Universidade de Aveiro Departamento de Biologia
2009

Ana Gabriela da Silva Cavaleiro Henriques

Biologia do Aβ e a sua contribuição para a doença de Alzheimer

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutora em Bioquímica, realizada sob a orientação científica da Doutora Odete Abreu Beirão da Cruz e Silva, Professora Auxiliar da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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Professora Auxiliar da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro

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palavras-chave

Doença Alzheimer, Aβ, metabolismo APP, AICD, Fe65, sinalização nuclear, secreção vesicular, citosqueleto, laminina, gelsolina, proteínas fosfatases.

resumo

A doença de Alzheimer (DA) é uma desordem neurodegenerativa progressiva patologicamente caracterizada pela presença de placas de amilóide (placas senis) insolúveis e também pela presença de tranças neurofibrilhares,formadas pela proteína Tau hiperfosforiladada. O principal constituinte das placas senis é o peptídeo beta-amilóide (Aβ), que deriva do processamento proteolítico da proteína precursora de amilóide de Alzheimer (APP). Embora Aβ exista como um agregado pouco solúvel nas placas senis, ele é secretado pelas células como uma molécula solúvel. O Aβ "per se" pode afectar o metabolismo da APP. Alguns autores sugerem que o Aβ exerce o seu efeito alterando o processamento ou catabolismo da APP, outros sugerem que ele também induz a transcrição da *APP*, onde aumentando os níveis da APP pode estar a contribuir para a sua própria produção (mecanismo de "feedback" positivo). Assim sendo, torna-se difícil consolidar todas estas observações e identificar as potenciais funções fisiológicas do Aβ "in vivo", ou as consequências da sua produção. Neste trabalho caracterizaram-se os efeitos do Aβ no metabolismo da APP. Os nossos estudos revelaram que um dos mecanismos induzidos pelo Aβ é a acumulação intracelular do fragmento neuroprotector sAPP (isAPPα) em estruturas com características vesiculares associadas ao citosqueleto. Estudos adicionais em culturas primárias revelaram que o Aβ estava a exercer o seu efeito ao nível da secreção vesicular, provavelmente interferindo com o transporte de APP/sAPP ao longo da rede do citosqueleto. Esta hipótese é sustentada pelo facto do Aβ estar a afectar a estabilidade e a polimerização de proteínas envolvidas na dinâmica do citosqueleto. Contrariamente a publicações anteriores o Aβ não induziu a transcrição da *APP*, na verdade em culturas primárias neuronais foi observado uma diminuição nos níveis de expressão da *APP*. Isto foi acompanhado por um aumento nos fragmentos C-terminais da APP (CTFs) e uma diminuição na localização nuclear do seu domínio intracelular (AICD), sugerindo alterações na sinalização nuclear da APP. O Aβ pode afectar outras vias de sinalização, particularmente alterando o balanço entre as actividades das proteínas cinases e fosfatases, o que pode ter consequências para o desenvolvimento da doença. Os dados obtidos indicam que o Aβ é capaz de inibir a actividade da proteína fosfatase1, a sua importância numa perspectiva de futuras terapias é discutida.

Devido à relevância da agregação do Aβ para a sua toxicidade, a formação de complexos com proteínas que promovem a sua desagregação/degradação e o seu efeito no processamento da APP foi avaliado. Na presença destes complexos observou-se uma reversão da acumulação isAPP, demonstrando o potencial terapêutico destas proteínas como moduladores do metabolismo da APP. Este trabalho permitiu compreender melhor os mecanismos envolvidos nos efeitos do Aβ no processamento da APP e descobrir algumas moléculas que podem ser relevantes numa perspectiva de diagnóstico e terapia na DA.

keywords

Alzheimer´s Disease, Aβ, APP metabolism, AICD, Fe65, nuclear signalling, vesicular secretion, cytoskeleton, laminin, gelsolin, protein phosphatases.

abstract

Alzheimer´s disease (AD) is a progressive neurodegenerative disorder pathologically characterized by the presence of extracellular deposition of insoluble amyloid plaques (senile plaques) and also by the appearance of neurofibrillary tangle-bearing neurons, mainly composed of hyperphosphorylated Tau protein. The major component of senile plaques is the amyloid-beta (Aβ) peptide, derived from proteolytic processing of the Alzheimer´s amyloid precursor protein (APP). Although Aβ exists as an aggregated, poorly soluble form in brain deposits, it is secreted from cells during normal metabolism as a soluble molecule. Aβ "per se" has been reported to affect APP metabolism, and while some authors suggest that it may exert its effects by altering APP processing/catabolism, others reported that it also induces *APP* transcription. This latter observation suggested a positive feedback mechanism resulting in increased APP levels, thus Aβ would stimulate its own production. Difficulties arise when trying to consolidate all these observations and to identify the potential "in vivo" physiological role of Aβ, or the consequences of its overproduction in AD. Hence, in this work we characterized the Aβ effects on APP metabolism. Our studies reveal that mechanistically Aβ leads to intracellular retention of the neuroprotective fragment (isAPPα) at vesicular-like densities associated with the cytoskeleton. Additional studies in primary neuronal cultures revealed that Aβ was exerting an inhibitory effect at the vesicular secretory level, probably by interfering with APP/sAPP transport along the cytoskeleton network. This hypothesis was supported by the fact that Aβ was affecting the stability and polymerization of proteins involved in cytoskeleton dynamics. Contrary to previous observations Aβ did not induce *APP* transcription. In fact for primary neuronal cultures we observed a decrease on *APP* expression levels. This was accompanied by an increase in APP C-terminal fragments (CTFs) and decreased APP intracellular domain (AICD) nuclear targeting, suggesting altered APP nuclear signaling. Aβ may affect other signaling cascades, for instance by altering the balance between kinase and phosphatase activities which may have consequences for the disease progression. Data obtained reveals that Aβ was able to inhibit protein phosphatase1 activity, and the importance of this for therapeutic approaches is discussed.

Further, it is also known that Aβ aggregation state is associated with its neurotoxicity. Hence, Aβ complex formation with proteins that promote its disaggregation/clearance and its effect on APP processing was evaluated. These complexes reverse isAPP retention, thus demonstrating the therapeutic potential of these proteins as modulators of APP metabolism. The work in this thesis allowed for a better understanding of the molecular mechanisms underlying Aβ effects on APP metabolism and identified molecules relevant for diagnosis and therapy in AD.

Abbreviations

aa	Amino acid
Aβ	Abeta
AD	Alzheimer's disease
ADAM	A desintegrin and metalloproteinase
ADDLS	Abeta-derived diffusible ligand
ADF	Actin-depolymerizing factor
AChE	Acetylcholinesterase
AICD/AID	APP intracellular domain
ANOVA	One way analysis of variance
APH-1	Anterior pharynx defective 1
APLP1/2	APP-Like Protein 1/2
APL-1	C. elegans APP homolog
APP	Alzheimer's Amyloid Precursor Protein
APP-BP1	APP-binding protein 1
APPL	Drosophila APP homolog
ApoE	Apolipoprotein E
ATP	Adenosine triphosphate
BACE	β-site APP cleaving enzyme
BBB	Brain blood barrier
BCA	Bicinchoninic acid
BChE	Bytyrylcholinesterase
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cDNA	Complementary DNA
CHO	Chinese Hamster Ovary cell line
CSF	Cerebral spinal fluid
CHX	Cicloheximide
CNS	Central nervous system
(α/β) CTF	APP Carboxy-terminal Fragment of α/β-secretase processing
CytD	Cytochalasin D
Dab1	Disabled 1
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential interference contrast microscopy
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N´,N´-tetraacetic acid
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's Disease
FBS	Fetal bovine serum
FDG	18F-2-Fluoro-2deoxy-D-Glucose
g	Gravitational acceleration
G_0	Heterotrimeric (α, β, and γ subunits) G0 protein
GDP	Guanosine
GLUT3	Glucose transporter3
GPI	Glycosylphosphatidylinositol
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
h/hr	Hour
H4	Histone 4
HEPES	4-(2-HydroxyEthyl)-1-PiperazineEthane sulfonic acid
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
IDE	Insulin-degrading enzyme
IgG	Immunoglobulin G
IgA	Immunoglobulin A
JIP	JNK interacting protein
JNK	Jun Kinase
KAI1	"Kang ai" (Chinese for anticancer) protein 1
KHC	Kinesin heavy chain
KLC	Kinesin light chain
KO	Knockout
KPI	Kunitz-type serine proteinase inhibitor
LRP	Low density lipoprotein receptor-related protein
LTD	Long-term depression
LTP	Long-term potentiation
MBP	Myielin basic protein
MDCK	Madine-Darby Canine Kidney cell line
MENA	Mammalian enabled
min	Minute
MINT	Munc-18-interacting protein
mRNA	Messenger ribonucleic acid
MRI	Magnetic resonance imaging
ND	Non-demented
NFT	Neurofibrillary tangle
NMDA	N-methyl-D-aspartate

NT2	Human neuron teratocarcinoma cell line
PAGE	PolyAcrylamide gel electrophoresis
PBS	Phosphate buffer saline (modified Dulbecco's)
PC12	Rat adrenal pheochromocytoma cell line
PCR	Polymerase chain reaction
PEN-2	Presenilin enhancer
PET	Positron emission tomography
PF	Protofibrils
PHF	Paired helical filament
PIB	Pittsburg compound-B
PKC	Protein kinase C
PM	Plasma membrane
PS1/2	Presenilin 1 and 2
PP1/2	Protein phosphatase type 1/2
PTB	Phospho tyrosine binding
PTM	Post-translational modification
RAGE	Advanced glycation end product receptor
RIP	Regulated intramembranar proteolysis
ROS	Reactive oxygen species
RP	Reserve pool
rpm	Rotations per minute
RRP	Readily releasable pool
RT-PCR	Reverse transcriptase-polymerase chain reaction
RT	Room temperature
SNARE	Soluble N-ethylmaleimide-sensitive fusion attachment protein receptor
(α/β)sAPP	Secreted APP of α/β-secretase processing origin
SAM	Substrate adhesion molecule
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TACE	Tumor necrosis factor-α converting enzyme
TBS	Tris buffered saline
TBS-T	TBS supplemented with Tween detergent
TEMED	N,N,N',N'-tetramethylethylenediamine
TGN	Trans-golgi network
Tip60	Tat interactive protein, 60 kDa
TM	Transmembrane
Tris-HCl	Tris (hydroxymethyl)-aminoethane chloride

Chapter I

INTRODUCTION

OPENING REMARKS

First described by the German pathologist Alois Alzheimer in 1906 (Alzheimer 1906), Alzheimer´s disease (AD) is a progressive neurodegenerative disorder that occurs predominantly in later life. The prevalence of the disease is below 1% in individuals aged 60-64 years old but it rises abruptly with age, so that in people aged 75 years or older, prevalence is about 19% and up to 30% above 85 (Blennow et al. 2006; Lambert and Amouyel 2007). As life span is increasing there is the possibility that this elderly population may develop AD, which will constitute a major public-health problem. Therefore, it is of extreme importance to understand the basic nature of the disease, so that effective preventive procedures can be developed. The latest worldwide estimate of AD prevalence shows that 26.6 million people were living with the disease in 2006. Researchers predict that global prevalence will quadruple by 2050 to more than 100 million, at which time 1 in 85 people worldwide will be living with the disease.

AD is a progressive neurodegenerative dementia that invariably leads to a complete loss of all cognitive abilities and ultimately to death. Initially AD patients exhibit subtle memory failure which becomes more severe. The short-term memory begins to decline when the cells in the hippocampus degenerate. As the cerebral cortex (the outer layer of the brain) becomes affected, judgment declines, emotional outbursts may occur, and language is impaired. Progression of the disease leads to death of nerve cells and subsequent behavioural changes, such as wandering and agitation. Thus, in the later stages perception and orientation are affected as well as loss of personality and intellect to a level that influences daily activities. The ability to recognize faces and to communicate is completely lost in the final stages of the disease. Patients will eventually need constant care, complete dependency may last for years before the patient dies. The average length of time from diagnosis to death is 4 to 8 years, although neurodegeneration is estimated to start 20-30 years before clinical symptoms become apparent (Bird 2007a). The overall lifetime risk of developing dementia is 10-12% (Goedert and Spillantini 2006).

1.1 Neuropathological Features of AD

AD is characterized by a variety of pathological features, including extracellular senile plaques and intracellular neurofibrillary tangles, synaptic loss, and brain atrophy (Selkoe 2000; Hardy and Selkoe 2002; Forman et al. 2004). The characteristic brain atrophy and mass loss in AD patients is due to extensive neuronal damage and death. There is an overall shrinkage of brain tissue, with the most affected areas being the hippocampus, the cerebral cortex and amygdala, regions of the brain that play a major role in memory, cognition and behaviour. Widening of the sulcus and shrinkage of the gyrus, the well-developed fold of the brain's outer layer, is evident. The ventricles, which are cavities or spaces in the brain that contain cerebrospinal fluid, are enlarged (Figure 1).

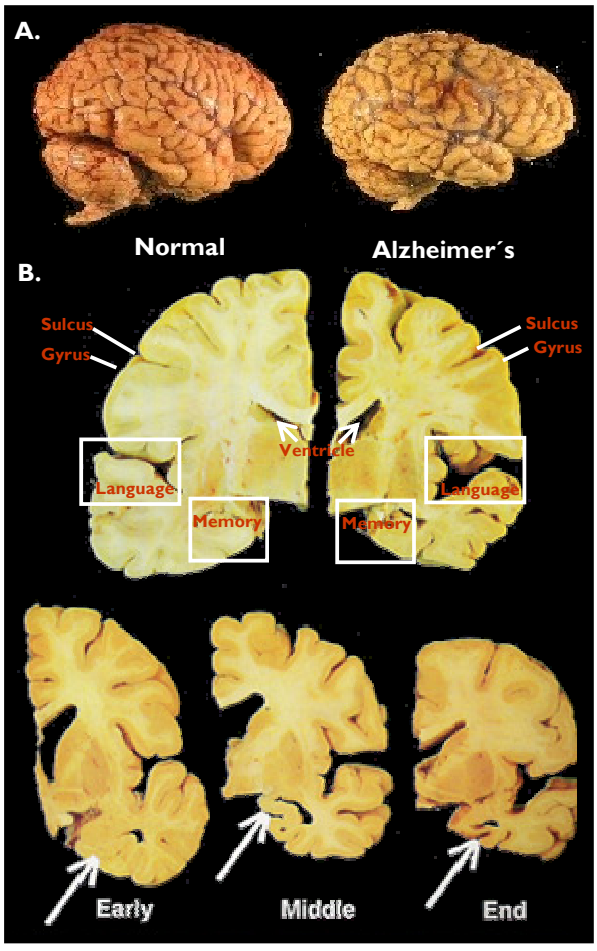
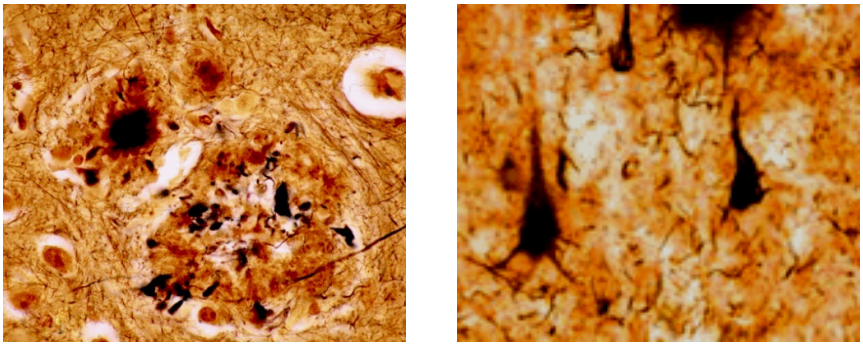


Figure 1. Normal *versus* Alzheimer´s brain. A. Brain volume reduction and atrophy characteristic of AD patients (right). From www.cienciahoje.pt/index.php?oid=17362&op=all. B. Brain areas affected in AD patients. Images represent brain cross sections of a normal individual (left) and of an AD patient (right). The bottom three pictures represent brain shrinkage as AD continues to evolute. The arrows demonstrate shrinkage in the region of the brain associated with short term memories. Adapted from www.alzheimer.sk.ca/english/Just4Kids/alz_dis...

The senile or neuritic plaques are mainly composed of Aβ (Allsop et al. 1983; Glenner and Wong 1984), while the neurofibrillary tangles (NFT) are intraneuronal bundles of paired helical filaments (PHF) consisting predominantly of hyperphosphorylated Tau protein (Goedert et al. 1992; Goedert et al. 1996) (Figure 2). While Aβ deposition is specific for AD, the NFTs are also seen in other degenerative disorders, but the coexistence of both lesions, along with cerebral atrophy and neuronal degeneration, are the conclusive hallmarks of the AD.



Senile plaques NFT

Figure 2. Signature lesions of AD. Plaques are extracellular deposits of Aβ surrounded by dystrophic neuritis, reactive astrocytes, and microglia, whereas neurofibrillary tangles (NFT) are intracellular aggregates composed of a hyperphosphorylated form of the microtubule-associated protein Tau. Bielschowsky silver stains (From www.neuropathologyweb.org/.../chapter9bAD.html).

NFTs - Tangles occur at dystrophic neuritis (small dendrites and axons with degenerative changes), and are mainly found in the pyramidal regions of the amygdala, the hippocampus and the neocortex (Haroutunian et al. 1999; Oddo et al. 2003). NFTs consist of pyramidal cells filled with paired helical and straight filaments of aggregated hyperphosphorylated Tau. Tau is usually quite soluble and a key normal function of this protein is to bind to axonal microtubules to stabilize the axonal cytoskeleton framework. Tau is known to aid in cell microtubule assembly and stabilization, by promoting tubulin polymerization and reducing dynamic instability of the microtubule (Smith et al. 1996). This binding of Tau protein increases the rate of association at the end of the microtubule and decreases the rate of dissociation at the growing end (Goedert et al. 1997). It was also discovered that this protein can acts as a regulator of intracellular vesicles and organelle traffic, by

interacting with cytoskeletal proteins, such as actin which also aid in cytoskeletal maintenance and trafficking (Drewes et al. 1998; Drouet et al. 2000). The extent to which Tau promotes its activity depends on its phosphorylation state (Lindwall and Cole 1984), with abnormal hyperphosphorylation interfering with its normal biological function (Gustke et al. 1992; Alonso et al. 1994). Problems arise when Tau becomes hyperphosphorylated at serine and threonine residues of the protein by a still unclear mechanism (Tanaka et al. 2000). Hyperphosphorylated Tau protein loses its ability to bind tubulin and stabilize microtubule assembly (Drouet et al. 2000), leading to microtubule breakdown into PHFs and NFTs. Hence, abnormal Tau phosphorylation may contribute to the formation of NFTs resulting in neuronal degeneration (Higuchi et al. 2002b; Sorrentino and Bonavita 2007).

Senile plaques - As already mentioned, another feature of AD brains are the neuritic plaques found distributed throughout the brain, but notably in the cerebral cortex and hippocampus of AD patients (Dickson 1997; Haroutunian et al. 1998). These plaques exhibit a central core of extracellular amyloid, surrounded of dystrophic neuritis, containing Tau aggregates (mostly in the straight filament form), and also reactive astrocytes and microglia, among other protein/peptides constituents. This central core is composed of aggregates of Aβ peptide of 40-43 amino acids (called $A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}43}$). The $A\beta_{1\text{-}40}$ peptides are most soluble and apparently less neurotoxic (majority of Aβ peptides), whereas the $A\beta_{1\text{-}42}$ peptides are more hydrophobic (less soluble), and exhibit a higher potential for aggregation and neurotoxicity than does $A\beta_{1\text{-}40}$. Although $A\beta_{1\text{-}42}$ peptides are less prevalent, overall they predominate in the central core of the plaques (Jarrett and Lansbury 1992; Jarrett et al. 1993). Aβ deposition occurs as oligomeric, protofibrilar, amylospheroid and fibrillar forms (Kuo et al. 1996; Lambert et al. 1998; Hartley et al. 1999; Walsh et al. 1999; Hoshi et al. 2003). The term "soluble Aβ" is generally applied either to newly generated, cell secreted Aβ, or to the fraction of tissue Aβ that is taken into the aqueous phase of a non-detergent-containing extraction buffer. "Misfolded" and "aggregated" Aβ are the terms used to describe very early, non-specific changes in Aβ folding states or solubility states, respectively. "Oligomeric" Aβ refers to

peptide assemblies with limited stoichiometry (e.g. dimmers, trimers, etc.), while protofibrils (PFs) are structures of intermediates preceding biologically inert amyloid fibrils that are found in plaques. The term "Aβ-derived diffusible ligand" (ADDLs) is also applied to pre-protofibrillar intermediates (Figure 3). Indeed, oligomers, PFs and ADDLs are believed to be the Aβ assembly states with the most potent toxicity, being therefore the mediators of Aβ induced neurotoxicity (Klein et al. 2001; Kayed et al. 2004). The final assemblies, named fibrils, are the basic insoluble building blocks of the amyloid plaques.

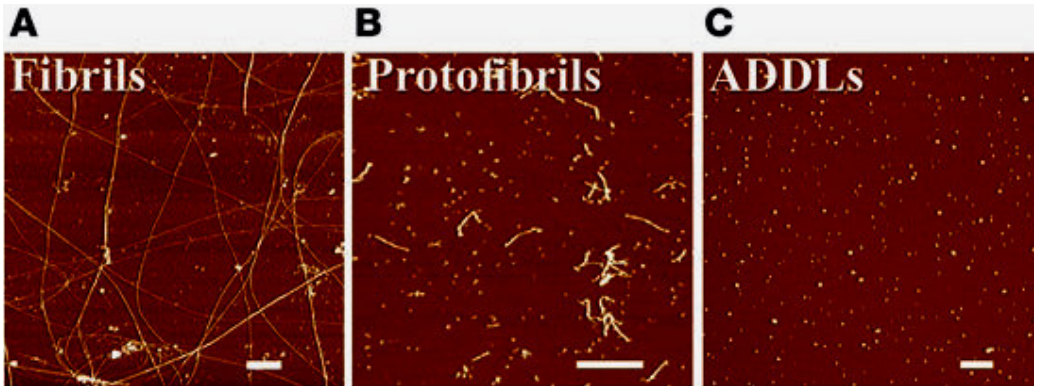


Figure 3. Different assembly (biophysical) states of Aβ. The assembled forms obtained from incubation with synthetic Aβ are highly sensitive to preparation and incubation. Widely different proportions of insoluble fibrils (A), soluble PFs (B), and oligomers (C), also known as ADDLs are revealed by atomic force microscopy. Scale bars: 200 nm. (Adapted from Gandy, 2005).

It has been hypothesized that the most dangerous Aβ form may be smaller groups of a "few pieces", rather than the large plaques themselves. The small clumps are suggested to synapse signal and possibly trigger immune system inflammation.

In addition to neuritic plaques, Aβ is also found in diffuse, non-fibrillar deposits, known as diffuse plaques, without accompanying dystrophic neuritis. Although these plaques may be found sometimes in large numbers in old, non-demented persons, and therefore not associated with dementia, they may also represent an early stage of AD plaques. Consistently, these plaques contain predominantly $A\beta_{1-42}$ and small levels of $A\beta_{1-43}$ rather than $A\beta_{1-40}$ (Gowing et al. 1994; Iwatsubo et al. 1994; Iizuka et al. 1995; Iwatsubo et al. 1995; Lemere et al. 1996).

Amyloid and NFTs - The unequivocal fact that both senile plaques and NFT are consistently found in the early-stages of the disease and increase as a function of disease severity have led a number of researchers to postulate a role for Aβ and Tau abnormalities in the pathogenesis of AD. Although it remains inconclusive whether Aβ or Tau initiates AD pathology, several evidences demonstrated that altered Aβ metabolism plays an essential role. Consistently, evidence points to amyloid deposition preceding and precipitating the formation of NFTs in some patients, with Aβ preceding Tau aggregation. In agreement with this, Tau deposition in transgenic mice is influenced by Aβ (Lewis et al. 2001; Oddo et al. 2003). Further, in young Down's syndrome patients, Aβ deposits exist in the absence of NFTs, notably in areas of the brain most affected by AD (Iwatsubo et al. 1995; Leverenz and Raskind 1998; Gouras et al. 2000). On the other hand, a number of Tau mutations result in familial forms of non-AD neurodegenerative dementia (Higuchi et al. 2002a), and neuropathological investigations of AD brains have indicated that filamentous Tau aggregates are more closely related to neuronal loss than Aβ plaques (Arriagada et al. 1992; Cummings and Cotman 1995; Gomez-Isla et al. 1996). By inducing Tau abnormalities, which promote disruption of neuronal structure and function leading to neuronal death, Aβ peptide may be placed in the centre of a molecular cascade of events that contributes to AD pathogenesis.

1.2 AD Clinical Diagnosis

AD is usually diagnosed only after clinical symptoms, such as memory loss and confusion, become apparent, symptoms that in most cases develop after Aβ begins to accumulate in the brain, and even then a diagnosis cannot be conclusive. Indeed, AD can only be diagnosed with entire certainty by examining post-mortem brain. Nowadays physicians can use brain imaging such as MRI (magnetic ressonance imaging) techniques, alongside with developed cognitive tests in order to identify and document specific changes in the brain as early as possible. More recently, neuroimaging techniques, such as PET (positron emission tomography) have been developed (Figure 4). Using PET, an Alzheimer's-predicting 18F-2-Fluoro-2deoxy-D-glucose (FDG)-PET scan, scientists can visualize and measure brain metabolic activity and try to predict future AD development (Mosconi 2005). Further, it is also possible to visualize Aβ inside the brain before the disease becomes debilitating, by using a chemical, named Pittsburgh Compound-B, or PIB for short, a novel PET biomarker. PIB can enter the brain in living humans, bind to the beta-amyloid plaques, and be detected by PET (Klunk et al. 2004). This compound can help determine the efficacy of anti-amyloid drug therapies in clinical trials, and in the future, it may also be used as a diagnostic agent for AD. The development of molecular imaging agents for AD is critically important in early diagnosis, neuropathogenesis studies and treatment of AD.

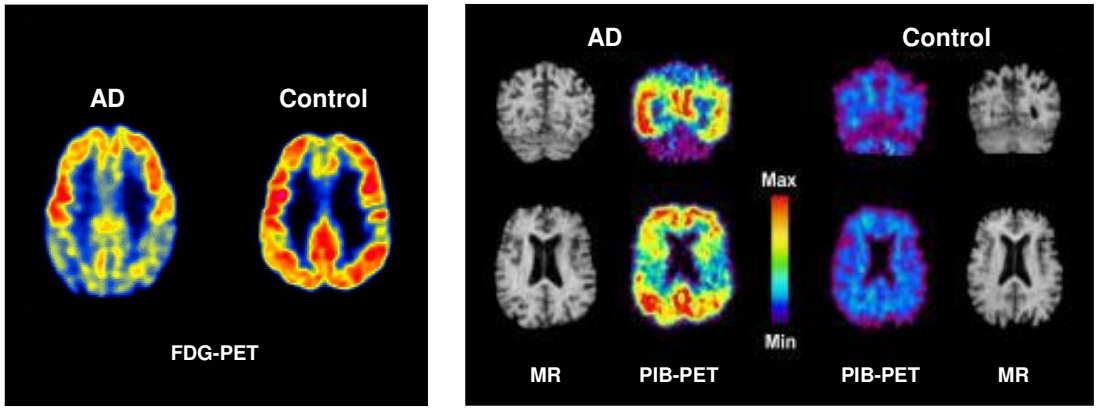


Figure 4. PET an Alzheimer´s predicting technique. FDG-PET (left) and PIB-PET (right) images show the regional distribution of the rate of glucose metabolism and of the amount of Aβ petide in AD brains, respectively. FDG-PET scans show the decline in metabolic activity in an Alzheimer's brain (AD) compared to a normal brain (Control). Because active neurons have a very high metabolic rate, FDG uptake is high in brains of healthy subjects, especially in the cortex. In contrast, FDG uptake in AD is greatly diminished, especially in the temporal and parietal regions of the brain. From www.researchmagazine.uga.edu/.../ra_slime2.htm and http://www.sciencedaily.com/releases/2004/01/040122084019.htm.

1.3 AD Sporadic and Genetic Risk Factors

Several pathogenic mechanisms that underlie the changes observed in AD have been extensively studied, including Aβ aggregation and deposition with plaque development, tau hyperphosphorylation with tangle formation, neurovascular dysfunction, and other mechanisms such as cell-cycle abnormalities, inflammatory processes, mitochondrial dysfunction, and oxidative stress. However to date, none of the known mechanisms alone are sufficient to explain all the biochemical and pathological alterations observed in AD. Nonetheless, the cause-and-effect relationship between Aβ deposition and AD pathology is once again strongly supported by the discovery of genetic mutations that are causative of familiar AD. Indeed, all identified mutations greatly alter the metabolic processing of Alzheimer´s amyloid precursor protein (APP) and Aβ production, resulting in decreased clearance and increased accumulation of fibrillary Aβ in the brain (Hardy and Selkoe 2002; Tsubuki et al. 2003).

From an etiological perspective AD forms have been characterized as sporadic or familial AD (FAD). FAD is considered when more that one person in a family has been affected, while sporadic refers to AD cases when no other cases in close family have been seen. Approximately 5% of all AD cases are associated to the familial form, with the remainder being sporadic (Bird 2007a). AD is further divided into early-onset (denotes onset of disease before age 65) and late-onset (denotes age onset after age 65). Most of the sporadic AD cases, which represent the vast majority, are late-onset. In most cases, AD is a complex multifactorial disease resulting from the interaction of several factors, principal genetic but also environmental.

1.3.1 Genetics of AD

From a genetic standpoint, AD is a heterogeneous disorder, with a number of genes that may increase the risk of developing the disease. The most well-established genetics link is in familial early-onset AD cases. Although these only represent a small percentage of all AD cases, their molecular and genetic study allows for improved knowledge about the etiology of the more abundant sporadic forms, since increased Aβ production and accumulation is a common feature in all cases.

Genes implicated in familial disease

Three genetic loci have been linked to early onset inherited forms of the disease namely genes encoding for APP located on chromosome 21, presenilin-1 (PS1) on chromosome 14 and presenilin-2 (PS2) on chromosome 1 (reviewed in Marambaud and Robakis, 2005). The *APP* gene encodes the APP which is normally cleaved to form Aβ. Within the *APP* gene twenty-three locations carrying mutations have been identified, however these only explain around 5-10% of familial early-onset cases (Bird 2007b). Although some of them are directly linked to AD, others are associated with cerebral haemorrhages (Figure 5 and see www.alzforum.org/res/com/mut/app). The first APP mutation discovered was Glu^{693} to Gln ("Dutch" mutation) within the Aβ sequence (Hardy and Allsop 1991). Synthetic peptides containing this mutation were shown to have high propensity to aggregate (Wisniewski et al. 1991; Clements et al. 1993). Subsequently, some families with early onset AD were found to have pathogenic mutations at APP Val^{717}, resulting in a change from Val^{717} to Ile, Gly or Phe (Chartier-Harlin et al. 1991; Goate et al. 1991; Murrell et al. 1991). This mutation was called the "London" mutation. The "Swedish" double mutation (Lys/Met^{670} to Asn/Leu, on the immediate Aβ N-terminus) results in secretion of larger amounts of total Aβ (Citron et al. 1992; Mullan et al. 1992; Cai et al. 1993). A more recently discovered pathogenic mutation, named the APP "Arctic" mutation (Glu^{693} to Gly), leads to decreased $Aβ_{1-40/1-42}$ levels in plasma and in cells conditioned media, but as a consequence a higher tendency of Aβ to aggregate. In fact, Aβ in the Arctic mutation, forms protofibrils at a much higher rate and in larger quantities than wild-type Aβ (Nilsberth et al. 2001). These mutations, all lye near or within the Aβ domain, result in

APP being more efficiently processed by secretases, thus generating increased amounts of Aβ that is more likely to form plaques (Citron et al. 1992; Cai et al. 1993; Haass et al. 1995; Goedert and Spillantini 2006), thereby promoting amyloidogenesis.

Further, the level of *APP* being expressed also appears to be an important aspect. For instance, in Down's Syndrome, caused by trissomy of chromosome 21, there is an extra copy of the *APP* gene. These individuals show increased levels of Aβ and invariably develop plaques and tangles in their brains, with clinical dementia in many cases before the age of 50 (Tanzi et al. 1987; Selkoe 1997; Esler and Wolfe 2001).

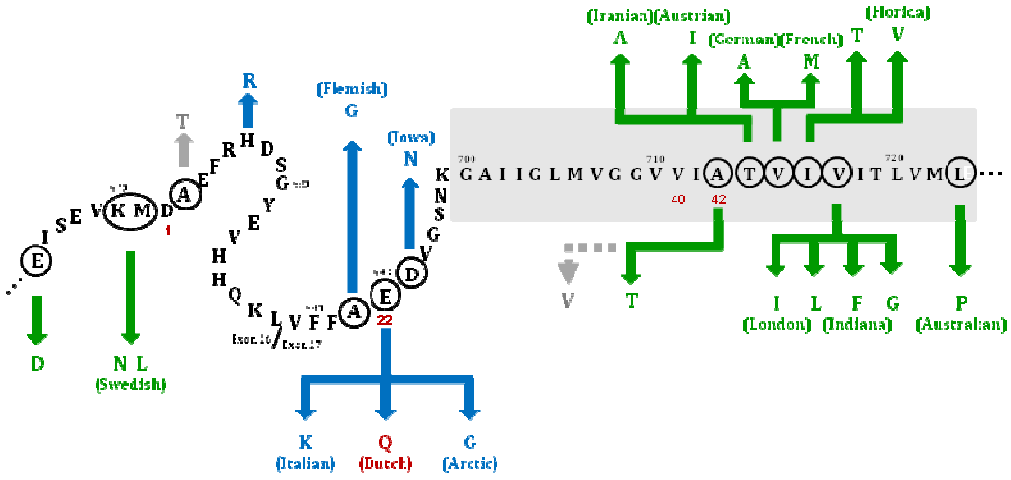


Figure 5. APP mutations lying near or in the Aβ domain. Location of the mutations within the fragment 665-723 of APP is highlighted with a circle in the corresponding amino acid sequence (APP_{770} isoform numbering). The most frequent Aβ peptides ($Aβ_{1-40}$ and $Aβ_{1-42}$) extend from position 672 to amino acid 711 (for $Aβ_{1-40}$) or 713 (for $Aβ_{1-42}$). Mutations indicated in green produce Alzheimer disease phenotypes; mutations notated in blue are primarily associated with cerebral amyloid angiopathy phenotypes. Mutations depicted in gray produce neither phenotype. Dutch mutation, the first APP mutation discovered, at position 22 of Aβ is highlighted in red. The gray box, spanning from positions 700 to 723, represents the location of the single transmembrane domain of APP. Adapted from www.nature.com/.../n9/fig_tab/nn0904-902_F1.html.

Mutations in the highly homologous *PS1* and *PS2* genes account for most cases of familial AD (Levy-Lahad et al. 1995; Sherrington et al. 1995). A total of 142 mutations have been found for *PS1* in 281 families (www.alzforum.org/res/com/mut/pre/table1.asp), which represents the gene with highest number of pathogenic mutations for AD. For PS2 10 mutations have been found in 16 families (www.alzforum.org/res/com/mut/pre/table2.asp). These genes encode for proteins that are involved in the normal cleavage of the APP protein, and mutations on these genes will result in increased $A\beta_{1-42}$ production (Citron et al. 1997; Xia et al. 1997; De Strooper et al. 1998). In particular, PS1 participates in the catalytic core of γ-secretase complex and its mutations induce relative amounts of $A\beta_{X-42}$ peptides (Wolfe et al. 1999). Some PS2 mutations, like those of PS1, were functionally associated with increased production of $A\beta_{X-42}$ peptides, while others did not modify either $A\beta_{X-40}$ or $A\beta_{X-42}$ peptide production (reviewed in Lambert and Amouyel, 2007).

Genes implicated in sporadic disease

Inheritance of ε4 allele of apolipoprotein E (*APOE ε4*) represents the greatest genetic risk factor in sporadic AD (Corder et al. 1993; Poirier 1994; Raber et al. 2004; Goedert and Spillantini 2006), although its mode of action in AD progression is unknown. The *APOE ε4* allele appears to operate mainly by modifying age onset (Meyer et al. 1998; Xiong et al. 2005), with each allele copy lowering the age of onset by almost 10 years, suggesting that *APOE ε4* association with AD may be related to longer disease duration in these cases (Basun et al. 1995). Further, there is no well described molecular mechanism underlying *APOE ε4* as an increased risk factor for AD. It is known that *APOE* acts as a cholesterol transporter in the brain, with *APOE ε4* being less efficient than the other variants in recycling membrane lipids and neuronal repair (Poirier 1994). On the other hand, *APOE* is essential for Aβ deposition, promoting Aβ aggregation and plaque formation (Holtzman et al. 2000; Holtzman 2001), possibly by acting as a pathological chaperone that binds to Aβ. Further, other genetic susceptibility factors have been proposed. Polymorphisms on genes encoding several proteins, including α2-macroglobulin, angiotensin I converting enzyme, Fe65 (Chapman et al. 1998; Alvarez et al. 1999; Kovacs 2000; Lambert et al. 2000), as well as some mitochondrial genetic polymorphisms (reviewed in Zhu et al. 2004) have been

associated with the disease. More recently, new susceptibility loci for late AD have been identified on chromosome 1, 9, 10, 12, and 13 (Bertram and Tanzi 2004). These risk factor genes are likely to affect one or more of the known pathogenic mechanisms (i.e. altered Aβ production, increased Aβ aggregation and inflammatory responses) which will result in decreased Aβ degradation/clearance and ultimately in neurodegeneration.

Genetic screening of AD

Genetic testing for the mutations associated with both familial and sporadic AD are available, although the circumstances under which testing is recommended differ. If there is suspicion of familial early-onset AD, genetic testing to detect gene mutations can and should be performed to identify the disease causing mutation and the molecular lesion (Bird 2007b). For *APOE*, and although a large number of patients with sporadic late-onset AD have at least one allele *APOE* ε4, the association of the mutation with the development of the disease is not strong enough to recommend that *APOE* genotyping be used as a predictive test in asymptomatic individuals. Instead *APOE* genotyping is most useful as an adjunct diagnostic test in individuals exhibiting symptoms of progressive dementia (Xiong et al. 2005; Bird 2007a).

1.3.2 Non-genetic factors contributing to sporadic AD

For AD sporadic forms, besides aging, which is the most obvious risk factor, epidemiological studies have proposed several other putative contributing factors. Some can be linked to decreased reserve brain capacity (including reduced brain size and number of neurons and their synaptic and dendritic arborisation) due to for instance brain injury, low educational and occupational attainment, low mental ability in early life, and reduced mental and physical activity during late life (Mayeux 2003; Mortimer et al. 2003; Jellinger 2004). Other risk factors are associated with vascular disease, including hypertension, hypercholesterolemia, atherosclerosis, coronary heart disease, smoking, obesity and diabetes (Mayeux 2003). Nonetheless is not clear if these are true causal risk factors that lead to the pathological features of the disease, or whether they induce cerebrovascular pathology that will add to the clinically silent disease pathology beyond the threshold for dementia. Evidence suggests that dietary intake of antioxidants, such as vitamin C and E; homocysteine-related vitamins (vitamin B12 and folate); unsaturated fatty acids; and also moderate alcohol intake, especially wine, could reduce the risk for AD (Luchsinger et al. 2007), but data so far does not support the recommendation of any specific diet for the prevention of AD. Although many environmental factors may increase the risk of developing sporadic AD, this form of the disease has been shown to have a significant genetic background.

1.4 A CENTRAL ROLE FOR Aβ IN AD NEURODEGENERATION

The association of the pathogenic mutations with alterations in APP processing pathways that relatively increase $A\beta_{1-42}$ production, together with the *in vitro* and *in vivo* observations of Aβ–induced neurotoxicity (for review see Canevari at al. 2004 and Smith et al. 2006) sustain that this peptide is at the heart of the disease process. These findings have lead to the proposal of the amyloid cascade hypothesis, which has been the basis of several research activities that have significantly contributed to the understanding of the molecular basis of AD (reviewed in Hardy 2006). In the cascade theory, Aβ is the central trigger for the pathological changes observed in AD brains, such as synapse loss, activation of inflammatory processes, the induction of NFTs and, ultimately, neuronal death. Therefore, all factors that can contribute to altered APP processing/metabolism resulting in increased Aβ production and/or aggregation, like APP and presinilin mutations, APOE ε4 allele, Trissomia 21, oxidative stress, environmental factors, and even normal aging will contribute to AD progression.

At the molecular cascade level diffuse amyloid deposits progress over time and eventually become neuritic plaques. One hypothesis is that deposition of $A\beta_{1-42}$ may form a "precipitation core" to which soluble $A\beta_{1-40}$ could aggregate, in an AD-specific process. The *in vivo* evolution of Aβ deposition and aggregation may trigger an oxidative inflammatory response that can be initiated due to the release of reactive oxygen intermediates, nitric oxide and inflammatory cytokines by activated microglia (Lukiw and Bazan 2000; Butterfield et al. 2001; Eikelenboom et al. 2008). Some of these pro-inflammatory molecules may be locally toxic to neuronal processes in the vicinity of amyloid plaques. Further, the relative overproduction of this peptide may lead, first to neurofibrillary degeneration and then to neuronal death (Hardy 1997).

The unequivocal involvement of Aβ in AD neurodegenerative process is summarized in the following figure.

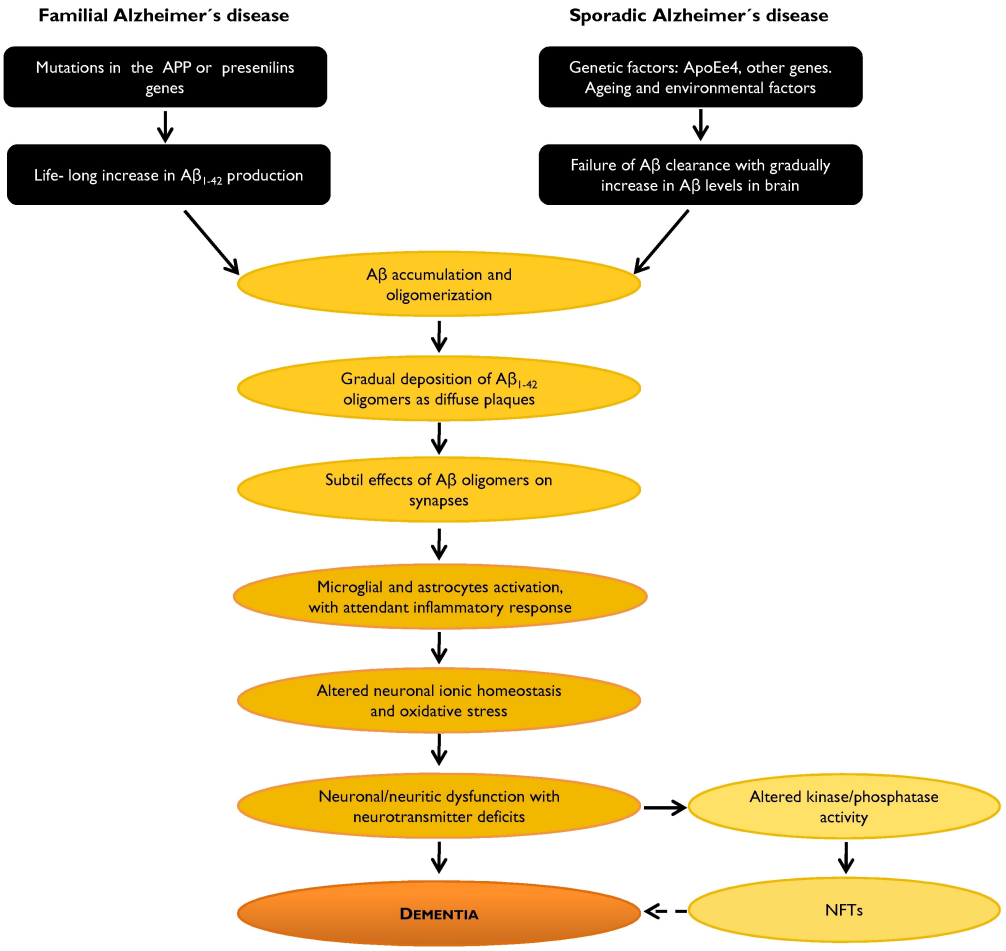


Figure 6. Aβ involvement in the AD neurodegenerative process: the amyloid cascade hypothesis. It is likely that during the disease process the balance between Aβ production and Aβ catabolism is altered, which results in increased Aβ production/accumulation. Adapted from http://www.alzforum.org/res/adh/cur/knowntheamyloidcascade.asp.

Nonetheless, this hypothesis, often considered a dogma, has some limitations and other pathophysiological mechanisms, not necessarily exclusive, are still under study. These include, for example alterations in cell trafficking (Naruse et al. 1998) or neuronal calcium homeostasis induced by mutant presenilins (Schneider et al. 2001), and also oxidative stress (reviewed in Chauhan and Chauhan 2006 and Hamel et al. 2008). Corroborating the latter hypothesis is the observation that genetic mutations in AD have lead to increased cellular vulnerability to oxidative stress and apoptotic insults. It is still unclear whether mutations result in Aβ deposition that then cause oxidative stress, or whether mutations cause oxidative stress that result in Aβ deposition. Indeed, while some studies demonstrated that Aβ can directly cause oxidative stress others shows that the reverse is also true. Obviously, whether Aβ is the culprit, as argued by the amyloid cascade, or just a promoter factor that may culminate in neuronal death needs to be further investigated to advance our understanding and contribute to the design of efficacious therapeutics for this disease.

1.5 ALZHEIMER´S AMYLOID PRECURSOR PROTEIN (APP)

1.5.1 APP gene family and alternatively spliced isoforms

Aβ is constitutively secreted by both neuronal and non-neuronal mammalian cells into the extracellular fluid (Selkoe 1994b) and arises from the proteolytic processing of APP, an integral transmembrane glycoprotein containing a membrane-spanning domain towards its carboxyl-terminus (Kang et al. 1987). The mammalian APP superfamily comprises APP and APP-like proteins, known as APLP1 and APLP2 (Sprecher et al. 1993; Wasco et al. 1993), of which APLP2 is the nearest relative (50% of homology). These three related proteins are well-conserved in evolution, functionally and structurally related, and share similar functions (Bayer et al. 1999; Coulson et al. 2000). The mammalian APP family members are type I integral membrane proteins that have relatively large extracellular domains and short intracellular domains. Of note is that APLP1 and APLP2 share homology at the amino acid sequence, domain structure and protein organization with APP, but lack the Aβ domain. Other known members of the APP superfamily are non-mammalian and include APPL in Drosophila (Rosen et al. 1989; Luo et al. 1992), APL-1 in *C. elegans* (Daigle and Li 1993) and an APP homologue protein in Xenopus (Okado and Okamoto 1992).

APP is encoded by a gene on chromosome 21 (21q21.3) and contains 18 exons (GenBank accession number D87675), with the Aβ sequence occurring between exons 16 and 17. Alternative post-transcriptional splicing of exons 7, 8 and 15 of the APP mRNA (Neve et al. 1988; Palmert et al. 1988; Koo et al. 1990b; Ohgami et al. 1993a; Sandbrink et al. 1994b) produces different isoforms of this protein ranging from 365-770 amino acid residues that differ in size extracellularly, but share the same cytoplasmic, transmembranar and Aβ peptide sequences. At least eight isoforms of APP have been described (Figure 7), numbered according to their length in amino acids: L-677, 695, L-696, 714, L-733, 751, L-752, 770 (Kitaguchi et al. 1988; Ponte et al. 1988).

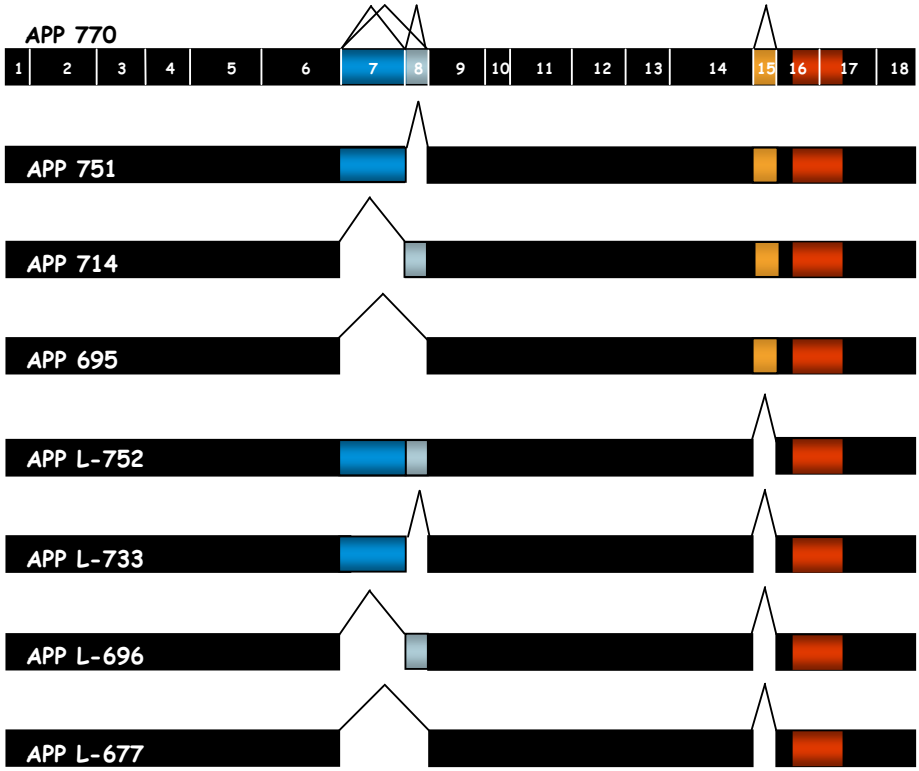


Figure 7. APP isoforms resulting from alternative splicing of the *APP* gene. Alternatively spliced exons are indicated in colour: dark blue, exon 7; light blue, exon 8; orange, exon 15. The Aβ sequence lying in exon 16 and 17 is indicated in red. Adapted from Sandbrink et al. 1994b.

APP is ubiquitously expressed in mammalian cells with a broad tissue distribution (Tanzi et al. 1987; Neve et al. 1988; Tanzi et al. 1988; Weidemann et al. 1989; Golde et al. 1990; Sisodia and Price 1995). Analysis of APP mRNA expression levels revealed that APP can be detected in almost all tissues examined, as well as in cultured cells. The tissue-specific pattern of APP mRNA splicing was studied by RT-PCR analysis (Sandbrink et al. 1994a). The less abundant L-APP isoforms, lacking exon 15, are mainly expressed in leukocyte cells, such as T-lymphocytes, macrophages and microglial cells. They are also ubiquitously expressed in rat tissues, including brain, but not in neurons (Ohgami et al. 1993b; Sandbrink et al. 1994b).

The three major isoforms expressed were found to be 695, 751 and 770 amino acids (APP_{695}, APP_{751} and APP_{770}, respectively). While the 751- and 770- amino acid spliced isoforms are predominantly expressed in peripheral tissues, the 695 is the APP isoform predominantly produced in the mammalian brain with the 695:751:770 mRNA ratios

being approximately 20:10:1 (Neve et al. 1988; Konig et al. 1989; Tanaka et al. 1989; Kang and Muller-Hill 1990). The exon 7-containing isoforms also predominate in cultured astrocytes (Gray and Patel 1993a; Gray and Patel 1993b; Rohan de Silva et al. 1997), with the 695:751:770 ratio being 1:4:2 (Gray and Patel 1993a; Gray and Patel 1993b). APP_{695}, lacking exon 7 and 8, is most highly expressed in neurons, representing 95% of total neuronal APP (Tanzi et al. 1987; Weidemann et al. 1989; LeBlanc et al. 1991), and is therefore often referred to as the "cerebral" or "neuronal" isoform. Due to APP_{695} predominance in the brain and CNS (Neve et al. 1988; Tanzi et al. 1993), this isoform has received considerable attention in AD research. Additional studies have indicated that alternative splicing of exons 7 and 8 changes in brain during aging and with AD, but results obtained are still inconsistent and controversial to consider altered alternative splicing as an AD risk factor (Sandbrink et al. 1994a; Rockenstein et al. 1995; Moir et al. 1998; Panegyres et al. 2000).

1.5.2 APP functional domains

The APP exon 7 encodes a 56 amino acid (aa) Kunitz-type serine protease inhibitor (KPI) domain, which inhibits proteases, such as trypsin or plasmin, and blood coagulation factors (Van Nostrand and Cunningham 1987; Kitaguchi et al. 1988; Ponte et al. 1988; Tanzi et al. 1988; Wagner et al. 1992) and thus may regulate the degradation of APP (Edelberg and Wei 1996). Exon 8 encodes a 19 aa domain with homologies to the MRC OX-2 antigen found on the surface of neurons and certain cells involved in the immune response such as thymocytes (Clark et al. 1985). Besides KPI and OX-2, several other structural domains have been identified within APP (Figure 8 and Reinhard et al. 2005). Several heparin-binding domains (Small et al. 1994), a collagen-binding site (Beher et al. 1996), an integrin-binding motif (amino acid sequence RHDS, Ghiso et al. 1992) and N-linked carbohydrate attachment sites (Weidemann et al. 1989) were also found in this region. Consistently, APP has been shown to bind heparin (Mok et al. 1997), collagen (Beher et al. 1996), and laminin (Kibbey et al. 1993). Additionally, two subdomains (328-332 and 444-612) were presumed to have a neuroprotective function, including the "RERMS" sequence with putative growth-promoting properties (Ninomiya et al. 1993).

The APP ectodomain also contains binding-sites for metals such as zinc (Bush et al. 1993; Bush et al. 1994) and copper (Hesse et al. 1994; Multhaup et al. 1996; Barnham et al. 2003) in the APP N-terminal but also within the Aβ domain. The C-terminus can be cleaved to release the APP intracellular domain (AICD). Almost all known APP binding proteins bind at its C-terminus, and specifically at one of two APP domains: YTSI and YENPTY. The latter is highly conserved from nematodes to humans, and has been shown to be responsible for several protein-protein interactions. Several intracellular proteins have been shown to bind to this domain including Fe65 (a phosphotyrosine binding domain-containing protein), X11 also known as LIN-10 or MINT (Munc-18-interacting protein), Dab1 (disabled homolog 1) and JIP-1b. Interacting proteins for YTSI domain included APP-BP1 (APP-binding protein 1), the microtubule associated protein PAT1 (protein interacting with APP tail 1), and kinesin-I (an axonal transport protein) (De Strooper and Annaert 2000; Van Gassen et al. 2000; King and Scott Turner 2004). Another domain, VTPEER, has not been implicated so far in APP binding to other proteins, but it includes a G_0 binding sequence. These intracellular domains can be classified according to their attributed functions and are thought to be involved in regulating APP rate of secretion, endocytosis, and Aβ production (Ando et al. 1999; Iijima et al. 2000; Mueller et al. 2000; Ando et al. 2001; Sabo et al. 2001; Roncarati et al. 2002).

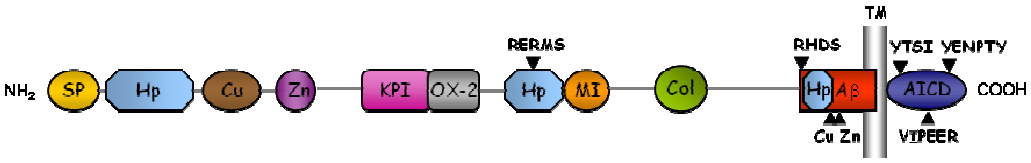


Figure 8. APP functional domains. *Yellow*, signal peptide (1-17); *light blue*, heparin-binding domains (28-123; 174-185; 391-412); *brown,* copper-binding domain (135-155); *violet*, zinc-binding domain (181-188); *pink,* KPI domain; *grey,* OX-2 domain; *"RERMS"*, putative growth-promoting motif (403-407); *orange*, gelateinase A (matrix metalloproteinase) inhibitor (407-417); *green*, collagen-binding site (523-540); *red,* Aβ; *"RHDS",* integrin-binding motif (aa 5-8 of Aβ); heparin-binding motif "VHHQK" (aa 12-16 of Aβ); *dark Blue*, APP intracellular domain (AICD) which include YTSI, YENPTY, VTPEER. APP_{770} isoform numbering. TM, transmembrane domain.

1.5.3 Putative functions for APP and APP cleaved fragments

The overall physiological function of APP has not yet been definitively determined but due to the structures and the specific characteristics of its domains several APP putative functions have been attributed and considered valid. These include cell surface receptor, cell adhesion molecule, precursor to growth factor and regulator of neuronal copper homeostasis. Besides that, other functions arise from many *in vitro* and *in vivo* studies which evaluated the involvement of APP and APP fragments resulting from APP processing (see section 1.5.4). These include neurotoxicity, neuritic outgrowth, synaptogenesis, involvement in learning and memory processes, and in cell signaling (for review see Zheng and Koo 2006; Senechal et al. 2006; Reinhard et al. 2005).

APP as a receptor molecule

Due to the type I integral membrane structure and due to it binding site to G_0 protein, via the intracellular tail, it has been suggested that APP might function as a cell surface G-protein coupled receptor (Kang et al. 1987; Okamoto et al. 1995). G_0 is a major GTP binding protein in brain involved in signal transduction cascades, such as adenylyl cyclase (Carter and Medzihradsky 1993), phospholipase C (Moriarty et al. 1990), voltage-dependent calcium channels (Hescheler et al. 1987) and pathways for apoptosis (Giambarella et al. 1997). Therefore, the activation of APP may contribute to one or more of these cascades, although the exact downstream mechanisms involving G_0 activation or inhibition by APP are unknown. Additionally, the analogy of the secondary structures and proteolytic processing profile between APP and Notch is also consistent with APP functioning as a cell surface receptor similar to Notch (Selkoe and Kopan 2003). Fibrillar forms of Aβ were reported to bind cell surface APP (Lorenzo et al. 2000), and Nogo-66 receptor was also shown to interact with the APP ectodomain, interaction which affects Aβ production (Park et al. 2006). Further evidence came from the Ho and Sudhof (2004) study, which demonstrated that the APP extracellular domain binds to F-spondin, a neuronally secreted glycoprotein, and that this interaction regulates APP cleavage and subsequent Aβ production and downstream signaling. More recently, Ma et al. (2008) identified TAG1 (a GPI-linked recognition molecule of CNS) as a functional

ligand for APP, and suggested that this TAG1–APP signaling pathway was involved in the modulation of neurogenesis.

APP involvement in cell adhesion

Convincing data places APP as a CAM (cell adhesion molecule) and SAM (subtrate adhesion molecule). Neuronal CAMs play an important role in neuronal plasticity and thus learning and memory processes may be closely linked to CAM function and any disruption in CAM interactions may have potential neuropathological consequences (Cotman et al. 1998). Cell surface APP has been described to enhance neuronal cell adhesion and neurite outgrowth (Breen et al. 1991). APP possesses several ectodomains (Figure 8) that promote binding to specific substrates such as heparin, collagen and laminin (extracellular matrix components), supporting its role in cell-substratum adhesion. The same sequences have been shown to be involved in cell-cell interactions. "RHDS" motif (Figure 8) within the Aβ sequence appears to also promote cell adhesion. In particular, cell surface APP has been recently reported to trans-interact with other APP (or APLP) molecules by homo- or hetero-dimer formation at the surface of adjacent cells, and that these trans-dimerizations promote trans-cellular adhesion *in vivo* (Soba et al. 2005). Furthermore, down-regulation of APP using antisense oligonucleotides also reduced neuronal adhesion to specific substrata, and APP overexpression in the neuronal-like B103 cells led to more rapid cellular adhesion (Schubert et al. 1993). Moreover, fibroblasts from FAD patients that were observed to have down-regulated APP mRNA levels, presented decreased cellular adhesiveness (Ueda et al. 1989), and Hep-1 cells expressing an APP FAD mutant cDNA also exhibited decreased cell adhesion properties (Kusiak et al. 2001).

APP and cell motility regulation

Via its intracellular domain APP can bind several proteins, including the adapter protein Fe65. The latter binds a second protein, MENA, which is a cytoskeletal protein expressed in active actin remodelling areas such as axonal growth cones. In H4 neuroglioma and MDCK cells, Fe65 has been found associated with MENA in active actin areas. The functional role of this ternary complex was documented in non-neuronal cells, in which co-expression of APP and Fe65 drastically increases cell motility, and this process appears to be partially dependent on the MENA/actin complex (Sabo et al. 2001). Subsequent analysis from the same group showed that in primary neurons, the APP and Fe65 complex is localized to the dynamic adhesion sites (actin-rich sites) in the growth cone (Sabo et al. 2003). Taken together these data supports a role for APP/Fe65 complex in cell motility and growth cone dynamics.

APP and sAPPα have a role in neurite outgrowth and synaptogenesis

This function is probably the most consistent and well documented since several overexpression and downregulation studies have addressed this issue. Neurotrophic and synaptogenic roles have been attributed to both APP and sAPPα (APP secreted fragment resulting from α-secretase cleavage, see section 1.5.4). As such APP may exert these activities in both an autocrine and a paracrine mode. APP is expressed at neuronal synapses and exhibits widespread expression in vesicular structures of cell bodies, axons and dendrites. It undergoes rapid anterograde transport and is targeted to the synaptic sites (Koo et al. 1990a; Sisodia et al. 1993; Yamazaki et al. 1995). During neuronal maturation and development APP expression has been described to be upregulated (Hung et al. 1992; Bibel et al. 2004), and correlated with periods of intense neuritic outgrowth and synaptogenesis (Loffler and Huber 1992; Moya et al. 1994). Moreover, cultured hippocampal neurons derived from APP knockout (KO) mice exhibit both reduced viability and neuritic outgrowth (Perez et al. 1997). Other studies directly demonstrated the importance of sAPPα (lacking APP C-terminal domain) in these functions. *In vivo* application of sAPPα causes neurite outgrowth in cultured fibroblasts (Saitoh et al. 1989;

Bhasin et al. 1991), PC12 cells (Milward et al. 1992), cortical and hippocampal neuronal cells (Araki et al. 1991; Qiu et al. 1995; Ohsawa et al. 1997) and human neuroblastoma cell lines (Wang et al. 2004). More recently, studies in mice overexpressing ADAM-10 (a secretase involved in APP cleavage to sAPPα) and perfused with exogenous sAPPα, were shown to present neurotrophic effects on cortical synaptogenesis (Bell et al. 2008). Of interest, the pentapeptide sequence "RERMS" located C-terminal to the KPI and OX-2 domains (Figure 8) was identified as the site responsible for the growth-promoting trophic role of sAPP (Ninomiya et al. 1993). Infusion of this peptide or sAPP into brain animals resulted in improved memory and increased synaptic density (Roch et al. 1994; Meziane et al. 1998). Further, neurotrophic and neuroprotective effects are induced by sAPPα approximately 100 times more strongly than by sAPPβ (resulting upon APP cleavage by β-secretase, section 1.5.4). Indeed, Li and colleagues (1997) showed that sAPPβ lowered neurite outgrowth below control levels. Although the conserved regions of both proteins contain domains that have been associated with these functional effects (such as "RERMS" region), the difference between these sAPP effects seems to subsist in their C-terminal region, where sAPPα contain 17 more aminoacids. This region of sAPPα contains a heparin-binding domain ("VHHQK" residues 12-16 of Aβ), that is lacking in sAPPβ, and appears to play a key role in mediating the neurotrophic effects (Furukawa et al. 1996).

APP, sAPP and Aβ functions in memory

APP appears to have an important role in the regulation of synaptic structure and neuronal function. Sequence-specific antibodies were used to block protein function and antisense oligonucleotides to prevent APP translation. APP association with improving memory was supported by studies were infusion of "RERMS" sequence rescued amnesia that was induced by anti-APP antibodies (Mileusnic et al. 2000). Additionally, intraventricular injection of anti-N-terminal APP antibodies (for example delivery of the 22C11 antibody targeting the N-terminal part of APP) in rat, close to the training period, has been shown to result in an impairment of rat memory in a passive avoidance task (Doyle et al. 1990; Huber et al. 1993; Gschwind et al. 1996; Turner et al. 2003). In the case of APP-null mutations, mice show a variety of alterations in neuronal structure and function, including gliosis, decreased neocortical and hippocampal levels of synaptophysin, reduced dendritic length in hippocampal neurons, reduced survival of cultured neurons and impaired long-term potentiation (LTP) (Perez et al., 1997; Dawson et al., 1999; Chapman et al., 1999; Seabrook et al., 1999; but see Phinney et al., 1999). However these effects could also be due to the loss of the neurotrophic sAPPα fragment. Consistently, exposure of hippocampal slices to sAPPα results in raising the threshold for long term depression (LTD) but facilitating LTP (Ishida et al. 1997) thus changing synaptic efficacy. Since memories are believed to be stored within synapses, LTP and its opposing process, LTD, are widely considered the major cellular mechanisms underlie learning and memory (Cooke and Bliss 2006).

Contrary to APP or sAPPα effects, Aβ peptide has been associated with synaptic dysfunction and inhibition of LTP (Cullen et al. 1997; Lambert et al. 1998; Hsia et al. 1999; Walsh et al. 2002a; Raymond et al. 2003; Turner et al. 2003), although downstream mechanisms are not well understood. Further, studies in transgenic mice models of AD have shown that Aβ immunization reduces plaque deposition and improves cognitive function (Bard et al. 2000; Lombardo et al. 2003; Oddo et al. 2004; Brendza et al. 2005; Buttini et al. 2005). All these data strengthen a role for APP and APP proteolytic fragments in learning and memory processes.

APP and sAPP involvement in brain repair and neuroprotection

Several studies have provided evidence that APP plays a role in the repair of adult brain following trauma. In fact, APP has been reported to be upregulated in response to neuronal injury and damage (Siman et al. 1989; Gentleman et al. 1993; Struble et al. 1998). Up-regulation of APP in response to neuronal injury appears to promote axonal arborization (Leyssen et al. 2005). Perez et al. (1997) showed that APP-deficient neurons exhibited shorter axons and branching processes. These effects were rescued when hippocampal neurons from APP-deficient mice were co-cultured with wild-type astrocytes, suggesting that APP contribute to axon and dendritic outgrowth and arborization. Consistent with a possible role for APP in axonal repair mechanisms, following traumatic brain injury *in vivo* administration of sAPPα has been shown to improve functional outcome and to reduce neuronal apoptosis and axonal injury (Thornton et al. 2006).

Metal ions such as copper are essential in brain, since it is an essential cofactor of oxidoreductive enzymes, including superoxide dismutase that is involved in cellular protection induced by oxidative stress. APP structure harbours several metal-binding domains for copper (Figure 8), in agreement with an APP involvement in the transport of this metal into the brain. Studies using APP KO mice have shown increased copper levels in the cerebral cortex, supporting a possible physiological role for APP in modulating copper neuronal homeostasis *in vivo* (White et al. 1999; Bayer and Multhaup 2005; Inestrosa et al. 2005; Maynard et al. 2005).

sAPP secreted fragment has also been implicated in neuroprotection (reviewed in Mattson 1997), including against Aβ effects and induced apoptosis. Stimulation of sAPPα secretion results in protective effects against Aβ-induced neurotoxicity (Levites et al. 2003). Also, in human keratinocytes, Wehner and colleagues (2004) have shown that at a nanomolar range sAPPα was able to protect these cells against induced apoptosis. Further, sAPPα was shown to increase expression levels of several neuroprotective genes and protect against Aβ induced-tau phosphorylation and neuronal death (Stein et al. 2004). Further, the APP N-terminal heparin binding domain was also reported to display a neuroprotective function (Small et al. 1999). Hence, it is clearly evident that

sAPPα fragments play a key role in neuroprotection and factors contributing to its decreased production or secretion will result in neuronal toxicity and death.

Aβ and CTF contribution to neurotoxicity and apoptosis

Several authors suggest that Aβ is at the heart of AD being either directly or indirectly responsible for the pathological mechanisms and altered cellular responses that culminate in the disease (Figure 6). This theory is supported by several studies where Aβ was shown to be toxic to various cultured cell types (Walsh et al. 2002b; Smith et al. 2006), and also to have long-term effects when injected directly into the brain (McKee et al. 1998). Of note is that Aβ is found in a variety of forms and aggregation states which affect its neurotoxic properties. Soluble oligomeric forms can produce sub-toxic effects on neuronal function (Klein et al. 2001; Walsh and Selkoe 2004), and the more extensively aggregated forms (fibrilar Aβ) have also been show to be potent neurotoxins able to impair neuronal function (Selkoe 1994a; Klein 2002). Both forms are usually present at the same time in brain, making it difficult to evaluate the effects of plaques and oligomers independently. Hence, debate arises regarding which Aβ state is more toxic, the soluble or the more aggregated one (Lue et al. 1999; Watson et al. 2005). Although initial fibrilar amyloid aggregates were considered the main neurotoxic forms, currently it is proposed that soluble olygomeric pools may be more important in synaptic dysfunction and consequent memory loss (Lue et al. 1999; McLean et al. 1999). It has been reported that gross amyloid deposition, although contributing to AD pathology, may be less toxic and, at least initially, does not impair memory (Walsh and Selkoe 2004; Lesne et al. 2008). Mechanisms underlying Aβ toxicity include mitochondrial dysfunction (reviewed in Chen and Yan 2007), disruption of calcium homeostasis, altered energy metabolism, and production of reactive oxygen species (ROS) that results in increased oxidative stress (reviewed in Chauhan and Chauhan 2006 and Hamel et al. 2008). Oxidative stress, induced by Aβ or other factors, can trigger apoptosis associated events, activating caspases or increasing expression of apoptotic related proteins. Concordantly, it has been demonstrated that $A\beta_{1-42}$ is toxic to human neurons through activation of p53 and Bax proapoptotic pathway (Zhang et al. 2002; Ohyagi et al. 2005).

Other potential mechanisms may contribute to neurotoxicity, including secretase cleavage to release both CTFs and AICD (section 1.5.4). CTFs have been associated with impaired calcium homeostasis and learning and memory through blocking LTP and overexpression of CTFs has the potential to elicit neurodegeneration *in vivo* (Sato et al. 1997; Kim et al. 2004; Chang and Suh 2005).

Signal transduction and AICD transcriptional regulation

Several groups have suggested the generation of signaling proteins by "regulated intramembrane proteolysis" (RIP) as a new paradigm of signal transduction. RIP occurs when a transmembrane protein is cleaved within the transmembrane domain, releasing an intracellular fragment that can act directly in the nucleus to activate gene expression. This occurs in two steps. Cleavage of the protein outside the membrane (usually in response to ligand binding) results in a conformational change, which in turn triggers a second intramembrane cleavage event that releases an active cytoplasmic fragment. The latter is able to translocate to the nucleus and activate gene expression (Ebinu and Yankner 2002). Consistently, following intramembranous γ-secretase cleavage, the APP intracellular domain (AICD) is released into the cytoplasm and tends to relocalize to the nucleus (Cupers et al. 2001b; Kimberly et al. 2001; Kinoshita et al. 2002) where it can function as a transcriptional regulator. AICD is very labile but it can be stabilized by the adaptor protein Fe65 (Kimberly et al. 2001). Additionally, Cao and Sudhof (2001) demonstrated that Fe65 can bind to a transcriptionally relevant protein, Tip60, which is a histone deacetylase enzyme that is involved in regulating chromosome structure and consequent gene expression (Kimura and Horikoshi 1998).

AICD is shown to form a transcriptionally active complex presumably in the nucleus together with Fe65 and Tip60 (Cao and Sudhof 2001), although AICD can bind Tip60, apparently independently of Fe65 (Kinoshita et al. 2002) also inducing gene expression. However, the nature of how AICD leads to activation of signaling pathways is unclear. More Recently, Cao and Sudhof (2004) showed that nuclear translocation of AICD is not required but may be indirect through Fe65 (Figure 9).

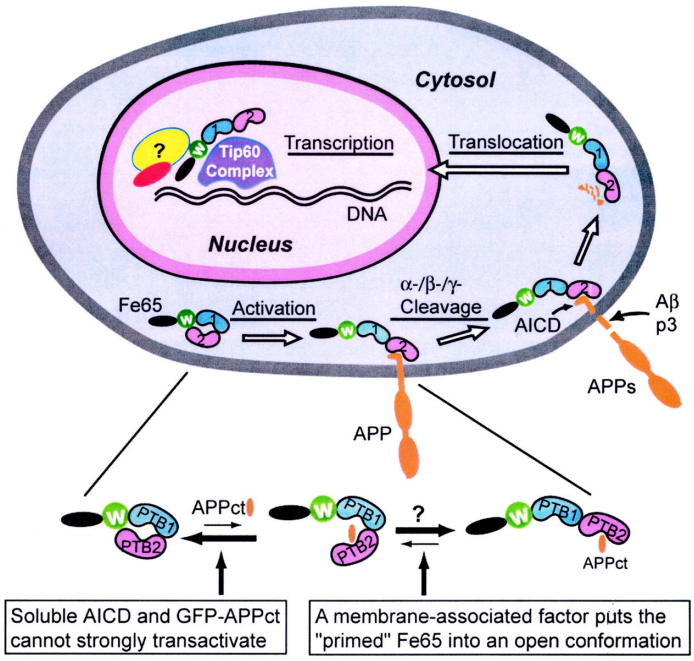


Figure 9. Cao and Sudhof proposed model for APP transcriptional activation.
From Cao and Sudhof 2004.

In this model the proposed AICD role in the transactivation process consists in activating Fe65, via conformational changes, for nuclear binding with Tip60. AICD binding to Fe65 at its PTB2 domain would disrupt Fe65 PTB2-WW intradomain binding, thus altering Fe65 initially closed conformation. The "primed" Fe65 open conformation will make available the Fe65 WW domain for its transactivator role upon nuclear translocation. Hass and Yankner (2005) also reported that AICD production is not required for the nuclear signaling activity as it proceeds normally even in the presence of γ-secretase/PS inhibitor treatment.

Finally, while some authors documented that the proposed signaling activity is executed by Fe65 and that APP is not required (Yang et al. 2006), others suggest that phosphorylation of APP at threonine 668 (Thr668, located in the ^{667}VTPEER^{672} APP motif) residue is required for Fe65-dependent gene transactivation (Nakaya and Suzuki 2006). In the latter study phosphorylation of APP liberated membrane-bound Fe65 allowing it to be translocated into the nucleus where it up-regulates gene transactivation, while AICD nuclear translocation does not required phosphorylation of this APP residue. Therefore,

phosphorylation of APP, but not AICD, modulates Fe65-dependent gene transactivation by AICD by regulating Fe65 intracellular localization.

Despite the controversy around the potential nuclear signaling activity, the trans-activating role of the APP/Tip60/Fe65 complex has been well documented, at least in overexpressing systems. Several downstream targets for AICD dependent gene regulation have been identified including, neprilysin, a neuronal endopeptidase with Aβ degrading activity (Pardossi-Piquard et al. 2005); *KAI1*, a tumor suppressor gene also thought to be involved in membrane receptor function regulation; glycogen synthase kinase-3β (*GSK3β)*, a kinase involved in Tau phosphorylation; *Tip60*; *BACE*, a secretase involved in APP processing; *APP* itself (reviewed in von Rotz et al., 2004); and *alpha2-actin* and *transgelin*, known to be involved in the organization and dynamics of actin cytoskeleton (Muller et al. 2007). A different AICD-containing complex, with Fe65 and the ternary complex CP2/LSF/LBP1, was also found to promote the expression of the GSK-3β (Kim et al. 2003). The regulation of both alpha2-actin and transgelin genes appears to be of relevance since cytoskeleton organization changes have been reported in AD brains (Pollak et al. 2003), but this will be further discussed. AICD transcriptional up-regulation of neprilysin, *APP* and *BACE* is of particular interest because it suggests that AICD release may regulate Aβ degradation (by increasing neprilysin expression) or production, in the latter case either by increasing APP substrate for secretases or by increasing amyloidogenic processing of APP by BACE (Kerr and Small 2005).

Aβ physiological concentration and physiological function

During the disease process the balance between Aβ production and Aβ catabolism is altered which results in increased Aβ accumulation and aggregation and subsequent neurotoxicity and neuronal death in specific brains areas. In AD brains soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations were in general drastically increased, by 100-fold in superior frontal gyrus (thought to contribute to cognitive functions and memory) and by more than 50-fold in entorhinal cortex (important memory center in brain), when compared to non-demented patients (Lue et al. 1999). For instance, in entorhinal cortex insoluble $A\beta_{1-40}$ concentrations were around 0.8 μg/g for the non-demented (ND) group versus 53.7 μg/g for the AD group, and insoluble $A\beta_{1-42}$ concentrations were 8.3 μg/g versus 117.3 μg/g, respectively. Concentrations of soluble $A\beta_{1-40}$ forms were around 1.9 pg/g in ND and 66.5 pg/g in AD cases, while for $A\beta_{1-42}$ they were 0 pg/g and 15.5 pg/g, respectively. In the same study CSF concentrations obtained for ND was 232.4 pg/ml and 162.9 pg/ml for AD. Consistently, in AD CSF soluble $A\beta_{42/40}$ ratio is decreased probably reflecting amyloid accumulation in brain blood vessels (Pirttila et al. 1996; Hock et al. 1998; Wiltfang et al. 2007).

Despite its altered production and accumulation in AD, Aβ is a naturally occurring peptide produced at low levels during normal APP metabolism (Haass et al. 1992), not only in CSF but also in brain. These data suggest that it may play a role in normal cell function, that may differ from it neurotoxic effects when is levels are elevated in AD. These peptides form a heterogeneous group of 38–43 amino acids in length, whereby $A\beta_{1–40}$ clearly predominates. Under physiological conditions, the fraction of $A\beta_{1-42}$ is only 10% of the total fraction (Haass et al. 1992b; Seubert et al. 1992).

Aβ is likely to have an important physiological role given that in primary neuronal cultures inhibition of endogenous Aβ production by secretase inhibitors induced neuronal apoptosis, and co-incubation of $A\beta_{1-40}$ with these inhibitors prevented this apoptotic effect (Plant et al. 2003). This effect appears to be neuron-specific since a variety of non-neuronal cells were unaffected by the same treatments. Some neurotrophic properties have also been attributed to this peptide (Tanimukai et al. 2002; Atwood et al. 2003).

Further, several lines of evidence indicate that Aβ may play a role in controlling synaptic plasticity. Kamenetz et al. (2003) showed that neuronal activity modulates the formation and secretion of Aβ peptides in neurons that overexpress APP. In turn, Aβ selectively depresses excitatory synaptic transmission onto neurons that overexpress APP. Hence, at physiological levels of APP, Aβ may provide a negative feedback. Without such depression, synaptic activity could become excessive, leading to excitotoxicity (reviewed in Pearson and Peers 2006). The underlying mechanisms still have to be clarified but it may involve inhibition of NMDA receptor mediated currents (Chen et al. 2002) or altered K+ channel expression (Kamenetz et al. 2003). K+ channels activity have been implicated in neuronal survival or death, in part because they govern excitability and hence the excitotoxicity of released glutamate, but also because cellular [K+] is a key determinant in apoptosis (Yu 2003). It would be predictable from these findings that APP KO transgenic animals show severe neurological deficits and lethality, but this is not the case. APP-null mice show reduced branching of dendrites and fewer synaptic buttons but no reduction in neuronal number despite an absence of Aβ (Dawson et al. 1999). Despite these *in vivo* observations, cell culture findings provided compelling evidence for an Aβ role in neuronal survival, at least at physiological levels. Hence, Aβ should not be regarded as a mere toxic factor that should be eradicated in order to avoid dementia. Therefore it is of extreme importance to understand the molecular mechanism underlying Aβ effects not only at physiological low levels but also at higher levels, in attempt to prevent Aβ neurotoxic and apoptotic effects.

1.5.4 APP proteolytic processing and Aβ genesis

APP metabolism is complex and can occur via several pathways (Nitsch et al. 1994; Checler 1995; Buxbaum and Greengard 1996; Selkoe et al. 1996) which have not been fully elucidated. In its simplest form APP processing can be described as occurring at least via two distinct pathways which differ in the proteases involved and in the resulting fragments generated (Figure 10 and Figure 11). These pathways are often referred as the non-amyloidogenic and the amyloidogenic pathways; the former precludes Aβ formation and the latter gives rise to the amyloidogenic peptide (Small and McLean 1999; Nunan and Small 2000; Sabo et al. 2001). APP proteolytic cleavage involves catalytic activity of three proteinases, termed α-, β- or γ-secretases, at specific sites (Esler and Wolfe 2001).

In the non-amyloidogenic pathway cleavage by α-secretase within the Aβ domain (Lys^{16}, also corresponding to α-site in Figure 11) results in the release of nearly the entire ectodomain, yielding a 612 amino acid long soluble APP derivate, sAPPα (Esch et al. 1990; Busciglio et al. 1993), and generation of membrane-associated 83 amino acid long C-terminal fragment (CTFα, with ~10 KDa). The latter is in turn processed by the γ-secretase to generate the so-called P3 peptide (3 KDa) and the APP intracellular domain (AICD, also referred to CTFγ). Alternatively, in the APP amyloidogenic pathway β-secretase cleavage (Figure 10) also results in the formation of a 595 amino acid long N-terminal fragment which is released from membrane (sAPPβ) and a membrane-bound 99 amino acid long C-terminal derivative (named CTFβ with ~12 KDa). Following extracellular cleavage CTFβ is processed by γ-secretase within transmembrane domain, releasing the Aβ peptide and generating the 59/57 amino acid long AICD (with ~4 KDa). Of note is that γ-secretase cleavage may generate different Aβ species. CTFβ may be processed by γ-secretase (Younkin 1998; Xu et al. 2002) to generate predominantly $Aβ_{1-40}$ (~90%) or $Aβ_{1-42}$ (~10%) peptides (Haass et al. 1992b; Seubert et al. 1992). Aβ species spanning from 1-38 to 1-43 amino acids long can also be produced, with fragments appearing to be more amyloidogenic with increasing length.

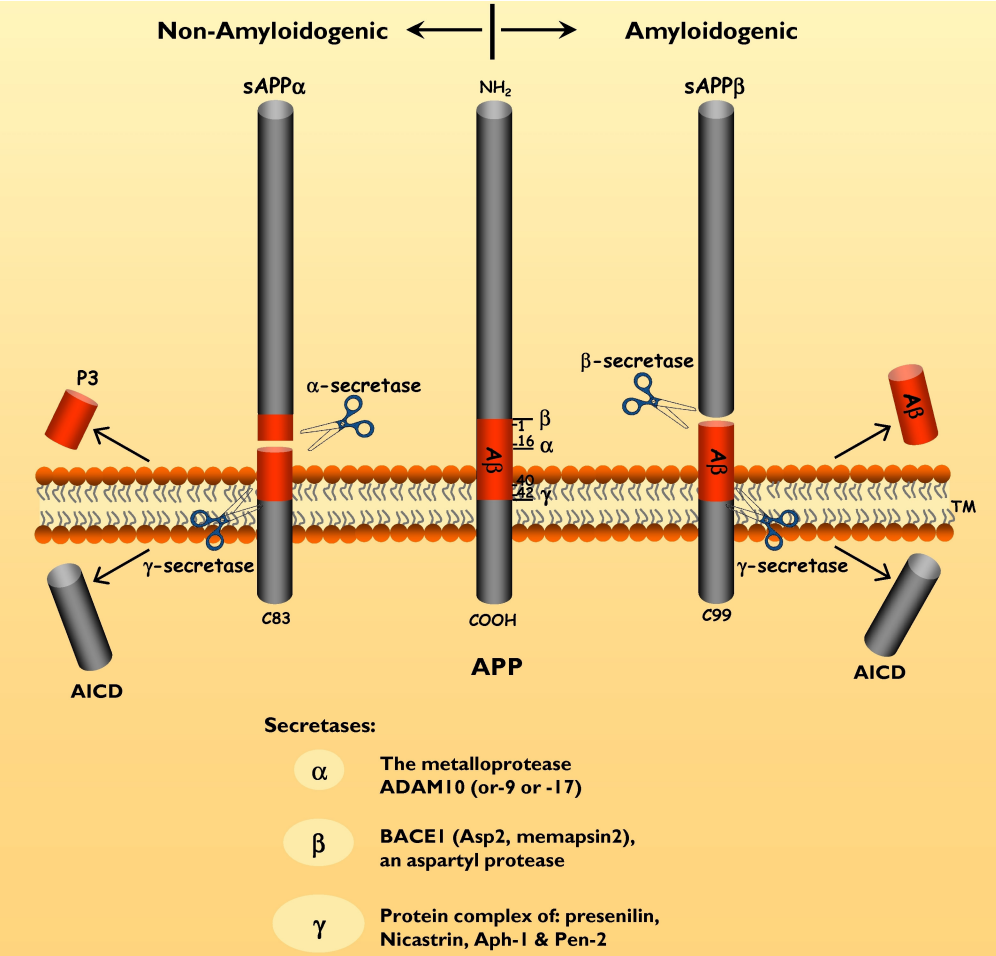


Figure 10. Schematic diagram of APP proteolytic processing. In its simplest form APP processing occurs via two distinct proteolytic pathways. One is known as the non-amyloidogenic pathway (left), precludes Aβ formation and involves sequential α- and γ-sectarese cleavage. The other is known as the amyloidogenic pathway (right) because it gives rise to Aβ production and involves β- and γ-secretase cleavage. TM, transmembrane domain. Aβ domain is highlighted in red.

Sequence analysis of the AICD fragment showed that γ-secretase cleaves APP at an additional site (ε-site) (Sastre et al. 2001), a few amino acids downstream of the γ-site (Figure 11). Even though both cleavages are independent, they may occur nearly simultaneously (for review Selkoe and Kopan 2003), with the ε-site cleavage thought to occur for better AICD membranar release. Simultaneous γ- and ε-cleavages yield a 50 amino acid long AICD peptide, making it difficult to know which AICD peptide (C59/57 or C50) is physiologically functional (Selkoe and Kopan 2003). Nonetheless, evidence points to the C50 AICD as the physiologically functional peptide (von Rotz et al., 2004).

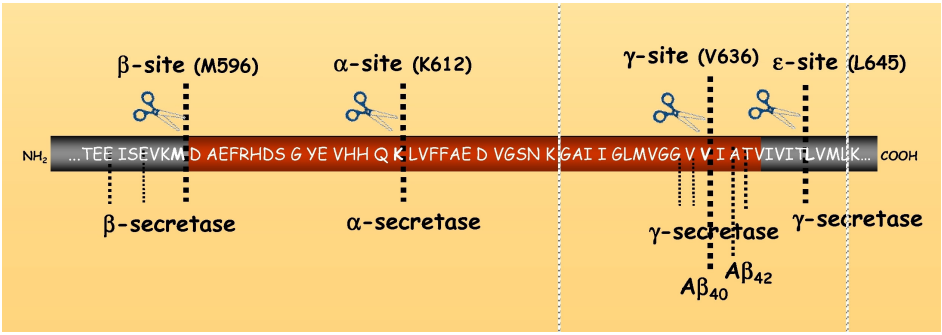


Figure 11. APP secretases cleavage sites. α-secretase cleavage precludes Aβ formation while sequential β-secretase and γ-secretase leads to the production of 38-43 amino acid long Aβ peptides (depending where γ-secretase cleavage occurs). The major secretase cleavage sites for APP are: Met^{596} for β-secretase, Lys^{612} for α-secretase, and Val^{636} (γ-site) and Leu^{645} (ε-site) for γ-secretase (APP_{695} isoform numbering). Aβ is highlighted in red and transmembrane domain defined by the two lightly lines.

Additional reports have revealed that APP can also be proteolytic processed at it C-terminus by caspases, like caspase-3 (Gervais et al. 1999), caspase-6 and -8 (Pellegrini et al. 1999), or caspase-9 (Lu et al. 2000). The target residue for the caspase-directed APP cleavage is Asp^{664} (Gervais et al. 1999; Weidemann et al. 1999; Zambrano et al. 2004). The resultant C-terminus C31 peptide is a potent inducer of apoptosis, and this cleavage was shown to reduce APP internalization but to have varying effects on the secreted levels of Aβ (Pellegrini et al. 1999; Lu et al. 2000; Soriano et al. 2001).

APP Secretases

Considerable work has been dedicated to identifying all the secretases involved in these pathways in an attempt to understand how secretase activities, APP processing and subsequent Aβ production may be regulated (for review see Nunan and Small 2000).

Likely candidates for ***α-secretase*** activity include several zinc metalloproteinases such as tumor necrosis factor α converting enzyme (TACE/ADAM17), ADAM9 (MCD9), ADAM10, which can cleave APP at the α-secretase site located within the Aβ domain (reviewed in Allison et al. 2003 and Marks and Berg 2008), thus precluding generation of intact Aβ. The aspartyl protease BACE2 has also been reported to have α-secretase like activity by efficiently cleaving APP within Aβ (Phe^{19} and Phe^{20}) (Yan et al. 2001). All these candidates have inherent α-secretase activity or are thought to be somehow involved in the regulation of α-secretase (Sabo et al. 1999). While the cleavage is constitutive and therefore depends on an enzyme that is active at all times, it can also be increased by substances that activate protein kinase C (PKC), such as metabotropic glutamate receptor agonists (Kirazov et al. 1997; Nitsch et al. 1997; Nakaya and Suzuki 2006) and phorbol ester activation (Buxbaum et al. 1990; Caporaso et al. 1992; da Cruz e Silva et al. 1993; Gabuzda et al. 1993; Marambaud et al. 1997). TACE ability to cleave APP constitutively was demonstrated using TAPI-1 inibitor in HEK293 cells, which lead to decrease sAPPα release (Slack et al. 2001). Further, TACE KO mice showed deficiencies in sAPPα secretion supporting that it may also cleave APP (Buxbaum et al. 1998; Merlos-Suarez et al. 1998). This data is in agreement with Checler et al. (2005) data who observed that 90-95% of sAPPα production was due to TACE activity in fibroblasts. Nitric oxide, a retrograde messenger in synaptic transmission, activates TACE *in vitro* and α-secretase *in vivo* (Zhang et al. 2000), consistent with TACE α-secretase activity and additionally supporting the APP ectodomain as a modulator of synaptic plasticity. Co-transfection of APP with ADAM10 or MDC9 results in increased (constitutive and regulated) sAPPα secretion. ADAM10 cleaves *in vitro* the Aβ peptide at the Lys^{16}-Leu^{17} bond (corresponding

to α-site in Figure 11, APP_{695} isoform numbering), and the Flemish mutation Ala^{21}-Gly reduces this cleavage (Lammich et al. 1999).

The major neuronal **β-secretase** is a transmembrane aspartyl protease, termed BACE1 (also termed Asp2 or memapsin2) for β-site APP cleaving enzyme expressed in neuronal tissues (Vassar et al. 1999; Yan et al. 1999; Bennett et al. 2000a; Vassar 2004). This aspartic proteinase is generated as a proenzyme, whose activation is mediated by furin or a furin-like enzyme immediately before full maturation (N-glycosilation) and trafficking trough the Golgi (Bennett et al. 2000b; Capell et al. 2000; Huse et al. 2000). β-secretase cleaves APP at the N-terminal side of Asp1 of the Aβ sequence (also corresponding to β-site in Figure 11, APP_{695} isoform numbering). It has been demonstrated that this APP cleavage occurs at positions 1 and 11, since cells overexpressing BACE1 exhibited an increase in secreted levels of $Aβ_{1-40/42}$, and $Aβ_{11-40/42}$ (Vassar et al. 1999). The former is the major cleavage site for β-secretase cleavage. Consistently with the β-secretase activity of BACE1, this membrane-anchored enzyme was reported to cleave APP carrying the FAD Swedish double mutation 10-fold more efficiently than wild-type APP (Yan et al. 1999). Furthermore, BACE1 KO mice showed a decrease in Aβ production and in β-cleavage (Cai et al. 2001; Luo et al. 2001), but given that rat were phenotypically normal (Roberds et al. 2001) it was proposed that inhibition of the β-site secretase pathway is not toxic and could be of therapeutic value. It was also recently demonstrated that *in vivo* BACE1 inhibition lead to a decrease in brain $Aβ_{1-40}$ and $Aβ_{1-42}$ levels, with a concomitant increase in sAPPα levels, without affecting neuregulin processing (Sankaranarayanan et al. 2008).
In humans, BACE2 has also been reported to cleave APP at Aβ position 1, however only BACE1 is significantly expressed in brain (Bennett et al. 2000a). Carboxypeptidase B was another β-secretase candidate identified by Matsumoto et al. (2000), which in contrast to BACE enzymes lacks a transmembrane domain. Carboxypeptidase B is a soluble enzyme located in the cytosol of various neurons and some microglial cells, especially in the hippocampus. *In vitro* or when overexpressed in cells it leads to increased CTFβ levels. Additional research may identify novel functions for this enzyme, since it is the only putative APP secretase that does not appear to be membrane-bound.

The **γ-secretase** activity is primarily executed by a multicomponent complex that contains at least 4 core components, including presenilin (PS1 or PS2), nicastrin, anterior pharynx defective 1 (APH1) and presenilin enhancer (PEN2) (Figure 12) (De Strooper 2003; Capell et al. 2005; Gandy 2005). The simultaneous expression of these components in yeast, an organism that lacks any endogenous γ-secretase activity, results in the reconstitution of γ-secretase complex formation and activity, demonstrating that the 4 membrane proteins are the core components of the complex (Edbauer et al. 2003).

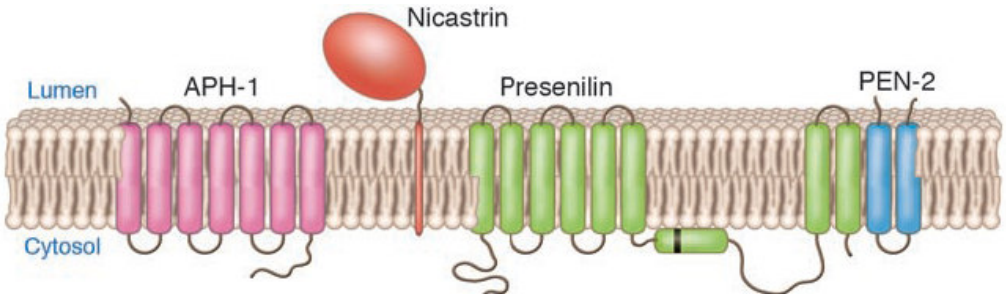


Figure 12. Topology of the 4 components that comprise the high molecular weight γ-secretase complex. Black bar represents the cleavage site for processing of the zymogen form of PS1 into the amino and carboxyterminal fragments that self associate and form the active enzyme (from Gandy 2005).

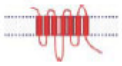
The γ-secretase complex is responsible for cleaving APP at the C-terminus of the Aβ fragment, a site which is located within the APP transmembrane domain (reviewed in Wolfe et al. 1999). This γ-secretase complex has also been responsible for cleaving the Notch protein, a well known signal transducer, at its intramembranous sequence (Berezovska et al. 2000; Selkoe and Kopan 2003). In an identical manner both Notch and APP apparently need proteolytic cleavage of the extracellular domain to become a substrate for γ-secretase (Mumm et al. 2000; Struhl and Adachi 2000; Berezovska et al. 2001).

The presenilins (PS) appear to provide the active core of the protease. Two mammalian homologs, PS1 and PS2, exist. The PS1 and PS2 are integral membrane proteins possessing at least eight putative transmembrane domains, expressed initially as full-length proteins that are thought to be inactive (Thinakaran et al. 1996). Two aspartate residues (Asp^{257} and Asp^{385}) located in transmembrane domains 6 and 7, respectively are essential for the catalytic activity of the protease. Cleavage between these transmembrane domains yields a C-terminal fragment and an N-terminal fragment, which remain associated with each other in a multiprotein complex of high molecular weight (minimal 200-250 kDa) (Kimberly et al. 2003). PS1 and PS2 are not found in the same complexes, but their tissue specific expression profiles can overlap (Saura et al. 1999). PS1 appears to be more responsible for CTF cleavage than PS2 (Herreman et al. 1999). Accordingly, PS1 KO mice have markedly reduced γ-secretase activity (De Strooper et al. 1998) as denoted by a lowered amount of Aβ and P3 production, consistent with membrane accumulation of the CTFs arising from APP β and α cleavage. In double KO mice for PS1 and PS2 γ-secretase activity was shown to be even more reduced (Donoviel et al. 1999; Herreman et al. 1999). Of note, in PS1/PS2 double KO mice there is a lack of $Aβ_{1-42}$, but not of $Aβ_{1-40}$, production in the early secretory pathway (Wilson et al. 2002), suggesting that there might be several γ-secretase enzymes with different selectivity for producing $Aβ_{1-40}$ and $Aβ_{1-42}$ (Wolfe and Haass 2001).

Another component of γ-secretase is nicastrin, which also affects the complex's enzymatic activity (Esler et al. 2000). This protein interacts with C-terminal fragments of APP and PS1 and PS2 (Yu et al. 2000). Nicastrin has four conserved cysteine residues near its extracellular N-terminus (Leem et al. 2002) and mutations in these residues lead to enhanced Aβ secretion, while deletion of the same region prevents Aβ secretion (Yu et al. 2000). Noticeably, it has been demonstrated that nicastrin trafficking and post-translational processing is affected in presenilin double KO mice, which provide evidence that presenilin is responsible for directing assembly of the γ-secretase complex (Leem et al. 2002).

The other proteins identified as γ-secretase complex components were APH-1 and PEN-2 (Francis et al. 2002; Steiner et al. 2002; for review De Strooper 2003). Recent reports provide evidence that the γ-secretase complex is formed as an APH/immature nicastrin complex at the ER, which is further matured at the Golgi. At the Golgi, APH/nicastrin first bind to PS, stabilizing the unstable and readily degraded PS pool, and following binding of PEN-2. PS endoproteolysis (called PS maturation) is required for the γ-secretase complex to become active (Kim and Sisodia 2005). Although the catalytic activity of γ-secretase complex resides in the 2 aspartate residues of PS1, the 4 proteins participate in the regulation of each other´s activation and/or maturation associated with the active γ-secretase complex (Table 1), and are required to reconstitute γ-secretase in mammalian cells (Kimberly and Wolfe 2003) and in yeast (Edbauer et al. 2003).

Table 1. γ-secretase complex members

Name (Generic)		*C. elegans*	Mammalian	Functions
Presenilin		HOP-1, SEL-12 (PSEN in *Drosophila*)	PS1, PS2	Catalytic subunit and assembly of γ-secretase (glycosylation of Nct, Pen-2 expression); β-catenin phosphorylation
Nicastrin		APH-2	Nct	Assembly of γ-secretase (stabilization of PS, Pen-2)
Aph-1		APH-1 (PEN-1)	Aph1aL and Aph1aS; Aph1b (Aph1c in rodents)	Assembly of γ-secretase (stabilization of PS/Nct)
Pen-2		PEN-2	Pen-2	Assembly of γ-secretase (proteolytic processing PS)

C. elegans components and mammalian homologs, and functions of the different γ-secretase components. (From De Strooper et al. 2003).

1.5.5 APP trafficking

APP processing events occur in various organelles during it normal secretory pathway and also at the cell surface. In both neuronal and non-neuronal cells, APP is known to be transported via the secretory pathway, a continuum transport in separate membrane-enclosed organelles that ultimately reach the cell surface. Throughout this secretory transport, post-translational modifications of the newly synthesized APP proteins may occur as well, which may influence APP cleavage and Aβ production.

APP subcellular trafficking and maturation

APP post-translational modifications include N- and O-linked glycosylation and tyrosine sulfation (Weidemann et al. 1989; Oltersdorf et al. 1990). In its mature form APP is both N- and O-glycosylated (Weidemann et al. 1989), while in its immature form APP is N-glycosylated only. Although APP N-glycosylation occurs at the endoplasmatic reticulum (ER) more carbohydrate chains can be further processed in the cis-Golgi, and thus immature N-glycated APP can be found in both subcellular organelles (Tomita et al. 1998). Nascent and immature APP can be degraded to a high degree by the proteasome, probably via the smooth ER (Yang et al. 1998; Kouchi et al. 1999), but if not degraded, APP maturation by O-glycosylation and tyrosyl-sulfation will occur while moving through the trans-Golgi network (TGN) (Weidemann et al. 1989) (Figure 13). Even though O-glycosylation does not depend on correct APP N-glycosylation to occur, it is a subsequent process since it occurs at the TGN (Pahlsson and Spitalnik 1996). Mature APP (N-, O-glycated and sulfated) is therefore located in compartments from the trans-Golgi to the plasma membrane (Tomita et al. 1998).

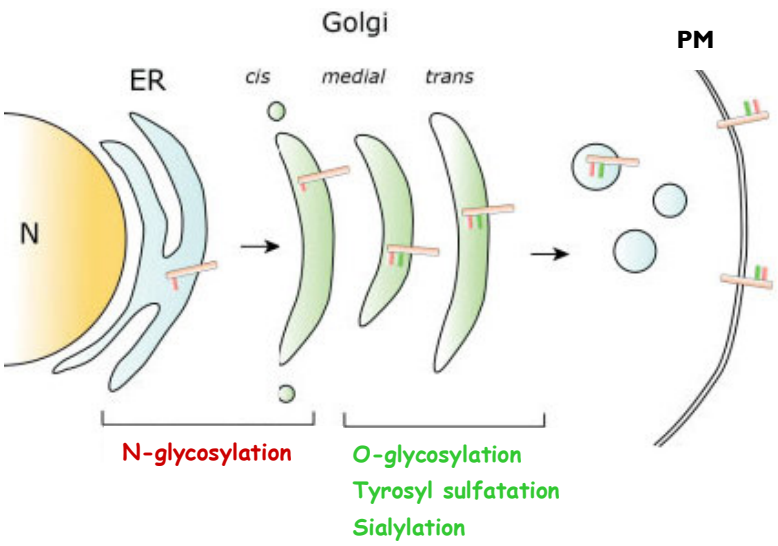


Figure 13. Post-translational modifications of APP along the classical secretory pathway. APP is subjected to N-glycosylation in the ER, O-glycosylation, tyrosyl sulfatation and sialylation in the Golgi apparatus, and then reaches the plasma membrane. N, Nucleus; ER, Endoplasmic reticulum; PM, Plasma membrane. (Adapted from Suzuki et al. 2006).

Maturation of metabolic labelled APP cell-associated proteins reached maximum intensity after 30 min of chase (Peraus et al. 1997). The pool of TGN mature APP in SH-SY5Y cells, takes 30 to 45 min to reach the plasma membrane (Cai et al. 2003). Alterations in the glycosylation state of APP have been reported to decrease secretion of the neuroprotective sAPPα (for review see Georgopoulou et al. 2001). Pahlsson et al. (1996) showed that APP N-glycosylation play a key role in APP biosynthesis and secretion. Additional, the majority of APP cleavage by secretases occurs after O-glycosylation, as shown by Tomita et al. (1998). In this report mutant APP defective in O-glycosylation accumulated in subcellular ER compartments and exhibited decreased cleavage to CTFα or to $A\beta_{1\text{-}40/1\text{-}42}$. Recently was described that APP sialylation enhanced extracellular levels of Aβ (Nakagawa et al. 2006). These data are in accordance with protein maturation playing a key role in APP processing and function.

In neurons, and due to their unique cell morphology with a long axon and a rich dendritic arbour, an elaborated protein trafficking exists enabling targeting of proteins to their designated compartments, or to be transported back (retrograde transport) to cell bodies. Protein processing and modifications are known to take place during the transit in axons and in dendrites. APP was detected in both pre- and post-synaptic sites, in the axoplasm of myelinated and unmyelinated nerve fibres, within vesicular structures in axonal, dendritic and synaptic compartments, as well as on the surface of axons and dendrites (Schubert et al. 1991; Ferreira et al. 1993; Allinquant et al. 1994; Caporaso et al. 1994; Simons et al. 1995). Presynaptically targeted full-length APP (and APLP2) occurs in its mature form (syalated and N- and O-glycosylated) (Lyckman et al. 1998).

Neuronal APP is transported in axons via fast anterograde transport machinery (Koo et al. 1990, Ferreira et al. 1993, Kaether et al. 2000), a process that requires direct or indirect association of APP with kinesin light chain (KLC) subunit, a component of kinesin-1 molecular motors (Kamal et al. 2000; Inomata et al. 2003; Matsuda et al. 2003; Lazarov et al. 2005) (see also section 1.6.1 and Figure 18).

Once at the cell surface APP can undergo two pathways (Figure 14): APP can be cleaved or undergo endocytosis if unprocessed, being retrogradely transported as a holoprotein from axonal synaptic compartments to neuronal cell bodies and dendrites (Simons et al. 1995; Yamazaki et al. 1995). In the endocytic pathway, APP can be recycled back to the membrane, by retrograde delivery to the TGN, or it can be incorporated into secondary endosomes, which will target APP to complete degradation at the lysosomes or to be recycled back to the TGN/Golgi (Tagawa et al. 1993; Koo et al. 1996; Yamazaki et al. 1996).

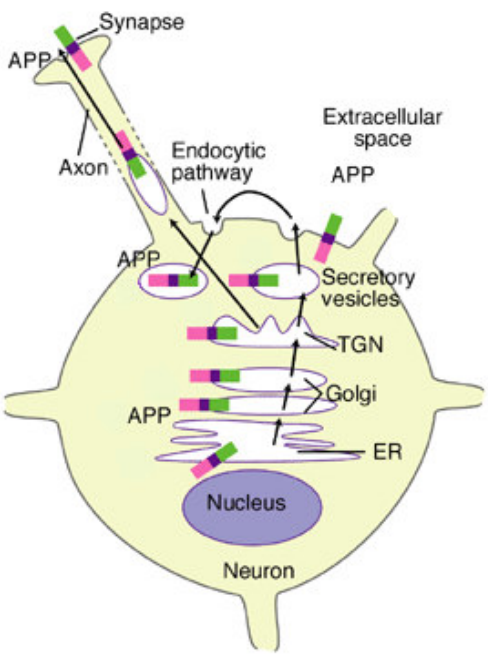


Figure 14. APP trafficking through the cellular secretory and endocytic pathways in neurons. APP domains are indicated in colours: *pink,* cytoplasmic domain; *violet,* Aβ domain; *green,* ectodomain (Gouras 2001).

APP processing during its intracellular trafficking

During its trafficking trough the different subcellular organelles besides maturating, APP can be proteolytic cleaved giving rise to the different APP fragments. Although most cell types appear to possess the same basic mechanisms for APP metabolism the relative contribution of the different pathways varies significantly, particularly between neurons and non-neuronal cell lines (Hartmann 1999). As already mentioned the majority of the APP cleavages occur after complete maturation of the protein, although some immature APP may also be cleaved by secretases at a low rate in the ER or the cis-Golgi subcellular compartments. Mature APP is processed rapidly (turnover of ~30-45 min) as it is transported to or from the cell surface via the secretory or endocytic pathways, respectively (Sambamurti et al. 1992; Kuentzel et al. 1993; Koo et al. 1996; Yamazaki et al. 1996; Cook et al. 1997; Hartmann et al. 1997). Further, only small amounts of APP were detected at the cell surface when compared to the total cellular pool (Kuentzel et al. 1993). This is due to rapid removal of cell surface APP, whose half-life was reported to be

less than 10 min (Lai et al. 1995), either by APP proteolytic cleavage or endocytosis. Approximately 30% of surface APP is cleaved to sAPP and secreted (Lai et al. 1995; Koo et al. 1996), while the remaining cell surface CTFs may be cleaved by γ-secretase locally, or in the endocytic pathway in endosomes, or further degraded in lysosomes (Tagawa et al. 1993; Mathews et al. 2002; Kaether et al. 2006). Both cell surface CTF cleavage product and unprocessed full-length APP are re-internalized via coated pits and vesicles by receptor-mediated endocytosis (Yamazaki et al. 1996). If endocytosed the half-life of internalized APP was estimated to be ~30 min (Koo et al. 1996), with a pool of endosomal APP being delivered to lysosomes.

Intracellular sites for Aβ precluding formation pathway

The intracellular sites for non-amyloidogenic APP cleavage are in aggrement with the subcellular organelles where the recently described α-secretases are localized and active. The α-secretases are synthesized as preproteins (Schlondorff et al. 2000; Anders et al. 2001) which turn into active proteolytic enzymes by cleavage of their inhibitory prodomain in late Golgi compartments, where they are thought to reside (Lammich et al. 1999; Tousseyn et al. 2006). The mature active form of TACE has been detected at the cell surface but also intracellularly, where it was mainly localized in a perinuclear compartment (for review, see Hooper and Turner 2002). The putative α-secretase, the ADAM10, was described to occur at the cell surface, nevertheless it was abundantly present in the Golgi apparatus and possibly in transport vesicles destinated to go to cell surface (Lammich et al. 1999; Gutwein et al. 2003).

APP processing by the non-amyloidogenic pathway has been described to occur intracellularly during its transport through the secretory pathway, before reaching the cell membrane. Several reports suggested that CTFα products were intracellularly detected before the appearance of sAPPα in the medium (Sambamurti et al. 1992; De Strooper et al. 1993; Kuentzel et al. 1993). APP cleavage by α-secretase was demonstrated to occur locally either in the trans-Golgi apparatus or in post-TGN vesicles (Sambamurti et al. 1992; Kuentzel et al. 1993). Additionally, in mice memory improvement models where α-secretase was up-regulated, it was observed that regulated α-secretase cleavage of APP

occurred mainly during its transport from the TGN to the plasma membrane (Sisodia 1992; Lammich et al. 1999). The pool of full-length mature APP that reaches the cell surface may locally undergo cleavage by α-secretase, living embayed to membrane the resulting CTFα (Sisodia 1992; Nordstedt et al. 1993; Koo and Squazzo 1994; Roberts et al. 1994; Parvathy et al. 1999). A more recent report demonstrated that α-secretase activities may also occur in the endoplasmic reticulum of a non-neuronal cell line (Shin et al. 2005). Nevertheless, it is known that the greater part of APP cleavage occurs after complete maturation and thus it is of reasonable consensus that the TGN/Golgi APP pool is the primarily source for α-secretase cleavage (Jolly-Tornetta and Wolf 2000; Hooper and Turner 2002).

The ratio between plasma membrane and intracellular α-secretase cleavage appears to be cell-specific. In fact, in N2a neuroblastoma cells (De Strooper et al. 1992; De Strooper et al. 1993), PC12 cells (Sambamurti et al. 1992), neuroblastoma H4 cells (Kuentzel et al. 1993), CHO cells (Jolly-Tornetta and Wolf 2000), the majority of APP cleavage to sAPPα was shown to occur intracellularly at the TGN or in post-TGN vesicles (probably just after sulfate incorporation), while in neurons α-secretase cleavage was reported to occur mainly at the cell surface (Parvathy et al. 1999). Additionally, in neuronal cells sAPPα may also be generated and released at synaptic terminals (McLaughlin and Breen 1999).

Intracellular sites for Aβ generating pathway

The major protease with known APP β-secretase activity is BACE1. The latter is expressed initially as a pro-enzyme and is cleaved after exiting the ER (Vassar et al., 1999). BACE1 is located intracellularly at the Golgi, TGN, and secretory vesicles, at the cell surface and in endosomes (Huse et al., 2000). This is in accordance with different pools of the secretase complexes regulating Aβ production and being present both at the plasma membrane and within the endocytic pathway. These intracellular localizations of β-secretase are in fact consistent with sites previously associated with Aβ production. Figure 15 illustrates the possible Aβ generating pathways.

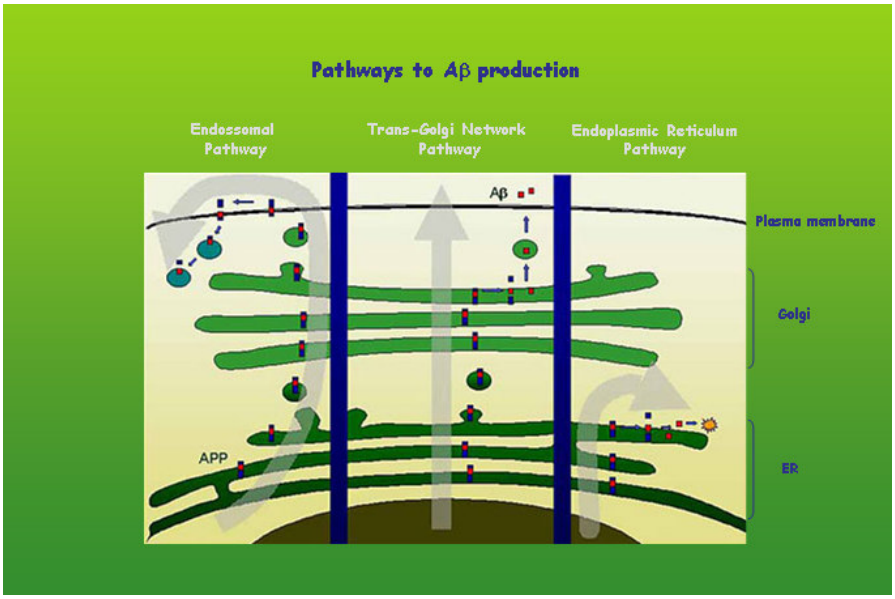


Figure 15. Three pathways leading to Aβ production. Aβ peptide is highlighted in red. ER, endoplasmic reticulum. (Adapted from Wilson et al. 1999).

Early on, it was suggested that most of the amyloidogenic β-cleavage events occurred in an endosomal compartment, following internalization of APP from the plasma membrane, leading to the generation of a large part of Aβ peptides in the endocytic pathway (Haass et al. 1992a; Haass et al. 1992b; Shoji et al. 1992; Peraus et al. 1997). This is corroborated by studies where mutations in the APP ^{682}YENPTY^{687} domain notably impair APP endocytosis and at the same time decrease Aβ levels (Perez et al. 1999). Additional studies identified that the secretory compartments ER and Golgi complex are also involved in Aβ generation (Cook et al. 1997; Hartmann et al. 1997; Tomita et al. 1998). In accordance with the last observations, Xia (2001) have reported that Aβ fragments can also be generated in the late Golgi compartment and packaged into post-TGN vesicles destined for extracellular secretion, during the APP secretory pathway. This Aβ production seems to occur at the TGN and does not need post-TGN vesicle formation (Xu et al. 1997).

Even though some authors defend that the γ-secretase complex is still not formed or active in the ER compartment (Kaether et al. 2006), it was reported that in neuronal cells $A\beta_{42}$ can also be produced inside this subcellular compartment (Cook et al. 1997;

Hartmann et al. 1997; Xia et al. 1998; Greenfield et al. 1999; Xia 2001). This pathway is considerably reduced in other cell types and consequently production of the Aβ species is also reduced (Wild-Bode et al. 1997; Skovronsky et al. 1998; Xia et al. 1998). Further, Chyung et al (1997) showed that β-secretase activity could be detected in neuronal but not in non-neuronal cells. Neurons exhibit higher levels of β-secretase activity (Busciglio et al. 1993; Simons et al. 1996) resulting in a higher Aβ/P3 ratio. Hence it is reasonable to deduce that Aβ production plays an important role in determining neuronal phenotype, particularly intracellular Aβ. This is well exemplified during the differentiation process of NT2 cells (human tetracarcinoma cells) into neurons (NTN2). One week following differentiation these cells exhibit soluble Aβ levels of 26fmol/mg and secrete 53fmolAβ/ml/24hr; at six weeks values rise to 44fmol/mg of soluble Aβ and secrete 266fmolAβ/ml/24hr (Turner et al. 1996).

Intracellular sites for γ-secretase cleavage

Evidently, the intracellular localization of the γ-secretase complex is consistent with the described intracellular sites for CTF cleavage and P3 or Aβ production. Presenilins were described to be predominantly localized to the ER, where it may also co-localize with nicastrin (Yu et al. 2000), and early Golgi (Kovacs et al. 1996), and in limited quantities at locations such as endosomes and plasma membrane (Weidemann et al. 1997; Xia et al. 1997). Interestingly, efficient γ-secretase cleavage of APP seems to occur in compartments that contain small amounts of PS1, while little γ-secretase activity is observed in compartments where abundant PS1 is residing (Cupers et al. 2001a). Hence, the main subcellular γ-secretase activity locations described for γ-secretase cleavage are the endosomes and the plasma membrane (Weidemann et al. 1997; Xia et al. 1997; Kaether et al. 2006). Mature nicastrin, PS1 and APP were found co-localized in the LAMP-1 positive endosome membranes, where an acidic γ-secretase activity was also present (Pasternak et al. 2003), suggesting a role of the the lysosomal/endosomal system in the generation of Aβ. More recently, Kaether et al. (2006) demonstrated that by blocking selective transport steps along the secretory pathway the main subcellular locations of AICD generation are the plasma membrane and the endocytic compartments.

1.6 Involvement of the Cytoskeleton Network in Alzheimer´s Disease

The cytoskeleton is a system of filaments found in the cytoplasm of cells that is responsible for the maintenance of and changes in cell shape, cell locomotion, movement of various elements within the cytoplasm, integration of the major cytoplasmic organelles, cell division, chromosomal organization and movement, and cell-cell or cell-surface adhesion. The primary cytoskeletons of eukaryotic cells are composed of microtubules (composed of α/β-tubulin heterodimers), actin and intermediate filaments (Figure 16). However, the presence of this system of filaments in all cells is extremely diverse in structure and cytoplasmic distribution.

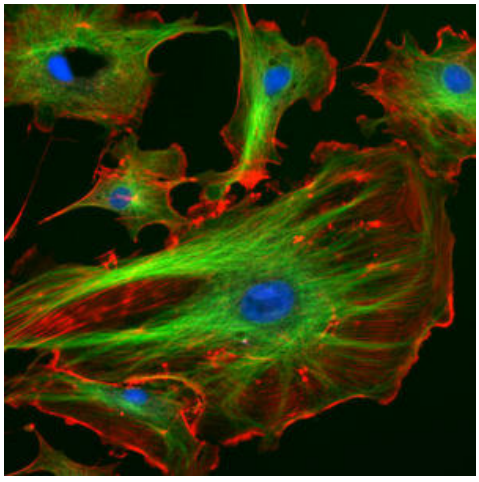


Figure 16. Eukaryotic cytoskeleton. Actin in red, tubulin in green, and nuclei in blue. From www.answers.com/topic/cytoskeleton.

Both microtubules and actin have important roles in the secretory and endocytic pathways (reviewed in Vliet et al. 2003 and Lanzetti et al. 2007). Unlike actin filaments and microtubules, intermediate filaments are the least understood part of the cytoskeleton (for review see Godsel et al. 2008). Nonetheless, intermediate filaments interact with microtubules contributing to cell shape maintenance (Chang and Goldman 2004) and appear to play an important role in astrocyte directional mobility of vesicles (Potokar et al. 2007). Therefore, alterations in the dynamics of the cytoskeleton components will ultimately lead to cytoskeleton organization abnormalities and defects in related processes, which have in fact been associated with several neurodegenerative disorders, including AD (Pollak et al. 2003).

1.6.1 Microtubule network in APP axonal transport

In neuronal cells the cytoskeleton consists of actin filaments, microtubules and neurofilaments (Figure 17).

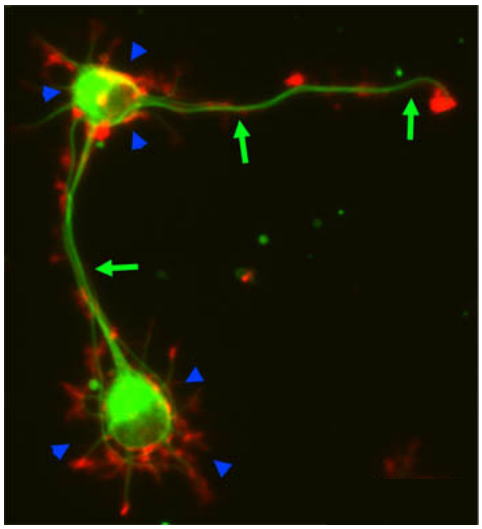


Figure 17. Neuronal cytoskeleton. Microtubules in green and actin in red. From http://neurobio.drexel.edu/GalloWeb/The%20Neuronal%20Cytoskeleton.htm.

The long neuritis and axonal processes constitute a challenge to the movement of proteins, vesicles and organelles between presynaptic sites and cell bodies. To overcome this challenge specialized transport machinery exists, consisting of cytoskeletal motor proteins that generate directed movement along cytoskeletal tracks.

Microtubules are the major tracks or "highways" along which motor proteins generate long-distance efficient axonal transport (Kreutzberg 1969; Kamal et al. 2000). Fast anterogade transport machinery, and axonal terminals are dependent on axoplasmic flow, a function that requires intact microtubules and motor proteins such as kinesin, dynein and dynamin. Evidence suggests that dynein may be the major motor protein powering microtubule retrograde transport, and that kinesin motor proteins are involved in anterograde axonal transport (for review see Stokin and Goldstein 2006). In fact it has been reported that kinesin molecular motor proteins are responsible for many of the major microtubule-dependent transport pathways in neuronal and non-neuronal cells (Goldstein 2001). The microtubule motor complex kinesin-I comprise two components: kinesin heavy chains (KHC) that have ATP- and microtubule-binding motifs essential for

vesicle transport, and kinesin light chains (KLC). KLC associates with KHC and tethers membrane vesicles containing proteins to be transported axonally from the neuronal soma to nerve terminals (Hirokawa et al. 1998).

The transport system is crucial to maintaining neuronal viability and differentiation, and failure of axonal transport may play a role in the development or progression of neurological diseases, including AD. Indeed, AD brains generally exhibit severe perturbations of several neurotransmitors and widespread synaptic and neuronal loss in specific brain areas. These changes are accompanied by severe disruption of the axonal as well as dendritic cytoskeleton, which suggests failed axonal transport as a contributing factor in the progression of the disease (Stokin and Goldstein 2006).

Many proteins associated with AD pathogenesis (including APP, BACE, PS1, nicastrin, Aph-1, synuclein and tau) have been observed in the axonal compartment of neurons, with many of them found at presynaptic terminal (Iwai et al. 1995; Beher et al. 1999; Capell et al. 2002; Siman and Salidas 2004). Therefore, transport is almost certainly necessary to deliver these proteins to their final destination (Koo et al. 1990a). APP axonal anterograde transport is proposed to be mediated by functional interaction between APP and the kinesin light chain (KLC) subunit, a component of kinesin-1 transport machinery (Kamal et al. 2000; Kamal et al. 2001). Nonetheless, recent evidence is consistent with the view that APP/KLC interaction may be enhanced/mediated through JIP-1, an adaptor protein member of JNK-interacting protein family known to interact with both KLC and APP (Taru et al. 2002; Inomata et al. 2003; Matsuda et al. 2003; Lazarov et al. 2005) (Figure 18).

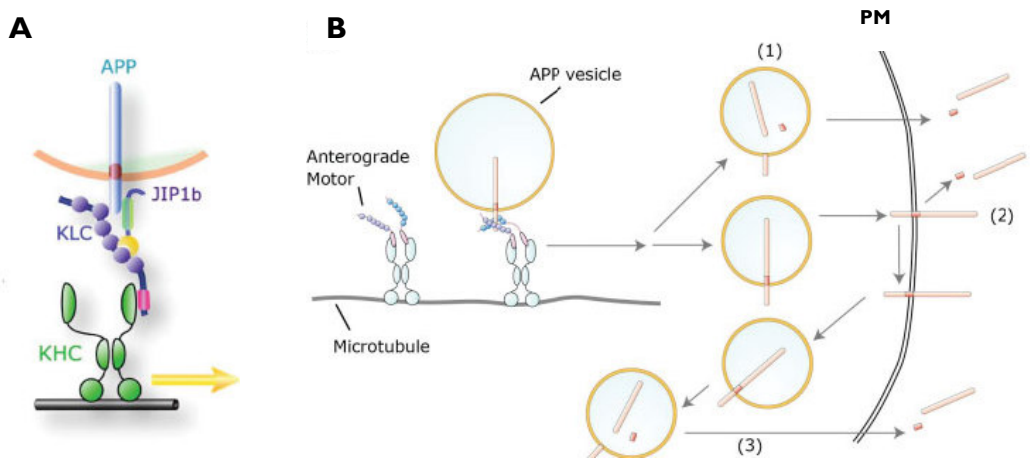


Figure 18. APP axonal anterograde transport and the kinesin motor protein. A. Association of APP with the kinesin-I motor. APP binds to KLC through an association with JIP-1b, but does not bind to KLC directly. Thus, APP serves as a cargo-receptor via JIP-1b. B. Anterograde transport of APP vesicles and metabolism of APP. APP is subjected to cleavage during axonal transport (1), on the plasma membrane (2), or in the endocytotic cycle (3), generating neurotoxic Aβ. (Adapted from Suzuki et al. 2006).

In mouse, kinesin-1 reduction enhances de development of axonal defects, increases aberrant Aβ generation, and enhances amyloid deposition (Gunawardena and Goldstein 2001; Stokin et al. 2005). Also, antisense treatment with oligonucleotides against KHC slows down APP axonal transport and increases the frequency of directional changes (Kaether et al. 2000). More recently, Stokin et al. (2005) observed axonal swellings containing motor proteins and vesicular elements in the brain of mouse models and patients with AD, and disturbed axonal transport resulted in increased Aβ generation.

Interestingly, Aβ *per se* is sufficient to induce formation of axonal abnormalities (Pike et al. 1992) and may directly contribute to the impairments in the axonal transport (Kasa et al. 2000; Hiruma et al. 2003). Whether Aβ is a consequence or a cause of altered axonal transport is still unclear. The fact is that alterations in microtubule network will ultimately result in altered APP axonal transport and increased Aβ production and aggregation, the latter being intimately associated with AD neurodegeneration.

Microtubule acetylation and neuronal transport

Key post-translational modifications of microtubules (PTM) may dictate microtubule dynamics and recruitment of microtubule associated proteins, microtubule stability and function (reviewed in Bloom, 2004). Several PTM of microtubules have been described including acetylation, polyglutamylation and tyrosination/detyrosination, all were shown to be reversible (reviewed in Bulinski, 2007 and Hammond et al., 2008). Although tubulin acetylation is not necessary for cell and organism survival, it is known that acetylation leads to microtubule stability. Further, recent work has suggested that α-tubulin acetylation plays a positive role in motor-based trafficking in mammals (Reed et al. 2006; Bulinski 2007; Dompierre et al. 2007). Of note, hyperacetylation of microtubules in neuronal cells using inhibitors of histone deacetylase 6 caused increased recruitment of the cytoplasmic motors dynein and kinesin-1 to microtubules and enhanced transport of brain derived neurotrophic factor vesicles (Dompierre et al. 2007).

Reed at al. (2006) showed that microtubule acetylation is sufficient to increase kinesin-mediated transport of JIP-1-containing vesicles to the tips of neurites (Figure 19, bold arrows), while detyrosination and poly-glutamylation appeared to have a smaller impact on transport (Figure 19, light arrows).

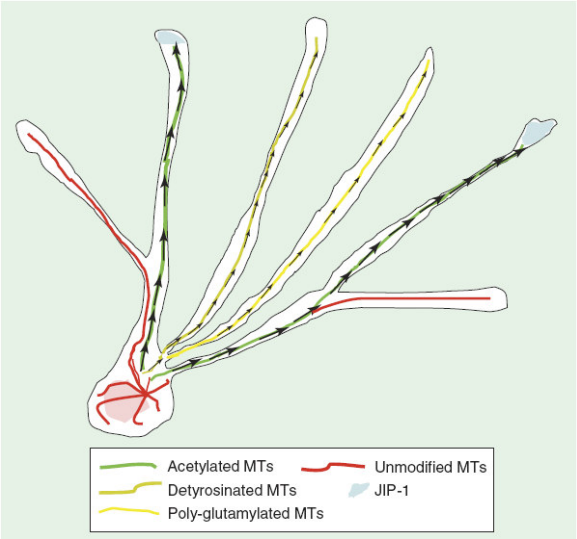


Figure 19. Microtubules post-translational processes in neurons. Neurons undergo multiple reversible post-translational modifications including acetylation, detyrosination and poly-glutamylation. Although microtubules in the same process actually carry multiple modifications, in the figure they are only shown to have one type of modification per process for simplicity's sake. Increased acetylation of all microtubules in hippocampal neurons was sufficient to target JIP-1 to neurite tips. Adapted from Bulinski, 2007.

Althought, the cellular functions of PTMs have only recently begun to be revealed, the data suggest that these modifications can affect microtubule dynamics. PTMs can influence the stability and/or structure of microtubule assemblies, either directly by affecting tubulin modification on microtubule structure, or indirectly by influencing the recruitment of microtubule-associated proteins, such as molecular motor proteins. In the case of motor-dependent transport it is possible that the PTMs serve as "road signs" to direct polarized trafficking such as axonal/dendritic trafficking in neuronal cells. It is also possible that PTMs simply mark stable microtubules for preferential transport, indicating to molecular motors the desirable microtubule track.

1.6.2 The role of actin remodelling in AD

Actin and the dendritic cytoskeleton

As already mentioned, AD is characterized by a progressive loss in the number of dendritic spines, as well as by alterations in the synaptic efficacy and damage at the synaptic terminal (Lippa et al. 1992; Masliah 1995), where decreased cortical synapse density correlates with cognitive decline in patients (Terry et al. 1991; DeKosky et al. 1996). These changes are accompanied by a severe disruption of the axonal as well as dendritic cytoskeleton, and culminate in alterations in axonal transport. The regulation of actin dynamics is considered to be the main mechanism underlying morphological changes in dendritic spines (Halpain 2000; Matus et al. 2000), and these pathological actin-induced alterations have been associated with an actin polymerized conformation (F-actin). Actin-depolymerizing factor (ADF) and cofilin are actin-binding proteins that critically control actin filament dynamics and reorganization by severing and depolymerizing actin filaments (Bamburg and Wiggan 2002). An imbalance in actin dynamics has been associated to the formation of cofilin-actin rods (Minamide et al. 2000), which are inclusion-like structures present in hippocampal and cortical neurons of post-mortem AD brain thought to mediate neurodegeneration. These rod-like inclusions are specially found in Alzheimer´s brain areas surrounding the amyloid plaque, supporting the idea that Aβ peptide may play a role in the imbalance of actin dynamics (Maloney et al. 2005). Further, under stress conditions induced by $A\beta_{1-42}$ it was reported that cofilin becomes active

(dephosphorylated) and forms actin rods. Surprisingly, however, in hippocampal neurons treated with high concentrations of $A\beta_{1-40}$ cofilin undergoes phosphorylation (inactivation), leading to dystrophic morphological changes, including accumulation of pretangle phosphorylated tau (Maloney and Bamburg 2007). Aβ *per se* has also been described to affect and accumulate in synapses (Takahashi et al. 2004), and this phosphorylation dependent regulation of cofilin by different Aβ peptides may provide a mechanistic explanation for the synaptic loss and plaque propagation in AD.

In agreement with these Aβ effects on actin dynamics it was recently reported that $A\beta_{1-42}$ could increase actin polymerization by increasing activity of Rac/Cdc42 Rho GTPases in hippocampal neurons (Mendoza-Naranjo et al. 2007). The Rho family of small GTPases (Rho, Rac and Cdc42) are regulators of F-actin polymerization (Bishop and Hall 2000), acting as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state. This system has also been implicated in the maintenance and reorganization of dendritic structures (Luo 2000; Nakayama et al. 2000). Rac1 and Cdc42 promote polymerization at the leading edge, orchestrating the formation of lamellipodia and membrane ruffles (Ridley et al. 1992), as well as peripheral actin microspikes and filopodia (Kozma et al. 1995; Nobes and Hall 1995).

A preceding study also demonstrated that Rac activation inhibits the formation of both dendritic spines and synapses (Zhang et al. 2003), and up-regulation of Rac/Cdc42 was observed in AD cases in comparison with an age-matched controls (Zhu et al. 2000). Further, Aβ was reported to destabilize Ca^{2+} regulation, and an increase in intracellular Ca^{2+} induces membrane translocation and activation of Rac, an event dependent on the activation of conventional protein kinase C (PKC) (Price et al. 2003). These results point to the actin cytoskeleton as a target for Aβ-induced neurodegeneration in AD.

The actin cytoskeleton in exocytosis of secretory vesicles

In neurons and endocrine cells, exocytosis of neurotrasnsmitters or hormones containing vesicles occurs upon specific stimuli. Exocytosis controls cell surface expansion and protein secretion by fusing secretory vesicles with the PM. These trafficking processes require actin remodelling, which is strictly regulated by time and localization in the process of membrane trafficking and provides the forces for movement.

In neurons, adrenal chromaffin cells and their tumor derived PC12 cells, synaptic vesicles/granules are organized in two pools: the reserve pool (RP) and the readily releasable pool (RRP) (Sudhof 2004; Dillon and Goda 2005). The RRP constitutes vesicles docked at the active zone, which are primed to undergo membrane fusion, whereas the RP is a cluster of vesicles present distally from the active zone. The traffick between these two pools is subjected to a fine regulation by the actin cytoskeleton. Actin was reported to act as a scaffold to impede vesicle dispersion. Indeed, in adrenal chromaffin cells, fodrin, an actin crosslinking protein, stabilizes the cortical actin network (below PM) and entraps secretory granules in the subplasmalemmal cytoskeleton severing as a reservoir for exocytotic demand (Aunis and Bader 1988; Malacombe et al. 2006). Additionally, the diverse effects of actin depolymerization on RP indicate that regulation of RP becomes dependent on actin under conditions when the RRP is depleted by stimulation (Dillon and Goda 2005). Actin depolymerization by cytochalasin D depletes vesicles in RP after high-frequency stimulation at neuromuscular junction (Kuromi and Kidokoro 1998), suggesting that the F-actin network serves to maintain a vesicle storage pool.

In the role of actin network in the docking and fusion process, it has been reported that actin depolymerization is required at docking stage to the PM, whereas actin polymerization is required at the stage of fusion to the PM (reviewed in Noda and Sasaki 2008). Nonetheless, actin remodeling in the docking and fusion processes are not fully understood.

It has been proposed that in the process of exocytosis actin may act into two distinct modes. Actin may provide a track that guides the movement of vesicles to the targeted PM domain, or cortical actin network forms a physical barrier that prevents exocytosis. In the delivery of synaptic vesicles to the active zone, evidence supports the first model in which actin polimerization plays a facilitator role in vesicle recruitment. For instance, actin depolymerization by latrunculin A delays vesicle recruitment at the Calyx of Held synapse (Sakaba and Neher 2003). Actin filaments may actively participate in the vesicle transport or it may serve as a track for the motor protein myosin to penetrate the actin cortical barrier, promoting actin-driven transport (reviewed in Noda and Sasaki, 2008). In the translocation of secretory granules in neuroendocrine cells, stabilization of actin inhibits exocytosis whereas actin depolymerization enhanced exocytosis, supporting the actin barrier model (Malacombe et al. 2006). Early on studies reported that under resting conditions, the actin cytoskeleton, prevented secretory granules from reaching their exocytic destination (Burgoyne and Cheek 1987; Aunis and Bader 1988; Trifaro and Vitale 1993). In PC12 cells the disruption of actin filaments by latrunculin increases granule motility (Ng et al. 2002; Desnos et al. 2003). Nonetheless, several reports showed a biphasic effect of actin on the exocytotic process. For instance, exocytosis in PC12 is promoted at low doses of latrunculin but inhibited at high concentrations (Gasman et al. 2004). These biphasic effects of actin depolymerization indicate the requirement of a minimal actin structure for exocytosis. The mechanisms regulating the actin barrier appear to be dependent on RhoA GTPase. RohA activation by G_0 or overexpression of constitutively RhoA mutant stabilizes the peripheral actin filaments and inhibits exocytosis in stimulated cells (Gasman et al. 1997; Frantz et al. 2002; Bader et al. 2004).

Aβ itself is suspected to be associated with alterations in the exocytic pathway. Uemura and Greenlee (2001) reported that Aβ contribute to decrease neuronal glucose uptake by preventing exocytosis in hippocampal neurons. Neuronal glucose uptake is regulated by SNARE complex-dependent docking and fusion of GLUT3 vesicles with the plasma membrane, and $A\beta_{25-35}$ decreased glucose uptake by inhibiting fusion of these vesicles with the PM.

All these findings implicate Aβ in altered axonal transport but also in actin dynamics, which suggests the existence of an interplay between altered axonal transport, damage signaling, synaptic and neuronal loss and the Aβ peptide in the progression of AD.

1.7 Current and Future Therapeutic Approaches in Alzheimer´s Disease

A number of clinical trials are currently in progress based on different therapeutic strategies and they should indicate which of these approaches will be efficacious for treatment of AD (reviewed in Klafki et al. 2006 and Wang et al. 2008). Since the predominant hypothesis to explain AD pathogenesis is the "amyloid cascade", several novel and promising current and future strategies are specifically targeting Aβ production and clearance.

1.7.1 Current strategies to ameliorate AD symptoms

Considerable efforts have been directed toward seeking or developing a variety of therapeutic strategies that seem to be able to produce modest symptomatic improvements in AD patients (reviewed in Cummings 2004), some of which will be herein discussed. At present, none of the available medications appear to be able to cure AD or to stop the disease progression. Current medications that have passed FDA approval for treatment of AD include acetylcholinesterase (AChE) inhibitors, for mild and moderate cases, and memantine, an NMDA-receptor antagonist for the treatment of moderate to severe Alzheimer´s dementia (Blennow et al. 2006; Hodges 2006; Klafki et al. 2006).

The "cholinergic hypothesis of AD" postulates that destruction of cholinergic neurons and the resulting deficit in central cholinergic transmission contributes substantially to the characteristic cognitive and non-cognitive symptoms observed in AD patients (Bartus et al. 1982; Cummings and Back 1998). Cholinergic neurons are the nerve cells that synthesize and release acetylcholine, an important neurotransmitter associated with neurological functions. When acetylcholine levels drop memory and other brain functions are affected. Impairment or loss of cholinergic neurons in the basal forebrain leads to a significant reduction in the level of acetylcholine and decrease activity in both AChE (the enzyme that degrades acetylcholine in the synaptic cleft) and choline acetyltransferase (the enzyme that synthesizes acetylcholine). Reductions in the activities of the latter enzymes were reported in brain tissues from AD patients (Bowen et al. 1976; Perry et al. 1977) and suggested a selective destruction of cholinergic neurons. This hypothesis

provides the rational for the development of the AChE inhibitors for AD therapy. Inhibition of AChE leads to an increase in the acetylcholine concentration in the synaptic cleft which is expected to enhance cholinergic transmission and ameliorate cholinergic deficit. Three different cholinesterase inhibitors, namely donepezil, galantamine and rivastigmine are commonly used for AD treatment. The former are selective inhibitors of AChE, while the latter also inhibits butyrylcholinesterase (BChE), which accounts for ~10% of the cholinesterase activity in normal human brain and appears to be predominantly associated with glia (Scarpini et al. 2003). Therefore, these drugs have been used to prevent the level of acetylcholine from dropping acutely in the brain.

Alternative approaches aimed to prevent glutamate-mediated neurotoxicity. It is believed that the neurotransmitter glutamate and NMDA receptors have crucial roles for memory and learning processes in brain. Glutamate represents the main excitatory neurotransmitter in the CNS and a physiological level of glutamate-receptor activity is essential for normal brain function (Kornhuber and Weller 1997). Excess glutamate is detrimental to neurons due to its overstimulation on NMDA receptors which is believed to play a role in neuronal death observed in AD (Hynd et al. 2004). Memantine that is an uncompetitive NMDA receptor antagonist can protect neurons from the cytotoxicity elicited by glutamate via partially blocking NMDA receptors. This drug was reported to have a beneficial effect on cognitive function and function decline in patients with moderated to severe AD. The positive clinical results of memantine monotherapy and the observation that memantine does not interact *in vitro* with the AChE inhibitors donepezil or galantamine (Wenk et al. 2000), suggested that the clinical combination therapy of memantine with cholinesterase inhibitors may be of therapeutic value.

Other potential treatments currently under investigation include vitamin E (antioxidant), Ginkgo biloba and Huperzine A (anti-inflammatory and anti-oxidant drug), calcium channel blockers, cholesterol lowering drugs, non-steroid anti-inflammatory drugs (NSAID) and metal interactions.

1.7.2 Therapeutic strategies targeting Aβ peptide

As mentioned above, the prevalent hypothesis to explain the mechanisms leading to AD is the "amyloid cascade hypothesis", which sates that Aβ plays a central role in the pathogenesis of the disease (Figure 6). It is believed that Aβ accumulation (in particular $A\beta_{1-42}$) initiates a cascade of events that ultimately leads to neuronal dysfunction, neurodegeneration and dementia (for review see Hardy 2006). Therefore, an enormous therapeutic effort is directed towards developing drugs that reduce Aβ burden or toxicity by inhibiting secretase cleavage, Aβ aggregation, Aβ toxicity, Aβ metal interactions or by promoting Aβ clearance. The steady levels of Aβ are determined by the balance between its production or clearance (Figure 20, for review see Wang et al., 2006). Dysfunction in Aβ clearance is crucial for the accumulation of Aβ in AD brains.

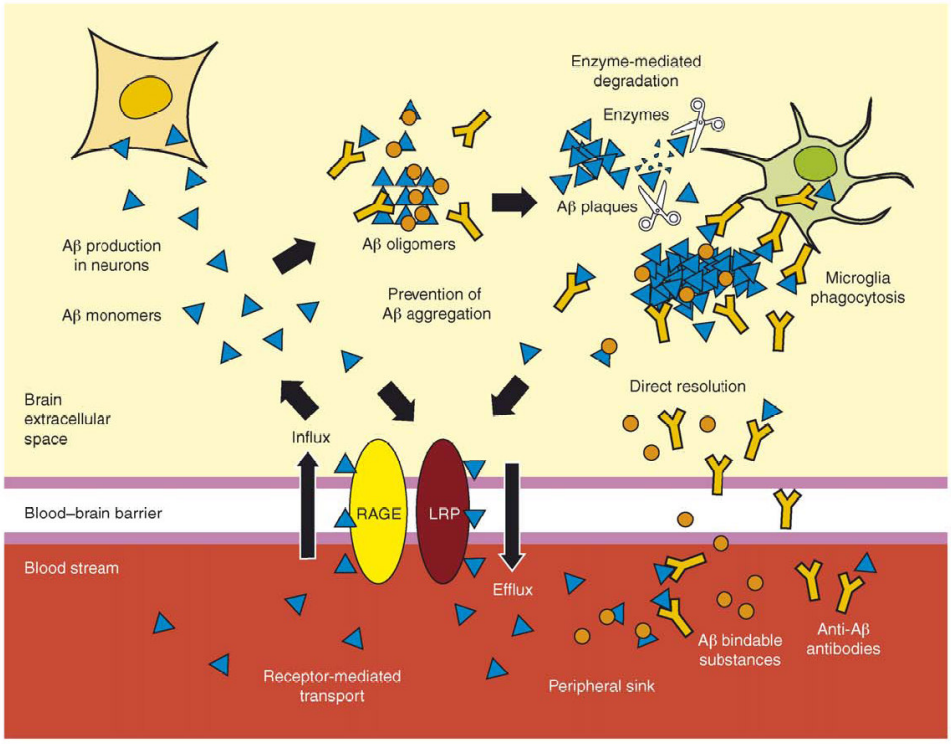


Figure 20. Mechanisms of Aβ clearance. The transport of Aβ across the blood–brain barrier (BBB) is mainly mediated by receptors [i.e. receptor for advanced glycation end products (RAGE) and lipoprotein receptor-related protein (LRP)] on endothelial cells. Aβ in the extra- and intra-cellular space can be degraded by enzymes [i.e. neprilysin and insulin-degrading enzyme (IDE)]. Peripheral anti-Aβ antibodies and Aβ-binding substances are able to enter the brain at low levels, where they prevent Aβ aggregation and resolve Aβ fibrils. By binding to peripheral Aβ they also act as a peripheral sink to promote the efflux of Aβ from the brain and disrupt the Aβ equilibrium between the brain and the blood, resulting in the clearance of Aβ from the brain. These mechanisms of Aβ clearance become potential targets for drug development in Alzheimer's disease. (From Wang et al., 2006).

Modulation of Aβ production by affecting secretase activity

Aβ peptide arise from proteolytic processing of APP by β- and γ-secretases, which places these two enzymes as prime targets in the active search for small molecule compounds that can reduce Aβ production by affecting one of these secretases. BACE1 KO mice were reported to produce only very small amounts Aβ confirming that BACE1 represents the primary β-secretase *in vivo*. For **β-secretase** the identification of specific small molecule inhibitors suitable for drug development appears to be difficult (Citron 2004a) because the size and the shape of the active site of this aspartyl protease presents a considerable challenge in the design of potent inhibitors that can also achieve good brain penetration.

In the case of **γ-secretase**, the situation is more complex, because its active site is intramembranous, and because PS requires additional membrane proteins for proteolytic activity. The γ-secretase inhibitor (LY450139) was reported to reduce Aβ levels in plasma but not in CSF at concentrations that did not produce significant side effects (Siemers et al. 2005). Another major concern regarding the therapeutic usefulness of γ-secretase inhibition and potential side effects comes from the identification of other γ-secretase substrates, including Notch 1 (De Strooper 2003). Nonetheless, the finding that certain NSAIDs can preferentially reduce the generation of the highly amiloidogenic species $A\beta_{1\text{-}42}$ without affecting Notch cleavage (Weggen et al. 2001), indicates the existence of a γ-secretase modulating mechanism which may be a potential drug target to modulate $A\beta_{1-42}$ production.

Cleavage of APP by **α-secretase** prevents Aβ production, and can be stimulated by muscarinic acetylcholine-receptor agonists (Wolf et al. 1995). The M1 muscarinic acetylcholine-receptor agonist (AF267B) was reported to ameliorate cognitive deficit and reduce both amyloid and tau pathologies in mice (Caccamo et al. 2006).

Inhibition of Aβ aggregation

There has been some disagreement regarding the exact structure of the aggregated species which are associated with toxicity. It is unclear if the Aβ species responsible for toxicity are the protofibrils (intermediate species observed during the formation of mature amyloid fibrils), the slowly sedimenting Aβ diffusible non-fibrillar species, some of other low molecular intermediates or spherical aggregates. Nonetheless, it is certain that the toxicity is associated with peptide structures that are part of an aggregation pathway associated with amyloid fibril formation (Wang et al. 2008). Therefore, preventing the formation of the presumed toxic oligomeric/fibril aggregates of Aβ by small molecules represents a promising approach for the development of novel therapeutic strategies for treating AD. Several biomolecules, such as proteins, proteoglycans, lipids, metals, among others have been reported to be associated with amyloid plaques in AD brains (Table 2). While it is possible that some of these physiologically relevant molecules may be related to secondary events in amyloid deposition, several proteins have been described to bind Aβ peptide both *in vitro* and *in vivo* thus regulating its aggregation and neurotoxicity, either by preventing Aβ fibril formation or by contributing to it fibril degradation.

A Canadian company (Neurochem Inc) has completed a Phase II clinical trial of their glycosaminoglycan mimetic Alzhemed that has been designed to bind Aβ peptides and thereby inhibits formation of Aβ aggregates. A Phase III trial was subsequently planned (reviewed in Citron 2004).

Metal ions like Cu^{2+} and Zn^{2+} may be involved in the mediation of Aβ aggregation and toxicity (Atwood et al. 1998). Cherny and colleagues (2001) showed a significant decrease in brain Aβ deposition in APP-transgenic mice after 9 weeks treatment with clioquinol (an antibiotic and Cu/Zn chelator that crosses the blood-brain barrier).

Table 2. Aβ-binding proteins found in amyloid plaques.

Modulator	Aβ secondary structure	Effect on Aβ aggregation	Aβ interaction domain
Plasma proteins			
Albumin	n/d	↓	1-28
α1-antitrypsin	n/d	↓	11-28
IgG	n/d	↓	n/d
IgA	n/d	↓	n/d
α1-antichymotrypsin	random	↑/↓	11-28, 29-42
α2-macroglobulin	random	↓	11-28
Serum amyloid A	n/d	↑	1-28
Glicosaminoglycans			
Heparan sulfate	β-sheet	↑	13-16
Keratan sulfate	β-sheet	↑	n/d
Dermatan sulfate	β-sheet	↑	n/d
Chondroitin sulfate	β-sheet	↑	13-16
Apolipoproteins			
ApoE	β-sheet	↑/↓	29-42
ApoJ	β-sheet	↑/↓	29-42
ApoA-I	n/d	↓	n/d
Acetylcholinesterase	n/d	↑	n/d
Base membrane components			
Laminin	n/d	↓	n/d
Entactin	random	↓	n/d
Phospholipids			
Phosphatidylserine	random	↑	29-42
Phosphotidylinositol	β-sheet	↑	29-42
Phosphotidylcholine	random	no change	n/d
Phosphotidylethanolamine	random	no change	n/d
Phosphatidic acid	random	↑	29-42
Inositol stereoisomers	α-helix	stabilize small aggregates	n/d
Metals			
Zn^{2+}	β-sheet	↑	His13, His14
Cu^{2+}	β-sheet	↑	His13
Fe^{3+}	β-sheet	↑	His 13

n/d – not definned

Another potential therapeutic approach to inhibiting Aβ aggregation is the use of peptidic inhibitors, consisting of shorter peptide fragments (KLVFF, GVVIN, RVVIA), which have been employed to prevent Aβ fibrillogenesis/aggregation. Due to their marked affinity for Aβ or critical involvement in Aβ aggregation, these shorter peptide fragments are able to interact with Aβ molecules, change Aβ structure and thus prevent the process of assembly (reviewed in Wang et al., 2008).

Promoting Aβ clearance via Aβ–binding substances

Penetration of Aβ-binding substances into the brain provides a chance of inhibiting the aggregation of soluble Aβ and/or resolution of Aβ fibrils. This would shift brain equilibrium of soluble and aggregated Aβ species towards soluble ones and finally facilitate Aβ clearance. Aβ-binding substances may sequester plasma Aβ, leading to Aβ clearance by promoting a net efflux of a rapidly mobilized soluble Aβ pool (peripheral sink hyphothesis, Figure 20). Peripheral treatment with gelsolin or GM1, an agent that has high affinity for Aβ, reduced the level of the peptide in the brain probably because of a peripheral action (Matsuoka et al. 2003). Another Aβ-binding substance, enoxaparin (a low-molecular-weight heparin), when administrated peripherally, lowered the number of cortical Aβ deposits and the total $A\beta_{1\text{-}40}$ concentration, possible by either impeding Aβ fibril formation or by sequestering the plasma Aβ peripherally (Bergamaschini et al. 2004).

Promoting Aβ clearance via up-regulation of Aβ degrading enzymes

Enhancement of Aβ-degradation enzymes represents a novel therapeutic strategy for the prevention and treatment of AD (Eckman and Eckman 2005). Gene transfer of both neprilysin and IDE reduces the accumulation of Aβ in the brain of AD animal models (Leissring et al. 2003; Marr et al. 2003; Iwata et al. 2004). Further, it has been reported that Aβ degradation by IDE can be enhanced by a small synthetic peptide substrate, without affecting the activity of this enzyme towards insulin (Song and Hersh 2005). Somatostatin can also regulate brain Aβ metabolism by up-regulating neprilysin. Aging induce downregulation of somatostatin expression, suggesting that somatostatin receptor

agonist may be useful in the prevention and treatment of AD. Neprilysin gene promoters can be transactivated by AICD (Pardossi-Piquard et al. 2005), which provides a potential physiological mode of modulate Aβ levels. Nevertheless, it should be considered that up-regulation of these Aβ degrading enzymes may also affect the physiological functions of other endogenous substrates, such as neuropeptides.

Promoting Aβ clearance via Aβ immunotherapy

Immunological approaches aim to reduce Aβ load in the brain by either active or passive immunization (for review see Wang et al. 2006). Two general approaches have been studied in AD mouse models: active vaccination with Aβ or fragments thereof and passive infusions of monoclonal antibodies. Both have worked well to clear plaques and lower Aβ levels in mice, and they have decreased neuritic dystrophy and even ameliorated learning deficits in these models. For instance, Bard et al. (2000) and co-workers, using peripheral antibody administration provided direct evidence that Aβ antibodies are sufficient to reduce amyloid deposition. These approaches have shown concomitant improvement in neuritic dystrophy and cognitive deficits in animal models (Schenk et al. 1999; Dodart et al. 2002; Bard et al. 2003; Wilcock et al. 2004; Billings et al. 2005; Maier et al. 2006).

Anti-Aβ therapy represents the most advanced disease-modifying attempt so far, in terms of experience in humans. The first clinical trials of $A\beta_{1-42}$ immunotherapy, also suggest that the active immunization with Aβ peptide is therapeutically effective, as demonstrated by eliciting amyloid plaque clearance, reduction in dystrophic neurites or reactive astrocytes compared with immunized controls. There is also decreased CSF tau levels and diminuished cognitive decline in patients (Lombardo et al. 2003; Nicoll et al. 2003; Ferrer et al. 2004; Oddo et al. 2004; Brendza et al. 2005; Gilman et al. 2005; Masliah et al. 2005). However, Aβ immunotherapy using aggregated $A\beta_{1-42}$ as antigen, had to be stopped in Phase II due to the developement of autoimmune meningoencephalitis in a significant number of patients. The condition appeared to be caused primarily by the infiltration of autoreactive T-lymphocytes into the brain in response to active immunization (Nicoll et al. 2003; Ferrer et al. 2004). T-lymphocytes are activated by T-cell epitopes mapped to $A\beta_{25-42}$, which is separated from the dominant B-cell epitopes identified in $A\beta_{1-15}$

(Monsonego et al. 2003). Currently, these findings concerning the adverse effects are being considered for the development of alternative approaches in order to avoid the unwanted T-cell response induced by Aβ peptide. Recent studies indicate that new vaccines composed of parts of the Aβ molecules, excluding the epitope that might provoke abnormal T-cell reactions are under development. Present findings indicate that immunization with $A\beta_{1-15}$ is effective at generating anti-Aβ antibodies in the absence of T-cell response and leads to reduction of cerebral-plaque Aβ burden and cognitive deficits in AD animal models (Agadjanyan et al. 2005; Maier et al. 2006). Antibodies generated against N-terminal of Aβ are able to inhibit Aβ fibrillogenesis and cytotoxicity, disaggregate pre-existing Aβ fibrils, and are most effective in clearance of amyloid plaque (McLaurin et al. 2002; Bard et al. 2003; Bussiere et al. 2004; Horikoshi et al. 2004). Recent data from the clinical Phase IIa study suggests that the predominant antibodies generated after immunization with $A\beta_{42}$ (AN1792) are primarily N-terminal (1–8) specific, independent of the presence of meningoencephalitis seen in a subset of immunized patients (Lee et al. 2005). These preclinical and clinical data provide the basis for an improvement of immunization antigens by selecting epitopes, eliciting beneficial immune response and avoiding a potentially deleterious cellular immune response. If these novel approaches will exhibit the expected safety profile, Aβ immunotherapy holds promise as a disease modifying AD therapy.

Nonetheless, and since AD is multifactorial disorder beside Aβ production/clearance other therapeutic targets have been addressed, being the identification of the various mechanisms associated with pathology critical for the development of potent disease modifying drugs.

1.8 AIM OF THE THESIS

AD is characterized neuropathologically by the presence of amyloid plaques, neurofibrillary tangles, synaptic loss and consequent neurodegeneration. APP can be cleaved to give rise to different fragments, including the neuroprotective sAPPα fragment and the neurotoxic Aβ peptide (a major constituent of amyloid plaques). The disclosure of the molecular mechanisms triggered by Aβ is crucial to understanding AD pathogenesis, as well normal APP function and processing. Aβ itself has been suggested to have an effect on APP metabolism, potentially affecting the balance of APP proteolytic fragments produced. Aβ-induced alterations on APP metabolism have been the focus of previous attention, although the data reported are controversial. While some authors suggested that Aβ exerts its effect solely on APP catabolism/proteolytic cleavage, others report that it also induces APP transcriptional activation.

The main aims of this thesis were to further characterize the Aβ-induced alterations on APP metabolism and to clarify the molecular mechanisms underlying Aβ function and neurotoxicity. The following specific objectives were addressed:

- the use of different model systems to study the effect of Aβ in terms of APP processing and expression;
- monitorization of the intracellular redistribution of APP protelolytic fragments in response to Aβ;
- evaluation of the nature of the identified intracellular sAPP accumulation in response to Aβ;
- monitorization of the nuclear targeting of proteolytic fragments, given the well documented role of APP as a signal transduction molecule;
- evaluation of the aggregation state of the Aβ peptide and its impact on neurotoxicity and APP processing;
- the effect of Aβ on signaling proteins, namely protein phosphatase 1, given the central role of signaling cascades in neuronal systems.

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Chapter II

Aβ AFFECTS sAPP SECRETION BY INTERFERING WITH CYTOSKELETAL ORGANIZATION

Chapter Outline

Aβ accumulation and deposition into amyloid plaques has been reported to be involved in the sequential events ultimately leading to the typical AD neurodegeneration. The Aβ peptide results from proteolytic processing of APP and thus factors contributing to altered APP processing will potentially lead to an imbalance in the generation of APP proteolytic fragments. This is of major importance if we consider the variety of functions described for both APP and APP fragments, namely sAPPα, which has a neuroprotective role. Aβ is produced in natural occurring conditions, particularly in neuronal cells, and so it is postulated that it might have a physiological function. Therefore several studies have been designed in an attempt to better understand the Aβ-mediated effects and consequently contribute to the understanding of the basis of the disease. However, Aβ has been reported to have inconsistent effects on APP metabolism, which may contribute to Alzheimer´s associated neurotoxicity. While some authors reported that Aβ leads to intracellular APP accumulation by inducing APP expression level, others link this observation with inhibition of APP catabolic/secretory processing (Le et al. 1995; Schmitt et al. 1997; Moreno-Flores et al. 1998; Carlson et al. 2000). The increase of APP transcription may suggest a possible positive feedback mechanism whereby Aβ can induce its own production. In terms of secretion, different data has been obtained, with Aβ having either an inhibitory or no effect on sAPP secretion, depending on the cell type analysed. For instance, Carlson et al. (2000) reported both inhibition in medium secreted sAPP and a concomitant accumulation of mature APP in human glioma cells exposed to $A\beta_{1-40}$ peptide. Interestingly, $A\beta_{25-35}$ induces cytoskeletal rearrangements in cultures astrocytes (Salinero et al. 1997). Mendoza-Naranjo (2007) showed that $A\beta_{1-42}$ was able to stimulate F-actin polymerization, indicating that Aβ may interfere with cytoskeletal network.

In this chapter Aβ-mediated effects on APP metabolism were addressed, in an attempt to elucidate the molecular mechanisms induced by Aβ and its potential relevance to AD. Experiments were carried out either in non-neuronal, neuronal-like and neuronal cells for comparative purposes. Results revealed that several cellular responses were induced upon exposure to the Aβ toxic peptide. Alterations could be detected in the processing of APP, and mechanistically an accumulation of the neuroprotective sAPPα fragment could be observed for all cell types. This observation is in agreement with Carlson et al. (2000). Nonetheless, novel contributions were made since we have shown that the accumulation of isAPP was associated with cytoskeletal structures in vesicular-like densities. The data also suggests that Aβ was affecting the vesicular secretory pathway, and this hypothesis was corroborated in primary cultures. Latter on, studies in both primary neuronal cultures and PC12 cells demonstrated that Aβ leads to abnormalities in cytoskeleton network-related proteins, which may explain the alterations observed in APP trafficking.

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Manuscript I - Intracellular sAPP retention in response to Aβ is mapped to cytoskeleton associated structures

Ana Gabriela Henriques, Sandra Isabel Vieira, Maria Elena Crespo-López[#], Márcio A. Guiomar de Oliveira, Edgar da Cruz e Silva[*] and Odete A.B. da Cruz e Silva.
Laboratório de Neurociências and [*]Laboratório de Transdução de Sinais, Centro de Biologia Celular, Universidade de Aveiro, Aveiro (Portugal), [#]current address Laboratório de Farmacologia Molecular, Universidade Federal Pará, Belém (Brasil).

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Running title: Aβ leads to intracellular cytoskeletal sAPP retention

Corresponding author: Odete A.B. da Cruz e Silva, Centro de Biologia Celular, Secção Autónoma das Ciências da Saúde, Universidade de Aveiro, 3810-193 Aveiro, Portugal. Phone: +351 234 370200 and Fax: +351 234 370983. E-mail: odetecs@ua.pt

ABSTRACT

Aβ contributes to neurodegeneration in Alzheimer's disease and provides a close association between molecular events and pathology, although the underlying molecular mechanisms are unclear. In the work here described Aβ did not induce APP expression, but APP processing/trafficking was markedly affected. In COS-7 cells Aβ provokes retention of intracellular sAPPα (isAPPα). Intracellular holoAPP levels remain unchanged and extracellular total sAPP increases, although extracellular sAPPα alone did not alter significantly. In primary neuronal cultures and PC12 cells isAPP also increased, but this was mirrored by a decrease in extracellular total sAPP. The isAPP retention, was particularly associated with the cytoskeletal fraction. The retention "per se" occurred in vesicular-like densities, negative for a C-terminal antibody and strongly positive for the 6E10 antibody, clearly showing abnormal intracellular accumulation of sAPPα in response to Aβ. Our data supports a dynamic model for intracellular retention of sAPPα as an early response to Aβ exposure. Particularly noteworthy was the observation that removal of Aβ reversed the isAPP accumulation. Mechanistically these findings disclose an attractive physiological response, revealing the capacity of cells to deal with adverse effects induced by Aβ.

Keywords: Aβ peptide, amyloid precursor protein (APP), Alzheimer's Disease, cytoskeleton.

INTRODUCTION

One of the principal hallmarks of Alzheimer's disease (AD) is the presence of senile plaques in the neocortex and hippocampus of affected individuals, where a small peptide termed Abeta (Aβ) predominates (Glenner and Wong 1984). Aβ is a product of proteolytic processing of Alzheimer´s amyloid precursor protein (APP), which can undergo proteolytic cleavage by β-secretase (mainly BACE1), yielding an N-terminal sAPPβ and a C-terminal fragment (CTFβ) (Vassar et al. 1999; Bennett et al. 2000; Yan et al. 2001). The latter is further cleaved by the γ-secretase complex, consisting of presenilin-1 or 2, nicastrin, aph1 or aph2, and pen2 (Li et al. 2000a; Li et al. 2000b; Esler et al. 2002; Lee et al. 2002; Steiner et al. 2002), giving rise to the Aβ peptide. Under pathological conditions this peptide can have neurotoxic effects (Gouras et al. 2005) and is able to aggregate into senile plaques. In a non Aβ-generating pathway, APP is cleaved by α-secretase [including ADAM 10 and TACE (Buxbaum et al. 1998; Lammich et al. 1999; Allinson et al. 2003)] within the Aβ domain, therefore precluding its production and generating an N-terminal APP fragment, termed sAPPα and a CTFα. Subsequent cleavage of CTFα by γ-secretase gives rise to a small fragment termed p3.

APP proteolytic processing and trafficking are closely related (reviewed in da Cruz e Silva and da Cruz e Silva 2003; Small and Gandy 2006), since APP cleavage may occur in several subcellular organelles during its intracellular transport. The major sites of Aβ production are the Golgi (Xu et al. 1997; Xia et al. 1998; Greenfield et al. 1999) and the endosomes (Perez et al. 1999; Vassar et al. 1999; Huse et al. 2000; Rebelo et al. 2007a).

The α-secretase cleavage can occur within TGN, post-TGN vesicles, and PM, with the ratio between intracellular and cell surface sAPPα production appearing to be cell-type specific (Sambamurti et al. 1992; Kuentzel et al. 1993; Parvathy et al. 1999; Jolly-Tornetta and Wolf 2000; Khvotchev and Sudhof 2004). The balance between α- and β-secretase pathways is of physiological relevance, since several AD patients exhibit abnormally low ratios of α-secretase APP processing (Citron et al. 1992; Felsenstein et al. 1994). Further, levels of both sAPPα and α-secretase ADAM 10 have been reported to be decreased in CSF of AD patients (Colciaghi et al. 2002). Hence, in an AD pathology perspective, not only is the presence of additional neurotoxic Aβ relevant, but also the decrease in sAPPα levels. The latter have themselves been associated with impaired memory and to

hippocampal and cortical neurodegeneration (Turner et al. 2003; Thornton et al. 2006). sAPPα is also able to counteract Aβ toxicity (Furukawa et al. 1996; Turner et al. 2003), and an increase in ADAM 10 activity is associated with a reduction in the production of Aβ peptides and their deposition in plaques (Postina et al. 2004).

Several studies have reported that exogenously added Aβ affects APP metabolism, either by altering APP processing/catabolism (Schmitt et al. 1997; Carlson et al. 2000), and/or by inducing APP expression (Le et al. 1995; Moreno-Flores et al. 1998). In fact, it was suggested that $A\beta_{1-40}$ can stimulate its own production in a neuronal hybrid cell line (Le et al. 1995), although the underlying mechanisms are controversial.

Consequently we addressed the effects of Aβ in a non-neuronal cell line, and compared it to primary hippocampal neurons and neuronal-like cells lines. We showed that Aβ dramatically affected APP processing/trafficking causing a specific increase in intracellular sAPP (isAPP). The reversible accumulation of isAPP may represent a cell survival mechanism in response to Aβ which deserves further investigation.

MATERIALS AND METHODS

Maintenance of cell cultures

Non-neuronal COS-7 cells (a monkey kidney cell line) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 3.7 g/l sodium bicarbonate and 10% foetal bovine serum (FBS). Neuronal-like PC12 cells (a rat pheochromocytoma cell line) were grown in RPMI 1640 (Gibco) supplemented with 0.85 g/l sodium bicarbonate, 10% horse serum and 5% FBS. SH-SY5Y cells (a human neuroblastoma cell line) were grown in a 1:1 combination of minimum essential medium (MEM, Gibco) and Ham's F12 medim (Gibco), with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.055 g/l sodium pyruvate and 1.5 g/l sodium bicarbonate. All cultures were maintained at 37°C and 5% CO_2.

Primary rat hippocampal neuronal cultures were established from 18 days embryos. Briefly, after dissociation with trypsin and deoxyribonuclease I (0.15 mg/ml) in Hank's balanced salt solution (HBSS) (0.75 mg/ml during 5-10 min at 37°C) cells were plated onto poly-D-lysine-coated dishes at a density of $1.0x10^5$ cells/cm^2 in B27-supplemented Neurobasal medium (Gibco), a serum-free medium combination (Brewer et al. 1993). The medium was further supplemented with glutamine (0.5 mM), gentamicin (60 μg/ml), and glutamate (25 μM). Cultures were maintained in an atmosphere of 5% CO_2 at 37°C for 9 days, before being used for experimental purposes.

Exposure to Aβ peptide

Cultures were incubated with 20 μM $A\beta_{25-35}$ (Sigma) in complete medium for 24 hr, which was replaced in the last 3 hr by serum-free medium with or without Aβ. This was the conditioned medium collected to monitor extracellular sAPP production. Additionally, for the indicated experimental conditions in COS-7 cells, 50 μg/ml of cycloheximide (CHX) were added during the last 5 hr and 30 min prior to cells and media collection. This period of exposure to CHX was previously determined (da Cruz e Silva et al. 2004; Henriques et al. 2007) as it minimizes adverse toxic effects and is sufficient to completely block APP 'de novo' protein synthesis. For the remaining cell lines, the experimental conditions were as described above but using the appropriate cell-specific medium. For hippocampal cultures, incubations were carried out in neurobasal medium without B27.

Northern blot analysis

Following exposure to Aβ, COS-7 cells ($2x10^6$ cells) were lysed using TRI REAGENT (Sigma) and total RNA was isolated according to the manufacturer's instructions. Normalized total RNA aliquots (15 μg) were separated by formaldehyde gel electrophoresis and transferred to nitrocellulose membranes. To evaluate APP expression levels, the blot was then hybridized with a [^{32}P]-labelled APP cDNA probe (25 ng, $1x10^6$ cpm/ng), as described in the MTN Blot User Manual (Clontech). The APP pan-isoform probe (756 bp) was obtained by *Age* I/*Bam* HI restriction digests of APP_{751} cDNA, labelled with [α-^{32}P]dCTPs (GE Healthcare) using the High Prime DNA Labelling Kit (Roche, Alfagene), and purified through NucTrap Probe Purification Columns (Stratagene). APP hybridizing RNA was detected using PhosphorImager software (Bio-Rad).

RT-PCR analysis

Total RNA was extracted from COS-7 cells and hippocampal cultures ($2x10^6$ cells in both cases) using the RNAgents® total RNA isolation kit (Promega). One microgram of total RNA was reverse transcribed to cDNA using $Oligo(dT)_{18}$ and Stratascript reverse transcriptase (Stratagene). Resulting cDNA was amplified by PCR in 50 μl of reaction mixture containing 2.5 U of Pfu Turbo DNA polymerase (Stratagene), 40 mM dNTPs, and 100 ng/μl primers. The primers used amplified the APP sequence between exon 7 (forward, AGA ACA ACC AGC ATT GCC ACC AC) and exon 9 (reverse, CCT CTC TTT GGC TTT CTG GAA ATG), generating fragments of 390 bp, 330 bp and 162 bp for APP_{770}, APP_{751} and APP_{695} isoforms, respectively. The cDNA was pre-denaturated for 1 minute at 95°C, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 3 min, and with a final extension for 10 min at 68°C. PCR products were resolved on a 2% agarose gel. The relative pattern of APP isoforms was determined as a ratio between the specific isoform level and the total amount of all APP isoforms amplified. At least three independent RT-PCR analyses were carried out for each condition.

Cell fractionation

SH-SY5Y cells were exposed to Aβ and fractions were prepared using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem). Sequential extraction steps yielded fractions containing cytosolic proteins ("Cytosol"), plasma membrane and organelle proteins ("Memb+Org"), nuclear proteins ("Nuclear") and finally cytoskeleton and cytoskeleton-associated proteins ("Cytosk"). Fractions obtained were separated on a 5-20% gradient SDS-PAGE gel and immunoblotted for specific proteins, as indicated.

Immunocytochemistry

COS-7 cells were plated onto coverslips at a confluency of approximately 50%. Following exposure to $A\beta_{25-35}$ for 24 hr, cells were fixed in 4% paraformaldehyde, permeabilized with methanol and blocked with 3% BSA. Subsequently, cells were immunolabelled with specific antibodies. In order to distinguish between KPI-positive sAPP/APP and KPI-negative sAPP/APP isoforms, or their cleaved fragments, we used an anti-KPI monoclonal (Chemicon) and an anti-APP C-terminus (rabbit anti-β-APP, Zymed) antibodies. To identify the intracellular sAPPα fragment, the 6E10 antibody (Sigma) was used. Organelle markers, namely for the endoplasmic reticulum (ER) (calnexin, Stressgen) and the early endosomes (Rab5, Stressgen) were also used. Primary antibody complexes were visualized using Texas Red- (Molecular Probes) and Fluorescein-conjugated (Calbiochem) secondary antibodies. Coverslips were mounted on microscope glass slides using antifading reagents containing or not DAPI for nucleic acids (Vectashield, Vector Laboratories). Epifluorescence and Differential Interference Contrast (DIC) images were acquired using a LSM 510-Meta confocal microscope (Zeiss), and a 63x/1.4 oil immersion objective. Argon laser lines of 405 and 488 nm were used to excite DAPI and Fluorescein, respectively, and a 561 nm DPSS laser was used to excite Texas Red. Microphotographs were acquired in a sole section in the z-axis (xy-mode), and represent a mean of 16 scans.

Sample collection and immunodetection

Conditioned media and cells were collected into boiling 1% SDS, sonicated and boiled. Protein determination of the cellular lysates was carried out using the BCA kit (Pierce). Samples normalized for protein content were separated on 7.5% SDS-PAGE and then electrophoretically transferred onto a nitrocellulose membrane. Immunoblotting was carried out using antibodies which detect holo APP (hAPP) but can also detect sAPP (22C11, Boehringer), APP C-terminal fragments (rabbit anti-β-APP, Zymed) and sAPPα (6E10, Sigma). In the subcellular fraction analysis, specific organelle markers used were pan-cadherin (membrane marker, Abcam), histone 4 (nuclear marker, Santa Cruz Biotechnology), HSP70(72) (cytosolic marker, Stressgen), calnexin (ER marker, Stressgen), and actin (enriched in cytosolic- and cytoskeleton-associated fractions, Stressgen). Primary antibody detection made use of horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia), for enhanced chemiluminescence (ECL and ECL plus Amersham Pharmacia Kits). ECL was used to detect N-terminal and C-terminal intracellular APP species, HSP70, pan-cadherin, calnexin and actin. ECL plus was used to detect extracellular sAPP, intracellular sAPPα, and C-terminal intracellular hAPP and histone 4 in cell fractionation assays.

Quantification and statistical analysis

Quantity One densitometry software (Bio-Rad) was used to quantify band intensity and correlate it to protein levels. Data are expressed as mean±SE determinations, from at least three independent experiments. Statistical analysis was carried out using one way analysis of variance (ANOVA). When the F values were significant, the Dunnett test was applied to compare all groups versus control. The level of significance accepted was $P<0.05$.

RESULTS

Aβ induces intracellular sAPPα accumulation

COS-7 cells exposed to $A\beta_{25-35}$ for 24 hr were lysed, prepared for immunoblotting and probed with the antibodies 22C11, C-terminal and 6E10 (Fig. 1). The former binds to the APP N-terminus, recognizing the full-length protein (hAPP) and the cleaved APP (sAPP) fragment. The C-terminal antibody binds the C-terminus of APP and only recognizes full-length hAPP (at the range of the blot being monitored), while 6E10 recognizes the Aβ domain and therefore can be used to monitor full-length APP and α cleaved sAPP (sAPPα). Comparison of the blots revealed striking responses to exogenously added Aβ. The N-terminal 22C11 antibody (Fig. 1A bands a, b and c) showed an increase in intracellular APP immunoreactive bands (3.09±0.21), whereas the C-terminus antibody which also revealed the same three bands did not detect any alterations in hAPP levels upon addition of Aβ (Fig. 1B). By comparing Fig. 1A with Fig. 1B we can directly deduce that the observed increment in APP is solely due to an increase in intracellular sAPP (isAPP) for $APP_{751/770}$ and APP_{695} (◀, Fig. 1A) which co-migrate with band a and b respectively. Corresponding conditioned medium also showed a significant increase in sAPP ($P<0.01$, 1.93±0.08, Fig. 1C). The reversible nature of this effect is evident given that Aβ removal in the last 3 hr of incubation yielded a decrease in isAPP (◀, Fig. 1A) and a further increase in total extracellular sAPP (2.97±0.09, Fig. 1C). Since Aβ withdrawal concomitantly decreases intracellular accumulated sAPP and increases medium secreted sAPP, the latter appears to result from the removal of an Aβ blocking effect on the secretion of intracellularly produced sAPP.

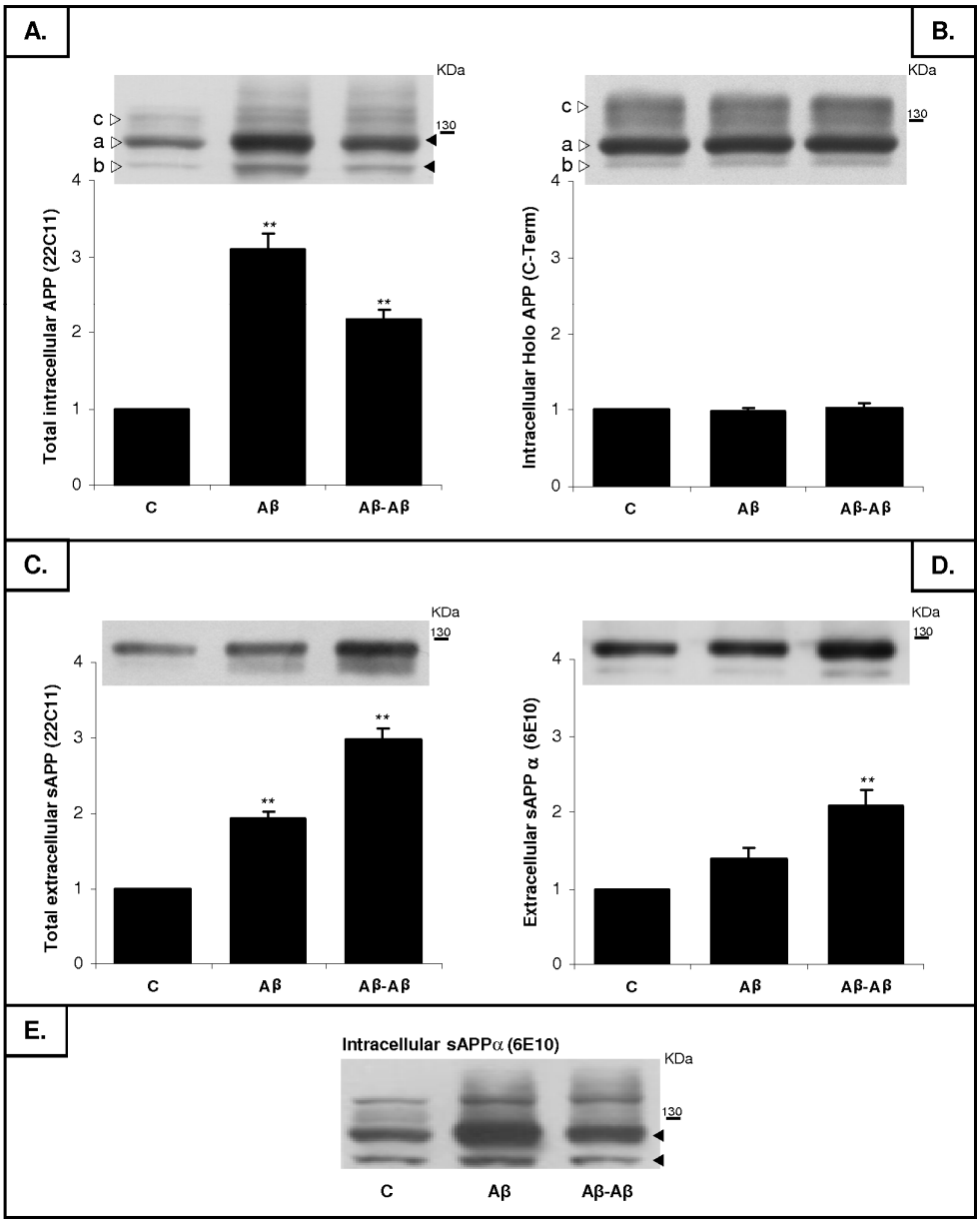

Fig. 1. Aβ induces alterations on APP metabolism in COS-7 cells. Cells were exposed to 20 μM $A\beta_{25-35}$ for a period of 24 hr. **(A)** Intracellular sAPP and **(B**) holo APP levels were determined using the 22C11 antibody and C-terminal antibodies respectively. (◁ a), predominantly immature $APP_{751/770}$; (◁ b), predominantly immature APP_{695}; (◁ c), mature $APP_{751/770}$. (upper arrowhead ◀), intracellular $sAPP_{751/770}$, and (lower arrowhead ◀), intracellular $sAPP_{695}$. **(C)** Total sAPP (22C11 antibody) and **(D)** sAPPα (6E10 antibody) secretion in conditioned medium. **(E)** Accumulation of intracellular sAPPα in cellular lysates using 6E10 antibody (◀ intracellular sAPPα). **P<0.01, significantly different from control; Dunnett *post hoc* test. All values are expressed as mean±SE from at least 3 independent experiments. C, Control; Aβ, Aβ exposure during 24 hr; Aβ-Aβ, Aβ removal in the last 3 hr.

In order to quantify the Aβ effect on the secretion of sAPPα specifically, being this the predominant cleavage product, COS-7 cells conditioned medium was also probed with the 6E10 antibody (Fig. 1D). Notably, following Aβ exposure, extracellular sAPPα levels did not increase as significantly (1.2 fold increase, Fig. 1D) as did total sAPP (2 fold increase, Fig. 1C). Similarly extracellular total sAPP increased comparatively more than extracellular sAPPα upon Aβ withdrawal (Fig. 1C, D). Consistently, when cell lysates were probed with the 6E10 antibody, a significant intracellular sAPPα retention was detected (Fig. 1E), but this decreased started to revert towards basal, following Aβ withdrawal in the last 3 hr; and correlated with the extracellular sAPPα increase (Fig. 1E, D). The sAPPβ species was not addressed directly as it represents a relatively small population, particularly difficult to monitor when endogenous protein levels are measured.

Aβ does not increase APP expression levels

To test if the observed increase in isAPP could be due to *APP* transcriptional activation, experiments were repeated in the presence of cycloheximide (CHX, a drug which inhibits "de novo" protein synthesis). Experimental conditions were modified, CHX was added in the last 5 hr and 30 min of the 24 hr exposure period to Aβ peptide. This period of CHX incubation was chosen given that it was previously shown to be sufficient to deplete APP levels in COS-7 cells (da Cruz e Silva et al. 2004). Simultaneous exposure of cells to Aβ and CHX still led to an increase in isAPP levels, when compared to CHX addition alone (Fig. 2A). Further, when Aβ was removed in the last 3 hr but CHX maintained, isAPP levels decreased and extracellular sAPP levels significantly increased (Fig. 2B) suggesting that Aβ, in fact, blocks sAPP secretion.

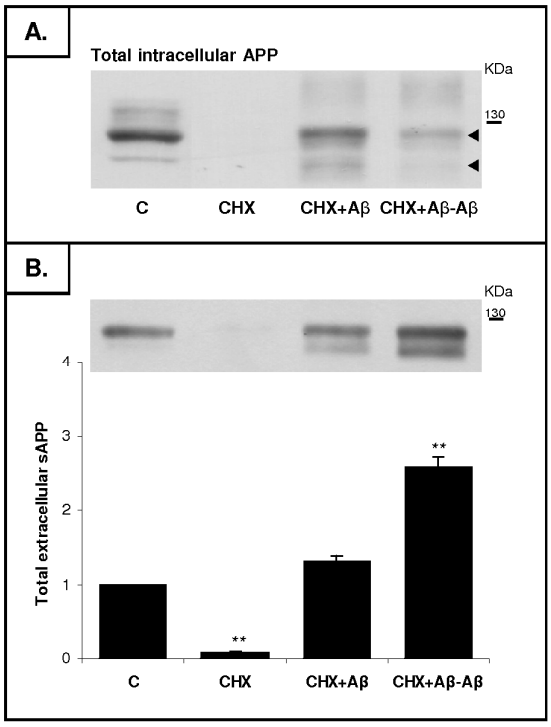

Fig. 2. Aβ response is maintained in the absence of "de novo" protein synthesis. (A) APP and sAPP (◀) intracellular sAPP levels and **(B)** sAPP secretion was monitored in the presence of cycloheximide (CHX) during the last 5 hr and 30 min of the 24 hr Aβ incubation period. **P<0.01, significantly different from control; Dunnett *post hoc* test. All values are expressed as mean±SE from at least 3 independent experiments. C - control; CHX, CHX in the last 5 hr and 30 min; CHXAβ, CHX and Aβ; and CHXAβ-Aβ, Aβ removal in the last 3 hr in the presence of CHX.

Further, Northern blot analysis indicated that Aβ exposure did not increase APP mRNA levels (Fig. 3A). This is consistent with results in Fig. 1B, where protein levels do not increase and with the hypothesis that *APP* transcriptional activation is not induced by Aβ exposure as has been previously observed for other cell types (Schmitt et al. 1997; Carlson et al. 2000). Isoform specific responses were also addressed using RT-PCR analysis and pan-APP primers. The relative abundance of the three major APP isoforms (APP_{770}, APP_{751} and APP_{695}) was unaltered by Aβ treatment, both in COS-7 cells and in primary hippocampal cell cultures (Fig. 3B). Hence it is reasonable to conclude that Aβ affects APP processing and does not induce APP transcription.

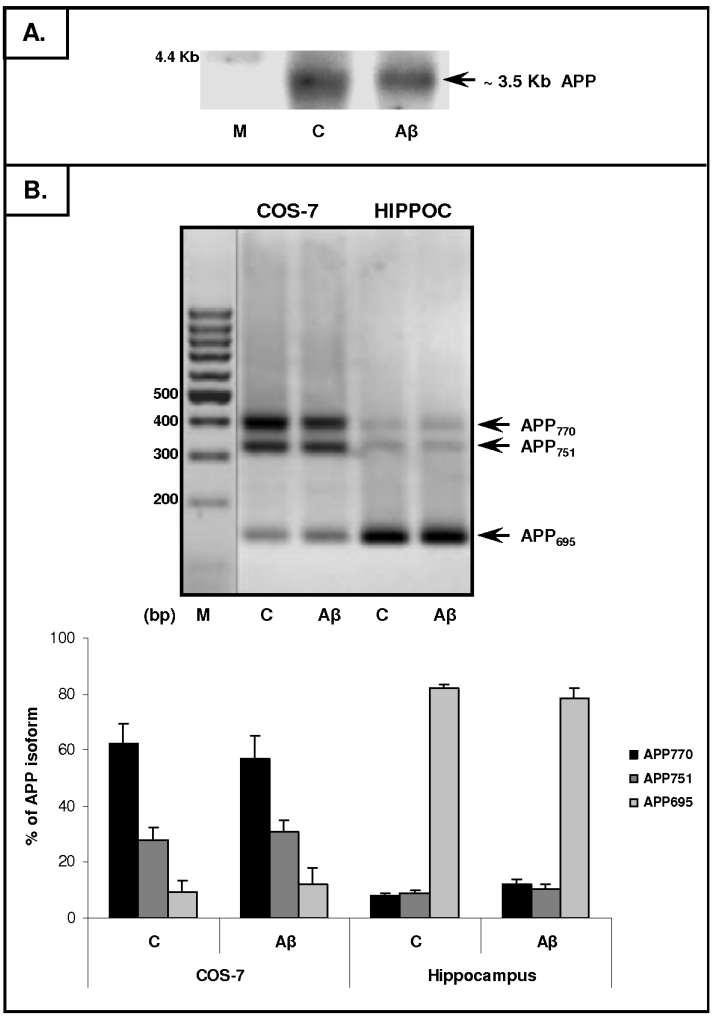

Fig. 3. Aβ effects are independent of *APP* transcriptional induction. Total RNA was purified from cells treated for 24 hr with 20 μM $A\beta_{25-35}$, and either processed for Northern blotting or RT-PCR analyses. **(A)** Evaluation of *APP* transcription in COS-7 cells, Northern blotting was performed as described in the methods. **(B)** After cDNA synthesis, RT-PCR was carried out using specific primers for the three major APP isoforms in COS-7 cells (that mainly express the $APP_{751/770}$ isoforms) and in hippocampal cultures (Hippoc, enriched in the APP_{695} isoform). Results are based on an APP isoform to total APP isoforms ratio. All values are expressed as mean±SE of 3 independent experiments. M - Molecular weight markers; C, Control; Aβ, Aβ treatment.

Aβ effect on sAPP secretion is cell type dependent

Since AD is a neurodegenerative disorder, the evaluation and comparison of Aβ effects in non-neuronal and neuronal cells is of particular interest. Several other cell types were incubated with 20 μM $A\beta_{25-35}$ peptide for 24 hr, and in all cases isAPP accumulation was observed (◀, Fig. 4A). Surprisingly, however, a wide range of responses occurred when sAPP secretion into the medium was monitored (Fig. 4A).

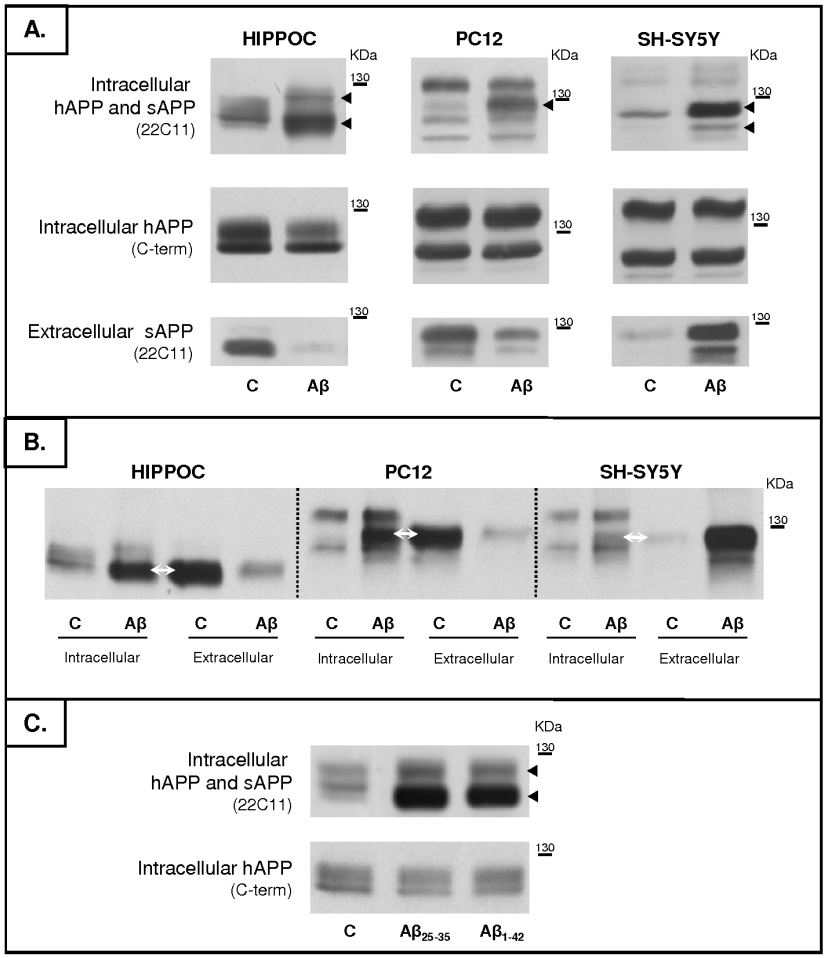

Fig. 4. Aβ effect on sAPP secretion is cell type dependent. **(A)** $A\beta_{25-35}$ effects on APP intracellular levels and sAPP secretion in primary hippocampal neurons (Hippoc) and in neuronal–like cell lines (PC12 and SH-SY5Y). Intracellular hAPP and sAPP (N-terminal 22C11 antibody) and intracellular hAPP (C-terminal antibody) were detected using ECL. (upper arrowhead ◀), intracellular $sAPP_{751/770}$; and (lower arrowhead ◀), intracellular $sAPP_{695}$. Total extracellular sAPP was detected using the ECL plus chemiluminescence reagent. **(B)** Comparison of migration profile for intracellular sAPP and extracellular sAPP (↔) with the antibody 22C11. **(C)** Intracellular sAPP accumulation in response to exposure to different Aβ peptides (20 μM) for primary neuronal cultures. C, Control; Aβ, $A\beta_{25-35}$ treatment.

While for hippocampal primary cultures, cortical cultures (data not shown) and PC12 cells, Aβ led to a dramatic inhibition of sAPP extracellular secretion, a contrasting response was observed for COS-7 (Fig. 1) and SH-SY5Y cells, where Aβ caused an increase in sAPP released into the medium. Nonetheless, in all cases tested, subsequent Aβ removal induced a further increase in extracellular sAPP levels (data not shown), as previously observed for COS-7 cells (Fig. 1C). As for COS-7 cells, this was not an APP isoform-dependent effect, as all isoforms responded similarly in each cell type. Co-migration of isAPP and extracellular secreted sAPP was confirmed by running both samples (lysates and medium) on the same gel system (Fig. 4B, double headed arrow). The physiological relevance of isAPP accumulation was further supported by the observation that $A\beta_{1-42}$ yields the same results (Fig. 4C). In summary, one can deduce that Aβ exerts its effect not in an isoform specific manner, but possibly on different secretory APP/sAPP trafficking pathways. Hence, subcellular fractionation studies and immunofluorescence assays were performed in either SH-SY5Y or COS-7 cells.

sAPP is retained in cytoskeletal-associated structures

Differential separation of proteins according to their subcellular localization was carried out following incubation of SH-SY5Y cells with Aβ. Subcellular organelle markers were used to confirm the enrichment of the different fractions (Fig. 5). The cytosolic fraction was enriched in HSP70 and cytosolic actin. The membranar fraction contained plasma membrane (pan-cadherin enriched) and organelles (ER marker, calnexin), with the nuclear fraction being enriched in histone 4, as expected. The cytoskeleton/microtubule fraction was actin and pan-cadherin-positive and free of organelle and nuclear markers (Fig. 5).

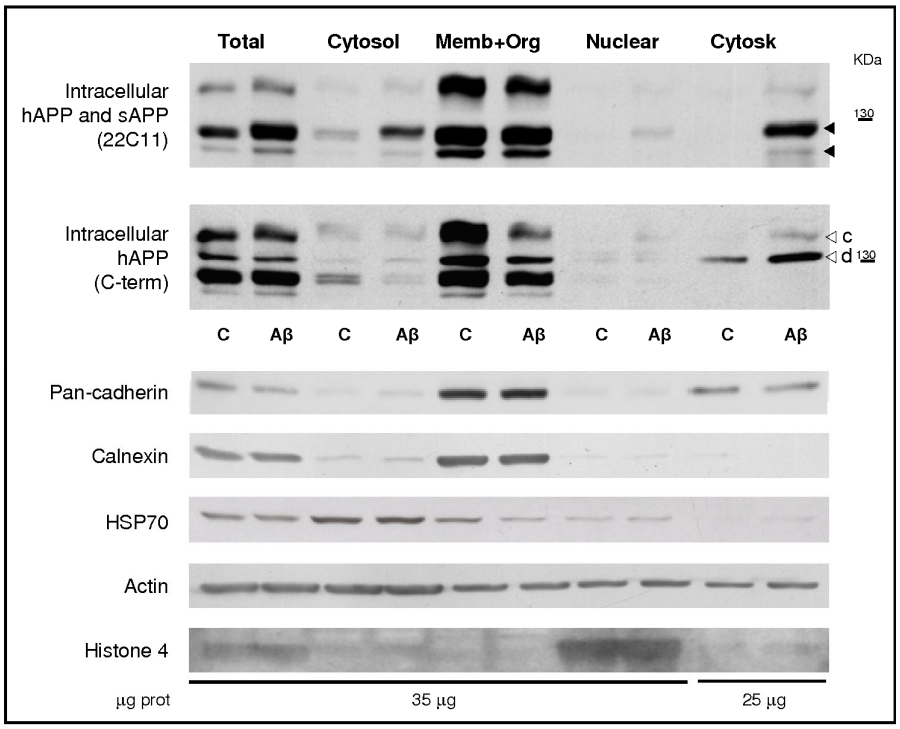

Fig. 5. APP subcellular distribution in response to Aβ treatment. Following incubation with $A\beta_{25-35}$, SH-SY5Y cells were fractionated as described and the resulting fractions analyzed by immunoblotting. Total – total cell lysate; Cytosol – cytosolic protein fraction; Memb + Org – membrane and organelle enriched fraction; Nuclear – nuclear enriched fraction; and Cytosk – Cytoskeleton protein fraction. (◀), isAPP forms; (◁ c), mature $hAPP_{751/770}$; and (◁ d), potential mature APP_{695} and also APLP forms. Intracellular sAPP and APP isoforms (N-terminal 22C11 antibody), pan-cadherin (membrane marker), calnexin (ER marker), HSP70 (cytosolic marker) and actin (cytosolic and cytoskeleton marker) were detected using ECL; intracellular hAPP (C-terminal antibody) and histone 4 (nuclear marker) were detected using the highly sensitive ECL plus reagent. C, Control; Aβ, Aβ treatment.

Immunoblot analysis of subcellular fractions with 22C11 or C-terminal antibodies allowed us to distinguish hAPP (◁) from sAPP (◀). A substantial accumulation of isAPP in the cytoskeletal fraction was evident, and a less marked increase could also be detected in the cytosolic fraction. A decrease in membrane-associated mature hAPP following Aβ exposure was demonstrated, as determined with both C- and N-terminal antibodies, and this was accompanied by an increase in mature $hAPP_{751/770}$ C-terminal positive isoforms in the cytoskeletal fraction (◁ c, Fig. 5). Other APP species, potentially mature $hAPP_{695}$ and/or APLP forms (◁ d, Fig. 5), which have a higher molecular weight than the N-terminal isAPP fragments (◀ isAPP, Fig. 5), also appeared to be retained in cytoskeletal-associated structures. In essence, Aβ exposure leads to a decrease in mature hAPP species in plasma membrane (PM), ER and Golgi-enriched membranar fraction, and to an

increase in the cytoskeletal fraction. Simultaneously, isAPP accumulates in the same cytoskeletal fraction and in the cytosolic fraction, fractions known to be associated with cytoplasmic vesicles. This suggests that mature hAPP is cleaved intracellularly before reaching the PM. These results strengthen the previous hypothesis that Aβ is exerting its blocking effect at the secretory level, with this being further investigated by immunofluorescence analysis.

Aβ induces accumulation of sAPPα-containing vesicular-like densities

To identify the intracellular site of sAPP retention, a C-terminal antibody that recognizes all endogenous hAPP isoforms and CTFs, and an anti-APP KPI antibody (specific for the N terminus of $hAPP_{751/770}$ - the most abundant isoforms in COS-7 cells - and their cleaved fragments $sAPP_{751/770}$) were used (Fig. 6). Under basal conditions, the co-localization of C-terminal positive and KPI-positive $hAPP_{751/770}$ was evident in small vesicles distributed throughout the cytoplasm (yellow/orange specs). C-terminal-positive and KPI-negative staining (APP CTFs and APP_{695}/APPLPs) was detected in the nucleus, vesicles, and in the Golgi (typical APP distribution). The latter are known subcellular sites of APP cleavage, and subsequent production of CTFs and sAPP.

Strikingly, Aβ treatment led to a dramatic accumulation of KPI-positive and C-terminal negative, green only clusters (white arrow, Fig. 6). Given the immunoreactive profile of these clusters, it is reasonable to conclude that they are $sAPP_{751/770}$–containing vesicular structures and therefore consistent with these being the subcellular structures where isAPP accumulates. These clusters were found concentrated in the cytoplasm and near the PM.

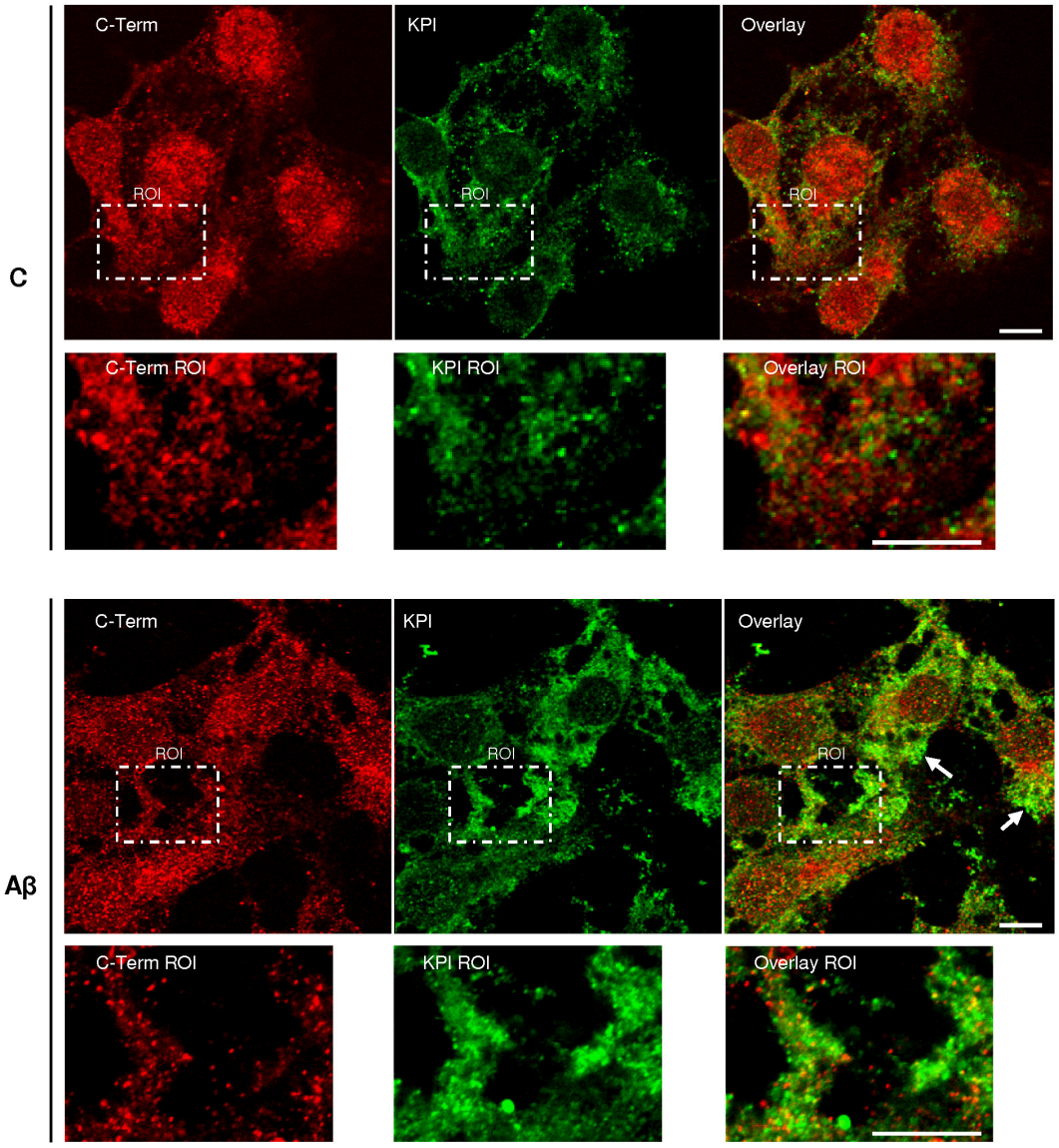

Fig. 6. **APP and sAPP intracellular distribution in response to Aβ**. COS-7 cells were treated with $A\beta_{25-35}$ and endogenous APP and sAPP subcellular localizations were addressed using either a KPI specific or the C-terminal antibody. ROI, region of interest (dashed box). Epifluorescence images where acquired using a Zeiss confocal microscope. Representative examples are shown for each experimental condition. C, Control; Aβ, Aβ treatment. Bar, 10 μm.

Given that the ER is a possible alternative pathway of sAPP production/secretion (Shin et al. 2005; Rebelo et al. 2007b), co-localization studies were carried out with anti-APP KPI and anti-calnexin (ER marker) antibodies. Exposure to Aβ did not render any visible effect on the ER morphology itself as is evident from the consistency of calnexin staining (red cytoplasmic and perinuclear staining). KPI(APP and sAPP)/calnexin(ER) co-localization was maintained in cytoplasmic and ER regions even in the presence of Aβ (Fig. 7, intermediate focus plane, yellow/orange staining in overlay), supporting the existence of an alternative sAPP ER-secretory pathway. Confocal microscopy at the nuclear focus plane, under basal conditions (Fig. 7) revealed APP KPI-positive green staining at the perinuclear ER (calnexin positive, red staining).

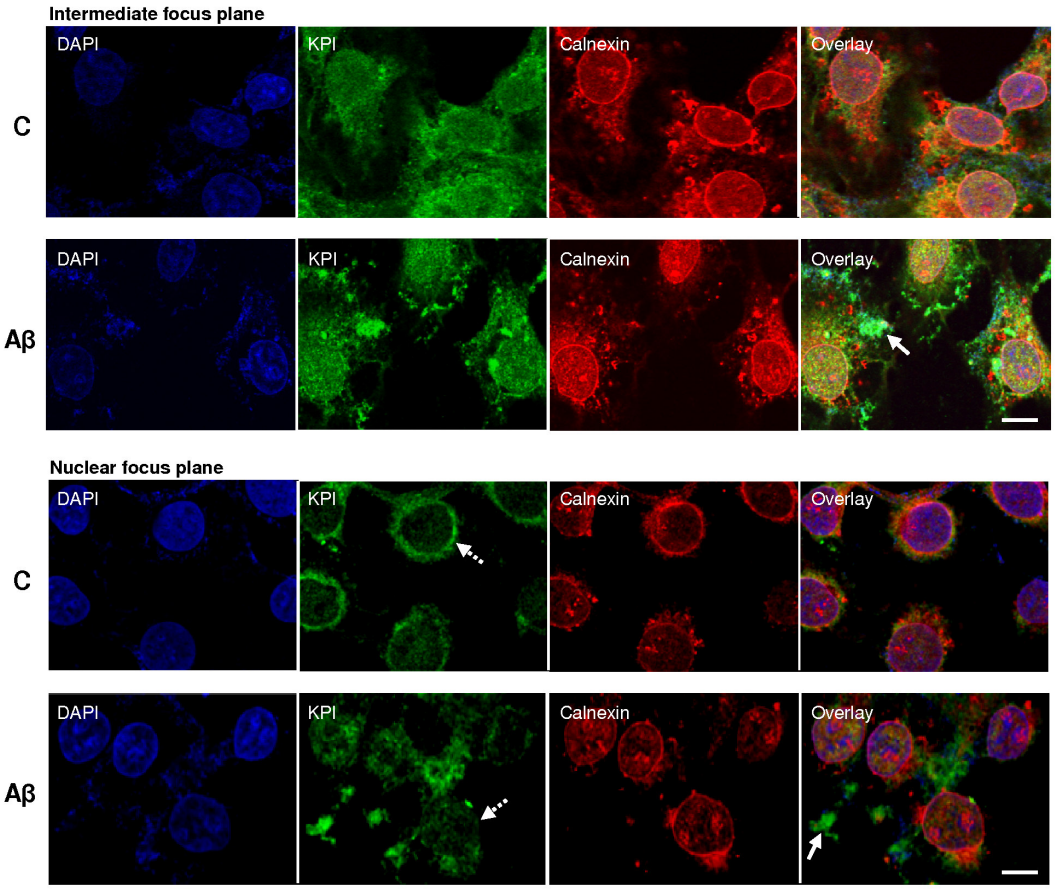

Fig. 7. ER integrity remains unaltered upon Aβ treatment. Following Aβ exposure as previously described, perinuclear and cytoplasmic APP (KPI antibody) co-localizations with an endoplasmic reticulum protein marker (calnexin) were evaluated at intermediate (above nucleus and below cell surface) and nuclear focus planes. Intense green fluorescent isAPP clusters are evident, solid arrow. Epifluorescence images where acquired using a Zeiss confocal microscope. C, Control; Aβ, Aβ treatment. Bar, 10 μm.

However, upon Aβ addition the green perinuclear staining decreased (dashed arrow), consistent with a decrease in hAPP observed for the "Memb+Org" fraction (Fig. 5). As described above, accumulation of green clusters were again evident in response to Aβ addition (solid arrow). Of note is that the cells are not apoptotic, as revealed by the DAPI staining (Fig. 7). Additional studies revealed that the vesicular-like densities, mainly negative with the C-terminal antibody but positive with the KPI antibody, are also 6E10 positive, thus characterizing the APP fragment retained intracellularly as mainly isAPPα (Fig. 8A).

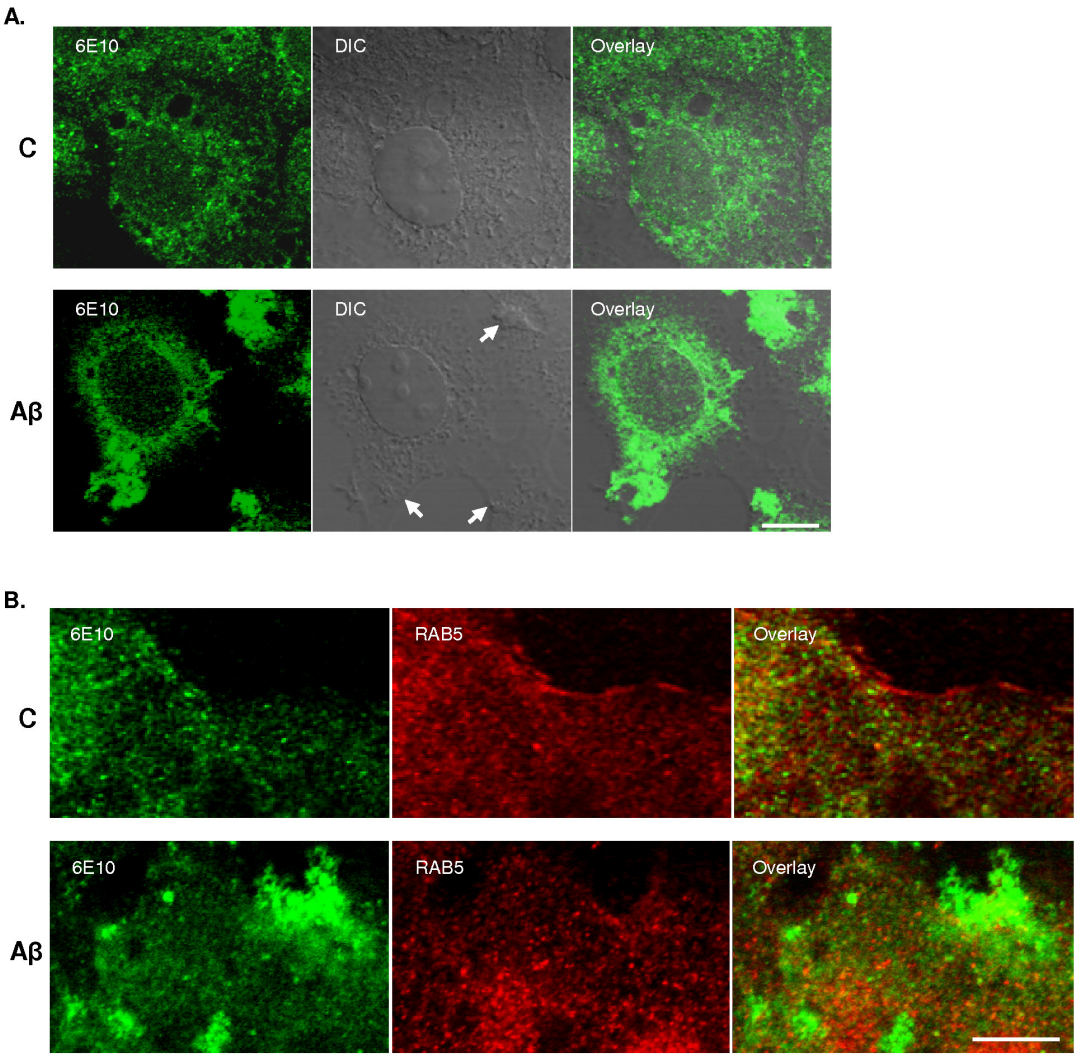

Fig. 8. sAPPα subcellular localization following Aβ exposure. sAPPα intracellular localization following Aβ treatment was determined using either the anti-APP 6E10 antibody and DIC microscopy (white arrows) **(A)** or the 6E10 and Rab5 and (an early endossomal marker) antibodies and epifluorescence confocal microscopy **(B)**. Intense green fluorescent isAPP clusters are evident upon Aβ treatment. C, Control; Aβ, Aβ treatment. Bar, 10 μm.

Moreover, DIC microscopy along with 6E10 immunofluorescence staining showed that although the isAPPα accumulation could be detected throughout the cytoplasm it occurred mainly near the PM (white solid arrows, Fig. 8A). Immunofluorescent studies obtained using the 6E10 and Rab5 (an early endosome marker) antibodies, showed virtually no co-localization of the isAPPα containing densities (Fig. 8B), revealing that isAPP is not accumulating in endocytic vesicles. In conclusion, the observed isAPPα accumulation in cytoplasmic vesicular-like densities near the cell surface is consistent with the subcellular fractionation data (Fig. 5) and with Aβ having and inhibitory effect on the APP Golgi-to-PM secretion via post-TGN vesicles.

DISCUSSION

Aβ is produced under physiological conditions, but various reports have shown it to be potentially neurotoxic. Aβ was added to cells in culture at concentrations where induction of apoptosis was minimal. Under conditions tested Aβ altered APP processing in a reversible manner. This response was addressed in different cells types using the biological active domain $A\beta_{25-35}$, which shows similar properties to naturally occurring $A\beta_{1-40}$ and $A\beta_{1-42}$ (the principal component of AD senile plaques) (Pike et al. 1995; Xu et al. 2001a; Xu et al. 2001b; Liao et al. 2007). Hence, $A\beta_{25-35}$ represents a good experimental model to study Aβ-downstream molecular mechanisms that may be involved in AD. Several authors have reported that Aβ itself can alter APP metabolism, with the underlying mechanisms potentially involving Aβ-dependent induction of APP mRNA (Le et al. 1995; Moreno-Flores et al. 1998), or altered APP processing/catabolism (Davis-Salinas et al. 1995; Yang et al. 1995; Schmitt et al. 1997; Carlson et al. 2000). In our work, we observed that COS-7 cells exposed to $A\beta_{25-35}$ exhibited an increase in APP intracellular levels, as detected with an N-terminal antibody that recognizes both hAPP and sAPP, confirming previous reports (Davis-Salinas et al. 1995; Le et al. 1995; Schmitt et al. 1997; Moreno-Flores et al. 1998; Carlson et al. 2000). However, by probing with a C-terminal antibody no differences could be detected in hAPP levels, allowing us to deduce that the observed accumulation resulted from a rise in an intracellular sAPP pool (isAPP). This has only been suggested by Carlson et al. (2000). Removal of the Aβ peptide during the last 3 hr of the 24 hr incubation period (Aβ-Aβ) reverted this effect, resulting in a significant isAPP decrease and in increased sAPP secretion. Thus, it seemed reasonable to deduce that mechanistically Aβ does not inhibit sAPP production but rather blocks its secretion/release to the extracellular milieu. Further, given that upon addition of Aβ the expression levels of APP in COS-7 cells are largely unaltered and that both isAPP as well as total sAPP in conditioned medium increase, it would appear that Aβ also has the capacity to induce sAPP production.

The use of specific antibodies allowed us to elucidate that Aβ was particularly affecting sAPPα secretion/release. Using the 6E10 antibody (Fig. 1E) we observed a high $A\beta_{25-35}$-induction of intracellular sAPPα retention in the cellular lysates but only marginal increases in sAPPα medium secretion (Fig. 1D). Levels were partially reverted following

Aβ withdrawal, again reinforcing a mechanism whereby Aβ blocks sAPPα secretion. This is consistent with previous observations of $A\beta_{1-40}$ on isAPPα (Carlson et al. 2000), and may explain the different fold increases in total sAPP (Fig. 1C) and sAPPα secretions (Fig. 1D). These differences suggest that APP cleavage by β-secretase is also enhanced and that sAPPβ is well secreted, leading to higher levels of total sAPP secreted when compared to sAPPα only. Noticeably, contrary to previous reports by other authors, increased accumulation of APP proteolytic fragments was not due to enhanced *APP* transcription (Figs 2, 3).

Aβ-induced isAPP retention was also demonstrated for SH-SY5Y and PC12 cells and primary neuronal cultures (Fig. 4). Differences however were observed for extracellular sAPP secretion. For COS-7 (Fig. 1) and SH-SY5Y there was an increase, whereas for PC12 and primary neuronal cultures a decrease was observed. Dissimilar effects on sAPP secretion have been reported by several authors upon exposure of different cell lines to Aβ peptides (Davis-Salinas et al. 1995; Schmitt et al. 1997; Carlson et al. 2000). We propose that these different responses in the levels of secreted sAPP could be due to cell type-specific processing pathways. Neuronal and even undifferentiated PC12 cells are well known models of highly regulated secretion, presenting not only a constitutive secretory vesicular pathway but also stimuli-sensitive regulated vesicular secretion (Greene and Tischler 1976; Burgess and Kelly 1987; Martin and Grishanin 2003). In contrast, besides the constitutive vesicular pathway, no regulated vesicular secretion has been described for COS-7 cells, and in undifferentiated SH-SY5Y cells, only residual regulated vesicular machinery appears to exist (Goodall et al. 1997), and not reported to be associated with APP/sAPP secretion. Our data suggest that sAPP/APP targeting to secretory pathways may be under tight control in neurons and PC12 cells, while it may be less regulated in COS-7 and undifferentiated SH-SY5Y cells, where other non-vesicular secretory routes may be used. A novel APP α-secretase cleavage pathway was recently described for COS-7 cells, involving the cytoplasmic-spanning smooth ER (Shin et al. 2005). These authors also observed that sAPP deriving from an APP mutant with and ER-retrieval signal did not follow the normal post-TGN vesicular secretory pathway. For that mutant, sAPP secretion was diminished but not absent, and sAPP appeared to be media secreted via the smooth ER, as deduced from strong sAPPα/ER co-localization. Further, in our laboratory, an APP phosphomutant with impaired vesicular secretion was observed to produce and

secrete normal levels of sAPP, apparently through the cytoplasmic ER (Rebelo et al. 2007b). Accordingly, in the immunocytochemistry experiments here described, some isAPP could be observed to co-localize with the ER marker calnexin (Fig. 7, intermediate plane), even upon addition of Aβ. The calnexin positive structures (potentially the smooth ER) were themselves largely unaffected by Aβ and did not co-localize with the identified sAPP clusters near the PM. Therefore, we postulate that the major APP/sAPP secretory pathway (vesicular) is compromised and, since the ER integrity remained unaltered, that in the presence of Aβ, sAPP of Golgi/ER origin could be redirected to an alternative, usually less used, ER-to-PM secretory pathway. This explains why under Aβ exposure we could still observe secretion of a pool of sAPP (Fig. 1). Further, the specificity of the sAPP retention effect on sAPPα may derive from the different pools of sAPP production. The α-secretase pathway is mainly associated with the TGN, post-TGN vesicles and the PM, while the β-secretase pathway mainly occurs at the Golgi and endosomes. Mechanistically Aβ appeared to be particularly affecting the vesicular secretory pathway, and in COS-7 cells, sAPP pools produced at the Golgi and ER (before post-TGN vesicles packaging) could still be secreted through the ER.

Further data supported the hypothesis of an Aβ-dependent inhibition of sAPP vesicle secretion. The subcellular fractionation data revealed that isAPP retention was associated with the cytoskeleton (◀, Fig. 5). As the cytoskeleton network has long been associated with vesicular motility (Meyer and Burger 1979; Hamm-Alvarez and Sheetz 1998; Buss et al. 2004; Lanzetti 2007; Potokar et al. 2007), this is in agreement with an Aβ blocking effect at a sAPP/APP secretory vesicular level. Immunofluorescent assays were therefore performed in COS-7 cells, and isAPP accumulation was detected in cytoplasmic vesicular-like structures often aggregated into clusters mainly found just below the PM (Figs 6, 7, 8). Further, the pattern of APP/Rab5 co-localizing vesicles was apparently unchanged upon Aβ treatment, and isAPPα-positive clusters did not co-localize with this early endosome marker (Fig. 8B). Therefore, our results support that isAPP accumulation in response to exogenous Aβ is derived from the secretory pathway, where sAPPα generation is known to occur. Altered intracellular trafficking in response to Aβ had already been suggested to account for increased cellular APP (Carlson et al. 2000). Possible underlying mechanisms proposed included Aβ interference with cytoskeleton-mediated transport and associated proteins (Salinero et al. 1997; Carlson et al. 2000; Mendoza-Naranjo et al. 2007). Our

results validate this hypothesis and are consistent with Aβ impairing vesicle motility along the cytoskeleton and/or inhibiting vesicle docking/fusion at the PM, leading to isAPP accumulation within vesicular-like structures before reaching the cell membrane. In parallel, hAPP at the Golgi and ER is a potential substrate for α- and β-secretases, with the resultant sAPP being free to be transported via the ER and subsequently medium secreted.

Our data supports a model where intracellular retention of sAPPα may be an early cellular response to Aβ exposure. This is of particular relevance to AD pathology when considering that the secretion of this neuroprotective fragment is affected in this disease. Indeed, it has been well described that the levels of the memory-related and potential regenerating sAPPα fragment are decreased in AD brain and CSF (Colciaghi et al. 2002; Olsson et al. 2003). Furthermore, we have shown that Aβ exerts an effect in the APP secretory vesicular route, impairing the normal Golgi-to-PM sAPP/APP traffic and blocking sAPP secretion. The involvement of cytoskeleton-associated alterations, producing vesicular movement abnormalities, is now being further evaluated in our laboratory. Removal of Aβ reverses isAPP accumulation thus representing the ability of cells to deal with adverse effects. However, continued exposure to Aβ would contribute to progressive neurodegeneration. Of note, Aβ had a more dramatic effect in inhibiting sAPP secretion in cells known to have highly regulated vesicular secretion, such as neurons. These differential Aβ responses in neuronal and non-neuronal cells are of particular interest if one considers that AD is a neurodegenerative condition essentially affecting the brain and sparing most of the peripheral tissues.

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Manuscript 2 - Aβ affects cytoskeleton dynamics with consequences for neuronal sAPP vesicular traffick

Ana Gabriela Henriques, Sandra Isabel Vieira, Edgar F. da Cruz e Silva* and Odete A.B. da Cruz e Silva.
Laboratório de Neurociências and *Laboratório de Transdução de Sinais, Centro de Biologia Celular, Universidade de Aveiro, Aveiro (Portugal).

Manuscript in preparation

Running title: Aβ affects neuronal APP/sAPP vesicular traffick

Corresponding author: Odete A.B. da Cruz e Silva, Centro de Biologia Celular, Secção Autónoma das Ciências da Saúde, Universidade de Aveiro, 3810-193 Aveiro, Portugal. Phone: +351 234 370200 and Fax: +351 234 370983. E-mail: odetecs@ua.pt

ABSTRACT

In neurons, APP is anterogradelly transported in a kinesin–associated manner to axonal vesicular compartments. The major properties attributed to Aβ are its neurotoxic properties which may be responsible for AD neurodegeneration. However, Aβ can also affect APP processing, and here we show that it can induce the accumulation of intracellular sAPP (isAPP), in primary neuronal cultures. Retention of sAPP (positive for an APP N-terminal antibody and negative for an APP C-terminal antibody) was localized to cytoskeleton associated vesicular-like structures along the neurite processes. These structures were also positive for kinesin light chain (KLC). Furthermore Aβ affected both actin and microtubule networks, in particular by increasing F-actin polymerization and decreasing α-tubulin acetylation. The use of cytoskeleton affecting drugs reversed the Aβ-induced effects on sAPP secretion. The data here presented supports that Aβ causes isAPP retention by inducing alterations in the cytoskeleton network, thus contributing to impaired APP vesicular transport. Moreover, the data strengthens the hypothesis of Aβ induced neurodegeneration, since impaired vesicular and axonal transport have been linked to AD pathology.

Keywords: Aβ peptide, amyloid precursor protein (APP), Alzheimer´s Disease, secretory vesicular pathway, neuroprotection, subcellular localization

INTRODUCTION

Alzheimer´s Disease (AD) is a neurodegenerative disorder of the CNS, prevalent in the elderly. Neuropathologically AD is characterized by the presence of amyloid plaques (Glenner and Wong, 1984), mainly composed of Aβ peptide, and neurofibrillary tangles, formed by hyperphosphorylated tau aggregates (Goedert et al., 1992), along with widespread synaptic and neuronal loss in distinct brain areas such as the neocortex and hippocampus (Hyman et al., 1984; Masliah et al., 1991; Terry et al., 1991). These changes have been reported to be accompanied by severe disruption of both axonal and dendritic cytoskeleton, suggesting failure in axonal transport in AD. In fact, abnormal axons precede amyloid deposition in some mouse models, suggesting that axonal defects may play a crucial role in the earliest stages of AD pathogenesis (Stokin et al., 2005). Further, AD axonal pathology includes atypical axons that exhibit abnormal accumulations of the amyloid precursor protein (APP) (Cras et al., 1991) and its fragments (Sennvik et al., 2004; Takahashi et al., 2004), consistent with altered or blocked APP axonal transport. In AD there is also impaired transport of neurotransmitters and neuropeptides (reviewed in Bell and Claudio Cueelo, 2006), processes essential for neuronal viability and function.

KLC-driven APP axonal transport has been suggested, either by direct APP interaction with KLC (Kamal et al., 2000), or indirectly via complex formation with JIP-1 (Inomata et al., 2003; Lazarov et al., 2005; Matsuda et al., 2003). Additionally, kinesin-1 reduction enhances the development of axonal abnormalities, as well as enhancing aberrant Aβ generation and amyloid deposition (Gunawardena and Goldstein, 2001). The latter derives from the amyloidogenic processing of APP, as it is sequentially cleaved by β-secretase (mostly BACE-1) (Vassar et al., 1999; Yan et al., 2001) and the γ- secretase complex (comprising presenilin, nicastrin, APH1 and PEN2) (Esler et al., 2002; Lee et al., 2002; Li et al., 2000; Steiner et al., 2002). Generation of Aβ also leads to the extracellular release of sAPPβ and intracellular APP C-terminal domain (AICD). In neurons, APP was found in an axonal vesicular compartment which is anterogradelly transported in a kinesin-dependent manner (Kamal et al., 2001), a potential subcellular compartment for Aβ generation. Alternatively, APP can be processed in a non-amyloidogenic pathway by α-secretase (Allinson et al., 2003; Buxbaum et al., 1998; Lammich et al., 1999) and the γ-secretase complex, hence precluding Aβ formation and giving rise to the peptide p3 and

sAPPα. Contrary to the Aβ peptide, the latter fragment has been reported to have neurotrophic and neuroprotective functions when added to cells in culture (Thornton et al., 2006; Turner et al., 2003).

Alterations in the intracellular transport of APP may affect the extent to which APP is available for β- or α-secretase cleavage in specific subcellular compartments, thus influencing the balance between the two pathways and subsequent generation of the different fragments. Since, APP anterograde transport has been associated either directly or indirectly with kinesin-1 complex and microtubules, APP trafficking is susceptible to alterations in the cytoskeleton network and its related proteins. Interestingly, Aβ peptide was reported to induce cytoskeleton reorganization and morphological alterations in astrocytes (Salinero et al., 1997). Additionally, our previous studies in a non-neuronal cell line, also demonstrated that Aβ leads to intracellular sAPP (isAPP) retention in vesicular-like densities associated with the cytoskeleton enriched fraction (Henriques et al., 2009a, in press). We demonstrated that the neuroprotective sAPPα fragment was particularly affected, being found in high density clusters in the cytoplasm. sAPP intracellular retention was observed in non-neuronal and neuronal-like cell lines and primary cultures, as confirmed in the study here described. From the evidence available one can deduce that Aβ interferes with APP processing and sequential release of proteolytic sAPP fragments. As explained above, in neurons APP is axonally transported and this is tightly associated with the secretory vesicular pathway. Further, APP axonal trafficking has been associated with kinesin-driven transport along the microtubule network. Thus, we went on to characterize Aβ-induced effects on neuronal APP processing and vesicular trafficking and to relate these to altered neuronal cytoskeleton network dynamics.

MATERIALS AND METHODS

Cell culture

Primary rat cortical and hippocampal neuronal cultures were established from 18 days rat embryos as previously described (Henriques et al., 2007). Following dissociation with trypsin and deoxyribonuclease I (0.15 mg/ml) in Hank's balanced salt solution (HBSS) (0.45 mg/ml for cortical cultures or 0.75 mg/ml for hippocampal cultures during 5-10 minutes at 37°C), cells were plated onto poly-D-lysine coated dishes at a density of $1.0x10^5$ cells/cm^2 in B27-supplemented Neurobasal medium (Gibco), a serum-free medium combination (Brewer et al., 1993). The medium was further supplemented with glutamine (0.5 mM), gentamicin (60 μg/ml), and glutamate (25 μM, for hippocampal cultures only). Cultures were maintained in an atmosphere of 5% CO_2 at 37°C for 9 days, before being used for experimental purposes.

Neuronal-like PC12 cells (a rat pheochromocytoma cell line) were grown in RPMI 1640 medium (Gibco) supplemented with 0.85 g/l sodium bicarbonate, 10% horse serum and 5% FBS, and maintained at 37°C and 5% CO_2. For experimental procedures, cells were plated at a density of $5.0x10^5$ cells/cm^2 in poly-L-ornithine coated dishes.

Exposure to Aβ

Both primary neuronal cultures and PC12 cells were incubated with 20 μM $A\beta_{25-35}$ (Sigma) during 24 h in the appropriate medium, which was replaced in the last 3 h by serum free medium with or without Aβ peptide. This was the conditioned medium collected to monitor extracellular sAPP production. For primary neuronal cultures experiments were carried out in Neurobasal medium-free of B27.

Sample collection and immunodetection

Following Aβ treatment, conditioned media and cells were collected as previously described (Amador et al., 2004). Cells were harvested into boiling 1% SDS, sonicated and boiled for 10 minutes. Protein determination of the cellular lysates was carried out using the BCA kit (Pierce). Samples normalized for protein content were separated on 7.5%

SDS-PAGE and then electrophoretically transferred onto a nitrocellulose membrane. Immunoblotting detection of APP and sAPP was carried out using antibodies that permit distinguishing between holo APP (hAPP)/APP CTFs (C-terminal fragments) and sAPP. The antibodies used were an APP N-terminal antibody (22C11, Boehringer) and an APP C-terminal antibody (rabbit anti-β-APP, Zymed).

Conditioned media were also processed to detect secreted proteins. Briefly, samples separated by SDS-PAGE, processed as described above, were fixed in a solution containing 50% methanol and 5% acetic acid for 30 minutes. The fixing solution was replaced with 50% methanol for 15 minutes, after which sensitizing solution [0.02% (w/v) sodium thiosulphate] was added for 1 minute. Subsequently, a staining solution of 0.2% (w/v) of silver nitrate chilled to 4°C was added to the gel and allowed to incubate for 25 minutes. Finally, the gel was incubated with developing solution of 3% sodium carbonate and 0.025% (v/v) of 37% formaldehyde and developed for a maximum of 10 minutes, using a 1.4% (w/v) sodium EDTA solution to stop color development.

Quantification and statistical analysis

Quantity One densitometry software (Bio-Rad) was used to quantify band intensity and correlate it to protein levels. Data are expressed as mean±s.e.m. determinations, from at least three independent experiments. Statistical analysis was carried out using one way analysis of variance (ANOVA). When the F values were significant, the Dunnett test was applied to compare all groups versus control. The level of significance accepted was $P<0.05$.

Subcellular fractionation

Primary neuronal cultures and PC12 were exposed to Aβ as previously described and subcellular fractions were prepared using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem). The differential solubility of the subcellular compartments in specific reagent mixtures enables the differential extraction of proteins according to their subcellular localization. Sequential extraction steps yielded fractions containing cytosolic proteins ("Cytosol"), plasma membrane and organelle proteins ("Memb+Org"), and cytoskeleton and cytoskeleton-associated proteins ("Cytosk"). Fractions obtained were

separated on a 5-20 % gradient SDS-PAGE gel and immunoblotted for specific proteins, as indicated.

For the subcellular fractionation studies, specific organelle markers used included HSP70(72) (cytosolic marker, Stressgen) and syntaxin 6 (Golgi marker, BD Biosciences). Actin (Stressgen) and β-tubulin (Zymed) confirmed subcellular enrichment in cytosolic- and cytoskeleton-associated fractions. Primary antibody detection made use of horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia) for enhanced chemiluminescence detection (ECL, Amersham Pharmacia). ECL Plus was used to detect extracellular sAPP, syntaxin, carboxipeptidase E (CPE) and intracellular hAPP.

APP and sAPP localization

For immunofluorescence analysis, cells were plated onto coverslips at a confluency of approximately 50%. Following exposure to $A\beta_{25-35}$ for 24 h, cells were fixed in 4% paraformaldehyde, permeabilized with methanol and blocked with 3% BSA. Subsequently, cells were immunolabelled with specific antibodies. To distinguish between APP and sAPP, or other APP cleaved fragments, primary cultures were incubated with an antibody against APP N-terminal (22C11, Boheringer) and an anti-APP C-terminus antibody (rabbit anti-β-APP, Zymed). The kinesin light chain (KLC) antibody (Santa Cruz Biotechnology) was used as a marker for the APP vesicular anterograde transport.

Primary antibody complexes were visualized using Texas Red- (Molecular Probes) and Fluorescein-conjugated (Calbiochem) secondary antibodies. Coverslips were mounted on microscope glass slides using antifading reagents (Vectashield, Vector Laboratories) containing in some cases DAPI for nucleic acid staining. Epifluorescence images were acquired using a Zeiss LSM 510-Meta confocal microscope and a 63x/1.4 oil immersion lens. Argon laser lines of 405 and 488 nm were used to excite DAPI and Fluorescein, respectively, and a 561 nm DPSS laser was used to excite Texas Red. Microphotographs were acquired in a sole section in the z-axis (xy-mode) and represent a mean of 16 scans.

Labbeling F-actin and acetylated α-tubulin

Cells fixed with paraformaldehyde were permeabilized with a solution of acetone at -20 °C for 3 min. Subsequently, cells were washed with PBS and then incubated with PBS containing 1% BSA for 1 hour. This blocking solution was removed and phallotoxin staining solution (labels F-actin) was added to cells (1.5 U/100 μl in PBS containing 1% BSA) for 30 min at RT. After PBS washing, cells were incubated with an anti-acetylated α-tubulin antibody (Zymed) for 2 h and primary complexes visualized using Fluorescein conjugated secondary antibody (Calbiochem). Coverslips were mounted and visualized as previously described.

Biochemical assays on cytoskeleton polymerization

Cells were incubated with $A\beta_{25-35}$ for 21 hour, followed by a 3 h incubation period with fresh medium with or without Aβ in the presence or absence of 10-40 μM cytochalasin D (a compound that drives actin depolymerization) or 20-40 μM taxol (that drives microtubule stabilization).

RESULTS

Aβ-induced intracellular sAPP accumulation in primary neuronal cultures is associated with the cytoskeleton

Primary neuronal cultures and PC12 cells were incubated with $A\beta_{25-35}$ peptide for 24 h, and in the last 3 h of the incubation, the medium was replaced by fresh medium containing Aβ (Aβ) or without Aβ (Aβ-Aβ). To distinguish between cleaved APP (sAPP) and the full-length holoAPP (hAPP) protein, two antibodies were used, an anti-APP N-terminus 22C11 antibody, which recognizes both holo APP and sAPP, and an anti-APP C-terminal antibody, that recognizes hAPP, were used. We observed that in both primary cortical and hippocampal neuronal cultures, Aβ induced an increase in APP intracellular levels (Fig. 1A) of 2.0±0.18 and 1.6±0.13 fold, respectively. Conversely, Aβ decreased hAPP levels (C-terminal antibody) to 0.6±0.01 for cortical cultures and 0.7±0.05 for hippocampal cultures, of control levels. In agreement with these observations, a decrease in APP mRNA was observed for neuronal cultures (Henriques et al. 2009b, in press). Under the same conditions, extracellular sAPP secretion was markedly decreased in both neuronal cultures, to 0.2±0.04 (cortical) and 0.1±0.04 (hippocampal). Further, if Aβ was removed during the last 3 h of incubation, the secretory block was released (Fig. 1A), leading to an increase in extracellular sAPP, reaching control levels in both hippocampal (1.1±0.09) and cortical neurons (1.3±0.14). This confirms that isAPP retention/sAPP secretory block in primary neuronal cultures are indeed Aβ-specific inhibitory effects. Additionally, analysis of the conditioned media demonstrated that the Aβ inhibitory effect on secretion was not generalized since many secreted proteins were unchanged after Aβ treatment, as revealed by silver staining proteins present in the conditioned medium (Fig. 1B). In parallel, similar data were obtained for PC12 cells, both in terms of intracellular APP accumulation and sAPP secretion (Fig. 1C).

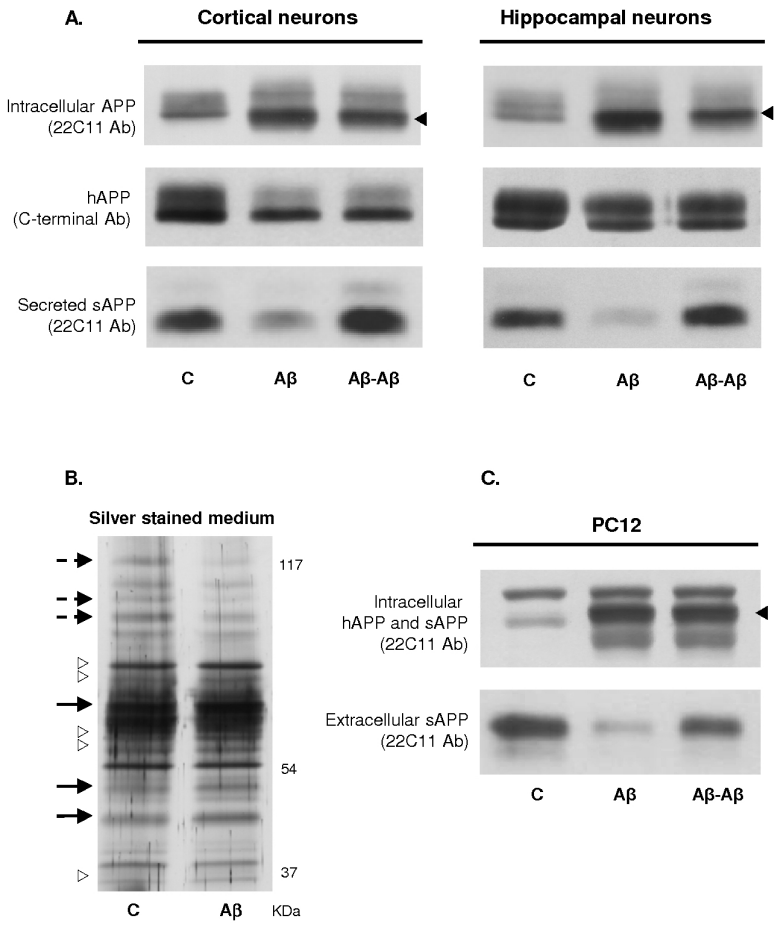

Fig. 1. Aβ induces intracellular sAPP retention in primary neuronal cultures and PC12 cells. Immunoblot analysis of cellular lysates and conditioned medium was performed with the specified antibodies (A and C). (◀), isAPP. B. The total protein content of primary neuronal cultures conditioned medium was analyzed by silver staining. Full arrows: bands with increased intensity upon Aβ treatment. Dashed arrows: bands with decreased intensity upon Aβ treatment. Unfilled arrowheads: roughly unchanged bands. (C), control cells; (Aβ), cells exposed to Aβ for 24 h; (Aβ-Aβ), cells exposed to Aβ for 21 h, and further incubated in Aβ-free medium for 3 h.

In order to characterize the nature of this isAPP retention we performed both subcellular fractionation analysis and immunofluoresecence analysis. We observed the association of isAPP with cytoskeleton structures, for both PC12 cells and for primary cortical neurons (Figs 2A, B). The cytoskeleton/microtubule containing fraction was actin and β-tubulin-positive and largely free of organelle markers (Fig. 2), while the cytosolic fractions were enriched in HSP70. Immunoblot analysis of the subcellular fractions with 22C11 or C-terminal antibodies allowed us to distinguish hAPP (unfilled arrowheads, ◁) from sAPP (solid arrowheads, ◀).

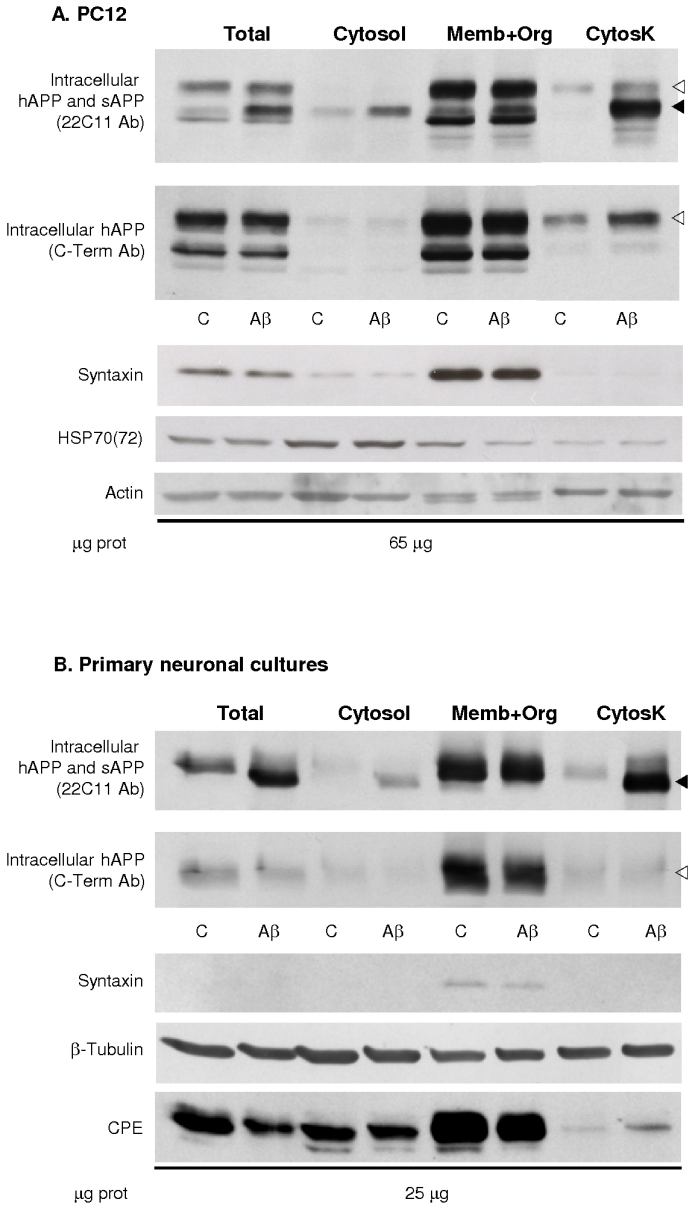

Fig. 2. Intracellular cytoskeletal sAPP retention. Following incubation with $A\beta_{25-35}$, PC12 cells (**A**) and primary cortical cultures (**B**) were fractionated as described, and the resulting fractions analyzed by immunoblotting. Total – total cell lysate; Cytosol – cytosolic protein fraction; "Memb+Org" – membrane and organelles enriched fraction; and Cytosk – cytoskeleton protein fraction. (◀), isAPP forms; (◁), mature hAPP. Intracellular sAPP and APP levels (N-terminal 22C11 antibody), HSP70 (cytosolic marker) and actin and β-tubulin (cytosolic and cytoskeleton marker) were detected using ECL; intracellular hAPP (C-terminal antibody), CPE (carboxypeptidase E, marker of regulated vesicular secretion), and syntaxin-6 (Golgi marker) were detected using the highly sensitive ECL plus reagent. (C), Control; (Aβ), Aβ exposure.

For PC12 cells, Aβ exposure led to some isAPP (solid arrowheads, ◀) accumulation in the cytosolic fraction, but much more markedly in the cytoskeleton enriched fraction. This was accompanied by an increase in mature $hAPP_{751/770}$ C-terminal positive isoforms in the cytoskeleton fraction (arrowhead ◁, Fig. 2A). For primary neuronal cortical cultures similar data were obtained: isAPP accumulated mainly in the cytoskeleton fraction (Fig. 2B, solid arrowheads, ◀). Further, carboxypeptidase E (CPE), a protein involved in regulated vesicular secretion, was also found to increase in the cytoskeletal fraction in response Aβ to treatment, although its total intracellular levels were decreased. The observed isAPP accumulation in cytoskeleton and cytosolic fractions, known to be associated with cytoplasmic vesicles, suggested that hAPP was being cleaved intracellularly before reaching the plasma membrane. These results strengthen the hypothesis that Aβ exerts an inhibitory effect at the vesicular secretory level, leading to intravesicular isAPP retention.

isAPP retention occurs in secretory vesicles

To characterize the location of intracellular sAPP retention in primary hippocampal neurons we performed immunofluorescence microscopy analyses. The vesicular axonal transport of APP has been linked, directly or indirectly, with the microtubule-associated motor protein kinesin 1. The latter is known to be responsible for a wide range of protein vesicular secretion and is composed of two subunits of KHC and two subunits of KLC. Co-localization of KLC with APP/sAPP was therefore addressed under basal and Aβ exposure conditions. These studies were carried out with the 22C11 (N-terminal APP) and C-terminal antibodies to distinguish between hAPP and sAPP.

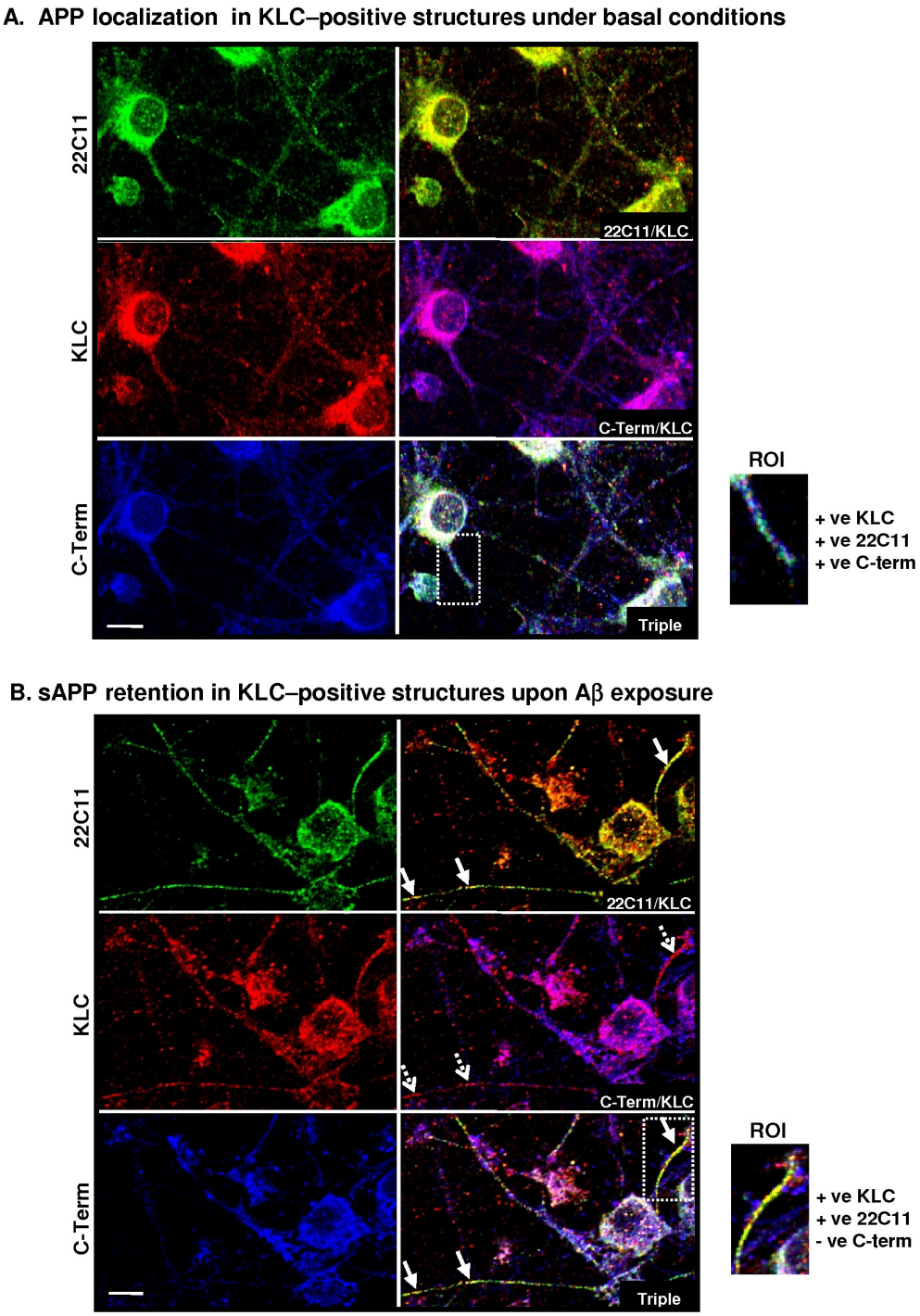

Fig. 3. Aβ induces intracellular sAPP retention in secretory vesicles. Following Aβ treatment, primary hippocampal cultures were prepared for immunofluorescence analysis. Both 22C11 (Fluoresceine-labelled, green) and C-terminal (labelled with Alexa 350, blue) antibodies allowed to discriminate between hAPP and the sAPP. KLC antibody was used as a marker of secretory vesicles (Texas Red-labelled, red). **A**. Represents hAPP localization under basal conditions. **B**. Represents sAPP localization under Aβ treated conditions. Solid arrows indicate KLC+ve/22C11+ve structures, and dashed arrows indicate KLC+ve/C-terminal–ve structures. Images were acquired using a Zeiss confocal microscope. (C), control cells; (Aβ), cells exposed to Aβ for 24 h. ROI (region of interest). Bar, 10 μm.

Under basal conditions 64%±3 of the 22C11 positive population co-localizes with the C-terminal immunoreactive population (hAPP), while with Aβ treatement only 44%±5 of the 22C11 positive population co-localizes with the C-terminal antibody, indicating that the remaining 22C11 positive population is sAPP (36% under basal and 56% with Aβ). This is in agreement with increased isAPP retention upon Aβ addition. We also observed that under basal conditions both 22C11 and C-terminal antibodies co-localize with KLC in the majority of the neurites observed (Fig 3A). Thus we can deduce that hAPP and KLC co-localize intracellularly (Fig. 3A, triple co-localization). However, Aβ exposure led to decreased C-terminal immunoreactivity and diminished KLC co-localization at the neurites (Fig. 3B, dashed arrows). In contrast, the 22C11 and KLC co-localization signal was maintained or increased in most neurites (Fig. 3B, solid arrows). This is consistent with isAPP accumulating in neuritic KLC-positive vesicles (Fig. 3B, mainly double co-localization).

Aβ interferes with the cytoskeleton network

The results described above indicate that Aβ affects KLC-driven APP vesicular transport which is known to be associated with the cytoskeleton network and dependent on microtubule tracks. Therefore, alterations in vesicular secretion may be associated with altered cytoskeletal dynamics. Thus, we addressed Aβ effects on the major protein constitutents of the cytoskeleton network, actin and tubulin. As the polymerization/depolymerization dynamics of these proteins are key processes in transport, we evaluated F-actin polymerization (filamentous actin deriving from globular actin polymerization) and α-tubulin acetylation (a measurement of microtubule stability) in both primary neuronal cultures and PC12 cells (Fig. 4).

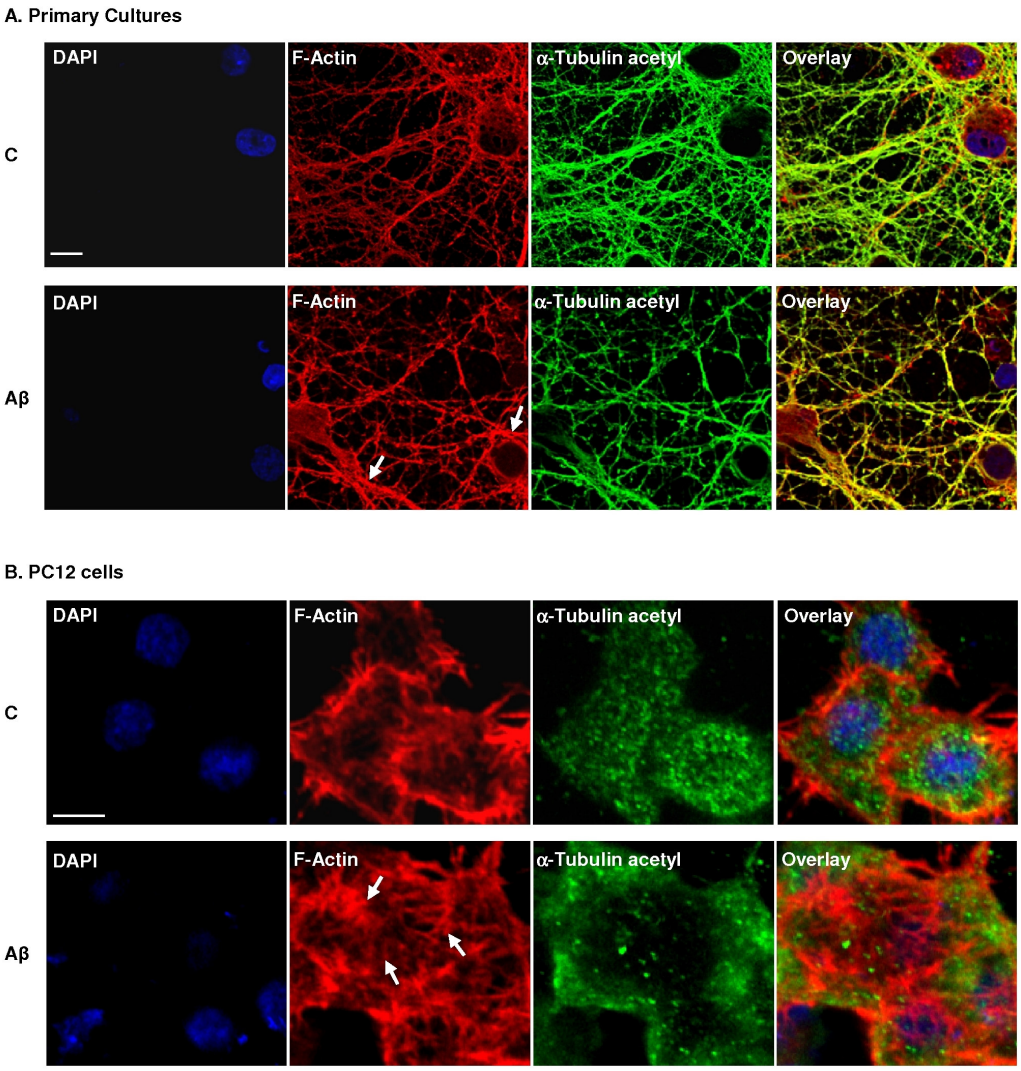

Fig. 4. Aβ leads to alterations in the cytoskeletal networks of neuronal cultures and PC12 cells. Upon Aβ treatment, cells (**A.**, primary hippocampal cultures and **B.**, PC12 cells) were stained with Texas red conjugated phallotoxin solution (labelled filamentous F-actin) and with acetylated α-tubulin antibody (labeled with Fluoresceine, green staining). Confocal images were acquired using a Zeiss confocal microscope. (C), control cells; (Aβ), Aβ for 24 h. Arrows indicate F-Actin polymerization. Bar, 10 μm.

For primary neuronal cultures, exposure to $A\beta_{25-35}$ leads to a dramatic decrease on α-tubulin acetylation (measure of microtubule stability). For PC12 cells a redistribution of α-tubulin acetylation to the periphery could be observed. With respect to F-actin polymerization, Aβ exposure lead to a significant increase, as is evident for PC12 cells (Fig. 4B). In fact increased polymerization is clearly visible around the PM and in cytoplasm (solid arrows). This effect is less obvious for primary neuronal cultures (Fig. 4A).

The Aβ blocking effect on APP/sAPP trafficking can be reversed by altering cytoskeleton dynamics

The Aβ induced isAPP retention and its relation with altered cytoskeleton dynamics was further established using an actin depolymerization agent (cytochalasin D, cytD) and a microtubule stabilization drug (taxol). In PC12 cells, although co-incubation of Aβ and cytD in the last 3 h had no noticeable effect on isAPP retention (data not shown), it reversed considerably the Aβ block on sAPP secretion (Fig. 5A), similarly to the effect obtained with Aβ removal in the last 3 h (Fig. 1A). Higher concentrations of the F-actin depolymerizing agent did not further increased sAPP secretion. Likewise, co-incubation with Aβ and taxol, could also partial reverse Aβ-induced effect on sAPP secretion (Fig. 5B). The results indicate that the Aβ inhibitory effect on sAPP secretion could be partially reversed by drugs which affect both the actin and microtubule networks.

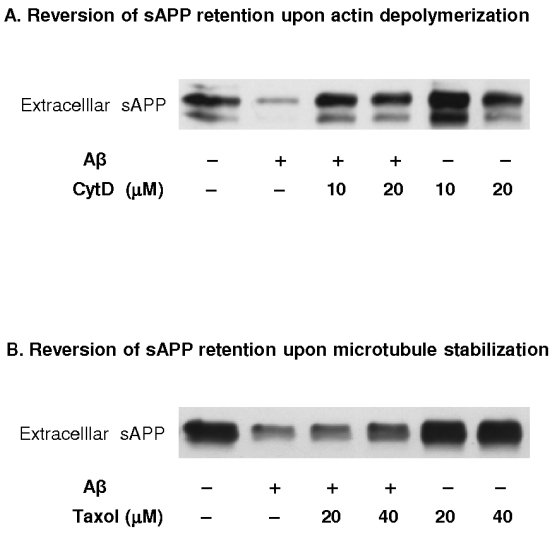

Fig. 5. Aβ effects can be reversed by drugs actin on cytoskeleton dynamics. Extracellular sAPP secretion levels were evaluated upon co-incubation of Aβ with drugs able to modulate cytoskeleton dynamics. PC12 cells were treated with Aβ and cytochalasin D (**A**) or taxol (**B**) during the last 3 h of the 24 h incubation period. (C), Control; (Aβ, Aβ treatment during 24 h; (CytD), Cytochalasin D.

DISCUSSION

Due to its ability to trigger a set of biochemical and cellular alterations, Aβ has been described as a key player in the amyloid cascade hypothesis leading to progressive neurotoxicity and neuronal death observed in AD (Hardy and Selkoe, 2002; Hardy and Higgins, 1992). Besides the well described neurotoxic/apoptotic effects, Aβ also provokes alterations in APP metabolism (Carlson et al., 2000; Davis-Salinas et al., 1995; Schmitt et al., 1997). Previous work from our laboratory demonstrated that Aβ also exerts an effect on APP trafficking/processing leading to isAPPα accumulation in cytoskeleton-associated vesicular-like structures in a non-neuronal cell line. This isAPP retention was observed in non-neuronal cell lines as well as in primary hippocampal and PC12 cells (Henriques et al. 2009a, in press). Nonetheless, differences could be observed at the level of sAPP secretion between these cell lines. Hence, we went on to evaluate the specificity and the origin of this potential pathological intracellular sAPP retention and to characterize the cellular structures mediating this Aβ response in neurons and in PC12 cells. Aβ-induced inhibition of sAPP secretion was considerable in both primary cortical and hippocampal cultures (Fig. 1A). This effect on sAPP secretion was relatively specific, since other medium secreted proteins were unaffected by Aβ treatment (Fig. 1B).

Intracellular sAPP retention was associated with cytoskeleton structures, as determined by subcellular fractioning studies in both PC12 and primary neuronal cultures (Fig. 2). It is well stablished that vesicular motility is intimately associated with cytoskeleton network (Hamm-Alvarez and Sheetz, 1998; Lanzetti, 2007; Meyer and Burger, 1979; Potokar et al., 2007), which in the light of our data, may explain the observed isAPP retention in this subcellular fraction. Of note, mature hAPP was also observed to accumulate in the cytoskeleton fraction (Fig. 2, more visible for PC12 cells). It appears then, that as a response to Aβ exposure, sAPP is intracellularly produced and retained in cytoskeleton-associated secretory vesicles. Indeed, co-immunocytochemistry studies located isAPP retention to KLC-positive secretory vesicles in Aβ treated cells (Fig. 3). Our data clearly shows that isAPP and KLC localize to the same subcellular structure, of course this is also in accordance with a direct or indirect interaction between KLC and APP in the late anterograde transport. However, from the results presented in Fig. 3B it is tempting to conclude that APP/KLC interaction, is not direct. Previous reports have suggested that

this interaction is mediated via the cytoplasmic tail of APP (Kamal et al., 2000). Our data does not exclude this possibility, but retention of isAPP which correlates with KLC immunoreactivity, does not support this hypothesis.

Since vesicular transport is related to the cytoskeleton network, hindered APP/sAPP vesicular transport induced by Aβ could be mediated by altered cytoskeleton dynamics. In fact, immunocytochemistry and immunoblot studies revealed that $A\beta_{25-35}$ affected the dynamics of polymerization/depolymerization of both actin and tubulin. $A\beta_{25-35}$ was previously shown to affect axonal transport by inducing neuronal actin polymerization and aggregation (Hiruma et al., 2003). Induction of F-actin polymerization by fibrillar $A\beta_{1-42}$ was also reported by Mendoza-Naranjo (2007) in hippocampal neurons. We have detected not only an increase in F-actin polymerization but also a decrease in α-tubulin acetylation in response to $A\beta_{25-35}$, for both primary neurons and PC12 cells (Fig. 4). α-tubulin acetylation is an indirect measure of the amount of the tubulin polymer and of microtubule stability (Black et al., 1989; Bloom, 2004). Taken together the data suggests that Aβ altered vesicular trafficking by affecting cytoskeleton dynamics. Indeed, as observed with Aβ withdrawal (Fig. 1), the effects induced by Aβ on sAPP secretion could be partial reversed both by inducing actin depolymerization or microtubule stabilization, clearly correlating Aβ effects on sAPP retention/secretion with altered cytoskeleton dynamics.

In synthesis, this work reveals that in neurons Aβ impairs APP/sAPP vesicular anterograde transport and exocytosis, in a mechanism mediated by alterations in the cytoskeleton dynamics of both microtubule and actin networks. The results described allow the disclosure of Aβ-mediated mechanisms that may contribute to neurodegeneration. Microtubule destabilization has been reported to be associated with neurotoxicity, and Aβ-induced neurodegeneration could be prevented by microtubule stabilization drugs (Michaelis et al., 2005; Michaelis et al., 1998; Seyb et al., 2006). An important consequence of the Aβ effects here reported resides in altered APP/sAPP neuritic transport and in decreased sAPP secretion. As extracellular sAPPα has potential neurotrophic and neuroprotective properties, this depletion is of extreme importance in a background of neuronal damage and loss as in AD. We hope that our results will improve the

understanding of the molecular basis of the disease, and might provide fundamental insights necessary for the development of effective therapeutic strategies.

ACKNOWLEDGMENTS

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Chapter III

Aβ ALTERS SIGNAL TRANSDUCTION MECHANISMS

Chapter Outline

One of the functions most recently attributed to APP is a role as a signal transduction molecule involved in transcriptional activation. This function was reported to be mediated by the APP intracellular domain (AICD), a product of APP CTFs intramembranous γ-secretase cleavage which is released into the cytoplasm and can to relocalize to the nucleus (Cupers et al. 2001; Kimberly et al. 2001; Kinoshita et al. 2002). At this subcellular location, AICD can function as a transcriptional regulator. Indeed, AICD was shown to form a transcriptionally active nuclear complex together with Fe65 and Tip60 (Cao and Sudhof 2001), which is able to activate several downstream target genes, including APP itself (von Rotz et al. 2004). The studies performed in primary neuronal cultures presented in the previous chapter, revealed a decrease in holoAPP intracellular levels upon exposure to Aβ peptide. This led us to speculate that besides having an effect on APP processing, Aβ could also have consequences on APP transcriptional activation. Therefore, a set of experiments were designed to investigate the effects of Aβ on APP nuclear signaling. Interestingly, under our experimental conditions, Aβ induced a decrease in APP expression that together with the observations of increased APP CTFs and decreased APP-C-terminus nuclear staining (immunofluorescence analysis), suggest a decrease in both AICD production and subsequent transcriptional activation. This Aβ effect on APP nuclear targeting is of particular importance since this peptide may induce a cascade of events which consequently decrease its own production.

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Manuscript 3 - Aβ hinders nuclear targeting of AICD and Fe65 in primary neuronal cultures

Henriques, A.G., Vieira, S.I., da Cruz e Silva, E.F.* and da Cruz e Silva, O.A.B.
Laboratório de Neurociências and *Laboratório de Transdução de Sinais, Centro de Biologia Celular, Universidade de Aveiro, Aveiro (Portugal).

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Running title: Aβ hinders nuclear target of AICD and Fe65

Corresponding author: Odete A.B. da Cruz e Silva, Centro de Biologia Celular, Secção Autónoma das Ciências da Saúde, Universidade de Aveiro, 3810-193 Aveiro, Portugal. Phone: +351 234 370200 and Fax: +351 234 370983. E-mail: odetecs@ua.pt

ABSTRACT

The intracellular domain of the Alzheimer´s amyloid precursor protein (AICD) has been described as an important player in the transactivation of specific genes. It results from proteolytic processing of the Alzheimer´s amyloid precursor protein (APP), as does the neurotoxic Aβ peptide. Although normally produced in cells, Aβ is typically considered to be a neurotoxic peptide, causing devastating effects. By exposing primary neuronal cultures to relatively low Aβ concentrations, this peptide was shown to affect APP processing. Our findings indicate that APP C-terminal fragments (CTFs) are increased with concomitant reduction in the expression levels of APP itself. AICD nuclear immunoreactivity detected under control conditions was dramatically reduced in response to Aβ exposure. Additionally, intracellular protein levels of Fe65 and GSK3 were also decreased in response to Aβ. APP nuclear signaling is altered by Aβ, affecting not only AICD production but also its nuclear translocation and complex formation with Fe65. In effect, Aβ can trigger a physiological negative feedback mechanism that modulates its own production.

Keywords: Aβ peptide, APP RIP signaling, Alzheimer´s Disease, Fe65, CTFs, AICD

INTRODUCTION

Aβ was originally defined as a pathogenic peptide associated with Alzheimer's Disease (AD), but it is now known to be produced during normal intracellular processing of the Alzheimer's amyloid precursor protein (APP) (Haass et al., 1992; Haass et al., 1993; Selkoe, 1993; da Cruz e Silva et al., 2004). Consecutive APP cleavage by α-secretase (Sisodia, 1992; Buxbaum et al., 1998; Allinson et al., 2003) and the γ-secretase complex (Li et al., 2000; Sastre et al., 2001; Esler et al., 2002; Lee et al., 2002; Capell et al., 2005) precludes Aβ production and produces a smaller fragment termed P3, whereas cleavage by β-secretase and γ-secretase results in the production of Aβ peptides, mainly in Golgi and endosomes (Vassar et al., 1999; Yan et al., 2001; Rebelo et al., 2007). The former non-amyloidogenic cleavage pathway also leads to the production of sAPPα, while the latter results in sAPPβ production. Proteolytic sAPP products are typically secreted, although intracellular sAPP (isAPP) production has been detected (Carlson et al., 2000; Henriques et al. 2009, in press). Resulting APP C-terminal fragments (CTFs), the products of α– and β– secretase activities, may be cleaved intracellularly by γ-secretase at γ- and ε-sites, giving rise to the APP intracellular domain (AICD) (Sastre et al., 2001). Cao and Sudhof (2001) have shown that AICD exhibits transcriptional activity, enhanced by the formation of a transcriptional active complex comprising AICD, Fe65 and the histone acetylase Tip60. This trimeric complex was reported to localize to multiple spherical nuclear compartments (von Rotz et al., 2004). AICD and Fe65 localize together at the nucleus (Kimberly et al., 2001; Minopoli et al., 2001; Walsh et al., 2003), and nuclear AICD-containing complexes were reported to activate the transcription of several genes, including *APP* itself, *BACE*, *Tip60* (von Rotz et al., 2004), *GSK3β* (Kim et al., 2003; Ryan and Pimplikar, 2005), *KAI1* (Baek et al., 2002) and *Neprilysin* (Pardossi-Piquard et al., 2005). However, it is still unclear how the translocation of Fe65 and AICD from the cytoplasm and/or membrane into the nucleus is accomplished.

APP/Fe65 interaction is also known to modulate APP metabolism, including sAPP secretion and Aβ production (Sabo et al., 1999; Ando et al., 2001). Sabo et al. (1999) reported that in MDCK cells stably expressing APP_{695}, Fe65 increased APP translocation to the plasma membrane (PM), which was accompanied by an increase in Aβ and sAPPα secretion. Recently Xie et al. (2007) showed that Fe65 RNAi silencing leads to an

increase in CTF levels and a decrease in Aβ levels, thus suggesting a role for Fe65 as a positive regulator of γ-secretase activity.

The present work focuses on the effect of exogenously added Aβ on APP metabolism in primary neuronal cultures, and its effects on AICD/Fe65 nuclear signaling. The data obtained supports the hypothesis that Aβ plays a role in APP processing and RIP signaling, by altering APP intracellular proteolytic cleavage and by decreasing both APP and Fe65 intracellular and nuclear levels. The intracellular Aβ effects appear to include decreased AICD production, given the increase in CTFs production, and decreased targeting and nuclear co-localization of AICD/Fe65.

MATERIALS AND METHODS

Preparation and maintenance of primary neuronal cultures

Rat cortical and hippocampal cultures were established from embryonic day 18 embryos as previously described (Henriques et al., 2007). After dissociation with trypsin (0.45 or 0.75 mg/ml for cortical or hippocampal cultures, respectively, for 5-10 min at 37°C) and deoxyribonuclease I (0.15 mg/ml) in Hank's balanced salt solution, cells were plated on poly-D-lysine-coated dishes at a density of $1.0x10^5$ cells/cm^2 in B27-supplemented Neurobasal medium (GIBCO), a serum-free medium combination (Brewer et al., 1993). The medium was supplemented with glutamine (0.5 mM), gentamicin (60 μg/ml), and with or without glutamate (25 μM) for hippocampal or cortical cultures, respectively. Cultures were maintained in an atmosphere of 5% CO_2 at 37°C for 9 days before being used for experimental procedures.

Incubation with Aβ peptide

$A\beta_{25-35}$ peptide (Sigma Aldrich) was dissolved in distilled water to prepare a 1 mM stock. Rat primary neuronal cultures were incubated for 24 h in Neurobasal medium free of B27 containing 20 μM $A\beta_{25-35}$, with the medium being replaced during the last 3 h of incubation by fresh medium with or without $A\beta_{25-35}$.

Sample collection and immunoblotting

Following exposure to Aβ, conditioned media and cells were collected in boiling 1% SDS and the lysates were homogenized as previously described (Amador et al., 2004). Protein determination was carried out using the BCA kit (Pierce). Samples normalized for protein content were separated on 7.5% or 5-20% gradient SDS-PAGE gels and then electrophoretically transferred onto nitrocellulose membranes for immunoblotting. Intracellular APP/isAPP and extracellular sAPP detection was carried out using the 22C11 mouse monoclonal antibody directed against the APP N-terminus (Boehringer), while for holoAPP and endogenous C-terminal fragments an APP C-terminal antibody was used (rabbit polyclonal anti-β-APP C-terminus, Zymed). Detection of total GSK3 was achieved using a rabbit polyclonal anti-glycogen synthase kinase 3 antibody (Chemicon). For Fe65

detection the antibody clone 3H6 (Upstate) was used, and tubulin detection was carried out using the monoclonal anti-β-tubulin antibody (Zymed). Following incubation with the primary antibodies, immunodetection made use of horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs secondary antibodies (Amersham Pharmacia) and for visualization enhanced chemiluminescence detection (ECL) was employed (Amersham Pharmacia). The ECL Plus reagent was used for extracellular sAPP, CTFs and Fe65 detection.

Quantification

Quantity One Densitometry software (Bio-Rad) was used to quantify band intensity and correlate it to protein levels. Data are expressed as mean±SEM of at least three independent experiments. Statistical analysis was carried out using one way analysis of variance (ANOVA). When significantly different, the Dunnett test was applied to compare all groups to the control. The level of statistical significance accepted was $P<0.05$.

Northern blot analysis

Total RNA was isolated from control primary cortical cultures ($3.0x10^6$ cells) following Aβ treatment (TRI REAGENT, Sigma). Normalized total RNA aliquots (10 μg) were separated by formaldehyde gel electrophoresis and transferred to nitrocellulose membranes using standard laboratory protocols (da Cruz e Silva et al., 2008). The blot was then hybridized with a [^{32}P]-labelled APP cDNA probe (25 ng, $1x10^6$ cpm/ng) to evaluate APP expression levels. The APP probe used (756 bp) was obtained by *Age*I/*Bam*HI restriction enzyme digestion of the APP_{751} cDNA, and labelled with [α-^{32}P]dCTP (GE Healthcare) using the High Prime DNA Labelling Kit (Roche, Alfagene). Purification of the probe through NucTrap Probe Purification Columns (Stratagene, Alfagene) was performed prior to hybridization APP hybridizing RNA was detected using Kodak Biomax XAR film (Sigma).

Monitoring nuclear targeting of APP C-terminal proteolytic products

For evaluating nuclear targeting of APP C-terminal proteolytic products and Fe65, cells were fixed with 4% paraformaldehyde, permeabilized with methanol for 2 min and blocked with 3% bovine serum albumin in PBS for 1 h, and further incubated with primary antibody (C-terminal APP antibody and Fe65 antibody) for 3 h. The antibody 4G8 (anti-Abeta 17-24 aa antibody, Chemicon) was used in co-localization studies with the APP C-terminal antibody, to rule out nuclear CTFs and confirm the identity of AICD at the nucleus. After washing with PBS, Texas Red-conjugated goat anti-rabbit (Molecular Probes) or Fluoresceine-conjugated goat anti-mouse (Calbiochem) secondary antibodies were added for 2 h at room temperature. Coverslips were mounted on microscope glass slides using FluoroGuard (BioRad) as an antifading reagent or Vectashield (Vector Laboratories), an antifading reagent containing DAPI for nucleic acid labelling. Nuclear targeting and co-localization studies of APP C-terminal fragments and Fe65 was carried out by immunofluorescence analysis. Acquisition of epifluorescence images made use of a LSM 510-Meta confocal microscope (Zeiss) and a 63x/1.4 oil immersion objective. The argon laser lines of 405 nm (DAPI), 488 nm (Fluorescein) and a 561 nm DPSS laser (Texas Red) were used. Microphotographs were acquired in a sole section in the z-axis (xy-mode), and represent a mean of 16 scans.

RESULTS AND DISCUSSION

Aβ affects APP expression levels

Aβ effects on APP metabolism are unclear, with some reports suggesting that Aβ may be affecting *APP* transcription, while others suggest it to have an effect at the APP processing/catabolic levels (Davis-Salinas et al., 1995; Schmitt et al., 1997; Carlson et al., 2000). In our work, primary neuronal cultures were incubated with and without $A\beta_{25-35}$ during 24 h and total RNA extracted for Northern blot analysis (Fig. 1A).

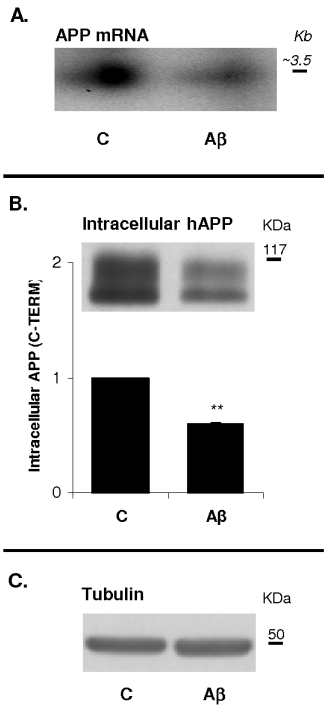

Fig. 1. Aβ decreases APP expression in primary neuronal cultures. Primary cortical neuronal cultures were treated with 20 μM $A\beta_{25-35}$ for 24 h as described in the methods. **A.** Total mRNA was isolated and APP mRNA expression in primary cultures was monitored in response to Aβ exposure. **B.** Intracellular holoAPP (hAPP) protein detection was carried out using an antibody to the APP C-terminus. **C.** Tubulin was used as a control. (C), Control; (Aβ), Aβ treatment for 24 h. **P<0.01, significantly different from control using Dunnett *post hoc* test.

Although Aβ induction of *APP* transcription was previously reported in a neuronal hybrid cell line and in cultured astrocytes (Le et al., 1995; Moreno-Flores et al., 1998), under our experimental conditions Aβ treatment lead to a clear decrease in *APP* expression in primary cortical cultures. Accordingly, APP intracellular protein levels also dropped a concordant 0.4 fold below control levels upon Aβ exposure (Fig. 1B). Similar results were obtained for hippocampal cultures, with Aβ leading to a decrease in APP intracellular levels, as detected by using an APP C-terminal antibody (Fig. 2A), again supporting a role for Aβ in modulating *APP* transcriptional levels in primary hippocampal cultures.

Aβ induces accumulation of APP C-terminal fragments

Our data shows that $A\beta_{25-35}$ provokes a decrease in intracellular holoAPP (hAPP, Fig. 2A), detected using the APP C-terminal antibody in primary neuronal cultures. Concomitantly, using the APP N-terminal antibody 22C11 we could observe a decrease in extracellular sAPP secretion (esAPP, Fig. 2A) and an increase in APP intracellular levels. The latter represents intracellularly accumulated sAPP (isAPP, Fig. 2A) since hAPP levels decreased. This isAPP retention was previously reported by us (Henriques et al. 2009, in press) in various cell types, and $A\beta_{1-42}$ elicits a similar response. Retention of isAPP was also observed with the physiological $A\beta_{1-40}$ peptide (Carlson et al., 2000). In this case, increases in medium secreted proteins, such as IL-8, concomitant with decreases in sAPP secretion, by 10-30%, were reported.

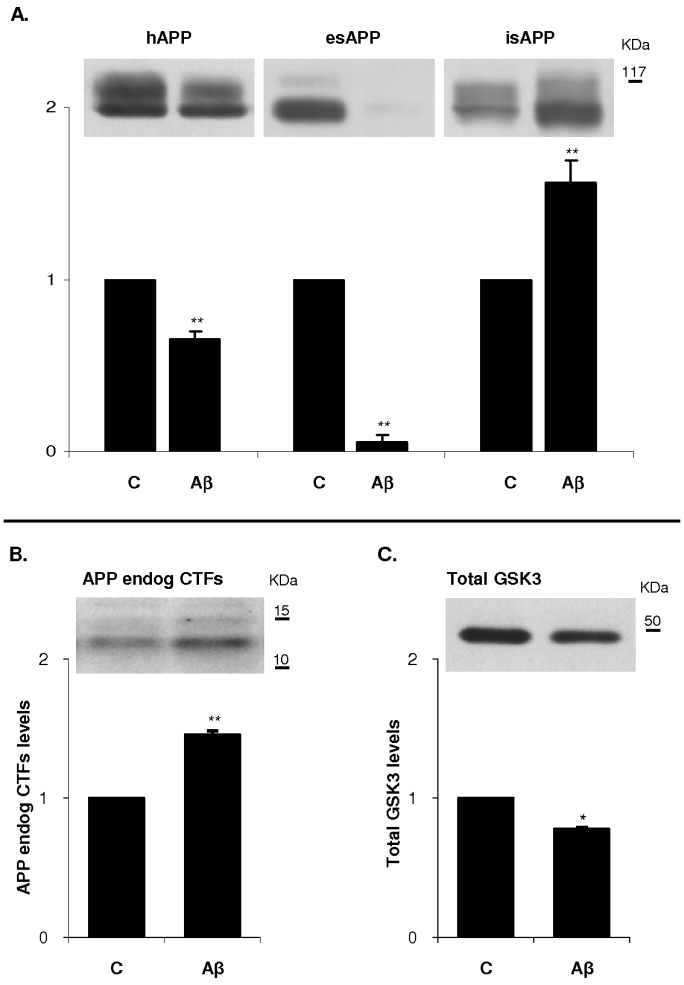

Fig. 2. Effect of Aβ on CTF production. Following incubation of hippocampal neurons with Aβ peptide, cell lysates and conditioned medium were collected and analyzed. **A.** Endogenous intracellular holoAPP (hAPP), extracellular secreted sAPP (esAPP) and intracellular sAPP (isAPP). APP and sAPP were distinguished using the APP C-terminal antibody and the APP N-terminal antibody. **B.** APP C-terminal fragments (APP CTFs) were detected with an anti-β-APP C-terminal antibody. **C.** Total GSK3 expression levels. *P<0.05 and **P<0.01, significantly different from control using Dunnett *post hoc* test. Values are expressed as mean±SEM from 3 independent experiments. (C), Control; (Aβ), Aβ treatment for 24 h.

Given that Aβ was clearly altering APP processing (Fig. 2), we monitored the levels of other APP proteolytic fragments and observed that these too were affected. The levels of endogenous CTFs produced by APP proteolytic processing increased with Aβ treatment (Fig. 2B). This suggests that γ-secretase activity was inhibited by Aβ and/or that CTFs were not accessible for γ-secretase cleavage. However, direct measurement of γ-secretase activity did not yield any significant alteration (data not shown), suggesting that

CTF cleavage by γ-secretase activity *per se* was not hindered. An accumulation of amyloidogenic APP CTFs in response to $A\beta_{1-42}$ exposure was previously observed by Yang et al. (1995) in APP transfected HEK293 cells. In neuronally derived cells, γ-secretase cleavage was described to occur at the plasma membrane and/or early endosomes (Kaether et al. 2006). Thus, the neuronal CTF increase observed (Fig. 2B) probably reflects a block in the transport to plasma membrane and a subsequent decrease in proteolytic cleavage of CTFs. This correlates well with our findings of isAPP retention within cytoskeleton-associated vesicular-like structures (Henriques et al. 2009, in press). Hence increased accumulation of CTFs also correlates with decreased AICD production.
AICD has been described as an APP nuclear signal peptide that can form a transcriptional active complex with Fe65 (Cao and Sudhof, 2001). AICD-containing complexes were reported to induce transcriptional activation of several genes, including *APP* itself and *GSK3β* (Kim et al., 2003; von Rotz et al., 2004; Ryan and Pimplikar, 2005). Thus, increased CTFs and concomitant decreased AICD levels would predict decreases in the AICD nuclear pool and signaling. This is consistent with the observed decrease in APP expression levels (Fig. 1A) and a significant decrease in total GSK3 levels (Fig. 2C). Direct measurements of AICD were not possible given that the endogenous levels in primary cultures are difficult to detect.

Aβ decreases nuclear targeting of APP C-terminal proteolytic products and Fe65

Our observation that Aβ increased APP CTF levels, decreased *APP* transcriptional activation and GSK3 expression levels (Fig. 1 and 2), is consistent with a decrease in AICD production and subsequent decreased transactivation of AICD-downstream genes. Hence, we focussed on AICD/Fe65 nuclear targeting and complex formation. The nuclear targeting of APP C-terminal proteolytic products was clearly hindered in the presence of Aβ (Fig. 3A, Texas Red staining). The APP C-terminal peptides detected in the nucleus and positive for the APP C-terminal antibody were negative for the 4G8 antibody, reinforcing the identity of the nuclear targeted APP C-terminal peptides as AICD proteolytic fragments (Fig. 3C). Under our experimental conditions Aβ exposure decreased not only the intensity of nuclear APP C-terminal punctuate immunoreactivity (Fig. 3A, Texas Red staining), but also Fe65 nuclear intensity and targeting (Fig. 3A, green

Fluoresceine staining). As a consequence, the co-localization of nuclear AICD-Fe65 was also compromised.

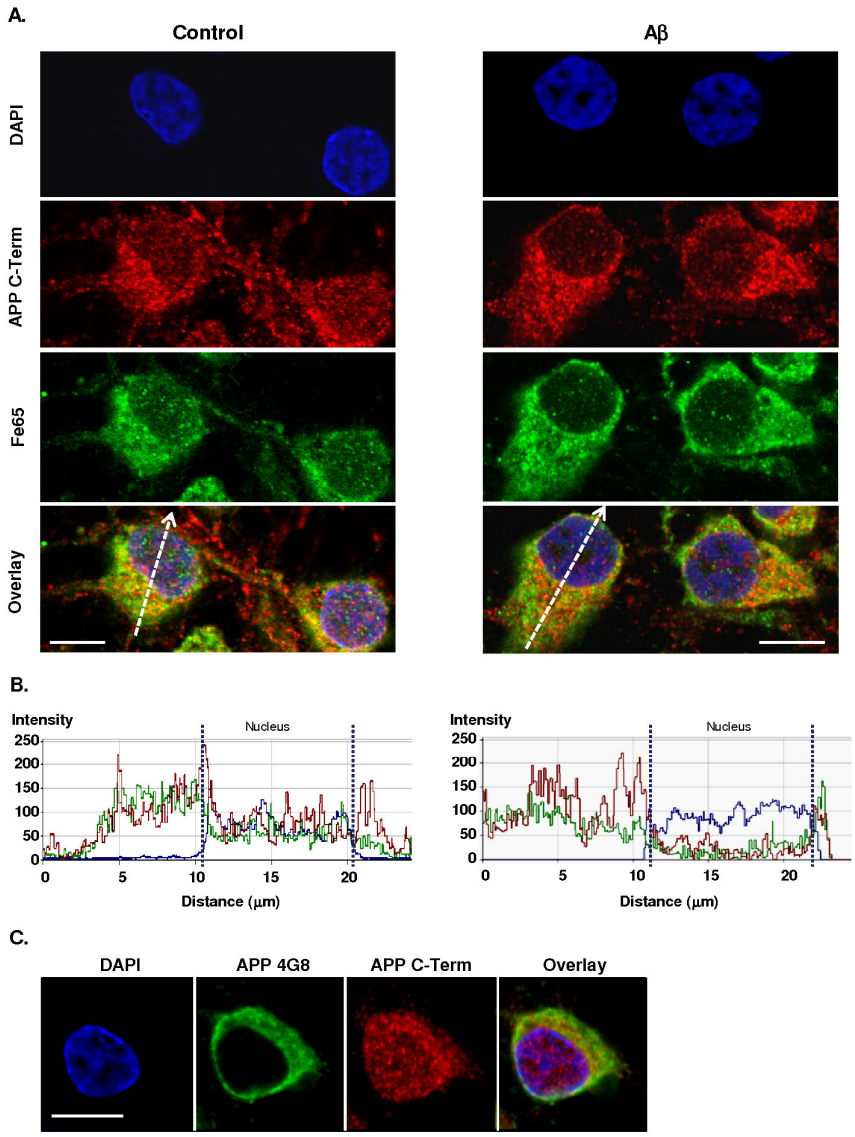

Fig. 3. Aβ induces alterations in AICD and Fe65 nuclear targeting. Hippocampal neuronal cultures were incubated with $A\beta_{25-35}$ for 24 h and subsequently fixed with paraformaldehyde. Immunofluorescence analyses were carried out using anti-β-APP C-terminal and anti-Fe65 antibodies, labelled with a Texas Red-conjugated (red staining) or with a Fluoresceine-conjugated (green staining) antibody, respectively. Cells were mounted with an antifading reagent containg DAPI for nuclei acids staining. **A.** AICD and Fe65 immunoreactivity was analyzed by confocal microscopy. **B.** Representative staining profiles. Fluorescence intensity profiles represent the voxels through the white lines indicated in the merged image shown in A. **C.** The identity of AICD fragments at the nucleus was shown by the positive immunoreactivity for the APP C-terminal antibody (Texas Red-conjugated, red staining) and the negative immunoreactivity for the 4G8 antibody (Fluoresceine-conjugated, green staining). (C), Control, (Aβ), Aβ treatment for 24 h.

Confocal profiling demonstrated that both proteins dramatically decreased in the nucleus (DAPI-positive organelle) following Aβ treatment (Fig. 3B). Hence the blue trace (denotes the nucleus) is sustained with Aβ exposure, whereas the red and green traces for AICD and Fe65, respectively, drop similarly. Of note, this analysis was carried out in non-apoptotic cells, as denoted by nucleus morphology (DAPI staining).

Detailed co-localization studies of AICD and Fe65 immunoreactivity in the nucleus using Zeiss confocal co-localization software (Table 1) also confirmed Aβ induced alterations in the nuclear targeting of both proteins. The percentage of both AICD and Fe65 positive pixels present in the nucleus, relative to the total neuronal soma population, showed a decrease of 42% and 56%, respectively.

Table 1. AICD and Fe65 nuclear targeting and co-localization in response to Aβ.

	Nuclear targeting as a % of total population			% of each protein co-localizing to the complex in the nucleus		
	C	Aβ	**% Dec + Aβ**	C	Aβ	**% Dec + Aβ**
AICD	46 ± 3.0	27 ± 1.7	**42**	33 ± 2.0	21 ± 1.9	**36**
Fe65	36 ± 2.0	16 ± 1.4	**56**	22 ± 1.1	20 ± 1.6	**10**

Analysis was carried out using Zeiss confocal microscospe co-localization software. The % of nuclear AICD and Fe65 immuno-positive pixels were determined relative to the total neuronal cell populations, excluding dendrites and axons ("nuclear targeting as a % of total population"). The "% of each protein co-localizing to the complex in the nucleus" represents the % of each protein (AICD or Fe65) co-localizing to the other, and relative to its total nuclear population. "% Dec + Aβ", percentage decrease upon Aβ addition. Data is presented as mean±SEM of 40 analysed cells.

Focusing on the nuclear population alone, we were able to determine that the AICD nuclear population co-localizing with Fe65 dropped from 33% to 21% upon addition of Aβ, which represents a 36% decrease. There was no significant difference in the percentage of the Fe65 nuclear population that co-localized with AICD (22% and 20%). Additionally we determined that the AICD population, as a percentage of the total APP C-terminal immunoreactivity, co-localizing to Fe65 in the nucleus decreased from ~15% (33% of the 46% of the nuclear targeted population, see Table 1) to ~5% (21% of 27%)

upon exposure to Aβ. Likewise we determined the values for Fe65, and the decrease was from ~7% (22% of 36%) to ~4% (20% of 16%).

Given that the nuclear abundance of both AICD and Fe65 were affected, we also tested the latter directly by immunoblotting and imunofluorescence. Our data showed a clear decrease in Fe65 intracellular levels in response to Aβ (Fig. 4A). This was also evident by confocal microscopy at a focus plane above the nucleus and just below the plasma membrane, where Fe65 immunoreactivity decreased overall, being particularly visible along neurites (Fig. 4B).

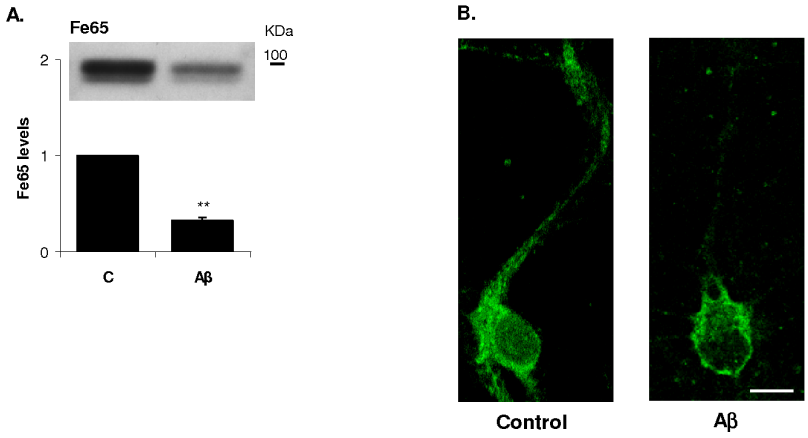

Fig. 4. Aβ effect on Fe65. Fe65 intracellular levels were evaluated using both immunoblotting (**A.**) and immunofluorescence (**B.**). Fe65 immunoreactivity was analysed by confocal microscopy at a focus plane above the nucleus and just below the plasma membrane. **P<0.01, significantly different from control using Dunnett *post hoc* test. (C), Control; (Aβ), Aβ treatment for 24 h.

Taken together, our results suggest that Aβ is affecting AICD production, its nuclear translocation, and nuclear complex formation with Fe65, whose nuclear targeting is itself decreased. A decrease in AICD production and in the formation of AICD/Fe65 transactivation complexes, potentially leads to altered APP nuclear signaling in the presence of Aβ, leading to impaired gene transcriptional activation.

In summary, we propose that Aβ leads to reduced *APP* expression and consequential diminished Aβ production, which is important for cells exposed to an Aβ saturated environment. It is attractive to postulate that the above mentioned mechanisms

congregate to reduce intracellular accumulation of Aβ, and that exogenous Aβ appears to induce a set of concerted cellular responses to prevent its own production, including reduced AICD/Fe65 nuclear targeting. We hypothesize that a physiologically relevant negative feedback mechanism may be operating, tightly coordinating the levels of APP expression, and AICD and Aβ production. Further, as APP CTF levels progressively decrease in AD (Sergeant et al., 2002), this feedback mechanism may be lost with the progression of the disease. This would be a physiologically relevant process given that neurons exhibit higher levels of Aβ production. Nonetheless we cannot exclude that non-physiological Aβ concentrations may trigger neuronal stress mechanisms which may in turn affect APP metabolism and Aβ production, future research will address this question.

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Chapter IV

THE BIOCHEMICAL BASIS OF Aβ BASED THERAPEUTICS

Chapter Outline

Histopathologically AD is characterized by the presence of both extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). Several studies in transgenic mice suggest that Aβ deposition develops prior to Tau pathology (Lewis et al. 2001; Oddo et al. 2003). Hence, amyloid deposition would precede and may lead to formation of NFTs. More, in Down´s syndrome patients the Aβ deposits exist in the absence of NFTs, in brains areas most affected by AD (Leverenz and Raskind 1998; Gouras et al. 2000). NFTs are mainly composed of hyperphosphorylated Tau protein and this has been attributed to an imbalance of cellular kinase/phosphatase activities, which may be triggered by Aβ.

The aggregation state of the Aβ peptide also appears to be a factor associated with the set of AD pathogenesis. Although Aβ aggregated forms were shown to induce damage to cultures neuronal cells (Pike et al. 1993; Lorenzo and Yankner 1994), subsequent studies suggested that rather than highly aggregated species, oligomeric Aβ may represent the principal neurotoxic entity that causes synaptic dysfunction (Lambert et al. 1998; Hartley et al. 1999).

Clearly, it appears that Aβ is able to trigger, directly or indirectly, mechanisms that ultimately lead to neuronal degeneration, and several novel therapeutic strategies are based on lowering Aβ levels or by preventing the formation of the neurotoxic oligomeric or more aggregated Aβ species. Other therapeutic approaches under development have focused on the prevention of Tau-induced neurodegeneration.

Therefore, due to the importance of the Aβ aggregation state to its neurotoxic properties, we address whether Aβ binding proteins (involved either in Aβ disaggregation or clearance) could affect the latter in terms of its neurotoxicity and effect on APP metabolism. The observation that these proteins could partially reverse the neurotoxicity and the isAPP accumulation induced by Aβ, suggested their therapeutic potential as modulators of APP processing and neuroprotectors.

Further, and according to the "amyloid cascade hypothesis", Aβ may trigger a set of signaling cascades which ultimately lead to neurodegeneration. Key players in signal transduction mechanisms are the protein kinases and phosphatases. One possible explanation for abnormal Tau phosphorylation involves abnormal kinase and/or phosphatase activities. Previous studies have shown that Aβ activates various PKC isoforms in different cell lines (Nakai et al. 2001, Tanimukai et al. 2002). Here we focus on Aβ effects on protein phosphatase 1 (PP1) activity. Our studies reveal for the first time the existence of an inhibitory effect on the different PP isoforms, suggesting that Aβ can interfere with neuronal signal transduction events.

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Manuscript 4 – Aβ-disaggregating proteins as modulators of APP processing

Henriques, A.G.; Gomes, B.; Ruivo, R.; da Cruz e Silva, E.F.* and da Cruz e Silva, O.A.B.
Laboratório de Neurociências, Centro de Biologia Celular, Universidade de Aveiro, Aveiro (Portugal). *Laboratório de Transdução de Sinais, Centro de Biologia Celular, Universidade de Aveiro, Aveiro (Portugal).

Manuscript in preparation

Running tittle: Aβ fibril formation and APP processing

Corresponding author: Odete A.B. da Cruz e Silva, Centro de Biologia Celular, Secção Autónoma das Ciências da Saúde, Universidade de Aveiro, 3810-193 Aveiro, Portugal. Phone: +351 234 370200 and Fax: +351 234 370983. E-mail: odetecs@ua.pt

ABSTRACT

Aβ induces several cellular responses associated with neurotoxicity. Of note, it was also shown that the fibrillar/aggregated species of Aβ are far more toxic. Thus, therapeutic strategies aimed at Aβ depolymerization represent attractive avenues of intervention. Laminin and gelsolin provide such a model, given that both prevent Aβ fibril formation, giving rise to Aβ-laminin and Aβ-gelsolin complexes. By forming these complexes the neurotoxic effects of Aβ are attenuated. Additionally, we have recently shown that Aβ can lead to intracellular accumulation of sAPP; pre-incubation of Aβ with laminin and gelsolin also renders it less potent in this respect. In conclusion, our data lead us to conclude that Aβ-laminin and Aβ-gelsolin complexes are less neurotoxic and less potent at inducing intracellular sAPP retention than fibrillar Aβ. These findings validate the potential of both proteins as therapeutic targets, preventing Aβ-induced effects, both at the neurotoxic and at the APP metabolic levels.

Keywords: Aβ, fibril inhibition, aggregation, APP processing.

INTRODUCTION

The deposition and accumulation of aggregates of fibrillar Abeta (Aβ) peptides into amyloid plaques is considered one of the key processes underlying neuronal loss in Alzheimer´s Disease pathology. The amyloid plaques are mainly composed of Abeta (Aβ) (Glenner and Wong 1984), but also include a number of other compounds such as inflammatory molecules, proteoglycans, metal ions, antioxidant proteins and protease and clearance related compounds (for review see Atwood et al. 2002).

Several investigators have reported that Aβ neurotoxicity is related to the degree of fibrillization, the β-sheet structure and the size of the peptides (Pike et al. 1993; Lorenzo and Yankner 1994; Dahlgren et al. 2002). The molecular mechanisms responsible for the passage of normal soluble Aβ forms to fibrils are not well understood, nonetheless, it is known that Aβ binds several proteins that can modulate its aggregation or its fibrillar state. Among these are laminin (Bronfman et al. 1996; Drouet et al. 1999; Monji et al. 1999; Morgan et al. 2002) and gelsolin (Chauhan et al. 1999; Ray et al. 2000; Qiao et al. 2005; Chauhan et al. 2008). Both have been shown to bind and form complexes with Aβ. Laminin is a major basement membrane protein shown to inhibit Aβ fibril formation and to disaggregate preformed Aβ fibrils (Monji et al. 1999; Morgan and Inestrosa 2001; Morgan et al. 2002). Gelsolin is found both as an intracellular protein (Tanaka and Sobue 1994; Ji et al. 2008) - that is able to modulate actin assembly and disassembly (Janmey et al. 1985; Howard et al. 1990) - and as a secreted plasma and CSF protein (Kwiatkowski et al. 1988; Paunio 1994; Kulakowska et al. 2008). Like laminin, it was reported to prevent Aβ fibrillogenesis, being also able to defibrillize preformed fibrils (Ray et al. 2000; Chauhan et al. 2008).

Aβ is an intracellular product of the proteolytic processing of Alzheimer´s amyloid precursor protein (APP). It is generated by sequential cleavage of β-secretase (mainly BACE1) (Vassar et al. 1999; Yan et al. 2001) and the γ-secretase complex (Li et al. 2000; Esler et al. 2002; Lee et al. 2002; Steiner et al. 2002). The physiological function of Aβ is unknown although it triggers many cellular responses. Among the most devastating is its ability to trigger several apoptotic mechanism and hence cellular death (Kienlen-Campard et al. 2002; Pereira et al. 2004). Other reports have associated Aβ to altered APP metabolism (Davis-Salinas et al. 1995; Schmitt et al. 1997; Moreno-Flores et al. 1998;

Carlson et al. 2000; Henriques et al. 2009a, in press). The latter is likely to be of physiological significance, given that Aβ may modulates APP processing and trafficking (Henriques et al. 2009a, in press), and decrease AICD production with consequences in decreased APP expression levels (Henriques et al. 2009b, in press), thus keeping "in check" its own production.

In the work here described we addressed the effects of Aβ aggregation on APP processing. Further, and since fibrillization is such an important event in AD pathogenesis, we also evaluated the effect of proteins involved in Aβ depolymerization and its complexes on APP processing. Our data demonstrated that inhibition of Aβ fibril formation by either laminin or gelsolin, was able to partially revert Aβ effects on APP metabolism, thus suggesting a therapeutic potential for these proteins in AD treatment.

MATERIALS AND METHODS

Materials

Laminin (from basement membrane of Engelbreth-Holm-Swarm mouse carcinoma) and gelsolin (from bovine plasma) were purchased from Sigma. Gelsolin was prepared as an 10 μM stock dissolved in 25 mM Tris-HCl, pH 7,5. Aβ peptide, corresponding to residues 25-35 (Sigma), $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides (American Peptide Company) and $A\beta_{25-35}$ with a scrambled sequence ($A\beta_{scrb,}$ from NeoMPS) were dissolved in sterile distilled water to obtain a 1mM stock solution. Thioflavine-T (Sigma) was prepared as a 30 mM stock in distilled water.

The monoclonal antibody 22C11 directed against the APP N-terminus (Boehringer) was used in this study to detect APP and total sAPP. Holo APP was achieved using the APP C-terminal antibody (rabbit polyclonal anti-β-APP C-terminus, Zymed). The monoclonal anti-β-tubulin antibody (Amersham Pharmacia) was used as a control protein. Anti-mouse IgG horseradish peroxidase-linked whole secondary antibody (Amersham Pharmacia) was used for enhanced chemiluminescence (ECL or ECL plus).

Aβ aggregation

Aβ aggregation was achieved by incubating the different peptides for 48 hr at 37°C. These preparations were shown to contain Aβ fibril forms (Drouet et al. 1999; Ray et al. 2000 and data not shown).

For co-incubation assays 100 μM $A\beta_{25-35}$ was incubated in the presence of either laminin at 0,5 μM (laminin/Aβ molar ration of 1:200) or gelsolin at 2,2 μM (gelsolin/ Aβ molar ratio of 1:45) in PBS (phosphate buffer saline, pH 7,4) for 48 hr at 37°C.

Fibrillar peptide and peptide complexes were then diluted before addition to the cells in culture (see Aβ treatment section).

Thioflavine T fluorimetric (TH-T) assay

$A\beta_{25-35}$ fibril formation in the presence or absence of the proteins mentioned above was monitored by a TH-T fluorometric assay as described by H. LeVine (1993), with a few modifications. TH-T fluorescence was measured at 0 hr and 48 hr of incubation at 37°C, using a Jasco FP-777 spectrofluorimeter. TH-T measurements used 20 μl of incubated sample mixed with 980 μl 15 μM TH-T in PBS. Samples were monitored at: λ_{Ex} = 450 nm, 5 nM bandpass and λ_{Em} = 482 nm, 10 nM bandpass. The background fluorescence of TH-T and buffer alone were subtracted from the fluorescence obtained value for each sample.

Cell culture

Rat cortical neuronal cell cultures were prepared from embryonic day 18 Wistar rat foetuses as previously described (Henriques et al. 2007). Briefly, after dissociation with trypsin (1.0 mg/ml; 10 min; 37°C) the cells were plated on poly-D-lysine-coated dishes at a density $1{,}0x10^5$ cells/cm^2 in B27-supplemented Neurobasal medium (GIBCO), a serum-free medium combination (Brewer et al. 1993), supplemented with glutamine (0.5 mM), gentamicin (60 μg/ml). Cultures were maintained in an atmosphere of 5% CO_2 at 37°C during 9 days before being subject to various experimental conditions.

Aβ treatment

Exposure of cells to Aβ was preceded by an aggregation step. For Fig. 1 the effect of different Aβ peptides and concentrations were evaluated. Subsequent experiments were carried out by pre-incubating $A\beta_{25-35}$ peptide alone or in the presence of the different proteins (laminin and gelsolin) during 48 hr at 37°C; 100 μl of each preparation were then diluted in 900 μl of the complete corresponding medium and added to the cells for a 24 hr period. Under these conditions Aβ was used in a final concentration of 10 μM, laminin of 50 nM and gelsolin of 220 nM.

Monotoring of apoptosis

In order to morphologically access apoptosis, cells were plated at a density of $0,8x10^5$ cells/cm^2 on poly-D-lysine-coated coverslips. After the appropriate treatments cells were fixed with 4% paraformaldehyde diluted in media (1:1) for 2 minutes, and then incubated for 15 minutes in undiluted paraformaldehyde. After 3 washes with PBS, cells were incubated with Hoescht dye 33258 (5 mg/ml stock, Sigma) at 5 μg/ml in PBS for 3 minutes to allow nuclear staining. For quantitative analysis of apoptosis, cell nuclei were visualized using a DAPI filter in an Olympus IX-81 inverted epifluorescence microscope. To evaluate the percentage of apoptotic cells, 10 independent fields were analyzed (approximately 400 cells) per experimental condition. Under control conditions primary neuronal cultures exhibited approximately 10% of apoptotic cells.

Sample collection and protein analysis

Following appropriate treatments, conditioned media and cells were collected into boiling 1% SDS and the samples were homogenized as previously described (Amador et al. 2004). Protein determination of the lysates was carried out with the BCA kit (Pierce). Samples normalized for protein content, were run on 7.5% SDS-PAGE and then electrophoretically transferred onto a nitrocellulose membrane. Immunoblotting was carried out by incubating overnight with 22C11 primary antibody for the detection of APP and total sAPP, or anti-β-tubulin antibody for tubulin detection. Detection was achieved using ECL or ECL plus detection systems.

Quantification and statistical analysis

Quantity One Densitometry software (Bio-Rad) was used to scan and quantify immunoblot intensity. Data are expressed as means ± SEM of triplicate determinations, from at least three independent experiments. Statistical analysis was carried out using one way analysis of variance (ANOVA). When the F values were significant, the Tukey test was applied to compare all pairs of groups. Statistical differences have been determined at $P<0,05$.

RESULTS

Effect of different aggregated Aβ peptides on intracellular sAPP accumulation

Previous data from our laboratory showed that "fresh" $A\beta_{25-35}$ peptide preparations led to intracellular sAPP accumulation (isAPP) in non-neuronal, neuronal-like and neuronal cells (Henriques et al. 2009a, in press). This was confirmed using both APP N-terminal (22C11) and APP C-terminal antibodies which permit distinction between APP and sAPP. For cells, particularly those neuronal in origin, alterations in APP expression levels could also be detected (Henriques et al. 2009b, in press). In this study we addressed the effect of $A\beta_{25-35}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ aggregated peptides on APP metabolism in neuronal cortical cells. Similar to "fresh" $A\beta_{25-35}$, aggregated $A\beta_{25-35}$ and $A\beta_{1-42}$ led to a significant increase in APP intracellular levels, as detected using the 22C11 antibody (Fig. 1A). Of note, the more aggregated $A\beta_{25-35}$ state (as denoted by TH-T assay, Fig. 2) resulted in a higher fold increase in intracellular APP levels (approximately 4.5 fold, Fig. 1A), than the "fresh" Aβ (2 fold, Henriques et al. 2009b, in press). At 20 μM concentration $A\beta_{1-40}$ peptide was much less effective in inducing an increase in APP intracellular levels that $A\beta_{25-35}$ and $A\beta_{1-42}$ peptides.

As previously observed, medium secreted sAPP decreased upon exposure to Aβ for both non aggregated "fresh" (Henriques et al. 2009b, in press) and aggregated $A\beta_{25-35}$ forms (Fig. 1). The other peptides $A\beta_{1-40}$ (20 μM), $A\beta_{1-42}$ (5 μM), $A\beta_{1-42}$ (20 μM) increased systematically in toxicity. This peptide species and concentration dependent effect was mirrored in isAPP retention (Fig. 1A). The scramble $A\beta_{25-35}$ peptide was used as a control, and confirmed the specificity of the Aβ response.

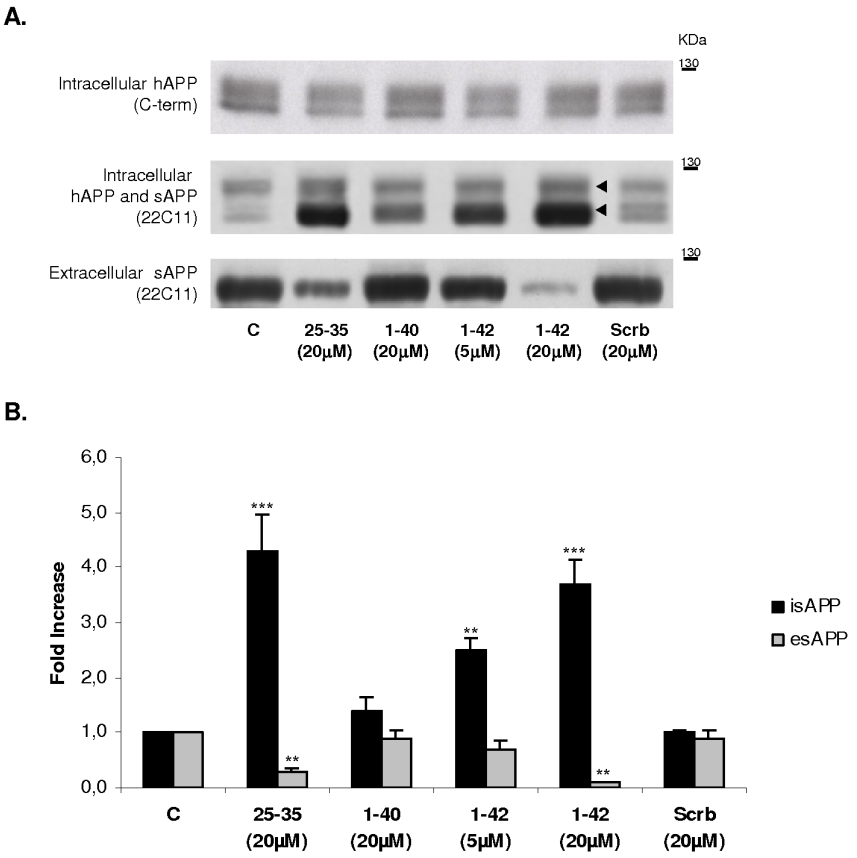

Fig. 1. Effect of different aggregated peptides on APP processing. $A\beta_{25-35}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ were aggregated for 48 hr at 37°C. Following aggregation peptides were added to primary neuronal cultures at different concentrations for 24 hr. Scramble 25-35 peptide was used as a control (Scrb). **(A)** Intracellular holo APP protein was detected using a C-terminal antibody. The levels of intracellular APP and extracellular sAPP were detected using the N-terminal antibody 22C11. **(B)** Quantitative results are expressed as mean±SEM of 3 independent experiments. isAPP, intracellular sAPP and APP levels; esAPP, extracellular secreted sAPP; ◄, intracellular sAPP retention. **P<0,01 and ***P<0,001 significantly different from control; Tukey *post hoc* test.

Inhibition of $A\beta_{25-35}$ aggregation by laminin and gelsolin

Previous observations showed that both laminin (Drouet et al. 1999; Morgan et al. 2002) and gelsolin (Ray et al. 2000; Qiao et al. 2005) are able to inhibit fibril formation or to depolymerize the preformed fibrils of both $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides. Here we addressed the effect of both proteins on $A\beta_{25-35}$ aggregation and subsequent effects on APP metabolism. We quantified the extent of $A\beta_{25-35}$ aggregation using the TH-T fluorescence assay. Although it has been reported that $A\beta_{25-35}$ spontaneously aggregates in solution, 48 hr after incubation of the latter at 37°C, revealed an increase in the relative fluorescence of the TH-T emission intensity (Fig. 2), indicating the existence of a more Aβ aggregated form.

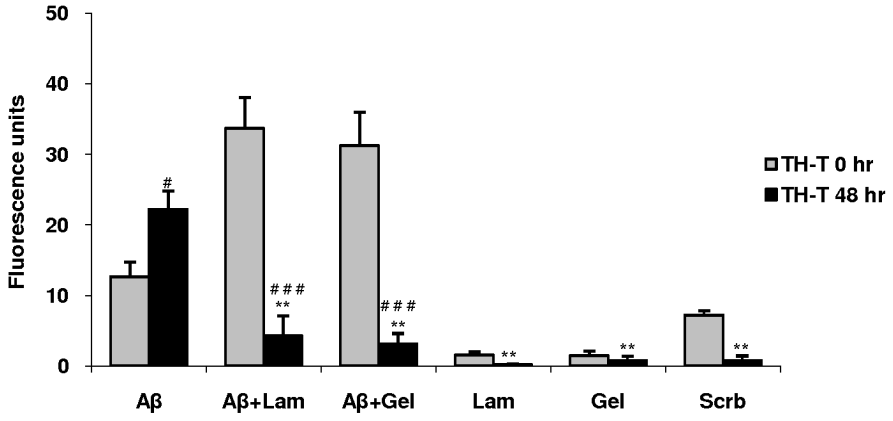

Fig. 2. Measurement of Aβ aggregation. $A\beta_{25-35}$ was incubated at 37°C for 48 hr either alone or in the presence of 0,5 μM laminin (Lam) or 2,2 μM gelsolin (Gel). The fluorescence intensity of TH-T was measured at λex = 450 nm, λem = 482 nm. Results are expressed as mean±SEM of three independent experiments. **P<0,01, significantly different from 48 h control; ##P<0,05 and ###P<0,001 difference between 0 h and 48 h treatment, for each condition, Tukey *post hoc* test.

Under these conditions laminin was used in a molar ratio of 1:200 (Aβ:laminin) and gelsolin of 1:45 (Aβ:gelsolin). By comparing to incubation of Aβ alone, we obtained a significant decrease in the fluorescence of TH-T when Aβ was co-incubation with laminin or gelsolin for 48 hr (from 22 fluorescence units to 4 and 3, respectively). These data indicate that in the presence of these two proteins $A\beta_{25-35}$ fibril formation was inhibited.

Neurotoxicity of $A\beta_{25-35}$ when complexed with laminin and with gelsolin

The neurotoxicity of Aβ and Aβ-complexes was evaluated, by analysis of cell nuclei following Hoescht staining. Representatative images of each condition are shown in Fig. 3A. The percentage of apoptosis was estimated, for primary cortical cultures when subjected to 10 μM of $A\beta_{25-35}$, both in the absence and presence of laminin (50 nM) or gelsolin (220 nM) for 24 hr (Fig. 3B). The apoptosis induced by Aβ peptide alone was low (~15% above control levels). However, the levels could be decreased to ~5% above control if Aβ was pre-incubated with both laminin and gelsolin. Thus, indicating that laminin and gelsolin can attenuate the toxic effects of Aβ as deduced from the decrease in apoptotic levels in the presence of both proteins.

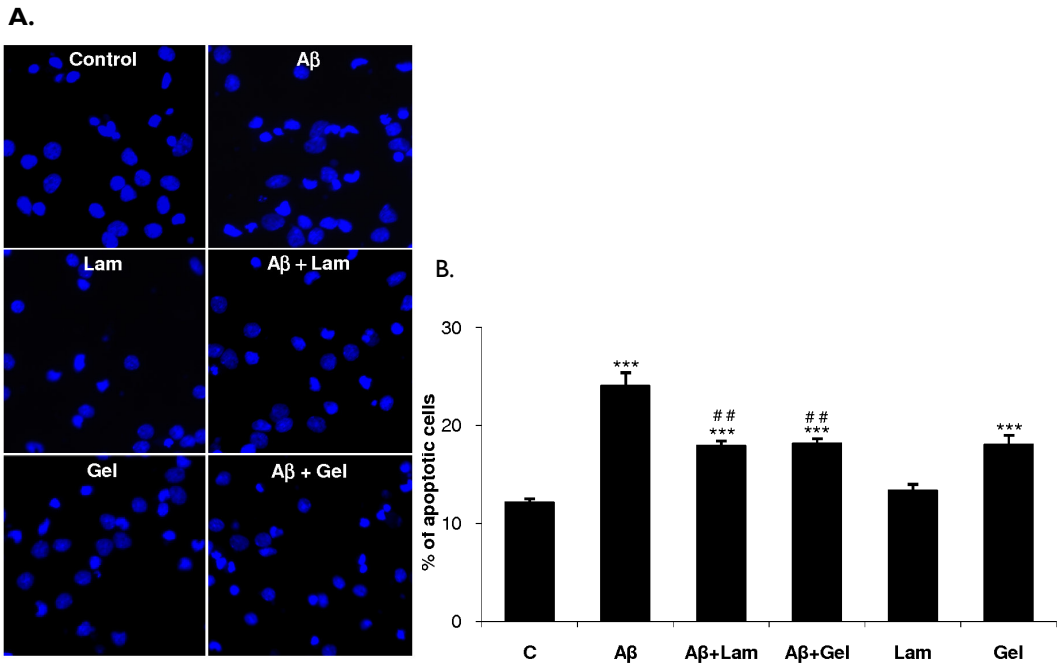

Fig. 3. $A\beta_{25-35}$ apoptotic effects in primary cortical cultures. (A) Hoescht stain was used to visualize cell nuclei in order to address the effects of 10 μM $A\beta_{25-35}$ incubation during 24 hr, either alone or in the presence of laminin (Lam) and gelsolin (Gel) on the viability of neuronal cortical cultures. **(B)** Quantitative results of the percentage of apoptotic cells for each condition. Ten independent fields of microscope were analyzed (approximately 400 cells) per experimental condition. ***P<0,001, significantly different from control; ##P<0,01, significantly different from Aβ; Tukey *post hoc* test.

Effect of Aβ-laminin and Aβ-gelsolin complexes on APP metabolism

As discussed above the Aβ binding proteins (laminin and gelsolin) prevent fibrillogenesis rendering Aβ less neurotoxicity. Consequently we took advantage of this phenomenon to evaluate whether APP anomalous processing was also affected by Aβ aggregation state. Hence these Aβ-protein complexes were added to cultured cells. When primary cortical cultures were treated with 10 μM of aggregated Aβ for 24 hr we detected an approximate 4,0 fold increase in isAPP levels (Fig. 4).

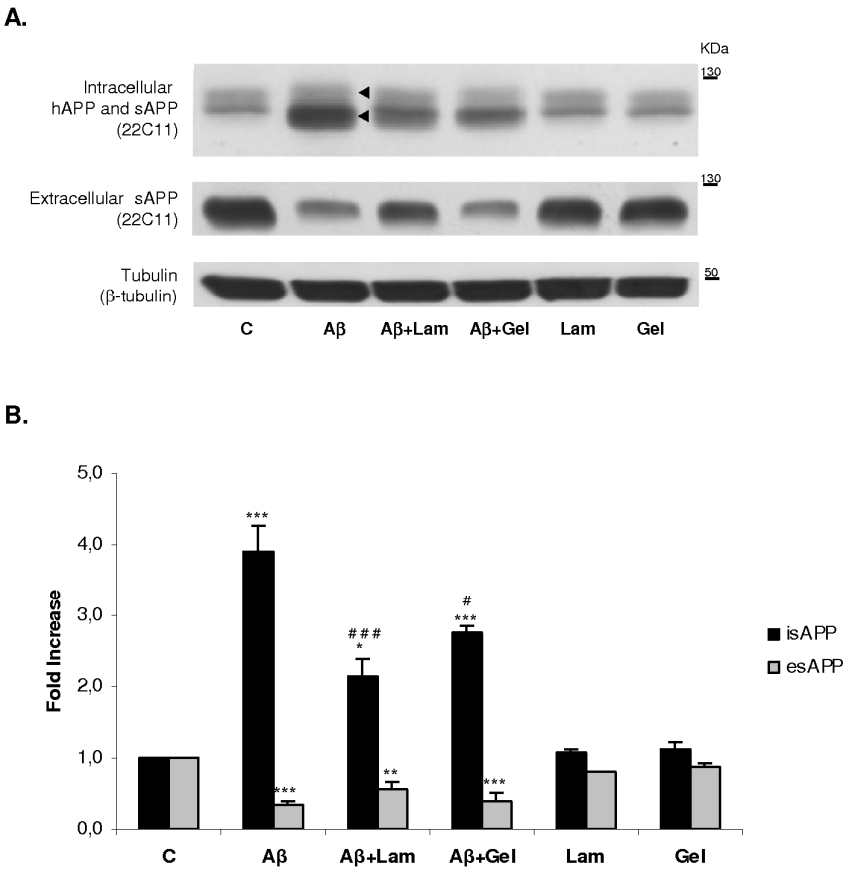

Fig. 4. Effect of Aβ-laminin and Aβ-gelsolin complexes on APP processing in primary cortical cultures. Primary cortical cultures were incubated during 24 hr with 10 μM of Aβ in the presence or absence of various proteins: laminin (Lam) and gelsolin (Gel). **(A)** Intracellular APP and total extracellular sAPP were detected using the 22C11 primary antibody. β-tubulin was used as a control protein. (B) Quantitative results are expressed as mean±SEM of 3 independent experiments. isAPP, intracellular sAPP and APP levels; esAPP, extracellular secreted sAPP; ◄, intracellular sAPP retention. *P<0,05; **P<0,01; ***P<0,001, significantly different from control; #P<0,01 and # # #P<0,01, significantly different from Aβ; Tukey *post hoc* test.

In the presence of Aβ-laminin and Aβ-gelsolin complexes, the levels of isAPP decreased to 2,0 fold and 2,5 fold, respectively, when compared to Aβ treatment alone. These data suggest that these proteins which decrease Aβ fibrillogenesis, favour a less toxic Aβ species resulting in a decrease in Aβ-induced intracellular sAPP accumulation. Laminin and gelsolin alone showed no effect on APP intracellular levels. With respect to medium secreted sAPP we observed that aggregated $A\beta_{25-35}$ resulted in decreased total extracellular sAPP levels, as previously reported. For the proteins tested it would be expected that inhibition of isAPP retention resulted in increased sAPP secretion. Nonetheless, only co-incubation of Aβ and laminin induced a slight increase on secreted sAPP (from 0,3 to around 0,5) showing a tendency of this protein to revert the inhibition of sAPP secretion induced by fibrillar Aβ alone. Aβ-gelsolin complexes had no significant effect on sAPP extracellular levels. Of note, both laminin and gelsolin alone slightly decreased sAPP extracellular secretion.

DISCUSSION

We previously reported that "fresh" $A\beta_{25-35}$ peptide leads to increased isAPP accumulation in non-neuronal cells (mainly isAPPα retention) and neuronal cultures (Henriques et al. 2009a, in press). Since the sAPPα proteolytic fragment has been shown to hold neurotrophic and neuroprotective properties when added to cells in culture, we proposed that retention of sAPP may be a physiologically relevant mechanism. The neurotoxic effects of the Aβ peptide *per se* appear to be associated with its polymerization state (Pike et al. 1993; Lorenzo and Yankner 1994; Dahlgren et al. 2002). From the data here presented it is reasonable to deduce that the aggregated $A\beta_{25-35}$ peptide showed a higher potential to induce isAPP retention when compared to "fresh" Aβ (4,5 fold versus 2,0 fold). This indicates that increasing Aβ aggregation leads to an enhanced toxic effect.

Given that Aβ toxicity is closely associated with its fibril state, proteins which can modulate the latter may therefore prevent some of the Aβ amyloidogenic effects. Both laminin and gelsolin have been shown to inhibit fibril formation and also to promote defibrillization of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides, and this was associated with a decrease in Aβ-induced neurotoxicity (Morgan et al. 2002; Qiao et al. 2005). We evaluated the effect of laminin and gelsolin on $A\beta_{25-35}$ aggregation. In agreement with previous observations for the longer peptides, these proteins were able to inhibit the aggregation of $A\beta_{25-35}$ as showed by TH-T assay (Fig. 2) and to prevent $A\beta_{25-35}$ neurotoxic effects (Fig. 3). Thus the $A\beta_{25-35}$ region must comprise at least one binding site for laminin and gelsolin. The resulting Aβ-laminin and Aβ-gelsolin complexes were effective in decreasing anomalous isAPP retention induced by the Aβ peptide alone. For medium secreted sAPP, only laminin was able to marginally provoke an increase, thus reverting the effect of fibrillar Aβ. Laminin was previously shown to decrease APP secretion and to affect intracellular APP biogenesis and catabolism (Monning et al. 1995; Bronfman et al. 1996).

Presently it is difficult to interpret the significance of the decrease in isAPP levels induced by these Aβ-protein complexes, because the metabolism and clearance of the compounds tested are not completely understood. Nonetheless, we postulate that the laminin- or gelsolin- Aβ complexes might be inhibiting Aβ signaling and/or be intracellularly targeted

for degradation, therefore preventing Aβ-induced responses. Peripheral administration of plasma gelsolin resulted in a reduction of $A\beta_{1-40}$/$A\beta_{1-42}$ and Aβ load in the brains of APP/PS transgenic mice that exhibited elevated intraneuronal $A\beta_{1-42}$ levels (Matsuoka et al. 2003). Moreover, in similar transgenic mouse models of AD, peripheral delivery of plasmid DNA of plasma gelsolin, also lead to a reduction in Aβ levels in the brain (Hirko et al. 2007). The reduction in Aβ pathology appeared to be related with an increase in activated and reactive microglia and with soluble oligomeric forms. Our study suggest that by interacting with Aβ peptide, laminin and gelsolin may be protective with respect to Aβ induced neurotoxicity and may play a role as modulators of APP metabolism. Further, due to their depolymerization properties and their capacity to bind Aβ these proteins could alter the Aβ periphery/brain load leading to its reduction in brain. This may be of therapeutic value to the development of future drugs designed to act at the level of Aβ fibrillogenesis.

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Manuscript 5 - PP1 inhibition by Aβ peptide as a potential pathological mechanism in Alzheimer´s disease

Ana Paula B. Vintém[1], Ana Gabriela Henriques[2], Odete A.B. da Cruz e Silva[2] and Edgar F. da Cruz e Silva[1*]

[1]Laboratório de Transdução de Sinais and [2]Laboratório de Neurociências, Centro de Biologia Celular, Universidade de Aveiro, Aveiro, Portugal

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***Corresponding author:** E-mail: edgar@ua.pt; Fax: +351-234-370-983

ABSTRACT

Abnormal protein phosphorylation has been associated with several neurodegenerative disorders, including Alzheimer's Disease (AD). Aβ is the toxic peptide that results from proteolytic cleavage of the Alzheimer's amyloid precursor protein, a process where protein phosphatases are known to impact. The data presented here demonstrates that protein phosphatase 1 (PP1), an abundant neuronal serine/threonine-specific phosphatase highly enriched in dendritic spines, is specifically inhibited by Aβ peptides both 'in vitro' and 'ex vivo'. Indeed, the pathologically relevant $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides, as well as $A\beta_{25-35}$, specifically inhibit PP1 with low micromolar potency, as compared to inactive controls and other disease related peptides (e.g. the prion related $Pr_{118-135}$ and $Pr_{106-126}$). Interestingly, PP1 inhibition is increased by Aβ aggregation, indicating a possible direct neurotoxic effect of the aggregated peptide. PP1 involvement in processes like long-term depression, memory and learning, and synaptic plasticity, prompt us to suggest that PP1 may constitute an important physiological target for Aβ and, therefore, increased Aβ production and/or aggregation may lead to abnormal PP1 activity and likely contribute to the progressive neuropsychiatric AD condition. Thus, PP1 activity and levels constitute potential biomolecular candidates for diagnostics and therapeutics.

Keywords: Phosphatase, PP1, Amyloid peptide, Alzheimer's disease, phosphorylation, Abeta aggregation

1. INTRODUCTION

Protein phosphorylation is a key post-translational modification that regulates numerous physiological processes. Thus, protein kinases and protein phosphatases are key pivotal players in signal transduction cascades, modulating many biological functions. Altered signal transduction leads to a variety of disorders, including neurodegenerative conditions. Protein phosphatase 1 (PP1) is the most widely expressed and highly regulated member of the serine/threonine-specific phosphatase family. The three known mammalian PP1 catalytic subunit genes (PP1α, PP1β and PP1γ) are abundantly expressed in the mammalian brain [6], sustaining the importance of PP1 in the control of brain functions. Both PP1α and PP1γ_1 were shown to be highly and specifically concentrated in dendritic spines [29], placing them at the centre of neuronal signaling. Indeed, PP1, via regulation by the dopamine- and cAMP-regulated phosphoprotein, DARPP-32 [18], in the neostriatum [30], mediates the action of several neurotransmitters, like dopamine, serotonin and glutamate [26]. Perturbations of these neurotransmitter systems are known to contribute to the etiology of several neuropsychiatric disorders and play an important role in the actions of drugs of abuse.

Alzheimer's disease (AD) is characterized clinically as a dementia of insidious onset and inexorable progression, and pathologically by the presence of large numbers of neuritic plaques and neurofibrillary tangles. The neuritic plaques are largely extracellular lesions consisting of deposits of 40–42/43 amino acids long peptides, termed Aβ, derived from the proteolytic processing of the Alzheimer's amyloid precursor protein (APP) [15,17]. Direct APP phosphorylation and phosphorylation-dependent APP processing have been shown to regulate Aβ formation [7,31], and thus altered cellular phosphorylation may lead to increased neuronal amyloid production and accumulation. Indeed, protein kinase C (PKC) and protein phosphatase 2B (PP2B) were reported to bidirectionally regulate Aβ formation in cell-free preparations [12]. Additionally, PP1 is also known to stimulate the secretion of APP, suggesting that it plays a central role in this process [5]. As mentioned above, AD patients also present characteristic neurofibrillary tangles in their brains, composed largely of hyperphosphorylated tau protein. Altered PP1 and protein phosphatase 2A (PP2A) activities are both thought to contribute to tau hyperphosphorylation [2]. Further, the cognitive decline observed in AD is largely thought to be related to the marked decrease observed in synaptic contacts [11,34],

where PP1 plays a central role since it is highly enriched in post-synaptic dendritic spines. Not only is PP1 involved in the control of long-term depression and synaptic plasticity [24], but also in aging-related memory defects [14]. The abnormal phosphorylation of key proteins linked to neurodegeneration and the demonstrated neurotoxicity of Aβ led us to investigate the effect of this peptide on PP1. Our previous work showed that PP1 was involved in secreted APP production [5] and that its intracellular levels were altered after incubation of cells in culture with Aβ peptides [1]. Herein we demonstrate that the activity of different isoforms of PP1 is highly and specifically inhibited by Aβ and that this effect is potentiated by Aβ aggregation. Our results provide novel insights into the molecular mechanisms associated with Alzheimer's disease that lead to neurodegeneration and dementia.

2. MATERIALS AND METHODS

2.1. Materials

Phosphorylase *b* and phosphorylase kinase were purchase from Sigma, [γ-^{32}P]ATP (3000Ci/mmol) from Amersham Pharmacia Biotech (Portugal), and PP1α and inhibitor I_2 from New England Biolabs (USA). PP1γ_1 and PP1γ_2 were expressed and purified, essentially as previously described [35].

Aβ peptide fragments 25-35, 1-40 and 1-42 (American Peptide Company, USA) were reconstituted in distilled water to concentrations of 1 and 5mM. The peptides used as controls were $A\beta_{40-1}$ (Bachem, Belgium) and $A\beta_{25-35}$ with a scrambled sequence, $A\beta_{SCR}$ (NeoMPS, France). The control peptides were reconstituted in distilled water to 5 and 10mM. $A\beta_{1-42}$ pre-treated with 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from rPeptide (USA) and reconstituted in DMSO to 1mM. The prion peptide $Pr_{106-126}$ and its scrambled control, $Pr_{106-126SCR}$ (NeoMPS, France), were reconstituted in water to 5mM. $Pr_{118-135}$ (American Peptide Company, USA) was first dissolved in DMSO and then brought to 5mM with water. All reconstituted peptides were immediately frozen and stored at –20°C.

2.2. Methods

2.2.1. Determination of peptide IC_{50} against PP1. The effect of all peptides on the activity of purified PP1 isoforms was assessed using ^{32}P-phosphorylase *a* and myelin basic protein (MBP) as substrates. The preparation of substrate and the assays using ^{32}P-MBP were carried out as described by the manufacturer (New England Biolabs, USA). ^{32}P-phosphorylase *a* was prepared from phosphorylase *b* using [γ-^{32}P]ATP and phosphorylase kinase as previously described [35]. Peptide stock solutions were diluted in inhibitor buffer [50mM Tris-HCl (pH7.5), 0.1mM EGTA, 0.03% (v/v) Brij-35], just before use. PP1 catalytic subunits diluted in phosphatase buffer [50mM Tris-HCl (pH7.5), 1mM $MnCl_2$, 0.1mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1mg/ml BSA] were incubated with the prepared peptides for 5min at 30°C. The reaction was started with the addition of ^{32}P-phosphorylase *a* (3mg/ml) substrate. After 10min, 100µl of ice-cold 20% (w/v) trichloroacetic acid (TCA) were added and the mixture was centrifuged at 12000g for

2min at room temperature. The ^{32}P-phosphate released into the supernatant was measured using a scintillation counter (kept below 20% of total substrate radioactivity for the control in order to ensure linearity). For each peptide, an appropriate range of concentrations was used and the IC_{50} calculated using the BioDataFit 1.02 software. The standard deviation was calculated from at least three independent experiments.

2.2.2. Effect of peptide aggregation on PP1γ_1. For Aβ oligomer preparation [32], 0.1mM $A\beta_{1-42}$ (HFIP) or 1mM $A\beta_{1-40}$ were incubated for 24h at 4°C. For fibrillar Aβ preparation [32], the same concentrations of $A\beta_{1-42}$ (HFIP) and $A\beta_{1-40}$ in 10mM HCl were incubated for 24h at 37°C. The acid was then neutralised and both preparations used in the phosphatase assay. The prion peptide $Pr_{118-135}$ remains soluble if kept at –20°C [3]. Fibrillar $Pr_{118-135}$ was prepared by incubation of 1mM of peptide for 72h at room temperature [3].

2.2.3. Incubation of PC12 cells with $A\beta_{25-35}$. PC12 cells were cultured in RPMI1640 medium (Alfagene, Portugal) supplemented with 5% fetal bovine serum and 10% horse serum. Cells were plated onto poly-L-ornithine coated dishes at a density of $5x10^5$ cells/cm^2 and washed with serum-free medium prior to $A\beta_{25-35}$ treatment. Cells were then exposed to 20 or 50μM Aβ for 2 or 24h in serum-free medium and after incubation were washed in cold Tris-HCl/EDTA buffer [50mM Tris-HCl (pH7.5), 0.1mM EDTA] and resuspended in homogenizing buffer [50mM Tris-HCl (pH7.5), 0.1mM DTT, 0.1mM EDTA, 0.1mM EGTA, 0.1mM PMSF, 1mM benzamidine, 150mM NaCl and 5μg/ml leupeptin]. Cell homogenates were normalized for total protein content, as determined by the BCA method (Pierce, USA), before being used in the phosphatase assay.

2.2.4. Phosphatase activity assay of cell extracts. The total phosphatase activity of control and Aβ-treated cell extracts was determined by assaying appropriate dilutions in phosphatase buffer with ^{32}P-phosphorylase *a*. PP2A specific activity was determined by pre-incubating the cell extracts with 200nM of I_2 for 15min at 30°C before substrate addition. The PP1 specific activity was calculated as the difference between the total phosphatase activity and the PP2A specific activity.

3. RESULTS

3.1. Aβ peptides inhibit PP1 at low micromolar concentration

The effect of the different Aβ peptides and their controls ($A\beta_{25-35}$, $A\beta_{1-40}$, $A\beta_{1-42,}$ $A\beta_{SCR}$ and $A\beta_{40-1}$) on the activities of the two main neuronal PP1 isoforms (PP1α and PP1γ_1) was assessed by determining the respective IC_{50} values (Table 1).

Table 1. Comparison of PP1 isoform inhibition by various Aβ and prion peptides

	IC_{50} (μM)[a]	
Peptides	PP1α	PP1γ_1
$A\beta_{25-35}$	8.2 ± 0.5	4.1 ± 0.3
$A\beta_{1-40}$	2.8 ± 0.4	1.3 ± 0.7
$A\beta_{1-42}$	2.3 ± 0.7	1.9 ± 0.5
$A\beta_{25-35\ SCR}$	Nd	>1000[b]
$A\beta_{40-1}$	Nd	137
$Pr_{106-126}$	Nd	122 ± 8
$Pr_{118-135}$	Nd	~500[c]

[a] Values are expressed as mean±S.E.M. of at least three independent experiments using phosphorylase *a* as substrate.
[b] Concentration yielding 20-30% inhibition.
[c] Concentration yielding ~50 % inhibition for three independent experiments.
Nd - not determined.

PP1γ_1 inhibition by $A\beta_{1-40}$ and $A\beta_{1-42}$ was very similarly (IC_{50} of 1.3±0.7μM and 1.9± 0.5μM, respectively), while inhibition by $A\beta_{25-35}$ was about two fold less potent (IC_{50} of 4.1±0.3μM). In contrast, the control peptide $A\beta_{40-1}$ was around 100-fold less potent than its $A\beta_{1-40}$ counterpart, and $A\beta_{SCR}$ yielded only a maximum of about 20% inhibition at 1mM (the highest concentration tested). Comparing the effect of the Aβ peptides on the two PP1 isoforms, the data obtained indicates that PP1γ_1 is approximately twice as sensitive to inhibition by all three Aβ peptides, compared to PP1α.

3.2. PP1 is relatively insensitive to inhibition by prion peptides

The accumulation in the brain of a pathological form of the normal cellular prion protein (PrP) is associated with the neurodegeneration of the transmissible spongiform encephalopathies. Fragments of PrP, like $Pr_{106-126}$ and $Pr_{118-135}$, can polymerise into amyloid-like fibrils [3,10]. In order to test the specificity of PP1 inhibition by Aβ, we determined the IC_{50} values for $Pr_{106-126}$ and $Pr_{118-135}$ against PP1γ_1 (Table 1). The IC_{50} values obtained for the prion peptides were approximately 100 times higher than that of the Aβ peptides, confirming the specificity of Aβ inhibition of PP1 activity.

3.3. Aβ inhibition of PP1 is not dependent on the substrate used

The PP1 catalytic subunit can dephosphorylate multiple substrates both *in vivo* and *in vitro*. In order to assess if the observed inhibitory effect was a substrate dependent phenomenon, we further compared the IC_{50} values of the Aβ peptides using MBP and phosphorylase *a*. Table 2 shows that PP1 inhibition by Aβ does not differ substantially when using different substrates. However, the use of a different lot (from the same supplier) of the $Aβ_{25-35}$ peptide yielded a slightly different IC_{50} value. Discrepancies between different lots of Aβ peptides have been widely reported [23], possibly related to their state of aggregation in solution.

Table 2. Comparison of PP1γ_1 inhibition by Aβ peptides using different substrates

	IC_{50} (μM)[a]	
Peptides	Phosphorylase *a*	MBP
$Aβ_{25-35}$	1.1 ± 0.1[b]	0.24 ± 0.18
$Aβ_{1-42}$	1.9 ± 0.5	6.5 ± 0.4

[a] Values are expressed as mean±S.E.M. of at least three independent experiments.
[b] This value was obtained using a different lot of Aβ peptide

Given that Aβ aggregation is thought to play a major role in AD development, this led us to investigate how this might affect Aβ inhibition of PP1 activity.

3.4. Aβ aggregation increases its potency against PP1

The IC_{50} values of the fibrillar forms of the peptides were compared to those obtained with their oligomeric forms, and the fold effect was calculated (Table 3).

Table 3. Aβ fibril formation increases its inhibitory potency against PP1

Peptide	Fold effect (Oligomer/Fibril)
$A\beta_{40-1}$	1
$A\beta_{1-40}$	7
$A\beta_{1-42}$	9

Each peptide was processed as described to form oligomers or fibrils and assayed against PP1γ_1. The fold effect of fibril formation on the corresponding IC_{50} values was determined graphically from at least three independent experiments.

Aggregation of $A\beta_{1-40}$ and $A\beta_{1-42}$ significantly augmented their potency against PP1γ_1, whereas at the tested peptide concentrations the aggregating conditions applied to $A\beta_{40-1}$ did not affect its PP1 inhibition curve significantly. Fibril formation of $A\beta_{1-40}$ and $A\beta_{1-42}$ decreased their IC50 against PP1γ_1 by approximately 7-fold and 9-fold, respectively (Table 3). Aggregating conditions were also tested for $Pr_{118-135}$ but, like for $A\beta_{40-1}$, no significant effect was observed on PP1γ_1 inhibition (data not shown), thus confirming the specificity of the effect for the Aβ peptide.

3.5. Effect of Aβ on phosphatase activity in PC12 cells

PC12 cells were exposed to $A\beta_{25-35}$ (20 and 50μM) for 2 and 24h, and PP1 and PP2A activities were determined (Fig. 1).

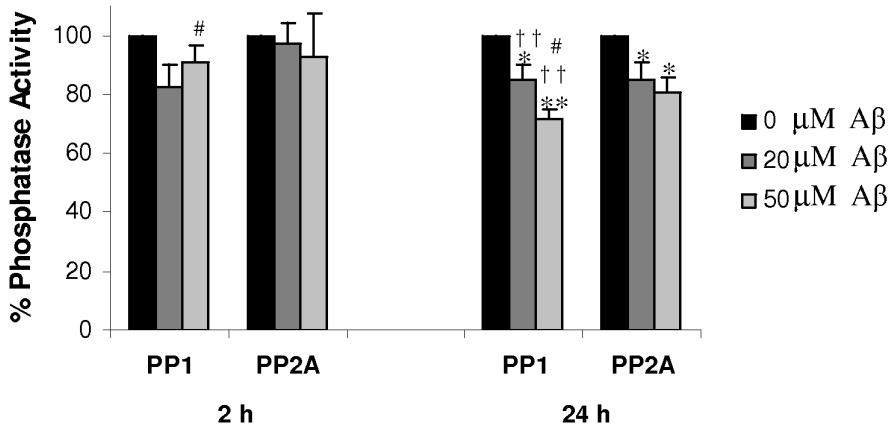

Fig. 1. Effect of $A\beta_{25-35}$ on PP1 and PP2A activities in PC12 cells. PC12 cells were incubated with 20 and 50μM $A\beta_{25-35}$ for 2 and 24h and the respective homogenates were assayed as described using ^{32}P-phosphorylase *a*. The values obtained from control cells incubated without Aβ were taken as 100%. Values statistically different from control [*P*<0.05 (*) and *P*<0.01 (**)] and between themselves [*P*<0.02 (#) and *P*<0.01 (††)] are indicated.

Overall, in this cell line, PP1 activity was significantly more affected by Aβ exposure than PP2A activity. Statistically significant decreases in activity were observed at 24h exposure. Moreover, a time and concentration dependent decrease of PP1 activity was observed. In particular, a decrease in activity was seen with time of exposure with 50μM Aβ (*P*<0.05) and also with increasing Aβ concentrations at 24h (*P*<0.01). Therefore, this "*ex vivo*" data support the "*in vitro*" data, showing that Aβ is capable of exercising an inhibitory effect on PP1 activity.

4. DISCUSSION

Altered signal transduction is thought to contribute significantly to the development of neurodegenerative conditions like AD. Indeed, several studies have reported abnormal protein kinase [19] and protein phosphatase [16] activities in AD brains, compared to normal aged controls. Moreover, APP processing is a phosphorylation-dependent event [13], in which protein kinase C (PKC) and PP1 appear to be central players by affecting intracellular trafficking events [4]. Furthermore, a potential PKC phosphorylation site within the cytoplasmic domain of APP was shown to be phosphorylated *in vivo* [28]. Besides its neurotoxic actions, Aβ might also have a feedback regulatory role on APP processing. Previous studies have shown that Aβ activates various PKC isoforms in different cell lines [27,33]. Here we focussed on PP1, demonstrating that Aβ also directly affects phosphatase activity. Indeed, Aβ peptides inhibit different PP1 isoforms at low micromolar concentrations. As aggregation of Aβ appears to be important for its neurotoxic effects, we investigated how Aβ aggregation affects PP1 activity. Fibril formation of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ significantly increased their inhibitory potency against PP1. $PP1\gamma_1$ was inhibited at concentrations up to 9-fold lower compared to their oligomeric state, thus making the fibrillar forms even more potent phosphatase inhibitors. Interestingly, aggregation of prion peptides did not increase their inhibitory effect on PP1, demonstrating specificity for the Aβ peptide aggregation effect on PP1 activity. In PC12 cells, 24h incubation with Aβ also affected both PP1 and PP2A activities in a dose-dependent manner. These results, taken together with the fact that Aβ causes alterations in PP1 intracellular levels in mammalian cells [1], lead us to conclude that Aβ can interfere with normal neuronal signal transduction events. Thus, concomitant activation of PKC and inhibition of PP1 may explain the observed hyperphosphorylation of key proteins associated with Alzheimer's disease (i.e. APP and tau). However, since a direct link between altered phosphorylation processes and Aβ neurotoxicity remains to be conclusively established, our results provide interesting support for further investigation.

Nevertheless, given the critical role played by PP1 in LTD, age-related memory and learning and synaptic plasticity, it is not surprising that some of the neurotoxic effects of Aβ might be related to its direct effect on PP1. Indeed, a two-week continuous infusion of Aβ into the cerebral ventricles of adult rats leads to an impairment of learning and memory compared to control animals [25]. However, LTP impairment induced by Aβ

oligomers can be reversed by PP1 inhibition *in vitro* [20]. It is unclear how those observations might be related to our demonstration of PP1 inhibition by Aβ. Also, since APP phosphorylation affects its own processing [8] and PP1 modulates APP secretion [5], Aβ might also be affecting its own production through modulation of PP1 activity. Further, as PP1 is known to dephosphorylate tau [22], the reported Aβ oligomer induction of tau hyperphosphorylation [9] might occur via inhibition of PP1 activity. PP1 inhibition by Aβ may explain not only tau hyperphosphorylation in AD brains, but also the reported hyperphosphorylation of APP itself in those patients [21].

The contribution of Aβ to the development of AD may be related to physiological inhibition of PP1 activity and thus underlie age-related and/or AD-related biochemical abnormalities, given the ubiquitous expression and wide variety of physiological roles of PP1. Although it is unclear what concentration of Aβ peptides occur in the brain, the production of Aβ in intracellular sites where PP1 is abundant may be sufficient to upset the exquisite balance that controls cellular health and vitality, especially if Aβ production is increased due to age-related or disease-related factors. The potential usefulness of PP1 for diagnostic purposes needs to be investigated in AD patients, although it may prove more interesting as a therapeutic target. Since PP1 occurs in complexes with other proteins that confer upon it physiological specificity, the development of drugs targeting specific PP1 binding proteins involved in APP processing may constitute potential therapeutic agents for AD. Work is currently underway to identify such proteins.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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Chapter V

DISCUSSION

5.1 Aβ-MEDIATED EFFECTS ON APP PROCESSING AND TRAFFICKING

Aβ peptide is the major component of senile plaques (Glenner and Wong 1984), one of the principal hallmarks of AD. Although it exists physiologically (Haass et al. 1992; Haass et al. 1993), in AD it aggregates and deposits in specific brain regions (Arnold et al. 1991; Braak and Braak 1991), thus interfering with crucial neuronal processes. Several *in vitro* and *in vivo* studies have shown that Aβ fibril formation can trigger several neurodegenerative mechanisms, including cytoskeletal associated morphological alterations of neurites (Pike et al. 1992; Busciglio et al. 1995; Grace et al. 2002; Mendoza-Naranjo et al. 2007).

Aβ derives from the amyloidogenic processing of APP, hence factors contributing to altered APP processing may potentially lead to increased Aβ production. Aβ itself has been described to alter APP metabolism although controversy exists. While some authors suggest that Aβ affects APP processing/catabolism (Davis-Salinas et al. 1995; Yang et al. 1995; Schmitt et al. 1997; Carlson et al. 2000) others reported that it can also induce APP expression (Le et al. 1995; Moreno-Flores et al. 1998). Therefore, the first chapters of this work were aimed at characterizing these Aβ effects on APP processing/trafficking and expression in different cell types.

Inhibition of sAPP secretion due to altered cytoskeletal dynamics

Aβ-induced cellular responses were addressed using the biological active peptide $A\beta_{25-35}$, which shows similar properties to naturally occurring $A\beta_{1-40}$ and $A\beta_{1-42}$ (Pike et al. 1995; Xu et al. 2001a; Xu et al. 2001b; Liao et al. 2007) and thus represents a good experimental model to study Aβ-downstream molecular mechanisms that may be involved in AD. We addressed the effects of exogenously added Aβ on APP trafficking and metabolism in non-neuronal, neuronal-like and neuronal cells (Chapter II). Aβ was reported to be efficiently internalized (Saavedra et al. 2007; Nielsen et al. 2008; Yamada et al. 2008) thus making possible the induction of a set of intracellular responses. In non-neuronal COS-7 cells we showed that this peptide leads to intracellular sAPPα (isAPPα) retention, in agreement with a previous report by Carlson et al. (2000). Interestingly, and despite isAPP retention, an increase in extracellular sAPP secretion could also be

observed. We postulated that sAPP secretion could partially occur via an alternative endoplasmic reticulum (ER) secretory pathway. This was based on the observation that isAPP co-localized to the ER and that the latter maintained its integrity upon Aβ treatment. Concordant with this hypothesis, Shin et al. (2005) observed that sAPP deriving from an APP mutant with an ER-retrieval signal, did not follow the normal post-TGN vesicular secretory pathway in COS-7 cells. For this mutant, sAPP secretion was diminished but not absent, and sAPP appeared to be media secreted via the smooth ER, as deduced from strong sAPPα/ER co-localization. Further, data from our laboratory showed that an APP phosphomutant expressed in COS-7 cells, exhibited impaired vesicular secretion, but still produced and secreted normal levels of sAPPα (Rebelo et al. 2007).

Aβ-induced isAPP retention was also demonstrated for SH-SY5Y and PC12 cells and primary neuronal cultures. Nonetheless, differences were observed in the degree of extracellular sAPP secretion, with both PC12 and neuronal cells being particularly affected. Neuronal and even undifferentiated PC12 cells are well known models of highly regulated secretion, presenting not only a constitutive secretory vesicular pathway but also stimuli-sensitive vesicular secretion (Greene and Tischler 1976; Burgess and Kelly 1987; Martin and Grishanin 2003). In contrast, besides the constitutive vesicular pathway, no regulated vesicular secretion has been described for COS-7 cells, and in undifferentiated SH-SY5Y cells only residual regulated vesicular machinery appears to exist (Goodall et al. 1997). Thus it seems reasonable to deduce that, Aβ compromises the major APP/sAPP TGN-to-PM vesicular secretory pathway and that, at least for non-neuronal cells (i.e. COS-7), sAPP of Golgi/ER origin can be redirected to an alternative, usually less used ER-to-PM pathway. Hence, sAPP is still secreted. This shift does not occur in PC12 cells and primary cultures, for the reasons described above, and the net effect is a decrease in extracellular sAPP. These differential Aβ responses in neuronal and non-neuronal cells are of particular importance given that AD is a neurodegenerative disorder essentially affecting the brain and sparing most of the peripheral tissues. It is worth mentioning that sAPPα levels are decreased in cerebrospinal fluid (CSF) in AD patients (Colciaghi et al. 2002; Olsson et al. 2003), which is consistent with our data on primary cultures, that is isAPP retention and decreased sAPP secretion, in response to excess Aβ. Due to the sAPPα neurotrophic/neuroprotective putative functions (Turner

et al. 2003; Thornton et al. 2006), extracellular depletion of this fragment may represent a pathogenic mechanism by which Aβ leads to neurotoxicity, thus contributing to the neuronal degeneration and death observed in AD. Of note, Aβ and sAPPα have been described to have opposite effects in several cellular mechanisms. For instance, while Aβ was reported to inhibit glucose receptors exocytosis and glucose uptake as well as glutamate transport, these effects were attenuated by sAPPα (Mattson et al. 1999; Uemura and Greenlee 2001). Therefore, an imbalance in Aβ/sAPPα intracellular/extracellular levels may underlie alterations in fundamental survival processes, contributing to progressive neurodegeneration.

Interestingly, we associated isAPP retention in response to Aβ exposure, to vesicular-like densities in COS-7 cells. Further, for all cells, subcellular fractionation assays revealed that isAPP accumulation was predominantly associated with cytoskeleton fraction. Since vesicular trafficking has long been associated with the cytoskeleton network (Hamm-Alvarez and Sheetz 1998; Gross 2004; Lanzetti 2007; Nakata and Hirokawa 2007; Potokar et al. 2007), the data here presented corroborates the hypothesis that Aβ impairs vesicle motility along the cytoskeleton, and/or inhibits vesicle docking/fusion at the PM. Thus we observe isAPP production/retention within cytoskeletal-associated vesicular-like structures and subsequent inhibition of sAPP secretion.

In neurons, KLC-driven APP vesicular axonal transport has been reported, either by direct APP interaction with KLC (Kamal et al. 2000), or indirectly via complex formation with JIP-1 (Matsuda et al. 2003; Lazarov et al. 2005). Despite the controversy around the mode of interaction between APP and KLCs, the presence of isAPP in kinesin-associated vesicles in neuronal cultures (represented in Figure 1, page 256 and 257), supports KLC mediated axonal transport of APP. Interestingly, this data clearly shows that APP can be intracellularly cleaved giving rise to isAPP production, which due to hindered APP axonal transport accumulates in the cytoskeletal fraction.

Clearly, Aβ affects APP/sAPP balance. Since alterations in cytoskeleton dynamics may account for altered protein axonal transport and vesicular secretion, and even neurodegeneration, we analysed Aβ effects on both microtubule and actin networks.

Aβ led to an increase in F-actin polymerization for both PC12 cells and in primary neuronal hippocampal cultures. This is consistent with previous observations showing that Aβ can interfere with the actin network (Song et al. 2002; Hiruma et al. 2003; Mendoza-Naranjo et al. 2007) thus contributing to neurodegeneration (Maloney and Bamburg 2007). Possible underlying mechanisms may include increased F-actin polymerization in a Rac1/Cdc42 dependent way (Mendoza-Naranjo et al. 2007) or through p38 mitogen-activated protein kinase pathway (Song et al. 2002).

Interestingly, our results also showed that Aβ was interfering with the microtubule network as demonstrated by changes in α-tubulin acetylation, which is an indirect measure of microtubule stability (Black et al. 1989; Bloom 2004). In particular, neuronal hippocampal cells exhibited a marked decrease in α-tubulin acetylation along neurites, as shown by immunofluorescence analyses. For PC12 cells, we also observed a decrease and an apparent redistribution of α-tubulin acetylation in response to Aβ peptide exposure. Of note, a recent study showed that tubulin acetylation could play a key role in microtubule-based transport, by demonstrating that α-tubulin acetylation can influence the binding and the motility of the microtubule motor kinesin-1 (Reed et al. 2006). Additionally, Gardiner et al. (2007) hypothesized that increased α-tubulin acetylation was associated with increased transport along microtubules. It follows that, by decreasing α-tubulin acetylation and increasing F-actin polymerization Aβ is potentially decreasing sAPP vesicular transport/secretion.

To confirm that Aβ disrupts cytoskeleton dynamics/stability and consequently sAPP secretion, experiments were repeated in the presence of an actin depolymerizing drug (cytochalasin D, CytD) or by interfering with microtubule stability (taxol). In PC12 cells, the use of CytD led to a robust reversion on sAPP secretion when compared to Aβ treatment alone. Likewise CytD, the microtubule stabilization drug, could also partially reverse the Aβ-induced inhibition of sAPP secretion. These results suggested that, for PC12 cells, sAPP secretion is more dependent on actin than on the microtubule network. These differences, in terms of reversion of sAPP secretion, may be associated with cell specific cytoskeleton organization of the actin and tubulin networks. For PC12 cells the actin cytoskeleton is very important for both vesicular motility and exocytosis (Martin and Grishanin 2003; Lanzetti 2007).

In summary, these novel findings clear show that Aβ is affecting both networks (illustrated in Figure 1, page 256 and 257), since both drugs could partially reverse the impaired sAPP secretion. Therefore, the Aβ-mediated effects on cytoskeleton dynamics/networks can account for the alterations in APP vesicular transport and decreased sAPP secretion associated with the disease. Highly relevant is the report that Aβ-induced neurodegeneration could be prevented by microtubule stabilizing drugs (Michaelis et al. 1998; Michaelis et al. 2005; Seyb et al. 2006). These results together with our data support the potential use of drugs targeting cytoskeletal integrity/dynamics as therapeutic strategies to prevent/ameliorate failures in axonal transport and neurodegeneration characteristic of AD.

5.2 Aβ-MEDIATED EFFECTS ON APP EXPRESSION

As explained above, previous reports documented that Aβ can induce APP expression levels. However, as described in Chapter II, and for the cell lines we tested, the increased immunoreactive associated with antibodies such as 22C11 is related with increased isAPP. In particular for primary neuronal cultures, holoAPP levels decreased in response to Aβ, rather suggesting reduced APP transcription. Further, APP has been described to be processed by "regulated intramembrane proteolysis" (RIP), giving rise to an active fragment, the APP intracellular domain (AICD), which plays a role in the regulation of nuclear signaling events. Hence, it became critical to address how Aβ affected APP expression levels, AICD production and nuclear targeting in primary neuronal cultures (Chapter III).

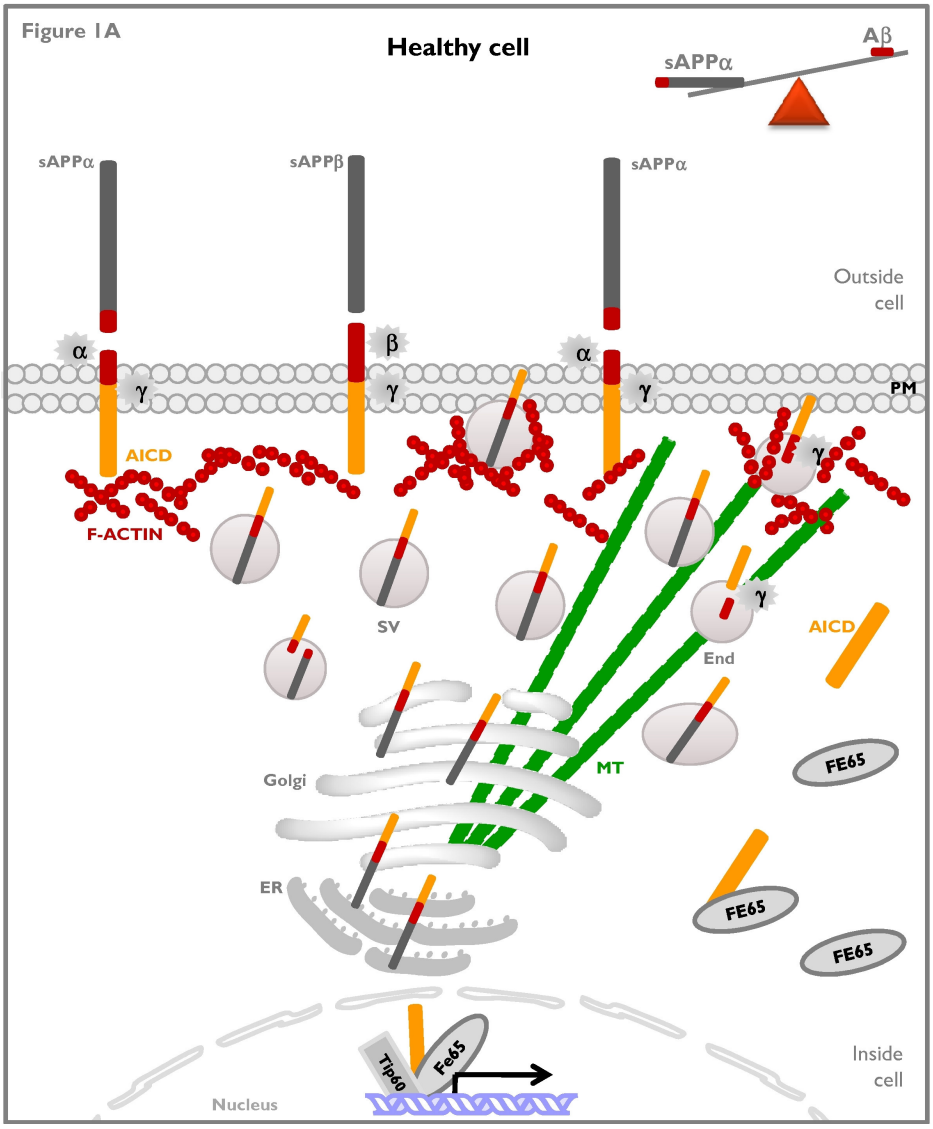
Regulation of AICD and Fe65 nuclear targeting

The AICD provides a mechanism by which APP can regulate its own expression. This APP proteolytic fragment is nuclear targeted and exhibits transcriptional activity, being a component of transcriptional active complexes such as the one comprising AICD, Fe65 and the histone acetylase Tip60 (Cao and Sudhof 2001). Several lines of evidence indicated that the adaptor protein Fe65 modulates the stability of AICD, thereby potentiating its subsequent nuclear translocation (Kimberly et al. 2001; Kinoshita et al.

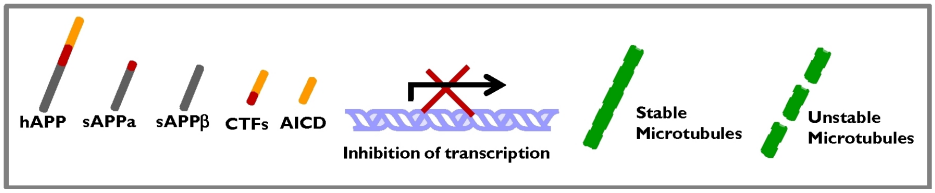
2002; Walsh et al. 2003). Nuclear AICD/Fe65-containing complexes were reported to activate the transcription of several genes, including *APP* itself, *BACE*, *Tip60* (von Rotz et al. 2004), *GSK3β* (Kim et al. 2003; Ryan and Pimplikar 2005), *KAI1* (Baek et al. 2002) and *Neprilysin* (Pardossi-Piquard et al. 2005).

Neuronal data obtained in Chapter II, led us to hypothesize that besides having an effect on APP trafficking/processing, Aβ could also have consequences on APP transcriptional activity. Indeed, a decrease in hAPP intracellular levels in Aβ-treated neuronal cells was observed, suggesting decreased APP mRNA expression. Subsequent experiments revealed that Aβ was in fact inducing a decrease in neuronal APP expression. Additionally, at the processing level we observed an increase in APP CTFs implying decreased AICD production. Interestingly, CTF accumulation is consistent with APP retention in intracellular vesicles that do not reach the PM. γ-secretase cleavage has been shown to occur mainly in endosomes and also at the PM (Fukumori et al. 2006; Kaether et al. 2006). If APP/CTFs are not reaching the PM, then subsequent Aβ and AICD production is expected to decrease. Diminished AICD production correlates well with decreased AICD nuclear targeting and reduced APP transcriptional activation. Consistently, we detected a decrease in the nuclear targeting of both AICD and Fe65, along with a decrease in the AICD/Fe65 nuclear complexes. Down-regulation of Fe65 was clearly evident, which is in agreement with previous observations that Fe65 affects APP processing. A report by Xie et al. (2007) showing that RNAi of Fe65 leads to increased CTFs accumulation is also consistent with our data. Further, Fe65 overexpression was associated with increased APP translocation to the cell surface, as well as increased sAPPα and Aβ secretion (Sabo et al., 1999). Hence, reduced Fe65 levels may account at least in part for the intracellular retention of APP/sAPP. At the transcriptional activation level, the results suggest that Aβ is affecting AICD production, its nuclear translocation and its nuclear complex formation with Fe65, whose nuclear targeting is also decreased. A potential decrease in AICD production and in the formation of AICD/Fe65 transactivator complexes lead us to conclude that APP nuclear signaling is altered in the presence of Aβ, leading to impaired specific gene transcription (illustrated in the following Figure). This effect on APP/Fe65 nuclear targeting is of particular importance, placing Aβ as a pivotal regulator of its own production.

Given the data discussed above for APP metabolism and trafficking in reponse to Aβ, a putative model for neuronal cultures was schemed in Figure 1A, representing basal conditions, and in Figure 1B integrating Aβ-induced alterations.



Symbol identity



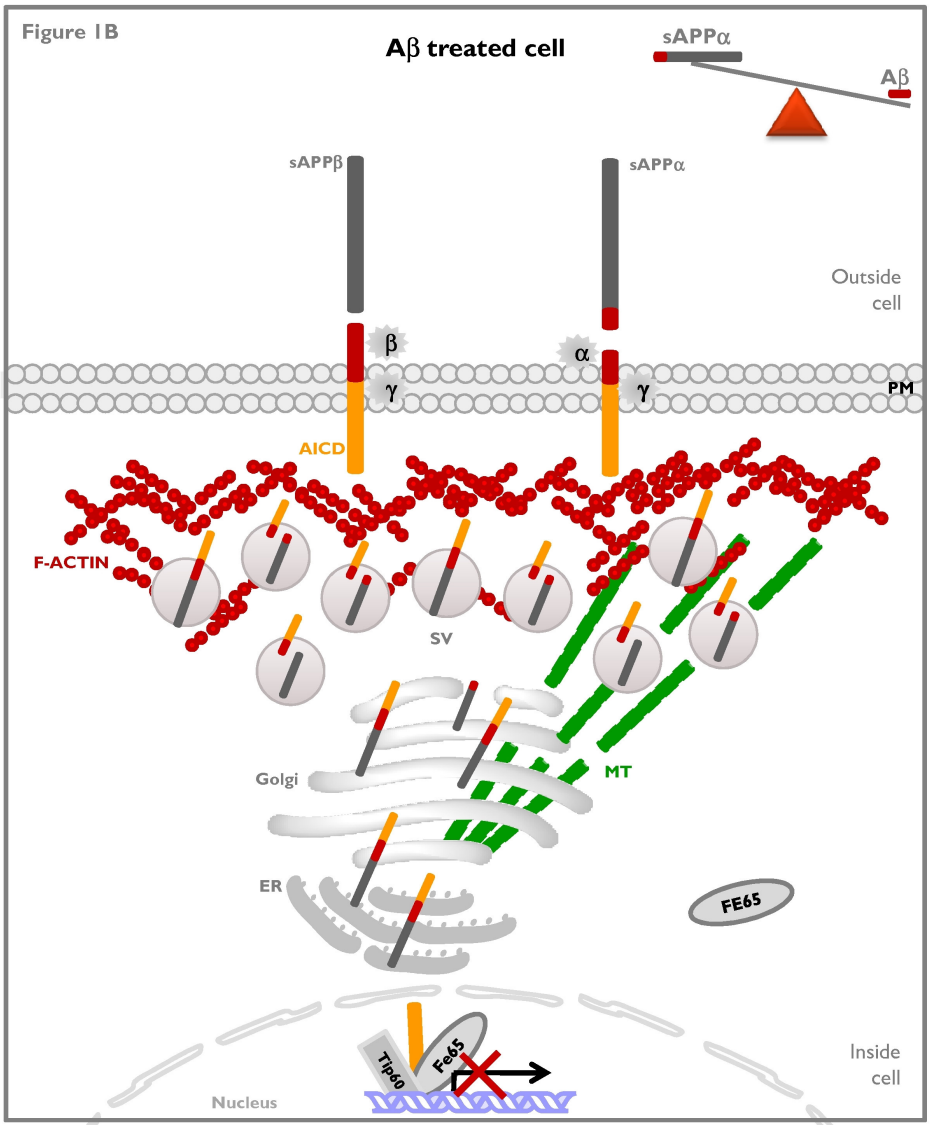


Figure 1. Putative model for Aβ–induced responses in primary neuronal cultures. A. Under basal conditions APP follows the normal and well described TGN-to-PM secretory pathway. At the cell surface APP can be proteolytic cleaved, mainly by α-secretase pathway giving rise to sAPPα production and secretion The latter is described to be neurotrophic and neuroprotective, and to be able to counteract Aβ toxicity. At the PM or upon internalization via the endocytic pathway, APP/CTFs can be cleaved by γ-secretase, giving rise to AICD and Aβ production. B. Upon Aβ exposure, abnormal Aβ-induced alterations of cytoskeleton networks occurs, i.e. increased F-actin polymerization and decreased α-tubulin acetylation (resulting in microtubule destability - represented by fragmented microtubule). These alterations on cytoskeleton dynamics lead to impaired APP vesicular trafficking and targeting. Consequently, sAPP and CTFs are intracellularly produced and accumulated in secretory vesicles, that do not reach PM. Decreased AICD production will hinder nuclear targeting, and potential inhibition of extracellular sAPPα secretion will contribute to progressive neurodegeneration. sAPPα was not directly measured in neurons, but this pathway is the most abundant under physiological conditions. Further, sAPPα was shown in the work herein presented to be highly retained in non-neuronal cells. PM, Plasma membrane; SV, secretory vesicles; End, endocytic vesicles; ER, endoplasmic reticulum; MT, microtubule network.

5.3 THERAPEUTIC APPROACHES TARGETING Aβ-MEDIATED EFFECTS

In the last decades, the treatment of AD patients focused mainly on symptomatical strategies. Nonetheless, improved knowledge of the mechanisms underlying AD pathology, in particularly the molecular causes and consequences of the disease, contribute to the development of novel therapeutic approaches. Considering that AD is a multifactorial disorder it is likely that combined therapeutic approaches will be most effective in future strategies for AD treatment.

Early evidence indicated that fibrillar aggregates of Aβ are neurotoxic (Lorenzo and Yankner 1994; Grace et al. 2002). Nonetheless, other reports demonstrated that Aβ soluble oligomers and protofibrils are also neurotoxic and potentially a major cause of neurodegeneration in AD (Hartley et al. 1999; Chimon et al. 2007; Cerpa et al. 2008). Eventhough the neurotoxicity, with respect to the different forms of Aβ is controversial, effective therapeutics are being directed towards both oligomeric and fibrillar species of Aβ. Several novel therapeutic strategies are based in decreasing or inhibiting Aβ production, stimulating the clearance of aggregated Aβ or preventing the aggregation of Aβ into amyloid plaques. All these interventions congregate to find drugs that are effective in lowering Aβ levels in the brain, thus preventing Aβ-induced neurodegeneration.

Prevention of isAPP retention by inhibiting Aβ fibril formation

Due to the importance of Aβ aggregation on its neurotoxicity, in Chapter IV we evaluated the effect of Aβ aggregation on isAPP retention. Our data demonstrated that 10 μM of Aβ aggregated, results in higher isAPP retention than 20 μM of freshly added Aβ (4.5 and 2 fold, respectively). These data are consistent with previous observations where Aβ neurotoxicity is related to its degree of fibrillization, the β-sheet structure and the size of the peptides (Lorenzo and Yankner 1994; Grace et al. 2002).

The molecular mechanisms responsible for the passage of normal soluble Aβ forms to fibrils are not well understood, but it is known that Aβ binds several proteins that can modulate its aggregation or fibril state (Table 2, Chapter I).

Since Aβ aggregation and/or clearance may be dependent on its interacting proteins we evaluated if Aβ effects could be quenched by Aβ binding proteins - involved either in Aβ disaggregation or clearance. Among these are laminin and gelsolin, two proteins known to be involved either in preventing Aβ fibrillogenesis or in promoting disaggregation and clearance (Ray et al. 2000; Morgan et al. 2002). Consistent with previous observations, preceding Aβ co-incubation with these proteins led to a decrease in the aggregation state of the peptide, as revealed by the TH-T assay. Subsequent addition of Aβ-complexes to primary neuronal cultures lead to a reduced accumulation of isAPP when compared to aggregated Aβ alone. This is in agreement with the capacity of these proteins to bind Aβ and promote disaggregation or decrease Aβ levels. Further, the preformed laminin- or gelsolin-Aβ complexes might be inhibiting Aβ signaling and/or be intracellularly targeted for degradation, therefore preventing Aβ-induced isAPP retention. In agreement with the last hypothesis, peripheral expression of plasma gelsolin in an AD transgenic mouse model lead to reduced Aβ load in the brain (Hirko et al. 2007). Of note, the decreased intracellular sAPP accumulation in the presence of these proteins was accompanied by a slight decrease in neuronal apoptotic levels, indicative of the anti-apoptotic capacity of these Aβ binding proteins.

At the secretory level, laminin was able to marginally increase sAPP extracellular secretion, thus partially reversing the Aβ inhibitory effect. Nonetheless, this reversion did not reach control levels. Laminin was previously shown to decrease APP secretion and to affect intracellular APP biogenesis and catabolism (Monning et al. 1995; Bronfman et al. 1996). Likewise, gelsolin treatment also leads to a decrease in medium secreted sAPP. Another possible explanation for the diminished sAPP secretion even in the presence of these proteins, may be due to non-complexed Aβ, which is still available to block sAPP trafficking/secretion, particularly if laminin and gelsolin concentration are limiting factors. It is worth mentioning that the metabolism and clearance of both Aβ binding proteins is unclear, making it difficult to interpret the differences in extracellular and intracellular sAPP levels. Nonetheless the data here discussed points to a therapeutic potential for these proteins in terms of modulating Aβ induced effects on APP metabolism and subsequent neurotoxicity.

PP1 as a potential therapeutic target for Aβ-induced responses

Of particular interest is the observation that altered signal transduction has been linked to the development of many neurodegenerative disorders, such as AD. Alterations in signal transduction cascades may be related to abnormal balance of protein kinases and phosphatases, which are key players in the reversible phosphorylation mechanism, involved in many cellular regulatory processes. Accordingly, abnormal activities of these proteins have been reported in AD brains (Gong et al. 1993; Jin and Saitoh 1995) when compared to normal aged controls. Of note, is PP1, a Ser/Thr-phosphatase widely expressed in adult mammalian brain both in neurons and glia (da Cruz e Silva et al. 1995b). Although ubiquitously expressed through the periphery, the three known catalytic subunits (PP1α, PP1β and PP1γ) are particularly abundant in mammalian brain, sustaining the importance of PP1 in brain function. Moreover, the cognitive decline observed in AD is thought to be related to the decrease in synaptic contacts (DeKosky and Scheff 1990; Terry et al. 1991), where PP1 plays a role since it is highly enriched in post-synaptic dendritic spines (Ouimet et al. 1995). PP1 was also shown to be involved in the control of LTD and synaptic plasticity (Morishita et al. 2001), as well as in aging-related memory defects (Genoux et al. 2002), thereby influencing learning and memory. This places PP1 at the center of neuronal signaling cascades. Furthermore, many AD pathogenic alterations may be associated with abnormal phosphorylation events. For instance, abnormal Tau phosphorylation involves decreases in protein phosphatase 1 (PP1) and phosphatase 2A (PP2A) activities (Bennecib et al. 2000; Liu et al. 2005). Moreover, APP processing can also be modulated by specific protein phosphatase inhibitors, resulting in increased sAPP secretion (da Cruz e Silva et al. 1995a). Thus protein phosphatases can modulate APP processing and consequent Aβ production. Adding complexity to the signaling cascades, Aβ was previously shown to induce alterations in PP1 intracellular levels (Amador et al. 2004), suggesting that Aβ can interfere with normal neuronal signaling events. Recently, Aβ oligomers were also shown to induce Tau hyperphosphorylation in hippocampal neurons (De Felice et al. 2008).

The abnormal phosphorylation of key proteins linked to AD and the possibility that Aβ mediated effects on APP metabolism may be associated with abnormal phosphorylation lead us to investigate the direct effect of this peptide on PP1. Our studies confirm the existence of an inhibitory effect on different PP isoforms directly provoked by Aβ both *in vitro* and *in vivo*. Additionally, and since aggregation of Aβ appears to be important for its neurotoxic properties, we investigated how Aβ aggregation affects PP1 activity. Fibril forms of Aβ peptides increased their inhibitory effect on PP1 activity. Hence, the abnormal production of Aβ in intracellular sites where PP1 is abundant may be sufficient to alter the equilibrium of phosphatase activities and lead to a set of abnormal signal transduction pathways. It therefore appears reasonable to conclude that PP1 may constitute an important physiological target for Aβ, and that physiological inhibition of PP1 activity by Aβ is likely to contribute to progressive neurodegeneration characteristic of AD. Nonetheless, since PP1 occurs in complexes with other proteins that confer it physiological specificity, the development of drugs targeting specific PP1 binding proteins, might constitute potential therapeutic agents for AD.

CONCLUDING REMARKS

The study of Aβ, often viewed as a neurotoxic peptide and the central culprit in AD, may in fact hold some important leads in terms of AD diagnostic and therapeutic advances. The work here described identified putative approaches for intervention. Defining, for the first time, isAPP retention at cytoskeletal-associated vesicles as a response to Aβ exposure brings the former proteolytic fragment to the centre stage of biomarkers in AD. The same experimental data set substantiates future efforts directed at finding putative therapeutic strategies in AD. These include identification of cytoskeletal–associated and Aβ-clearance drugs, which may modulate Aβ downstream effects.

The work carried out in this project also offers a molecular basis which may link the two neuropathological hallmarks in AD. Aβ can alter APP processing but can also affect signal transduction cascades; namely by interfering with protein phosphorylation processes. In the previous sections the consequences on APP metabolism was discussed, but Tau is phosphorylated in AD. Aβ via its effects on protein phosphatases may be a contributing factor to this hyperphosphorylation state, this deserves further investigation. Nonetheless, Aβ has the potential to be a common factor of pathological relevance which can contribute to the formation of amyloid plaques and NFTs.

In closing, it has been rewarding to acknowledge that molecular and cellular studies are of direct relevance to Alzheimer´s disease, and provide the basis for future strategies in the fight against this debilititating disease.

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Appendix

Appendix I

METHODS AND KITS

(The medium and solutions composition used for the different techniques are listed on Appendix II).

1. CELL CULTURE AND RAT PRIMARY CULTURES

Cell culture maintenance

Non-neuronal COS-7 cells (a monkey kidney cell line) were grown in complete DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco).

Neuronal-like PC12 cells (a rat pheochromocytoma cell line) were grown in RPMI 1640 (Gibco) supplemented with 10% horse serum and 5% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco).

SH-SY5Y cells (a human neuroblastoma cell line) were grown in a 1:1 combination of minimum essential medium (MEM, Gibco) and Ham's F12 medim (Gibco), supplemented with 10% FBS.

All cultures were plated in 100 mm diameter plates or 6-well plates (35 mm diameter), and grown in a humidified incubator at 37 °C and 5% CO_2. Cells were subcultured whenever ~95% confluence was reached.

Rat cortical/hippocampal primary cultures

Rat cortical neurons were isolated from cortex or hipoccampus of Wistar Hannover 18 days rat embryos whose mother was killed by rapid cervical dislocation. After brain dissection, tissues were dissociated with trypsin (0.45 mg/ml for cortical cultures or 0.75 mg/ml for hippocampal cultures) and deoxyribonuclease I (0.15 mg/ml) in Hank's balanced salt solution (HBSS), supplemented with BSA (Merck), during 5-10 min at 37°C. Cells were washed with HBSS supplemented with 10% FBS to stop trypsinization, centrifuged at 1,000 rpm for 3 min, and further washed and centrifuged with HBSS for serum withdraw.

Cells pellet was ressuspended in complete Neurobasal medium, which is supplemented with 2% B27. Viability and cellular concentration were assessed by using the Trypan Blue excluding dye [0.4% Trypan Blue solution (Sigma)], and cells with (dead) or without (living) intracellular blue staining were counted in a hemocytometer chamber. Cellular viability was calculated and normally higher than 95%. These neuronal cells were plated onto 100 μg/ml poly-D-lysine pre-coated glass coverslips at a density of 1.0 x 10^5 cells/cm^2. Cells were maintained in 2 ml of complete Neurobasal medium and three and seven days after plating, 500 μl of cultured medium was replaced with 500 μl of fresh medium. Cultures were maintained in an atmosphere of 5% CO_2 at 37°C for 9 days, before being used for experimental purposes.

Culture cells fixation and immunocytochemistry

Fixation

COS-7 and neuronal primary cells were grown in 1M HCl pre-treated glass coverslips pre-coated with 100 μg/ml poly-L-ornithine or poly-D-lysine, respectively. After the experimental procedures, cells were washed three times with 1 ml of the serum-free DMEM, after which 1 ml of a 1:1 DMEM/4% paraformaldehyde fixative solution was gently added and allowed to stand for 1-2 min. Subsequently, 1 ml of fixative solution was gently added for 25 minutes. Finally, cells were washed 3 times with PBS for 10 min, being ready for immunocytochemistry procedures or to be directly mounted on glass microscope slides for confocal microscopy analysis.

Immunocytochemistry

For immunocytochemistry procedures, a permeabilization step with methanol was taken (2 min at RT) and cells were immediately washed four times with PBS. Afterward, cells were incubated with primary antibody diluted in 3% BSA in PBS for 2-4 h at RT. The primary antibody was removed by washing the coverslips 3 times with PBS and a secondary antibody (also diluted in 3% BSA in PBS) was added for 2 h at RT. After washing 3 times with PBS the coverslips were mounted with one drop of the antifading reagent on a glass slide.

Labbeling of F-actin

Following paraformaldehyde fixation cells were permeabilized with a solution of acetone at ≤-20 °C for 3 min. After that, cells were washed 2 times with PBS and then incubated with PBS containing 1% BSA for 20 min. This blocking solution is removed and pallotoxin staining solution is then added to cells (1.5 U/100 μl in PBS containing 1% BSA) for 30 min at RT. After washing 3 times with PBS were mounted with one drop of the antifading reagent on a glass slide.

2. PROTEINS MANIPULATION

Protein assay kit (BCA, Pierce)

Samples total protein measurements were performed with Pierce's BCA protein assay kit, following the manufacturer's instructions. The method combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the biuret reaction) with a sensitive colorimetric detection of the Cu^{+} cation using a reagent containing bicinchoninic acid (BCA). The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one Cu^{+} ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration over a working range of 20 μg/ml to 2000 μg/ml.

Working Reagent (W.R.)

The W.R. was prepared by mixing X ml of BCA reagent A with Y ml of BCA reagent B in the proportion of 50:1.

Samples preparation

A microtube per sample was prepared to be assayed with 25 μl of each sample plus 25 μl of the solution in which the sample was collected (1% SDS).

Standard curve

Microtubes with standard protein concentrations were prepared as described below (Table 1).

Table 1 – Standards used in the BCA protein assay method. BSA, Bovine serum albumin solution (2 mg/ml).

Standard	BSA (µl)	10% SDS (µl)	H_2O (µl)	Protein mass (µg)	W.R. (ml)
P_0	-	5	45	0	1
P_1	1	5	44	2	1
P_2	2	5	43	4	1
P_3	5	5	40	10	1
P_4	10	5	35	20	1
P_5	20	5	25	40	1
P_6	40	5	5	80	1

Incubation and absorbance measurement

1 ml of W.R. was rapidly added to each microtube (standards and samples) and the microtubes were incubated at 37 °C exactly for 30 min. Tubes cool to RT and immediately measure their absorbance at 562 nm.

Samples concentration

A standard curve is prepared by plotting BSA standard absorbance vs. BSA concentration, and used to determine the total protein concentration of each sample.

SDS-PAGE (for Western blotting)

SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations were carried out using well established methods (Laemmli, 1970), where proteins are separated by their molecular weight and negative net charge due to SDS-amino acid binding. The gels percentage and size chosen depend on the molecular weight of the proteins to be separated in the gel. Gels were prepared by mixing several components (Appendix I). The resolving gel solution was immediately and carefully pipetted down the spacer into the gel sandwich, leaving free space for the stacking gel. Water was carefully added to cover the top of the gel and the gel was allowed to polymerize for 1 h. Stacking gel solution was prepared according to Appendix I. The water was poured out and the stacking gel was added to the gel sandwich; a comb was inserted and the gel allowed to polymerize for 1 h. In parallel, samples were prepared by adding to the protein sample solution ¼ volume of 4X LB (Loading Buffer). Samples microtubes were boiled and spinned down, the combs removed and the gels wells filled with Tris-Glycine running buffer. The samples were carefully loaded into the wells, and electrophoretically separated using a 90 mA electric current. Molecular weight markers (Kaleidoscope Prestained Standards or Prestained SDS-PAGE Standards – Broad Range, Bio Rad) were also loaded and resolved side-by-side with the samples.

Proteins electrotransfer

Through the Western Blotting technique, proteins that were electrophoretically separated by SDS-PAGE can be transferred to membranes (nitrocellulose membranes, for instance), while keeping their positions. 3MM blotter papers and a nitrocellulose membrane were used to build up the transfer sandwich. The gel was removed from the electrophoresis device and the stacking gel discarded. A transfer sandwich was assembled under transfer buffer, in the following order: sponge, 3MM blotter paper, gel, nitrocellulose membrane, 3MM paper, sponge. The cassette was placed in the transfer device, previously filled with transfer buffer, oriented so that the negatively charged proteins migrate towards the anode. Electrotransfer was allowed to proceed for 18 h at 200 mA, after what the membrane was allowed to dry on a clean paper.

Immunoblot analysis

After proteins electrotransfer, the nitrocellulose membranes could be used immediately, and membranes were initially soaped in 1X TBS for 10 min. Blocking of possible non-specific binding sites of the primary antibody was performed by immersing the membrane in 5% (w/v) non-fat dry milk in 1X TBST solution for 1-4 h. Further incubation with primary antibody was carried out for the specified times, ranging from 2 h to overnight incubation at 4 °C with agitation. After three washes with 1X TBS-T, of 10 min each, the membrane was further incubated with the appropriate secondary antibody for 2 h with agitation. All primary and secondary antibodies used were diluted in 1X TBS-T/non-fat dry milk (3% w/v). Membranes were additional washed three times with 1X TBST, before being submitted to one of the following detection methods:

Enhanced chemiluminescence detection (ECL and ECL plus Kits, Amersham Pharmacia)

ECL™ Western blotting from Amersham Pharmacia Biotech is a light emitting non-radioactive method for detection of immobilised specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies (Fig. 1).

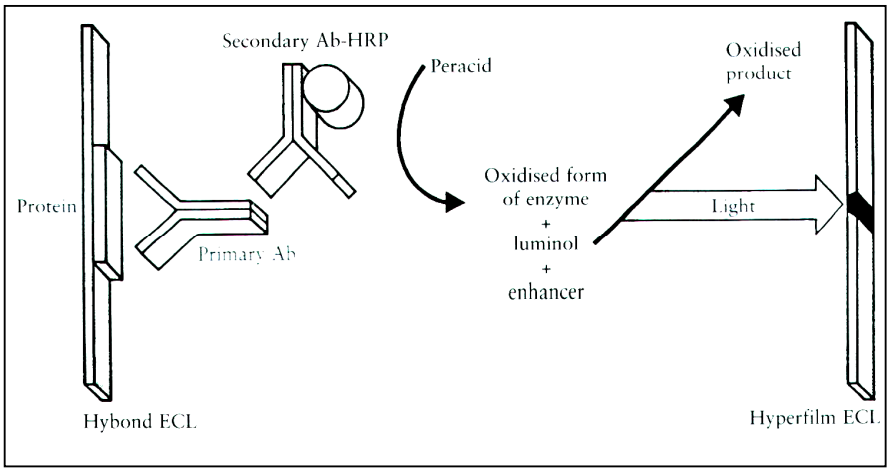

Fig. 1. The ECL analysis system detects the presence of an antibody labelled with horseradish peroxidase by catalysing the oxidation of luminol, leading to the emission of light, which can be detected by an autoradiography film (From the manufacturer datasheet).

The membrane was incubated for 1 min at RT with the ECL detection solution or for 5 min with the ECL+ detection solution. These solutions were prepared fresh following the manufacturer's instructions. ECL/ECL+ detection solution in excess was drained by touching the edge of the membrane against tissue paper and the membrane was gently wrapped in cling-film, eliminating all the air bubbles. In a dark room, an autoradiography film (XAR-5 film, Kodak, Sigma Aldrich) was placed on the top of the membrane, inside a film cassette. The cassette was closed and the blot exposed for an appropriate period of time. The film was then removed and developed in a developing solution (Kodak, Sigma Aldrich), washed in water, and fixed in a fixing solution (Kodak, Sigma Aldrich).

Table 1. List of the antibodies used and specific dilutions for each assay.

Target Protein	Primary Antibody	Assay/Dilution
APP/sAPP	22C11 (Boehringer)	**WB/1:250** **IF/ 1:50**
APP/CTFs	Anti-β-APP (Zymed)	**WB/1µg/ml** **IF/ 2.5µg/ml**
APP/sAPPα/Aβ	6E10 (Sigma)	**WB/1:1000** **IF/ 1:200**
$APP_{751/770}$/$sAPP_{751/770}$	KPI (Chemicon)	**WB/1:1000** **IF/ 1:200**
APP/CTFα/Aβ	4G8 (Chemicon)	**WB/1:1000** **IF/ 1:200**
Actin	Actin (Stressgen)	**WB/1:1000**
ER	Calnexin (Stressgen)	**WB/1:500** **IF/1:200**
Fe65	Fe65, clone 3H6 (Upstate)	**WB/1:5000** **IF/1:500**
GSk3 total	Glycogen synthase kinase 3 (Chemicon)	**WB/1:1000**
Histone 4	Histone H4 (Santa Cruz Biotechnology)	**WB/1:200**
HSP70	HSP70 (Stressgen)	**WB/1:10000**
KLC	KLC (Santa Cruz Biotechnology)	**WB/1:1000** **IF/ 1:200**
Pan- cadherin	Pan-cadherin (Abcam)	**WB/1:200**
Rab5	Rab5 (Stressgen)	**IF/1:400**
Syntaxin 6	Syntaxin 6 (BD Biosciences)	**WB/1:350**
Acetylated α-Tubulin	Acetyated α-tubulin (Zymed)	**WB/1µg/ml** **IF/1µg/ml**
β-tubulin	β-tubulin (Zymed)	**WB/1:5000**

WB - Western blotting

IF - Immunofluorescence

Subcellular fractionation

For subcellular fractionation the Calbiochem ProteoExtract Subcellular Proteome Extraction Kit was used. It allows subcellular extraction of proteins according to their subcellular localization, i.e. from the cytosolic, organelle and membrane, nuclear and cytoskeletal fractions. It takes advantage of the differential solubility of proteins in different subcellular compartments and utilizes highly specialized extraction buffers to target specific subcellular compartments and simultaneously preserve the structural integrity of the proteins before and during each sequential extraction.

As the extraction buffers contain components that might interfere with protein quantification, each fraction was overnight precipitated with acetone, centrifuged 15 min at 15 000 g, ressuspended in SDS1% and normalized for protein content using BCA assay.

Protein silver stain

Silver staining is one of the procedures available for detecting proteins separated by gel electrophoresis. Switzer et al., introduced silver staining in 1979, a technique that today provides a very sensitive tool for protein visualization with a detection level down to the 0.3-10 ng level (Switzer et al. 1979). The basic mechanisms underlying silver staining of proteins in gels are relatively well understood. Basically, protein detection depends on the binding of silver ions to the amino acid side chains, primary the sulfhydril and carboxiyl groups of proteins (Switzer et al. 1979; Oakley et al. 1980; Merril et al. 1981; Merril et al. 1986), followed by reduction to free metallic silver (Rabilloud, 1990; Rabilloud, 1999). The proteins are visualized as bands where the reduction occurs and, as a result, the image of protein distribution within the gel is based on the difference in oxidation-reduction potential between the gel's area occupied by proteins and the free adjacent sites.

Protein silver was performed according to the protocol adapted from Blum et al. (1987). This protocol stain is useful for protein concentrations ranging from <1ug to >1ng.

Gel staining protocol: Everything Fresh!

	solution		V = 100 ml	operation	time
1	sol A	50% methanol 5% acetic acid		fix	30 min
2	sol B	50% methanol		incubate	15 min
3		milli-Q H_2O		wash 5 times	5 x 5 min
4	sol. C	sodiumthiosulphate ($Na_2S_2O3.5H_2O$)	0.2 g/L fresh!	Incubate	60 sec
5		milli-Q H_2O		wash 2 times	2 x 60 sec
6	sol D	silver nitrate (chilled to 4C) ($AgNO_3$)	0.2 g/100 ml	Incubate	25 min
7		milli-Q H_2O		wash 2 times	2 x 60 sec
8	sol E	sodium carbonate anhydrous (Na_2CO_3) 37% HCOH	3 g/100 ml 25ul/100ml	develop	max 10 min
9	sol F	Na_2-EDTA	(14g / L)	stop develop	10 min
10		milli-Q H_2O		wash	2X1min

3. MEASUREMENT OF PROTEIN PHOSPHATASE ACTIVITY

Sample and Substrate preparation

Peptide stock solutions were diluted in inhibitor buffer, just before use. PP1 catalytic subunits were diluted in phosphatase buffer.

"In vitro" assay

Effects of all peptides on purified PP1 isoforms activity were assessed using ^{32}P-phosphorylase *a* and (myelin basic protein) MyBP as substrates. The preparation of substrate and the assays using ^{32}P-MyBP were done as described by the phosphatase kit supplier (Neb). ^{32}P-phosphorylase *a* was prepared from phosphorylase *b* using [γ-^{32}P]ATP and phosphorylase kinase as previously described in MacKintosh reference.

PP1 catalytic subunits were incubated with previously prepared peptides for 5 min at 30 °C. The reaction started with the addition of ^{32}P-phosphorylase *a* (3 mg/ml) to a final volume of 30 µl. After 10 min, 100 µl of ice-cold 20% (w/v) TCA were added and the mixture centrifuged at 12000 g for 2 min at room temperature. The ^{32}P-phosphate released into the supernatant was measured in a scintillation counter. The control phosphatase activity was 10-20% of total substrate radioactivity to ensure linearity. For each peptide, an appropriate range of concentrations was used, and the IC_{50} calculated using the BioDataFit 1.02 software.

"In vivo" assay

PC12 cells were plated in poly-L-ornithine coated dishes at a density of 5x10^{5}cells/cm^2 and washed twice with serum free medium prior $A\beta_{25-35}$ treatments. Cells were exposed to 20 or 50 µM Aβ during 2 or 24 h in serum-free medium. After incubation cells were washed twice in cold Tris-HCl/EDTA buffer and ressuspended in homogenizing buffer. The total phosphatase activity of the control and Aβ-pre-incubated cell extracts was determined by reacting the appropriate dilution in phosphatase buffer with ^{32}P-phosphorylase *a*. The PP2A activity was determined by pre-incubating the same dilution of cell extracts with 200 nM of I_2 for 15 min at 30 °C before adding the substrate. The PP1 activity in the cell extracts was calculated as the total phosphatase activity minus the PP2A activity towards ^{32}P-phosphorylase *a*.

4. RNA EXTRACTION

Solutions/plastic material used for this RNA extraction was done/treated with Nuclease-Free Water (DEPC- treated H_20).

Isolation of total RNA from monolayer cells

Total RNA extraction was performed according to TRI Reagent™ (Sigma) protocol. COS-7 cells were plated in the day before RNA extraction at a density of (2.0x10^6 cells). For primary neuronal cultures cells were plated at a density of (3.0x10^6 cells). Following

extraction samples were used either for analysis of APP expression levels (Northern blot) or for analysis of the APP isoform pattern (RT-PCR).

Sample preparation

Cells were directly lysated on the culture dish, using 1 ml of the TRI REAGENT per 10 cm^2 of glass culture plate surface area. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. After homogenization, homogenate is centrifuged at 12.000 g for 10 min at 4ºC to remove insoluble material (extracellular membranes, polysaccharides, and high molecular weight DNA). The supernatant contains RNA and protein. The clear supernatant was transferred to a fresh tube. To ensure complete dissociation of nucleoprotein complexes, samples stand for 5 minutes at room temperature, and then 0.2 ml of chloroform per ml of TRI REAGENT was added. After, shake vigorously for 15 seconds and allow to stand for 2-15 min at room temperature, samples were centriguged at 12.000 g for 15 min at 4 °C. Centrigugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).

RNA Isolation

The aqueous phase was then transferred to a fresh tube and added 0.5 ml of isopropanol per ml of TRI REAGENT (used in Sample Preparation). After mixing, samples stand for 5-10 minutes at room temperature, and were then centrifuged at 12.000 g for 10 minutes at 4 °C. The RNA precipitate forms a pellet on the side and bottom of the tube. Then, supernatant was removed and the RNA pellet washed by adding 1 ml (minimum) of 75% ethanol per 1 ml of TRI REAGENT (used in Sample Preparation). Samples were vortexed and then centrifuge at 7,500 x g for 5 minutes at 4 °C. After briefly dry of the pellets for 5-10 min under vacuum, the RNA pellets were dissolved in RNAse free water and if not used immediately stored at -70 °C.

The final preparation of RNA is free of DNA and proteins if 260/280 ratio is ≥1.7.

Electrophoretic analysis of RNA

An agarose gel was prepared by melting the appropriate amount of agarose in water, cooling it to 60°C, and adding 5X formaldehyde gel-running buffer and formaldehyde to produce a final concentration of 1X and 2.2M, respectively. The gel was then cast in a chemical hood and allowed to set for 30 min at room temperature.

The RNA samples were prepared by mixing the following components in a sterile microtube:

- RNA (≈10μg) 4.5 μl
- 5X formaldehyde gel-running buffer 2.0 μl
- 17.5% formaldehyde 3.5 μl
- 50% formamide 10.0 μl

The samples were incubated for 15 min at 65°C, chilled on ice and centrifuged to deposit all of the fluid in the bottom of the tubes. Before applying the samples the gel was pre-run for 5 min at 5V/cm submerged in 1X formaldehyde gel-running buffer. After loading the samples the gel was run at 3V/cm for 4h.

Northern blot analysis

Probe isolation - The DNA to be labelled was cut with the appropriate restriction enzymes in order to release the appropriate fragment, which will allow the detection of APP mRNA. Then the fragments were separated by electrophoresis on 1.5% low melting agarose. After staining the gel in ethidium bromide (0.2 μg/ml) for 30 min, the desired band was cut out of the gel and placed in a pre-weighted microtube. After, 3 ml of dH_2O were added per gram of gel slice and placed in a boiling water bath for 7 min. The sample was stored at –20°C. Prior to using the DNA in a labelling reaction the DNA was denatured by boiling for 10 min and kept at 37°C.

Labelling reaction - 25 ng of template DNA was dissolved in a final volume of 8 μl dH_2O and denatured in a boiling water bath for 3 min. Then, the following components were added to the DNA: 4 μl of High Prime reaction mixture (Roche) containing random

primer mixture, Klenow polymerase and reaction buffer followed by 1 μl of dATP, 1 μl of dGTP and 1μl of dTTP and 5μl (50μCi) [α^{32}P]dCTP (3000Ci/mmol). This mixture was incubated for 2 h at 37°C. The reaction was stopped by adding 2 μl 0.2 M EDTA (pH 8.0) and gelsolining at 65°C for 10 min.

Probe purification - Stratagene's NucTrap probe purification columns were used to rapidly separate unincorporated nucleotides from the radiolabeled DNA, according to the manufacturer's instructions.

Membrane hybridization - ExpressHyb Solution Clontech was warmed at 65°C to completely dissolve any precipitate. The membrane was rehydrated in DEPC-H_20 and then prehybridized in 10 ml of ExpressHyb Solution with continuous shaking at 68°C for 5 h. Meanwhile, the radioactively labelled DNA probe was denatured at 100°C for 5 min and chilled on ice. After, the ExpressHyb Solution was replaced with the fresh solution containing the radiolabeled probe and the membrane was incubated overnight.
The blot was then rinsed at 65°C in solution I (2X SSC/ 0.05% SDS), 2 times for 10 min plus 1 time for 20 min. Then, 2 washes with Solution I at 50°C for 30min were performed. Finally, the blot was covered with plastic wrap and exposed for 3 days in using an autoradiography film (XAR-5 film, Kodak, Sigma Aldrich). The film was then developed and fixed with the appropriate solutions.

RT-PCR (reverse transcriptase polymerase chain reaction) analysis

RT-PCR analyses were performed using the ProSTAR™ Ultra HF RT-PCR system (Stratagene), which is a two-step system designed for high-fidelity, high-efficiency cDNA synthesis and PCR. First, cDNA is synthesized from total RNA in a reaction primed with oligo(dT) primer. Second, a portion of the cDNA synthesis reaction is transferred to a new tube and then amplified by PCR using APP isoform specific primers. PCR amplification was achieved by employing *PfuTurbo*® DNA polymerase. This system can amplify cDNA of 0.1-8 Kb in length synthesized from 10-500 ng of total RNA.

cDNA synthesis and RT-PCR

cDNA synthesis - The control and experimental reactions were prepared by adding the following components to separate microtubes in order:

5.9 µl RNase-free water (not DEPC-treated water)
1.0 µl 10X StrataScript RT buffer (Strategene)
0.6 µl oligo(dT) primer (100 ng/µl)
1.0 µl dNTP mix (40 mM)
1.0 µl RNA (100 ng) or 1.0 µl control mRNA

Reactions were incubated at 65°C for 5min and then cooled at RT for 5min to allow the primers to anneal to the RNA. After, 0.5 µl of StrataScript Reverse Transcriptase (20U/µl) were added to each reaction. The tubes were incubated for 30 min at 42°C. At the end the completed first-strand cDNA synthesis reactions were placed on ice for subsequent use in the PCR amplification.

PCR amplification – controls and experimental condition were preparing by adding the following components to separate microtubes in order:

41.0 µl RNase-free water (not DEPC-treated water)
5.0 µl 10X Ultra HF PCR buffer
1.0 µl dNTP mix (40mM)
1.0 µl of upstream primer (100 ng/µl) or 0 µl for negative control
1.0 ml of downstream primer (100 ng/µl) or 0 µl for negative control
1.0 µl of experimental first-strand cDNA reaction
1.0 µl of *PfuTurbo* DNA polymerase (2.5 U/µl)

The PCR conditions were as follows:

95°C, 5 min
95°C, 30 sec
60°C, 30 sec } 40 cycles
68°C, 1 min
68°C, 10 min

PCR products were run in a 2% agarose gel containing 2 μg/ml of ethidium bromide, and further analyzed using Quantity One quantitation software (Bio-Rad).

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Appendix II

REAGENTS AND SOLUTIONS

1. CELL CULTURE AND RAT PRIMARY NEURONAL CULTURES

Cells seeding and maintenance

■ ***DMEM medium (COS-7 cells)***

For a final volume of 1 L, dissolve one pack of DMEM powder (with L-glutamine and 4500 mg glucose/L, Gibco) in deionised H_2O and add:

- $NaHCO_3$ (Sigma) 3.7 g

adjust to pH 7.4 and before sterilizing add:

- Fetal Bovine Serum (FBS) (Gibco) 100 ml (10% v/v)

■ ***RPMI 1640 medium (PC12 cells)***

For a final volume of 1 L, dissolve one pack of RPMI 1640 powder (with L-glutamine and 4500 mg glucose/L, Gibco) in deionised H_2O and add:

- $NaHCO_3$ (Sigma) 0.85 g

adjust to pH 7.4 and before sterilizing add:

- Fetal Bovine Serum (FBS) (Gibco) 50 ml (5% v/v)
- Horse Serum (HS) (Gibco) 100 ml (10% v/v)

■ ***MEM:Ham´s F12 medium (SH-SY5Y cells)***

1:1 combination of minimum essential medium (MEM) and Ham's F12 medim.

For a final volume of 1 L of deionised H_2O add:

- MEM
- Ham´s F12
- $NaHCO_3$ (Sigma) 1.5 g
- Na 0.055 g
- L-glutamine 2 mM
- Non-essential aminoacids 0.1 mM

adjust to pH 7.4 and before sterilizing add:

- Fetal Bovine Serum (FBS) (Gibco) 100 ml (10% v/v)

Notes:
- Prior to pH adjustment add 100 U/ml penicillin and 100 mg/ml streptomycin [10 ml Streptomycin/ Penicilin/ Amphotericin solution (Gibco)].
- Sterilize all mediums by filtering through a 0.2 μm filter and store at 4 °C.
- FBS and HS were heat-inactivated for 30 min at 56 °C.

■ ***PBS (1x)***

For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionised H_2O. Final composition:

- Sodium Phosphate	8 mM
- Potassium Phosphate	2 mM
- NaCl	140 mM
- KCl	10 mM

Sterilize by filtering through a 0.2 μm filter and store at 4 °C.

■ ***Complete Neurobasal medium (Cortical primary cultures)***

This serum-free medium (Neurobasal; Gibco, BRL) is supplemented with:

- B27 supplement (Gibco, BRL)	2%
- L-glutamine (Gibco, BRL)	0.5 mM
- Gentamicine (Gibco, BRL)	60 μg/ml
- Phenol Red (Sigma Aldrich, Portugal)	0.001%

Adjust to pH 7.4. Sterilize by filtering through a 0.2 μm filter and store at 4 °C.

■ ***Complete Neurobasal medium (Hippocampal primary cultures)***

This serum-free medium (Neurobasal; Gibco, BRL) is supplemented with:

- B27 supplement (Gibco, BRL)	2%
- L-glutamine (Gibco, BRL)	0.5 mM
- L-glutamate (Gibco, BRL)	25 μM
- Gentamicine (Gibco, BRL)	60 μg/ml
- Phenol Red (Sigma Aldrich, Portugal)	0.001%

Adjust to pH 7.4. Sterilize by filtering through a 0.2 μm filter and store at 4 °C.

■ ***Hank's balanced salt solution (primary neuronal cultures)***

This salt solution is prepared with deionised H_2O. Final composition:

- NaCl	137 mM
- KCl	5.36 mM
- KH_2PO_4	0.44 mM
- $Na_2HPO_42H_2O$	0.34 mM
- $NaHCO_3$	4.16 mM
- Glucose	5 mM
- Sodium pyruvate	1 mM
- HEPES	10 mM

Adjust to pH 7.4. Sterilize by filtering through a 0.2 μm filter and store at 4 °C.

Cells fixation and Imunocytochemistry

■ ***1 mg/ml Poly-L-ornithine solution (10x) (COS-7 cells)***

To a final volume of 100 ml, dissolve in deionised H_2O 100 mg of poly-L-ornithine (Sigma-Aldrich, Portugal).

■ ***10 mg/ml Poly-D-lysine stock (100x) (rat primary neuronal cultures)***

To a final volume of 10 ml, dissolve in deionised H_2O 100 mg of poly- D-lysine (Sigma-Aldrich).

■ ***Borate buffer (at primary neuronal cultures)***

To a final volume of 1 L, dissolve in deionised H_2O 9.28 g of boric acid (Sigma-Aldrich). Adjust to pH 8.2, sterilize by filtering through a 0.2 μm filter, and store at 4 °C.

■ ***Poly-D-lysine solution (neuronal cells)***

To a final volume of 100 ml, dilute 1 ml of the 10 mg/ml poly-D-lysine stock solution in borate buffer.

■ ***4% Paraformaldehyde Fixative solution***

For a final volume of 100 ml, add 4 g of paraformaldehyde to 25 ml deionised H_2O. Dissolve by gelsolining the mixture at 58 °C while stirring. Add 1-2 drops of 1 M NaOH to clarify the solution and filter (0.2 μm). Add 50 ml of 2X PBS and adjust the volume to 100 ml with deionised H_2O.

2. PROTEINS MANIPULATION

SDS-PAGE

■ ***LGB (Lower gel buffer) (4x)*** *(1 L)*

- Tris	181.65 g
- SDS	4 g

Shake until the solutes have dissolved. Adjust the pH to 8.9 with HCl and adjust the volume to 1 L with deionised H_2O.

■ ***UGB (Upper gel buffer) (5x)***

Per litre, to 900 ml of deionised H_2O add 75.7 g of Tris base. Shake until the solute has dissolved. Adjust the pH to 6.8 with HCl and adjust the volume to 1 L with deionised H_2O.

■ ***30 % Acrylamide / 0.8 % Bisacrylamide solution***

Per 100 ml, to 70 ml of deionised H_2O add:

- Acrylamide	29.2 g
- Bisacrylamide	0.8 g

Shake until the solutes have dissolved. Adjust the volume to 100 ml with deionised H_2O. Filter through a 0.2 μm filter and store at 4 °C.

■ ***10 % APS (ammonium persulfate)***

In 10 ml of deionised H_2O dissolve 1 g of APS. Note: prepare fresh before use.

■ ***10 % SDS (sodium dodecilsulfate)***

In 10 ml of deionised H_2O dissolve 1 g of SDS.

■ ***Loading (sample) buffer (4x)*** *(10 ml)*

- 1M Tris solution (pH 6.8)	2.5 ml (250 mM)
- SDS	0.8 g (8%)
- Glicerol	4 ml (40%)
- β-Mercaptoetanol	2 ml (2%)
- Bromofenol blue	1 mg (0.01%)

Adjust the volume to 10 ml with deionised H_2O. Store in darkness at RT.

■ ***1 M Tris (pH 6.8) solution***

For a final volume of 250 ml, dissolve 30.3 g of Tris base in 150 ml of deionised H_2O, adjust pH to 6.8, and adjust final volume to 250 ml.

■ ***Running buffer (10x)*** *(1 L)*

- Tris	30.3 g (250 mM)
- Glycine	144.2 g (2.5 M)
- SDS	10 g (1%)

Dissolve in deionised H_2O, adjust pH to 8.3, and adjust volume to 1 liter.

■ ***Resolving (lower) gel solution*** *(60 ml)*

	7.5%	5% and	20% for gradient gels
- H_2O	29.25 ml	17.4 ml	2.2 ml
- 30% Acryl/0.8% Bisacryl solution	15.0 ml	5 ml	20 ml
- LGB (4x)	15.0 ml	7.5 ml	7.5 ml
- 10% APS	300 µl	150 µl	150 µl
- TEMED	30 µl	15 µl	15 µl

■ ***Stacking (upper) gel solution*** *(20 ml)* ***3.5%***

- H_2O	13.2 ml
- 30% Acryl/0.8% Bisacryl solution	2.4 ml
- UGB (5x)	4.0 ml
- 10% SDS	200 µl
- 10% APS	200 µl
- TEMED	20 µl

Western Blotting

■ ***Electrotransfer buffer (1x)***

Per litre, to 700 ml of deionised H_2O add:

- Tris	3.03 g (25 mM)
- Glycine	14.41 g (192 mM)

Mix until solutes dissolution. Adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with deionised H_2O. Just prior to use add 200 ml of methanol (20%).

■ ***TBS (Tris Buffered Saline) (10x)***

Per litre, to 700 ml of deionised H_2O add

- Tris	12.11 g (10 mM)
- NaCl	87.66 g (150 mM)

Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionised H_2O.

■ ***TBS-T (Tris Buffered Saline + Tween) (10x)***

For a final volume of 1 L, to 700 ml of deionised H_2O add

- Tris	12.11 g (10 mM)
- NaCl	87.66 g (150 mM)
- Tween 20	5 ml (0.05%)

Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionised H_2O.

■ ***Blocking solution*** *(100 ml)*

- TBS-T stock solution (10x)	10 ml
- non-fat milk (dry powder)	5 g

Dissolve in deionised H_2O and adjust volume to 100 ml.

■ ***Antibody solution (25 ml)***

- TBS-T stock solution (10x)	2.5 ml
- non-fat milk (dry powder)	0.75 g

Dissolve in deionised H_2O and adjust volume to 25 ml. Add antibody, mix gently without vortex, and store at -20 °C.

■ ***Alkaline Phosphatase (AP) Reaction Solution (1 L)***

- Tris-HCl (pH 9.5)	12.11 g (100 mM)
- NaCl	5.85 g (100 mM)
- $MgCl_2$	1.02 g (5 mM)

Dissolve Tris base in deionised H_2O and adjust solution to pH 9.5 with HCl. Dissolve the other solutes and adjust volume to 1 L.

■ ***AP Stop Solution*** *(1 L)*

- Tris-HCl (pH 9.5)	2.42 g (20 mM)
- EDTA	1.86 g (5 mM)

Dissolve Tris in deionised H_2O and adjust with HCl to pH 9.5. Add EDTA after and adjust volume to 1000 ml.

■ ***Membranes Stripping Solution*** *(500 ml)*

- Tris-HCl (pH 6.7)	3.76 g (62.5 mM)
- SDS	10 g (2%)
- β-mercaptoethanol	3.5 ml (100 mM)

Dissolve Tris and SDS in deionised H_2O and adjust with HCl to pH 6.7. Add the mercaptoethanol and adjust volume to 500 ml.