Y.)* 258: 304-7

Noureddine H, Schmitt C, Liu W, Garbay C, Massoulie J, Bon S. 2007. Assembly of acetylcholinesterase tetramers by peptidic motifs from the proline-rich membrane anchor, PRiMA: competition between degradation and secretion pathways of heteromeric complexes. *The Journal of biological chemistry* 282: 3487-97

Nuydens R, Dispersyn G, de Jong M, van den Kieboom G, Borgers M, Geerts H. 1997. Aberrant tau phosphorylation and neurite

retraction during NGF deprivation in PC12 cells. *Biochemical and biophysical research communications* 240: 687-91

Oh YS, Turner RJ. 2005. Topology of the C-terminal fragment of human presenilin 1. *Biochemistry* 44: 11821-8

Ordentlich A, Barak D, Kronman C, Flashner Y, Leitner M, et al. 1993. Dissection of the human acetylcholinesterase active center determinants of substrate specificity. Identification of residues constituting the anionic site, the hydrophobic site, and the acyl pocket. *The Journal of biological chemistry* 268: 17083-95

Osenkowski P, Li H, Ye W, Li D, Aeschbach L, et al. 2009. Cryoelectron microscopy structure of purified gamma-secretase at 12 A resolution. *J Mol Biol* 385: 642-52

Paoletti F, Mocali A, Vannucchi AM. 1992. Acetylcholinesterase in murine erythroleukemia (Friend) cells: evidence for megakaryocyte-like expression and potential growth-regulatory role of enzyme activity. *Blood* 79: 2873-9

Parks AL, Curtis D. 2007. Presenilin diversifies its portfolio. *Trends in genetics : TIG* 23: 140-50

Pedersen WA, Kloczewiak MA, Blusztajn JK. 1996. Amyloid beta-protein reduces acetylcholine synthesis in a cell line derived from cholinergic neurons of the basal forebrain. *Proceedings of the National Academy of Sciences of the United States of America* 93: 8068-71

Perrier AL, Massoulie J, Krejci E. 2002. PRiMA: the membrane anchor of acetylcholinesterase in the brain. *Neuron* 33: 275-85

Perrier NA, Kherif S, Perrier AL, Dumas S, Mallet J, Massoulie J. 2003. Expression of PRiMA in the mouse brain: membrane anchoring and accumulation of 'tailed' acetylcholinesterase. *The European journal of neuroscience* 18: 1837-47

Perrier NA, Salani M, Falasca C, Bon S, Augusti-Tocco G, Massoulie J. 2005. The readthrough variant of acetylcholinesterase remains very minor after heat shock, organophosphate inhibition and stress, in cell culture and in vivo. *Journal of neurochemistry* 94: 629-38

Perrier NA, Salani M, Falasca C, Bon S, Augusti-Tocco G, Massoulie J. 2006. Readthrough acetylcholinesterase expression remains minor after stress or exposure to inhibitors. *Journal of molecular neuroscience : MN* 30: 75-6

Perry EK, Gibson PH, Blessed G, Perry RH, Tomlinson BE. 1977. Neurotransmitter enzyme abnormalities in senile dementia. Choline acetyltransferase and glutamic acid decarboxylase activities in necropsy brain tissue. *Journal of the neurological sciences* 34: 247-65

Perry EK, Smith CJ, Atack JR, Candy JM, Johnson M, Perry RH. 1986. Neocortical cholinergic enzyme and receptor activities in the human fetal brain. *Journal of neurochemistry* 47: 1262-9

Pettit DL, Shao Z, Yakel JL. 2001. beta-Amyloid(1-42) peptide directly modulates nicotinic receptors in the rat hippocampal slice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21: RC120

Podlisny MB, Citron M, Amarante P, Sherrington R, Xia W, et al. 1997. Presenilin proteins undergo heterogeneous endoproteolysis between Thr291 and Ala299 and occur as stable N- and C-terminal fragments in normal and Alzheimer brain tissue. *Neurobiology of disease* 3: 325-37

Qi-Takahara Y, Morishima-Kawashima M, Tanimura Y, Dolios G, Hirotani N, et al. 2005. Longer forms of amyloid beta protein: implications for the mechanism of intramembrane cleavage by gamma-secretase. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25: 436-45

Qian S, Jiang P, Guan XM, Singh G, Trumbauer ME, et al. 1998. Mutant human presenilin 1 protects presenilin 1 null mouse against embryonic lethality and elevates Abeta1-42/43 expression. *Neuron* 20: 611-7

Rabano A, Jimenez-Huete A, Acevedo B, Calero M, Ghiso J, et al. 2005. Diversity of senile plaques in Alzheimer's disease as revealed by a new monoclonal antibody that recognizes an internal sequence of the Abeta peptide. *Current Alzheimer research* 2: 409-17

Racchi M, Mazzucchelli M, Porrello E, Lanni C, Govoni S. 2004. Acetylcholinesterase inhibitors: novel activities of old molecules. *Pharmacological research : the official journal of the Italian Pharmacological Society* 50: 441-51

Ramirez-Bermudez J. 2012. Alzheimer's disease: critical notes on the history of a medical concept. *Archives of medical research* 43: 595-9

Rao A, Craig AM. 2000. Signaling between the actin cytoskeleton and the postsynaptic density of dendritic spines. *Hippocampus* 10: 527-41

Rapoport M, Dawson HN, Binder LI, Vitek MP, Ferreira A. 2002. Tau is essential to beta -amyloid-induced neurotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* 99: 6364-9

Razon N, Soreq H, Roth E, Bartal A, Silman I. 1984. Characterization of activities and forms of cholinesterases in human primary brain tumors. *Experimental neurology* 84: 681-95

Rees T, Hammond PI, Soreq H, Younkin S, Brimijoin S. 2003. Acetylcholinesterase promotes beta-amyloid plaques in cerebral cortex. *Neurobiology of aging* 24: 777-87

Roberson MR, Harrell LE. 1997. Cholinergic activity and amyloid precursor protein metabolism. *Brain research. Brain research reviews* 25: 50-69

Rosen C, Hansson O, Blennow K, Zetterberg H. 2013. Fluid biomarkers in Alzheimer's disease - current concepts. *Molecular neurodegeneration* 8: 20

Rossner S, Ueberham U, Schliebs R, Perez-Polo JR, Bigl V. 1998. The regulation of amyloid precursor protein metabolism by cholinergic mechanisms and neurotrophin receptor signaling. *Progress in neurobiology* 56: 541-69

Rotundo RL, Thomas K, Porter-Jordan K, Benson RJ, Fernandez-Valle C, Fine RE. 1989. Intracellular transport, sorting, and turnover of acetylcholinesterase. Evidence for an endoglycosidase H-sensitive form in Golgi apparatus, sarcoplasmic reticulum, and clathrin-coated vesicles and its rapid degradation by a non-

lysosomal mechanism. *The Journal of biological chemistry* 264: 3146-52

Saez-Valero J, Barquero MS, Marcos A, McLean CA, Small DH. 2000a. Altered glycosylation of acetylcholinesterase in lumbar cerebrospinal fluid of patients with Alzheimer's disease. *J Neurol Neurosurg Psychiatry* 69: 664-7

Saez-Valero J, de Ceballos ML, Small DH, de Felipe C. 2002. Changes in molecular isoform distribution of acetylcholinesterase in rat cortex and cerebrospinal fluid after intracerebroventricular administration of amyloid beta-peptide. *Neuroscience letters* 325: 199-202

Saez-Valero J, Fodero LR, Sjogren M, Andreasen N, Amici S, et al. 2003. Glycosylation of acetylcholinesterase and butyrylcholinesterase changes as a function of the duration of Alzheimer's disease. *Journal of neuroscience research* 72: 520-6

Saez-Valero J, Mok SS, Small DH. 2000b. An unusually glycosylated form of acetylcholinesterase is a CSF biomarker for Alzheimer's disease. *Acta neurologica Scandinavica. Supplementum* 176: 49-52

Saez-Valero J, Sberna G, McLean CA, Masters CL, Small DH. 1997. Glycosylation of acetylcholinesterase as diagnostic marker for Alzheimer's disease. *Lancet* 350: 929

Saez-Valero J, Sberna G, McLean CA, Small DH. 1999. Molecular isoform distribution and glycosylation of acetylcholinesterase are altered in brain and cerebrospinal fluid of patients with Alzheimer's disease. *Journal of neurochemistry* 72: 1600-8

Sato T, Diehl TS, Narayanan S, Funamoto S, Ihara Y, et al. 2007. Active gamma-secretase complexes contain only one of each component. *The Journal of biological chemistry* 282: 33985-93

Saura CA, Choi SY, Beglopoulos V, Malkani S, Zhang D, et al. 2004. Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. *Neuron* 42: 23-36

Saura CA, Tomita T, Davenport F, Harris CL, Iwatsubo T, Thinakaran G. 1999. Evidence that intramolecular associations between presenilin domains are obligatory for endoproteolytic processing. *The Journal of biological chemistry* 274: 13818-23

Sberna G, Saez-Valero J, Beyreuther K, Masters CL, Small DH. 1997. The amyloid beta-protein of Alzheimer's disease increases acetylcholinesterase expression by increasing intracellular calcium in embryonal carcinoma P19 cells. *Journal of neurochemistry* 69: 1177-84

Sberna G, Saez-Valero J, Li QX, Czech C, Beyreuther K, et al. 1998. Acetylcholinesterase is increased in the brains of transgenic mice expressing the C-terminal fragment (CT100) of the beta-amyloid protein precursor of Alzheimer's disease. *Journal of neurochemistry* 71: 723-31

Schellenberg GD, Montine TJ. 2012. The genetics and neuropathology of Alzheimer's disease. *Acta neuropathologica* 124: 305-23

Selkoe DJ. 1998. The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends in cell biology* 8: 447-53

Selkoe DJ. 2002. Alzheimer's disease is a synaptic failure. *Science (New York, N.Y.)* 298: 789-91

Shankar GM, Walsh DM. 2009. Alzheimer's disease: synaptic dysfunction and Abeta. *Molecular neurodegeneration* 4: 48

Shen J, Bronson RT, Chen DF, Xia W, Selkoe DJ, Tonegawa S. 1997. Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell* 89: 629-39

Shirotani K, Edbauer D, Capell A, Schmitz J, Steiner H, Haass C. 2003. Gamma-secretase activity is associated with a conformational change of nicastrin. *The Journal of biological chemistry* 278: 16474-7

Shirotani K, Tomioka M, Kremmer E, Haass C, Steiner H. 2007. Pathological activity of familial Alzheimer's disease-associated mutant presenilin can be executed by six different gamma-secretase complexes. *Neurobiology of disease* 27: 102-7

Sihlbom C, Davidsson P, Sjogren M, Wahlund LO, Nilsson CL. 2008. Structural and quantitative comparison of cerebrospinal fluid glycoproteins in Alzheimer's disease patients and healthy individuals. *Neurochemical research* 33: 1332-40

Silveyra MX, Evin G, Montenegro MF, Vidal CJ, Martinez S, et al. 2008. Presenilin 1 interacts with acetylcholinesterase and alters its enzymatic activity and glycosylation. *Molecular and cellular biology* 28: 2908-19

Silveyra MX, Garcia-Ayllon MS, de Barreda EG, Small DH, Martinez S, et al. 2012. Altered expression of brain acetylcholinesterase in FTDP-17 human tau transgenic mice. *Neurobiology of aging* 33: 624 e23-34

Simon S, Krejci E, Massoulie J. 1998. A four-to-one association between peptide motifs: four C-terminal domains from cholinesterase assemble with one proline-rich attachment domain (PRAD) in the secretory pathway. *The EMBO journal* 17: 6178-87

Sindou P, Couratier P, Barthe D, Hugon J. 1992. A dose-dependent increase of Tau immunostaining is produced by glutamate toxicity in primary neuronal cultures. *Brain research* 572: 242-6

Sklan EH, Lowenthal A, Korner M, Ritov Y, Landers DM, et al. 2004. Acetylcholinesterase/paraoxonase genotype and expression predict anxiety scores in Health, Risk Factors, Exercise Training, and Genetics study. *Proceedings of the National Academy of Sciences of the United States of America* 101: 5512-7

Small DH, Michaelson S, Sberna G. 1996. Non-classical actions of cholinesterases: role in cellular differentiation, tumorigenesis and Alzheimer's disease. *Neurochem Int* 28: 453-83

Small DH, Nurcombe V, Moir R, Michaelson S, Monard D, et al. 1992. Association and release of the amyloid protein precursor of Alzheimer's disease from chick brain extracellular matrix. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 12: 4143-50

Small DH, Reed G, Whitefield B, Nurcombe V. 1995. Cholinergic regulation of neurite outgrowth from isolated chick sympathetic

neurons in culture. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15: 144-51

Snow AD, Mar H, Nochlin D, Kimata K, Kato M, et al. 1988. The presence of heparan sulfate proteoglycans in the neuritic plaques and congophilic angiopathy in Alzheimer's disease. *Am J Pathol* 133: 456-63

Soreq H, Seidman S. 2001. Acetylcholinesterase--new roles for an old actor. *Nature reviews. Neuroscience* 2: 294-302

Steiner H, Fluhrer R, Haass C. 2008. Intramembrane proteolysis by gamma-secretase. *The Journal of biological chemistry* 283: 29627-31

Steiner H, Romig H, Pesold B, Philipp U, Baader M, et al. 1999. Amyloidogenic function of the Alzheimer's disease-associated presenilin 1 in the absence of endoproteolysis. *Biochemistry* 38: 14600-5

Sternfeld M, Ming G, Song H, Sela K, Timberg R, et al. 1998. Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18: 1240-9

Sternfeld M, Shoham S, Klein O, Flores-Flores C, Evron T, et al. 2000. Excess "read-through" acetylcholinesterase attenuates but the "synaptic" variant intensifies neurodeterioration correlates. *Proceedings of the National Academy of Sciences of the United States of America* 97: 8647-52

Struhl G, Adachi A. 2000. Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. *Molecular cell* 6: 625-36

Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, et al. 1991. Atomic structure of acetylcholinesterase from Torpedo californica: a prototypic acetylcholine-binding protein. *Science (New York, N.Y.)* 253: 872-9

Suzuki K, Hayashi Y, Nakahara S, Kumazaki H, Prox J, et al. 2012. Activity-dependent proteolytic cleavage of neuroligin-1. *Neuron* 76: 410-22

Sych M, Hartmann H, Steiner B, Mueller WE. 2000. Presenilin I interaction with cytoskeleton and association with actin filaments. *Neuroreport* 11: 3091-8

Takami M, Nagashima Y, Sano Y, Ishihara S, Morishima-Kawashima M, et al. 2009. gamma-Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 13042-52

Tanzi RE. 2013. A brief history of Alzheimer's disease gene discovery. *Journal of Alzheimer's disease : JAD* 33 Suppl 1: S5-13

Taylor P, Radic Z. 1994. The cholinesterases: from genes to proteins. *Annu Rev Pharmacol Toxicol* 34: 281-320

Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, et al. 1996. Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. *Neuron* 17: 181-90

Thinakaran G, Koo EH. 2008. Amyloid precursor protein trafficking, processing, and function. *The Journal of biological chemistry* 283: 29615-9

Thinakaran G, Parent AT. 2004. Identification of the role of presenilins beyond Alzheimer's disease. *Pharmacological research : the official journal of the Italian Pharmacological Society* 50: 411-8

Tischer E, Cordell B. 1996. Beta-amyloid precursor protein. Location of transmembrane domain and specificity of gamma-secretase cleavage. *The Journal of biological chemistry* 271: 21914-9

Trillo L, Das D, Hsieh W, Medina B, Moghadam S, et al. 2013. Ascending monoaminergic systems alterations in Alzheimer's disease. translating basic science into clinical care. *Neurosci Biobehav Rev* 37: 1363-79

Tu H, Nelson O, Bezprozvanny A, Wang Z, Lee SF, et al. 2006. Presenilins form ER Ca2+ leak channels, a function disrupted by familial Alzheimer's disease-linked mutations. *Cell* 126: 981-93

Ulrich J, Meier-Ruge W, Probst A, Meier E, Ipsen S. 1990. Senile plaques: staining for acetylcholinesterase and A4 protein: a comparative study in the hippocampus and entorhinal cortex. *Acta neuropathologica* 80: 624-8

Verdile G, Gnjec A, Miklossy J, Fonte J, Veurink G, et al. 2004. Protein markers for Alzheimer disease in the frontal cortex and cerebellum. *Neurology* 63: 1385-92

Vetrivel KS, Cheng H, Lin W, Sakurai T, Li T, et al. 2004. Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. *The Journal of biological chemistry* 279: 44945-54

Vidal R, Calero M, Piccardo P, Farlow MR, Unverzagt FW, et al. 2000. Senile dementia associated with amyloid beta protein angiopathy and tau perivascular pathology but not neuritic plaques in patients homozygous for the APOE-epsilon4 allele. *Acta neuropathologica* 100: 1-12

Wakabayashi T, De Strooper B. 2008. Presenilins: members of the gamma-secretase quartets, but part-time soloists too. *Physiology (Bethesda, Md.)* 23: 194-204

Wang H, Li R, Shen Y. 2013a. beta-Secretase: its biology as a therapeutic target in diseases. *Trends in pharmacological sciences* 34: 215-25

Wang JZ, Xia YY, Grundke-Iqbal I, Iqbal K. 2013b. Abnormal hyperphosphorylation of tau: sites, regulation, and molecular mechanism of neurofibrillary degeneration. *Journal of Alzheimer's disease : JAD* 33 Suppl 1: S123-39

Wang Y, Greig NH, Yu QS, Mattson MP. 2009. Presenilin-1 mutation impairs cholinergic modulation of synaptic plasticity and suppresses NMDA currents in hippocampus slices. *Neurobiology of aging* 30: 1061-8

Watanabe N, Tomita T, Sato C, Kitamura T, Morohashi Y, Iwatsubo T. 2005. Pen-2 is incorporated into the gamma-secretase complex through binding to transmembrane domain 4 of presenilin 1. *The Journal of biological chemistry* 280: 41967-75

Weingarten MD, Lockwood AH, Hwo SY, Kirschner MW. 1975. A protein factor essential for microtubule assembly. *Proceedings of the National Academy of Sciences of the United States of America* 72: 1858-62

Weintraub S, Wicklund AH, Salmon DP. 2012. The neuropsychological profile of Alzheimer disease. *Cold Spring Harbor perspectives in medicine* 2: a006171

Wiley JC, Smith EA, Hudson MP, Ladiges WC, Bothwell M. 2007. Fe65 stimulates proteolytic liberation of the beta-amyloid precursor protein intracellular domain. *The Journal of biological chemistry* 282: 33313-25

Wischik CM, Novak M, Edwards PC, Klug A, Tichelaar W, Crowther RA. 1988a. Structural characterization of the core of the paired helical filament of Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America* 85: 4884-8

Wischik CM, Novak M, Thogersen HC, Edwards PC, Runswick MJ, et al. 1988b. Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America* 85: 4506-10

Wolf BA, Wertkin AM, Jolly YC, Yasuda RP, Wolfe BB, et al. 1995. Muscarinic regulation of Alzheimer's disease amyloid precursor protein secretion and amyloid beta-protein production in human neuronal NT2N cells. *The Journal of biological chemistry* 270: 4916-22

Wolfe MS. 2013. Toward the structure of presenilin/gamma-secretase and presenilin homologs. *Biochim Biophys Acta* 1828: 2886-97

Wolfe MS, Xia W, Moore CL, Leatherwood DD, Ostaszewski B, et al. 1999a. Peptidomimetic probes and molecular modeling suggest that Alzheimer's gamma-secretase is an intramembrane-cleaving aspartyl protease. *Biochemistry* 38: 4720-7

Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ. 1999b. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature* 398: 513-7

Xie HQ, Leung KW, Chen VP, Chan GK, Xu SL, et al. 2010. PRiMA directs a restricted localization of tetrameric AChE at synapses. *Chemico-biological interactions* 187: 78-83

Yu G, Chen F, Levesque G, Nishimura M, Zhang DM, et al. 1998. The presenilin 1 protein is a component of a high molecular weight intracellular complex that contains beta-catenin. *The Journal of biological chemistry* 273: 16470-5

Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, et al. 2000. Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. *Nature* 407: 48-54

Zakut H, Matzkel A, Schejter E, Avni A, Soreq H. 1985. Polymorphism of acetylcholinesterase in discrete regions of the developing human fetal brain. *Journal of neurochemistry* 45: 382-9

Zhang C, Wu B, Beglopoulos V, Wines-Samuelson M, Zhang D, et al. 2009. Presenilins are essential for regulating neurotransmitter release. *Nature* 460: 632-6

Zhang Y, Chen L, Shen G, Zhao Q, Shangguan L, He M. 2014. GRK5 dysfunction accelerates tau hyperphosphorylation in APP (swe) mice through impaired cholinergic activity. *Neuroreport*

Zheng L, Calvo-Garrido J, Hallbeck M, Hultenby K, Marcusson J, et al. 2013. Intracellular localization of amyloid-beta peptide in SH-SY5Y neuroblastoma cells. *Journal of Alzheimer's disease : JAD* 37: 713-33

Zimmermann M, Borroni B, Cattabeni F, Padovani A, Di Luca M. 2005. Cholinesterase inhibitors influence APP metabolism in Alzheimer disease patients. *Neurobiology of disease* 19: 237-42

Zlokovic BV. 2013. Cerebrovascular effects of apolipoprotein E: implications for Alzheimer disease. *JAMA Neurol* 70: 440-4

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“Two things are infinite:
the universe and human stupidity;
and I'm not sure about the universe.”

Albert Einstein

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