Universidade de Aveiro Secção Autónoma de Ciências da Saúde

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Abeta dependent tau phosphorylation

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Odete da Cruz e Silva, Professora Auxiliar da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro, e co-orientação da Professora Doutora Sandra Rebelo, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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Dedico esta dissertação de mestrado aos meus pais que sempre me apoiaram em todas as etapas da minha vida.

o júri

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palavras-chave

Doença de Alzheimer, Proteína precursora de amilóide de Alzheimer, Abeta, tau, fosforilação da tau, inibidores das fosfatases, proteínas de ligação à tau.

resumo

A doença de Alzheimer (DA) é uma doença neurodegenerativa caracterizada pela presença de duas características histopatológicas: as placas senis na matriz extracelular compostas por Beta-amilóide (Abeta) e as tranças neurofibrilhares intracelulares contendo proteína tau hiperfosforilada. Assim, o Abeta e a proteína tau são importantes moléculas associadas à DA e evidências sugerem que o Abeta possa mediar a hiperfosforilação da tau levando á disrupção da rede neuronal e consequentemente ao processo de neurodegeneração. No presente estudo, em culturas primárias neuronais de córtex e hipocampo de rato, verificou-se que a exposição a $Abeta_{1-42}$ agregado por longos períodos diminui a fosforilação da tau nos resíduos Ser202 e Thr205 e, em contraste, aumenta a fosforilação no resíduo Ser262.

Pensa-se que a hiperfosforilação da tau na DA pode estar relacionada com alterações nas vias de sinalização celular envolvidas no processo de fosforilação da tau, tais como alterações na regulação das cinases e das fosfatases. Deste modo, é também de extrema importância determinar as cinases e fosfatases envolvidas neste processo. Por tratamento de neurónios corticais com diferentes concentrações de ácido ocadéico (AO), um inibidor das fosfatases, verificamos o envolvimento da PP1 na desfosforilação da tau nos resíduos Ser202 e Thr205, bem como o envolvimento da PP1 e PP2A na desfosforilação do resíduo Ser262.

Um outro aspecto importante do metabolismo da tau são as proteínas de ligação, e actualmente já foram descritas várias proteínas que interagem com a tau *in vitro* e *in vivo*. O interactoma da tau é regulado pela sua fosforilação e portanto é crucial estabelecer uma relação entre a tau normal e a tau patológica hiperfosforilada no que diz respeito às proteínas de ligação. Por co-imunoprecipitação de neurónios corticais pretendemos identificar proteínas de ligação à tau e especificamente à tau fosforilada, e ainda avaliar o efeito do Abeta neste interactoma. O interactoma da tau dependente da fosforilação e do Abeta é de particular relevância para a compreensão da DA.

keywords

Alzheimer´s Disease, Alzheimer´s amyloid precursor protein, Abeta, tau, tau phosphorylation, protein phosphatase inhibitors, tau binding proteins.

abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of two histopathological hallmarks: the extracellular amyloid plaques (APs) composed of beta-amyloid protein (Abeta) and intracellular neurofibrillary tangles (NFTs), containing hyperphosphorylated tau protein. Therefore, Abeta and tau are important molecules associated with AD and evidence suggests that Abeta may initiate the hyperphosphorylation of tau, which by disrupting neuronal network leads to the process of neurodegeneration. In the present study, using rat primary cortical and hippocampal neuronal cultures, it was shown that exposure to aggregated $Abeta_{1-42}$ for prolonged periods decreased tau phosphorylation at Ser202 and Thr205 residue, but in contrast increased at Ser262 residue. Tau hyperphosphorylation in AD may be related to alterations in signal transduction pathways involving tau phosphorylation, such as an imbalance in the regulation of protein kinases (PKs) and protein phosphatases (PPs). Thus it is also important to determine which specific PKs and PPs are involved in this process. We observed the involvement of PP1 in the dephosphorylation of tau at Ser202 and Thr205, and the involvement of PP1 and PP2A at the Ser262 residue.

An important aspect of tau metabolism are its binding proteins, and to date many such proteins have already been described both in vitro and in vivo. The interactome of tau is shaped by its phosphorylation and so is crucial to map the crosstalk between normal and pathologically hyperphosphorylated tau. By co-immunoprecipitation we intend to identify proteins that interact with tau and more specifically with phosphorylated tau (p-Tau). Furthermore the effect of Abeta on this interactome should be forthcoming, which is relevant for AD tau pathology.

Abbreviations

Abeta	Amyloid peptide
AD	Alzheimer´s disease
ADAM	A Disintegrin And Metalloproteinase
AICD	Amyloid precursor protein intracellular domain
AP	Amyloid plaques
APS	Ammonium persulfate
Aph1	Anterior pharynx-defective 1
APLP1	APP like protein 1
APLP2	APP like protein 2
ApoE	Apolipoprotein E
APP	Alzheimer´s amyloid precursor protein
ATP	Adenosine triphosphate
BACE	Beta-site APP-cleaving enzyme
BCA	Bicinchoninic acid
BSA	Bovine serum albumine
CaMPKII	Ca^{2+}/Calmodulin-dependent protein kinase II
Cdc2	Cyclin-dependent protein kinase 2
Cdk5	Cyclin-dependent protein kinase 5
CSF	Cerebrospinal fluid
CNS	Central nervous system
CT	Computarized tomography
C-terminal	Carboxyl-terminal
CTF	Carboxyl-terminal fragment
DTT	Dithiothreitol
E	Exon
EC	Extracellular domain
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
FAD	Familial Alzheimer´s disease
FBS	Fetal bovine serum
Fc	Fragment crystallizable
GSK3	Glycogen synthase kinase 3
HBSS	Hank´s balanced salt solution
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
IB	Immunoblotting
IC	Intracellular domain
IC50	50% inhibition concentration
IgG	Immunoglobulin G

IP	Immunoprecipitation
JIP1b	JNK-interacting protein 1b
JNK	Jun N-terminal kinase
KLC	Kinesin light chain
KPI	Kunitz-type serine protease inhibitor
LB	Loading buffer
LC-MS/MS	Liquid chromatography-mass spectrometry
LGB	Lower gel buffer
MAP	Microtubule associated proteins
MAPK	Mitogen activated protein kinases
MAPT	Microtubule Associated Protein Tau
MARK	Microtubule-affinity regulating kinase
MOPS	3-(N-morpholino)propanesulfonic acid
MRI	Magnetic resonance imaging
NFT	Neurofibrillary tangles
NMDA	N-Methyl-D-aspartic acid
NPDPK	Non - Proline directed protein kinases
NSAID	Nonsteroidal anti-inflammatory drug
N-terminal	Amino-terminal
OA	Okadaic acid
ON	Overnight
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline - tween
PDPK	Proline directed protein kinases
PEN-2	Presenilin enhancer 2
PET	Positron emission tomography
PHF	Paired helical filaments
Pin1	Protein interacting with NIMA
PK	Protein kinase
PKA	Cyclic-AMP-dependent kinase
PKC	Protein kinase C
PLC	Phospholipase C
PP	Protein phosphatase
PSEN1	Presenilin 1
PSEN2	Presenilin 2
p-tau	Phosphorylated tau
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic acid
RT	Room temperature
SAP	Stress-activated protein
sAPP	Secreted APP
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error

Ser	Serine
SH3	SRC homology
TBS	Tris Buffered Saline
TBS_T	Tris Buffered Saline - tween
TGN	Trans-Golgi network
Thr	Threonine
TRIS	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
TM	Transmembrane domain
UGB	Upper gel buffer
WR	Working reagent

INDEX

1. Introduction

1.1. Alzheimer ’s disease (AD)

Alzheimer´s disease (AD) is a neurodegenerative disorder first described in 1906 by Dr. Alois Alzheimer[5,9]. AD is the most common form of dementia among the elderly, and currently affects about 18 million people worldwide, 7.3 million in Europe and about 90.000 people in Portugal[2-4,10-11]. Dementia is the loss of cognitive functions such as thinking, remembering and reasoning, that interfere with a person´s daily life and activities[2,12]. AD is characterized by memory, language, learning and other cognitive impairments, severe enough to interfere with social, occupational and personal functions[3-5,12-14]. Besides this cognitive decline, behavioral, emotional and psychiatric symptoms are also frequent in AD[14].

1.1.1. Epidemiology and Genetics

The most prevalent forms of AD are sporadic, with the symptoms beginning after 65 years of age, and are called late-onset AD. But there is a small percentage of cases (less than 5%) with an early onset (30-60 years), generally inherited in an autosomal-dominant manner – the familial AD (FAD). There are some risk factors for developing sporadic AD (late-onset AD) such as advancing age, the presence of certain alleles of the Apolipoprotein E gene (*ApoE*), gender, level of education and head trauma. The single most important risk factor is age, since the rate of occurrence of the disease doubles approximately every five years after the age of 65[4,14-15]. Apolipoprotein E gene (*ApoE*) is localized to chromosome 19, encodes a glycoprotein involved in cholesterol transport and alters the risk of developing AD but does not cause it. The gene codifying this protein has three possible alleles: ε2, ε3 and ε4. The ε3 is the most common allele in the general population and ε2 the less common. The ε4 allele is connected with an increased risk of developing AD. The mechanism through which this allele increases the risk is unknown but it appears that the ε4 allele along with other proteins may influence Abeta metabolism and its aggregation in the Central nervous system (CNS)[16-18].

Additionally mutations in three different genes: presenilin-1 gene (*PSEN1*) located on chromosome 14, presenilin-2 gene (*PSEN2*) located on chromosome 1 and amyloid precursor protein gene (*APP*) on chromosome 21, are known to cause 90% of the FAD[14-15,18]. The APP gene encodes a transmembrane glycoprotein abundant in the nervous system that is proteolytically cleaved to produce Abeta. As this gene is localized to chromosome 21, this explains the observation that patients with Trisomy 21, which possess an extra copy of the APP gene, develop early in life (40 years) the neuropathological features associated with AD (like Abeta deposition). Studies have allowed to conclude that most of the APP mutations alter the proteolytic processing of APP resulting in increased Abeta production[16-18]. The *PSEN1* and *PSEN2* genes encode two highly homologous transmembrane proteins whose normal functions are not yet known. However the products from proteolytic cleavage of these proteins are known to be essential to gamma-secretase complex formation. The majority of mutations in *PSEN1* and *PSEN2* are missense and increase the activity of gamma-secretase and consequently Abeta production. From all early-onset AD cases 50% are linked to the *PSEN1* gene and only a few cases are associated to the *PSEN2* gene[16-17]

1.1.2. Histopathological Hallmarks

In AD there is neuronal and synaptic loss associated with two histopathological hallmarks: the extracellular amyloid plaques (AP, Fig. 1) and intracellular neurofibrillary tangles (NFTs, Fig. 1), in distinct brain areas including the neocortex and hippocampus [15,19].

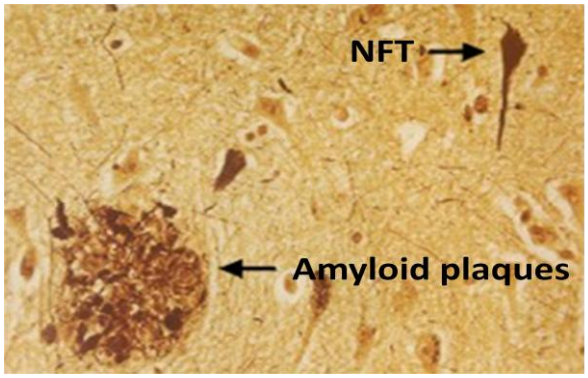

Figure 1 – Two histopathological hallmarks of AD: Amyloid plaques (AP) and Neurofibrillary tangles (NFTs)[20].

Amyloid plaques are extracellular insoluble deposits of a protein fragment, the beta-amyloid (Abeta) generated by proteolytic cleavage of the amyloid precursor protein (APP), surrounded by dystrophic neurites. The Abeta peptide is a physiological soluble cellular metabolite that comprises two predominant forms, the $Abeta_{1-40}$ and the $Abeta_{1-42}$ which differ in their C-terminal[15]. The $Abeta_{1-40}$ is the most predominant and presumably is not neurotoxic while the $Abeta_{1-42}$ is less prevalent, more hydrophobic and more toxic. The $Abeta_{1-42}$, is also proportionally increased in patients with AD and has a major propensity to aggregate and to form oligomers and fibrils that ultimately generate amyloid plaques[6]. The steady state level of Abeta is controlled by its production, degradation and clearance. In AD it is proposed that the cause of Abeta accumulation is a defect that leads to its over-production or decreased clearance[4]. Although, actually, it remains unclear what triggers these alterations in APP and Abeta metabolism causing this increased production and aggregation of Abeta peptide. However it is known that Abeta accumulation and aggregation results in organelle and membrane damage, which in turn leads to the disruption of cellular processes and also oxidative stress, inflammation and cell death[6].

The NFTs are intracellular aggregates composed of bundles of paired helical filaments (PHF) whose major protein component is the microtubule-associated tau protein. In PHF the tau protein is abnormally hyperphosphorylated and aggregated[14,18,21-22] and so has a reduced ability to bind to microtubules and to promote their assembly. As a consequence the axonal cytoskeleton is disturbed, axonal transport disrupted and finally neuronal viability compromised[23]. For

example, synapses are very vulnerable to these perturbations in the axonal transport system since it causes dysfunction in neurotransmission and signal propagation leading to synaptic degeneration.[24] Thus, the number of NFT are positively correlated with the degree of AD, and the same does not occur with amyloid plaques[24].

1.1.3. Diagnosis and Treatment

Currently, the diagnosis and treatment of AD is limited and insufficient. As such definitive diagnosis of AD is still only possible after death, with an examination and pathological analysis of brain tissue during an autopsy, where the presence of the senile plaques and neurofibrillary tangles is confirmed. However, there are many current tools that are used to diagnose AD patients or even to exclude this pathology. These tools included detailed patient medical history, information obtained from family members, physical and neurological examinations, laboratory tests, neuropsychological tests to measure, for example language and memory skills and a variety of other approaches, including neuroimaging studies such as computerized tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). The neuroimaging techniques are of great help since they provide regional structural and functional details of the brain, as well as assist in the identification of the biochemical profile of brain dysfunction[25]. Although there are significant advances in these neuroimaging techniques, the use and identification of novel AD biomarkers is necessary since they give more direct and convenient information to detect the preclinical stages of AD, as well as assisting in the study of disease progression[25-26]. The most important potential sources of AD biomarkers are cerebrospinal fluid (CSF), plasma and urine[25]. Currently the quantification of Abeta and tau, both total and phosphorylated tau, in the CSF is the more appropriate to detect early AD patients[26]. An early diagnosis of AD is beneficial, as it facilitates the efficient treatment with new generation of disease modifying drugs[12].

Regarding the treatment for AD, it is a complex disease and no single treatment is likely to prevent or cure it. Thus current treatments focus on helping patients maintain mental function, managing behavioral symptoms and delaying the

disease[14]. Actually the therapies for AD can be based in symptomatic approaches or based in neuroprotective approaches. Therefore there are five major categories of drugs used in AD treatment: the acetylcholinesterase inhibitors, antiglutaminergic treatment, vitamins and antioxidants, anti-inflammatory drugs and pharmacological management of behavioral disturbances[27]. From these, the most successful AD drugs to date are the acetylcholinesterase inhibitors since in AD there is a deficiency in cholinergic neurotransmission that plays a major role in the expression of cognitive functional and behavioral symptoms of AD. Thus acetylcholinesterase inhibitors act by stopping or slowing down the action of acetylcholinesterase, a catabolic enzyme that breaks down acetylcholine, the neurotransmitter involved in memory formation, prolonging its action at cholinergic synapses[14,27-28]. The antiglutaminergic treatment, for example the memantine, is another therapeutic approach, but in this case it blocks glutamatergic neurotransmission, since it is an uncompetitive antagonist of NMDA receptors avoiding its hyperstimulation which causes neuronal dysfunction and death[14,27]. Evidence that free radicals may accumulate in AD brains, due to the existence of oxidative stress, has led to interest in the use of antioxidants such as vitamin E. Nonsteroidal anti-inflammatory drugs (NSAIDs) may have a protective role against the development of AD but this effect does not extend once AD is established[27]. Concerning the management of behavioral disturbances it can be achieved by using nonpharmacological (music, light exercise, relaxation exercise) or pharmacological approaches using anxiolytic, antidepressive or antipsychotic drugs. The future of therapies in AD will be based on the understanding of AD pathophysiology and could be achieved with anti-amyloid therapies that are being studied. The development of these therapies has two main approaches: reduce the production of Abeta that can be achieved by inhibiting beta- and gamma-secretase, or increase its clearance by anti-amyloid immunotherapy. The main goal of these new approaches is to modify the progression of the disease; these are called disease-modifying drugs[14,27].

1.2. Alzheimer´s amyloid precursor protein (APP)

APP is a ubiquitously expressed membrane-spanning glycoprotein with a large N-terminal extracellular domain (EC) and a small cytoplasmic domain (IC) [21,29]. It is a member of a conserved family of type I membrane proteins including APP like protein 1 (APLP1) and APP like protein 2 (APLP2) in mammals. While the APP and APLP2 are ubiquitous but with highly expression in neurons, the APLP1 is brain-specific[29-30]. APP is encoded by a gene localized on the mid-portion of the long arm of human chromosome 21 (21q21) and contains 18 exons[30-31]. Alternative splicing of the exons 7, 8 and 15 generates eight APP isoforms that range from 365-770 amino acids. The most abundant APP isoforms are APP_{695}, APP_{751} and APP_{770} (Fig. 2)[15,32-33]. APP_{751} and APP_{770} are largely expressed by non-neuronal cells and contain a domain homologous to the Kunitz-type serine protease inhibitor (KPI), encoded by exon 7, whereas the APP_{695} is expressed at higher levels in neurons and does not have the KPI domain[30-31,34]. KPI-containing APP isoforms are thought to be more amyloidogenic and their levels increase in the brains of AD patients[15]. However the cause and the functional significance for this tissue-specific isoforms is still not fully understood.

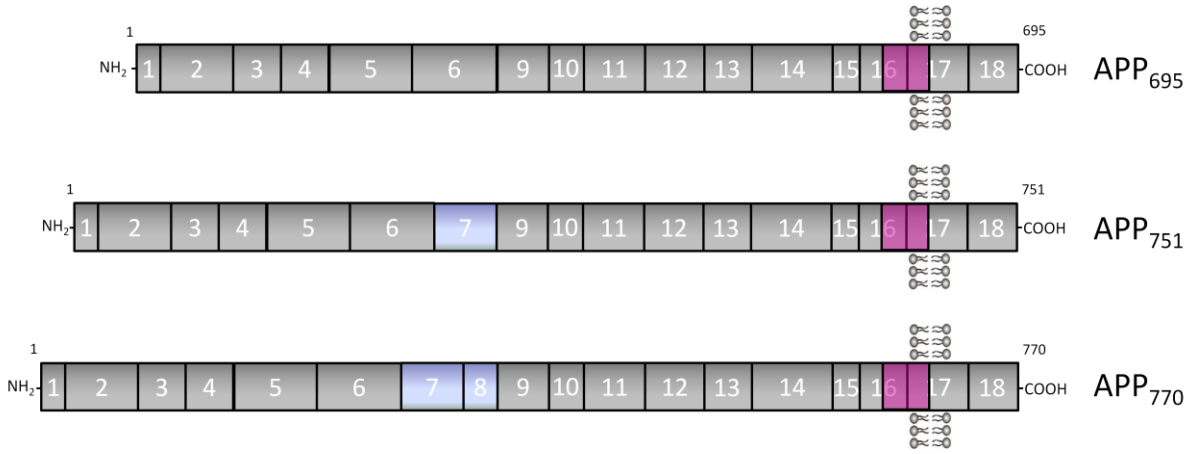

Figure 2 – Schematic representation of the three major APP isoforms in mammalian tissues. Numbers and vertical lines indicate the corresponding exons. The most abundant neuronal isoform, comprising 695 amino acids, is APP_{695}. APP_{751} and APP_{770} are alternatively spliced isoforms that differ from APP_{695} in the expression of exons 7 and 8, as shown. The sequences encoded by the APP gene exons are indicated approximately to scale. The solid pink region represents the Abeta peptide region, whose sequence is divided between exons 16 and 17. Adapted from Cruz e Silva and Cruz e Silva, 2003.

In neuronal and non-neuronal cells, APP is generated in the endoplasmic reticulum (ER) and is known to be transported via the secretory pathway reaching cell surface. During its transit from the ER to the plasma membrane, APP undergoes post-translational modifications that include N- and O- glycosylation and tyrosine sulfation[30,33]. Therefore mature APP is located in compartments from the trans-Golgi to the plasma membrane, being that, only a small fraction is present at the plasma membrane. At the cell surface, APP can be cleaved or rapidly internalized via endocytosis to be recycled back to the membrane, retrogradely delivered to the trans-Golgi-network (TGN) or incorporated into secondary endosomes. The majority of mature APP is proteolytically cleaved either via the alpha-secretase or beta-secretase pathway. It is thought that this proteolytic processing occurs through the secretory pathway, on the plasma membrane and/or in the endocytic cycle [30,35-36]. Moreover, in neurons, APP is rapidly and anterogradely transported along peripheral and central axons[36].

Another important aspect is the fact that the trafficking, metabolism and even the functions of APP are regulated through interactions with several cytoplasmic proteins, for example, the relatively well analyzed Fe65, X11 and X11L, JIP1b (JNK-interacting protein 1) and KLC (kinesin light chain) proteins[15,37] . All these proteins bind to the APP intracellular domain (AICD) at specific binding domains.

1.2.1. APP Proteolytic processing

APP can be cleaved by two major proteolytic processing pathways: the beta-secretase and alpha-secretase pathway, also called amyloidogenic and non-amyloidogenic, respectively. In the beta-secretase pathway the APP is first cleaved by the beta-secretase, releasing the ectodomain (sAPPbeta) while a C-terminal fragment with 99 amino acids (C99 or beta-CTF) remains membrane bound. Then C99 is cleaved by the gamma-secretase complex to produce Abeta peptide and the AICD. Alternatively, in the alpha-secretase pathway, alpha-secretase primarily cleaves APP releasing the ectodomain (sAPPalpha) and a membrane bound C-terminal fragment with 83 amino acids (C83 or alpha-CTF), which then is also cleaved by gamma-secretase into p3 peptide and the AICD (Fig. 3)[15,34,36]. The majority of APP is

processed by the alpha-secretase pathway and so there is a balance between these two proteolytic pathways[21].

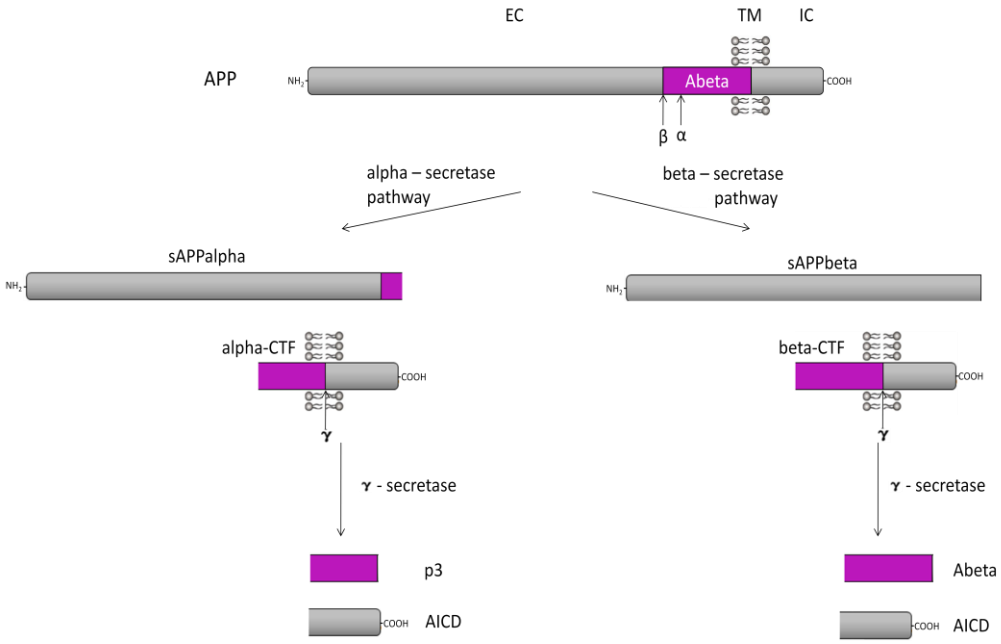

Figure 3 – Proteolytic processing of APP[30]. EC: extracellular domain; TM: transmembrane domain; IC: intracellular domain. The scheme is not to scale.

Thus, there are three proteases involved in the cleavages of APP: the alpha-, beta- and gamma-secretases. The candidates for the alpha-secretase activity are members of the ADAM family of desintegrin and metalloproteases. BACE (beta-site APP-cleaving enzyme) is a type I transmembrane aspartic protease, has two homologues, BACE1 and BACE2 that are the major beta-secretase in neurons. The gamma-secretase is a multimeric complex with proteolytic activity formed at least by four proteins: PSEN1 or PSEN2, Nicastrin, PEN-2 and Aph1[36-37].

1.2.2. APP phosphorylation

Protein phosphorylation is an important cellular regulatory mechanism that is increased in AD. APP can be phosphorylated at multiple sites in both extracellular and intracellular domains. In the intracellular domains 8 putative phosphorylation residues are described: Tyr653, Thr654, Ser655, Thr668, Ser675, Tyr682, Thr686 and Tyr687[29,34,38-39]. Since these residues are located in specific protein interacting sites its phosphorylation may interfere with protein binding and thus interfere with APP and AICD function.[29] Previous studies point to an important role for the Thr668 and Tyr682. The phosphorylation of both these residues is increased in AD brains: the Tyr682 is important for APP interactions with the cytosolic proteins and can promote or abolish them; the Thr668 phosphorylation allows Pin1 (a prolyl isomerase) binding and reduces Fe65 binding to APP and thus it alters APP processing and Abeta production[40]. In the APP ectodomain, phosphorylation at Ser198 and Ser206 residues are present, and occurs in a post-Golgi secretory compartment and at the cell surface.[41] All of these APP phosphorylation sites are represented in Fig. 4.

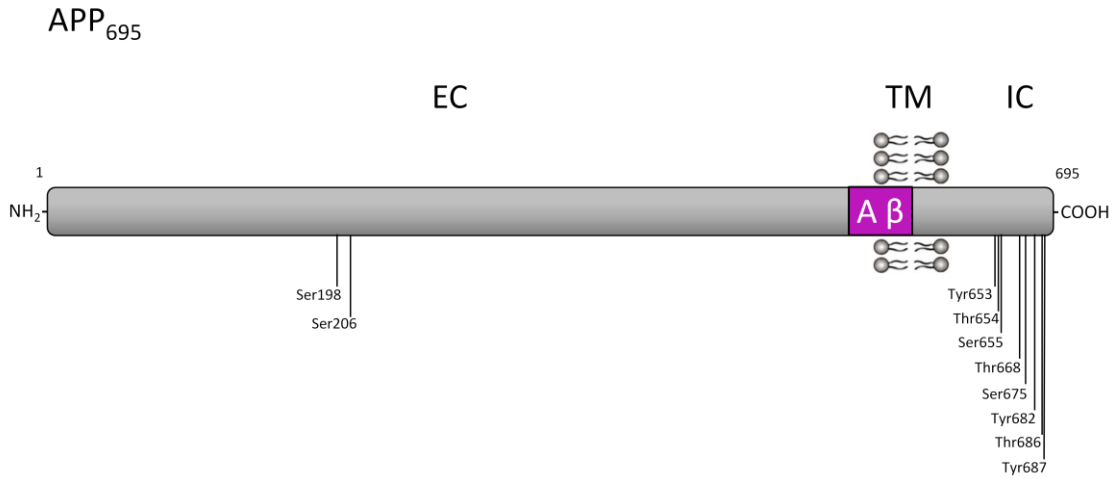

Figure 4 – Schematic representation of the phosphorylation residues present in the APP_{695} isoform protein: the two phosphorylated Ser residues present in the APP ectodomain, and the eight putative phosphorylated residues in the APP intracellular domain. EC: extracellular domain; TM: transmembrane domain; IC: intracellular domain[29,41].

1.2.3. APP and APP fragments functions

The precise roles of APP are unknown, although the overall structure of the protein suggests a role as a receptor or growth factor[5]. However some studies describe more putative roles for APP and its fragments in development, cell growth, intercellular communication, signal transduction, nuclear signaling and structural and functional plasticity[29-30,42]. Table 1 summarizes some of these putative roles for APP and its fragments that are produced during APP metabolism.

Table 1 - APP and APP fragments (sAPP, Abeta and AICD) putative functions.

APP	sAPP	Abeta	AICD
- membrane receptor[1-2] - cell adhesion[1-2] - stimulation of neurite outgrowth[1] - synaptogenesis[1,3] - promotion of cell survival[2] -neuroprotection[2,8] -axonal transport[3]	- promotion of neurite outgrowth[1-2] - synaptogenesis[1] - synaptic plasticity[5] - modulation of neuronal excitability and axon growth[1] - neuronal activation[1] - neuroprotection[2] - promotion of neuronal survival[2] -regulates calcium homeostasis[3]	**Physiological concentrations**: - promotion of neurite outgrowth[2] - synapse function[4] - homeostatic plasticity[3] - neuronal survival[4] -cholesterol homeostasis[3] **Pathological concentrations**: - neurotoxic[1,6] - synaptic dysfunction[1] - negatively affects neuronal viability[1]	- gene transcription[3] - synaptic function[3] - synapse remodeling[7]

1.3. Microtubule-associated tau protein

Tau protein belongs to the microtubule-associated protein (MAP) family and was first isolated in 1975 as a protein that co-purifies with tubulin and has the ability to promote microtubule assembly *in vitro*[24,43]. Tau is mainly a neuronal protein, although it can be expressed in non-neuronal cells[43]. The human tau gene, *MAPT*, is located on the long arm of chromosome 17 at band position 17q21 where it occupies over 100 kb [43-44]. The tau primary transcript contains 16 exons but three of them (exons 4A, 6 and 8) are never present in any mRNA of the human brain (Fig. 5)[44]. Exon 1 is part of the promoter and is transcribed but not translated, as is the case for exon 14. Exons 1, 4, 5, 7, 9, 11, 12 and 13 are constitutive, but exons 2, 3 and 10 are alternatively spliced, and exon 3 never appears in the absence of exon 2 [43-44]. Therefore, the transcript produced by alternative splicing of these three exons yields six different mRNA species that are then translated in six different isoforms of tau which range from 352 to 441 amino acids (Fig. 5) [22,43].

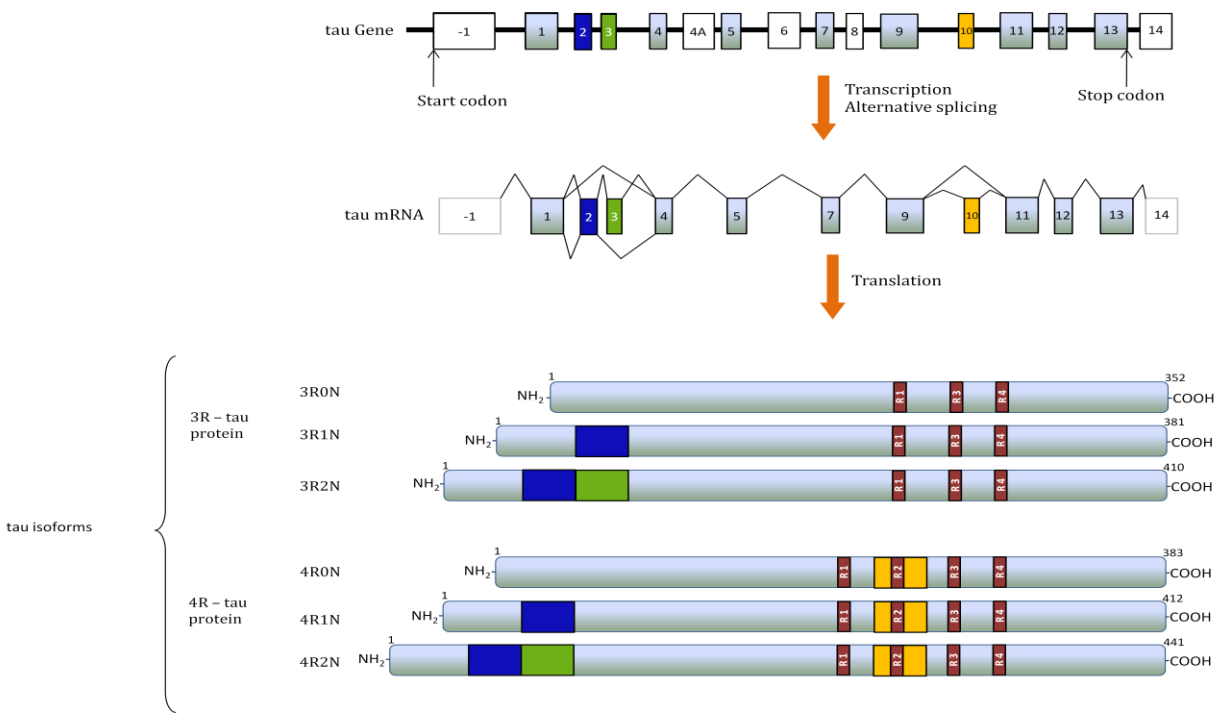

Figure 5 – Schematic representation of the human tau gene, mRNA and different protein isoforms[24]. The human tau gene is located over 100kb on the long arm of chromosome 17 at position 17q21. It contains 16 exons; exon -1 is a part of the promoter. The tau primary transcript contains 13 exons since exons 4A, 6 and 8 are not transcribed in human. Exons -1 and 14 are transcribed but not translated. Exons 1, 4, 5 ,7, 9, 11, 12, 13 are constitutive, and exons 2, 3 and 10 are alternatively spliced, giving rise to six different mRNAs, translated in six different tau isoforms. These isoforms differ by the absence or presence of one or two 29 amino acids inserts encoded by exon 2 and 3 in the amino-terminal part, in combination with either three (R1, R3 and R4) or four (R1, R2, R3 and R4) repeat-regions in the carboxyl-terminal part.

The isoforms differ by the absence or presence of one or two acidic inserts (0N, 1N or 2N, respectively) at the amino-terminal (N-terminal) part of the molecule and whether they contain three or four repeats of a conserved tubulin binding motif (3R or 4R) at the carboxyl-terminal (C-terminal) region, and they can be designated as 3R0N, 3R1N, 3R2N, 4R0N, 4R1N and 4R2N[44-48]. Thus, the longest isoforms in the CNS has four repeats and two insert (441 residues), and the shortest isoforms has three repeats and no inserts (352 residues) (Fig. 5). The later isoform (3R0N), the smallest form of tau protein, is the only one expressed in fetal tissue while the six isoforms are expressed in adult brain[46]. It is thought that tau isoforms have specific physiological roles since they are differentially expressed during development[43].

Tau isoforms have two domains: the projection domain and the microtubule-binding domain, that have been proposed to have distinct roles. The projection

domain contains the N-terminal two-thirds of the molecule and can be further subdivided into the acidic N-terminal region and a basic proline-rich region. The projection domain of tau determines spacings between axonal microtubules, interacts with other cytoskeletal proteins, for example, spectrin and actin filaments which allow microtubules to interconnect with other cytoskeletal components (Fig. 6). This domain may also allow interaction of tau with proteins associated with the neural plasma membrane and cytoplasmatic organelles, such as mitochondria, and there is some data indicating that tau proteins may interact with src-family non-receptor tyrosine kinases and phospholipase C-ϒ (PLC-ϒ), which suggests that tau may have a role on signal transduction pathways involving these two proteins. Moreover, the interaction of this domain with cytoskeletal and plasma membrane elements is only possible because this part of the molecule projects from the microtubule surface.

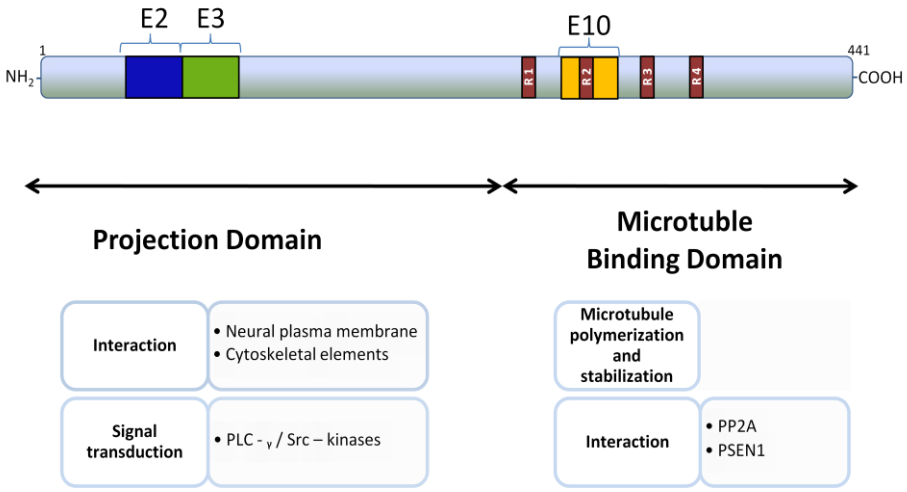

Figure 6 – Summary of biological functions of tau associated with respective functional domain. E: exon; R: repeat domains. Adapted from Buée et.al, 2000.

The microtubule-binding domain contains the C-terminal one-third of the molecule and, likewise the projection domain, has been subdivided into the basic tubulin-binding domain region and the acidic C-terminal region (Fig. 6). As its name suggests, this domain is responsible for the binding of tau to microtubules and more specifically, tau binds microtubules through repetitive regions present in this domain. The repetitive regions are the repeat domains (R1, R2, R3 and R4) encoded by exons 9, 10, 11 e 12 and with sequences of 31 or 32 residues very similar but not identical since they are composed by an 18 amino-acid sequence highly conserved and a less conserved sequence composed by 13 or 14 amino-acid sequence. The 18 amino-acid sequence is responsible for binding to microtubules, promoting microtubule polymerization and stabilization. For this reason tau isoforms with 4R (R1, R2, R3 and R4) binds more efficiently to microtubules than the isoforms with 3R (R1, R2 and R3). Besides microtubule assembly recent data suggests that the microtubule-binding domain can also modulate the phosphorylation state of tau proteins since it can bind directly with the protein phosphatase 2A (PP2A) and in consequence microtubules can inhibit PP2A activity by competing for binding to tau at this domain [43-44,46,49].

1.3.1. Posttranslational modifications of tau protein

Like many other proteins that are implicated in human disease, tau protein is posttranslationally modified. Several modifications have been described for tau protein such as phosphorylation, glycosylation, ubiquitination, glycation, truncation and deamination. Of all posttranslational modifications the most important is phosphorylation because it is an important cellular regulatory mechanism.

1.3.1.1 Tau phosphorylation

Tau is a phosphoprotein that possesses a large number of potential phosphorylation sites mainly serine, threonine and tyrosine residues[45,49]. On the longest brain tau isoform (441 amino-acids) there are 45 serine and 35 threonine putative phosphorylation sites, and at least 35 phosphorylation sites have already been described (Fig. 7)[43].

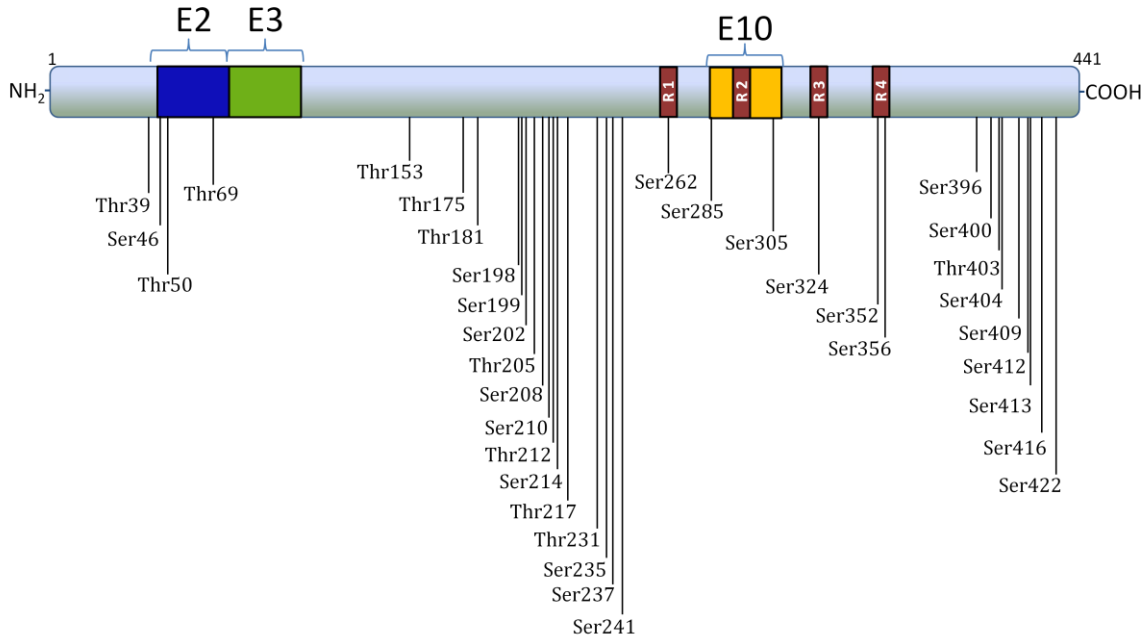

Figure 7 – Representation of phosphorylation sites already described on the longest brain tau isoform using phosphorylation-dependent monoclonal antibodies against tau, mass spectrometry and sequencing. All of these sites are localized outside microtubule-binding domains with the exception of Ser262, Ser285, Ser305, Ser324, Ser352 and Ser356[43]. E: exon; R: repeat domain.

The level of tau phosphorylation is a dynamic process controlled by several protein kinases and protein phosphatases (summarized in table 2). Interestingly, tau phosphorylation is developmentally regulated as fetal isoforms are more phosphorylated during neurogenesis and synaptogenesis and then phosphorylation decreases during development due to phosphatases activation[43].

Table 2- Protein kinases and protein phosphatases most probably involved in tau protein phosphorylation and dephosphorylation, respectively [13,43-44,47-50].

Kinases	**Proline directed protein kinases (PDPK)**
	Glycogen synthase 3 (GSK3) Mitogen activated protein kinase (MAPK) Tau-tubulin kinase Cyclin-dependent Kinases (cdc2 and cdk5) Stress-activated protein kinases (SAP)
	Non-proline directed protein kinases (NPDPK)
	Microtubule-affinity regulating kinase (MARK) Ca^{2+}/calmodulin-dependent protein kinase II (CaMPKII) Cyclic-AMP-dependent kinase (PKA) Casein Kinase II Protein kinase C (PKC**)**
Phosphatases	Protein phosphatase 1 Protein phosphatase 2A Protein phosphatase 2B (calcineurin)

The majority of the kinases involved in tau phosphorylation belong to the Proline-directed protein kinases (PDPK) which comprise glycogen synthase 3 (GSK3), mitogen activated protein kinase (MAPK), tau-tubulin kinase, cyclin-dependent kinases such as cdk2 and cdk5, and stress-activated kinases (SAP kinases). Another group named non-PDPK or NPDPK comprises microtubule-affinity regulating kinase (MARK), Ca^{2+}/calmodulin-dependent protein kinase II (CaMPKII), cyclic-AMP-dependent kinase (PKA), casein kinase II and protein kinase C (PKC)[13,43,47-49]. GSK3 is one of the protein kinases that has gained significant attention as a tau kinase and comprises the highly homologous proteins GSK-3α and GSK-3β[46]. GSK-3β is highly expressed in brain and associates with microtubules[47]. Some studies in which tau and GSK-3β are co-transfected into non-neuronal cells showed an increase in tau phosphorylation and impairment in the binding of tau to microtubules[46,51-52]. Interestingly, this kinase can phosphorylate numerous sites on tau protein, not all

having an impact on tau function[51]. Other important tau kinases are the MAPKs in which some of its members can phosphorylate tau: p42^{mapk}, p44^{mapk}, pk40^{erk2} and p49^{3F12}. It was shown in several studies that these MAPKs phosphorylate tau protein in cultured neurons mainly via activation of tyrosine kinase receptors and protein kinase cascades[46]. Cdk5 is a member of cyclin-dependent kinase family and its activity is highest in neurons due to selective expression of its regulator p35 in these cells[51]. This kinase induces phosphorylation of tau protein *in vitro,* maybe not directly but instead by regulating the kinases and phosphatases that act on tau[47]. The NPDPK cyclic-AMP-dependent kinase (PKA) is shown to phosphorylate tau *in vivo[47].* Since many kinases are likely to be involved in tau phosphorylation one possibility is that tau might be primed by one kinase before subsequent phosphorylation by a second kinase that recognizes a nearby phosphorylated residue[53].

Regarding protein phosphatases (PP) several studies have shown that three major PPs: PP1, PP2A and PP2B (calcineurin), but not PP2C can dephosphorylate tau in vitro[44,49-50]. All of these PPs are present in the brain and are developmentally regulated[43]. The PP2A is the most probable phosphatase that acts on most phosphorylation sites and it is also associated with microtubules[46,49-50,54]. It was shown that, in cultured neurons treated with okadaic acid and calyculinA (phosphatase inhibitors) at concentrations sufficient to inhibit PP2A, phosphorylation of tau was increased[54]. Similar studies using PP2B inhibitors also suggest that PP2B is involved in the dephosphorylation of tau protein but at different sites of the PP2A[43]. PP1 involvement in tau phosphorylation was also demonstrated by inhibition of phosphatases in neuronal cell lines. Although, PP2C has been reported to dephosphorylate tau phosphorylated by PKA *in vitro,* these dephosphorylations did not affect PHF tau[46]. Table 3 summarizes the phosphorylation residues in human tau associated with its tau-directed protein kinases and phosphatases.

Table 3 – Tau-directed protein kinases and phosphatases and respective phosphorylation residues in human tau [44,46]. In bold are the residues addressed in this study.

	Residues on human tau
Kinases	
GSK3β	Ser46, Thr50, Thr181, Ser184, Ser195, Ser198, Ser199, **Ser202**, **Thr205**, Thr212, Thr217, Thr231, Ser235, **Ser262**, Ser356, Ser396, Thr403, Thr404
GSK3α	Ser199, **Ser202**, Thr212, Thr231, Ser235, **Ser262**, Ser324, Ser356, Ser396, Ser404
Cdc2	Ser195, **Ser202**, **Thr205**, Thr231, Ser235, Ser396, Ser404
Cdk5	Thr181, Ser195, Ser199, **Ser202**, **Thr205**, Thr212, Thr214, Thr217, Thr231, Ser235, Thr373, Ser396, Ser404
MAPK	Ser46, Ser199, **Ser202**, Ser235, Ser396, Ser404, Ser422
PKA	Ser214, Ser234, **Ser262**, Ser293, Ser324, Ser356, Ser409, Ser416
PKC	Thr123, **Ser262**, Ser324
CaM Kinase II	Thr135, Ser137, Thr212, Ser214, **Ser262**, Ser356, Ser409, Ser416
Casein kinase II	Ser396, Ser404
Phosphatases	
PP1	Ser199, **Ser202**, Thr231, Ser396, Ser404
PP2A	Ser46, Thr231, Thr181, Ser199, **Ser202**,**Thr205**, **Ser262**, Ser396, Ser404

1.3.2.1. Other posttranslational modifications of tau protein

Glycosylation is an enzymatic process through which oligosaccharides are covalently attached to the side chain of polypeptides. There are two types of glycosylation according to the nature of glycosidic bonds: O-glycosylation and N-glycosylation. In tau protein, both types have been reported, but O-glycosylation occurs in unmodified tau whereas N-glycosylation occurs in hyperphosphorylated tau. It was reported that the inhibition of protein phosphatases, which induces tau hyperphosphorylation, also decreased O-glycosylations[49]. Thus, later, a reciprocal relationship between the O-glycosylation and phosphorylation was established, in which O-glycosylation negatively regulates tau phosphorylation[44].

Ubiquitination consists in the association of ubiquitin, a stress protein, with misfolded or damaged proteins to be degraded in an ATP-dependent manner. The tau protein can be ubiquitinated, however it has only been thus found when in NFTs. Despite the PHF-tau being highly ubiquitinated, it is not degraded and instead it is deposited as NFTs in AD brain[44,49].

In tau isolated from PHF the glycation was present which refers to a non-enzymatic linkage of a reducing sugar to a polypeptide. This glycation might be involved in the insolubility/aggregation of PHFs into NFTs since a cross-linking reaction leading to the formation of insoluble aggregates of proteins is often described as a consequence of proteins glycation. It was also found that glycated tau can also induce neuronal oxidative stress by generating oxygen free radicals[43,49].

Truncation in PHF-tau which consists in the cleavage of tau at the glutaminic acid residue 391 has also been observed. This modification could facilitate aberrant tau aggregation[44,49].

Lastly, the deamination is a chemical reaction in which an amide functional group is removed, that in tau protein is at aspargine or glutamine residues. This tau modification can also have a role in tau aggregation[49].

1.3.2. The physiological role and the pathological effects of tau phosphorylation

Phosphorylation at specific sites and when it is properly coordinated is the predominant mechanism that regulates the different roles of tau protein both in physiological and pathological conditions (Fig. 8).

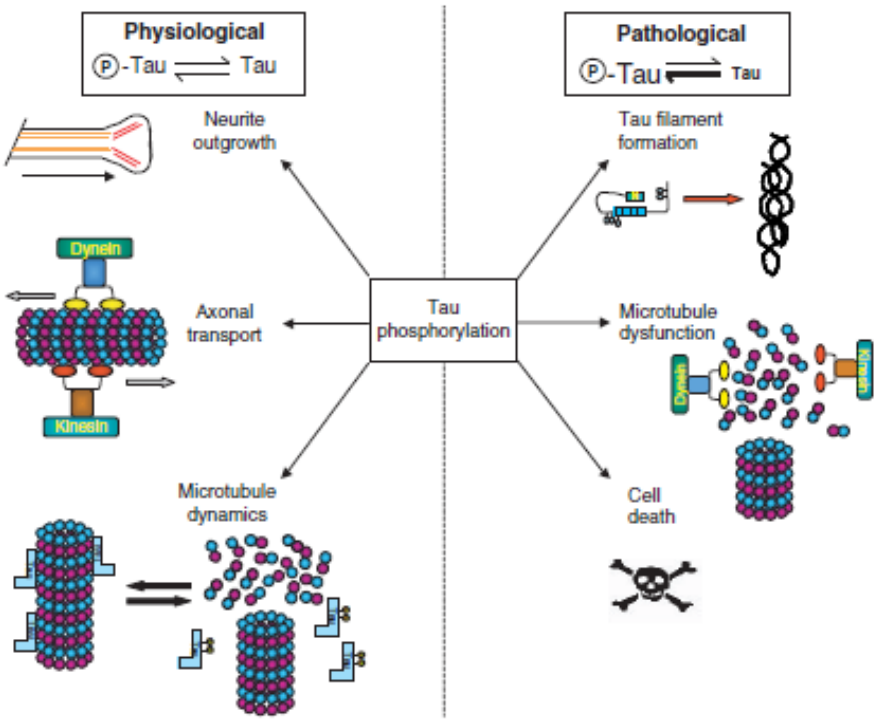

Figure 8 - Physiological and pathological roles of tau phosphorylation in the cell. Taken from Johnson et al. 2004.

Therefore, tau phosphorylation at normal physiological conditions controls a variety of processes such as microtubule binding and microtubule assembly[47,55], neurite outgrowth[56], axonal transport[57] and cell sorting[43]. As already mentioned tau protein binds to microtubules through the microtubule-binding domains. However the phosphorylation within this microtubule-binding domain, at the KXGS motifs, has been shown to reduce the binding of tau to microtubules which in physiological conditions facilitates the formation of cellular processes[47]. More specifically is the phosphorylation at residues Ser262 and Ser356 that is required for 'breaking' the binding between tau and microtubules[58]. Additionally, phosphorylation at Thr231 by GSK-3beta also plays a role in diminishing the ability of tau to bind to microtubules[55]. Tau phosphorylation, probably by GSK-3beta, controls the axonal transport given that when tau is phosphorylated the affinity to microtubules decreases which makes it less effective at competing with kinesin (a protein belonging to a class of motor proteins) for binding sites at microtubules and results in a proper anterogradely

organelle transport in neurons[49,57]. It is also thought that tau is involved in the regulation of neurite outgrowth and neuronal polarization[47]. This tau function is also controlled by phosphorylation, in the KXGS motifs, since phosphorylation at the proximo-distal gradient in neurons[56] can be verified. Tau is present in all cell compartments, but depending on the cell compartment the levels of tau phosphorylation also vary, thus contributing to the cell sorting[43]. It is also important that tau phosphorylation might have a developmental-specific role since it is more heavily phosphorylated in fetal than in adult brain[52].

However, under pathological conditions, tau is hyperphosphorylated, meaning that it is phosphorylated to a higher degree at normal, physiological sites, and at additional "pathological" sites which can affect its physiological role. Probably this hyperphosphorylation is due to an increase in kinase activity and/or a decrease in phosphatase activity that causes an imbalance in the phosphorylation/dephosphorylation of tau [43]. Actually, tau obtained from the brain of Alzheimer patients has 40 phosphorylation sites, 28 serines, 10 threonines and 2 tyrosines, the majority of which can be modified by GSK3[53]. This hyperphosphorylation seems to occur in a sequential manner in AD brain. Indeed, the phosphorylation of determined sites such as Ser262, Ser202, Thr205 and Thr231 was frequently observed in the brain of patients at an early stage of the disease[59-61].

Hyperphosphorylation of tau, in addition to facilitating tau assembly into PHF, causes a change in the stabilization of microtubules due to decreased microtubule binding which affects the overall organization leading to its dysfunction. Thus the localization and organization of other subcellular structures are affected, and ultimately increase cell death[22].

1.3.3. Tau binding proteins

Another important aspect of tau metabolism is its interacting partners, and actually many proteins have already been described as interacting with tau both *in vitro* and *in vivo* (Table 4). These include proteins such as tubulin[62], spectrin[63], calmodulin[63], actin[64], kinases involved in tau phosphorylation[49] such as GSK-3beta[65],

PP1 and PP2A[49], protein interacting with NIMA 1 (Pin1)[24,66], PSEN1[67-68], alpha-synuclein[69-71], fyn tyrosine kinase[72], 14-3-3ξ[73], the heat shock proteins HSP70 and HSP90[74-75] and ferritin[76].

The most well known tau binding protein is the tubulin as already mentioned above. When tau is phosphorylated its affinity to tubulin is reduced which contributes to self association and the formation of NFT[62]. Calmodulin is another tau binding protein that only binds tau in the presence of Ca^{2+} which prevents tau from interacting with tubulin leading to an inhibition of microtubule assembly. Tau also binds to spectrin, an important protein in the maintenance of plasma membrane integrity, but more studies are needed to clarify the physiological relevance of this interaction. Interestingly it has also been demonstrated that spectrin binds calmodulin, however a possible formation of a complex between these three proteins (spectrin-calmodulin-tau) still remains to be elucidated[49,63]. The interaction between tau protein and actin was demonstrated and this interaction of actin occurs through the tubulin-binding motif of tau[77]. This interaction might affect actin polymerization and modulation of its dynamics. It is also possible that this interaction helps in the organization of the cytoskeletal network through the interaction of microtubules to actin[78]. Another important interaction of tau protein is with Pin1, which is a member of the peptidyl-prolyl cis-trans isomerase group of proteins, and can regulate tau phosphorylation and facilitates its dephosphorylation by PP2A. In this case there is a particularity, since the interaction between these two proteins depends on the phosphorylation of tau: Pin1 only binds tau when phosphorylated at Thr231[24,49]. Besides Pin1, the PS1 can also regulate tau phosphorylation. So, PS1 binds directly to tau and also to the GSK-3beta, and both bind PS1 in the same domain[68]. It was observed that in PS1 mutants there is an increase in the association with the GSK-3beta that leads to increased phosphorylation of tau[65]. The alpha-synuclein is another known tau binding protein that stimulates tau phosphorylation through protein kinase A (PKA) and more specifically the interaction is between the C-terminal of alpha-synuclein and the microtubule-binding domain of tau. Since this interaction modulates tau phosphorylation, indirectly affecting the stability of microtubules[71].

Table 4 – Tau binding proteins and remarks of these interactions.

Protein	Remarks	Reference
Tubulin	- Promotes microtubule assembly	Tseng *et al.*, 1999
Calmodulin	- Block tau and tubulin interaction - Inhibition of microtubule assembly	Carlier *et al.*, 1984 Avila *et al.*, 2004
Spectrin	- Physiological relevance of this interaction is unknown	Carlier *et al.*, 1984
Actin	- Interaction affects actin polymerization and modulates its dynamics - Organization of cytoskeleton network	Yu *et al.*, 2006 Correas *et al.*, 1990
Pin-1	- Regulates tau phosphorylation - Facilitates dephosphorylation of tau by PP2A - Pin-1 binds to p-tau at Thr231	Gendron *et al.*, A Agarwal-mawal *et al.*, 2003 Avila *et al.*, 2004
PS1	- Regulates tau phosphorylation - Binds to GSK-3β	Ramirez *et al.*, 2001 Shepherd *et al.*, 2004 Takashina *et al.*, 1993
α-synuclein	- Stimulates PKA tau phosphorylation - Indirectly affects microtubules stability	Shepherd *et al.*, 2004 Jellinger *et al.*, 2011 Jensen *et al.*, 1999
Fyn tyrosine kinase	- Induces tyrosine phosphorylation of tau - Allows signals transduced through fyn to alter microtubule cytoskeleton	Lee *et al.*, 1998 Klein *et al.*, 2002
14-3-3ξ	- Stimulates tau phosphorylation through cAMP-dependent protein kinase	Hashiguchi *et al.*, 2000
HSP70 and HSP90	- Promotes tau solubility - Promotes tau binding to microtubules - Prevents tau aggregation	Dou *et al.*, 2003 Jinwal *et al.*, 2009

The fyn tyrosine kinase, a src-family non-receptor tyrosine kinase, interacts with a PXXP motif in the proline rich region of tau through its SH3 domains. The interaction results in the tyrosine phosphorylation of tau which results in the alterations on microtubules by signals transduced through fyn[64,79]. 14-3-3ξ is another protein involved in the abnormal phosphorylation in AD since it is an effector of tau protein phosphorylation. More specifically, this protein binds to microtubule-binding domain of tau (phosphorylated and nonphosphorylated) and stimulates tau phosphorylation through cAMP-dependent protein kinase[73]. Increasing levels of heat shock proteins (HSP70 and HSP90), which interact with tau, promote tau solubility and binding to microtubules preventing aggregation[74].

Currently, all the tau interacting proteins and all the role of these interactions have not been described.

1.4. Relationship between Abeta peptide and tau phosphorylation

An important issue in the pathogenesis of AD that is not clear is the association between the two histopathological hallmarks of the disease: amyloid plaques and neurofibrillary tangles. Currently, the most accepted hypothesis is the Amyloid hypothesis in which the accumulation of the Abeta peptide is a central event in the pathogenesis of AD (Fig. 9)[80-82]. According to this hypothesis the pathological processing of APP leads to an increased Abeta concentration in brain that is the main component of the amyloid plaques. The plaques lead to neuronal death and secondarily to tau pathology[80].

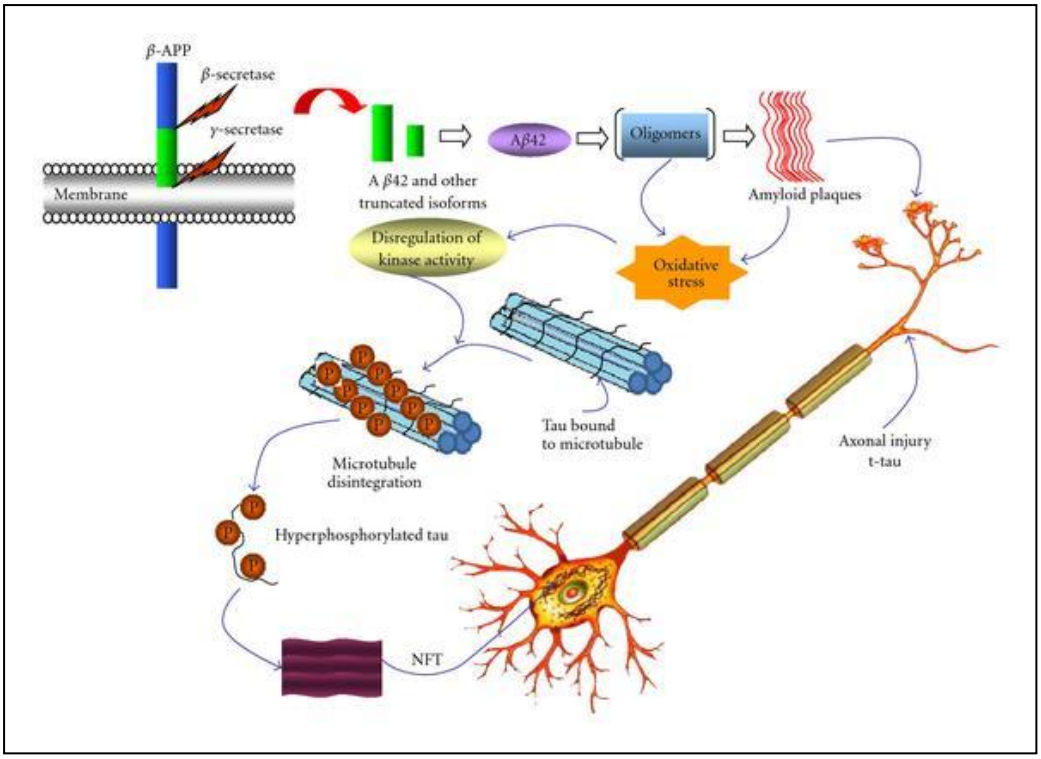

Figure 9 – Pathological cascades of AD. Primarily APP is cleaved by beta-secretase followed by gamma-secretase to produce Abeta$_{1-42}$ and other shorter fragments. Subsequently Abeta$_{1-42}$ aggregates resulting in oligomers and amyloid fibrils that eventually are deposited as amyloid plaques. The toxicity of oligomers and amyloid plaques can lead to the cascade of tau hyperphosphorylation. Tau normally binds to microtubules promoting stability. Following phosphorylation, tau dissociates from microtubules and instead aggregates into NFT which in turn can eventually cause increased cytoskeleton flexibility and neuronal death. Taken from Anoop et al., 2010.

Indeed, an earlier study from Takashima et al. described that 20µM of Abeta$_{1-43}$ and Abeta$_{25-35}$ are toxic for rat primary hippocampal cultures and induces an increase on tau phosphorylation mediated by the activation of the GSK-3beta and tau tubulin kinase[65]. In 2002, Zheng et al. reported that aggregated Abeta$_{25-35}$ induces tau phosphorylation at Thr181, Ser202 and Thr205 residues in a time and concentration dependent manner in rat septal cultured neurons and activated the MAPK and GSK-3beta[82]. Furthermore, Sul et al. showed that Abeta$_{25-35}$ increased tau phosphorylation at disease-relevant sites, such as Ser202, and then induced aggregation of tau proteins into NFTs, mediated by GSK-3beta. In this study, PC12 cells were exposed to 10µM of Abeta$_{25-35}$ for 24 hours[83]. In marked contrast to these findings, Davis et al. found that 100µM of aggregated Abeta$_{25-35}$ in 8-day-old rat primary cortical neuronal

cultures induced no obvious changes in the phosphorylation state of tau at Ser202 and Thr205 residue even if there is an evident toxic effect [84].

A more recent study, revealed that 10μM of Abeta$_{1-42}$ can also potentiate hyperphosphorylation of tau at Ser202 in differentiated PC12 cells in a time-dependent manner being that maximal increase could be achieved within 24 hours[85]. Another report from Bulbarelli et al. also showed that in hippocampal neurons the tau phosphorylation at Ser262 residue progressively increased during Abeta$_{1-42}$ (2,5μM) treatment and death significantly increased in a time-dependent manner reaching 60% in 24hours[86].

Moreover, it was shown that 5μM of Abeta$_{1-40}$ activates c-Abl tyrosine kinase for 30 minutes and 3 hours of exposure in rat hippocampal neurons, with a higher fold increase at the 30 minutes time point. Then c-Abl participates in Abeta-induced tau phosphorylation through cdk5 activation, by its Tyr15 phosphorylation [87]. Besides the effects of Abeta in mediating the activation of kinases involved in tau phosphorylation, it has also been described by Vintém et al. that Abeta (Abeta$_{1-40}$, Abeta$_{1-42}$ and Abeta$_{25-35}$) specifically inhibits different PP1 isoforms at low micromolar (20 and 50μM) concentrations both *in vitro* and *ex vivo*[88].

Thus, it is thought that Abeta binds to certain cell receptors and interacts with the signaling pathways that regulate the phosphorylation of tau protein, and multiple kinases and phosphatases are likely to be involved (Fig. 10) [89]. Furthermore, degradation of hyperphosphorylated tau by proteasome is inhibited by the actions of Abeta [87].

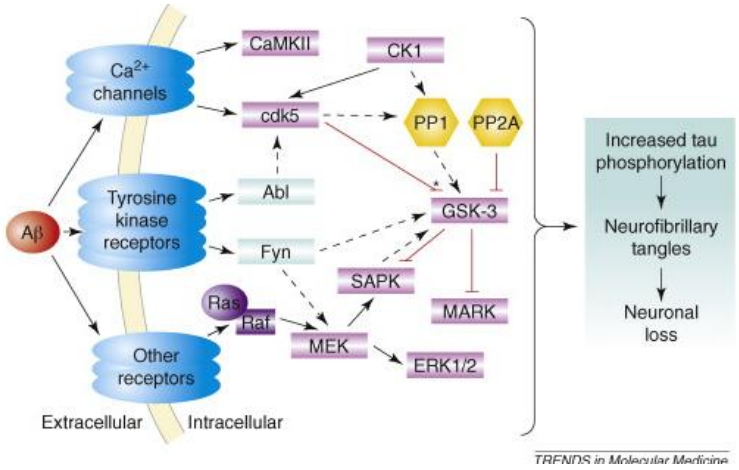

Figure 10 – Involvement of multiple interacting candidate tau kinases and phosphatases in Abeta-induced neurodegeneration. Extracellular Abeta activates candidate protein kinases through several different mechanisms, including those represented in this summary. Numerous interactions between protein serine/threonine (pink) and tyrosine (Abl and Fyn, pale blue) kinases as well as phosphatases (PP1 and PP2A, yellow) have been reported. Dashed and solid lines indicate indirect and direct interactions, respectively, and red lines indicate inhibitory relationships between enzymes. CaMKII: calcium-calmodulin kinase II; MARK: microtubule affinity-regulating kinase; MEK: mitogen-activated protein kinase; SAPK: stress-activated protein kinase. Taken from Hanger et al. (2009)[53].

Thus although several phosphorylation relevant events have been studied, the cross talk between signaling cascades and all the phosphatase and kinases involved have not been fully elucidated. Clarification of these aspects would undoubtedly be an important step towards developing novel effective therapeutic strategies.

2. Aims of the dissertation

Alzheimer´s disease is a neurodegenerative disorder characterized by two major histopathological hallmarks: extracellular AP and intracellular NFTs. The latter is primarily composed of hyperphosphorylated tau protein. According to the amyloid cascade hypothesis, the formation of AP precedes tau pathology, which in turn is induced by Abeta oligomers.

However some questions remain regarding the pathological hyperphosphorylation of tau protein. Another important feature of tau metabolism is its interacting proteins; more importantly the proteins that interact specifically with phosphorylated tau (p-tau). The different tau interacting proteins provide relevant information with respect to pathological and protective pathways that are active at different stages of the disease process. These pathways are attractive targets for therapeutical intervention.

Thus the specific aims of this dissertation are to:

- Determine the role of Abeta on tau phosphorylation;
- Establish the protein phosphatases involved in tau dephosphorylation.
- Identify the proteins that interact with the tau protein and with phosphorylated tau (p-tau) and evaluate the effects of Abeta on these interactions;

3. Materials and Methods

3.1. Antibodies

The following primary antibodies were used: rabbit polyclonal p-tau Ser262 antibody (Santa Cruz Biotechnology, Inc) directed against the phosphorylated tau at Ser262; mouse monoclonal anti-phosphorylated tau antibody, clone AT8 (Pierce) which specifically recognizes phosphorylated tau at Ser202 and Thr205; mouse monoclonal anti-tau antibody, clone Tau-5 (Millipore) to detect all phosphorylated and non-phosphorylated isoforms of tau; and mouse monoclonal anti-β-Tubulin antibody (Invitrogen) directed against β-Tubulin (table 5).

Horseradish peroxidase-conjugated anti-mouse (1:5000) and anti-rabbit (1:5000) IgGs were used as secondary antibodies (Amersham Pharmacia) for immunoblotting (table 5).

Table 5- Summary of the antibodies used to detect target proteins and specific dilutions used for the different assays. The specific dilutions used for the different assays are also indicated. IB: Immunoblotting; IP: immunoprecipitation.

Antibody	Target Protein/Epitope	Dilution	Expected bands site (KDa)
p-tau Ser262	p-tau at Ser 262	IB dilution: 1:1000 IP dilution: 1:150	46-68
AT8	p-tau at Ser202 and Thr205	IB dilution: 1:1000	46-68
Tau-5	total tau	IB dilution: 1:500 IP dilution: 1:100	46-68
Anti - β-Tubulin	β-Tubulin	IB dilution: 1:1000	50

3.2. Cell culture

3.2.1. Primary Neuronal Cultures

Rat cortical and hippocampal cultures were established from Wistar Hannover 18 days rat embryos whose mother was euthanized by rapid cervical dislocation. After cortex and hippocampus dissection, tissues were dissociated with trypsin (0.23 or 2.25 mg/ml for cortical or hippocampal cultures, respectively) and deoxyribonuclease I (0.15 or 1.5 mg/ml for cortical or hippocampal cultures, respectively) in Hank's balanced salt solution (HBSS) for 5 minutes at 37^{o}C. Cells were washed with HBSS supplemented with 10% FBS to stop trypsinization, centrifuged at 1000 rpm for 2 minutes, and further washed and centrifuged with HBSS for serum withdraw. Cells pellet was ressuspended in complete Neurobasal medium (Gibco), a serum-free medium combination, which is supplemented with 2% NB27 (Gibco). The medium was also supplemented with glutamine (0.5 mM), gentamicin (60µg/ml) and with or without glutamate (25µM) for hippocampal or cortical cultures, respectively. Viability and cellular concentration were assessed by using the Trypan Blue excluding dye (Sigma). For immunoblotting analysis cortical and hippocampal primary neuronal cultures were plated on poly-D-lysine-coated six-well plates at a density of $0.8x10^6$ cells per well. For immunoprecipitation analysis, cortical primary neuronal cultures were plated on poly-D-lysine-coated 100 mm plates at a density of $6.0x10^6$ cells per plate. Cells were maintained in 12 ml of Neurobasal medium in 100 mm plates and 2 ml of Neurobasal medium in six-well plates in an atmosphere of 5% CO_2 at 37^{o}C for 10 days before being used for experimental procedures. Five days after plating, ¼ of medium was replaced with glutamate-free complete Neurobasal medium for both cortical and hippocampal cultures.

3.3. Cortical and hippocampal neurons treatment with Abeta

To evaluate the effects of Abeta on tau phosphorylation at residues Ser202, Thr205 and Ser262, 10 days cortical and hippocampal neurons were incubated with different concentrations of Abeta peptides for different periods of time.

Synthetic $Abeta_{1-42}$, $Abeta_{42-1}$ and $Abeta_{25-35}$ peptides (American Peptide) were dissolved in water to prepare 1mM stock solutions. Exposure of cells to Abeta was preceded by an aggregation step, which was achieved by incubating the different peptides for 48 hours at 37^{o}C with PBS at concentration of 100µM. 10 days cortical and hippocampal neurons were used and washed twice with PBS before Abeta treatments. Cells were then incubated for 30 minutes, 3 hours and 24 hours in Neurobasal medium free of B27 with different Abeta concentrations: 0,5µM $Abeta_{1-42}$, 2µM $Abeta_{1-42}$, 10µM $Abeta_{1-42}$ and 20µM $Abeta_{1-42}$ for immunoblotting analysis.

After the specific treatments, media and cells were collected. The media were centrifuged at 300 g for 5 minutes, the supernatant transferred to a new microtube and then made up to 1% SDS and boiled for 10 minutes. The cells were collected with RIPA buffer and sonicated twice during 5 seconds. RIPA buffer was used because enables an efficient cell lysis and protein solubilization while avoiding protein degradation and interference with the protein´s immunoreactivity and biological activity.

3.4. Cortical and hippocampal neurons treatment with protein phosphatase inhibitors

In order to establish the protein phosphatases (PPs) involved on tau dephosphorylation at residues Ser202, Thr205 and Ser202, rat primary neuronal cultures were incubated with a PPs inhibitor: okadaic acid..

Stock solution of okadaic acid (0.5µM, Calbiochem) was prepared and used for the following incubations. Rat primary cortical neuronal cultures were plated as described above (section 3.2.1.) and washed twice with PBS before okadaic acid treatments. 10 days cortical and hippocampal neurons were incubated with okadaic

acid in Neurobasal medium free of B27 for 30 minutes and 3 hours at different concentrations in order to inhibit specifically different PPs (table 6).

After the appropriate treatments, media and cells were collected as described in section 3.3.

Table 6 – Range of IC50 values of protein phosphatase inhibition. All Values expressed as nanomolar (nM). PP, protein phosphatase; IC50, 50% inhibition concentration. Adapted from Swingle et al., 2007 [90].

Inhibition of Ser/Thr Protein Phosphatase activity (IC_{50})						
Drug	**PP1**	**PP2A**	**PP2B**	**PP4**	**PP5**	**PP7**
Okadaic acid (OA)	15-50	0.1 – 0.3	4000	0,1	3.5	>1000

3.5. BCA protein quantification assay

Protein content determination of the cellular lysates was carried out using the BCA Protein Assay (Pierce). This assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for colorimetric detection and quantification of total protein. The method combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the biuret reaction) with high sensitivity and selective colorimetric detection of the cuprous cation (Cu^{+}) using a unique reagent containing bicinchoninic acid. The purple-coloured reaction product of this assay is formed by chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration over a working range of 20µg/ml to 2000µg/ml. The standards were prepared as described in table 7, and final volume of each was equal to 50µL.

Table 7 - Standard preparation; BSA, bovine serum albumin; WR, working reagent.

Standards	BSA (µL)	Extraction buffer (µL)	Protein Mass (µg)
P0	0	50	0
P1	1	49	2
P2	2	48	4
P3	5	45	10
P4	10	40	20
P5	20	30	40
P6	40	10	80

Briefly, the quantitative analyses were carried out using 10 µL of the collected cell lysates (IB) and 5 µL of the collected cell lysates (IP) (final volumes of 50 µL were adjusted with extraction buffer). After preparation of both samples and standards they were incubated at 37^{o}C for 30 minutes with 1 ml of working reagent, which is prepared with 50 parts of reagent A to 1 part of the reagent B. After incubation the tubes were cooled to RT and the absorbances were then measured at 562 nm. A standard curve was prepared by plotting the O.D. value for each BSA standard against its concentration. Using this standard curve the protein concentration of each sample was determined. Duplicates of samples and standards were always prepared.

3.6. SDS - Polyacrylamide gel electrophoresis

SDS–Polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical technique of electrophoresis of proteins on polyacrylamide gels under conditions that ensure dissociation and characterization of proteins and peptides in mixtures. In SDS-PAGE proteins are separated according their molecular weight and negative net charge due to SDS-amino acid binding since SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone, conferring a negative charge to the polypeptide in proportion to its length.

Samples were subjected to 5-20% gradient SDS-PAGE in a Hoefer electrophoresis system. The gradient gel were prepared and allowed to polymerize

for 45 minutes at room temperature. Subsequently, the stacking gel solution was prepared and loaded on the top of gradient gel. A comb was inserted and the gel was left to polymerize for 30 minutes at room temperature. Prior to loading, the samples were prepared by the addition of ¼ volume of 4x Loading buffer (LB), boiled for 10 minutes and spinned down. The samples were carefully loaded into the wells, and gels were run at 90 mA for approximately 3 hours. Molecular weight markers (Kaleidoscope Prestained Standards and Dual Colour Prestained Standards – Broad range, Bio Rad) were also loaded and resolved side-by-side with the samples.

3.7. Immunoblotting

After electrophoresis, proteins can be transferred from a gel to a solid support, while keeping their positions and then can be visualized with specific antibodies. In this work, proteins were electrophoretic transferred to nitrocellulose membranes (Whatman®) for 18 hours at 200 mA. After transfer the proteins were detected using specific antibodies against the proteins of interest. Once the immunoblotting protocol is antibody specific, the protocols used were summarized in the table 8.

Table 8 – General immunoblotting protocol used for each antibody. ON, overnight; RT, room-temperature; min, minutes; h, hours.

Antibody	Hydration	Blocking Agent	Primary Antibody	Washings	Secondary Antibody	Washings	Detection method
p-tau Ser262	-1x TBS -5 min	-5% BSA in 1x TBS-T -4 h RT	- 5% BSA in 1x TBS-T - 4 h RT + ON at 4ºC	-1x TBST - 3 times - 10 min	-5% BSA in 1x TBST - 2 h RT	-1x TBST - 3 times - 10 mi	ECL Plus
AT8	- 1x TBS - 5 min	-5% low fat dry milk in 1x TBS-T - 4 h RT	- 5% low fat dry milk in 1x TBS-T - 4 h RT + ON at 4ºC	-1x TBST - 3 times - 10 min	-3% low fat dry milk in 1x TBS-T - 2 h RT	-1x TBST - 3 times - 10 min	ECL Plus
Tau-5	- 1x TBS -5 min	- 5% BSA in 1x TBS-T - 4 h RT	- 5% BSA in 1x TBS-T - 4 h RT + ON at 4ºC	-1x TBST - 3 times - 10 min	-5% BSA in 1x TBS-T - 2 h RT	-1x TBST - 3 times - 10 min	ECL
β-Tubulin	- 1x TBS -5 min	-5% low fat dry milk in 1x TBS-T - 4 h RT	- 5% low fat dry milk in 1x TBS-T - 2 h RT	-1x TBST - 3 times - 10 min	-3% low fat dry milk in 1x TBS-T - 2 h RT	-1x TBST - 3 times - 10 min	ECL

Immunoblotting of the transferred proteins was performed by initially soaking the membranes in 1x TBS for 5 minutes and then blocking non-specific binding sites of the primary antibody by incubating the membrane with 5% non-fat dry milk/5% BSA in 1x TBST for 4 hours. The membrane was further incubated with the primary antibody, washed with 1x TBST, incubated with secondary antibody, washed again in 1x TBST and then incubated for 1 minute at RT with ECL detection kit or for 5 minutes with the ECL plus detection kit (GE Healthcare) in a dark room, as described in table 8 for each specific primary antibody.

ECL™ Western blotting from GE Healthcare is a chemiluminescent (light emitting non-radioactive) method for detection of immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. The ECL reaction is based on the oxidation of the cyclic diacylhydrazide luminal and ECL plus utilizes a technology based on the enzymatic generation of an acridinium

ester, which produces a more sensitive light emission of longer durations than ECL. After exposure to X-Ray film (Kodak) films were developed and fixed with appropriate solutions (Kodak).

3.8. Co- immunoprecipitation and mass spectrometry analysis

To evaluate the effects of Abeta on tau binding proteins, more specifically on p-tau binding proteins rat primary cortical neuronal cultures were incubated with the Abeta peptide and okadaic acid for 3 hours.

In our experimental procedure the immunoprecipitation was carried out using Dynabeads® Protein G (Invitrogen) because magnetic handling is fast and easier, efficient, extremely gentle on our target proteins and eliminate background since there are less non-specific binding. The principle was the same for other immunoprecipitation procedures: primary antibody is added to the Dynabeads® Protein G and during a short incubation the antibody will bind to the Dynabeads via their Fc region. The tube is then placed on a Dynal magnet, where the beads will migrate to the side of the tube facing the magnet and allow for easy removal of the supernatant. The bead-antibody complex may now be used for immunoprecipitation. Bound material is easily collected utilizing the unique magnetic properties of the Dynabeads® (Fig. 11). Magnetic separation facilitates washing, buffer changes and elution.

Stock solution of aggregated $Abeta_{1-42}$ and okadaic acid were prepared as described in section 3.3 and 3.4. Cells were plated as described above (section 3.2.1), washed twice with PBS and incubated for 3 hours with 10µM $Abeta_{1-42}$ and 0.25µM okadaic acid in Neurobasal medium free of B27. After the appropriate treatments, the media were removed and cells were gently scrapped off the culture plate with MOPS lysis buffer and the lysates collected. The lysates were sonicated three times during 10 seconds. The MOPS buffer was used because according the recommendations from Kinexus the lysis must be performed with a pH buffering agent that do not contain reactive amine groups, and for example, the TRIS cannot be used.

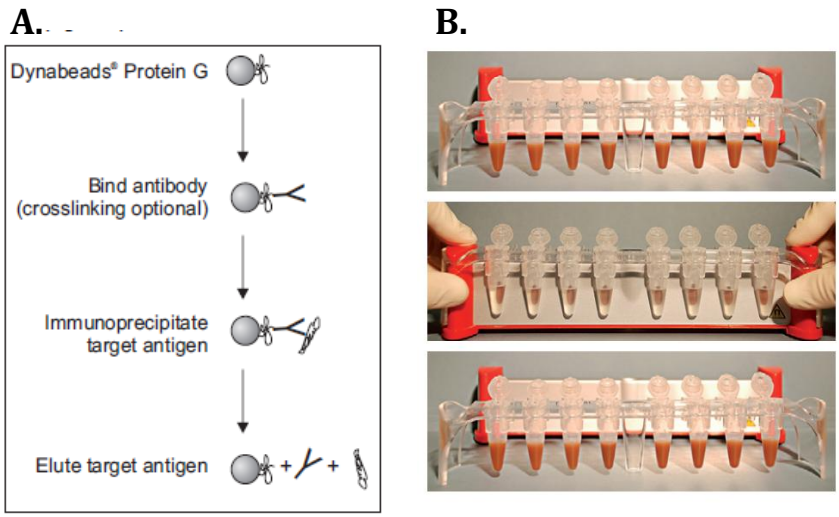

Figure 11 –**A**. Principle of immunoprecipitation of antigen using Dynabeads Protein G; **B**. Example of a Dynal magnet. Taken from the manufacturer datasheet (Invitrogen).

Thus after BCA protein quantification of samples (as described above) mass normalizes lysates were precleared with 15 µL Dynabeads protein G for 1 hour at 4^{0}C with agitation. Then the supernatant was transferred to a new microtube and 60 µL of Dynabeads protein G plus the primary antibody (Tau-5 and p-Tau Ser262 at respective dilutions) was added and incubated overnight with shaking at 4^{0}C. The supernatant was then removed and the beads washed four times with 400 µL of washing solution (3% BSA/PBS) for 15 minutes with agitation at 4^{0}C. After the last wash the supernatant was fully discarded and the beads were resuspended in 45 µL of 1xLB (Loading buffer) and boiled for 10 minutes. The immnunoprecipitates were frozen at -80^{0}C and shipped to Kinexus to Mass Spectrometry Services PIMS (Protein ID by mass spectrometry, Fig.11). Our immunoprecipitates were subjected to SDS-PAGE and the gel stained with Coomassie blue. Then the bands of interest were excised from the SDS-PAGE gel, subjected to trypsin digestion and analyzed by high-resolution mass spectrometry, performed on an LC-MS/MS (Thermo Electron LTQ – Orbitrap), to determine their accurate masses. Finally, a search of the appropriate protein sequence databases using a Mascot search was performed to determine a matching mass pattern that can lead to a definitive identification of the protein.

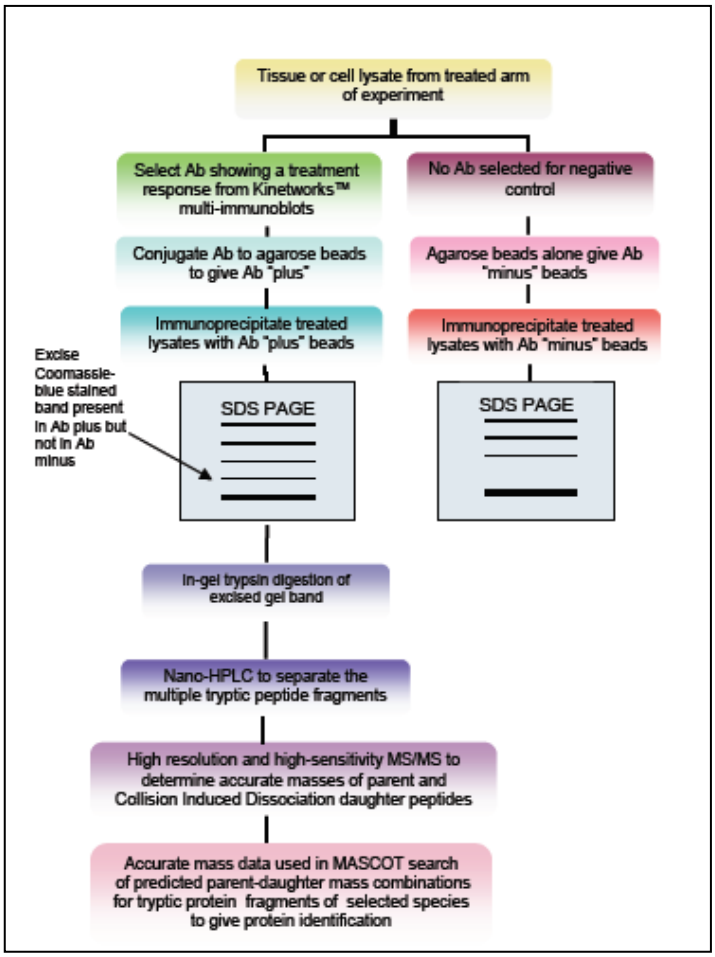

Figure 12 – Methodology of protein identification by mass spectrometry using agarose beads without antibody as a negative control (Taken from the manufacturer datasheet). In our experimental procedure we performed the immunoprecipitations, using tau specific antibodies and Dynabeads protein G instead of agarose beads (Aveiro Laboratory). As negative control we use Dynabeads without antibody (Aveiro laboratory). The immunoprecipitates were sent to Kinexus and were further analyzed by SDS-PAGE followed by LC-MS/MS (Kinexus).

3.9. Quantification and statistical analysis

Quantitative analyses of immunoblots were performed using the Quantity One densitometry software (Bio-Rad). This system quantifies band intensity and correlates it to protein levels.

4. Results

4.1. Abeta effects on tau phosphorylation at Ser202, Thr205 and Ser262 residues

4.1.1. Rat primary cortical neuronal cultures

Recent studies have provided new evidence showing that Abeta peptide and tau reciprocally interact in mediating neurodegenerative processes. However, the functional relevance between Abeta and hyperphosphorylated tau in the pathway leading to neurofibrillary pathology is unclear. To determine whether Abeta could promote tau phosphorylation, we exposed rat primary cortical neurons to aggregated $Abeta_{1-42}$ at increasing concentrations (0.5, 2, 10 and 20μM) for 30 minutes, 3 and 24 hours. Following this period the cell lysates were collected and further analyzed by SDS-PAGE and immunoblotting using Tau5, AT8 (phospho-tau Ser202 and Thr205), p-tau Ser262 and β-Tubulin antibodies. The results are presented in Fig. 13 and Fig. 14, the antibodies used in each of the figures is indicated in figure legend.

As indicated in Fig. 13A, when cortical neurons were incubated with increasing concentrations of aggregated $Abeta_{1-42}$ for 30 minutes and 3 hours, the levels of total tau protein detected with Tau5 antibody did not fluctuate markedly although there was a tendency to decrease. In contrast for the 24 hour period a decrease in total tau protein levels was detected, particularly with the two higher Abeta concentrations.

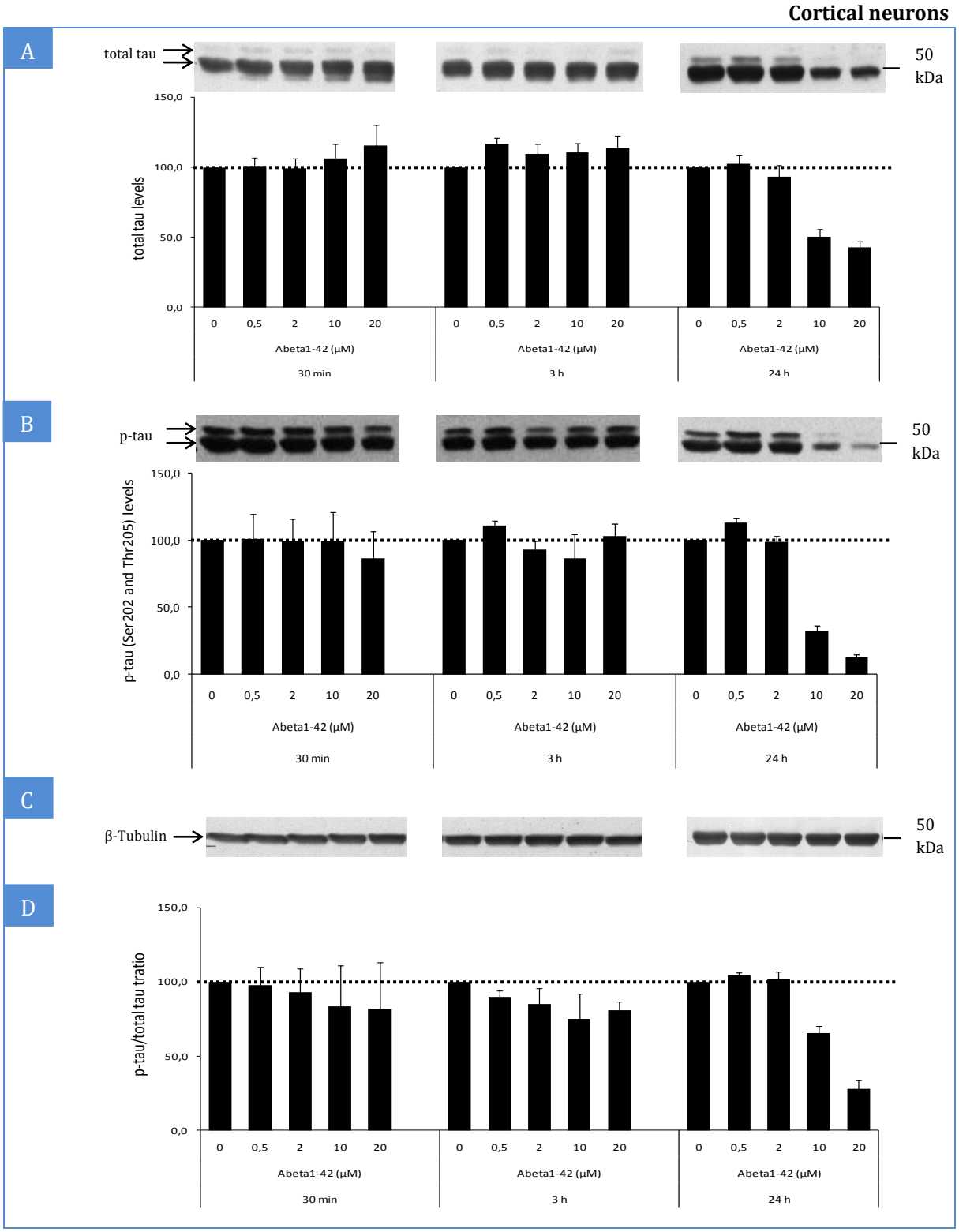

Figure 13 - Abeta effects on tau phosphorylation at both Ser202 and Thr205 residues. Rat primary cortical neuronal cultures were incubated at 37ºC in Neurobasal medium free of B27 for 30 minutes, 3 hours and 24 hours with aggregated $Abeta_{1-42}$ (0.5, 2, 10 and 20μM). Cell lysates were collected and analyzed by immunoblotting with Tau5 antibody which recognizes total tau (**A**), AT8 antibody which recognizes tau phosphorylated at Ser202 and Thr205 residues (**B**) and β-Tubulin antibody (**C**). **D** – Ratio between phospho-tau and total tau. Data was obtained from triplicate experiments (n=3).

The state of tau phosphorylation (Fig. 13B) at both Ser202 and Thr205 residues (recognized by AT8 antibody), upon treatment for 30 minutes and 3 hours with aggregated $Abeta_{1-42}$ remains almost the same as the controls. However, at the 3 hour time point of incubation, with 0.5μM of aggregated $Abeta_{1-42}$, we observed a slight increase of tau phosphorylation of approximately 10% and this increase is also evident at the 24 hour time point. Additionally, after 24 hours of Abeta treatment, there is a robust decrease of tau phosphorylation with 10μM and 20μM of aggregated $Abeta_{1-42}$, approximately 70% and 85%, respectively (Fig. 13B). β-Tubulin was used as loading control (Fig. 13C). In order to have a clear idea of the phosphorylation pattern on these 2 residues, following $Abeta_{1-42}$ treatment we calculated the ratio of phosphorylated tau protein (p-tau) versus total tau protein (total tau), Fig. 13D. The analysis of this ratio revealed that tau phosphorylation upon $Abeta_{1-42}$ treatment, Ser202 and Thr205 exhibits a tendency to decrease after 30 minutes and 3 hours. In contrast after 24 hours of treatment with $Abeta_{1-42}$ the tendency to decrease is evident at the higher $Abeta_{1-42}$ concentrations (10μM and 20μM).

Regarding the levels of tau phosphorylation at Ser262, residue recognized by p-tau Ser262 antibody (Fig. 14B), quantifications revealed that it remains almost unvaried upon $Abeta_{1-42}$ treatment for 30 minutes. However, with 3 hours after Abeta treatment we observed a different pattern. At 0.5μM and 2μM $Abeta_{1-42}$ concentrations, tau phosphorylation at Ser262 increased by approximately 10-20%, but at 20μM $Abeta_{1-42}$ concentration the response was reversed and tau phosphorylation decrease to 70% of control levels. At 24 hours of treatment the pattern of tau phosphorylation is similar to that observed at 3 hours, although this biphasic response is more marked.

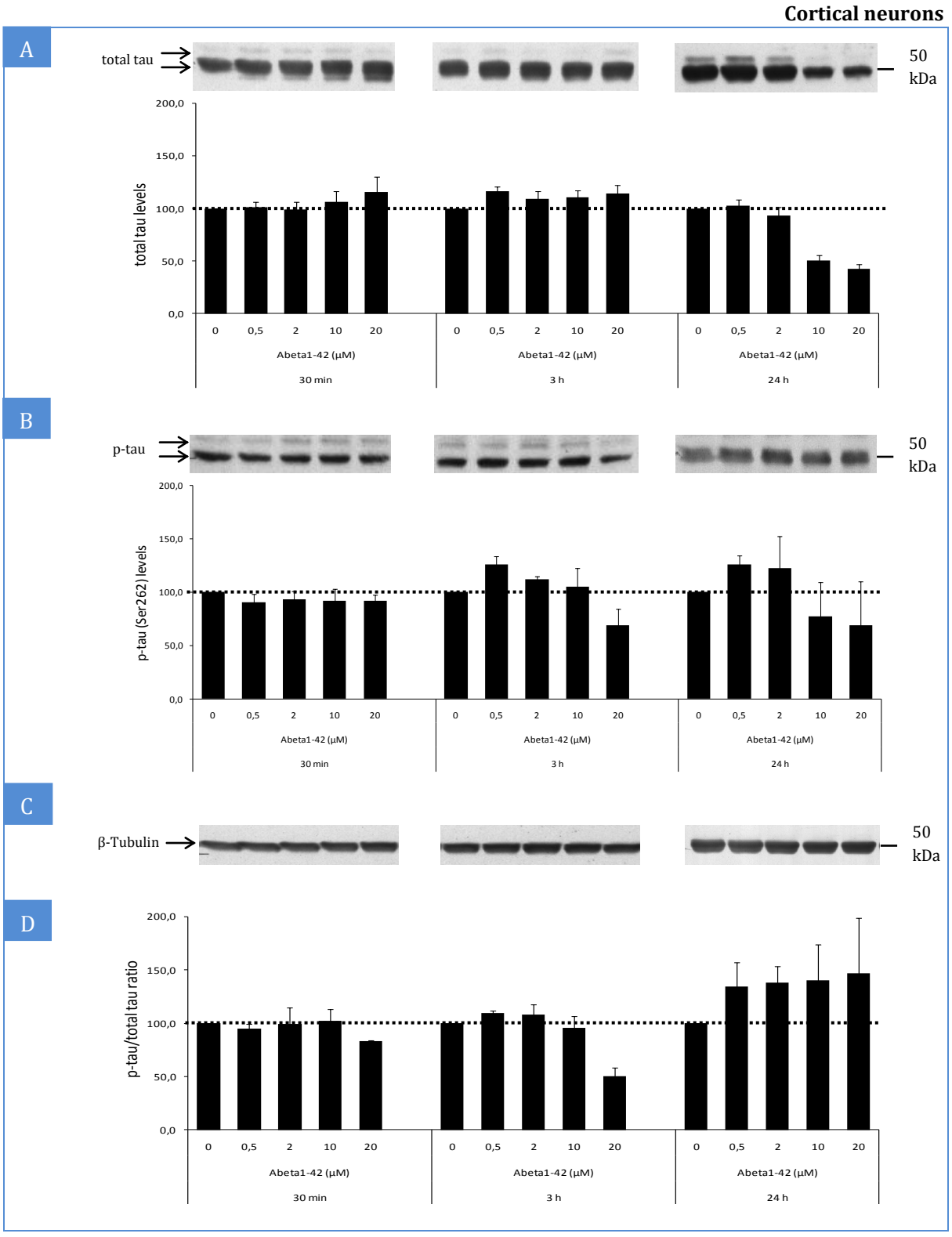

Figure 14 - Abeta effects on tau phosphorylation at Ser262 residue. Rat primary cortical neuronal cultures were incubated at 37ºC in Neurobasal medium free of B27 for 30 minutes, 3 hours and 24 hours with aggregated $Abeta_{1-42}$ (0.5, 2, 10 and 20μM). Cell lysates were collected and analyzed by immunoblotting with Tau5 antibody which recognizes total tau (**A**) p-tau Ser262 antibody which recognizes tau phosphorylated at Ser262 residue (**B**) and β-Tubulin antibody (**C**). **D** – Ratio between phospho-tau at residue Ser262 and total tau. Data was obtained from triplicate experiments (n=3).

Consequently the ratio of phospho-tau (Ser262) versus total tau was calculated, Fig. 14D. This ratio shows that upon 30 minutes and 3 hours of Abeta treatment phosphorylation at the residue drops only for the higher $Abeta_{1-42}$ concentration. However, when we treat the cortical neurons for 24 hours the increase of tau phosphorylation on Ser262 residue increases as a percentage of total tau (Fig. 14D). At this time point β-Tubulin (Fig. 14C) remains constant.

4.1.2. Rat primary hippocampal neuronal cultures

To evaluate the effects of Abeta peptide on tau phosphorylation in rat primary hippocampal neuronal cultures, we treated these cells as for cortical neuronal cultures described in section 4.1.1. The results regarding the effect of $Abeta_{1-42}$ treatment on tau phosphorylation on Ser202 and Thr205 residues are presented in Fig. 15. In Fig. 15A we can observe that upon 30 minutes of exposure to 0.5, 2 and 10μM of aggregated $Abeta_{1-42}$ the levels of total tau protein detected with Tau5 antibody decrease in a dose-dependent manner, although there is an increase similar to control with 20μM of $Abeta_{1-42}$. This pattern is the same after 24 hours of exposure, although the decrease is maintained for all $Abeta_{1-42}$ concentrations. At 3 hours of exposure the increasing Abeta concentrations do not affect the total tau protein levels.

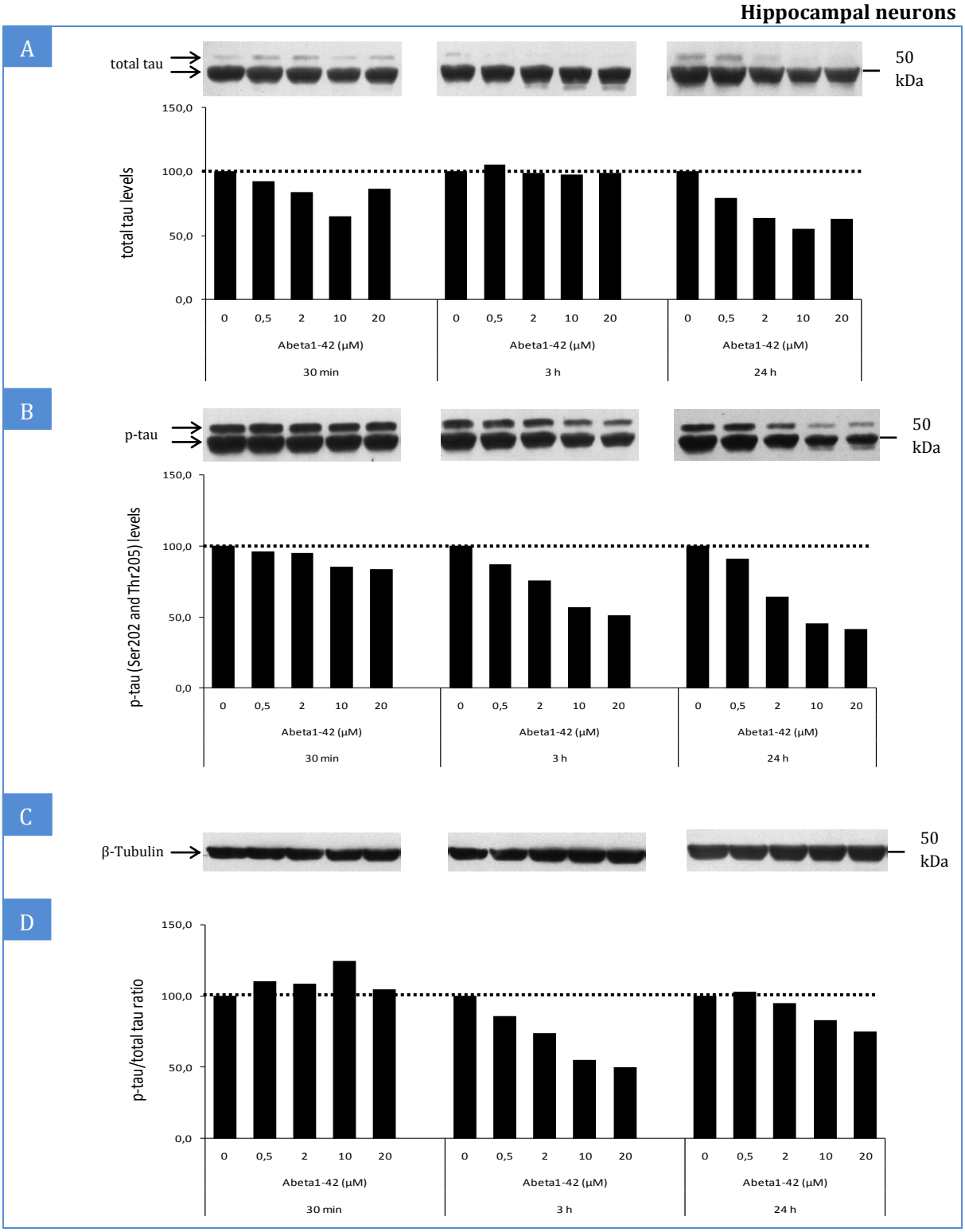

Figure 15 - Abeta effects on tau phosphorylation at both Ser202 and Thr205 residues. Rat primary hippocampal neuronal cultures were incubated at 37ºC in Neurobasal medium free of B27 for 30 minutes, 3 hours and 24 hours with aggregated $Abeta_{1-42}$ (0.5, 2, 10 and 20μM). Cell lysates were collected and analyzed by immunoblotting with Tau5 antibody which recognizes total tau (**A**), AT8 antibody which recognizes tau phosphorylated at residues Ser202 and Thr205 (**B**) and β-Tubulin (**C**). **D** – Ratio between phospho-tau at residues Ser202 and Thr205 and total tau. Data was obtained from duplicate experiments (n=2).

For hippocampal neurons treated with $Abeta_{1-42}$, the immunoblots were probed with the AT8 antibody (Fig. 15B) and we can observe that levels of tau phosphorylated at Ser202 and Thr205 residues decreased in a concentration dependent manner at all the three time points, being more evident for the 24 hour treatment. The actual pattern of percentage tau phosphorylation at both these residues is revealed by the p-tau/total tau ratio (Fig. 15D). Therefore, upon 30 minutes of treatment we can observe a tendency of increased tau phosphorylation with 0.5, 2 and 10μM of $Abeta_{1-42}$. With 3 and 24 hours of treatment there is a decrease on tau phosphorylation, as a percentage of total tau, in a dose- dependent manner, that is more evident at the 3 hour time point.

The absolute phosphorylation levels of tau protein at residue Ser262 are shown in Fig. 16B. These decrease upon treatment with $Abeta_{1-42}$, in a dose-dependent manner, for the three periods of time, being more evident at 3 hours of exposure. However, when is taken into account the phosphorylation levels in proportion to the total tau protein expression (Fig. 16D) the pattern is similar to that observed with p-tau alone (Fig. 16B), being that at both conditions, 30 minutes and 3 hours of treatment, it remains almost the same with exception of the incubation with 10μM of $Abeta_{1-42}$ for 30 minutes, where we can observe a slight increase on phosphorylated tau levels. When hippocampal neurons are exposed to increasing concentrations of $Abeta_{1-42}$ for 24 hours it causes an increase on tau phosphorylation that is dose-dependent until the 10μM and not so evident with 20μM.

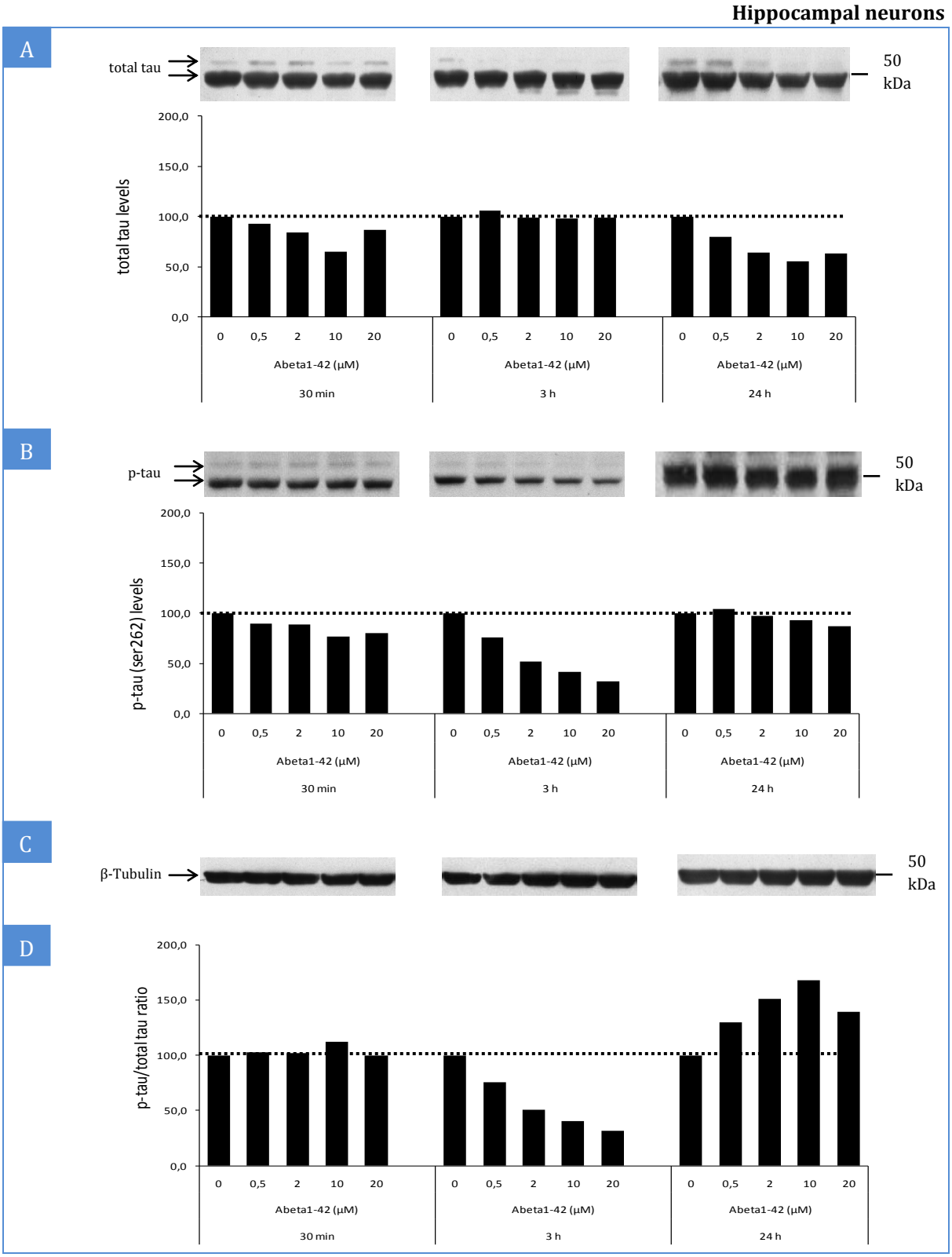

Figure 16 - Abeta effects on tau phosphorylation at Ser262 residues. Rat primary hippocampal neuronal cultures were incubated at 37ºC in Neurobasal medium free of B27 for 30 minutes, 3 hours and 24 hours with aggregated Abeta$_{1\text{-}42}$ (0.5, 2, 10 and 20μM). Cell lysates were collected and analyzed by immunoblotting with Tau5 antibody which recognizes total tau (**A**), p-tau Ser262 antibody which recognizes tau phosphorylated at Ser262 (**B**) and β-Tubulin antibody (**C**). **D** – Ratio between phospho-tau at residue Ser262 and total tau. Data was obtained from duplicate experiments (n=2).

4.2. Protein phosphatases involved in tau dephosphorylation at Ser202, Thr205 and Ser262 residues

4.2.1. Rat primary cortical neuronal cultures

To elucidate the probable protein phosphatases (PPs) involved on tau dephosphorylation at Ser 202, Thr205 and Ser262 residues, we examined the effect of a PP inhibitor, okadaic acid (OA). Cortical neurons were exposed to different concentrations of OA for two periods of time (30 minutes and 3 hours). As described above, cell lysates were collected and separated by SDS-PAGE, and further analyzed by immunoblotting with Tau5, AT8, p-tau Ser262 and β-Tubulin antibodies.

4.2.1.1. Okadaic acid

Upon 30 minutes and 3 hours exposure of cortical neurons to increasing concentrations of OA (0.1, 0.25, 50, 500 and 5 μM) the levels of total tau protein do not significantly differ from the control (Fig. 17A). There is, however, for the two higher concentrations of OA (500 and 5 μM) for both 30 minutes and 3 hours of exposure, a shift that is indicative of a significant difference in electrophoretic mobility, consistent with increased levels of tau phosphorylation, as already described[54]. Relative to phosphorylated tau at Ser202 and Thr205 residues (Fig. 17B) we can observe that lower concentrations of OA (0.1 and 0.25μM), when PP2A is inhibited, are not sufficient to induce alterations in the level of phosphorylation for both time points.

Cortical neurons

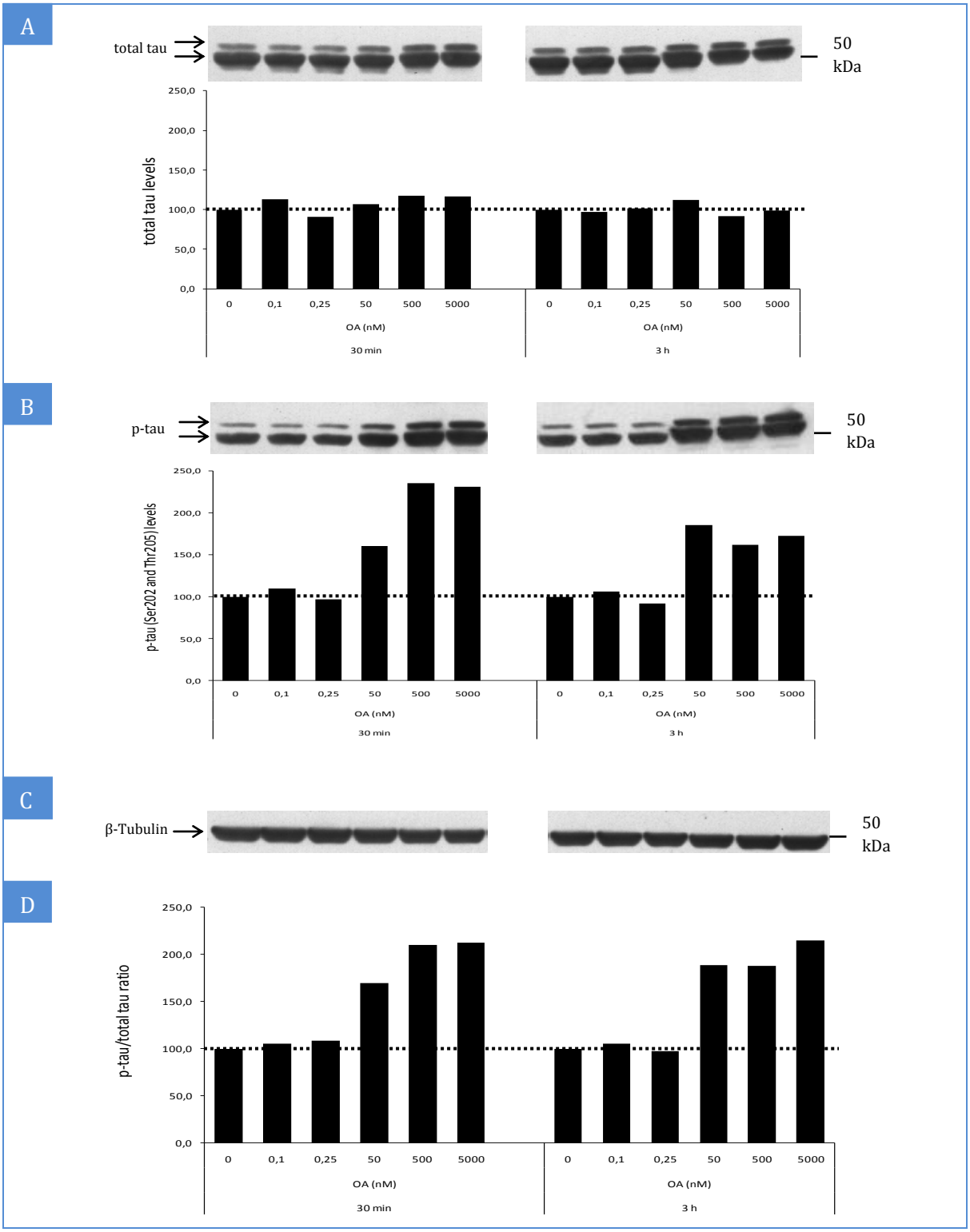

Figure 17 - Okadaic acid effects on tau phosphorylation at both Ser202 and Thr205 residues. Rat primary cortical neuronal cultures were incubated at 37ºC in Neurobasal medium free of B27 for 30 minutes and 3 hours with okadaic acid (0.1, 0.25, 50, 500 and 5000 nM). Cell lysates were collected and analyzed by immunoblotting with Tau5 antibody which recognizes total tau (**A**), AT8 antibody which recognizes tau phosphorylated at Ser202 and Thr205 residues (**B**) and β-Tubulin antibody (**C**). **D** – Ratio between phospho-tau at Ser202 and Thr205 and total tau. Data was obtained from duplicate experiments (n=2).

Exposure to 50 nM, 500 nM and 5 μM of OA already induces a marked increase on tau phosphorylation both at 30 minutes and 3 hours, suggesting an involvement of PP1 on tau dephosphorylation at both these residues (Ser202 and Thr205). Since the total tau protein expression does not significantly alter, as mentioned above, the pattern of tau phosphorylation observed in the phospho-tau/total tau ratio (Fig. 17D) is similar to the pattern already discussed (Fig. 17B).

The pattern of protein phosphatase inhibition with respect to tau phosphorylation of Ser262 is different to the situation described above. At 0.1 nM OA concentration there appears to be an inhibition characteristic for PP2A inhibition (table 6, section 3.4.). This is true for 30 minutes and 3 hours (Fig. 18B) and also when data is expressed as a percentage of the total tau (Fig. 18D). The involvement of PP1 is also evident given that increased tau phosphorylation is detected when OA is added at 50 nM (30 minutes, Fig. 18B and Fig. 18D). Prolonged exposure to OA, for 3 hours, confirmed the involvement of PP2, and excluded the possibility of PP2B, given that no further increase were obtained at OA concentrations of 5μM. Thus, with respect to residue Ser262, PP1 is clearly involved, PP2A also appears to be relevant but PP2B does not appear to be involved.

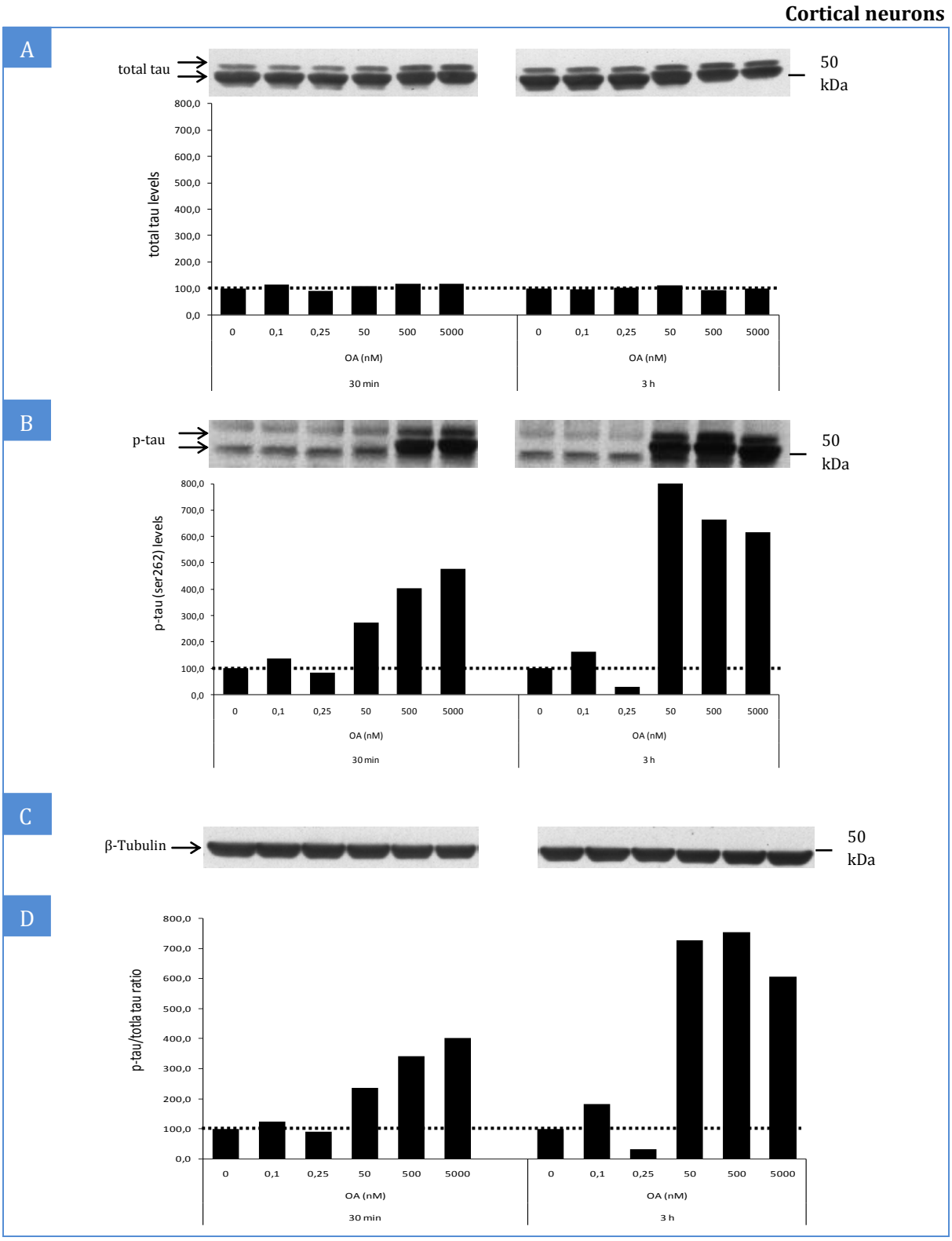

Figure 18 - Okadaic acid (OA) effects on tau phosphorylation at Ser262 residue. Rat primary cortical neuronal cultures were incubated at 37ºC in Neurobasal medium free of B27 for 30 minutes and 3 hours with okadaic acid (0.1, 0.25, 50, 500 and 5000 nM). Cell lysates were collected and analyzed by immunoblotting with Tau5 antibody which recognizes total tau (**A**), p-tau Ser262 antibody which recognizes tau phosphorylated at Ser262 (**B**) and β-Tubulin antibody (**C**). D – Ratio between phospho-tau at residue Ser262 recognized and total tau. The respective quantitative data is also presented. Data was obtained from duplicate experiments (n=2).

4.3. Abeta effects on tau and p-tau binding proteins

4.3.1. Rat primary cortical neuronal cultures

In order to accomplish this aim of identifying the tau binding proteins and more specifically the phosphorylation dependent tau binding proteins, co-immunoprecipitation assays were carried out using control cortical neuronal cultures and treated with 10μM of aggregated $Abeta_{1-42}$ or 0.25μM of okadaic acid for 3 hours. Co-immunoprecipitations were performed using the Tau5 and p-tau Ser262 antibodies. The immunoprecipitates were sent to Kinexus Company where they were subjected to SDS-PAGE and further stained with Coomassie blue (Fig. 19). After comparison the results of Tau5 and p-tau Ser262 immunoprecipitations with negative control immunoprecipitation we observe bands that specifically appear with Tau5 antibody (bands 1-6, Fig. 19) and others with p-tau Ser262 antibody (bands 7-9, Fig. 19). These nine bands of interest (1-9; Fig. 19) were excised from the SDS- PAGE gel, subjected to trypsin digestion and are now being analyzed by high-resolution mass spectrometry for protein identification (Kinexus Company).

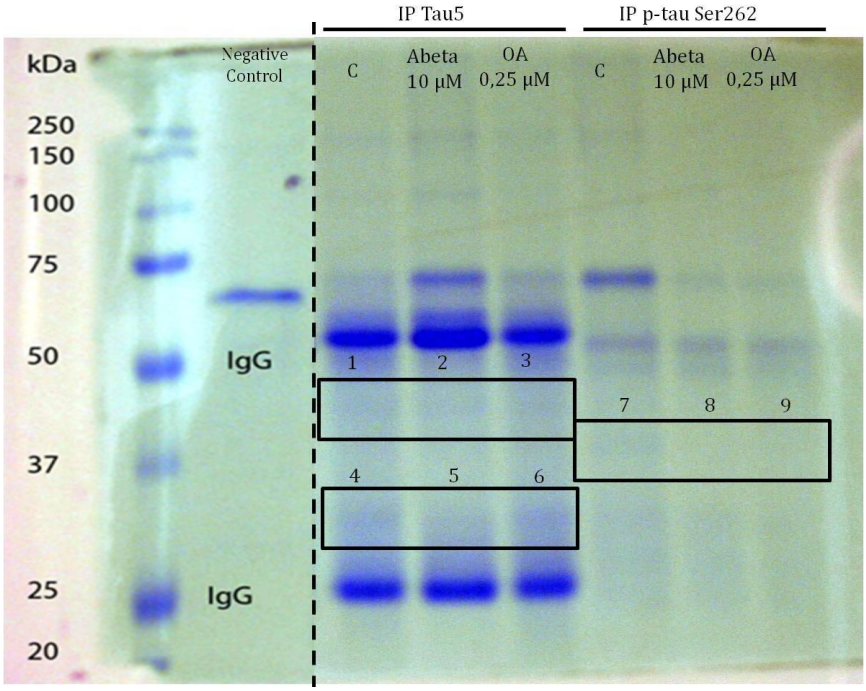

Figure 19 - Coomassie blue-stained gel of proteins co-immunoprecipitated with Tau5 antibody and p-tau Ser262 antibody from rat primary cortical neurons. Rat primary cortical neurons were treated with 10µM of Abeta$_{1-42}$ or 0.25 µM of okadaic acid (OA) for 3 hours. Cleared cell lysates were immunoprecipitated with Tau5 antibody directed against total tau protein and with p-tau Ser262 antibody directed against phosphorylated tau at Ser262 residue and resolved by SDS-PAGE. The gel was stained with Coomassie blue. Molecular masses of protein size markers are indicated (KDa). The black boxes indicate the bands of interest excised for enzymatic digestion by trypsin and subsequent protein identification by high-resolution mass spectrometry. C, control; IgG, immunoglobulins; IP, immunoprecipitation.

5. Concluding remarks

Abeta effects on tau phosphorylation

In cortex:

- Total tau levels decreased with prolonged $Abeta_{1-42}$ exposure;
- $Abeta_{1-42}$ (10μM and 20μM) slightly decreased tau phosphorylation at residues Ser202 and Thr205, following a 24 hour exposure;
- Consequently the tau phosphorylation, as a percentage of total tau, at both these residues also decreased;
- In absolute terms tau phosphorylation at residue Ser262 also decreased with exposure to higher $Abeta_{1-42}$ concentrations;
- Tau phosphorylation at Ser262 increased as a percentage of total tau upon exposure to $Abeta_{1-42}$.

In hippocampus:

- Total tau levels decreased with $Abeta_{1-42}$ exposure;
- $Abeta_{1-42}$ decreased tau phosphorylation at residues Ser202 and Thr205;
- $Abeta_{1-42}$ treatment appeared to provoke a slight increase on tau phosphorylation (percentage of total tau) at residues Ser202 and Thr205 upon 30 minutes of exposure;
- In absolute terms tau phosphorylation at Ser262 decreased with exposure to $Abeta_{1-42}$;
- Tau phosphorylation at Ser262 increased as a percentage of total tau after prolonged periods of exposure;

Protein phosphatases (PPs) involved on tau dephosphorylation

- PP1 is involved in tau dephosphorylation at Ser202 and Thr205 residues;
- PP2A and PP1 are involved in tau dephosphorylation at Ser262 residue.

6. Discussion and Conclusion

Tau is a phosphoprotein, which in pathological conditions, such as AD, is found hyperphosphorylated. This is turn affects its physiological role due to loss of its ability to bind and consequently stabilize the microtubules. Another interesting aspect in tau biology is its relationship with Abeta, and has been proposed by amyloid hypothesis defenders, that Abeta may induce a series of neuronal signal transduction alterations, such as PKs and PPs activation and/or inactivation. As a consequence, tau becomes hyperphosphorylated, which in turn promotes disruption of neuronal structure and function leading to neuronal death consistent with neurodegeneration. Therefore these mechanisms, potentially mediated by Abeta deserve further investigation. We addressed the effects of Abeta on tau phosphorylation at Ser202, Thr205 and Ser262 residues. The latter are all relevant AD epitopes, since they are reported to be hyperphosphorylated in an early stage of AD[59-61]. In addition, Ser262 has been proposed to be more injurious than other sites, since it is localized in the microtubule-binding domain of tau protein thus decreasing tau biological activity of promoting and stabilizing microtubules.

Exposure of primary cultures to $Abeta_{1-42}$ provoked responses in terms of tau phosphorylation, which appear to be consistent with respect to AD pathology. Residues phosphorylated in tau include; Ser202, Thr205 (analyzed together) and Ser262. The tau phosphorylation profiles for the above mentioned residues were quite different upon exposure to Abeta, and further complicated given that it alters with respect to the length of time of exposure. Finally, cell type is also relevant, in terms of tau phosphorylation levels, as a consequence of exposure to Abeta. Hence, at 30 min exposure to $Abeta_{1-42}$, tau phosphorylation remained unchanged except for phosphorylation at Ser202 and Thr205 in hippocampal neuronal cultures, which increased. By 3 hours, phosphorylation decreased at these two residues but more so for hippocampal neurons (Table 9). Suggesting once again that the latter are probably more sensitive to $Abeta_{1-42}$ or respond more markedly to the latter. The tendency for decreased phosphorylation at these two residues, as analyzed together, upon $Abeta_{1-42}$ exposure was augmented following the 24 hour exposure period. Thus tau phosphorylation at Ser202 and Thr205 decreases upon exposure to Abeta in a concentration and time dependent manner and hippocampal neurons appear to

respond more readily than cortical neurons (Fig 13 and Fig 15). For this data set, it did not go unnoticed that at the 30 minute time point and $Abeta_{1-42}$ 10μM concentration there was a slight increase in tau phosphorylation in hippocampal neurons, and this deserves to be further addressed. Overall these results are discordant to those described by Hu et al. that observed an induced tau phosphorylation at Ser202 mediated by $Abeta_{1-42}$ (10μM) in a time-dependent manner in differentiated PC12 cells, with a maximal increase at 24 hours. One possible explanation maybe that a different cell type was used in this study and as explained above different responses may occur with respect to cell type.

Tau phosphorylation on Ser262 upon exposure to Abeta resulted in a more complex response. At 30 minutes, overall values of absolute tau phosphorylation started to decrease and this was clearly evident by 3 hours (Fig. 14 and Fig 16). However following 24 hour exposure the percentage of tau phosphorylated at this residue increases significantly. Again, hippocampal cultures appear to respond more readily. This dual response appears to be related to length of exposure time. It is plausible to hypothesize that at short periods of exposure, physiological mechanisms come into play, in order to overcome the effects of exposure to the toxic Abeta peptide as described below. However upon longer periods of incubation, intracellular regulatory mechanisms 'break down' and processes equivalent to a pathological situation come into play, resulting in phosphorylation at Ser262. This is consistent with tau being hyperphosphorylated in AD. These results are consistent with previous observations indicating that $Abeta_{1-42}$ induces tau phosphorylation at this residue in a time-dependent manner, since phosphorylation of tau increases at higher periods of exposure[86]. Interestingly, increased tau phosphorylation induced by $Abeta_{1-42}$ was achieved at Ser262 residue, that plays a critical role in $A\beta_{1-42}$-induced tau toxicity[91] since this has a strong effect on microtubules.

Table 9 - Summary of results obtained for the tau phosphorylation analyzed following cortical and hippocampal neurons exposure to Abeta$_{1-42}$. Data is based on calculated ratio p-tau/total tau.

	Tau phosphorylation levels	
	Ser202 and Thr205	Ser262
Cortical neurons	- **30 min**: no changes; - **3 h**: slight decrease; - **24 h**: robust decrease with higher Abeta$_{1-42}$ concentrations.	- **30 min**: no changes - **3 h**: decrease with higher Abeta$_{1-42}$ concentration; - **24 h**: tendency to increase with all Abeta$_{1-42}$ concentrations.
Hippocampal neurons	- **30 min**: increase with all Abeta$_{1-42}$ concentrations; - **3 h**: decrease with Abeta$_{1-42}$ in a dose-dependent manner; - **24 h**: decrease with Abeta$_{1-42}$ in a dose-dependent manner.	- **30 min**: no changes - **3 h**: decrease with Abeta$_{1-42}$ in a dose-dependent manner - **24 h**: increase with all Abeta$_{1-42}$ concentrations.

As already mentioned, in AD tau is aberrantly hyperphosphorylated and actually accumulating evidence suggests that PKs and PPs activity are altered in AD brain. Therefore it follows that tau hyperphosphorylation is likely to be due to an imbalance of PKs and PPs regulation. Many studies have been devoted to elucidating the protein kinases involved in tau phosphorylation, however less effort has been devoted to elucidating the protein phosphatases involved. Therefore, we went on to study the protein phosphatases responsible for tau dephosphorylation at residues Ser202, Thr205 and Ser262.

Our experiments revealed that inhibition of PP1 alone could result in increased tau phosphorylation at Ser202 and Thr205. In this way it seems that the major PP involved in Ser202 and Thr205 dephosphorylation is PP1, which is

consistent with previous observations[44]. On the contrary Merrick et al. described PP2A as being involved in dephosphorylation at these residues[92]. For tau phosphorylation at Ser262, this increased when PP2A and PP1 were inhibited, and thus it seems that both are involved in tau dephosphorylation at this residue. Thus the major PP that appears to be involved in tau dephosphorylation at Ser262 is PP2A. Actually the expression and activity of both these PPs, PP1 and PP2A, are decreased in AD brain[13], suggesting an involvement of these PPs on tau hyperphosphorylation which comprises PHFs of neurofibrillary tangles. However Liu et al. reported that PP1, PP2A and PP2B, all dephosphorylate tau at Ser202, Thr205 and Ser262, but with different efficiencies toward different sites and thus further studies to clarify the PPs actually involved in tau dephosphorylation are required.

Another important feature of tau metabolism are its binding proteins, and actually many proteins have already been found to interact with tau both in vitro and in vivo (table 4, section 1.3.3.). The interactome of tau is shaped by its phosphorylation and so is crucial to mapping the crosstalk between normal and pathologically hyperphosphorylated tau. Therefore our aim is to also identify proteins that interact with tau and more specifically assess the role of Ser262 phosphorylation in shaping this interactome, while also evaluating the $Abeta_{1-42}$ effects on this interactome. Our approach was based on co-immunoprecipitation of total tau and phosphorylated tau at Ser262, using Tau5 antibody and p-tau ser262 antibody, respectively, their resolution in SDS-PAGE and then mass spectrometry analysis. Hence the immunoprecipitates, which were already subjected to SDS-PAGE were stained with Coomassie blue and the bands of interest were excised and are now being analyzed by mass spectrometry analysis. A preliminary analysis of the SDS-PAGE gel revealed some differences, in terms of band intensity and the appearance of novel bands, indicating that probably the phosphorylation at Ser262 residue modulates the tau interactome. Full identification of these proteins will thus be important.

In closing, it is evident that tau phosphorylation can be modulated by Abeta. This is not a generic response given that different residues exhibit different phosphorylation profiles following a period of incubation with Abeta. It is therefore reasonable to deduce that the latter is specifically affecting signaling cascades, resulting in specific phosphorylation/dephosphorylation signaling events. Further from the work here presented PP1 and PP2A are key protein phosphatases. These findings are consistent with previous reports from the laboratory by Vintém et al showing that Abeta inhibits PP1. Mechanistically one can propose that Abeta production affects PP1 causing its inhibition which in turn favors tau hyperphosphorylation. This model is also consistent with the pathological model of AD where senile plaques proceed neurofibrillary tangles. Thus these findings deserve further investigation and are important in terms of clearly identifying the molecular sequential events in Alzheimer's disease.

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Appendix

I. Culture media and Solutions

Cell Culture Solutions

- **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:
- 8 mM Sodium Phosphate
- 2 mM Potassium Phosphate
- 140 mM Sodium Chloride
- 10 mM Potassim Chloride

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

- **10 mg/ml Poly-D-lysine stock (100x)**

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

- **Borate buffer**

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**

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

- **Hank's balanced salt solution (HBSS)**

This salt solution is prepared with deionised H_2O. Final Composition:
- 137 mM NaCl
- 5.36 mM KCl
- 0.44 mM KH_2PO_4
- 0.34 mM $Na_2HPO_42H_2O$
- 4.16 mM $NaHCO_3$

- 5 mM Glucose
- 1 mM Sodium pyruvate
- 10 mM HEPES

Adjust to pH 7.4. 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) is supplemented with:

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

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) is supplemented with:

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

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

- **RIPA buffer**

To 6.5 ml of RIPA buffer (Sigma-Aldrich) add:

- 40.3 µL NaF
- 65 µL NaOrt
- 65 µL Protease inhibitor cocktail (Sigma-Aldrich)

SDS-PAGE and Immunoblotting Solutions

- **LGB (Lower gel buffer) (4x)**

To 900 ml of deionised H_2O add:
- Tris 181.65 g
- SDS 4 g

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

- **UGB (Upper gel buffer) (5x)**

To 900 ml of deionised H_2O add:
- Tris 75.69 g

Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1 L with deionised H_2O.

- **30% Acrylamide/0.8% Bisacrylamide**

To 70 ml of deionised H_2O add:
- Acrylamide 29.2 g
- Bisacrylamide 0.8 g

Mix until the solute has dissolved. Adjust the volume to 100 ml with deionised water. 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 Gel Buffer (4x)**

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

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

- **1 M Tris (pH 6.8) solution**

To 150 ml of deionised H_2O add:
- Tris base 30.3 g

Adjust the pH to 6.8 and adjust the final volume to 250 ml.

- **10x Running Buffer**

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

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

- **Resolving (lower) gel solution** (for gradient gels, 60 ml)

	5%	**20%**
- H_2O	17.4 ml	2.2 ml
- 30% Acryl/0.8% Bisacryl solution	5 ml	20 ml
- LGB (4x)	7.5 ml	7.5 ml
- 10% APS	150 µL	150 µL
- TEMED	15 µL	15 µL

- **<u>Stacking (upper) gel solution</u>** (20 ml)

		3.5%
-	H_2O	13.2 ml
-	30% Acryl/0.8% Bisacryl solution	2.4 ml
-	UGB (5x)	4.0 ml
-	10% APS	200 µL
-	10% SDS	200 µL
-	TEMED	20 µL

- **<u>1x Transfer Buffer</u>**

- 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%).

- **<u>10x TBS (Tris buffered saline)</u>**

- 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 1L with deionised H_2O.

- **<u>10x TBST (TBS+Tween)</u>**

- 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 1L with deionised H_2O.

- **<u>Membranes Stripping Solution</u>** (500 ml)

- Tris-HCl (pH 6.7) 3.76 g (62.5 mM)
- SDS 10 g (2%)
- Beta-mercaptoetanol 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.

Immunoprecipitation solutions

- **Lysis Buffer**

- 2M MOPS (pH 7.0)	300 µL (20 mM)
- 250 mM EGTA	240 µL (2 mM)
- 250 mM EDTA	600 µL (5 mM)
- NaF 50 mg/ml	900 µL (30 mM)
- 100 mM NaOrt	300 µL (1 mM)
- Triton X-100 100%	300 µL (1%)

- **Lysis Buffer + Protease Inhibitors** (30 ml)

Add to 28819µL of lysis buffer the following quantities for a final volume of 30 mL:

- 100 mM PMSF	300 µL (1 mM)
- 200 mM Benzamidine	450 µL (3 mM)
- Pepstatin A 1mg/ml	102 µL (5 µM)
- Leupeptin 5 mg/ml	28,56 µL (10 µM)
- 0.1 M Dithiothreitol (DTT)*	300 µL (1mM)

* prepare fresh before use.

- **Dithiothreitol (DTT)0.1 M**

To 1ml of deionised H_2O add 0.0154 g of DTT.

- **Blocking solution**

To 20 ml of PBS 1x add 0.6 g of Bovine Serum Albumine (BSA).