**Universidade de Aveiro** Secção Autónoma de Ciências da Saúde
**2013**

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# Análise bioinformática do fosfoproteoma neuronal

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Tese 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 Ana Gabriela Henriques, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro e co-orientação da Professora Doutora Odete da Cruz e Silva, Professora Auxiliar da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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**o júri**

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**palavras-chave**

Doença de Alzheimer, fosforilação, ácido ocadáico, Aβ, fosfoproteoma neuronal.

**resumo**

A fosforilação anormal de proteínas é uma das características chave da Doença de Alzheimer (DA) que pode estar envolvida tanto na patogénese como na progressão da doença. A fosforilação reversível de proteínas representa um importante mecanismo regulador que envolve a atividade de fosfoproteínas fosfatases (FPF) e proteínas cinases (PC). Um desequilíbrio intracelular entre a actividade de FPF e PC pode alterar a atividade, localização subcelular e interacções de proteínas, contribuindo para a desregulação da função e sinalização neuronal e, consequentemente para a neurodegeneração. Assim, o estudo do fosfoproteoma neuronal da DA torna-se relevante tanto do ponto de vista fisiológico como patológico. Culturas primárias corticais foram expostas ao ácido ocadáico (AO, um inibidor de PPP) ou ao péptido β amilóide (Aβ) para mimetizar as condições da DA. Os lisados celulares foram aplicados numa coluna de afinidade para fosfoproteínas. As frações enriquecidas em fosfoproteínas foram analisadas por espetrometria de massa tendo sido desenvolvido um script em linguagem python (http://sourceforge.net/projects/protdb/) para análise das proteínas identificadas. Os resultados provenientes das condições Controlo vs AO indicam que o tratamento com este inibidor de FPF leva a um aumento do número de fosfoproteínas (174 vs 242 proteínas totais e 32 vs 100 proteínas exclusivas). Os resultados do tratamento com Aβ indicam uma alteração qualitativa do fosfoproteoma neuronal (174 vs 166 proteínas totais) com um número considerável de proteínas exclusivas (42 vs 34 proteínas exclusivas). Subsequentemente, para a obtenção de informação detalhada e caracterização das proteínas identificadas em cada condição, foi realizada uma análise exploratória das fosfoproteínas organizando-as por classe proteica, processos biológicos, localização subcelular e funções moleculares. Os tratamentos com AO e Aβ levam a alterações em proteínas envolvidas em processos celulares que se encontram comprometidos na DA, tais como a actividade das PC e FPF, degradação proteica, stress oxidativo, folding proteico, dinâmica do citoesqueleto, síntese proteica e apoptose. A caracterização do fosfoproteoma neuronal da DA pode revelar ou elucidar os mecanismos moleculares subjacentes à transdução de sinais anormal associada com a patogénese da doença. A análise das fosfoproteínas exclusivas poderá, também, contribuir para a identificação de potenciais novos biomarcadores ou alvos terapêuticos para a DA.

**keywords** Alzheimer's Disease, phosphorylation, okadaic acid, Aβ, neuronal phosphoproteome.

**abstract** Abnormal protein phosphorylation is a characteristic hallmark of Alzheimer's disease (AD) and may be implicated both in pathogenesis or disease progression. Reversible protein phosphorylation represents a key regulatory mechanism involving the activity of protein phosphatases (PPP) and protein kinases (PK). Imbalanced PPP and PK activity can alter protein action, subcellular localization and protein interactions, thus contributing to abnormal neuronal function and signaling and consequently to neurodegeneration. Hence, the study of the AD neuronal phosphoproteome is of physiological and pathological relevance. Primary cortical cultures were exposed to okadaic acid (OA, a PPP inhibitor) or amyloid-β peptide (Aβ), in order to mimic AD conditions. Cell lysates were applied to a phosphoprotein affinity column and phosphoprotein enriched fractions analyzed by mass spectrometry. A protein database management framework (http://sourceforge.net/projects/protdb/) was set up allowing for the development of a script to analyze the identified proteins. Data from Control vs OA conditions indicates that OA treatment leads to an increase in phosphoproteins (174 vs 242 proteins and 32 vs 100 exclusive proteins). Data indicates that Aβ treatment leads to a shift in neuronal phosphoproteome pool (174 vs 166 proteins) with noteworthy alterations in the exclusive neurophosphoproteome (42 vs 34 exclusive proteins). Subsequently, analysis of the protein classes, biological processes, subcellular localization and molecular functions allowed for detailed information regarding the proteins obtained in the different groups. Upon treatments an alteration in the proteins involved in critical processes impaired in AD such as PK and PPP activities, protein degradation, oxidative stress, protein folding, cytoskeleton network dynamics, protein synthesis and apoptosis was observed. The characterization of AD neuronal phosphoproteome may reveal or elucidate the molecular mechanisms underlying abnormal signal transduction associated with AD pathogenesis. Further, by analyzing the pool of exclusive proteins, this work may also contribute to identify potential novel biomarker candidates or AD targets for therapeutic intervention.

# Table of Contents

## ABBREVIATIONS

| | |
|---|---|
| 1D | 1 dimension |
| 1DE | 1 dimension gel electrophoresis |
| AA | Acrylamide |
| ACN | Acetonitrile |
| AD | Alzheimer's disease |
| ADAM | A disintegrin and metalloproteinase |
| ADAS-Cog | Alzheimer's Disease Assessement Scale-Cognitive |
| ADL | Activities of dailly living |
| AICD | Amyloid precursor protein intracellular domain |
| AMBIC | Ammonium bicarbonate |
| Aph-1 | Anterior pharynx defective 1 |
| APOE | Apolipoprotein E |
| APP | Amyloid precursor protein |
| APS | Amonium persulfate |
| Aβ | Amyloid β peptide |
| BACE1 | β-secretase |
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| CaMK-II | Calcium-calmodulin-dependent kinase II |
| cAMP | 3'-5'-cyclic adenosine monophosphate |
| cdk5 | Cyclin-dependent kinase 5 |
| CIs | Colinesterase inhibitors |
| CNS | Central nervous system |
| CRMP-2 | Collapsin response mediator protein 2 |
| CSF | Cerebrospinal fluid |
| Dyrk1A | Dual-specific tyrosine (Y) regulated kinase 1A |
| EBI | European Bioinformatics Institute |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| EOAD | Early-onset Alzheimer's disease |
| ER | Endoplasmic reticulum |
| ERK1 | Extracellular regulated kinase-1 |
| ERK2 | Extracellular regulated kinase-2 |

| | |
|---|---|
| FBS | Fetal bovine serum |
| FDG | Fluorodeoxyglucose |
| GO | Gene ontology |
| GSK3β | Glycogen synthase kinase 3 β |
| HBSS | Hanks balanced solution |
| HCl | Hydrogen chloride |
| I1 | Inhibitor 1 |
| I2 | Inhibitor 2 |
| IPIs | International protein index |
| JNK | c-Jun NH(2)-terminal kinase |
| KPI | Kunitz protease inhibitor |
| LC | Liquid chromatography |
| LDS | Lithium dodecyl sulfate |
| LOAD | Late-onset Alzheimer's disease |
| LTP | Long term potentiation |
| MAP1B | Microtubules-associated protein 1 B |
| MAPKs | Mitogen-activated protein kinases |
| MAPs | Microtubule-associated proteins |
| MCI | Mild cognitive impairment |
| MMSE | Mini-Mental State Examination |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| MRI | Magnetic resonance imaging |
| mRNA | messenger ribonucleic acid |
| MS/MS | Tandem mass spectrometry |
| NB | Neurobasal |
| Nct | Nicastrin |
| NFTs | Neurofibrillary tangles |
| NMDA | N-methyl-D-aspartate |
| OA | Okadaic acid |
| p53 | Tumor protein 53 |
| PANTHER | Protein Analysis Through Evolutionary Relationships |
| PBS | Phosphate buffered saline |
| Pen2 | Presenilin enhancer 2 |
| PET | Positron emisson tomography |
| PHF | Paired helical filaments |
| PI3K | Phosphatidylinositol 3-kinase |
| PIR | Protein Information Resource |

| | |
|---|---|
| PKA | cAMP-dependent protein kinase |
| PKC | Protein kinase C |
| P-kit | Phosphoprotein enrichment kit |
| PMAC | Phosphate Metal Affinity Chromatography |
| PPP1 | Phosphoprotein phosphatase 1 |
| PPP2 | Phosphoprotein phosphatase 2 |
| PPP5 | Phosphoprotein phosphatase 5 |
| PSEN1 | Presenilin 1 |
| PSEN2 | Presenilin 2 |
| PSENs | Presenilins |
| P-tau | Phosphorylated tau |
| PTEN | Phosphatase and tensin homolog |
| PTK | Protein tyrosine kinase |
| sAPPα | Secreted amyloid precursor protein α |
| sAPPβ | Secreted amyloid precursor protein β |
| SDS | Sodium dodecyl sulfate |
| SIB | Swiss Institute of Bioinformatics |
| SPs | Senile plaques |
| TEMED | Tetramethylethylenediamine |
| TFA | Trifluoroacetic acid |
| TGN | Trans-Golgi network |
| T-tau | Total tau |
| Uniprot | Universal Protein Resource |
| UniprotKB | Universal Protein Resource Knowledgebase |

# 1. Introduction

## 1.1. ALZHEIMER'S DISEASE

### 1.1.1. Overview of Alzheimer's disease

In last decades neurodegenerative disorders have gained a prominent place within the scientific community. This group of disorders, known as hereditary or sporadic conditions, is characterized by progressive nervous system dysfunction, often related with atrophy of the affected structures. Among the neurodegenerative disorders is Alzheimer´s disease (AD), the most common form of dementia (50% to 80% of all cases) with an estimated prevalence of 30 million people worldwide, a number that is expected to quadruple in 40 years (1). Alzheimer Europe estimates the number of European citizens with dementia at 7.3 million. For Portugal the number of people with AD is estimated at over 90 000 (2).

In clinical terms, AD is characterized by progressive cognitive impairment, commonly beginning as memory loss with difficulties to remember newly learned information. In later stages, the disorder spreads through the brain affecting multiple cognitive and behavior domains, leading to severe symptoms including disorientation, mood and behavior changes; expanding confusion about occasions, time and place; speculative worries about family and friends; more serious memory loss and personality fluctuations; besides difficulty speaking, swallowing and walking (1,3).

Since AD is a multifactorial disorder, several hypotheses have been proposed to explain the pathogenesis and progression of AD. Among the most noteworthy hypotheses are: the amyloid cascade, the oxidative stress and environment-polygenic risk disorder hypothesis (4–6). The initiating events of AD are until now unknown but it is though that this neurodegenerative disorder is a consequence of the combination of aging, environmental and genetic risk factors with different epigenetic events (7).

### 1.1.2. Hallmarks of AD

Neuropathologically, the key hallmarks of AD (Figure 1), first described by Alois Alzheimer in 1906 (8) and at about the same time by Oskar Fischer, (9) are the amyloid or senile plaques (SPs) and the neurofibrillary tangles (NFTs). SPs are mainly constituted by amyloid beta peptide (Aβ), which results from the proteolytic cleavage of amyloid precursor protein (APP). Accordingly to amyloid cascade hypothesis, the deposition of Aβ is the initial and crucial pathological trigger in AD, which subsequently leads to neuronal death and dementia (10). For this reason, Aβ and its precursor (APP) will be focused later on.

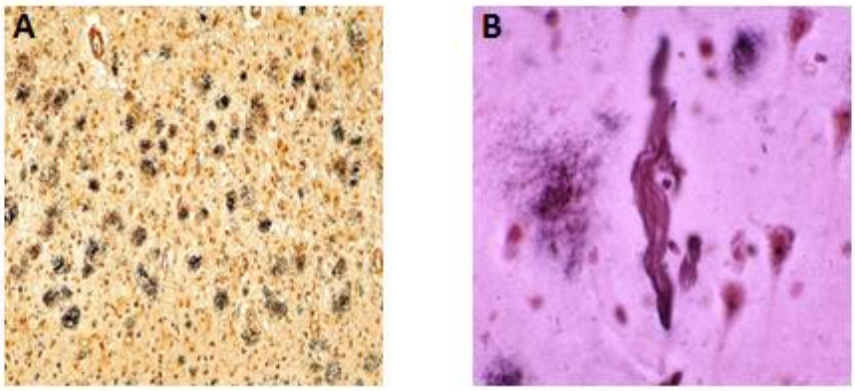


**Figure 1 - Neuropathological hallmarks of AD.** A - The senile plaques (SPs) observed in Alzheimer's brain. B - The neurofibrillary tangles (NFTs) in AD brains. Taken from (11).

The NFTs are intracellular structures present in neuronal cell bodies mainly composed of abnormal hyperphosphorylated and aggregated form of tau protein, a microtubule binding protein. All the studies developed focusing the NFTs strongly suggest that the neurofibrillary pathology contributes to neuronal dysfunction and correlates with the clinical progression of AD. NFTs formation seems to occur in several steps with different morphological stages: pre-NFT, intra-neuronal and extra-neuronal NFT. NFTs occurs in a stereotypical hierarchical distribution, with certain cytoarchitectural regions affected, as example, the neurons in layer II of the entorhinal cortex are among the first affected (1,12,13).

In addition, other neuropathological and neurochemical hallmarks of AD have been described, including extensive neuronal and synapse loss in specific brain areas - neocortex and hippocampus - (14,15) as well as alterations in certain neurotransmitters (16). Beyond the pathophysiological hallmarks of AD, the disease is also associated with oxidative stress, decreased glucose metabolism, mitochondrial deficit, increased protein misfolding and decreased protein turnover (17).

### 1.1.3. Genetic Factors of AD

Most cases of AD are sporadic and idiopathic however, genetic factors have an important role in understanding the pieces of the complex AD puzzle. AD can be classified as early-onset AD (EOAD) or late-onset AD (LOAD) based on the age of onset. Mutations in 3 genes: APP, presenilin 1 (PSEN1), and presenilin 2 (PSEN2) are responsible for a fraction of EOAD (<60 years) that accounts for approximately 5% of all AD cases. The identification of these 3 genes has been crucial to understand the molecular mechanisms underlying AD pathology. On the other hand, the presence of the ε4 allele of the gene encoding apolipoprotein E (APOE) is considered the major genetic risk factor for LOAD,

which represents 95% of all AD cases, and also has been linked to autosomal dominant familial disease in different populations (18,19).

Mutations in PSEN1, mainly missense mutations, lead to the most severe forms of AD with complete penetrance (autosomal dominant inheritance). Moreover, mutations in this gene account for 18-50% of autosomal dominant EOAD cases. Despite EOAD mutations occur in 3 different genes located on 3 different chromosomes, they all share a common biochemical event: altered Aβ production leading to a relative abundance of the Aβ species. Ultimately, this phenomenon causes neuronal death and dementia providing the connection between the long-known familial aggregation and the increase in Aβ production observed on AD brains (which originally gave rise to the amyloid cascade hypothesis of AD) (18,19).

### 1.1.4. Diagnosis of AD

To date, the definitive diagnosis of AD can only be made after clinical diagnosis with postmortem histopathological examination of the brain (20). However, many progresses have been made in this area. In 2011, the National Institute of Neurological and Communicative Disorders Association criteria updated and presented 3 different types of AD dementia: probable - according to the presence of the core clinical criteria only; possible – according to an atypical course without differential diagnosis; or definitive – according to neuropathological evidence (21). A fundamental part of AD diagnosis continues to be the evidence of cognitive impairment, especially in the memory domain. The cognitive impairment is detected and diagnosed through a combination of clinical history analysis and objective cognitive assessment by means of a brief mental evaluation or comprehensive neuropsychological testing. For this purpose, the mini-mental state examination (MMSE), AD Assessment Scale-Cognitive (ADAS-Cog) and clock drawing test are frequently recommended (21,22).

Despite the importance of the cognitive testing, new trends in AD dementia diagnosis suggest the need to reflect upon the biological dimension of the disease, including biomarkers related to Aβ deposition in the brain and downstream neuronal degeneration (neuronal injury) (20). Remarkably, an early diagnosis with reliable biomarkers is essential to distinguish between AD, mild cognitive impairment (MCI) and other dementia types (23). The molecular composition of cerebrospinal fluid (CSF) can reflect biochemical changes in the brain. To date, a panel of CSF biomarkers have been used for differential diagnosis of dementia, including AD diagnosis and progression, since in early stages of AD low Aβ42, elevated total tau (T-tau), and elevated phosphorylated tau (P-tau) are

denoted (24). Of note, due to CSF collection limitation many research have been focused on biomarker identification on more peripheral and accessible fluids (e.g plasma, saliva, urine).

In addition to CSF biomarkers already validated, other imaging biomarkers are being used: positive retention of tracer in positron emission tomography (PET), decreased fluorodeoxyglucose (FDG) uptake on PET reflected in the temporoparietal cortex and the presence of patterns of atrophy on magnetic resonance imaging (MRI), involving medial, basal and lateral temporal lobes and medial parietal cortex (21). New potential biomarkers are emerging, and CSF or plasma marker profiles may eventually become part of the clinician's toolkit for accurate AD diagnosis and management (25).

For enhancing the accuracy of diagnosis and screening AD drug therapies it is crucial to achieve a definitive diagnosis of AD linking both molecular and clinical data to demonstrate dementia, SPs and NFTs, pursuing the detection of the neurodegenerative condition even before the onset of clinical symptoms (25).

### 1.1.5. Treatment of AD

As previously mentioned, AD cases are expected to quadruple in 40 years (1). Hence, an enormous research focused on the discovery of drugs for primary, secondary or tertiary prevention of the disease has been made. The primary prevention aims to prevent or slow the neuropathology of AD acting before symptoms arise. The second prevention represents the early detection and treatment or control of the disease. At last, the aim of the tertiary prevention is to prevent the complications of the disease when symptoms have become apparent and to reverse the excess disability of patients. Despite all scientific efforts, there are no effective pharmacotherapeutic options for prevention and treatment of AD (26,27).

The current treatments are only symptomatic in nature, trying to counterbalance the typical neurotransmitter disturbance of the disease. For the treatment of mild to moderate AD patients, 3 cholinesterase inhibitors (CIs) are approved and represent the standard and first-line of treatment. Early in AD progress the cholinergic systems in the basal forebrain are affected resulting in memory loss and deterioration of cognitive and noncognitive functions. As CIs act in the cholinergic systems, they delay the decline in cognitive function, global clinical rating, behavior and activities of daily living (ADL) (28,29). Another therapeutic option is available for moderate to severe AD patients named N-methyl-D-aspartate (NMDA) antagonist – memantine. It is though that this uncompetitive, moderate-affinity NMDA antagonist protects neurons from glutamate excitotoxicity leading to improvement in cognition, ADL and behavior (30).

Neuropsychiatric and psychological symptoms are common in all clinical stages of AD. As a result, among the most efficient antidepressants to treat comorbid depression is the serotonin reuptake inhibitors. Other antidepressants like mirtazapine and venlafaxine, which are combined selective noradrenalin and serotonin inhibitors, are widely used in AD patients. (31,32).

On the basis of AD pathogenesis findings, novel treatments under development aim to interfere with the pathogenic steps attempting to block the course of the disease in its early stages. For this reason, they have been termed disease-modifying drugs. The aims of these new drugs include decrease Aβ production, interfere with Aβ deposition, promote Aβ clearance, inhibit β- and γ-secretase, potentiate α-secretase and interfere with tau deposition and phosphorylation (26).

The new strategies investigate the neuroprotective potential of disease-modifying drugs in the presymptomatic stages of AD, with biomarkers support, which predict disease progress before development of evident dementia (26).

## 1.2. AMYLOID PRECURSOR PROTEIN (APP)

### 1.2.1. The APP structure

Among the conserved type-I membrane proteins is the APP, which is abundantly expressed in human brain. The APP gene is located on the long arm of chromosome 21 and comprises 18 exons. Alternative splicing of 3 exons (7, 8 and 15) generates APP mRNAs encoding several isoforms ranging from 365 to 770 amino acid residues. The major isoforms of APP are APP695, APP751 and APP770 (containing 695, 751 and 770 amino acids, respectively). APP751, encoding cDNA lacking the gene sequence from exon 8, and APP770, comprising all 18 exons, are expressed in most tissues whereas APP695 isoform, lacking the gene sequence from exons 7 and 8, is predominantly expressed in neurons (33,34).

APP contains a large extracellular portion, a hydrophobic transmembrane domain and a short C-terminus named APP intracellular domain (AICD) (Figure 2). The extracellular portion holds E1 and E2 domains besides a Kunitz protease inhibitor (KPI) domain with 57 amino acids that is missing in one isoform of APP, the APP695. The KPI domain, a 57-amino-acid insert with striking homology to the Kunitz family of serine protease inhibitors, seems to have an involvement in the amyloid cascade hypothesis through the interaction with multiple proteins that play a key role in the sequence of AD molecular events. Moreover, it is though that the KPI domain plays a major role in APP dimerization (35–37).

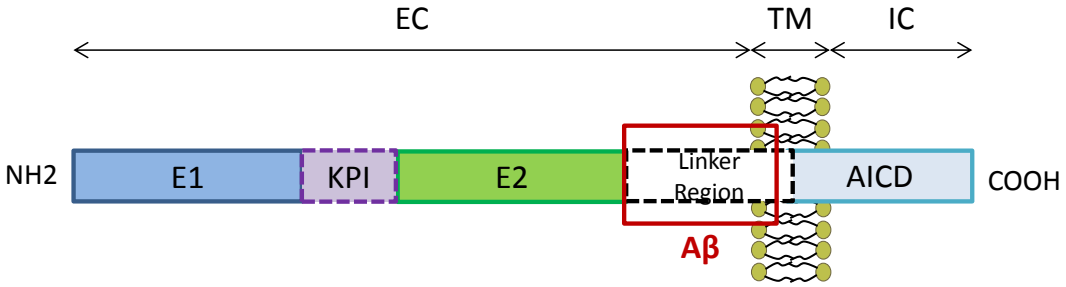


**Figure 2 - Schematic representation of APP domains**. In the extracellular portion the kunitz protease inhibitor (KPI) domain is missing in the APP695 isoform. EC = extracellular portion. TM = transmembranar portion. IC = intracellular portion.

APP plays an important role in AD pathogenesis since Aβ peptide, the major component of SPs, is a cleavage product derived from the transmembrane domain of this protein. Furthermore, it is observed a splicing shift in neurons from APP695 to KPI-containing APP isoforms. As a result, the protein and mRNA levels of the APP isoforms with KPI domain are elevated in AD brain and are associated with increased Aβ production (36,38). Therefore, it is important to study APP processing, trafficking and physiological functions to understand the pathogenesis of AD and pursue potential biomarkers for early diagnosis and therapy.

### 1.2.2. The APP processing and trafficking

APP is synthesized in the endoplasmic reticulum (ER) and then transported through the Golgi apparatus to the trans-Golgi network (TGN) where the highest concentration of APP is found in neurons at steady state. From the TGN, APP can be distributed to different cell compartments: it can be transported in TGN-derived secretory vesicles to the cell surface or re-internalized via an endossomal/lysosomal degradation pathway (39).

This protein undertakes post-translational processing through two proteolytic pathways: the non-amyloidogenic and the amyloidogenic pathway (Figure 3), both involving different secretases and proteases (38,40).

In the non-amyloidogenic pathway, APP is sequentially cleaved by α- and γ-secretase complex. The proteolytic cleavage by α-secretase inside the Aβ peptide sequence (at the 17$^{th}$ amino acid) releases a large secreted extracellular domain (sAPPα) and a membrane-associated C-terminal fragment consisting of 83 amino acids (C83). The C83 fragment is further cleaved by γ-secretase complex to generate a P3 peptide and the AICD, which are both rapidly degraded (38,41,42). Interestingly, it seems that the generation of sAPPα is more associated to the TGN-derived secretory vesicles, which transport APP from TGN to the cell surface (39).

In the amyloidogenic pathway, APP is proteolytic cleaved firstly by β-secretase (BACE1) (43) and secondly by the γ-secretase complex (44). The BACE1 cleavage occurs at the β-site, in the 1$^{st}$ or 11$^{th}$ residue of the Aβ sequence, shedding sAPPβ and generating a membrane associated C-terminal fragment consisting of 99 amino acids (C99). The C99 fragment has the same fate of C83, however, the cleavage by the γ-secretase complex releases the amyloidogenic Aβ peptide and also the AICD. Since γ-secretase can cleave APP at multiple sites and in sequential steps, Aβ peptides of different lengths can be generated. The majority of Aβ peptides produced are 40 amino acids long (Aβ40), nevertheless, peptides ranging from 38 to 43 amino acids are found *in vivo* (38,40). Contrasting to the generation of sAPPα, the Aβ peptide is generated in the ER and Golgi/TGN (45). It was also proposed that it can be generated via an endosomal/lysosomal degradation pathway, if APP is re-internalized by this degradation system (46).

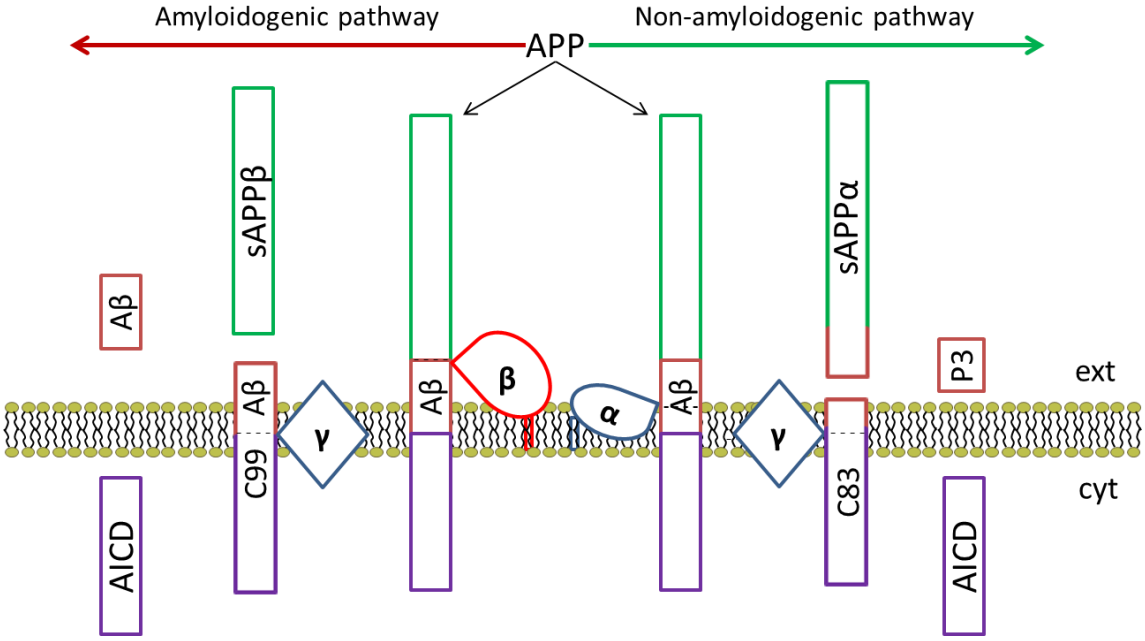


**Figure 3 - APP processing and cleavage products.** The non-amyloidogenic pathway of APP (right) involves cleavages by α- and γ-secretases resulting in the generation of a long secreted form of APP (sAPPα) and C-terminal fragments (C83, p3, and AICD). The amyloidogenic pathway of APP (left) involves cleavages by β- and γ-secretases resulting in the generation of a long secreted form of APP (sAPPβ), C-terminal fragment (C99) and Aβ. ext = extracellular, cyt = cytosol. Adapted from (40).

**The secretases involved in APP processing**

There are evidences that APP cleavage by α-secretase implicates at least four members of the family of disintegrin and metalloproteinase domain proteins (ADAM): ADAM-9, ADAM-10, ADAM-17 and ADAM-19 (47). ADAMs are type I integral membrane proteins that belong to the zinc protease superfamily and have been associated with the control of cytokine and growth factor shedding (38).

The BACE1 secretase is a type I transmembrane aspartyl protease with its active site on the luminal side of the membrane. BACE1 undergoes alternative splicing resulting in 4 different transcripts. BACE1, constituted by 501 amino acids (BACE1-501), is mainly expressed in perinuclear post-Golgi membranes, vesicular structures throughout the cytoplasm as well as on the cell surface. The other 3 minor transcripts (BACE1-476, 457 and 432) have reduced β-secretase activity and subcellular localization different from those of BACE1-501 (48).

Distinctive from the others secretases, the γ-secretase complex is constituted by a multiprotein complex including, at least, 4 proteins: PSEN1 or PSEN2, nicastrin (Nct), presenilin enhancer 2 (Pen2) and anterior pharynx defective 1 (Aph-1) (49). Presenilins (PSENs) are multi-transmembrane proteins necessary for γ-secretase activity. Nct, a type I membrane glycoprotein with a large ectodomain, interacts with PSENs. Pen2 mediates endoproteolysis of PSENs enhancing γ-secretase activity. Aph-1 is a multiple transmembrane protein that interacts with immature Nct and PSENs to form a relative pre-complex that is further maturated.

### 1.2.3. Physiological functions of APP and its fragments

**APP**

The main physiological function of APP *per se* remains essentially irresolute. A role for APP has been suggested in neurite outgrowth, synaptogenesis, cell adhesion, transmembrane signal transduction, neuronal protein trafficking along the axon, calcium metabolism and as a membrane receptor (50–55). During brain development, APP plays an important role promoting the adhesion of neurons to glia cells (56). More widely accepted, APP has been described as a protein contributing to cell adhesion through its extracellular domain. Studies have confirmed that E1 and E2 regions of APP can interact with extracellular matrix domains (50). Nevertheless, more recently, other studies advocate APP as a synaptic adhesion molecule (52,55).

**sAPPα and sAPPβ**

The key function of sAPPα is poorly understood and its exact mode of action is still unrevealed. It appears that it has neuroprotective and memory-enhancing properties. Several studies demonstrate that sAPPα has critical influence in promoting neuronal survival, neurite outgrowth, synaptogenesis, cell adhesion and enhances long-term potentiation (LTP) (57–61). Furthermore, in dividing cells of epithelial origin (including embryonic and adult neural stem cells) sAPPα has growth promoting functions and also plays a role in brain development (40).

Contrary to the neuroprotective effects of sAPPα, sAPPβ was found to be critically involved in pruning of synapses during development of both central and peripheral neurons (62). Although unclear, other possible role of sAPPβ include suppression of neuronal stem cell differentiation in favor of glial differentiation (63).

**AICD**

AICD is generated either in the amyloidogenic or in the non-amyloidogenic pathways of APP cleavage. The γ-secretase cleavage of C99 or C83 fragments, release AICD with various lengths (59, 57, 53 or 50 residues), since APP holds many cleavage sites for this secretase. After the γ-secretase cleavage AICD is rapidly degraded turning the biochemical features and physiological functions of AICD difficult to study *in vivo*. Therefore, most of the information on AICD is deduced from exogenous systems. The consensus motif YENPTY is present in all forms of AICD and it is thought to be crucial for the binding of AICD to adapter proteins such as Fe65, and subsequent biologic actions promoted by the recruitment of the histone deacetylase TIP60 (forming a transcriptionally-active complex) and nuclear translocation. Inside the nucleus, AICD is a significant player in the transactivation of specific genes including p53, GSK3β, neprilysin, EGFR and APP itself (38). Controversially, other studies demonstrated that AICD-mediated conformational change in Fe65 is sufficient and that the nuclear translocation of AICD is not required for gene expression (64). Another study has proposed that the nuclear translocation of AICD is independent of Fe65 being a result of its phosphorylation at Threonine 668 (T668) (65). However, since no consensus has been reached, the model of how AICD functions remains controversial.

**Aβ**

The presence of Aβ in the CSF of non-demented individuals and in media from neuronal cell cultures (66,67) demonstrates that Aβ has a function in the normal physiology of the central nervous system (CNS). There are evidences that Aβ might play a role in controlling synaptic activity, through protection against excessive glutamate toxicity release (68–70); in monitoring cholesterol transport

(71) and may function as a transcription factor in its own right (72). Other findings suggested that Aβ has a role in excitability and neuronal survival. The underlying mechanism remains to be resolute, but may implicate altered expression of $K^+$ channels (73,74). Nonetheless, it is important to note that the complete physiological role of Aβ remains to be elucidated.

### 1.2.4. Aβ and senile plaques formation

An early and invariant neuropathological feature of AD are SPs, which are composed by the accumulation of molecules in the extracellular space of the brain (10). These plaques are mainly characterized by insoluble fibrillar deposits of Aβ peptides (a 38-43 amino acid peptide), which are the major proteinaceous component of the plaques (75,76).

The oligomerization and fibrillization of Aβ are key events for the development of SPs (Figure 4). A primary stage is represented by the conformational switching of Aβ peptide from an α-helix or random coil to a β-sheet structure. Through the generation of intermolecular β-sheet structures, noncovalent interactions are generated between individual monomers units that held together to form oligomers, which in turn lead to fibrils formation (77). Besides the fibrils that are neurotoxic structures, Aβ also assembles into soluble forms like small oligomeric intermediates and protofibrils, which can also induce neurotoxicity (78,79).

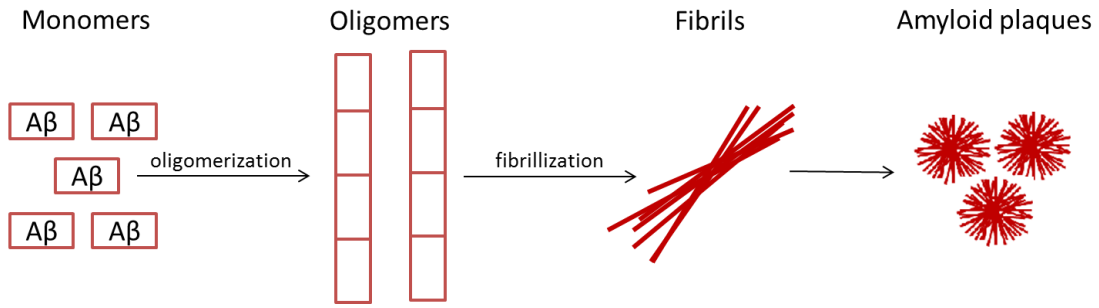


**Figure 4 – Aβ oligomerization, fibrillization and deposition into senile plaques**. In AD patients, Aβ monomers can self-associate to form dimers, trimers and larger oligomers. Aβ fragments oligomerize and fibrillize leading to AD pathology trough formation of the senile plaques. Adapted from (80).

The Aβ peptides family (Aβ38, Aβ39, Aβ40, Aβ42 and Aβ43), as mentioned above, is derived from the proteolytic cleavage of APP (42). In non-pathological conditions, the most abundant Aβ peptide produced in the brain and found in the CSF is Aβ40. However, several studies demonstrated that mutations in APP, PSEN1 and PSEN2 leads to increased production of Aβ42 and to early onset of AD (81). Consequently, in AD brains are found Aβ40 and Aβ42 peptides, which differ structurally by the absence or presence of two C-terminal amino acids (82). A number of *in vitro* and *in vivo* studies

showed that Aβ42 is more toxic than Aβ40 (83). Aβ42 is generally present in tissues and body fluids at levels ranging from 5-10% of those of Aβ40, appearing to be vital to initiating Aβ aggregation since it is more hydrophobic and prone to aggregate than Aβ40 (1). Additionally, Aβ42 oligomers, initially formed as a seed, accelerate Aβ40 aggregation to form the SPs that ultimately lead to neurodegeneration (84). Within SPs, Aβ is present in aggregated forms including fibrils and oligomers (1).

### 1.2.5. Aβ role in AD pathogenesis

In order to improve the knowledge of the cellular and molecular processes involved in AD it is vital to elucidate the roles of Aβ in this neurodegenerative disease. All the hypotheses proposed to explain AD pathogenesis, including the amyloid cascade (Figure 5), excitotoxicity, oxidative stress and inflammation hypotheses, are remarkably based on the role of Aβ to some extent (82,85).

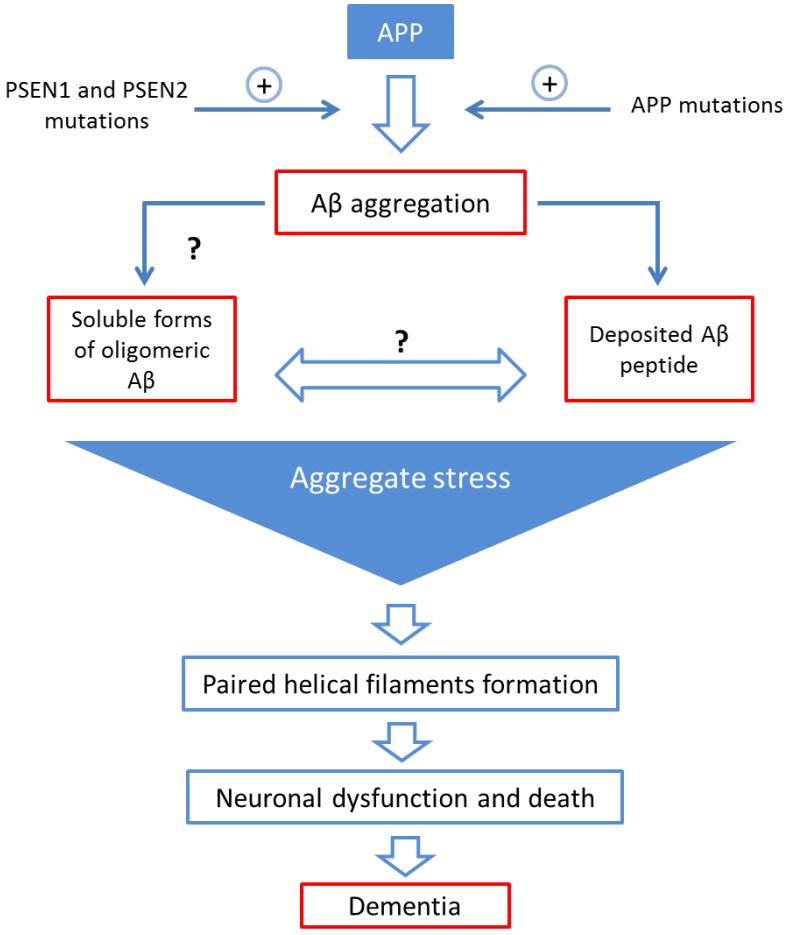


**Figure 5 – The amyloid cascade hypothesis for AD.** Adapted from (86).

The ability of toxic Aβ peptides to induce protein oxidation and to inhibit the activity of oxidation-sensitive enzymes is consistent with the hypothesis that Aβ can induce severe oxidative damage. A number of oxidatively modified brain proteins were identified using redox in AD and MCI patients and in *in vitro* models of AD, which places Aβ in the center of a number of biochemical and cellular processes such as energy metabolism, protein degradation, synaptic function, neuritic growth, neurotransmission, cellular defense and memory formation (87).

Other studies demonstrated that, in neurons, Aβ impairs APP/sAPP vesicular anterograde transport and exocytosis through a mechanism mediated by altered cytoskeleton dynamics of both microtubule and actin networks. Aβ-mediated cytoskeleton abnormalities contribute to impaired protein vesicular secretion of neurotransmitters and substances needed for neuronal survival and consequently leads to AD neurodegeneration. On the other hand, Aβ decreases AICD production, its nuclear translocation and nuclear complex formation with Fe65 leading to altered APP nuclear signaling and impaired gene transcriptional activation (88–90).

## 1.3. PHOSPHORYLATION IN AD

A crucial mechanism involved in cellular signaling of multiple biological processes is protein phosphorylation on tyrosine, threonine and serine residues which require a strict control by protein kinases and protein phosphatases (Figure 6) (7).

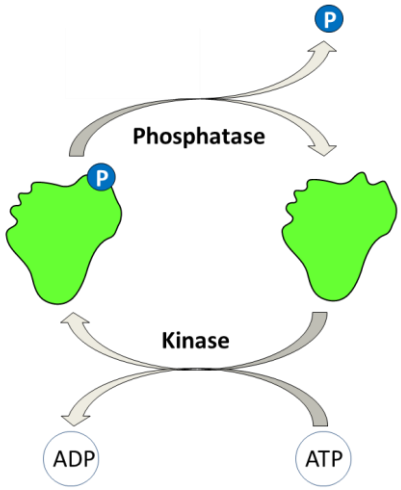


**Figure 6 – Phosphorylation-dephosphorylation process as a regulatory mechanism of proteins.** The protein kinase catalyzes the reaction in which a phosphoryl group (phosphate), donated by ATP, is transferred to an acceptor protein (protein phosphorylation). The protein phosphatase removes a phosphate group from phosphoproteins (protein dephosphorylation). Adapted from (91).

Furthermore, phosphorylation denotes one of the most important post-translational modifications in proteins. Abnormal protein phosphorylation has been linked with numerous human diseases including AD. As previously mentioned, one of the specific hallmarks of AD is the presence of NFTs composed of tau protein abnormally hyperphosphorylated (7). Interestingly, in addition to tau, several other brain proteins such as neurofilaments, microtubule-associated protein 1B (MAP1B), dynein, CRMP-2, β-tubulin and β-catenin are also found to be hyperphosphorylated, suggesting that the protein phosphorylation/dephosphorylation mechanism is dysregulated in AD (92–95). Corroborating this knowledge, several studies, gathering high consensus, reveal alterations in protein kinase and phosphatase activities in AD (Table 1), as well in phosphorylation levels of key proteins involved in AD pathogenesis as tau protein and APP.

**Table 1 – Altered expression and/or activity of kinases and phosphatases in AD brain.** Adapted from (96).

| Protein | Expression and/or activity in AD |
|---|---|
| **Protein kinases** | |
| **GSK3β** | Increased |
| **Cdk5** | Increased |
| **Dyrk1A** | Increased |
| **ERK1/2** | Increased |
| **JNK** | Increased |
| **p38MAPK** | Increased |
| **CKI** | Increased |
| **Akt/PKB** | Increased |
| **PKA** | Decreased |
| **PKC** | Decreased |
| **Phosphoprotein phosphatases** | |
| **PPP1** | Decreased |
| **PPP2** | Decreased |
| **PPP5** | Decreased |
| **PPP3 (calcineurin)** | Increased |
| **Cdc25A** | Increased |
| **Cdc25B** | Increased |
| **PTEN** | Decreased |

**Kinases and phosphatases**

As observed in Table 1, a number of kinases are upregulated with increased expression and/or activity in AD brains. Among the upregulated kinases are glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (cdk5), dual-specific tyrosine (Y) regulated kinase 1A (Dyrk1A), mitogen-activated protein kinases (MAPKs), c-Jun NH(2)-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) (93). GSK3β is implicated in the pathogenesis of AD through Aβ-induced neurotoxicity

and interaction with PSEN1, as well as in tau hyperphosphorylation (97,98). The cdk5-mediated phosphorylation of tau, APP and PSEN1 could also be partially behind tau hyperphosphorylation, neurofibrillary pathology, increased Aβ levels and neurodegeneration (99–101). In respect to Dyrk1A, it has been proved that its overexpression leads to learning and memory deficits, tau hyperphosphorylation and elevated Aβ levels (102–104). Finally, it is also known that MAPKs phosphorylate tau protein *in vitro* while the activation of JNK and p38, was specifically associated with age-dependent amyloid plaque deposition (105–107). Consistent with increased levels of Aβ peptides, protein kinase C (PKC) has reduced activity in AD patients. The signaling pathways of this kinase regulate the α and β-secretase-mediated cleavage of APP, resulting in a reduction of Aβ peptides production (108).

On the other hand, the more relevant tau phosphatase in the brain, the phosphoprotein phosphatase 2 (PPP2), is decreased both in activity and expression in selected areas of AD brain along with other phosphatases as phosphoprotein phosphatase 1 (PPP1), phosphoprotein phosphatase 5 (PPP5) and phosphatase and tensin homolog (PTEN) (93,96). The downregulation of PPP2 may underlie the hyperphosphorylation of tau in AD brains, what can partially be due to the upregulation of two endogenous PPP2 inhibitors, I1 and I2 (109). PPP2 can also be behind the activation of several PPP2-regulated protein kinases, including calcium/calmodulin-dependent kinase II (CaMK-II) and MAPKs (93).

**Tau protein**

The gene of tau is localized on the long arm of chromosome 17 (17q21) and contains 16 exons. Tau protein is a member of the microtubule-associated proteins (MAPs) group and consists of a prototypical “natively unfolded” protein (110,111). In the CNS there are 6 isoforms of tau which result from alternative splicing of exons 2, 3 and 10, varying from 352 to 441 amino acids. The isoforms are differentially expressed during brain development since each of them has specific physiological roles (112). Tau is synthetized and produced in all neurons. The main functions of tau consist in binding to tubulin via its microtubule-binding domains (located at the C-terminal half of the molecule) and promote the assembly and stabilization of microtubules which is important to proper function of neurons (93,113). In AD, the insoluble form of tau results from its hyperphosphorylation state, causing its dissociation from microtubules and consequent formation of paired helical filaments (PHF) previously mentioned. These PHF have propensity to self-aggregate forming NFTs in cell bodies and dystrophic neurites (114,115). The abnormally hyperphosphorylated tau detached from microtubules also causes increased intraneuronal soluble tau concentration due to

sequestration of normal tau from microtubules, which further facilitates tau aggregation into PHFs (93).

Microtubules contribute to several cellular processes such as cell morphogenesis, cell division and intracellular trafficking. Since tau binds to microtubules and its phosphorylated state regulates the affinity to microtubules, tau hyperphosphorylation affects axonal transport altering intracellular traffic (75,78,79). The breakdown of the microtubule network in the affected neurons compromises axonal transport and leads to retrograde degeneration, which in turn results in neuronal death and dementia (93).

The pattern of tau phosphorylation correlates with the loss of neuronal integrity (116). Overall tau phosphoprotein is at least three to fourfold more hyperphosphorylated in AD brains than control normal brains (117). It has been proved that this abnormal phosphorylation is a vital event that triggers the pathological aggregation of tau in AD (113,118,119). An upregulation of tau kinases - GSK3β, cdk5, cAMP-dependent protein kinase (PKA), extracellular regulated kinase-1 (ERK1) and ERK2 and CaMK-II - or a downregulation of tau phosphatases - PPP1, PPP2, PPP3 and PPM1 - can be behind the abnormal protein phosphorylation (87,120,121).

**APP**

APP itself has several putative phosphorylation residues in its short cytoplasmic domain and can be phosphorylated both *in vivo* and *in vitro* (122,123). Lee et al. (124) demonstrated that in AD patients seven of the eight potentially phosphorylatable residues in the intracellular domain of APP were phosphorylated, namely Tyrosine 653 (Tyr653), Serine 655 (Ser655), Threonine 668 (Thr668), Serine 675 (Ser675), Tyrosine 682 (Tyr682), Threonine 686 (Thr686) and Tyrosine 687 (Tyr687), according to APP695 isoform numbering. The Tyr687 lies within a typical internalization signal motif for membrane-associated receptor proteins, the $^{682}$YENPTY$^{687}$ domain (125–127). This domain has an important role in protein-protein interactions, as for example with X11 (128). Accordingly, it was discovered that APP sorting and processing are modulated through signal transduction mechanisms regulated by protein phosphorylation (129–131). Remarkably, PKC seems to be a vital component in signaling pathways that control APP metabolism since the stimulation of this kinase activates sAPPα production and concomitantly inhibits generation of Aβ fragment (132,133). PKC phosphorylates APP both *in vitro* and *in vivo* on Ser655 within the cytoplasmic domain (123,134). Different PKC isoforms have different roles in controlling the generation of sAPP and Aβ fragments. It is though that PKCα increases soluble APP secretion by enhancing APP metabolism and PKCε increases sAPP secretion probably through increasing APP expression (135). The same kinases responsible for the

phosphorylation of tau such as GSK3β and cdk5, seem to be also involved in the phosphorylation of APP (96). Since kinases are always associated with phosphatases, these ones must have a role in APP secretion too. The inhibition of PPP1 stimulates secretion of APP which implicates PPP1 in APP metabolism (136).

Several studies were performed in order to understand the role of the phosphorylation at specific residues of APP. In one of those studies, it was found that the subcellular localization of APP, proteolytic cleavage and its incorporation into vesicular structures is Tyr687 phosphorylation dependent through mimicking phosphorylation/dephosphorylation of this residue. It was also discovered that Aβ production can be modulated directly by APP phosphorylation (137).

Other important role of phosphorylation in AD pathogenesis is that accordingly to the phosphorylated state of APP, the proteins that bind and interact with APP are different (138). For example, the phosphorylation of APP at Thr668 affects its binding to Fe65 (65). This fact has implications in the molecular mechanisms underlying AD pathogenesis since the interactions of APP can precipitate events that culminate in neuronal damage.

**Aβ as a link between tau and APP abnormal phosphorylation**

Aβ holds an important role in the phosphorylation of proteins involved in AD including tau protein and APP, which are key players in the development of the disease (Figure 7). As example, Aβ peptide fibrils induce an increase of the c-Abl activity in rat hippocampal neurons. The cytoplasmic, nonreceptor tyrosine kinase c-Abl activation is involved in cell signals that regulate neuronal death and consequently, may be a downstream apoptotic mechanism induced by Aβ in neurons (139). Other studies of the effects of Aβ include activation of the protein tyrosine kinase (PTK) Lyn in microglial cells (140), increased tyrosine phosphorylation (141), activation of Src family protein kinases, activation of phosphatidylinositol 3-kinase (PI3K) (142) and cAMP response element-binding protein phosphorylation (143). The rapid changes, initiated by Aβ, in phosphorylation of neuronal proteins, including the cytoskeletal ones, may be critical early pathogenic events in AD, which culminate with neuronal death and neurodegeneration (144). Furthermore, stress and apoptotic proteins are increased after treatment with Aβ, what is in agreement with well-known neurotoxic and apoptotic effects of this peptide (145).

Aβ can induce a number of biochemical changes in neurons (Figure 7) including the stimulation of tau kinases previously mentioned, which contribute to hyperphosphorylation of tau (120,140,146–149). Aβ also directly affects phosphatase activity inhibiting both PPP1 and PPP2 activities in a dose-dependent manner (150). Ultimately, Aβ may prompt the production of PHF through increasing

expression of tau phosphorylation kinases and inhibiting the activity of tau phosphatases, what will induce formation of NTFs preceding neurodegeneration (147).

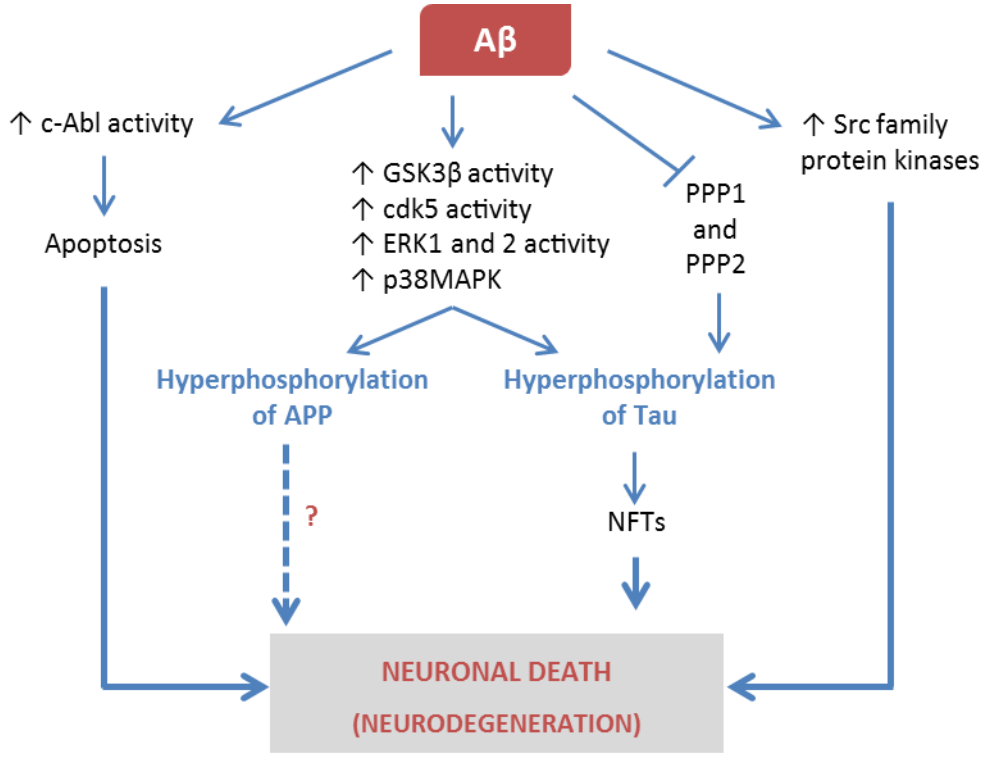


**Figure 7 - Aβ effects on phosphorylation.** Aβ enhances c-Abl activity (139), tau and APP kinases (140,147,151–154) and Src family protein kinases (144). On the other hand PPP1 and PPP2 are inhibited (149,150).

The reported hyperphosphorylation of APP itself in AD patients may be explained by the Aβ inhibition of PPP1 (124). Aβ also inactivates PPP2 leading to the phosphorylation of APP at Thr668 (155). In turn, phosphorylation of APP at Thr668 residue has been linked to increased amyloidogenic processing of APP (122,153). Aβ itself affects the phosphorylation of APP and other proteins through increasing percentage of kinases and phosphatases (145).

Given the involvement of protein phosphorylation and imbalanced protein phosphatases and kinases activities in AD pathogenesis, is urgent to develop work that highlight the vital importance of abnormal phosphorylation state observed in AD patients. This purpose is crucial to understand the molecular mechanisms of AD as to identify potential future biomarkers and therapeutic targets.

# 2. Aims

Alzheimer's disease is the most common form of dementia among the neurodegenerative disorders and is neuropathologically characterized by the presence of SPs and NFTs in AD brains. Senile plaques are mainly composed by aggregates of Aβ peptide and NFTs are intracellular hallmarks formed by hyperphosphorylated tau protein. All the hypotheses proposed to explain the molecular basis of AD, including the amyloid cascade, are based on Aβ accumulation and deposition that will contribute to neuronal degeneration. Another important event during the progression of the disease is the abnormal protein phosphorylation, as in NFTs. Interestingly, Aβ also holds a role in protein phosphorylation in AD brains. Therefore, a link between Aβ, abnormal phosphorylation and AD pathogenesis can be made. Hence, the study of Aβ effects on neuronal proteins phosphorylation is important and may help in the understanding of the molecular events underlying AD. Further, by looking at Aβ effects in the neurophosphoproteome we can also identify novel candidate biomarkers, which may assist in AD differential diagnosis, or putative therapeutic targets for the AD treatment.

Therefore, the main objectives of this thesis were:

- Development of a script to organize and characterize the phosphoproteins identified;
- Characterization of the neuronal phosphoproteome in hyperphosphorylation conditions;
- Characterization of the neuronal phosphoproteome in AD mimicking conditions;
- Identification of potential novel phosphospecific biomarkers candidates for AD.

# 3. Materials and Methods

In order to identify and analyze the phosphoprotein profile upon OA and Aβ treatments, a workflow was designed. The experiments were conducted in primary neuronal cultures. Neuronal cells were obtained from rat embryos. After 10 days in culture, primary cortical neurons were treated with or without (Control) Okadaic Acid (OA) or Aβ. Upon the indicated treatment, a phosphoprotein enrichment kit (P-kit) was used to isolate and purify the phosphoproteins from the rat neurons. To identify the purified phosphoproteins in each condition a tandem mass spectrometry (MS/MS) analysis was performed. Before MS/MS analysis, samples obtained from the P-kit were subjected to 1 dimension gel electrophoresis (1DE) to separate the phosphoproteins. The phosphoproteins were trypsin digested and extraction of peptides was performed for MS/MS analysis. Finally, to choose the relevant phosphoproteins and to characterize them, a script was developed (Figure 8).

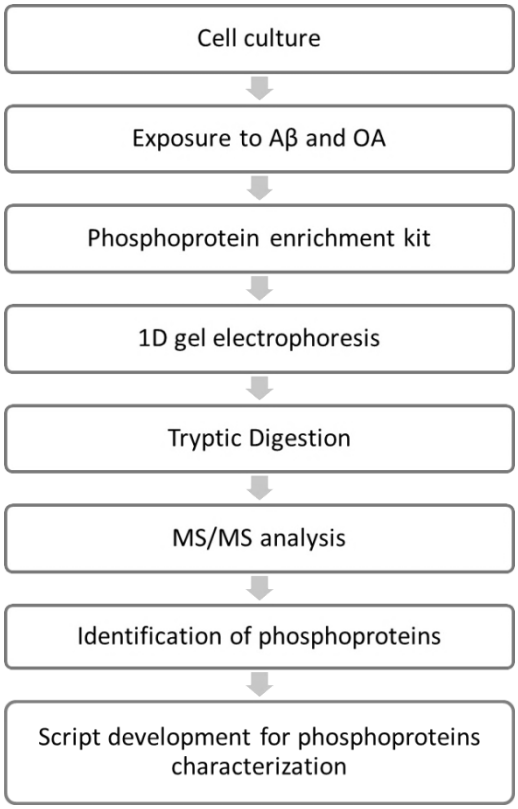


**Figure 8 – Workflow of the method used to achieve the main objective of this project.**

## 3.1. ISOLATION AND CELL CULTURE

The experiments were carried out in primary rat neuronal cultures, from Wistar Hannover rat embryo at 18$^{th}$ day of gestation as described in Henriques et al. (156). Cerebral cortex was dissected and dissociated with trypsin (0.23 mg/mL) and desoxyribonuclease I (0.15 mg/mL) in Hanks balanced solution (HBSS). In order to stop trypsinization, cells were washed with HBSS supplemented with 10% fetal bovine serum (FBS), centrifuged at 1000 rpm for 2 minutes and further washed and centrifuged again with HBSS for serum withdraw. Cells were plated onto poly-D-lysine coated dishes at a density of 6x10$^6$ cells/cm$^2$ and cultured for 10 days in Neurobasal medium (Gibco) supplemented with a serum-free medium combination, 2% of B27 (final concentration). Additionally, the medium was supplemented with glutamine (0.5 mM) and gentamicin (60 µg/mL). Cells were maintained in an atmosphere of 5% $CO_2$ at 37ºC and observed under an inverted optical microscope. Five days after plating, 25% of culture medium was replaced with complete Neurobasal medium. On the 10$^{th}$ day of culture cells were used for experimental procedures.

## 3.2. EXPOSURE TO OA AND Aβ

In order to mimic AD conditions the cells were exposed to OA or Aβ. Before treatment with OA or aggregated A$\beta_{1-42}$, cells were plated and washed twice with phosphate buffered saline (PBS). The Control cultures received PBS vehicle. Then, cells were exposed to 0.5 µM of OA, which is a protein phosphatase inhibitor, for 3 hours in a B27-free Neurobasal medium combination (NB-B27). Aggregated A$\beta_{1-42}$ was obtained through reconstitution of A$\beta_{1-42}$ in $H_2O$ ultrapure (1mM stock) and aggregated in PBS for 48 hours at 37ºC (100 µM aggregated stock). Finally, cells were incubated with 10 µM of Aβ1-42 concentration for 3 hours in a NB-B27 medium.

## 3.3. PHOSPHOPROTEIN ENRICHMENT KIT

The P-kit was used to separate phosphoproteins from the non-phosphorylated proteins through a Phosphate Metal Affinity Chromatography (PMAC) resin (purification step). This PMAC resin allows proteins that contain a phosphate group on any amino acid (including serine, threonine or tyrosine) to be selectively bound to it. The non-phosphorylated proteins and other contaminants pass through the column. At the end, a solution enriched in phosphoproteins is obtained and eluted from the column. This method is extremely selective and gives high resolution.

Three hours after the exposure to OA and Aβ the cells were collected to performed the extraction of phosphoproteins. They were removed from the NB-B27 medium and washed twice with 8mL of PBS. After washes, PBS was added to each plaque and cells removed using a scraper. To obtain a pellet, cells were centrifuged 2 times at 500xg for 5 min and the weight of the pellet determined. Depending on the weight of the pellet in each sample, the extraction Buffer (30µL of Buffer A per 1mg of pellet) was added. Sodium Fluoride (a phosphatase inhibitor) was also added to a final concentration of 10mM. The samples were incubated at 4ºC for 10 min and afterwards centrifuged at 10000xg, for 20 min at 4ºC. At this point the phosphoprotein affinity columns begun to be prepared:

1. Columns were positioned at room temperature in an upright position until the resin to settles out of suspension;
2. The caps of the columns were removed to allow the drain of the storage buffer;
3. Columns were washed firstly with 5mL of distilled water and then 3 times with 5 mL of Buffer A to equilibrate the columns;
4. The pH of the columns was measured and it was equal to 6.0;
5. Columns were closed with the caps and were ready to receive the samples.

The supernatants resultant from the last centrifugation represent the total extract of phosphoproteins to be analyzed, thus they were added to the respectively columns (Control, OA and Aβ). Columns were closed and gently agitated at 4ºC for 20 min on a platform shaker to allow the phosphorylated proteins to bind to the column. After that, the columns were positioned in the upright position to allow the resin to settle out of suspension. The caps of the columns were removed and the non-adsorbed material flowed through. Columns were further washed 4 times with 5mL of Buffer A. Finally, to obtain the phosphorylated proteins, 1 mL of elution Buffer (Buffer B) was added and the fractions of phosphorylated proteins collected on ice. This step was repeated 3 times resulting in 4 protein fractions for each column. To measure total protein concentration 2 aliquots (50µL) of the phosphoprotein fractions were removed and total protein content quantified using BCA assay (see section 3.3.1). Protein content will allow the selection of the fraction to be analyzed by MS/MS. Samples were stored at -80ºC.

- **Protein concentration determination**

Regarding the quantification of cell lysates obtained the BCA protein assay (Pierce) was used. BCA assay is based on the use of bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. Since proteins have the ability to reduce $Cu^{2+}$ to $Cu^{+}$ in an alkaline

environment (the biuret reaction), BCA produces a purple color in the presence of the reduced $Cu^{+}$ ion. This reduced ion results from chelation of two molecules of BCA with one cuprous ion. The soluble complexes formed exhibit a strong absorbance that can be read at 562 nm.

The quantitative analysis was carried out using 50 µL of the collected phosphoprotein fractions. A standard curve was prepared by plotting Bovine Serum Albumin (BSA) standard absorbance vs BSA concentration (Table 2) and used to determine the total protein concentration of each sample. Both samples and standards were incubated with working reagent (prepared with 50 parts of reagent A to 1 part of reagent B). All samples were incubated at 37ºC during 30 minutes, cooled to room temperature, and directly measured at 562 nm.

**Table 2 – Standards used in BCA protein assay method.** BSA - Bovine Serum Albumin solution (2mg/mL).

| Standard | BSA (µL) | $H_2O$ (µL) | Protein Mass (µg) |
|---|---|---|---|
| **P0** | 0 | 40 | 0 |
| **P1** | 1.6 | 38.4 | 3.2 |
| **P2** | 3.2 | 36.8 | 6.4 |
| **P3** | 8 | 32 | 16 |
| **P4** | 16 | 24 | 32 |
| **P5** | 32 | 8 | 64 |

Accordingly to BCA results, the fraction 2 of each condition was the most phospho-enriched fraction and was consequently lyophilized to further MS/MS analysis.

## 3.4. Sample Preparation and MS/MS analysis

To prepare the phospho-enriched fractions for MS/MS analysis, a gel based method was performed (Figure 9).

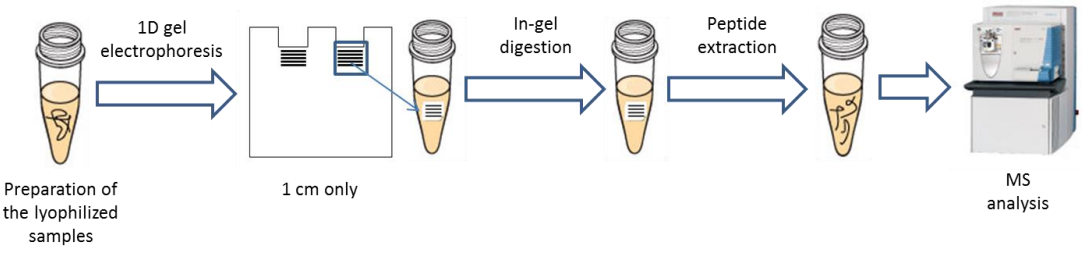


**Figure 9 – Sample preparation workflow for MS/MS analysis utilizing a gel based method.**

### 3.4.1. 1D Gel Electrophoresis (1DE)

In order to dissolve the lyophilized samples for 1DE, 60 µL of lithium dodecyl sulfate (LDS) 1-fold buffer were added. Following, samples were incubated for 10 min at 95ºC plus a sonication step of 5 min. At this stage samples were ready to be load on the 1DE.

The polyacrylamide gel was done as described in Table 3.

**Table 3 - Quantities used to do one polyacrylamide gel**. AA = acrylamide; Bis Tris = 2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol; $H_2O$ = water; TEMED = N,N,N',N'-Tetramethylethylenediamine; APS = Ammonium Persulfate.

| Substances | Quantities |
|---|---|
| **AA 30%** | 4 mL |
| **Bis Tris 7x (10%)** | 1.42 mL |
| **$H_2O$** | up 10 mL |
| **TEMED** | 2 µL |
| **APS 40%** | 10L |

Samples were loaded on the gel and the electrophoresis system run for 45 min at 50 V. After 1DE, the gel was stained with Coomasie Blue staining solution for 1 hour. To de-staining the gel, 3 washing steps of 30 min were performed with acetic acid (10%) and water. An additional wash step, only with water, for 20 min was carried out.

### 3.4.2. Band Excision and Tryptic Digestion of gel pieces

The gel bands of 1 cm of each lane were excised and cut in smaller pieces to further washes. The 10 min washes were performed utilizing 15 mL of Solution A (10mM of AMBIC buffer) and 15 mL of Solution B (7.5 mL of Acetonitrile (ACN) + 7.5 mL of AMBIC buffer) sequentially and repeated 3 times. The SpeedVacuum was used during 30 min to dry the samples finishing the preparation for tryptic digestion of the gel pieces.

For the tryptic digestion, 4 µL of stock solution of trypsin and 12 µL of AMBIC buffer (tryptic solution) were added to each sample and incubated overnight at 37ºC.

### 3.4.3. Peptide Extraction

To extract peptides from gel slices the next steps were repeated twice in the previously incubated samples:

1) 60 µL of the extraction solution (trifluoroacetic acid (TFA) 0.1% + ACN - 50:50) were added to each sample;

2) Samples were sonicated for 15 min;

3) Supernatant were collected in a new microtube.

Supernatant of each sample were subjected to the SpeedVacuum for 1 hour. After that, 30 µL of TFA 0,1% was added and samples stored at -30ºC. The samples were then ready for MS/MS analysis.

### 3.4.4. MS/MS analysis and identification of the proteins

The proteolytic samples (40 µL) were injected in the Q Exactive- Orbitrap LC-MS/MS System (Thermo Scientific). After the acquisition of the MS/MS spectra of each peptide present in the samples (Figure 10), bioinformatic tools were used to identify the phosphoproteins. Through Proteome Discoverer Software (Thermo Scientific), a qualitative analysis of the data was carried out using:

1) International Protein Index (IPI) as database for protein search;
2) Mascot as MS/MS database search tool and protein score (proteins with score below 65 were excluded);
3) Rattus novergicus as organism model.

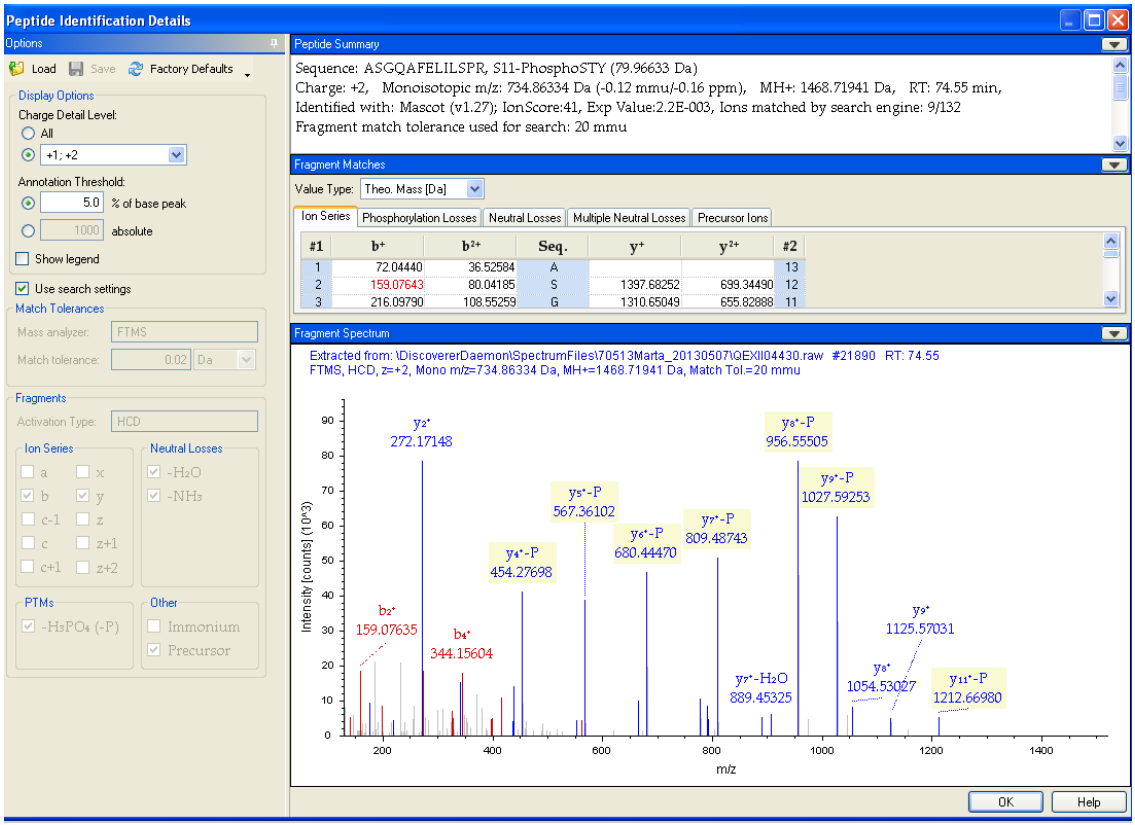


**Figure 10 – An example of the MS/MS spectra of a peptide.**

## 3.5. CHARACTERIZATION OF THE PHOSPHOPROTEINS AND THE APPLICATION OF A SCRIPT

The software associated with the MS/MS analysis provided a list of protein names with an international protein index (IPI) identifier. The IPI is a protein database associated to the European Bioinformatics Institute (EBI) that describes the proteome of higher eukaryotic organisms including rat (http://www.ebi.ac.uk/IPI/IPIhelp.html). Consequently, after the MS/MS analysis a list with names and the IPI of each protein was generated. The number of proteins obtained in each condition was massive to be individually analyzed (around 800 proteins). In order to process all data, a bioinformatic tool - a script - was developed to characterize the proteins. The script, named **ProtDB**, was developed in Python language with the collaboration of the Physic Engineering Cristóvão da Cruz e Silva. This tool is available at http://sourceforge.net/projects/protdb/files/. Furthermore, a neural network prediction of phosphorylation sites (NetPhos 2.0 Server) was also used to validate the proteins as phosphoproteins.

To characterize properly the proteins a GeneOntology (GO) analysis based on the protein features (biological process, cellular component and molecular function) was carried out. Therefore, the first step of the ProtDB script was to convert proteins IPI into Universal Protein Resource (UniProt) identifiers. UniProt is a comprehensive resource for protein sequence and annotation data that includes the UniProt Knowledgebase (UniProtKB). The UniProtKB is divided in two branches, a reviewed database (UniProtKB/Swiss-Prot) and an unreviewed database (UniProtKB/TrEMBL). As UniProt is a collaboration between the EBI, the Swiss Institute of Bioinformatics (SIB) and the Protein Information Resource (PIR) it is a powerful database that comprises crucial information about the proteins, including GO information (http://www.uniprot.org/help/about). Essentially, the ProtDB script allows the separation of the proteins (gives information about common and exclusive proteins in each condition) and, through the conversion of database identifiers, provides information that leads to the characterization of the proteins, including sequence, recommended name, motifs, biological process, cellular component and molecular function. Through specific commands wrote in Python language (Figure 11), the ProtDB script provided the protein characterization.

```
1  from ProtDB import ProtDB
2  from Library.Motifs import Motifs
3
4  lib = ProtDB()
5  lib.loadXLS_IPI("proteinas.xls")
6  lib.loadDB(other = "RAT")
7
8  lib.followXRefs("IPI", "UPSP")
9  lib.followXRefs("IPI", "UPTr")
10
11 lib.savePickle("proteinas.pickle")
12 #lib = lib.loadPickle("proteinas.pickle")
13 print len (lib)
14
15
16 #Proteinas com o Grupo A positivo (Controlo +)
17 lib2 = lib.findScreening("Grupo A")
18 print len(lib2)
19 lib3 = lib2.findGO("GO:0005488", "UniProtKBSwissProt")
20 lib3.saveXLS("proteinasGO:binding.xls")
21 lib3.saveXLSGO("proteinasinfo:binding.xls", "UniProtKBSwissProt")
22 print len(lib3)
23
24 #Proteinas apenas presentes no Controlo
25 lib11 = lib2 - (lib2.findScreening("Grupo B") + lib2.findScreening("Grupo C"))
26 print len(lib11)
27
```

**Figure 11 – Examples of commands used in the ProtDB script to find specific information**. From line 4 to line 10 is the conversion of the protein IPIs to Uniprot identifiers. After saving the pickle file (line 11) the number of proteins in specific conditions can be accessed, as examples: the number of proteins in the all file (proteinas.xls) - line 13; the number of proteins in a specific group (Group A) – line 18; the number of the proteins with a specific GO term (GO:0005488 –binding) – line 22. All proteins information (name, sequence, molecular function, etc) present in each specific search done can be saved and analyzed in an excel file through the commands saveXLSGO and saveXLS (as lines 20 and 21).

# 4. Results

## 4.1. PHOSPHOPROTEINS OVERVIEW

Abnormal phosphorylation is a fundamental event associated with many pathological signaling cascades and consequent with many alterations observed in AD.

In order to uncover key molecules and pathways, whose phosphorylation can be altered in AD, imbalanced phosphorylation conditions were induced. In one of the approaches, primary rat neuronal cultures were treated with OA, a potent phosphatase inhibitor, to exacerbate abnormal phosphorylation. Additionally, to mimic the pathogenic AD condition, Aβ was added to neuronal cultures. Moreover, Aβ *per se* is able to alter phosphatase and kinase activities (139–154). In both cases, alterations in the neuronal phosphoproteome were determined.

The experiments were carried out 6 times and untreated rat neuronal cultures were used as controls. From the 6 replicas performed, two of them were excluded due to experimental errors. Namely the total protein number was low, probably due to the lyophilization process under bad thermal conductivity or the sublimation was not properly controlled which would render the poor outcomes with a possible loss of many proteins. It was also observed that 1D gel bands for those experiments were very tenuous corroborating the low concentration of proteins. The remaining 4 replicas were analyzed comparing Control and OA conditions (Table 4) or Control and Aβ conditions (Table 5). In order to process all the data obtained, the ProtDB script was developed (section 3.5) and used to quantify the total number of proteins in each experiment, as well as to identify the exclusive and the shared proteins in each condition. In both cases the total phosphoproteome obtained was validated by using bioinformatic tools.

### 4.1.1. Upon OA treatment

In a general perspective, the number of phosphoproteins increased upon OA treatment with a similar global protein number between the 4 replicas (P1-P4) of each condition (Table 4).

**Table 4 - Total number, shared and exclusive phosphoproteins obtained for Control and OA conditions**.

| Control vs OA | P1 | P2 | P3 | P4 |
|---|---|---|---|---|
| **Total in Control** | 505 | 554 | 455 | 569 |
| **Total in OA** | 575 | 569 | 680 | 663 |
| **Shared** | 346 | 370 | 380 | 448 |
| **Control exclusives** | 159 | 184 | 75 | 121 |
| **OA exclusives** | 229 | 199 | 300 | 215 |
| **Global protein number (shared + exclusive)** | 734 | 753 | 755 | 784 |
| **% shared** | 47.1% | 49.1% | 50.3% | 57.1% |
| **% Control exclusives** | 21.7% | 24.4% | 9.9% | 15.4% |
| **% OA exclusives** | 31.2% | 26.4% | 39.7% | 27.4% |

In order to define a consistent pattern of phosphorylation changes typical of the OA model, only the phosphoproteins shared between the 4 replicas in each condition were considered for further analysis (Figure 12). These values were obtained applying the ProtDB script.

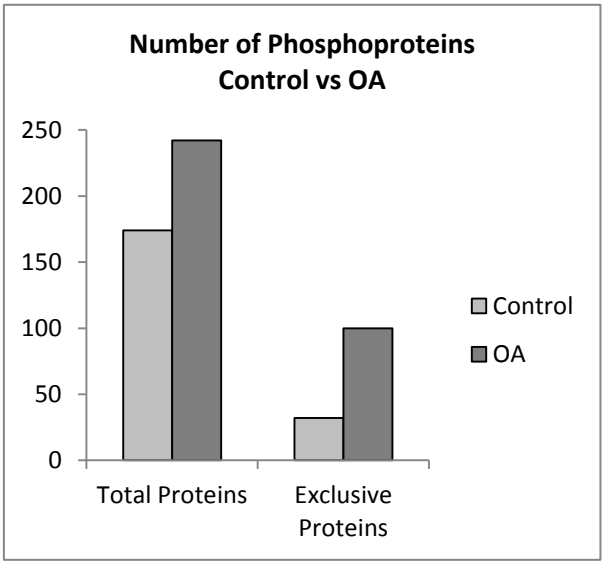


**Figure 12 - Shared phosphoproteins in Control and OA conditions**. The global number of proteins found was 274 proteins. The Control condition possesses 32 exclusive proteins from a total number of 174 proteins. On the other hand, OA condition has a total number of 242 proteins with 100 proteins being exclusive.

As expected, an increase is observed in the total and exclusive number of proteins for the OA condition (Figure 12). After this protein selection, the validation of these proteins as phosphoproteins was carried out (Figure 13), through research on the available literature, applying the ProtDB script (using phosphoprotein as keyword) and using NetPhos 2.0, a neural network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences (157).

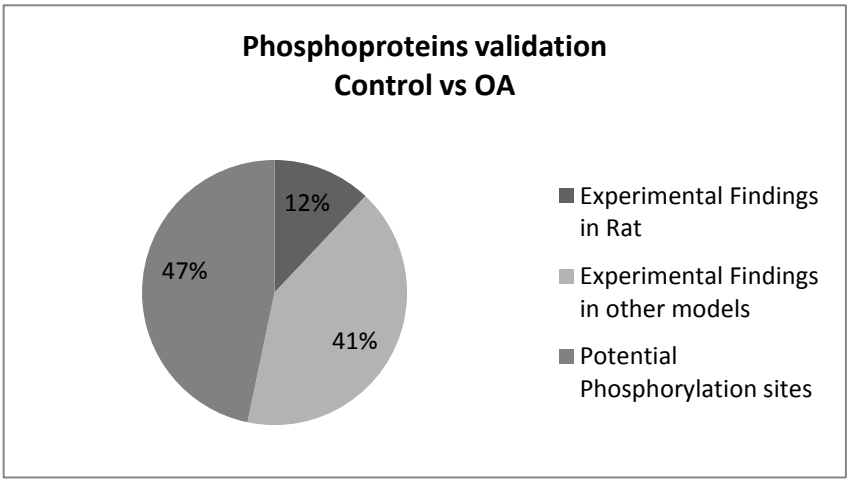


**Figure 13 - Phosphoproteins validation in Control vs OA conditions.** From a total of 274 proteins, 12% were confirmed in the literature as phosphoproteins relying upon experimental evidence through findings in Rat; 41% by experimental findings in the literature using other models and 47% using a neural network prediction server (NetPhos 2.0).

This *in silico* analysis reveals that all proteins obtained were already validated as phosphoproteins in experimental *in vitro* models or that at least contain potential phosphorylation sites. The validation of the uncharacterized proteins identified was also possible through the prediction of phosphorylation sites on the protein sequence by NetPhos 2.0.

### 4.1.2. Upon Aβ treatment

The total number of proteins in Control and Aβ is identical in the 4 replicas (P'1-P'4) as observed in Table 5.

**Table 5 – Total number, shared and exclusive phosphoproteins obtained for Control and Aβ conditions**.

| Control vs Aβ | P'1 | P'2 | P'3 | P'4 |
|---|---|---|---|---|
| **Total in Control** | 505 | 554 | 455 | 569 |
| **Total in Aβ** | 543 | 381 | 451 | 682 |
| **Shared** | 384 | 309 | 335 | 441 |
| **Control exclusives** | 121 | 245 | 120 | 128 |
| **Aβ exclusives** | 159 | 72 | 116 | 241 |
| **Global protein number (shared + exclusive)** | 664 | 626 | 571 | 810 |
| **% shared** | 58% | 50% | 58.7% | 54.4% |
| **% Control exclusives** | 18% | 39% | 21.0% | 15.8% |
| **% Aβ exclusives** | 24% | 11% | 20.3% | 29.8% |

Similar to what was done previously for Control vs OA conditions, the shared proteins from P'1 to P'4 replicas, were selected for further analysis in order to characterize the neuronal phosphoproteome upon Aβ treatment (Figure 14).

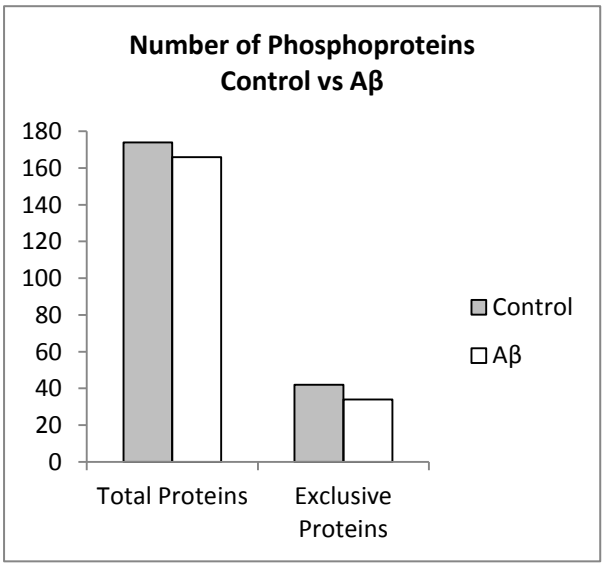


**Figure 14 – Shared phosphoproteins in Control and Aβ conditions**. From the 208 total proteins obtained, 174 and 166 proteins represent the total phosphoprotein pool (exclusives included) for Control and Aβ condition, respectively. Concerning the exclusive proteins, Control condition has 42 proteins whereas Aβ has 34 proteins.

In general, the total and exclusive number of proteins in Control is slightly higher than in Aβ condition. To validate the proteins as phosphoproteins, database search were accomplished (Figure 15) using the criteria mentioned above for Control vs OA conditions. Similar to Control and OA conditions, all proteins had already been validated in different experimental models or contain potential phosphorylation sites.

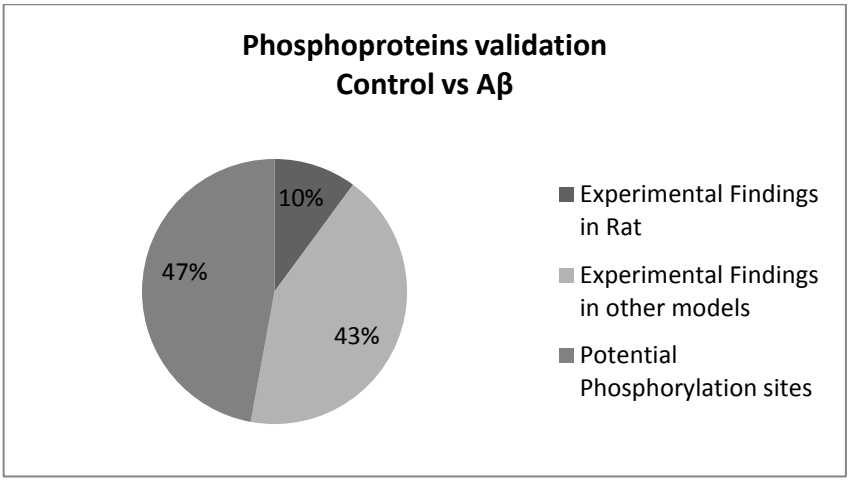


**Figure 15 – Phosphoproteins validation in Control vs Aβ conditions.** From a total of 208 proteins, 10% were confirmed in the literature as phosphoproteins relying upon experimental evidence through findings in Rat; 43% by experimental findings in the literature using other models and 47% using a neural network prediction server (NetPhos 2.0).

In summary, from the high number of proteins initially obtained, a more restrictive group of proteins (representing the shared phosphoproteins in the 4 replicas of each condition) was selected for subsequent neurophosphoproteome characterization. All of these proteins were bioinformatically validated as true (experimental evidence) or potential phosphoproteins.

## 4.2. OA EFFECTS IN THE NEUROPHOSPHOPROTEOME

To analyze the effects of OA in the neurophosphoproteome, 3 steps were observed: (1) general protein class sorting, in accordance with Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System (158); (2) GO analysis; and (3) comprehensive analysis of the exclusive proteins for each condition. However, not all proteins could be characterized since 36% of the pool of proteins in Control vs OA conditions holds information unreviewed or represent uncharacterized proteins.

### 4.2.1. Protein Classes

The phosphoproteins of Control and OA conditions were organized by classes in Figure 16. Remarkably, in the presence of OA, all protein classes increased, with exception of the isomerase, receptor and storage protein classes. Under these conditions a novel protein class, extracellular matrix protein, was evident.

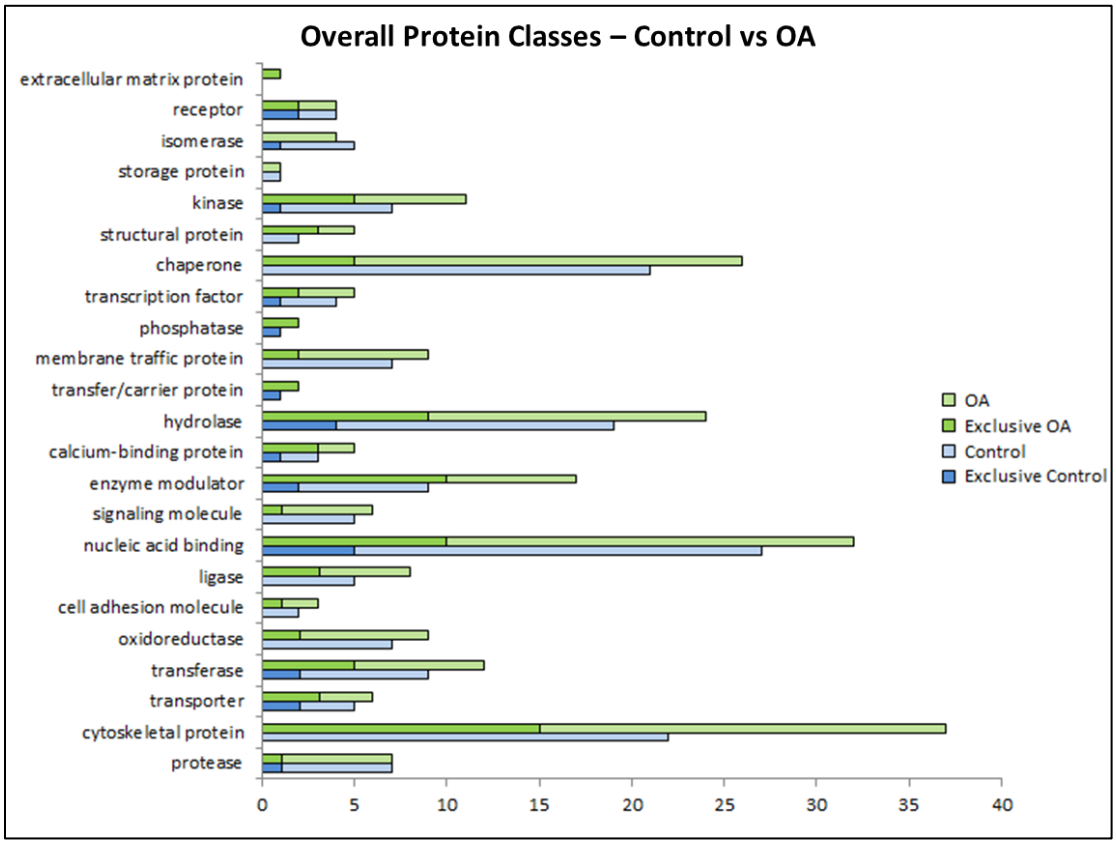


**Figure 16 - Overview of the protein classes presented in Control and OA conditions.**

### 4.2.2. Gene Ontology

The GO analysis was carried out to characterize and organize the phosphoproteins in three main domains: cellular component, biological process and molecular function.

- Cellular Components

The cellular component allows the organization of the proteins in terms of subcellular localization and was obtained through the use of the ProtDB script.

Figure 17 exhibits OA effects on the subcellular localization of the phosphoproteins. For all cellular components selected in this analysis the number of phosphoproteins increased with OA treatment.

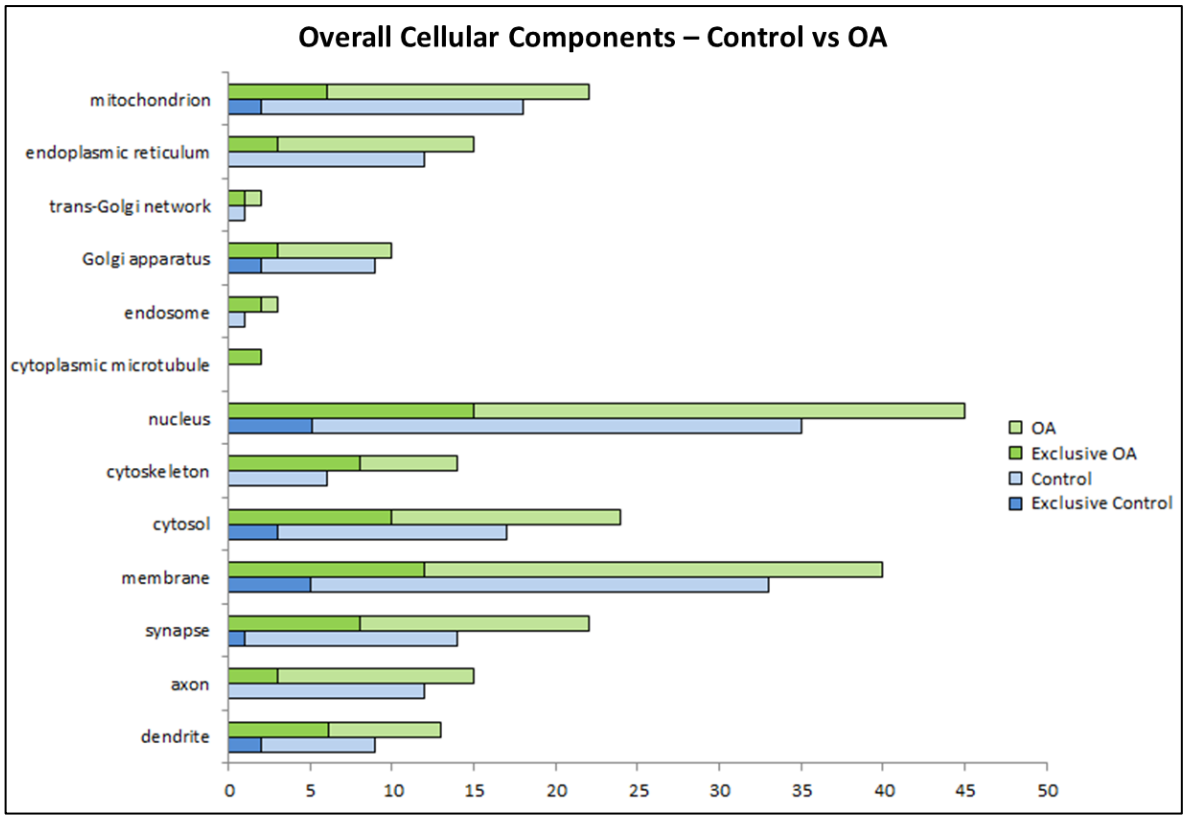


**Figure 17 - Overview of cellular component of Control and OA conditions.**

- Biological Processes

The Biological Process ontology of GO provides a general view of ordered assemblies of molecular functions and was systematized employing PANTHER Classification System (158).

The differences between the Control and OA conditions regarding biological processes were identified in Figure 18. The number of phosphoproteins augmented upon OA treatment in the majority of biological processes with the exception of two processes, homeostatic process and generation of precursor metabolites and energy that maintains the same number of phosphoproteins in OA condition.

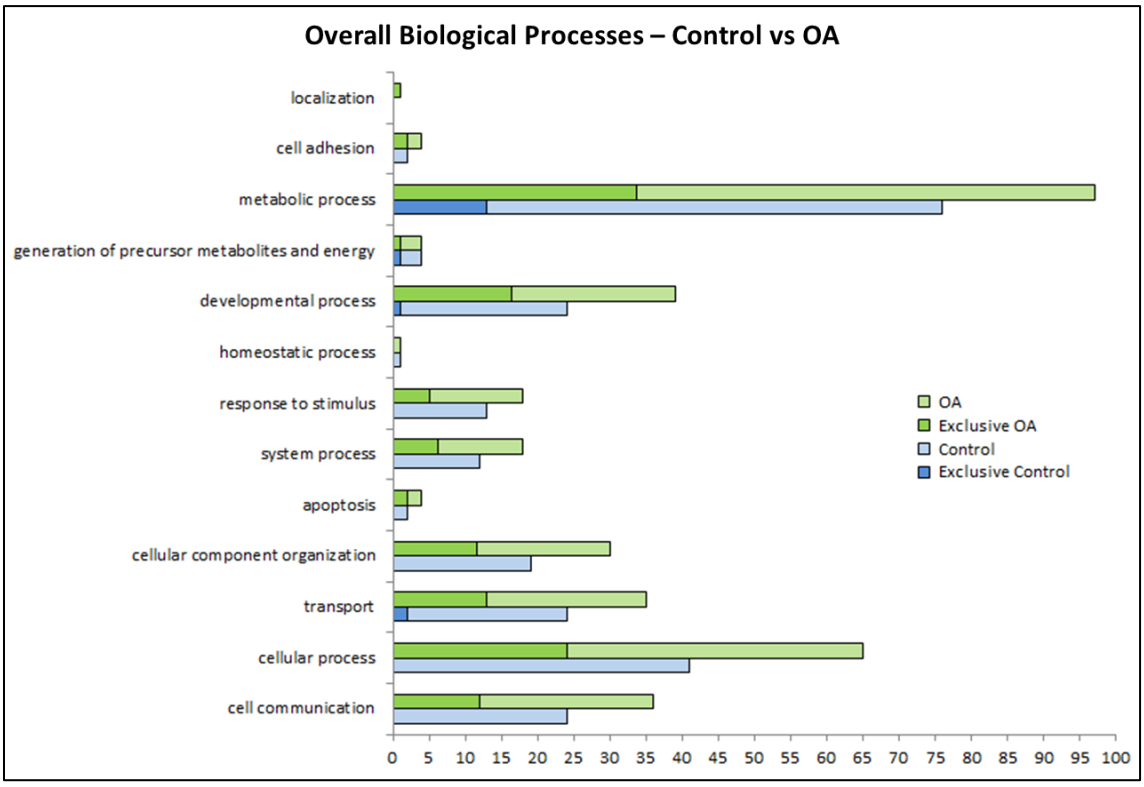


**Figure 18 - Overview of biological process involved in Control and OA conditions.**

- Molecular Functions

The molecular function ontology of GO analysis was obtained using the ProtDB script and the PANTHER Classification System (158). These data relate and describe the activities of the phosphoprotein pool at the molecular level.

Figure 19 compares Control and OA conditions, regarding the molecular function of the phosphoproteome pool. OA increases in a qualitative manner the phosphoproteins involved in binding, structural molecule, motor, catalytic, enzyme regulator, transcription, translation and transporter activities. The antioxidant activity is exclusive to the OA condition and the receptor and ion channel activities remain with the same number of phosphoproteins for both conditions but has exclusive phosphoproteins (darker color in graphic bars).

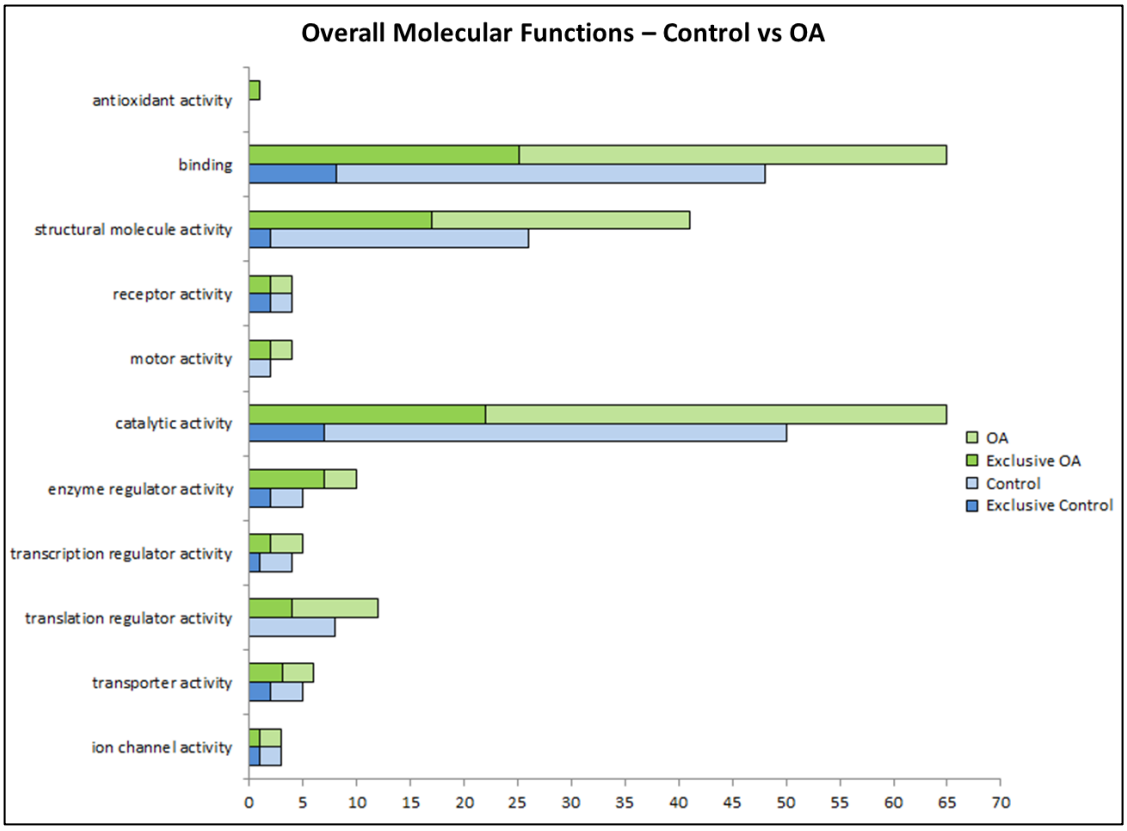


**Figure 19 - Overview of molecular functions involved in Control and OA conditions.**

## 4.3. Aβ EFFECTS IN THE NEUROPHOSPHOPROTEOME

The same approach used for characterization of the neurophosphoproteome upon OA treatment was used to characterize the neuronal phosphoproteins following Aβ exposure. Once more, as 37% of those obtained were uncharacterized proteins, not all proteins could be characterized in the Control vs Aβ pool.

### 4.3.1. Protein Classes

The first step to evaluate the effects of Aβ in the phosphoproteome comprised the organization of the proteins into classes (Figure 20).

Aβ treatment lead to an increase in the overall class of proteins of transfer/carrier and calcium-binding proteins when compared to Control, and a decrease in kinase, chaperone, transcription factor, cytoskeletal protein and protease classes. Eventhough, apparently, some proteins classes did not differ among Control and Aβ conditions, there are phosphoproteins exclusive to each condition, as in chaperone and protease classes (darker color in graphic bars).

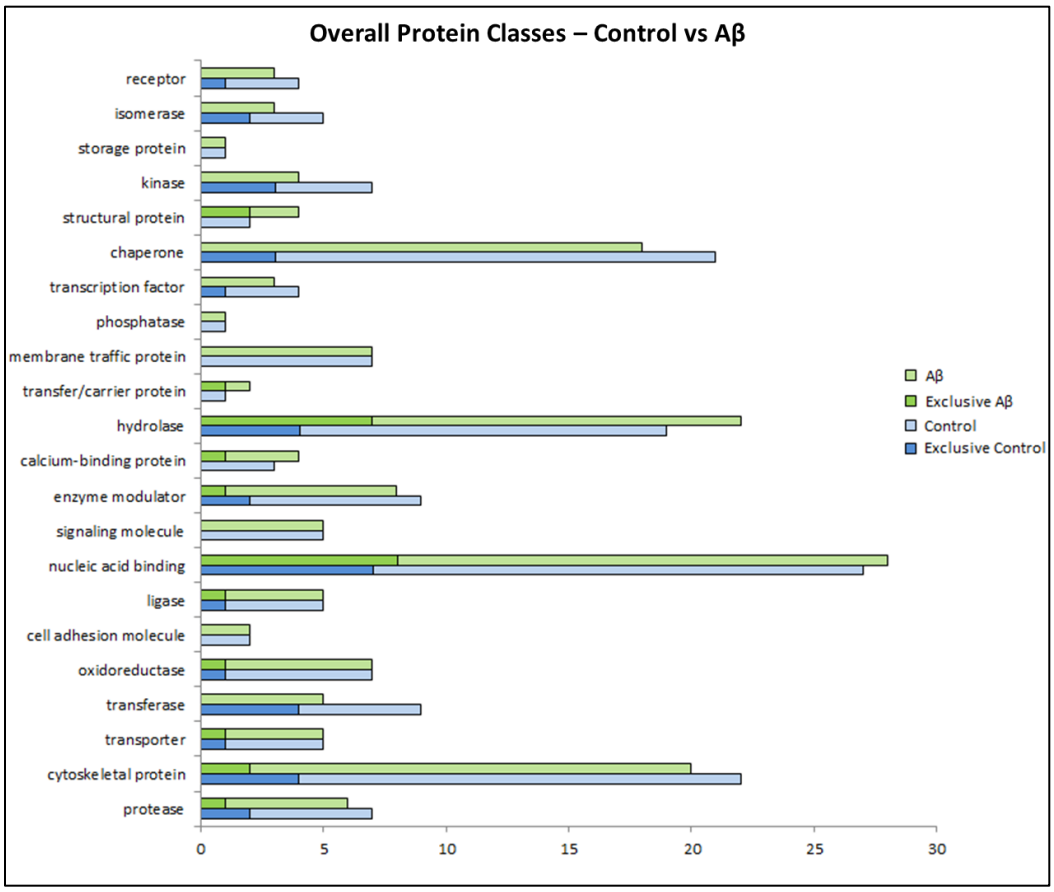


**Figure 20 - Comparison of the protein classes obtained in Control and Aβ conditions.**

### 4.3.2. Gene Ontology

- Cellular Components

The localization of the proteins in Control and Aβ conditions are represented in Figure 21. The number of phosphoproteins localized in mitochondrion, endoplasmic reticulum, Golgi apparatus, nucleus, cytosol and membrane diminished in Aβ condition. Nevertheless, upon Aβ treatment, the number of phosphoproteins increased in cytoskeleton and a new phosphoprotein was found in the cytoplasmic microtubules. On the other hand, in the Control condition there was a phosphoprotein that localizes to endosomes and disappeared upon Aβ treatment.

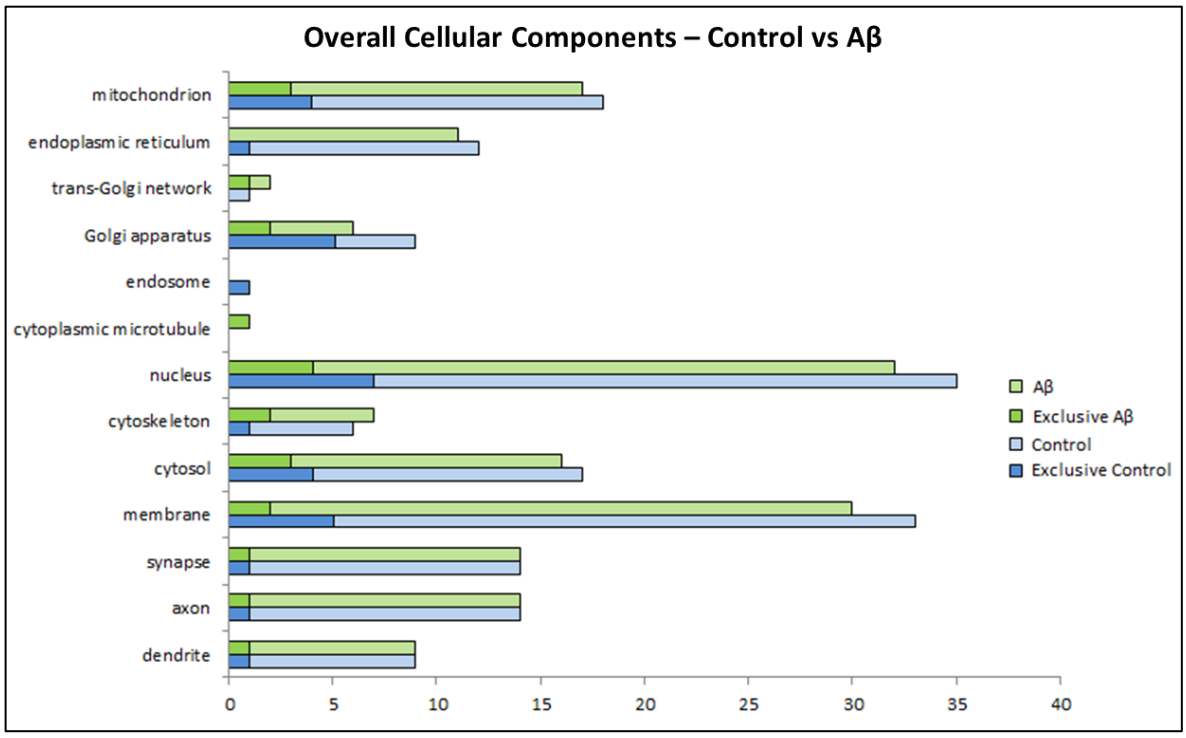


**Figure 21 - Overview of the phosphoprotein cellular component of Control and Aβ conditions.**

- Biological Processes

The effects of Aβ treatment with respect to biological processes in which the phosphoproteins obtained are involved were represented in Figure 22. Upon Aβ treatment the phosphoproteins involved in cellular processes increased, while phosphoproteins involved in response to stimulus, cellular component organization and transport decreased. Although the number of phosphoproteins in metabolic processes was identical, considering specific processes inside this overall class, the exclusive proteins of each condition (darker color in graphic bars) lead to alterations in the neurophosphoproteome of protein folding, translation, transcription and proteolysis. Unexpectedly, the apoptotic process does not suffer any alteration in the neurophosphoproteome.

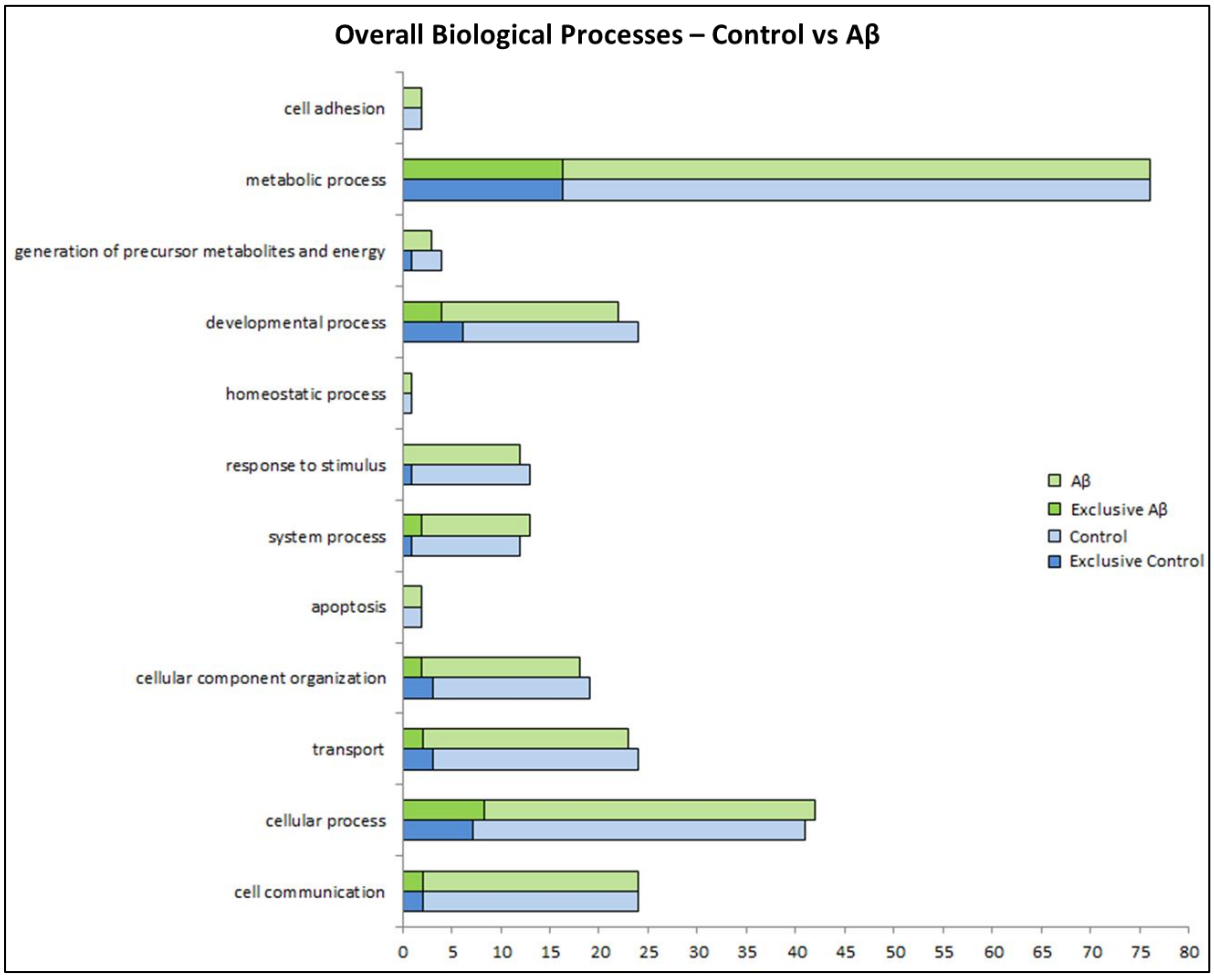


**Figure 22 - Overview of the biological processes of Control and Aβ conditions.**

- Molecular Functions

Figure 23 represents the qualitative differences in molecular functions between Control and Aβ conditions. While the catalytic activity and translation regulator activity pool of phosphoproteins increased with Aβ treatment, a decrease of phosphoproteins involved in the molecular functions of binding, structural molecule activity, receptor activity, motor activity, enzyme regulator activity, transcription regulator activity and ion channel activity could be observed. Further, a phosphoprotein involved in the antioxidant activity is present only in Aβ condition. The molecular function that maintains the same number of phosphoproteins in both conditions is the transporter activity, although there is a difference in the pool itself (darker color in graphic bars).

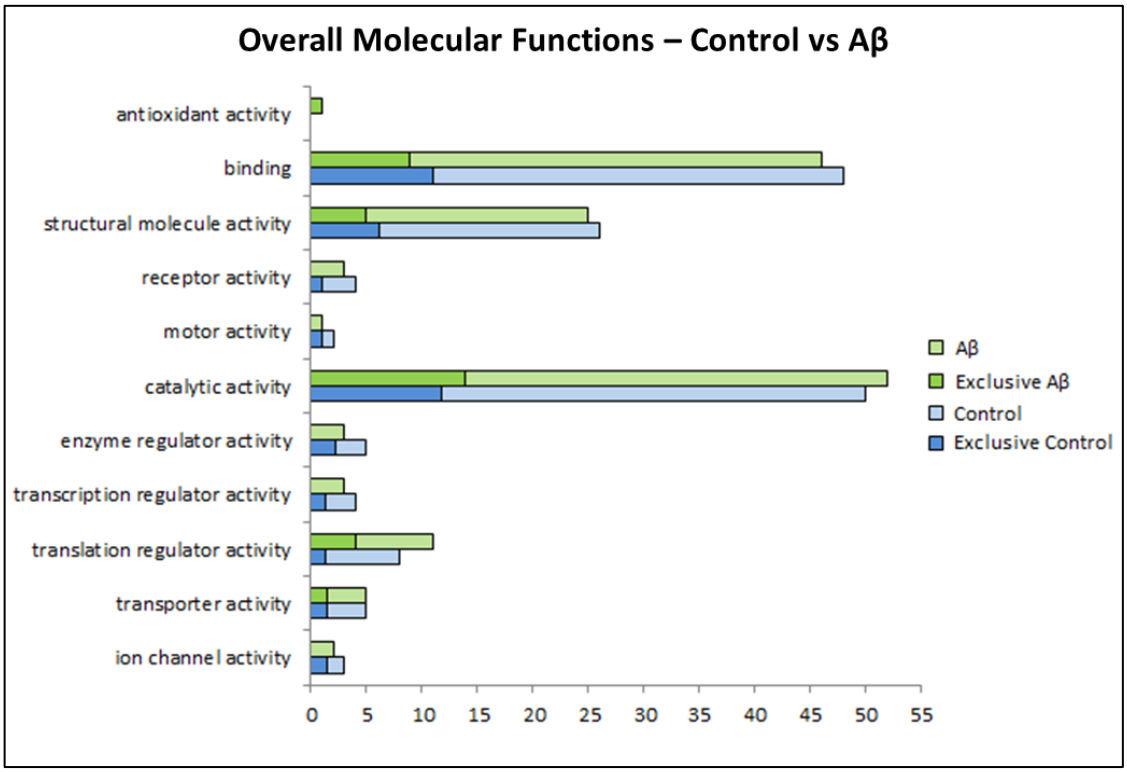


**Figure 23 - Overview of molecular functions of Control and Aβ conditions.**

## 4.4. EXCLUSIVE PROTEIN POOL OF CONTROL AND OA CONDITIONS

The exclusive protein pool analysis provides vital information for the differences observed between Control and OA conditions. The ProtDB script and the PANTHER Classification System were again used to organize the exclusive data of each condition.

### 4.4.1. Protein Classes

Figure 24 represents the protein classes of the exclusive phosphoproteins for Control and OA conditions.

The protein class with higher number of phosphoproteins in Control is nucleic acid binding (21%) whereas in OA it is cytoskeletal proteins (17%). The Control condition has a unique protein isomerase class that disappears with OA treatment. Overall, the number of proteins classes increased upon OA treatment.

The cytoskeletal protein class, similar to other protein classes (oxidoreductase, ligase, chaperone, structural protein, cell adhesion molecule, signaling molecule, membrane traffic protein and extracellular matrix protein) only appeared upon OA treatment.

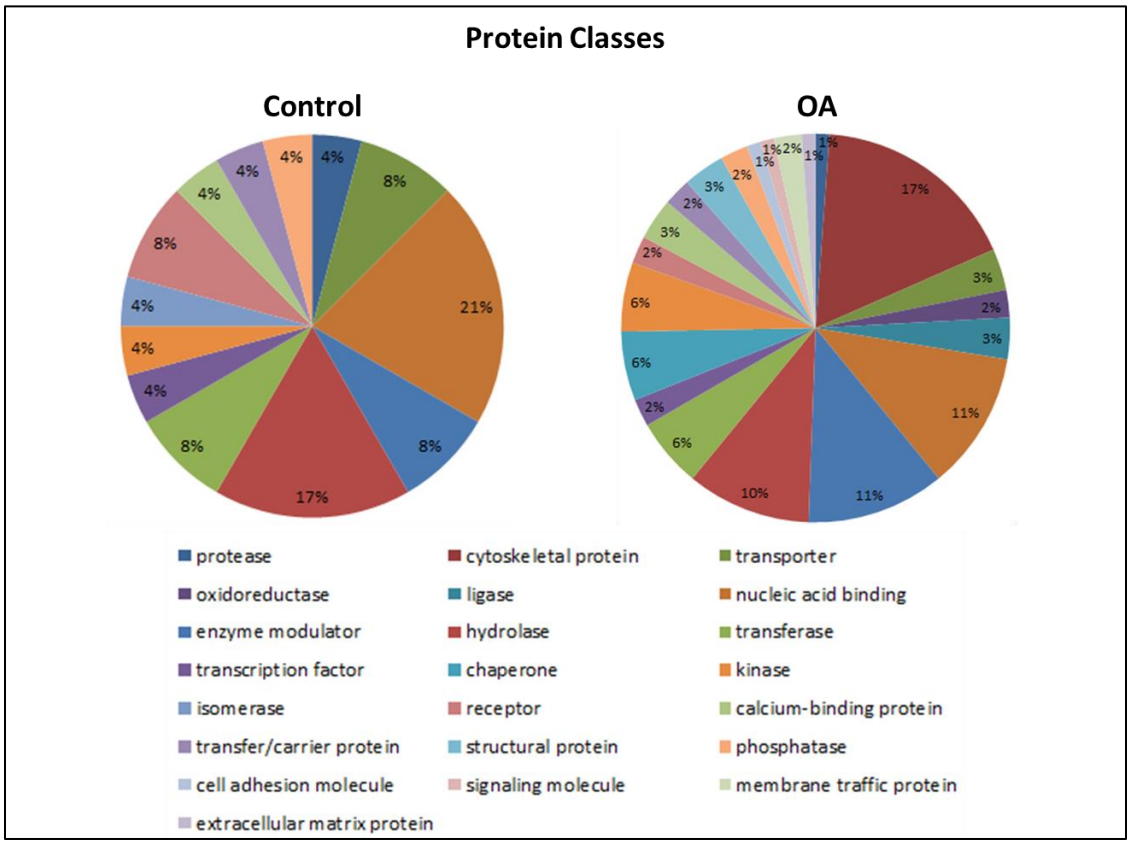


**Figure 24 - Protein classes of the phosphoproteins obtained exclusively for Control and OA conditions.**

### 4.4.2. Gene Ontology

- Cellular Components

The subcellular localization of the exclusive phosphoproteins of Control and OA conditions is represented in Figure 25.

In both conditions, the highest number of phosphoproteins occurs in the nucleus. Six cellular components (axon, cytoskeleton, endoplasmic reticulum, endosome, cytoplasmic microtubule and trans-Golgi network) only appear associated with the OA exclusive pool of phosphoproteins. The highest variations in the phosphoproteins subcellular localization were in the membrane and synapse, with 25% vs 15% and 5% vs 10%, respectively for Control and OA treatment.

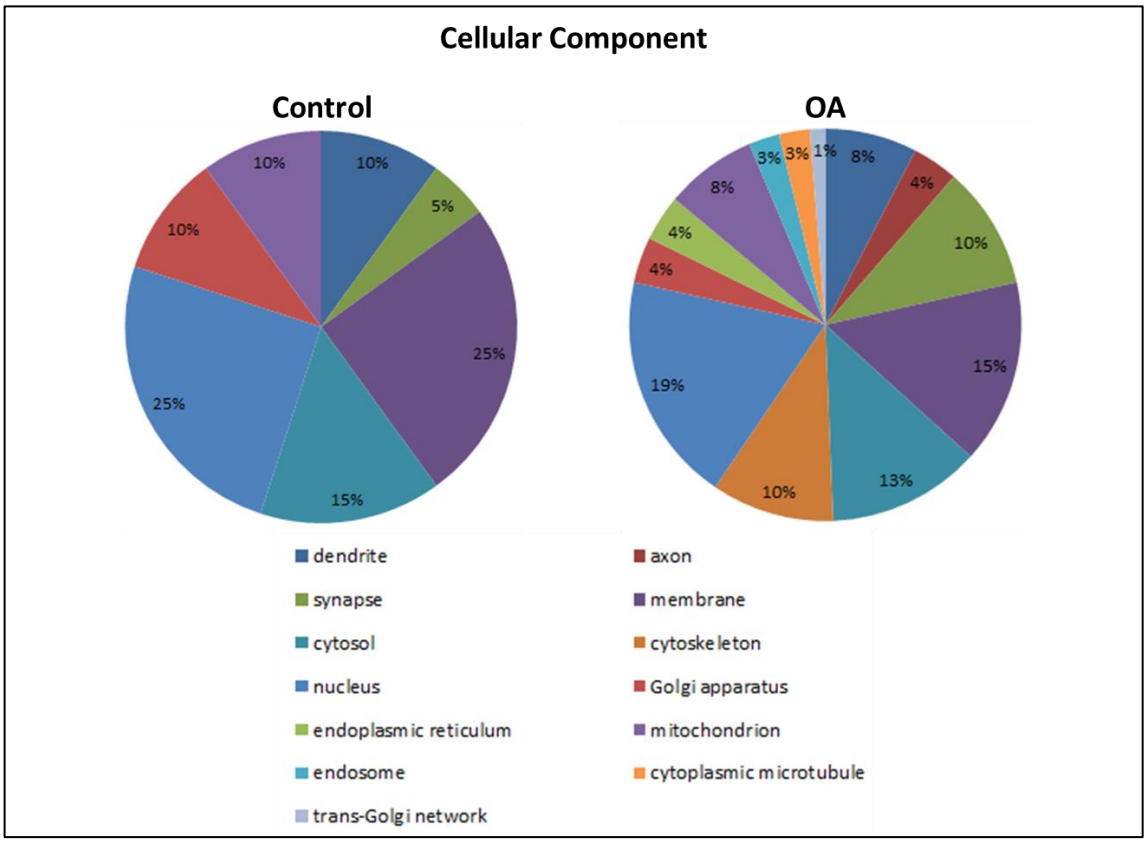


**Figure 25 - Overview of the exclusive phosphoproteins of Control and OA conditions organized by cellular component.**

- Biological Processes

Figure 26 emphasizes the biological processes implicated by the exclusive phosphoproteins of Control and OA conditions.

The exclusive phosphoproteins for the Control condition are involved in 4 biological processes: the metabolic process that reaches the highest number of phosphoproteins (76%), transport, developmental process and generation of precursor metabolites and energy. For OA, the phosphoproteins exclusive for this condition are involved in numerous biological processes, being the metabolic (23%) and the cellular (16%) processes the ones that comprise the higher number of phosphoproteins.

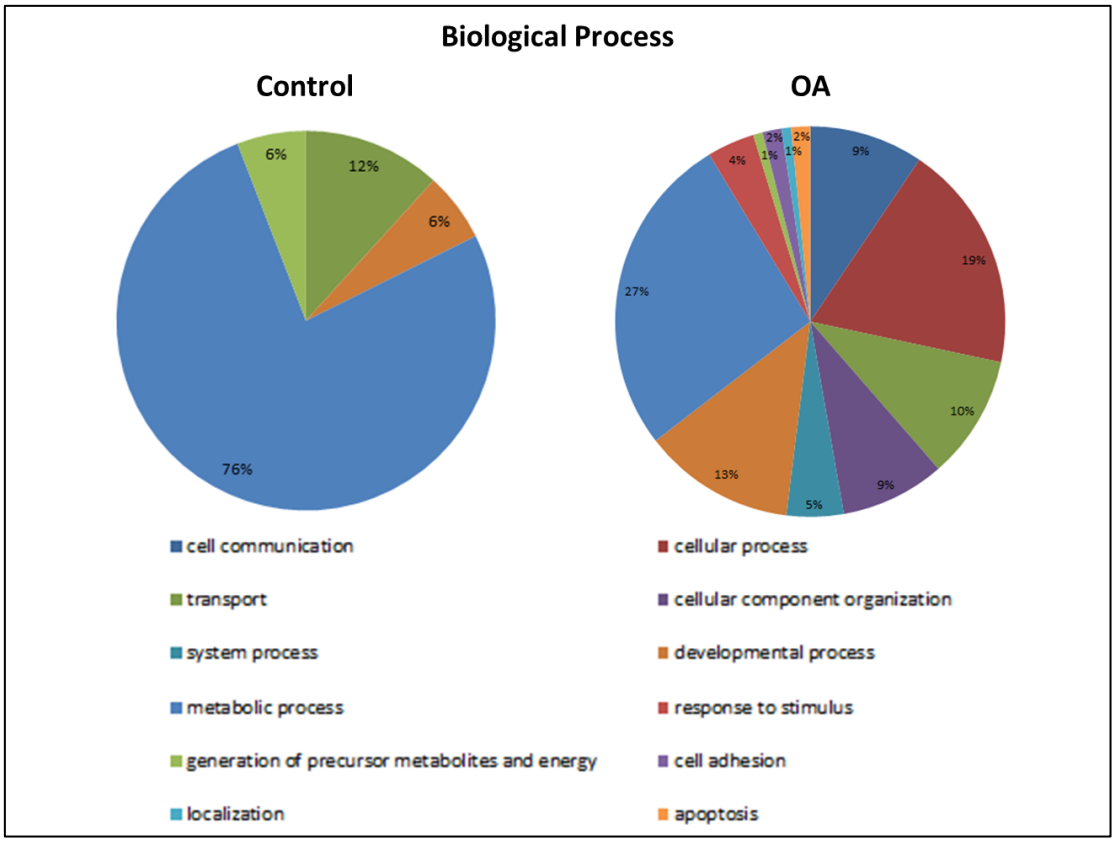


**Figure 26 - Exclusive phosphoproteins of Control and OA conditions displayed by biological processes.**

- Molecular Functions

The differences of the molecular functions associated with the exclusive phosphoproteins of Control and OA conditions are shown in Figure 27.

Binding is the function with the highest number of phosphoproteins in both conditions followed by the catalytic activity function. For OA condition, the exclusive phosphoproteins participate in three additional molecular functions (antioxidant, translation regulator and motor activities) when compared to Control condition. An increase in the structural molecular activity for OA could also be observed (8% to 20%).

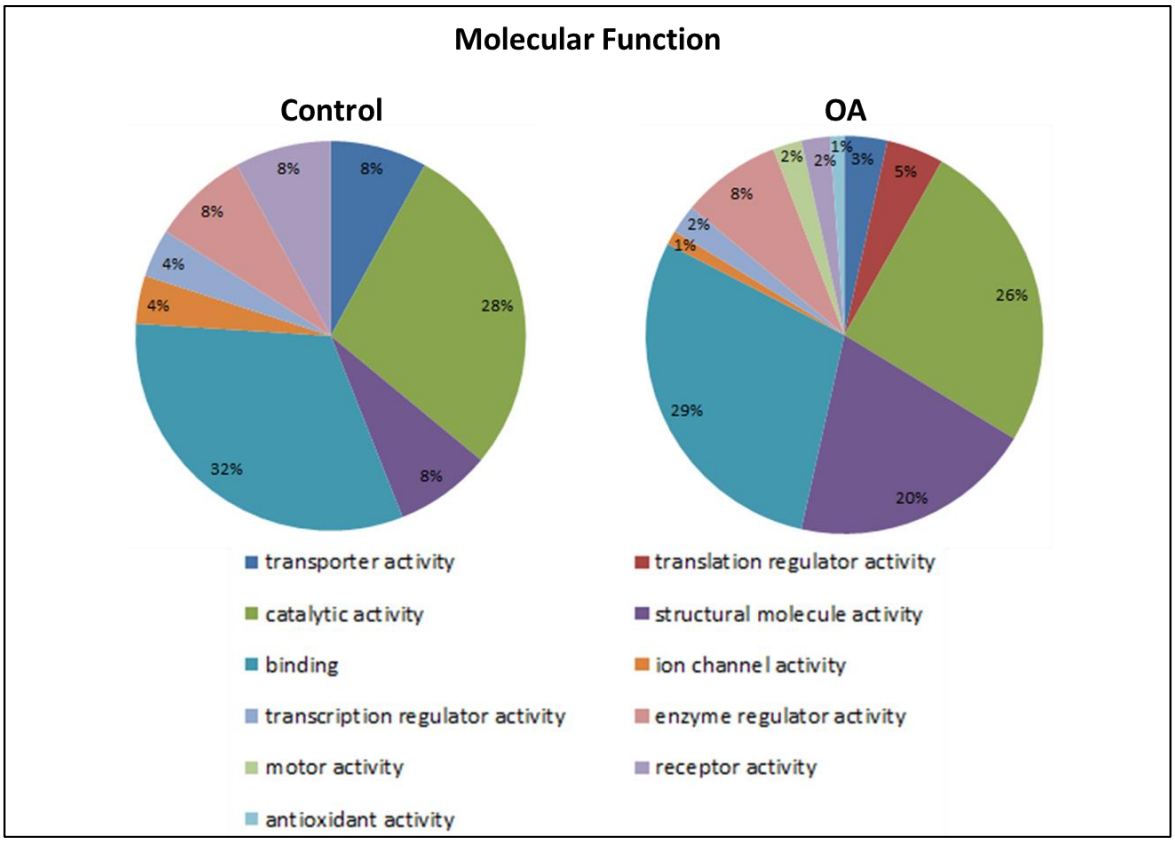


**Figure 27 - Molecular functions of the exclusive phosphoproteome pool in Control and OA conditions.**

## 4.5. EXCLUSIVE PROTEIN POOL FOR CONTROL AND Aβ CONDITIONS

The exclusive phosphoproteins for Control and Aβ were organized following the same criteria used for Control and OA phosphoprotein pool, such that differences between conditions could be detected.

### 4.5.1. Protein Classes

The protein classes of the exclusive phosphoproteins are represented in Figure 28 comparing Control and Aβ conditions.

The major protein class in both conditions is the nucleic acid binding class. Protein classes like calcium-binding, transfer/carrier and structural protein only appear in the Aβ condition. Of note, the phosphoprotein kinase, the chaperone and the transcription factor classes were completely abolished upon Aβ treatment. In Aβ conditions the cytoskeletal protein class also decreased when compared to Control (8% vs 11%, respectively).

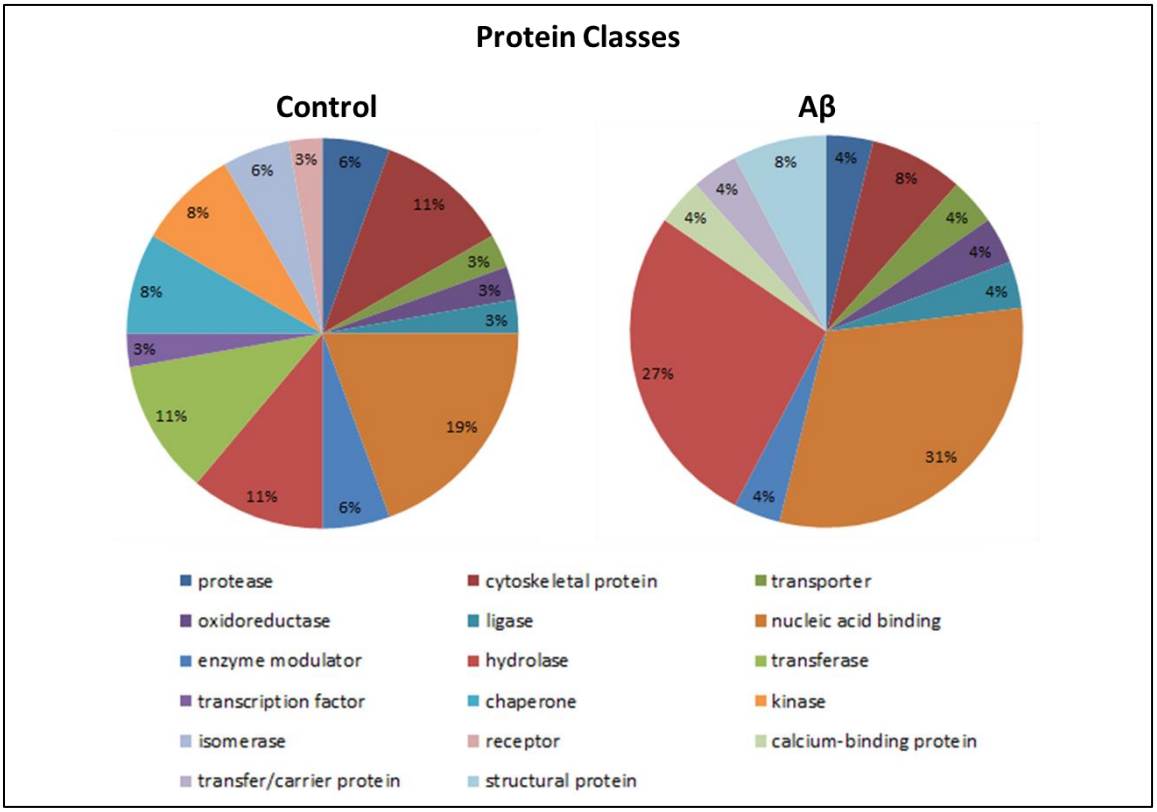


**Figure 28 - Protein classes of the phosphoproteins obtained exclusively for Control and Aβ conditions.**

### 4.5.2. Gene Ontology

- Cellular Components

The data obtained for the subcellular localization of the exclusive phosphoproteins for Control and Aβ conditions is represented in the Figure 29.

The nucleus was the subcellular organelle that has the highest number of exclusive phosphoproteins for both conditions. The phosphoproteins present in the endosome cellular component in Control condition disappear upon Aβ treatment. Nonetheless, cytoplasmic microtubule and trans-Golgi network are localizations exclusive to phosphoproteins from the Aβ condition. Among the other subcellular components, phosphoproteins were differently distributed between Control vs Aβ conditions, e.g. 16% vs 10% for Golgi apparatus, 16% vs 10% for membrane and 3% vs 10% for cytoskeleton.

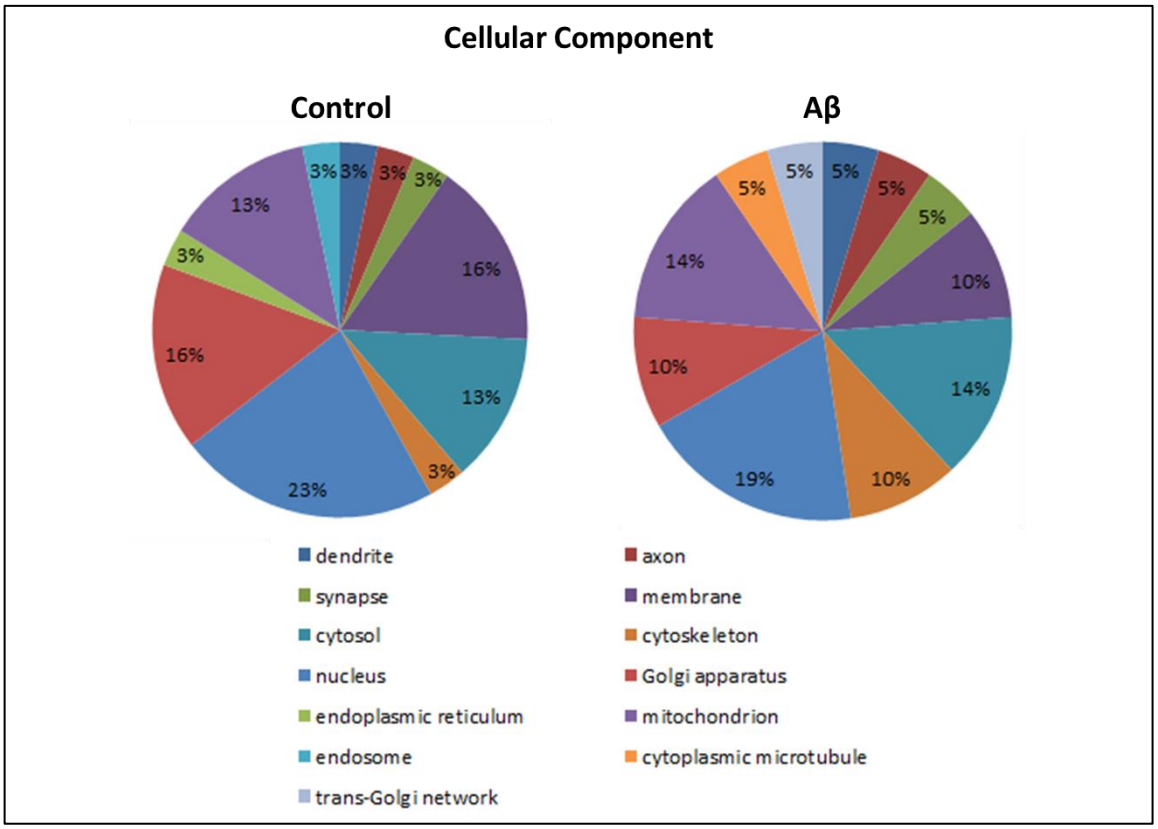


**Figure 29 - Overview of the exclusive phosphoproteins of Control and Aβ conditions organized by cellular component.**

- Biological Processes

The biological processes implicated by the exclusive phosphoproteins for each condition are shown in Figure 30.

The main biological process represented in both conditions is the metabolic process followed by the cellular process. Response to stimulus together with generation of precursor metabolites and energy are only represented in the phosphoprotein pool of Control condition.

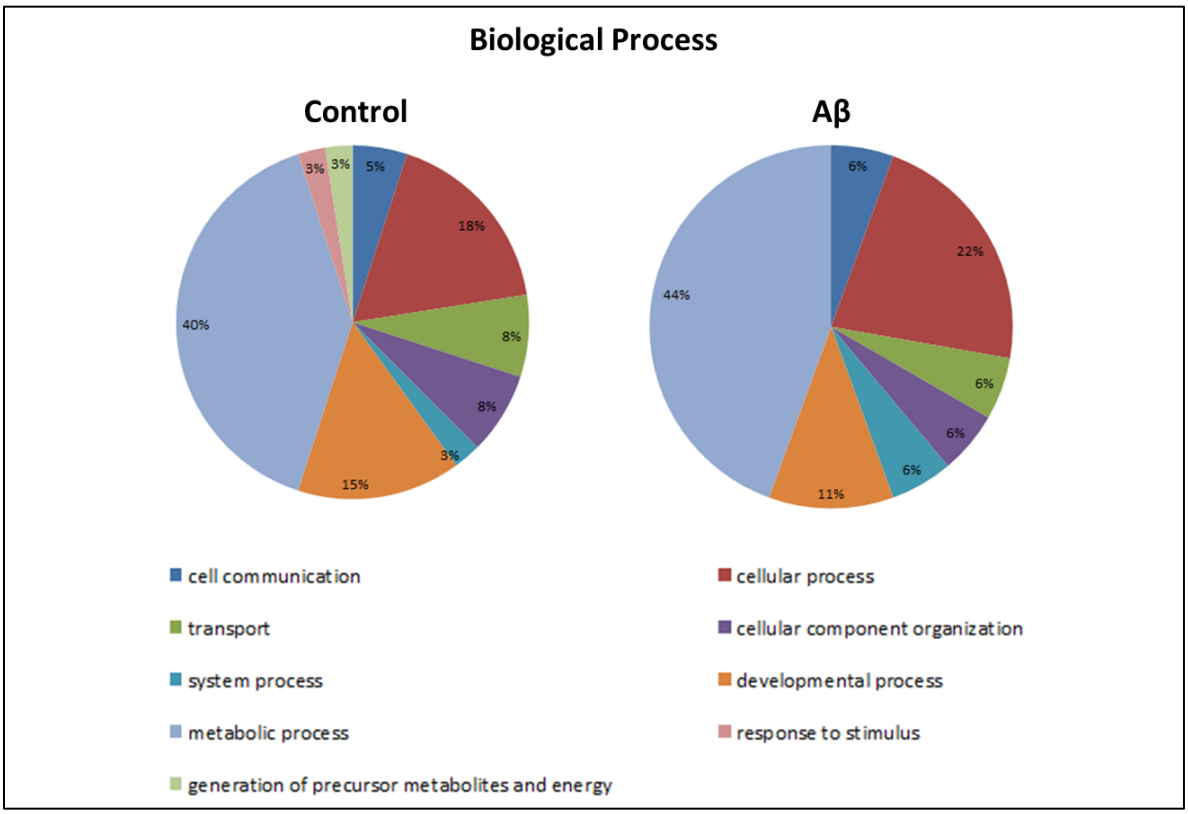


**Figure 30 - Exclusive phosphoproteins of Control and Aβ conditions structured by biological processes.**

- Molecular Functions

Figure 31 represents the differences of the molecular functions obtained for the exclusive phosphoproteome pool for Control and Aβ conditions.

For both conditions, the catalytic activity is the molecular function with more exclusive phosphoproteins followed by the binding function. There are only five molecular functions evident in the Control condition: ion channel, transcription regulator, enzyme regulator, motor and receptor activity; and one exclusively to the Aβ condition: antioxidant activity. Regarding Aβ treatment there is also an increase in the translational regulatory activity (12%) when compared to Control (3%).

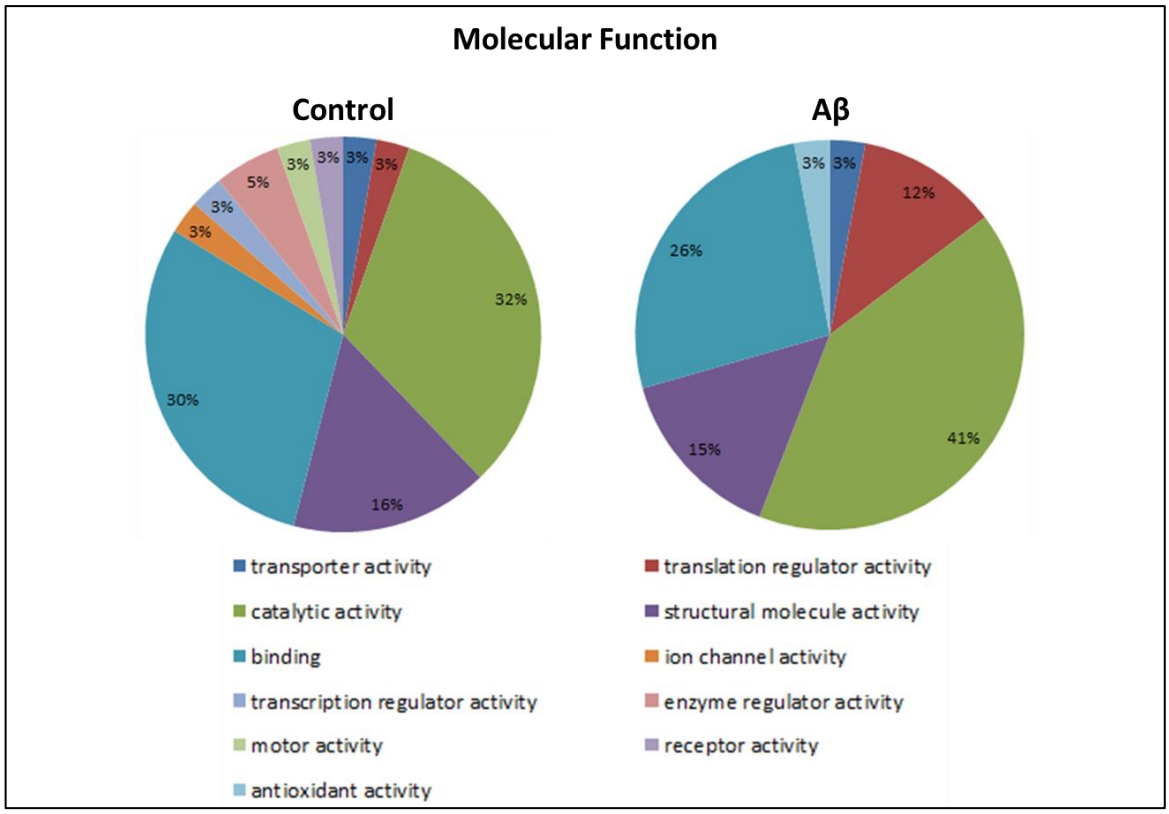


**Figure 31 - Molecular functions of the exclusive phosphoproteome pool in Control and Aβ conditions.**

## 4.6. MERGING EXCLUSIVE OA AND Aβ PHOSPHOPROTEIN POOLS

In the work herein presented we used two models to induce abnormal phosphorylation: OA as a general inducer of abnormal phosphorylation and Aβ as a more specific AD pathological model. In order to consider these two models together, a Venn diagram was constructed in Figure 32. We obtained 75 exclusive phosphoproteins upon OA treatment, 9 upon Aβ treatment and 25 phosphoproteins were common to both conditions.

The pool of exclusive proteins in each condition may constitute relevant biomarkers for AD and/or other neurodegenerative disorders in which phosphorylation is a relevant mechanism.

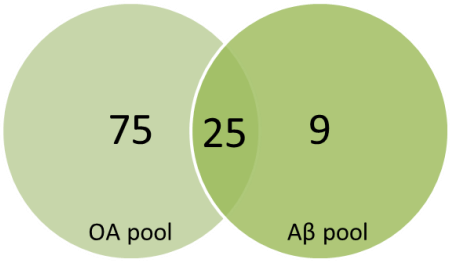


**Figure 32 – Venn diagram of the OA and Aβ exclusive phosphoproteins.**

# 5. Discussion

## 5.1. NEURONAL PHOSPHOPROTEOME OVERVIEW

Protein hyperphosphorylation is a characteristic hallmark of AD and had been implicated both in pathogenesis and disease progression. Hence, unrevealing the neurophosphoproteome altered in AD may allow for the understanding of molecular mechanisms underlying disease pathogenesis and contribute to the identification of novel biomarker candidates or therapeutic targets. Two models were adopted in order to mimic abnormal protein phosphorylation conditions: OA, a potent protein phosphatases inhibitor (159), was used to mimic an abnormal hyperphosphorylation condition; and Aβ was used to mimic AD related responses, thus providing a neurophosphoproteome for this neurodegenerative disorder. As Aβ was shown to affect kinase and phosphatase activities (139–154) both models will allow for the evaluation of abnormal phosphorylation in the neurophosphoproteome.

Tandem mass spectrometry, a powerful tool to analyze large scale proteomes, was used to assess the neurophosphoproteome. In particular for this case, this proteomics approach has some limitations in terms of phosphoprotein detection. Namely the use of the trypsin protease which can produce peptide fragments that are too small or too large, resulting in peptides which cannot be observed by MS/MS. Additionally, the lower detection efficiencies of phosphopeptides (compared with their unphosphorylated cognates) by the equipment used (157,160). These limitations of the MS/MS were minimized by including, in the workflow, an enrichment of the phosphoprotein. To have a good degree of assurance and relevance in the neurophosphoproteins, only the phosphoproteins repeated across the 4 experiments were considered for further phosphoproteome characterization.

Regarding Control vs OA results, the total number of neuronal phosphoproteins treated with the potent phosphatase inhibitor is higher than in Control (Figure 12), as expected. The concentration used in the experiments (0,5 μM) inhibits particularly PPP1 and PPP2 (161). Inhibition of phosphatases also permits the activation of downstream kinases, for example, after PPP2 inhibition, MAP-2 kinase becomes active (162). Therefore, by potentially blocking phosphatases activity, OA may also enhance kinase activity. Hence, OA promotes a hyperphosphorylation condition that explains the higher number of total (242 proteins) and exclusive (100 proteins) phosphoproteins found (Figure 12). Besides the qualitative effect, OA has a noteworthy quantitative effect on the neurophosphoproteome of the primary rat neuronal cultures.

The total number of phosphoproteins detected in the Aβ condition is nearly the same as the Control condition (Figure 14). Several studies concluded that Aβ enhances the activity of relevant kinases, for example GSK3β and cdk5 (140,141,147,152), and inhibits the activity of phosphatases, in

particular PPP1 and PPP2 (150,155). However, one should consider that these *in vitro* studies evaluate the Aβ effects directly on specific proteins, but they do not consider Aβ effects in a dynamic environment, where the total neurophosphoproteome may interact and result in activating downstream signaling cascades. It is known that phosphorylation is a fast process and a chemical equilibrium between the phosphorylated and non-phosphorylated form of the same protein. Even though the total number of proteins remains identical among the treatments, one should consider that their concentrations may be altered. Furthermore comparison of total protein number does not permit a detailed evaluation. Namely, when comparing Control to Aβ conditions, the total number of proteins was 174 and 166 respectively. However, 42 proteins were unique to Control and 34 unique to Aβ (Figure 14). Thus there is a dynamic alteration in the neurophosphoproteome profile in Control vs Aβ conditions although total phosphoprotein number does not change considerably.

Due to the above mentioned limitations of MS/MS to detect phosphoproteins and the possibility of detecting some non-phosphorylated proteins by the chromatography column, through physical interaction with phosphorylated proteins, a validation of the phosphoproteins was carried out for both Control vs OA (Figure 13) and Control vs Aβ (Figure 15) conditions, using the ProtDB script developed and a protein phosphorylation prediction server. The probability of false-positive predictions by NetPhos was reduced by eliminating predictions with an output score threshold below 0.8, which give consistency and robustness that all proteins selected for neurophosphoproteome characterization can be indeed phosphorylated.

## 5.2. NEURONAL PHOSPHOPROTEOME CHARACTERIZATION UPON OA TREATMENT

Generally, in the OA condition almost all protein classes, pathways, biological processes and molecular functions suffer an increase in the number of phosphoproteins. Therefore, OA neurophosphoproteome represents an hyperphosphorylation model due is powerful inhibition of PPP1 and PPP2. Starting with the analysis of kinases and phosphatases, it can be observed that either phosphorylated kinases or phosphatases change upon OA treatment (Figure 16). Interestingly, there was an exclusive pool of phosphorylated kinases found in the OA condition that can explain the hyperphosphorylation typical of the OA neurophosphoproteome (Figures 16 and 24). Besides that, the phosphorylated phosphatases found are exclusive to each condition (Figures 16 and 24). This data supports that phosphatase activities are highly altered by OA.

- Apoptosis and neuronal death

OA was reported to have cytotoxic properties which lead to neuronal death (163–165). In accordance, there is an alteration in the neurophosphoproteome of apoptotic process in response to OA (Figure 18), which can be an argument in line with the neuronal death promoted by OA. The results indicate a role of PPP1 and/or PPP2 in the apoptotic process as described by other studies (166,167).

- Reorganization of cytoskeleton

OA had a marked effect on the cytoskeleton network dynamics and organization as evident by the following increases in the number of: cytoskeletal phosphoproteins (Figure 16), phosphoproteins localized in cytoskeleton and cytoplasmic microtubules (Figure 17), phosphoproteins involved in cellular component organization and transport (Figure 18) and phosphoproteins with structural molecule and motor activities (Figure 19). PPP1 and/or PPP2 are involved in the cytoskeleton dynamics since its inhibition lead to alterations of the phosphoproteins. These results are supported by others studies in which OA leads to cytoskeleton reorganization trough PPP1 and PPP2 actions (168–170).

- Synaptic effects

The imbalance induced between protein phosphorylation and dephosphorylation by OA also allows addressing the role of protein phosphorylation on cellular events. In particular, focusing in synaptic transmission, it has been described that OA alters the phosphorylation of ion channels and other proteins involved in synaptic transmission, mimicking the AD effects on synapses (171). An increase in the phosphoproteins localized in synapses could be detected upon OA treatment (Figure 17). Moreover, despite the same number of phosphoproteins involved in ion channel activity there is one exclusive phosphoprotein in OA condition (Figures 19 and 27). These findings suggest an important role of OA-sensitive phosphatases in the regulation of phosphorylation of several proteins involved in synaptic transmission.

## 5.3. NEURONAL PHOSPHOPROTEOME CHARACTERIZATION UPON Aβ TREATMENT

It is necessary to bear in mind that the neurophosphoproteome herein analyzed results from the balance between kinase and phosphatase activities upon Aβ treatment. Figure 20 shows that the number of phosphorylated kinases suffers an alteration in response to Aβ. On the other hand, the phosphorylated phosphatases remain the same. However, it is not possible to know if these proteins involved in the reversible phosphorylation events are up or downregulated, which may explain the alterations in the Aβ neurophosphoproteome. A quantitative analysis will be useful in the future to further address this issue.

- Oxidative stress

Studies *in vitro* and *in vivo* have demonstrated that Aβ peptide induces protein oxidation (172,173). A new phosphoprotein with antioxidant activity appears only in the Aβ condition (Figure 23). This alteration in the neurophosphoproteome may be a protective mechanism against oxidative stress caused by Aβ treatment, and thus further analysis of this protein profile may be of interest. Furthermore, an alteration in the phosphoproteins involved in response to stress (inside the response to stimulus process) also corroborates a role for Aβ in oxidative stress (Figures 22 and 30). Upon Aβ treatment, some cellular processes and pathways can be activated to cope with oxidative stress whereas others can initiate or exacerbate the stress oxidative effects.

- Protein synthesis and protein degradation

The levels of protein synthesis are impaired in AD as described previously by other authors (174,175) and Aβ itself can alter the APP processing and consequently AICD production and nucleus translocation (89). Transcription factor class, plus transcription and translation regulator activities neurophosphoproteomes were altered as a result of the Aβ treatment (Figures 23 and 31) in agreement with a possible role for Aβ in protein synthesis and transcriptional changes observed in AD. Further, the differences observed in the phosphoproteins localized in the Golgi apparatus, trans-Golgi network and endosome (components involved in increased production of Aβ, Figure 21) can propose a protective mechanism to decrease the Aβ production in an environment where it already exists in excess.

Apart from protein synthesis, protein degradation is another process altered in AD (175). Indeed, in sporadic AD cases there are significant defects in the clearance of Aβ strongly supporting the idea that defective Aβ degradation may be operative in AD (176). Involved in proteolytic Aβ degradation

are several proteases which have a bidirectional interaction with this hallmark of AD. Upon Aβ treatment the protease class suffers an alteration in their neurophosphoproteome with exclusive proteins both in Control and Aβ conditions (Figures 20 and 28). The proteasome is in part responsible for the clearance of Aβ peptide (176). Interestingly, there are two exclusive proteins of the proteasome - proteasome subunits alpha (PSA) that only differ in the type between conditions. Moreover, an ubiquitin carboxyl-terminal hydrolase is only present in Control condition and it is known that Aβ inhibits ubiquitin-dependent protein degradation (177). Taken together these differences observed in the exclusive proteases may explain why Aβ can interfere with its own degradation and that of other proteins.

- Misfolded proteins

The accumulation of misfolded proteins is a pathological lesion characteristic of neurodegenerative diseases such as AD. The main proteins which possess a role in the intracellular handling of misfolded proteins are the chaperones (178). Aβ changes the phosphoproteome in terms of phosphorylated chaperones and there is a loss of three proteins that are Control exclusives (Figures 20 and 28). Furthermore, the metabolic process (Figure 22) that includes the protein folding reflects an alteration in the neurophosphoproteome in response to Aβ which is in agreement with the findings of the chaperone class. These observations support a role for Aβ in the accumulation of misfolded proteins. Remarkably, phosphorylated heat shock proteins (Hsps), known as professional chaperones, are more abundant in the neurophosphoproteome of the Control condition, what may indicates that Aβ leads to phosphoprotein alterations that may result in its own accumulation.

- Apoptosis

It has been extensively described that Aβ peptide causes apoptosis of neuronal cells (179–181). In these experiments, when looking at the apoptotic process (Figure 22) no differences could be observed in the neurophosphoproteome upon Aβ treatment. In order to understand if Aβ promotes neuronal death through change in the expression level of phosphoproteins a quantitative analysis of the phosphoproteins identified would be of interest as previously mentioned.

- Cytoskeleton abnormalities

Previous studies, including from our own group, have demonstrated that Aβ promotes alterations in the cytoskeleton network dynamics (88,90), which may contribute to neuronal death. The results obtained in this work are in agreement with this role for Aβ in AD pathogenesis.

Specifically, alterations in cytoskeletal phosphoproteins (Figure 20), changes in the number of phosphoproteins localized in the cytoskeleton and cytoplasmic microtubule (Figure 21) and modification of the phosphoproteins involved in cellular component organization and transport (Figure 22) are in accordance with previous observations.

- Calcium influx

Disturbances of intracellular calcium homeostasis have been implicated in AD neurodegeneration (182) with a role for Aβ in promoting calcium influx (183). The alterations in phosphoproteins involved in ion channel activity (Figure 23) and in the calcium-binding proteins (Figure 20) are in agreement with a conceivable role for Aβ in calcium influx through alterations in protein affinity to calcium and ion channels permeability.

## 5.4. EXCLUSIVE PHOSPHOPROTEINS – A COMPREHENSIVE ANALYSIS

The characterization of the exclusive phosphoproteins pool in response to OA and Aβ reveals that some cellular events are inactivated whereas others are activated. The phosphoproteins present may explain the differences observed in the biological processes (Figures 26 and 30) and the molecular functions (Figures 27 and 31) in each condition.

Also, it is evident that upon OA or Aβ treatment new phosphoproteins involved in critical processes of AD pathogenesis appeared, including metabolic processes (protein folding, transcription, translation, proteolysis), catalytic activity (kinase and ubiquitin-protein ligase activities), binding (cytoskeletal protein binding), cellular processes (signal transduction) and cytoskeletal proteins (Figures 24 to 31).

The exclusive pool of phosphoproteins present in OA and Aβ can reveal phosphospecific biomarkers for AD and potentially for other dementias where hyperphosphorylation is a characteristic hallmark (Figure 32). The OA exclusive phosphoprotein pool (75 proteins) may reveal useful biomarkers for AD and other dementia types in which phosphorylation is a relevant event for disease pathogenesis. On the other hand, the exclusive phosphoproteins of Aβ pool (9 proteins) can be relevant for other Aβ associated events. Finally, the common phosphoproteins between OA and Aβ exclusive pools (25 proteins) may reveal biomarkers to distinguish AD from other dementias since it represents a merge between two models that mimic AD.

# 6. Concluding Remarks

The experiments herein carried out allowed the characterization of the neuronal phosphoproteome in AD mimicking conditions and under abnormal phosphorylation. Of note are the following conclusions:

- OA is a good model for inducing neuronal phosphoproteome hyperphosphorylation;
- Aβ leads to a shift in neuronal phosphoproteome pool, although the final balance of phosphatase and kinase activities induced by Aβ is unknown;
- Both OA and Aβ induced alterations in neuronal biological processes and molecular functions relevant for AD;
- Despite the number of proteins, or even though the pool of proteins among conditions are identical, phosphoproteins expression can be altered with the different treatments; and as such the quantitative analysis of these phosphoproteins should also be considered;
- Both OA and Aβ treatments lead to the activation of an exclusive pool of phosphoproteins;
- The analysis of the exclusive phosphoproteins in OA and Aβ conditions may reveal novel potential biomarkers candidates for AD or other phosphorylation associated- pathologies;
- The knowledge of the neuronal phosphoproteome may allow for the understanding of molecular mechanisms underlying AD pathology.

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# 8. Appendix

Below is listed the equipment used and composition of buffers for the different techniques applied.

## CELL CULTURE AND EXPERIMENTAL MODELS

### EQUIPMENT

- Hera cell $CO_2$ incubator (Heraeus)
- Safety cabinet Hera safe (Haraeus)
- Inverted optical microscope (LEICA)
- Hemacytometer (Sigma)
- Sonicator (U200S (IKA)
- Bath SBB6 (Grant)

### REAGENTS

- 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 4ºC.

- Hank's balanced salt solution (HBSS)

  This salt solution is prepared with deionised H2O. 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 4ºC.

- Poly-D-lysine stock (Sigma-Aldrich)

  To a final volume of 10 ml at 10mg/ml, dissolve in deionized $H_2O$ 100 mg of poly-D-lysine. To prepare the poli-D-Lysine solution dilute 1 ml of the 10 mg/ml poly-D-lysine stock solution in borate buffer.

- Borate buffer

  To a final volume of 1 L, dissolve in deionised H2O 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 4ºC.

- PBS (1x)

  For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionised H2O. 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 4ºC.

- $A\beta_{1-42}$ (American Peptide)
- Okadaic Acid (Calbiochem)

## PHOSPHOPROTEIN EXTRACTION

### REAGENT

- Phosphoprotein Enrichment Kit Talon PMAC (Clontech)

## PROTEIN CONTENT DETERMINATION

### EQUIPMENT

- Spectrophotometer Cary 50 (Varian)

### REAGENTS

- BCA assay kit (Pierce, Rockford, IL)
- Bovine Serum Albumin (BSA) (Pierce)
- Working Reagent (50 Reagent A : 1 Reagent B)

  Reagent A: sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0,2N sodium hydroxide.

  Reagente B: 4% cupric sulfate.

## 1D GEL ELECTROPHORESIS

### EQUIPMENT

- Electrophoresis system (Hoefer SE600 vertical unit)
- Electrophoresis power supply EPS 1000 (Amersham Pharmacia Biotec)

### REAGENTS

- LDS (lithium dodecyl sulfate) sample buffer (4x)

  Final composition:

  - 40% glycerol
  - 4% lithium dodecyl sulfate (LDS)
  - 4% Ficoll*-400
  - 0.8 M triethanolamine-Cl pH 7.6
  - 0.025% phenol red
  - 0.025% coomassie G250
  - 2mM EDTA disodium.
- 30% Acrylamide
- APS (Ammonium Persulfate) 40%
- TEMED (N,N,N',N'-Tetramethylethylenediamine)
- Bis Tris (2,2-Bis(hydroxymethyl)-2,2',2"-nitrilotriethanol) 10% (7x)
- MOPS (3-(N-morpholino)propanesulfonic acid) running buffer (20x)

  Final composition:

  - 50 mM MOPS
  - 50 mM Tris Base
  - 0.1% SDS (sodium dodecyl sulfate)
  - 1 mM EDTA (ethylenediaminetetraacetic acid)
  - pH 7.7.
- Coomassie Brilliant Blue (Bio-Rad)

  Dissolve 1 g of Coomassie Brilliant Blue in 1 L of the following solution: Methanol (50% [v/v]), Glacial acetic acid (10% [v/v]) and $H_2O$ (40%). Stir the solution for 3-4 hours and then filter through Whatman filter paper. Store at room temperature.
- Acetic acid 10%

## TRYPTIC DIGESTION AND PROTEIN EXTRACTION

### EQUIPMENT

- SpeedVac (Thermo Scientific)
- Sonicator (Thermo Scientific)

### REAGENTS

- Solution A
  - AMBIC buffer (Amonium Hydrogen Carbonate)
- Solution B
  - ACN (Acetonitrile) + AMBIC buffer (50:50)
- Trypsin Stock Solution

  Final Composition:
  - 1 mg/ml trypsin
  - 1 mM HCl
  - 20 mM $CaCl_2$

  Divide the solution into small aliquots (~50 µl) and store frozen at -20ºC.
- AMBIC (ammonium bicarbonate) buffer

  For 100mM of AMBIC, weigh out 79mg of AMBIC and dissolve in 10 mL ultrapure water.
- TFA (Trifluoroacetic acid) 0,1%
- ACN (Acetonitrile)

## MASS SPECTROMETRY

### EQUIPMENT

- Q Exactive (Thermo Scientific)

# 8. Appendix

Below is listed the equipment used and composition of buffers for the different techniques applied.

## CELL CULTURE AND EXPERIMENTAL MODELS

### EQUIPMENT

- Hera cell $CO_2$ incubator (Heraeus)
- Safety cabinet Hera safe (Haraeus)
- Inverted optical microscope (LEICA)
- Hemacytometer (Sigma)
- Sonicator (U200S (IKA)
- Bath SBB6 (Grant)

### REAGENTS

- 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 4ºC.

- Hank's balanced salt solution (HBSS)

  This salt solution is prepared with deionised H2O. 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 4ºC.

- Poly-D-lysine stock (Sigma-Aldrich)

  To a final volume of 10 ml at 10mg/ml, dissolve in deionized $H_2O$ 100 mg of poly-D-lysine. To prepare the poli-D-Lysine solution dilute 1 ml of the 10 mg/ml poly-D-lysine stock solution in borate buffer.

- Borate buffer

  To a final volume of 1 L, dissolve in deionised H2O 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 4ºC.

- PBS (1x)

  For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionised H2O. 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 4ºC.

- $A\beta_{1-42}$ (American Peptide)
- Okadaic Acid (Calbiochem)

## PHOSPHOPROTEIN EXTRACTION

### REAGENT

- Phosphoprotein Enrichment Kit Talon PMAC (Clontech)

## PROTEIN CONTENT DETERMINATION

### EQUIPMENT

- Spectrophotometer Cary 50 (Varian)

### REAGENTS

- BCA assay kit (Pierce, Rockford, IL)
- Bovine Serum Albumin (BSA) (Pierce)
- Working Reagent (50 Reagent A : 1 Reagent B)

  Reagent A: sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0,2N sodium hydroxide.

  Reagente B: 4% cupric sulfate.

## 1D GEL ELECTROPHORESIS

### EQUIPMENT

- Electrophoresis system (Hoefer SE600 vertical unit)
- Electrophoresis power supply EPS 1000 (Amersham Pharmacia Biotec)

### REAGENTS

- LDS (lithium dodecyl sulfate) sample buffer (4x)

  Final composition:

  - 40% glycerol
  - 4% lithium dodecyl sulfate (LDS)
  - 4% Ficoll*-400
  - 0.8 M triethanolamine-Cl pH 7.6
  - 0.025% phenol red
  - 0.025% coomassie G250
  - 2mM EDTA disodium.
- 30% Acrylamide
- APS (Ammonium Persulfate) 40%
- TEMED (N,N,N',N'-Tetramethylethylenediamine)
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  Final composition:

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### EQUIPMENT

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