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Eventos mediados por fosforilação na descoberta de biomarcadores para a doença de Alzheimer

Phosphorylation mediated events in biomarker discovery for Alzheimer's disease

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biomedicina, realizada sob a orientação científica da Doutora Ana Gabriela Henriques, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro e coorientação da Doutora Odete Abreu Beirão da Cruz e Silva, Professora Associada com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro

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Dedico este trabalho aos meus pais, Glória e David, ao Mário e às minhas queridas filhas, Laura e Maria.

o júri

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palavras-chave

Doença de Alzheimer, peptídeo Aβ, ácido ocadaico, fosforilação de proteínas, proteína precursora da amiloide,Tau.

resumo

A doença de Alzheimer (DA) é uma das formas mais comuns de demência em todo o mundo representando 60-70% de todos os casos de demência. Esta doença neurodegenerativa é caracterizada pela presença de placas senis (PSs), emaranhados neurofibrilares (TNFs) e por perda sináptica. O peptídeo Aβ, principal constituinte das PSs, é fundamental na DA, dado que pode desencadear vários eventos patogénicos, como neurotoxicidade e ativação de cascatas inflamatórias e apoptóticas. Todos estes eventos estão associados à neurodegeneração e, consequentemente, ao declínio cognitivo gradual evidente nos doentes.

A fosforilação anormal é aceite como um dos principais mecanismos de transdução de sinais envolvidos na patogénese da DA. De facto, vários estudos relataram atividades anormais de proteínas cinase e fosfatase no cérebro de doentes, bem como níveis de fosforilação anormais das proteínas APP (proteína precursora da amiloide) e Tau, duas proteínas na génese das PSs e TNFs.

Esta tese teve como objetivo avaliar de que modo eventos mediados por fosforilação podem ter impacto no fosfoproteoma neuronal, com o intuito de revelar novos alvos moleculares da doença. Para tal, dois modelos distintos foram aplicados: um deles utilizava o ácido ocadaico (AO), inibidor de proteínas fosfatase e potente neurotoxina; e outro que usava o peptídeo neurotóxico Aβ, como modelo relevante e mais específico da DA. Em ambos as situações, várias fosfoproteínas foram identificadas. O tratamento neuronal com AO levou à recuperação de 245 fosfoproteínas significativamente aumentadas e 75 fosfoproteínas significativamente diminuídas, enquanto a exposição ao Aβ conduziu ao aumento de 73 proteínas fosforiladas e à diminuição de 68, em resposta ao peptídeo. Adicionalmente, devido ao papel relevante desempenhado pelo Aβ na DA, os seus efeitos na fosforilação de duas proteínas-chave na DA, APP (no resíduo Thr668) e Tau (nos resíduos Ser262 e Ser396), foram também avaliados. Os resultados mostram que o Aβ pode alterar o estado de fosforilação de ambas as proteínas e, ao afetar a fosforilação da APP e potencialmente o seu processamento, pode contribuir para sua própria produção.

Em conjunto, estes resultados sugerem que o Aβ desempenha um papel fundamental na fosforilação anormal de proteínas, ativando potencialmente cascatas de sinalização anormais e, consequentemente, contribuindo para a patologia da DA.

Este trabalho revelou vários candidatos que podem contribuir para a compreensão dos mecanismos moleculares subjacentes à patologia da DA; mas, mais ainda, identificou novos potenciais biomarcadores que podem abrir caminho para o desenvolvimento de futuras ferramentas de diagnóstico e terapêutica para a doença.

keywords

Alzheimer´s disease, amyloid-β peptide, okadaic acid, protein phosphorylation, amyloid precursor protein, Tau.

abstract

Alzheimer´s disease (AD) is one of the most common forms of dementia worldwide; representing 60-70% of all dementia cases. This neurodegenerative disorder is characterized by the presence of senile plaques (SPs), neurofibrillary tangles (NFTs) and synaptic loss. Amyloid-β peptide (Aβ), the major constituent of SPs, is a fundamental player in AD, since it can trigger several pathogenic events such as neurotoxicity and activation of inflammatory and apoptotic cascades. All these events associate with neurodegeneration and consequently gradual cognitive decline are evident in AD patients. Abnormal phosphorylation is accepted as one of the key signaling transduction mechanism involved in AD pathogenesis. In fact, several studies have reported abnormal protein kinase and protein phosphatase activities in AD brains as well as abnormal phosphorylation levels of both amyloid precursor protein (APP) and Tau protein, two proteins in the genesis of SPs and NFTs.

This thesis aimed to address how phosphorylation mediated events could impact the neuronal phosphoproteome, thereby unravelling novel disease molecular targets. To achieve this, two distinct models were employed: one using okadaic acid, a protein phosphatase inhibitor and a potent neurotoxin; and the other using the neurotoxic Aβ peptide as a relevant AD specific mimicking model. In both cases, several phosphoproteins were identified. Neuronal treatment with OA lead to the recovery of 245 phosphoproteins that significantly increased and 75 phosphoproteins that significantly decreased; while exposure to Aβ resulted in 73 phosphorylated proteins that increased, and 68 that decreased in response to peptide exposure. In addition, due to the key role played by Aβ in AD, its effect on the phosphorylation state of the two key proteins in AD, APP (at Thr668 residue) and Tau (at Ser262 and Ser396 residues), were also evaluated. Data shows that Aβ itself can impact the phosphorylation state of both proteins, and thus, by affecting APP phosphorylation and potential APP processing, it can contribute to its own production.

Taken together these findings suggest that Aβ plays a fundamental role in abnormal protein phosphorylation, potentially leading to abnormal signaling cascades, and consequently contributing to AD pathology.

Interestingly, this work also unravels several candidates that can contribute to our understanding of the molecular mechanisms underlying AD pathology, but more so it identified novel putative biomarker candidates that can open avenues for the development of future diagnostic and therapeutic tools.

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Table of Contents

Table of Figures

Table of Supplemental Figures

Table of Tables

Table of Supplemental Tables

Abbreviations

1DE	One-dimensional polyacrylamide gel
Aβ	Amyloid-beta peptide
Abl2	Abelson tyrosine-protein kinase 2
ACN	Acetonitrile
ACTN1	Actinin Alpha 1
AD	Alzheimer's disease
ADAM	A Disintegrin and Metalloproteinase
ADP	Adenosine diphosphate
AICD	APP Intracellular Domain
Akt	RAC-gamma serine/threonine protein kinase
ALDOA	Aldolase, Fructose-Bisphosphate A
APBB1	Amyloid Beta Precursor Protein Binding Family B Member 1
Apc	Adenomatous polyposis coli protein
APLP1/2	Amyloid Precursor Like Protein 1/2
ApoE 2/4	Apolipoprotein E allele 2/4
APP	Amyloid precursor protein
APP-BP1	Amyloid Precursor Protein-binding Protein 1
APPBP2	Protein interacting with amyloid precursor protein tail 1
APS	Ammonium Persulfate
ARF	ADP-ribosylation factor
ATF2	Activating transcription factor 2
Aβ	Amyloid-beta peptide
BACE	Beta-site APP Cleaving Enzyme
BCA	Bicinchoninic acid assay
Bid	BH3-interacting domain death agonist
BIN1	Bridging integrator 1
BP	Biological processes
BP1	Amyloid precursor protein binding protein 1
Brsk1	Serine/threonine protein kinase Brsk1
BSA	Bovine Serum Albumin
C83	α-C-terminal fragment of APP
C99	β-C-terminal fragment of APP
CaMK	Calcium/calmodulin-dependent protein kinase
CAMK2D	Calcium/Calmodulin Dependent Protein Kinase II Delta
cAMP	Cyclic adenosine monophosphate
Camk2α	Calcium/calmodulin-dependent protein kinase type 2 alpha
CAPPD	Central amyloid precursor protein domain (E2)
Cdc2	Cyclin-dependent kinase 2
Cdk5	Cyclin-dependent kinase 5
CHIP	Chaperone-associated ubiquitin ligase
CK2	Casein kinase 2

CNS	Central nervous system
CREBEA	Comissão responsável pela Experimentação e Bem-Estar Animal
CRMP-2	Neurofibrillary Tangle-associated Collapsin Response Mediator Protein-2
CSF	Cerebrospinal Fluid
Csnk1e	Casein kinase 1 isoform épsilon
CT	Computed tomography
CTF's	C-Terminal fragments
CuBD	Copper/metal binding domain
Dbnl	Drebrin Like
Dctn1	Dynactin Subunit 1
Dlg2/3/4	Disks large homolog 2/3/4
Dmn1	Dynamin 1
Dmn1l	Dynamin 1 like protein
DNA	Deoxyribonucleic acid
Dnm	Dynamin 1
Dpysl3	Dihydropyrimidinase-related protein 3
Dyrk1A	Dual-specificity tyrosine-regulated kinase 1A
E1	Cysteine-rich globular domain of amyloid precursor protein
E2	α-helix-rich domain of amyloid precursor protein
ECL	Enhanced Chemioluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic Reticulum
Erk1/2	Extracellular signal-regulated kinase 1/2
ESI-MS/MS	Electrospray ionization tandem MS
FA	Formic acid
FAD	Familial Alzheimer´s disease
Fyn	Tyrosine-protein kinase Fyn
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFLD	Growth Factor Like Domain
GO	Gene ontology
GpAβ	Group Aβ
GpC	Group Control
Grb2	Growth factor receptor-bound protein 2
GSK3α/β	Glycogen synthase kinase 3 alpha/beta
HBD	Heparin binding domain
HBSS	Hanks Balanced Solution
HPLC	High-performance liquid chromatography
HSC70	Heat shock cognate 71 kDa protein
HSP70/90	Heat shock protein of 70kDa and 90kDa, respectively
Hspd1	Heat Shock Protein Family D (Hsp60) Member 1
HSPG	Heparin Sulphate Proteoglycan binding-site
IDE	Insulin Degrading Enzyme

IGF-1	Insulin-like growth factor 1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IPI	International Protein Index
Itsn1	Intersectin 1
Jip 1b/2	C-Jun-amino-terminal kinase-interacting protein 1b/2
JNK3	C-Jun-amino-terminal kinase 3
Junk2	c-Jun N-terminal kinase 2
KPI	Kunitz Protease Inhibitor
LASP1	LIM And SH3 Protein 1
LDL	Low Density Lipoprotein
LDS	Lithium dodecyl sulfate
LGB	Lower Gel Buffer
Lyn	Tyrosine-protein kinase Lyn
MAGI2	Membrane Associated Guanylate Kinase, WW And PDZ Domain Containing 2
MAP1B	Microtubule Associated Protein 1B
MAP kinase	Mitogen-activated protein kinase
Map2K1	Mitogen-activated protein kinase kinase 1
Map4k4	Mitogen-activated protein kinase kinase kinase kinase 4
Mapk3	Mitogen-activated protein kinase3
Mapt	Microtubule Associated Protein Tau
MARK	Microtubule-affinity regulating kinase
Mark2	Serine/threonine-protein kinase Mark 2
MCI	Mid Cognitive Impairment
MDC	Metalloprotease/Disintegrin-like
Mek1/2	Dual specificity mitogen-activated protein kinase kinase 1/2 (Map2k1/Map2k2)
MHC II	Major histocompatibility complex II
MIP-1 α	Macrophage inflammatory protein-1 α
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
Nano-LC-MS/MS	Nanoscale liquid chromatography coupled to tandem MS
NB	Neurobasal
Ncam1	Neural cell adhesion molecule 1
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NFTs	Neurofibrillary tangles
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NPXY motif	AsparagiNes-Proline-any-tYrosina
NSAID's	Non-Steroids Anti-Inflammatory Drugs

OA	Okadaic acid
p38MAPK	p38 mitogen-activated protein kinases
PAK3	Serine/threonine-protein kinase PAK 3
PAT1	DNA topoisomerase 2-associated protein PAT1
PBS	Phosphate buffered saline
PDPK	Proline-directed protein kinase
PET	Positron Emission Tomography
Pgam5	Mitochondrial serine/threonine phosphatase Pgam5
PHFs	Paired Helical Filaments
Plcg1	Phospholipase C Gamma 1
PI3K	Phosphoinositide 3-kinase
PICALM	Phosphatidylinositol-binding clathrin assembly protein
PID	Phosphotyrosine Interaction Domain
PIN1	Peptidyl-prolyl cis-trans isomerase
PIN1P1	Putative PIN1 like protein
PIP	Protein phosphatase interacting protein
PK	Protein kinase
PK A/B/C	Protein Kinases A/B/C
PMAC	Phosphate metal affinity chromatography
PP	Protein phosphatase
PP1/2A/2B/4/5/7	Protein phosphatases 1/2A/2B/4/5/7
PP1R9A	Protein phosphatase 1 regulatory subunit 9A (Neurabin 1)
PPP1R9B	Protein phosphatase 1 regulatory subunit 9A (Neurabin 2)
PPP1R12A	Protein phosphatase 1 regulatory subunit 12A
Ppm1e	PP Mg2+/Mn2+ dependent 1e
Ppm1h	PP Mg2+/Mn2+ dependent 1h
Ppp1ca	Serine/threonine PP1 alpha catalytic subunit (PP1α)
Ppp1cb	Serine/threonine PP1 beta catalytic subunit (PP1β)
Ppp1cc	Serine/threonine PP1 gamma catalytic subunit (PP1γ)
Ppp2r2a/PP2R2A	Serine/threonine PP2A regulatory subunit B alpha isoform
Ppp2r5e	Serine/threonine PP2A regulatory subunit B epsilon isoform
Ppp3ca	Serine/threonine PP2B catalytic subunit alpha isoform
Ppp3cb	Serine/threonine PP2B catalytic subunit beta isoform
PPP6C	Protein Phosphatase 6 Catalytic Subunit
PRNP	Prion protein
PS/PSEN 1/2	Presinilin 1/2
Psmd13	Proteasome 26S Subunit, Non-ATPase 13
PSPs	Protein phosphatases serine and threonine
Ptk2b	Protein tyrosine kinase 2 beta
PTPs	Protein tyrosine phosphatase
Ptpn11	Tyrosine-protein phosphatase non-receptor type 11
RAB	Ras-related protein
Rab 5/6/10/11/11a	Ras-related protein 5/6/10/11/11a

RANBP9	Ran-binding protein 9
RIPA	Radio-Immunoprecipitation Assay
ROCK	Rho-associated protein kinase
S	Serine
SAD	Sporadic Alzheimer's disease
sAPP	Secreted APP fragment
sAPPα/β	Secreted APP fragment after α and β cleavage, respectively
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
Slc2a4	Solute Carrier Family 2 Member 4
Slc8a3	Solute Carrier Family 8 Member A3
Slc25a22	Solute Carrier Family 25 Member 22
Shc	SHC-transforming protein
Shtn1	Shootin-1
Snap25	Synaptosomal-associated protein 25
SNCA	α-Synuclein
SPs	Senile plaques
Src	Tyrosine-protein kinase Src
Synj1	Synaptojanin-1
SysBioTK	Systems Biology Toolkit
T	Threonine
Tau	Microtubule-associated protein tau
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween
Tf	Serotransferrin
TFA	Trifluoroacetic acid
TGF-α	Transforming Growth Factor-α
TGN	Trans-Golgi Network
Thr668	Threonine
TIP60	Histone acetyltransferase Tip60
TNF α	Tumour necrosis factor α
TRIS	Tris(hydroxymethyl)aminomethane
TrkA	Tropomyosin receptor kinase A
UGB	Upper Gel Buffer
Y	Tyrosine

CHAPTER I

Introduction

Protein phosphorylation is a major regulatory mechanism under physiological conditions but anomalies in this finely tuned post-translational modification can result in an array of pathological conditions. This is the case for several neuropathologies, among them Alzheimer's disease (AD). Abnormal phosphorylation is a key event impacting the two main AD histopathological hallmarks; the presence of senile plaques (SPs) and the formation of neurofibrillary tangles (NFTs), thus contributing to disease development. Manuscript 1 is a review on the critical role that protein phosphorylation plays in AD pathogenesis.

The importance of protein phosphorylation to AD underlies the basis for this thesis' working hypothesis; namely that abnormal protein phosphorylation events can provide a range of biomarkers than can be further explored for their biomarker potential.

Manuscript 1

Oliveira J.M., Costa M., Almeida M.C., da Cruz e Silva O.A.B. and Henriques A.G. (2017). Protein phosphorylation is a key mechanism in Alzheimer´s disease. J Alzheimers Dis, 58(4), 953-978. doi: 10.3233/JAD-170176 Review

Manuscript 1

Protein phosphorylation is a key mechanism in Alzheimer's Disease

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ABSTRACT

Altered protein phosphorylation states of several proteins are closely associated with Alzheimer's disease (AD). Among these are the Alzheimer's amyloid-β precursor protein (APP) and the TAU protein. In fact, altered protein phosphorylation states already provide strong biomarkers for AD diagnosis, as is the case with hyperphosphorylated TAU. It follows that modulating signaling cascades provides an attractive avenue for exploring novel therapeutic strategies. This review focuses on some of the major protein kinases and protein phosphatases relevant to AD. Of relevance, posttranslational modifications dynamically regulate protein activity, subcellular localization and stability. Protein phosphorylation states can mediate complex formation as well as regulate protein function and this is important for cellular physiology but can likewise contribute to the development of neuropathological conditions. Furthermore, applying a system's approach provides a more comprehensive understanding of the signaling events associated with AD and highlights possible convergence points that may contribute to the different AD pathological hallmarks.

INTRODUCTION

Post-translational modifications like protein phosphorylation and ubiquitination act as the gatekeepers of cellular processes. It is now widely accepted that aberrant protein phosphorylation is involved in the pathogenesis of a wide range of diseases among them neuropathological disorders (da Cruz e Silva et al., 2004; Wagey and Krieger, 1998). Neuropathologies are complex and anomalies can occur at the molecular and cellular level, affecting strategically distinct brain regions. There are hundreds of diseases of the nervous system, among them Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis, Creutzfeldt-Jakob disease, Familial Adenomatous Polyposis, Frontotemporal dementia, Huntington's disease, Machado–Joseph disease and Parkinson's disease.

AD has been associated with abnormal phosphorylation of specific key proteins, as has been well documented for TAU (microtubule-associated protein tau) (Goedert et al., 1992; Grundke-Iqbal et al., 1986). Of note, protein phosphorylation is recognized as the major post-translational modification through which numerous physiological processes are regulated. However, in AD the exact role played by abnormal protein phosphorylation in the disease aetiology, is not completely understood. It is particularly relevant that protein phosphorylation, via complex signaling cascades, can regulate neuronal plasticity, neurotransmission, and consequently compromise memory and learning. Ultimately protein phosphorylation can contribute to disease associated processes (Cohen, 2001; Gong et al., 2006; Wagey and Krieger, 1998). Signaling cascades are precisely controlled by dynamic reversible protein phosphorylation and depend on the precise balance between protein kinase (PK) and protein phosphatase (PP) activities. Human genome sequencing predicts more than 500 protein kinases and around 150 protein phosphatase genes (Lander et al., 2001; Venter JC, Adams MD, 2001). The kinases are subdivided into two families, the Ser/Thr-kinases with 428 members and the Tyr-kinases with 90 members (C. Manning, D.B. Whyte, R. Martinez, T. hunter, 2002; Johnson and Hunter, 2005). For the phosphatases, four distinct families have been described, the protein Tyr-phosphatases (PTPs) (Alonso et al., 2004), the specificity phosphatases dual specificity protein phosphatases (Patterson et al., 2009) and two families of protein phosphatases, serine and threonine (PSPs) (Barford, 2010), of these, around 107 are PTPs (Alonso et al., 2004) and around 40 are PSPs (Bollen, 2001; Ceulemans and Bollen, 2004). The apparent lower number of PSPs is compensated for by an array of regulatory and targeting subunits, which confer target and substrate specificity, with more than 200 of these phosphatase interacting proteins (PIPs), thus far identified (Esteves et al., 2012, 2013; Fardilha et al., 2010; Heroes et al.,

2013; Moorhead et al., 2008). Originally kinases were considered the regulators of signal transduction-mediated events, however it is now recognized that protein phosphatases have an equally important role. Together, kinases and phosphatases represent a central mechanism regulating major cellular events. Brain is the human tissue expressing the highest levels of protein kinases and phosphatases (da Cruz e Silva et al., 1995a; Ouimet et al., 1995). Consistently abnormal protein phosphorylation states are a hallmark in specific disease conditions, and consistently altered phosphatase and kinase activities have been reported in the brains of AD patients (Chung, 2009).

ALZHEIMER'S DISEASE SIGNS, HALLMARKS AND PROTEIN PHOSPHORYLATION

AD is a neurodegenerative multifactorial disorder first described in 1906 by the German pathologist Alois Alzheimer. Symptoms include progressive memory loss and decline in other cognitive functions. Affected individuals suffer personality changes, such as behavioural and emotional disturbances, and the capacity to execute social and personal activities declines. Initial memory impairment evolves to disorientation, speech abnormalities, hallucinations, apraxias, among other signs. At later stages, patients become completely dependent. Typically, other diseases can become lethal, in particular pneumonia, which is the principal cause of death in AD patients (Kukull et al., 1994).

The majority of AD cases are sporadic (SAD), but rare, familial, early-onset autosomal dominant forms of AD (FAD) have been described. Mutations or polymorphisms in genes encoding APP (Alzheimer´s amyloid precursor protein), PS1/PSEN1 (Presenilin-1) and PS2/PSEN2 (Presenilin-2) (Weggen and Beher, 2012) are related to FAD. Although more than 25 mutations in the APP gene have been described, these, as already mentioned, are related to FAD, but are rare when all AD patients are considered. Nonetheless, these mutations suggest that abnormal APP processing can contribute to the disease condition. Of note, these mutations occur in the flanking region of the Aβ domain. Duplication of the APP gene also appears to contribute to the early onset of the disease, explaining why individuals with Down's syndrome/trisomy 21 (chromosome encoding the APP gene) have an increased risk of developing AD (Head et al., 2012). Missense mutations in APP represent less than 0.1% of all the AD cases. Missense mutations, in the secretases, PS1 (chromosome 14) and PS2 (chromosome 1) genes are related to early onset (between 40 and 60 years) and aggressive AD forms. Studies revealed that mutations in these two genes shift the substrate specificity of PS1 and PS2 from Notch to APP; resulting in increased Aβ production (Kulic

et al., 2000). Additionally, the presence of two alleles APOE4 (allele 4) is one of the most important genetic risk factors for SAD (Corder et al., 1993). The presence of APOE4 precipitates the onset of the disease. Contrastingly, APOE2 (allele 2) seems to have a protective effect against the disease (West et al., 1994). Non-genetic factors, like regular use of NSAID's (Nonsteroidal anti-inflammatory drugs), wine and coffee consumption, and regular physical activity have been associated with a lower risk of developing AD; whereas aging and low educational levels, were associated with increased risk of AD incidence (Lindsay et al., 2002). Elevated serum levels of cholesterol and LDL also correlated with increased amounts of Aβ in the brain and subsequently increase the risk of developing AD (Kuo et al., 1998).

Senile plaques (SPs), intraneuronal neurofibrillary tangles (NFTs) and neuropil threads (abnormal neurites) have been extensively described as AD hallmarks. These lesions are found in specific brain regions associated with memory and learning processes namely the neocortex, entorhinal cortex and hippocampus. The presence and distribution of NFT, SP and synaptic degeneration correlate with the degree of cognitive decline (Davies et al., 1987; Guillozet et al., 2003).

Of particular relevance, both histopathological hallmarks of proteinacious deposits in AD; the NFTs and SPs, can be correlated to phosphorylation events. NFTs are a consequence of hyperphosphorylated TAU, whereas SPs have at their core the Aβ peptide, whose production can be modulated by the phosphorylation state of APP (Da Cruz e Silva and Da Cruz e Silva, 2003; Gandy et al., 1993; Rebelo et al., 2007a, 2007b). A third hallmark, which should be considered in AD, is synaptic dysfunction that appears to precede the deposition of NFTs and SPs (Scheff et al., 2006). Synaptic signaling cascades inevitably involve protein phosphorylation mediated events, thus the third hallmark, like NFTs and SPs involves anomalous phosphorylation processes. Consistently, post-mortem analysis of AD brains reveals a tendency towards decreased phosphatase levels, and increased kinases levels, together favouring conditions for hyperphosphorylated proteins (Avila, 2009; Chung, 2009; Gong et al., 2006).

TAU PROTEIN AND ALZHEIMER'S DISEASE

NFTs and neuropil threads are composed of aggregated abnormal paired helical filaments (PHFs) of hyperphosphorylated TAU protein. TAU is a microtubule-associated protein, essential in microtubule dynamics, neurite outgrowth and axonal transport. It is regulated in a phosphorylation dependent manner. In its hyperphosphorylated state, TAU sequesters normal TAU and other

microtubule-associated proteins, leading to microtubule destabilisation and depolymerisation. Consequently, axonal transport and neurotransmission are compromised, affecting particularly synapses and contributing to a decline in cognitive functions. Moreover, hyperphosphorylated TAU self-assembly (Gómez-Ramos et al., 2004) leads to small deposits (pretangles) that adopt a β-sheet conformation in PHFs. In turn, these assemblies form into large NFTs, whereby TAU undergoes additional modifications, namely, truncations, glycations and cross-linking by transglutaminases (Jakob-Roetne and Jacobsen, 2009; Martin et al., 2011). Therefore, signaling cascade alterations, leading to abnormal protein phosphorylation or aggregation can potentiate NFT formation and neuronal degeneration.

Although TAU is involved in many cellular functions; amongst the most important is perhaps tubulin polymerization. Protein phosphorylation regulates the binding of TAU to tubulin, whereby phosphorylation of the former alters its conformation and causing it to detach from microtubules (Fischer et al., 2009; Jho et al., 2010). Cross talk between TAU and APP is an intense area of research, this may involve protein phosphorylation, particularly if one considers that Aβ has been shown to influence TAU phosphorylation (Oliveira et al., 2015).

TAU possesses a large number of potential phosphorylation sites (Table I-1) at serine, threonine and tyrosine residues (Avila et al., 2004; Götz et al., 2010). For the longest brain TAU isoform (441 amino-acids) more than 80 phosphorylation sites have been described ((Avila, 2009) and Table I-1). TAU phosphorylation at normal physiological conditions controls a variety of processes such as microtubule binding and microtubule assembly (Cho and Johnson, 2004), neurite outgrowth (Mandell and Banker, 1996), axonal transport (Tatebayashi et al., 2004) and cell sorting (Litman et al., 1993). The proline-directed protein kinases (PDPK) are the major proteins involved in TAU protein phosphorylation. These include glycogen synthase kinase 3 (GSK-3), mitogen activated protein kinase (MAPK), TAU-tubulin kinase, cyclin-dependent kinases such as CDK2 and CDK5, and stress-activated kinases (SAP kinases). Kinases from the Non-PDPK group with activity towards TAU protein, include the microtubule-affinity regulating kinase (MARK), Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II), cyclic-AMP-dependent kinase (PKA), casein kinase II (CK2) and protein kinase C (PKC) (Avila et al., 2004; Buée et al., 2000; Chung, 2009; Metcalfe and Figueiredo-Pereira, 2010). Of note, TAU extracted from the brain of AD patients exhibited 45 phosphorylation sites, 29 serines (S), 13 threonines (T) and 3 tyrosines (Y) (Figure I-1), the majority of which can be modified by GSK-3 (D.P. et al., 2009). However, if one globally considers the findings published, identifying the TAU phosphorylation sites, many more have been documented and these are summarized in Table I-1.

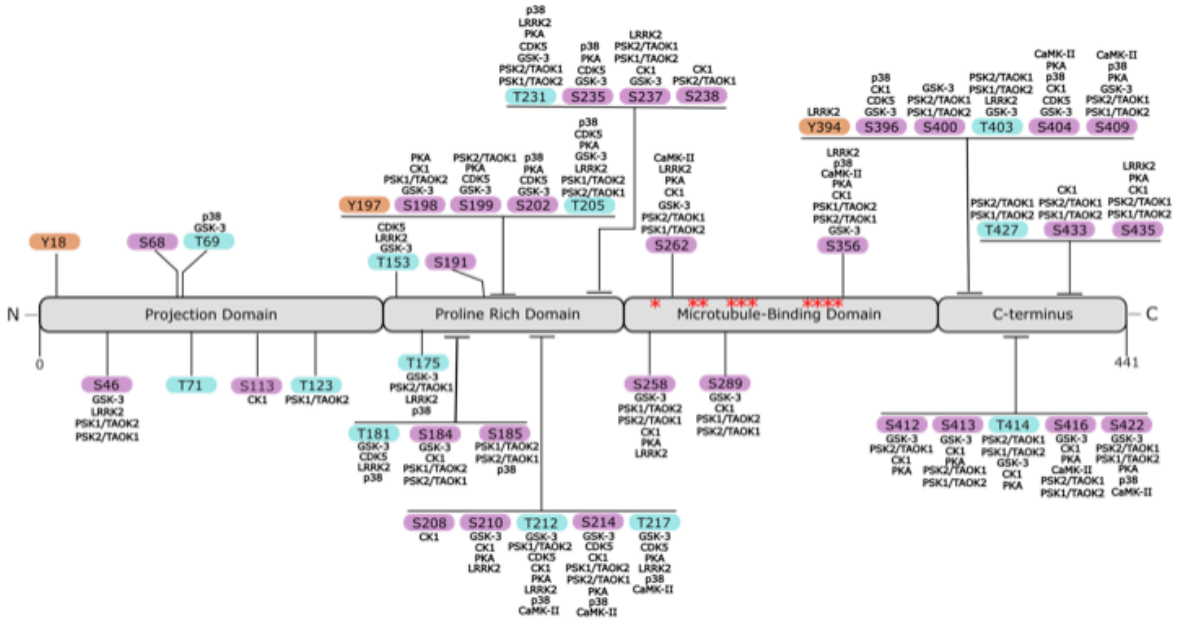

Figure I-1. **TAU phosphorylation sites identified in brains of AD patients.** Diagrammatic representation of the most common TAU residues (Y in orange; S in purple and T in blue) shown to be phosphorylated in the AD brain. The kinases responsible for the respective phosphorylations are also indicated (when the information was available). The KXGS motifs are indicated *259-QIGS-262, **290-QCGS-293, ***321-QCGE-324 and ****353-QIGS-356

Phosphorylation within the microtubule-binding domain, at the KXGS motifs (red asterisks Figure I-1), reduces the binding of TAU to microtubules. More specifically the phosphorylation at residues S262 and S356 'breaks' the binding between TAU and microtubules (Biernat and Mandelkow, 1999). Likewise, phosphorylation at T231 by GSK-3β also plays a role in diminishing the ability of TAU to bind to microtubules (Lin et al., 2007). Given that so many kinases are involved in TAU phosphorylation, TAU might be primed by a given kinase before subsequent phosphorylation by another kinase (D.P. et al., 2009). For instance, an example from Down's syndrome, identified that DYRK1A phosphorylates TAU at several sites including T181, S199, S202, T205, T212, T217, T231, S396, S400, S404, and S422; these phosphorylations can prime TAU for further phosphorylations by GSK-3 at T181, S199, S202, T205, and S208 but not by CDK5 and PKA (Liu et al., 2008; Ryoo et al., 2007; Wegiel et al., 2011). Sequential phosphorylations have long been shown to be relevant and a key event in modulating protein functions (Cohen, 2000).

Table I-1.Phosphorylated residues in TAU.

	Residue	Protein Kinase	Reference
AD brain	Y18	SYK; FYN	(Hanger et al., 2013; Lebouvier et al., 2008; Lee et al., 2004; Tavares et al., 2013)
	S68	N.D.	
	T69	GSK-3; ERK; p38	(D.P. et al., 2009; Hanger et al., 2013)
	T71	AMPK	(Hanger et al., 2013)
	S113	CK1	(D.P. et al., 2009; Hanger et al., 2013)
	T123	CHK1; PSK1/TAOK2	(Hanger et al., 2013; Mendoza et al., 2013)
	T153	GSK-3; CDK5; ERK; SAPK1γ; SAPK2; SAPK3; SAPK4; LRRK2	(Hanger et al., 2013)
	T175	GSK-3β; JNK; ERK2; p38; SAPK1γ; SAPK2; SAPK3; LRRK2; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013; Reynolds et al., 2000)
	S184	GSK-3β; CK1; SAPK2; SAPK3; PSK1/TAOK2; PSK2/TAOK1	(D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013)
	S185	p38; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013; Reynolds et al., 2000)
	S191	CHK1; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013)
	Y197	MET	(Hanger et al., 2013)
	S208	CK1δ; CHK1; TTBK1; TTBK2; AMPK	(D.P. et al., 2009; Hanger et al., 2013; Mendoza et al., 2013)
	S210	GSK-3; PKA; CK1*; LRRK2	(D.P. et al., 2009; Hanger et al., 2013)
	S214	GSK-3β; PKA; PKB; PKC; PKN; CaMK-II; CDK2; CDK5; CK1; CHK1; CHK2; p38; SAPK1γ; SAPK2; SAPK3; SAPK4; AMPK; MSK1; p70S6K; PSK1/TAOK2; PSK2/TAOK1; RSK1/2; SGK1; SRPK2	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Mendoza et al., 2013; Wang et al., 2007; Yoshimura et al., 2003)
	S237	GSK-3; CK1; PhK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013)
	S238	CK1; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013)
	S258	GSK-3β; PKA; PKC; PKN; CK1δ; CHK1; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013; Mendoza et al., 2013)
	S262	GSK-3α; GSK-3β; PKA; PKC; CaMK-II; CK1δ; CHK1; CHK2; MARK; PhK; AMPK; BRSK; LRRK2; MSK1; p70S6K; PSK1/TAOK2; PSK2/TAOK1; ROCK	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Mendoza et al., 2013; Wang et al., 2007; Yoshimura et al., 2003)
	S289	GSK-3β; CK1δ; CHK1; CHK2; AMPK; PSK1/TAOK2; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013; Mendoza et al., 2013)

		Residue	Protein Kinase	Reference
		S356	GSK-3α; GSK-3β; PKA; CaMK-II; CK1δ; JNK; MARK; ERK; CHK1; p38; SAPK1γ; SAPK2; SAPK3; SAPK4; PhK; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Mendoza et al., 2013; Reynolds et al., 2000; Yoshimura et al., 2003)
		Y394	c-Abl; LRRK2	(Hanger et al., 2013)
		T403	GSK-3β; CHK2; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Gong et al., 2005; Hanger et al., 2013; Mendoza et al., 2013)
		S409	GSK-3β; PKA; CaMK-II; CHK1; CHK2; p38; SAPK3; SAPK4; PSK1/TAOK2; PSK2/TAOK1; ROCK	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Mendoza et al., 2013; Wang et al., 2007)
		T414	GSK-3β; PKA; CK1δ; CK2**; CHK1; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013)
		S422	GSK-3β; PKA; CaMK-II; MAPK; JNK1; JNK2; JNK3; ERK2; p38; TTBK1; DYRK1A; SAPK4; PSK1/TAOK2; PSK2/TAOK1	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Hanger et al., 2013; Sato et al., 2006; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004)
		T427	PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013)
		S433	CK1δ; CHK2; PSK1/TAOK2; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013; Mendoza et al., 2013)
		S435	PKA; CK1δ; CHK2; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013; Mendoza et al., 2013)
AD & Normal brain		S46	GSK-3β; CK1*; MAPK; ERK2; p38 SAPK2; SAPK3	(Billingsley and Kincaid, 1997; Gong et al., 2005; Hanger et al., 2013; Reynolds et al., 2000)
		T181	GSK-3β; CDK5; JNK1; JNK2; JNK3; ERK2; p38; DYRK1A; SAPK1γ; SAPK2; SAPK3; SAPK4; LRRK2	(D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Reynolds et al., 2000; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004)
		S198	GSK-3β; PKA; CK1; TTBK1; PSK1/TAOK2	(D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Sato et al., 2006)
		S199	GSK-3α; GSK-3β; PKA; CDK5; CK2; MAPK; JNK1; JNK2; JNK3; ERK2; TTBK1; DYRK1A; SAPK4; PSK2/TAOK1	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Reynolds et al., 2000; Sato et al., 2006; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004)
		S202	GSK-3α; GSK-3β; PKA; CDK5; MAPK; JNK1; JNK2; JNK3; ERK2; p38; TTBK1; DYRK1A; SAPK1γ; SAPK2; SAPK3; SAPK4	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Pevalova et al., 2006; Reynolds et al., 2000; Ryoo et al., 2007; Sato et al., 2006; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004)
		T205	GSK-3β; PKA; CDK5; JNK1; JNK2; JNK3; ERK2; p38; DYRK1A; SAPK1γ; SAPK2; SAPK3; SAPK4; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Billingsley and Kincaid, 1997; Hanger et al., 2013; Pevalova et al., 2006; Reynolds et al., 2000; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004)

		Residue	Protein Kinase	Reference
		T212	GSK-3α; GSK-3β; PKA; CaMK-II; CDK5; CK1*; JNK1; JNK2; JNK3; ERK2; p38; DYRK1A; SAPK1γ; SAPK2; SAPK3; SAPK4; LRRK2; p70S6K; PSK1/TAOK2	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Pevalova et al., 2006; Reynolds et al., 2000; Ryoo et al., 2007; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004; Yoshimura et al., 2003)
		T217	GSK-3β; PKA; CaMK-II; CDK5; JNK1; JNK2; JNK3; ERK2; p38; DYRK1A; SAPK4; LRRK2	(D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Pevalova et al., 2006; Reynolds et al., 2000; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004)
		T231	GSK-3α; GSK-3β; PKA; CDK5; JNK; ERK2; p38; DYRK1A; SAPK1γ; SAPK3; SAPK4; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Reynolds et al., 2000; Wang et al., 2007; Wegiel et al., 2011)
		S235	GSK-3α; GSK-3β; PKA; CDK5; MAPK; JNK2; ERK2; p38; SAPK1γ; SAPK2; SAPK3; SAPK4; PhK; AMPK	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Pevalova et al., 2006; Reynolds et al., 2000; Yoshida et al., 2004)
		S396	GSK-3α; GSK-3β; CDK5; CK1; CK2; MAPK; JNK1; JNK2; JNK3; ERK2; p38; DYRK1A; SAPK1γ; SAPK2; SAPK3; SAPK4	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Pevalova et al., 2006; Reynolds et al., 2000; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004)
		S400	GSK-3β; CK2; CHK1; DYRK1A; AMPK; PSK1/TAOK2; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013; Mendoza et al., 2013; Reynolds et al., 2000; Wegiel et al., 2011)
		S404	GSK-3α; GSK-3β; PKA; CaMK-II; CDK5; CK1; CK2; MAPK; JNK1; JNK2; JNK3; ERK2; p38; DYRK1A; SAPK4	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013; Pevalova et al., 2006; Reynolds et al., 2000; Ryoo et al., 2007; Wang et al., 2007; Wegiel et al., 2011; Yoshida et al., 2004)
		S412	GSK-3; PKA; CK1; CK2**; PSK2/TAOK1	(D.P. et al., 2009; Hanger et al., 2013)
		S413	GSK-3β; PKA; CK1*; CK2**; PSK1/TAOK2; PSK2/TAOK1	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Hanger et al., 2013; Wang et al., 2007)
		S416	GSK-3; PKA; CaMK-II; CK1; CK2**; PSK1/TAOK2; PSK2/TAOK1	(Billingsley and Kincaid, 1997; D.P. et al., 2009; Gong et al., 2005; Hanger et al., 2013)
Normal brain		T17	CK1δ; CHK2; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013)
		Y29	N.D.	
		T39	CK2	(Hanger et al., 2013)
		T50	GSK-3β; CK1*; ERK	(Gong et al., 2005; Hanger et al., 2013)
		T52	CK2*	(Hanger et al., 2013)
		S56	CK2*	(Hanger et al., 2013)

Residue	Protein Kinase	Reference
T95	CK1	(Hanger et al., 2013)
T101	CK1*	(Hanger et al., 2013)
T102	CK1*	(Hanger et al., 2013)
T111	N.D.	
S131	CaMK-II; CK1	(Gong et al., 2005; Hanger et al., 2013; Yoshimura et al., 2003)
T135	CaMK-II; LRRK2	(Gong et al., 2005; Hanger et al., 2013; Yoshimura et al., 2003)
T149	GSK-3β; CK1δ; CHK1; LRRK2; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013)
T169	CK1δ; CHK1; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013)
S195	GSK-3β; PKA; CDK5; AMPK; PSK1/TAOK2; PSK2/TAOK1	(Billingsley and Kincaid, 1997; Gong et al., 2005; Hanger et al., 2013)
T220	GSK-3; PKA*; PSK1/TAOK2	(Hanger et al., 2013)
S241	GSK-3; CK1; AMPK; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013)
T245	GSK-3β; PKA; CHK1; CHK2; p38; SAPK2; SAPK3; SAPK4; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1; ROCK	(Hanger et al., 2013; Mendoza et al., 2013; Reynolds et al., 2000)
T263	CK1; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013)
S285	GSK-3β; CaMK-II; CK1δ; CHK2; PhK	(Hanger et al., 2013; Mendoza et al., 2013)
S293	PKA; PKC; CK1; CHK1; MARK; AMPK	(Hanger et al., 2013; Mendoza et al., 2013)
S305	GSK-3β; PKA; PKC; CK1δ; CHK1; MARK; p38; PhK; AMPK; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013; Reynolds et al., 2000)
S316	N.D.	
S320	PKA; PKN; CHK1; CHK2; MARK; p38	(Hanger et al., 2013; Mendoza et al., 2013)
S324	GSK-3α; GSK-3β; PKA; PKC; PKN; CHK1; MARK; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Billingsley and Kincaid, 1997; Gong et al., 2005; Hanger et al., 2013; Mendoza et al., 2013)
S341	CK1	(Hanger et al., 2013)
S352	GSK-3β; PKA; PKC; PKN; CK1δ; CHK1; CHK2; PhK; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013)
T361	CK1; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013)
T373	GSK-3; CDK5; CK1; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Gong et al., 2005; Hanger et al., 2013)
T386	CK1; CK2; CHK1; CHK2; AMPK; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013)
T30	CHK2; LRRK2; PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013; Mendoza et al., 2013)

		Residue	Protein Kinase	Reference
Not fully characterized		S61	PSK2/TAOK1	(Hanger et al., 2013)
		T63	PSK1/TAOK2	(Hanger et al., 2013)
		S64	N.D.	
		T76	PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013)
		S129	N.D.	
		S137	LRRK2	(Hanger et al., 2013)
		Y310	N.D.	
		T319	PSK1/TAOK2; PSK2/TAOK1	(Hanger et al., 2013)
		T377	PKC; CHK1; CHK2; LRRK2; PSK1/TAOK2; PSK2/TAOK1; ROCK	(Hanger et al., 2013; Mendoza et al., 2013)

* Indicates that only one of two closely spaced residues is phosphorylated and **only one of four closely spaced residues is phosphorylated.

Abbreviations: GSK – 3, Glycogen synthase kinase 3; PKA, Protein kinase A; PKB, Protein kinase B; PKC, Protein kinase C; PKN, Protein kinase N; CaMK-II, Calcium/calmodulin-dependent protein kinase II; CDK2, Cyclin-dependent kinase 2; CDK5, Cyclin-dependent kinase 5; CK1, Casein kinase 1; CK2, Casein kinase 2; MAPK, Mitogen Activated Protein Kinase; JNK1, c-Jun N-terminal Kinase 1; JNK2, c-Jun N-terminal Kinase 2; JNK3, c-Jun N-terminal Kinase 3; CHK1, Checkpoint Kinase 1; CHK2, Checkpoint Kinase 2; MARK, MAP/microtubule affinity-regulating kinase; ERK2, Extracellular signal–regulated kinases; p38, p38 mitogen-activated protein kinase; TTBK1, TAU tubulin kinase 1; TTBK2, TAU tubulin kinase 2; DYRK1A, Dual-specificity tyrosine phosphorylation-regulated kinase 1A; SAPK1γ, Stress-activated protein kinase 1 gamma; SAPK2, Stress-activated protein kinase 2; SAPK3, Stress-activated protein kinase 3; SAPK4, Stress-activated protein kinase 4; SYK, Spleen tyrosine kinase; FYN, Proto-oncogene tyrosine-protein kinase; PhK, Phosphorylase kinase; AMPK, 5’ adenosine monophosphate-activated protein kinase; BRSK, Brain-specific kinase 1/2; LRRK, Leucine-rich repeat kinase 2; MET, Met (tyrosine kinase); MSK1, Mitogen- and stress-activated protein kinase; P70S6K, Ribosomal protein S6 kinase beta-1; PSK1/TAOK2, Prostate-derived sterile 20-like kinase 1 alpha/beta; PSK2/TAOK1, Prostate-derived sterile 20-like kinase 2; ROCK, RHO-associated kinase; RSK1/2, 90 kDa ribosom al S6 kinase; SGK1, Serine/threonine-protein kinase; SRPK2, Serine/arginine-rich protein-specific kinase; N.D., Not Defined.

APP AND ALZHEIMER'S DISEASE

APP is one of three members of a gene family, which includes APLP1 (Amyloid-like protein 1) and APLP2 (Amyloid-like protein 2) where only APP contains the Aβ domain. This protein is a ubiquitously expressed type I transmembrane glycoprotein, encoded by a single gene on chromosome 21q21. Multiple isoforms exist resulting from alternative splicing of exons 7, 8 and 15 of the APP mRNA. There are three major APP isoforms (APP695, APP751, and APP770), consisting of 695, 751, and 770 amino acids, respectively (Tanzi et al., 1987). The 695 isoform is the only one that lacks a kunitz protease inhibitor (KPI) domain in its extracellular domain and is the predominant form in neuronal tissues.

APP can be processed by a non-amyloidogenic and an amyloidogenic pathway. In the non-amyloidogenic pathway, APP is cleaved by α-secretase within the Aβ domain. This results in the shedding of nearly the entire ectodomain (releasing a secreted APP fragment, sAPPα) and generation of a membrane anchored α-C-terminal fragment (C83). The latter is cleaved by the γ-secretase complex (Edbauer et al., 2003; Haass and Steiner, 2002; De Strooper, 2003), releasing a non-toxic p3 peptide and the APP intracellular domain (AICD) polypeptide fragment. APP cleavage by activation of α-secretase is the major and ubiquitous pathway of APP metabolism in most cells. Estrogen, testosterone, various neurotransmitters, growth factor and protein kinase C (PKC) can regulate the α-cleavage pathway (Ling et al., 2003). Zinc metalloproteases like TACE/ADAM17, ADAM9, ADAM10 and MDC-9 can all cleave APP at the α-secretase site (Allinson et al., 2003).

In the amyloidogenic pathway, particularly enriched in neurons, APP is first cleaved by β-secretase, releasing sAPPβ and β-C-terminal fragment (C99). Subsequently this fragment is cleaved by the γ-secretase complex; giving rise to the Aβ peptide and the AICD fragment. β-secretase can also cleave APP within the Aβ domain to produce a C89 truncated Aβ species (Zhang and Song, 2013). BACE1 (Beta-secretase 1) and BACE2 (Beta-secretase 2) are two enzymes capable of cleavage at the β-site. BACE 1 is the major β-secretase in the brain and is the key rate-limiting enzyme that initiates Aβ formation. Over-expression of BACE1 in cell culture has been shown to increase the amount of β-secretase cleavage products (Vassar et al., 1999). BACE2 shows similar substrate specificity but is not as highly expressed in the brain (Farzan et al., 2000).

APP can be phosphorylated at multiple sites in both extracellular and intracellular domains. In neuronal cells, APP695 is phosphorylated at serine, threonine and tyrosine residues. In the intracellular domain 8 putative phosphorylation residues have been described (Figure I-2): Y653, T654, S655, T668, S675, Y682, T686 and Y687 (Lee et al., 2003; Oishi et al., 1997; Zambrano et al.,

2001). In the extracellular domain two phosphorylatable residues have been identified, S198 and S206 (Walter et al., 1997). The consequence and physiological relevance of phosphorylation events at each of these residues is not clearly understood. However, correlations between phosphorylation at specific residues and APP fate are starting to be described. For example, phosphorylation at Y687 is relevant for APP endocytosis and subsequent Aβ production (Rebelo et al., 2007a). Likewise, S655 phosphorylation determines the fate of APP with respect to lysosomal targeting or retrograde transport to the Golgi via a retromer mediated process (Vieira et al., 2010), and T668 phosphorylation is important to regulate APP binding to other proteins (Barbagallo et al., 2011).

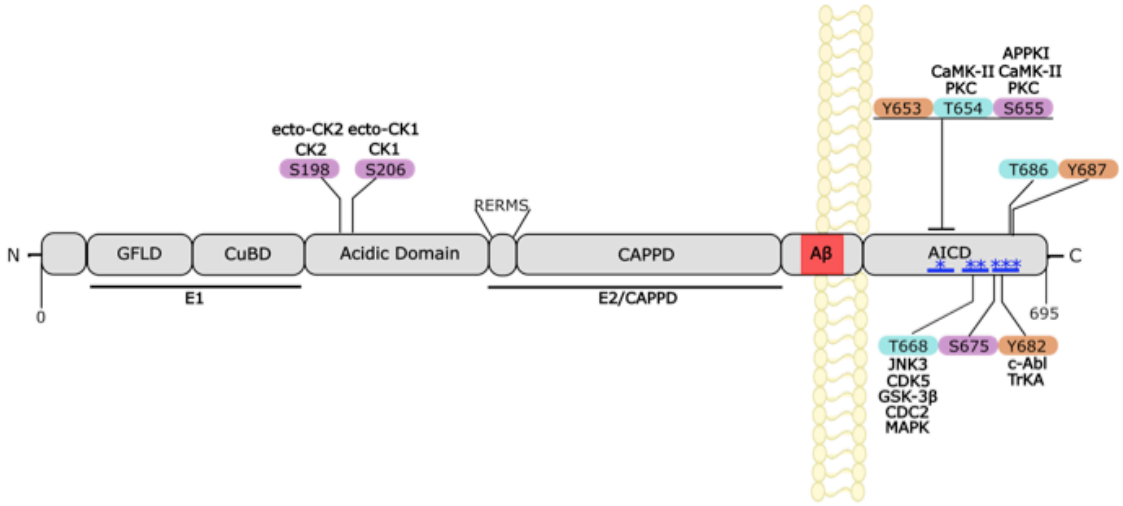

Figure I-2. APP phosphorylation sites identified in brains of AD patients. Diagrammatic representation of APP (isoform 695 numbering) and the residues (Y in orange, S in purple and T in blue) shown to be phosphorylated in the AD brain. The kinases responsible for the respective phosphorylations are also indicated (when the information was available). Several motifs are indicated; GFLD the growth-factor like domain, the E1 and E2 domain, CuBD the copper binding domain, the pentapeptide RERMS domain, CAPPD the central APP domain, the Aβ domain shown in red, the APP C-terminal fragment and the 3 C-terminal motifs *682-YENPTY-687, **667-VTPEER-672 and ***653-YTSI-656.

Several kinases have been shown to be relevant to APP biology, either by directly phosphorylating APP or by phosphorylating other relevant substrates. Amongst the first reported was protein kinase C (PKC) (Caporaso et al., 1992; Gillespie et al., 1992; Haass et al., 1992; Suzuki et al., 1992). Direct activation of PKC by phorbol esters, which mimics the effects of diacylglycerol, resulted in increased sAPP release and inhibited Aβ formation (Buxbaum et al., 1993; Caporaso et al., 1992; Gabuzda et al., 1993; Gillespie et al., 1992; Henriques and Vieira, 2007; Hung et al., 1993). APP can be phosphorylated on the C-terminal domain and a number of kinases fulfilling this function have been identified. PKC phosphorylates APP S655 (Edbauer et al., 2003), whereas CDK5

and CDC2 phosphorylate APP at T668 in neurons (Iijima et al., 2000; Suzuki et al., 1994). T668 can also be phosphorylated *in vivo* by a number of protein kinases, namely GSK-3, JNK3/SAPK1b, (Aplin et al., 1996; Iijima et al., 2000; Standen et al., 2001). *In vitro*, the tyrosine kinases, TRKA and c-Abl, have also been shown to phosphorylate APP on Y682 (Tarr et al., 2002; Zambrano et al., 2001).

OTHER MAJOR PHOSPHORYLATED PROTEINS IN ALZHEIMER'S DISEASE

Several other phosphorylatable proteins of relevance to AD have been described, among them the secretases. The γ-secretase complex consists of at least four different proteins: Presenilins (PS), Nicastrin, APH-1 and PEN-2 proteins. In order to form an active γ-secretase complex, Nicastrin, a type I transmembrane glycoprotein, and APH-1, form a dimeric subcomplex to which PS binds. Subsequently, PEN-2 is incorporated into the complex and PS is cleaved into two stable fragments; the N-terminal fragment and a C-terminal fragment. The active γ-secretase is thus formed (Parks and Curtis, 2007; Spasic and Annaert, 2008; Verdile et al., 2007). ERK1/2 appears to be an endogenous negative regulator of γ-secretase, probably via direct phosphorylation of Nicastrin (Kim et al., 2006). The γ-secretase can also be activated by tumor necrosis factor α (TNF-α), which promotes JNK phosphorylation of Nicastrin and PS1 (Kuo et al., 2008).

PS is responsible for the catalytic activity of the γ-secretase complex as described above. The former has two homologues, PS1 and PS2, mutations of which are the most common cause of early-onset FAD, as previously discussed. PS1 is an integral membrane protein, with nine predicted transmembrane domains, localized in the ER, Golgi and plasma membrane (Laudon et al., 2005). PS1 can be phosphorylated by several protein kinases (Kockeritz et al., 2006). Phosphorylation of PS1 at S353 and S357 by GSK-3β may influence binding to β-catenin (Prager et al., 2007). PS1 phosphorylation at S397 by GSK-3β regulates C-terminal fragment levels (Kirschenbaum et al., 2001). Physiologically distinct functions can occur via PKA-mediated phosphorylation, which strongly inhibits proteolytic processing of PS1 by caspase activity during apoptosis, reducing the progression of apoptosis (Fluhrer et al., 2004). To add further complexity to the system, studies have shown that PS1 can stimulate PI3K/AKT signaling, thus promoting phosphorylation and inhibiting GSK-3β with the effect of suppressing GSK-3β mediated TAU overphosphorylations found in AD. It appears that in FAD mutations PS1-dependent PI3K/Akt activation is inhibited favouring GSK-3β mediated TAU phosphorylation (Baki et al., 2004).

In the near future, many other phosphorylated proteins associated with AD are likely to be forthcoming. Recent work by Henriques et al (Henriques et al., 2016), showed that when cells were

exposed to Aβ the recovery of 73 phosphorylated proteins increased and that of 68 phosphorylated proteins decreased, in comparison to cell models not exposed to the toxic peptide. These candidates include protein kinases and phosphatases and provide a set of potential candidates for therapeutic and diagnostic strategies.

THE RELEVANCE OF PROTEIN PHOSPHATASES IN ALZHEIMER'S DISEASE

Given the dynamic nature of protein phosphorylation systems, it is perhaps not surprising that the association of protein phosphatases (PPs) with AD has been well established and is increasing. Of note, altered PP expression levels have been reported in AD. Most of the phosphatase activity can be attributed to the Ser/Thr PPs (Gong et al., 1993).

In the human brain PP2A, PP5, PP1 and PP2B have all been shown to regulate TAU phosphorylation, accounting for approximately 71%, 11%, 10% and 7%, of TAU dephosphorylation, respectively (Liu et al., 2005). Thus, PP2A is the major TAU phosphatase. In many cases, all the above-mentioned phosphatases can dephosphorylate the same TAU residues, albeit with different efficiencies towards different sites. TAU dephosphorylation by PP1, PP2A and PP5 exhibited K(m) values around 8-12 microm, whereas that for PP2B was around five times higher (Liu et al., 2005). In the AD brain PP1, PP2A and PP5 phosphatase activity were decreased whereas PP2B increased. Among these the most significant is PP2A, as it can regulate phosphorylation at multiple TAU sites. The relevance of PP2A was shown by using metabolically competent rat brain slices as a model, and inhibiting PP2A with okadaic acid (OA), induced AD like hyperphosphorylation and the accumulation of NFTs (Wang et al., 2001).

PP2A is also involved in Aβ production. OA induced inhibition of PP2A in N2a cells, increased APP phosphorylation at T668 and the secretion of both sAPPα and sAPPβ were enhanced. However, in some of these experiments it is not clear whether these effects are preferentially mediated by PP1 (da Cruz e Silva et al., 1995b) and/or PP2A (Holzer et al., 2000).

It is particularly noteworthy that PP1 is highly enriched in dendritic spines (Ouimet et al., 1995). This observation led to the discovery of spinophilin, which binds PP1, targeting it to the post-synaptic density. Of note, synaptic loss is presently the best correlation for AD, and the best post-synaptic marker is PP1. Subsequently, many PP1 interacting proteins (PIP) have been identified (Esteves et al., 2012, 2013), some of which are brain specific or enriched. Three mammalian isoforms, expressed in virtually all tissues, have been described, PP1α, PP1β and PP1γ1, (Shima et al., 1993). However, their expression levels vary in different brain regions where it appears they are

localized to specific subcellular compartments (da Cruz e Silva et al., 1995a; Rebelo et al., 2015). A recently described PIP that is of specific neuronal relevance, is FE65. The latter which binds APP was shown to also bind PP1 (Rebelo et al., 2013), forming the complex APP:FE65:PP1, thus bringing the phosphatase to the proximity of APP and modulating dephosphorylation of the latter. In essence, FE65 is an important bridging protein between APP and PP1 and even more so, when one considers that a brain specific FE65 has also been described (Domingues et al., 2011). Of all the above-mentioned phosphatases PP1 is an important phosphatase in AD related pathology, further strengthened by the finding that Aβ can inhibit PP1 (Vintém et al., 2009) and PP1 can bind both APP and TAU, as discussed below.

THE AMYLOID CASCADE HYPOTHESIS

As already discussed, SPs are extracellular deposits found in AD brains, mainly composed of Aβ peptide aggregates, although Aβ is associated with AD it is also found in normal aging. SPs are likewise observed in individuals with Mild Cognitive Impairment (MCI) at a higher level than in non-AD older adults (Haroutunian et al., 2009) and it is a strong predictive factor of conversion to AD. Aβ deposition spreads from regions with early deposits to regions which receive their neuronal input. A strong correlation exists between the degrees of Aβ deposition and clinical symptoms. Indeed, several studies support the ***amyloid cascade hypothesis*** which defends that Aβ accumulation in the central nervous system is the first event that initiates the pathogenic cascade culminating in neurodegeneration and neuronal death typical of AD (Gandy, 2010). Clearly, in this hypothesis (Figure I-3), APP and PS mutations contribute to higher Aβ production favouring disease onset (Goate et al., 1991). Longer forms of this peptide increase its aggregatory properties and seeding is an important aspect for peptide aggregation (Fritschi et al., 2014). Aβ accumulation involves oligomerization, aggregation processes and amyloid deposition; these events are related with the above mentioned histopathological (SPs) and clinical manifestations of the disease (Jakob-Roetne and Jacobsen, 2009). In the amyloid cascade hypothesis, the following three aspects are significant: Aβ deposition is central to the disease process; Aβ deposition is an early event; mutations in APP and other players involved in the cascade contribute to FAD and SAD.

According to this hypothesis, genetics, age and environment factors, can all lead to an imbalance in Aβ production and clearance. Amongst these, the greatest genetic risk factor is APOE4, which appears to contribute to late onset cases (Corder et al., 1993). Also central to the hypothesis are the secretases, particularly γ-secretase, which regulates levels of Aβ production. It is not less

important that the physiological responses of these molecular players can be regulated by their phosphorylation state. For instance, phosphorylation of γ-secretase can modulate its activity (Kim et al., 2006; Kuo et al., 2008) and APP phosphorylation can regulate the levels of Aβ production (Rebelo et al., 2007a). However, the amyloid cascade hypothesis cannot explain all the histopathological hallmarks associated with AD, like for instance NFTs, although some studies suggest that Aβ itself can render in NFT formation (Oddo et al., 2003; Takashima et al., 1998). Additionally, if one considers that, as explained above, Aβ can inhibit PP1 and or PP2A (Vintém et al., 2009), this would subsequently result in TAU hyperphosphorylation (dashed black arrow, Figure I-3) and thus the AD related hallmarks can all be explained by the amyloid cascade hypothesis (Figure I-3). Furthermore, Aβ can activate mitogen-activated protein kinase (MAPK) and (GSK-3β) that can, in turn, phosphorylate TAU (Zheng et al., 2002). This is consistent with the dual pathway hypothesis as described below. In fact. Aβ can influence many other molecular events, such as those related to cytoskeletal organization (Henriques et al., 2014, 2015), and this can also potentially contribute to AD.

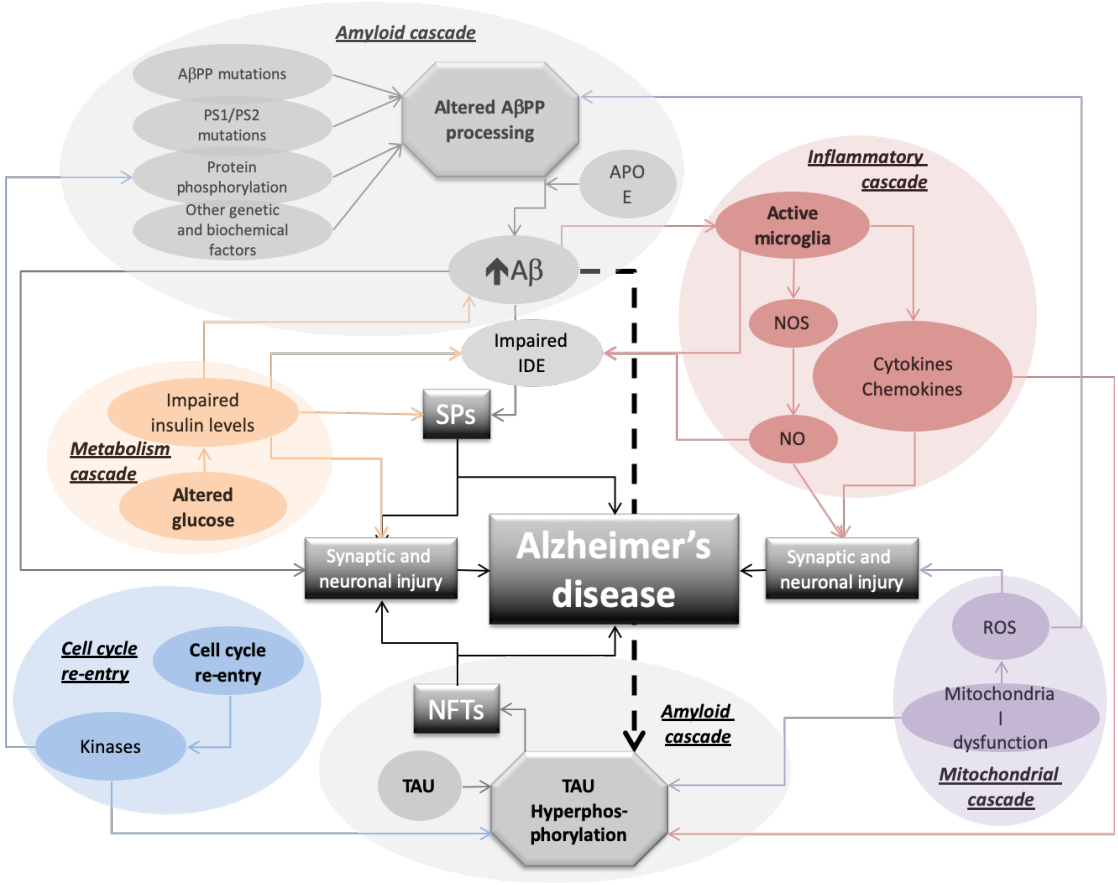

Figure I-3. Diagrammatic representation of AD pathology related hypotheses. Overview of the most documented cascade hypotheses for the onset of AD pathology. PS1- Presenilin 1; PS2- Presenilin 2; NOS – nitric oxide synthase expression; NO – nitric oxide; ROS – reactive oxygen species; IDE - insulin degrading enzyme; SPs- senile plaques; NFTs – neurofibrillary tangles. Dashed black line denotes that Aβ can influence TAU hyperphosphorylation.

Associated with Aβ deposition a variant of the amyloid cascade hypothesis has evolved (Walsh and Selkoe, 2007). The hypothesis defends that the formation oAβ (Aβ oligomers) is neurotoxic and can cause synaptic damage. The latter is a third AD hallmark which precedes SPs and NFTs deposits. This is attractive given that amyloid plaque deposition does not necessarily correlate with AD onset or neuronal loss (Delacourte et al., 1999; Gomez-Isla et al., 1996). This hypothesis places oAβ as a neurotoxic agent with the capacity to mediate toxic effects, and whereas SPs are neurotoxically inert oAβ is not. It is noteworthy that oAβ can affect several important neuronal receptors such as the insulin receptor and nicotinic receptors. For example, it can induce the reduction or abolition of long-term potentiation, and when injected into rodent brains it can induce impaired cognitive function (Ferreira and Klein, 2011; Haass and Selkoe, 2007; Hayden and Teplow, 2013; Hefti et al., 2013; Koffie et al., 2011; Mucke and Selkoe, 2012; Viola and Klein, 2015). Furthermore, the oAβ species may have a role in the seeding of amyloid plaques (reviewed in Walker and Jucker, 2015).

ALTERNATIVE AD HYPOTHESIS

Several other hypotheses have been proposed to explain AD (reviewed in Karran and De Strooper, 2016). Among them the ***dual pathway hypothesis*** (Small and Duff, 2008) that attempts to refine the amyloid cascade hypothesis, particularly with respect to SAD. The dual pathway hypothesis proposes that upstream factors may drive both Aβ and TAU mediated pathologies. With this in mind, downstream treatments targeting both Aβ and TAU pathology will ultimately be of therapeutic benefit. In this hypothesis Aβ and TAU deposits increase, although the authors of the model defend that a decrease in Aβ clearance could be operating. It is worth reinforcing, as stated above, that protein phosphorylation appears to be a key event in SPs and NFTs formation and thus of relevance to this model.

Another hypothesis is the ***mitochondrial cascade hypothesis***. The latter proposes that age-related mitochondrial dysfunction will lead to AD (Swerdlow and Khan, 2004; Swerdlow et al., 2014; Zhu et al., 2007a). In fact, several studies have confirmed mitochondrial damage in AD patients' brains (Cardoso et al., 2016; Lin and Beal, 2006). Consistently AD cybrid cells have increased Aβ production (Khan et al., 2000) and reactive oxygen species (ROS) (Cardoso et al., 2004). These AD cybrids include platelet mitochondria from an AD subject in a cell line (p0) blocked for mitochondrial DNA replication (King and Attardi, 1989). It appears that mitochondria trigger the abnormal onset of neuronal degeneration and cell death associated with AD (Moreira et al., 2006). A range of anomalies have been detected in AD patients, including mitochondrial dysfunction, increased

oxidative stress, and apoptotic neurons. Besides providing the cell with ATP, mitochondria play a significant role in regulating cell death and thus can contribute to the above-mentioned pathological AD hallmarks. Mitochondria perform electron transport via the electron transport chain (ETC) complex (I, II, III, and IV). This enables the harnessing of energy from mobilized free electrons and drives proton translocation. An additional complex (V) permits protons to re-access the matrix, coupling the energy from this proton flux to ADP phosphorylation. Thus, protein phosphorylation is also relevant in mitochondrial function (Mondragón-Rodríguez et al., 2013). Furthermore, mitochondrial function was shown to determine TAU phosphorylation. In fact, TAU phosphorylation is promoted in AD fibroblasts exposed to an ETC uncoupler (Blass et al., 1990). To summarize, the mitochondrial cascade hypothesis proposes that a mitochondrial deficiency in AD brains underpins an increase in Aβ production and can even promote TAU phosphorylation. However, despite genome wide association studies, genes encoding mitochondrial proteins have not yet been found (Lambert et al., 2009), and in fact this hypothesis cannot explain all the panoply of AD pathology. Furthermore, placing mitochondria at the apex of AD pathology remains controversial.

The ***metabolism cascade hypothesis*** (Hoyer, 1991; Hoyer et al., 1988) proposes that the underlying cause of AD is cerebral glucose hypometabolism. The authors developed a rat model in which injecting streptozotocin intracerebroventricularly (Lannert and Hoyer, 1998) associated with decreased glucose/energy brain metabolism and learning and memory deficits. Subsequent work showed that insulin signaling in the brain is significantly impaired in AD (Steen et al., 2005), leading to the term that AD is a 'Type 3 Diabetes'. Both insulin and IGF-1 (insulin/insulin-like growth factor I) stimulate Aβ release from neurons, and IGF-I promotes brain amyloid clearance. In addition, insulin and IGF-I levels are altered in AD and cell sensitivity towards insulin and possibly IGF appears to be reduced in these individuals. Insulin is likely to exert two distinct effects on brain Aβ. It stimulates neuronal Aβ release and at the same time contributes to extraneuronal Aβ accumulation by competing for insulin degrading enzyme (IDE). The latter enzyme can also regulate Aβ extracellular levels. Therefore, the net action of insulin is to increase brain Aβ. It appears that preserving synaptic connectivity requires insulin signaling and the latter may also play a role in neuronal stem cell activation and neuronal 'resilience'. The oAβ can bind to and antagonize components of the insulin signaling pathway resulting in increased activity of GSK-3β, a known TAU kinase (Morgen and Frölich, 2015). This is an attractive hypothesis; involving complex phosphorylation mediated responses, cognitive dysfunction and insulin resistance, and is presently the focus of much research (Stoeckel et al., 2016).

Microglia, astrocytes and even neurons appear to be involved in the inflammatory processes associated with AD, giving rise to the ***inflammatory cascade hypothesis***. Aβ was shown to activate microglia leading to increased cell surface expression of the major histocompatibility complex II (MHC II) and increased secretion of the pro-inflammatory cytokines; interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor α (TNF α), the chemokines- interleukin-8 (IL-8), macrophage inflammatory protein-1 α (MIP-1 α), and monocyte chemo-attractant protein-1 (Meraz-Ríos et al., 2013). Many other cytokines can be released in response to Aβ.

Additionally, microglia can participate in Aβ degradation by releasing IDE. At sites of Aβ deposition astrocytes will cluster and secrete a range of factors like interleukins, prostaglandins, coagulation factors, and protease inhibitors. Furthermore, Aβ induced a phagocytic response in microglia and nitric oxide synthase (NOS) expression, leading to increased nitric oxide (NO) and neuronal damage. In turn, NO can inhibit IDE activity (Kummer et al., 2012). The nonfibrillar $A\beta_{1-42}$ form is the predominant form found in diffuse plaques. Astrocytes in contact with diffuse plaques were shown to accumulate $A\beta_{1-42}$, but also cell debris, from degenerated synapses and dendrites. Microglia activation in AD brains appears to be involved not only in Aβ degradation ("good phagocytic phenotype"), but also in the production and release of ROS and pro-inflammatory cytokines and chronic microgliosis ("bad phagocytic phenotype") (Guillot-Sestier and Town, 2013). In turn inflammatory mediators can also affect Aβ deposition and even TAU phosphorylation levels (Domingues et al, submitted). These alterations appear to contribute to neuronal dysfunction and death and therefore to disease progression in a vicious cycle.

Neurons themselves can express high levels of classical pathway complement and pro-inflammatory products that trigger inflammatory processes. In essence, the complement system, cytokines, chemokines, and acute phase proteins appear to contribute to the inflammatory response in AD. Similar to other hypothesis mentioned above, protein phosphorylation can be an important factor. For instance, in FAD and SAD, GSK-3 over-activity accounts for memory impairment, TAU hyperphosphorylation, increased Aβ production, and inflammatory responses. Whether neuroinflammation is a primary cause or secondary effect in AD remains to be elucidated (Tuppo, 2005).

The observation that AD brains have a disorganized and reduced capillary and vascular network (De La Torre, 2004; de la Torre and Mussivand, 1993; Marchesi, 2011) lead to the ***vascular hypothesis***. The vascular hypothesis proposes that AD develops when advancing age and the presence of vascular risk factors converge. Cerebral microvascular pathology and cerebral hypoperfusion can potentially trigger cognitive and degenerative changes in AD. It is noteworthy

that AD risk factors include hypertension and diabetes, both exhibiting significant vascular morbidities (Rius-Pérez et al., 2015). Vascular damage and Aβ clearance do colocalize but more work is needed to establish if this is a chance or a neuropathological link, and for one to determine if amyloid plaques lead to vascular damage, or if vascular damage results in plaque formation. Additionally, according to this hypothesis TAU hyperphosphorylation and NFTs development may be triggered either by secondary consequences of cerebrovascular damage (eg. hypoperfusion/hypoxia insult), by Aβ mediated neurotoxicity or a concomitant action of both events (Sagare et al., 2012).

The ***cell cycle re-entry hypothesis*** is a general hypothesis whereby an age-related increase in neuronal DNA damage underlies neurodegenerative diseases. Given that neurons are post-mitotic cells they need to sustain their genomic integrity for life. Of note mitogen kinases, which play a key role in cell cycle control, have increased expression in the AD brain (Arendt et al., 1995). In fact, APP appears to have a role in activating neuronal cell cycle proteins and a failure in the regulation of this pathway occurs in neurons in AD brains. Also supportive of this hypothesis is the interaction of APP with the adaptor protein BP1 (amyloid precursor protein APP binding protein 1/APP-BP1), a cell cycle protein that regulates mitotic transition from S- to M-phase. Phosphorylation of APP at T668 is required for protein interaction. The complex APP: APP-BP1 activates a pathway leading to the conjugation of NEDD8 (Neural Precursor Cell Expressed, Developmentally Down-Regulated 8), which is a ubiquitin-like protein playing an important role in cell cycle control and embryogenesis. NEDD8 mediated neddylation of cullins enhances ubiquitin ligase activity, promoting polyubiquitination and proteasomal degradation of cyclins and other regulatory proteins. The neddylation pathway promotes APP-mediated cell cycle entry and apoptosis which appears to be relevant in FAD (Neve and McPhie, 2006). Of note overexpression of APP-BP1 pushes neurons into the S-phase causing DNA replication and expression of the cell cycle markers CDC2 and cyclin B1 (Bonda et al., 2011; Yang et al., 2001; Zhu et al., 2017). It has also been reported that phosphorylation of APP at T668, known to occur during the G2/M phase, is required for the interaction between APP and APP-BP1 (Joo et al., 2010). It would appear that cell cycle re-entry is pivotal in AD, given that these markers preclude the appearance of NFTs and SPs (Zhu et al., 2007b). In other words cell cycle re-entry may be a causal factor in AD (McShea et al., 1999).

Another interesting finding is that specific antibodies raised to PHF of TAU purified from AD brain, cross-reacted with epitopes in dividing cells (Vincent et al., 1996). Other lines of research showed that infecting differentiated neurons with oncogenes c-myc and ras, promoted them to initiate division and this resulted in DNA duplication and increases in anti-phospho-TAU immunoreactivity

(McShea et al., 2007). Of note, the concept that TAU phosphorylation promotes tangle formation in neurons attempting to re-enter the cell replication cycle has been well documented. Also consistent with the cell cycle re-entry hypothesis, is the finding that in postmitotic neurons, DNA repair defects can result in neurological abnormalities including neurodegeneration (McKinnon, 2013).

As briefly discussed above, there is significant evidence supporting the different AD hypotheses. However, a physiologically relevant process in all cases appears to be protein phosphorylation. Thus, by addressing protein phosphorylation dependent processes one may identify potential cues of diagnostic and therapeutic potential.

TAU INTERACTIONS AND PHOSPHORYLATION MEDIATED EVENTS

Signaling pathways involve a dense network of protein:protein interactions, and the reaction rates depend on protein concentrations and associations/dissociations, among other factors. Protein phosphorylation can modulate the nature and the strength of protein:protein interactions, consequently regulating protein binding and the signaling pathways. Protein phosphorylation at or near a binding site, may directly affect the binding energy of the complex. On the other hand, phosphorylation at a site outside a binding domain may cause a long-range conformational change. This allosteric mechanism can affect the binding of interacting proteins, as observed for glycogen phosphorylase (Jenal and Galperin, 2009; Lin et al., 1997). Recent advances in proteomics and the implementation of a systems biology approach have unraveled interactomes of many proteins. It is clear that protein:protein interactions are central to cellular functions and anomalies there for can contribute to pathological conditions, among them AD. Thus, analyzing the interactome of proteins relevant to this pathology will provide fundamental information regarding the phosphorylation dependent complexes formed and subsequently how these might contribute to the disease process.

The TAU protein is a dipole, possessing two domains of opposite charge (Sergeant et al., 2008) and these are important in determining internal folding and aggregation (Mandelkow and Mandelkow, 2011), as well as the protein's interaction with microtubules and other binding partners (Figure I-1 and I-4). The C-terminus binds to microtubules (Steiner et al., 1990) and the region of 150-240 residues is the 'proline-rich domain' that can serve as a target of proline-directed kinases as well as binding sites for proteins with SH3 domains (Mandelkow and Mandelkow, 2011). The N terminus does not bind microtubules, it is the 'projection domain' (Figure I-1) (Hirokawa et

al., 1988) and it interacts with other cytoskeletal elements, mitochondria and the neuronal plasma membrane (Al-Bassam et al., 2002; Brandt et al., 1995; Jung et al., 1993).

The TAU interactome revealed 35 interacting proteins (Figure I-4), which were retrieved from the public databases IntAct and MINT as well as from specific reports, as indicated below. The output from the databases refers the gene, but for consistency in the text, the interactions are discussed at the protein level. The TAU protein:protein interaction network has (Figure I-4) 12 nodes (red) with kinase activity, two that bind phosphoproteins (blue circumference), one with phosphatase activity (green node) and four nodes (green circumference) that interact with protein phosphatases. Analysis of the TAU interacting proteins (Figure I-4) clearly reveals that TAU binds to APP directly. These highlighted nodes represent proteins that bear some of the gene ontologies most overexpressed in the TAU interactome and are also relevant to protein phosphorylation events. The protein:protein interaction network reflects interactions for the human proteins, however two examples from the mouse Tau interactome were included. The PP2R2A (serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform), because the mouse Tau bound to the human PP2R2A, and PP2 is the most important TAU phosphatase, and the mouse interaction Tau:Ppp1ca was also included for the same reason, many TAU residues are dephosphorylated by PP1.

Among the 12 TAU interactors having kinase activity (Figure I-4 red nodes), the expected kinases like GSK-3, CDK5 and MARKs are present but among them is also FYN. TAU interacts with FYN (Reynolds et al., 2008), which is regulated by TAU phosphorylation (Usardi et al., 2011). It is plausible that TAU may localize FYN to the postsynaptic compartment, leading it to dephosphorylate the NMDA receptor causing increases in Ca^{2+}, and culminating in pathological neurotoxicity (Pritchard et al., 2011). Thus, one can draw direct parallels between the TAU interactome and AD associated anomalies.

Given the relevance of molecular chaperons in regulating TAU post-translational processing, it is not surprising that HSP90 (Tortosa et al., 2009), HSP70 and HSC70 (Jinwal et al., 2010) are found in the TAU interactome (Figure I-4). HSP90 regulates GSK-3β promoting TAU phosphorylation, potentially favoring hyperphosphorylation of the latter. Furthermore, HSP90 can, in combination with FKBP52, produce oligomeric TAU preventing clearance and increasing its aggregation potential. FKBP52 is another TAU binding protein (Chambraud et al., 2010). HSP70 has a dual role with TAU, stabilizing it to microtubules and promoting its degradation in association with CHIP (chaperone-associated ubiquitin ligase). HSC70 can likewise regulate TAU's microtubule association (Jinwal et al., 2010). It appears that HSC70 is important in maintaining TAU conformation as a

protective mechanism preventing the TAU self-assembly process associated with NFTs and a major histopathological hallmark in AD.

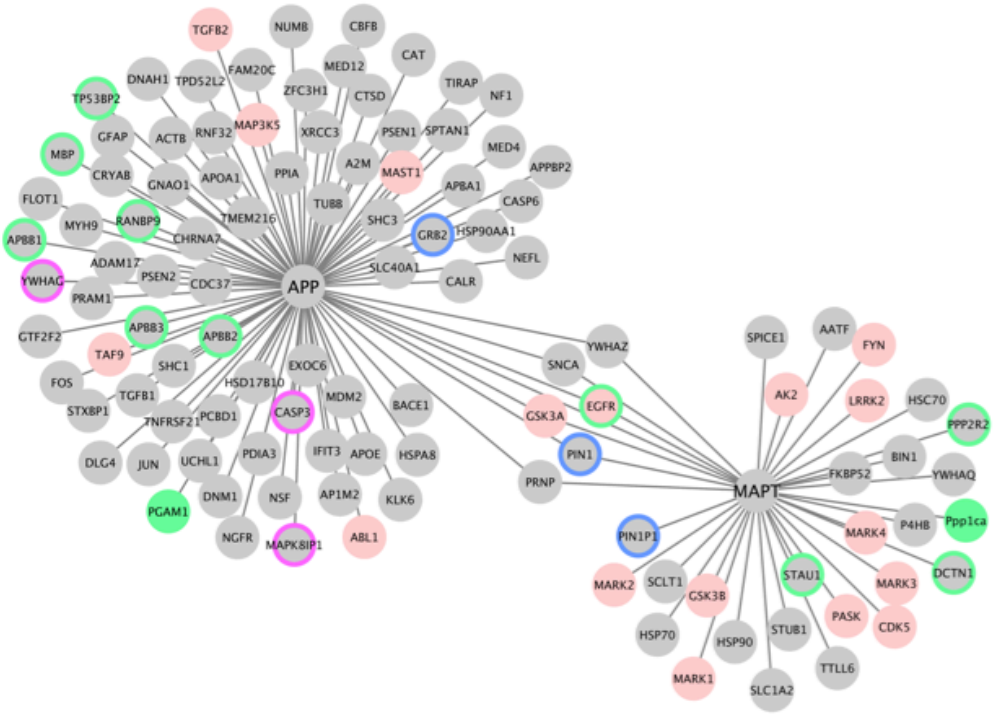

Figure I-4. Merged protein:protein interaction network for TAU and APP. Human interactors for the APP (gene APP) and the TAU (gene MAPT) were retrieved from the public databases (MINT and IntAct), further interactions were included as follows: Domingues et al.(Domingues et al., 2014) for RANBP9, Sumioka et al. (Sumioka et al., 2005) for YWHAG, Zheng et al. (Zheng et al., 1998) for APPBP2 and for the TAU interactors: PIN1 (Lu et al., 1999), HSP90 (Tortosa et al., 2009), HSP70 and HSC70 (Jinwal et al., 2010), FKBP52 (Chambraud et al., 2010) and SNCA (Jensen et al., 1999). Two nodes from the mouse interactome were also included; in one case the mouse Tau bound to the human PPP2R2 and in the other to the mouse Ppp1ca. The network was developed using Cytoscape 3.4 and the following gene ontologies for molecular function are indicated: GO 16301 kinase activity (17 red nodes); GO 16791 phosphatase activity (2 green node); GO 51219 phosphoprotein binding (3 nodes with a blue circumference); GO 4860 protein kinase inhibitor activity (3 nodes with a pink circumference); GO 19903 protein phosphatase binding (10 nodes with a green circumference). Gene ontologies were retrieved using the BINGO 'plug in', and the PP1 binding proteins from (Esteves et al., 2012, 2013).

APP INTERACTIONS AND PHOSPHORYLATION MEDIATED EVENTS

APP can integrate into the plasma membrane (Figure I-1) and interact both intracellularly and extracellularly to regulate various signal transduction mechanisms. It appears that the N terminal domain binds to phosphoinositide-rich domains on the surface of hippocampal neurons (Dawkins et al., 2014). The RERMS domain is also important from an interaction perspective and is briefly discussed. Many AICD binding proteins have been identified, and some of these may present novel potential targets for therapeutic intervention (Annaert and De Strooper, 2002; Russo et al., 1998).

The N-terminus of APP encompasses a cysteine-rich globular domain (E1), and an acidic α helix-rich domain (E2) (Reinhard et al., 2005). Two distinct regions have been identified within the E1 domain, the growth factor-like domain (GFLD) and the copper/metal binding domain (CuBD)(Rossjohn et al., 1999; Small et al., 1994). The GFLD corresponds to amino acid residues 23–128, this was crystallized several years ago (Rossjohn et al., 1999) with the copper/metal binding domain, the structure contains nine β-strands and one α-helix and is well conserved across the APP family. The disulfide bridge between the residues C98 and C105 stabilizes a β-hairpin loop, and is critical for neurite outgrowth (Small et al., 1994) and MAP kinase activation (Greenberg et al., 1995). Several basic residues within this domain give rise to the heparin binding domain (HBD) which is a hydrophobic pocket that could potentiate a protein-binding site or a dimerization site (Rossjohn et al., 1999). Although the precise function of APP is still unclear it may act as a receptor, as a growth factor (Rossjohn et al., 1999), or may bind to an extracellular matrix component (Small et al., 1994). The CuBD is adjacent to the HBD and can bind several metal ions (Bush et al., 1993). On its C-terminal the E1 domain has an acidic region of unknown function, which is rich in glutamic acid and aspartic acid residues and contains a stretch of seven threonine residues (Kang et al., 1987).

This acidic region connects the E1 to the E2 domain. The E2 domain, also called the central APP domain (CAPPD), can readily dimerize (Lee et al., 2011) and may be involved in APP self-association. This domain consists of six α-helices (Wang and Ha, 2004) and provides potential binding sites for other binding partners. The E2 domain contains the RERMS sequence, which may account for the growth-promoting properties of APP (Ninomiya et al., 1993). The E2 domain also has a highly conserved heparan sulfate proteoglycan (HSPG)-binding site (Clarris et al., 1997; Reinhard et al., 2005) and a number of putative metal-binding sites (Dahms et al., 2012; Multhaup, 1994). Metal binding is likely to hold the E2 domain in a rigid conformation. The longer isoforms of APP (APP751 and APP770) contain a Kunitz-type protease inhibitor (KPI) domain and an Ox-2 antigen domain, only in the latter. In general terms it appears that the APP N-terminus may have an important role in cell adhesion and extracellular interactions, but whether any of these events can be mediated by protein phosphorylation remains to be seen. From a phosphorylation mediated functional perspective, the AICD is the most interesting region (Kerr and Small, 2005; Schettini et al., 2010). It contains at least three functionally important motifs (Figure I-1) enabling APP interaction with several binding-partners, 86 of which are represented in Figure I-4. The interactome was retrieved from the public databases IntAct and MINT, additional interactors were included as individually identified throughout the text. Given the pivotal role of protein phosphorylation in the different biological processes played by APP, it is perhaps not surprising that its interactome with 86 nodes

includes seven nodes (red) with kinase activity, three with protein kinase inhibitor activity (nodes with pink circumference), two which bind phosphoproteins (blue circumference), one with phosphatase activity (green node) and seven nodes (green circumference) that interact with protein phosphatases.

Such protein interactions regulate APP/AICD function, localization, processing and Aβ production (Domingues et al., 2014; Henriques et al., 2009). The highly conserved 682-YENPTY-687 APP motif (APP 695 numbering) is a sorting motif involved in clathrin-mediated endocytosis and found in many tyrosine receptor kinases, non-receptor tyrosine kinases, low-density lipoprotein-receptor related family proteins and integrins (Bonifacino and Traub, 2003; Lemmon and Schlessinger, 2010). The YENPTY domain is recognized by proteins containing phosphotyrosine interaction domains (PID), namely, the FE65 (Figure I-4 APBB1) family, c-Jun N-terminal kinase interacting protein, X11 family (Figure I-4 APBA1), SHC family (Figure. I-4 SHC1 and SHC3). To the 667-VTPEER-672 APP motif, binds the protein 14-3-3γ (YWHAG) that is highly expressed in the brain, skeletal muscle and heart (Sumioka et al., 2005). Another important interaction is that with PAT1 (protein interacting with APP tail 1, also denoted APPBP2), a microtubule interacting protein, which binds to the 653-YTSI-656 motif (Zheng et al., 1998). The three above mentioned domains all contain T, S and/or Y residues which can be phosphorylated, thereby modulating protein:protein interactions as discussed below.

AICD has an unstable conformation in solution and the interaction with a binding-partner will stabilize its structure. This property has been termed binding promiscuity. The stability of the AICD-ligand complex can be influenced by AICD phosphorylation and this can influence the binding of interacting proteins at the above-mentioned domains, or vice versa. For instance, T668 phosphorylation is essential for FE65 (APBB1) binding to the YENPTY APP domain, and probably involves GSK-3 mediated phosphorylation (Chang et al., 2006). Subsequent work unravelled the existence of a trimeric complex (APP:FE65:PP1). The authors demonstrated that FE65 is the bridging protein in the complex formed, and therefore a PP1 interacting protein (Figure I-4 APBB1). This interaction correlated with APP T668 phosphorylation state, consistent with the role whereby PP1 was recruited and could dephosphorylate T668 (Rebelo et al., 2013). The same group also showed that the phosphorylation state of Y687 could influence the binding of FE65. The AICD also interacts with RANBP9 via the NPXY internalization motif (Domingues et al., 2014). Subsequent work, in cell culture, showed a RANBP9-TIP60 interaction that is important for nuclear targeting.

The JNK pathway is another signaling cascade highly relevant to AD, which typically targets c-Jun, ATF2 and Elk-1, JNK3 (SAPK1b/MAPK10). JIP-1b (Figure I-4 MAPK8IP1) can recruit JNK to APP,

and JNK3 causes phosphorylation of the latter on T668 (Inomata et al., 2003; Standen et al., 2001). Furthermore JIP-1b binds the TPR domain of kinesin light chain, thus APP can associate with kinesin-I via JIP-1b. Hence it would appear that JIP-1b is a linker protein between kinesin-I motor protein and the cargo receptor APP (Sumioka et al., 2005).

COMMON NODES AND CROSSTALK BETWEEN TAU AND APP

Considerable scientific effort has focussed on identifying molecular factors common to the development of SPs and NFTs (Figure I-3). It is particularly noteworthy that upon merging the protein:protein interaction networks of TAU and APP a sub-set of nodes common to both interactomes is evident. As for the individual interactomes, in the merged interactome, phosphorylation related gene ontologies are well represented. Of these 17 nodes (red) have kinase activity, three with protein kinase inhibitor activity (nodes with pink circumference), three that bind phosphoproteins (blue circumference), two with phosphatase activity (green node) and ten nodes (green circumference) that interact with protein phosphatases.

Six nodes were identified (Figure. I-4), exhibiting a direct interaction between TAU and APP. In fact, both proteins can bind to proteins involved in other neuropathological conditions, for instance SNCA (α-Synuclein) and PRNP (prion protein) (Figure I-4). The protein SNCA, found in Lewy bodies, is typically associated with Parkinson's disease; however, it may also be involved in AD (Korff et al., 2013). SNCA interacts with synaptic vesicles at presynaptic terminals, functionally resembling TAU protein, to which it binds, priming it for kinases. In particular, SNCA forms a complex with TAU and GSK-3β. Additionally SNCA interacts directly with TAU and stimulates its phosphorylation by PKA (Qureshi and Paudel, 2011). GSK-3α is another shared node, and as discussed throughout this review, this is an important kinase able to phosphorylate TAU and APP. Likewise YWHAZ (14-3-3 protein zeta/delta) binds to both TAU and APP. This is an adaptor protein involved in the regulation of a wide range of signaling pathways, with many interactors and typically recognizes a phosphoserine or phosphothreonine motif. YWHAZ can simultaneously bind AICD and FE65 facilitating FE65-dependent gene transactivation by enhancing the association of AICD with FE65 (Sumioka et al., 2005). In TAU, the binding with YWHAZ promotes the interaction of the former with a number of kinases (reviewed in Sluchanko and Gusev, 2011). Thus, YWHAZ acts as a facilitator.

PIN1 (peptidyl-prolyl cis-trans isomerase), is another node in the 'common sub-set'. Lu et al (Lu et al., 1999) reported TAU:PIN1 binding but in the database searches a PIN1P1 (putative PIN1-like protein) has been identified, thus both nodes are included in Figure I-4. Further research is needed

to clarify if these two nodes correspond to the same protein. PIN1 binds to hyperphosphorylated TAU (Hamdane et al., 2002; Lu et al., 1999), promoting its dephosphorylation at CDK5 phosphorylation sites, in particular S202, T205, S235, and S404 (Kimura et al., 2013). It is interesting to note that PIN1 can modulate PP2A activity, which is a major TAU phosphatase. PIN1 appears to regulate, not only the phosphorylation, but also the conformation of its substrates. PIN1 can catalyze prolyl isomerization of specific pS/T-P motifs both in CDC25C and TAU facilitating PP2A mediated dephosphorylation (Zhou et al., 2000). The relevance with respect to APP stems from the finding that neurons exposed to Aβ, exhibit PIN1 activation, with a consequential dephosphorylation of TAU on T231 (Bulbarelli et al., 2009). PIN1 is likewise responsible for the transient regulation of S199, S396, S400 and S404 TAU phosphorylation in response to Aβ. Furthermore, PIN1 can regulate Aβ production by binding to APP when the latter is phosphorylated on T668 (Pastorino et al., 2006; Tamayev et al., 2009). PIN1 promotes APP turnover by inhibiting GSK-3 activity (Ma et al., 2012). Likewise, relevant to the cell cycle re-entry hypothesis was the finding that PIN1 can upregulate cyclin D1 expression. This can, in turn, facilitate the transition of neuronal quiescent cells to the G1 phase (Hamdane et al., 2002). Given PIN1's essential role in the G0/G1 transition it may provide a good target for novel therapeutic strategies.

Another common node identified was EGFR (Epidermal growth factor receptor; also known as HER-1 or ErbB-1), although intrinsically associated with cancer, it has similarly been associated with neurometabolic conditions, like AD and diabetes. In neurodegeneration, altered EGFR expression levels have been observed. EGFR appears to mediate the effects of EGF/TGF-α, associated with neuronal differentiation (Anchan et al., 1991), survival (Alexi and Hefti, 1993; Morrison et al., 1987, 1988) and glial proliferation (Simpson et al., 1982) and appears to mediate Aβ related toxicity (Wang et al., 2012). PS1 regulates the EGFR pathway (Repetto et al., 2007). Mutations in the PS1 and PS2 genes contribute significantly to early onset FAD (Sherrington et al., 1995). In fact, EGFR levels increased in fibroblasts that were deficient in both PS1 and PS2, but transfecting with PS1 reversed this effect (Repetto et al., 2007). The loss of PS1 can stimulate the activation of EGFR and β-catenin pathways, contributing to neurodegeneration and aberrant cell cycle re-entry (Repetto et al., 2007).

As a consequence of EGFR signaling activation, the transcription of several genes and regulators can be altered. In particular, a panel of miRNAs essential for metastatic phenotypes and neurodegeneration appears to be involved. The miRNAs have been termed "guardians" of the genome, as they help maintain cellular genomic stability. Reports have identified two miRNAs, miR-221 and miR-222, as downstream targets of the EGFR-RAS-RAF-MEK pathway (Shah and Calin, 2011; Stinson et al., 2011). MiR-221/222 can modulate cell cycle progression by repressing cell cycle

inhibitor proteins p27/Kip1 and p57, which facilitates cell proliferation and self-renewal (Miller et al., 2008). The ability to modulate the cell cycle may explain the role of miR-221/222 in neurodegenerative disorders and in the apoptotic death of damaged neurons. Cell cycle control is amongst the hypothesis for AD (Figure. I-3) and preventing neurons from entering high-risk states may provide attractive therapeutic strategies. Given the central role of these miRNAs in neurodegenerative disorders, they provide potential novel molecular therapies to be explored in the future.

CLOSING REMARKS

The criteria for diagnosing dementia require the presence of multiple cognitive deficits in addition to memory impairment. The diagnostic approach for AD begins with collecting the clinical history, carrying out a physical examination and cognitive testing. At this phase there is a clinical recognition of a progressive memory decline, a decrease in the patient's ability to perform daily living activities, personality changes and behavioural problems may also become evident. During the diagnostic process, interviewing friends and family may be helpful and an important tool to assist in the diagnosis. Neuroimaging techniques are evermore used as tools for AD diagnosis, namely MRI (magnetic resonance imaging), CT (computerized tomography) and more recently PET (positron emission tomography). In MRI, structural brain changes can be visualized due to substantial neuronal loss. MRI exams are routinely requested and are helpful to rule out other possible causes of dementia such as brain tumour and vascular lesions (Mosconi et al., 2007). PET assesses the Aβ deposition in brain using a radioactive compound, where the S. Pittsburgh compound is the present gold standard (Fripp et al., 2008). This compound binds specifically to Aβ and does not bind to neurofibrillary tangles or Lewy bodies at the concentrations achieved during PET scan (Klunk et al., 2004). Nonetheless, diagnostic confirmation is based on post-mortem observation of the specific pathological lesions like NFT, SP and synapse dysfunction and loss. The present focus is on identifying molecular abnormalities that contribute to the deposition of SPs and NFTs as early as possible in the aetiology of AD.

Neurochemical biomarkers are increasingly used in assisting with the complementary AD diagnostics. The 'gold-standard' monitors levels of Aβ peptides (in particular $A\beta_{1-42}$) and phosphorylated TAU in the cerebrospinal fluid (Bibl et al., 2012; Otto et al., 2008). It is evident that protein phosphorylation contributes significantly to the occurrence of both these biomarkers; TAU by direct phosphorylation and as described above APP phosphorylation can influence levels of Aβ

production. Novel biomarkers are presently the subject of intense study as well as identifying novel biomarker candidates in peripheral tissues. Blood based biomarkers are particularly sought, given that lumbar puncture is an additional burden for the patient. Here too, investigating protein phosphorylation events may provide very promising candidates. As described above, given the number of kinases, phosphatases and the various substrates involved, predicting an outcome as a result of any single phosphorylation/dephosphorylation event can prove to be an inefficient exercise and it follows that we move towards a system's biology approach. It is clear however that the phosphorylation state of a key set of proteins may provide fundamental cues to anomalous cellular processes and thus serve as highly specific diagnostic biomarkers. In closing, it is worth emphasizing, that by merging the interactomes for TAU and APP a sub-set of nodes that are presently being pursued for their therapeutic value became evident. In fact, these candidates are particularly important as they bridge/interact with the proteins fundamental to the two central histopathological hallmarks associated with AD.

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Conflict of interest

The authors have no conflict of interest to declare.

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AIMS

AD is a complex neurodegenerative disorder neuropathologically characterized by the presence of SPs and NFTs, synaptic loss and consequently neurodegeneration. Altered signal transduction is one of the key molecular aspects in AD pathology. Phosphorylation of proteins is recognized as a fundamental mechanism by which the regulation of key intracellular events is achieved. Several studies have reported abnormal protein kinase and protein phosphatase activities in AD brains as well as abnormal phosphorylation levels of APP and Tau itself.

The main aim of this thesis is to study protein phosphorylation events in AD relevant conditions. The results obtained will contribute not only to the understanding of the molecular mechanism underlying the disease but also to the identification of potential AD biomarker candidates that can assist in early disease diagnosis. Hence, the specific aims of the thesis were:

Aim1: Address the impact of protein phosphorylation inhibition, induced by okadaic acid, on the neuronal phosphoproteome (Chapter II);

Aim2: Study the role of Aβ itself on the neuronal phosphoproteome (Chapter III);

Aim3: Evaluate the Aβ mediated effects on APP and Tau phosphorylation (Chapter IV).

CHAPTER II

Okadaic Acid-Induced Alterations in the Neuronal Phosphoproteome

Given the central role that protein phosphorylation plays in dementia related diseases and, in particular AD, it was deemed essential to evaluate, using a system's biology approach, how okadaic acid (OA) affected protein phosphorylation levels at the neuronal proteome level. OA is a toxin commonly used to inhibit protein phosphatases and has been extensively used to study AD related events. At different concentrations it can inhibit specific protein phosphatases, thus it has been used as a model to address the role of protein phosphatases in a range of diseases. AD *per se*, can exhibit altered phosphorylation of specific proteins, such as APP itself and hyperphosphorylated Tau; both these proteins can in turn be dephosphorylated by protein phosphatases. Hence, exposure to OA followed by identification of phosphorylated proteins is an important model to address AD related protein phosphorylation events. The data obtained is presented in manuscript 2.

Manuscript 2

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Manuscript 2

Toward Neuroproteomics in Biological Psychiatry: A Systems Approach Unravels Okadaic Acid-Induced Alterations in the Neuronal Phosphoproteome

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ABSTRACT

Neuroproteomics is an evolving field of postgenomic medicine, highlighting the convergence of psychiatry/neurology and proteomics, yet compared with neurogenetics, it has received little attention. This study in rat primary neuronal cultures provides an example of a neuroproteomic approach relevant to the study of psychiatric disease pathophysiology, focusing on Alzheimer's disease (AD). In this context, okadaic acid (OA) is routinely used in experimental designs to investigate phosphorylation-mediated events. It is a potent protein phosphatase (PP) inhibitor, particularly of PP1 and PP2A. Typically, a single protein and its phosphorylation level are monitored upon OA exposure. Although useful, this can be misleading as protein phosphorylation-mediated events involve complex signalling cascades and an array of kinases, phosphatases and substrates. Bearing in mind the involvement of multiple pathways and cascade cross talk, this study employed a systems approach to analyse OA-induced molecular responses through PP inhibition. We showed that upon OA exposure, the recovery rate of 245 phosphoproteins significantly increased, while that of 75 significantly decreased. The prominent biological processes affected included anatomical structural development, transport, cell differentiation, and signal transduction. The associated phosphointeraction networks identified nodes representing OA-responsive phosphoproteins. Many of these are key players of signalling cascades relevant to a range of pathologies. In summary, the data presented results from a neuroproteomic preclinical study offering an array of phosphoproteins as potential targets for future diagnostic and therapeutic strategies in biological psychiatry. We note, however, the nonspecificity of targeting PPs themselves and emphasize the need for future neuroproteomic approaches toward systems psychiatry.

INTRODUCTION

Okadaic acid (OA) has been broadly used as a model to study protein phosphorylation and, in particular, the role of protein phosphatases (PPs) in many diseases, including neurodegenerative disorders. It is a potent neurotoxin capable of binding and selectively inhibiting not only major brain serine/threonine PPs, namely PP1 and PP2A (Fernández et al., 2002), but also PP4, PP5, and PP2B (Swingle et al., 2007). PP inhibition results in dramatic increases in protein phosphorylations and cellular alterations, making OA an invaluable pharmacological tool in current use (Cruz et al., 2013).

The association between PPs and neurodegenerative disorders has been well established. *In vitro* and *in vivo*, OA was shown to mimic protein hyperphosphorylation and to reproduce altered phosphorylation states similar to those observed under pathological conditions, such as Alzheimer's disease (AD). This toxin promotes Tau hyperphosphorylation (Kim et al., 1999; Sun et al., 2003; Wang et al., 2001), amyloid-beta peptide (Aβ) deposition, neuronal degeneration, synaptic loss/dysfunction, and memory impairments (Kamat et al., 2012, 2014a; Sun et al., 2003). Imbalanced Aβ production and its accumulation into senile plaques, as well as Tau hyperphosphorylation and neurofibrillary tangle (NFT) formation, are hallmarks of AD pathology. Increased Aβ production derives from cleavage of the Alzheimer's amyloid precursor protein (APP), which can in turn can be modulated by APP phosphorylation (Ando et al., 2001; Rebelo et al., 2007) or the Aβ peptide itself (Henriques et al., 2009, 2010).

Consistent with this, PP inhibition by OA, in particular PP1, was shown to enhance APP processing (da Cruz e Silva et al., 1995a; Henriques et al., 2007). The abovementioned Tau is a microtubule-binding protein and a fundamental phosphoprotein in the central nervous system. Tau hyperphosphorylation correlates with NFT formation and neuronal toxicity (Augustinack et al., 2002; Kim et al., 1999). Several PPs are involved in Tau dephosphorylation (Liu et al., 2005) and inhibition of its activity renders a loss of its biological function compromising microtubule dynamics.

OA can likewise affect the activity of several protein kinases (PKs), involved in Tau pathology, such as glycogen synthase kinase 3 beta (Gsk3β), cyclin-dependent kinase 5 (Cdk5), and mitogen-activated protein kinases (Mapks) (Bennecib et al., 2000; Lim et al., 2010; Pei et al., 2003; Zimmer et al., 2012), therefore contributing to NFTs, neuronal toxicity, and degeneration (Kamat et al., 2013, 2014a). It appears that OA can impact PK activity through inhibition of PP2A and promote downstream effects in signaling cascades. It was reported that this neurotoxin can lead to activation of extracellular signal-regulated kinase 1/2 (Erk1/2) and Erk kinases, dual-specificity mitogen-activated protein kinase kinase 1/2 (Mek1/2), by inhibiting dephosphorylation of these kinases by

PP2A, consequently contributing to Tau hyperphosphorylation (Pei et al., 2003).

Furthermore, PP inhibition by OA was shown to alter glutamate cerebrospinal fluid concentrations (Zimmer et al., 2012), supporting the interplay between OA and the glutamatergic system, which is impaired in AD. Moreover, cerebral administration of OA increased the expression of subunits of the glutamatergic N-methyl-d-aspartate receptor (NMDAR) (Kamat et al., 2014b). Recently, it was proposed that increased glutamate levels prompted by OA could render NMDAR hyperactivation and Cdk5 dysfunction with consequences to Tau hyperphosphorylation (Zimmer et al., 2016). In summary, it is perhaps not surprising that OA has been widely used to model AD.

However, although it is clear that OA triggers neurotoxicity by different pathways, the exact mechanisms linking various signalling pathways are not completely understood. Hence, this study aims to contribute to the identification of novel OA-responsive molecular targets and signaling pathways prompted by this phosphatase inhibitor. Unraveling these neuronal responses, from a systems perspective, can be of extreme importance to the understanding of AD-related signaling cascades, or even other neuropathological conditions, where abnormal phosphorylation and PP inhibition play a central role.

MATERIAL AND METHODS

Primary neuronal cultures

Cortical neurons were obtained from embryonic Wistar rats (Harlan Interfauna Ibérica) at gestation day 18, as previously described (Oliveira et al., 2015). In brief, upon dissection of cerebral cortex, cells were dissociated with trypsin (0.23 mg/mL)/deoxyribonuclease I (0.15 mg/mL) in Hanks balanced solution and plated onto poly-d-lysine-coated dishes (6 × 106 cells/100 mm) in B27-supplemented Neurobasal (NB) medium (Gibco). Neuronal cultures were maintained *in vitro* at 37°C and 5% CO_2 for 10 days before being subjected to the experimental procedures.

For animal experimentation, the European legislation (2010/63/EU) was observed and no specific ethics approval under European Union (EU) guidelines was required for this project. The Council Directive 86/609/EEC was followed, and during the procedure, all measures were taken to ameliorate animal suffering. Procedures were approved and supervised by the Institutional Animal Care and Use Committee: Comissão Responsável pela Experimentação e Bem-Estar Animal (CREBEA).

Okadaic acid treatment

Primary cortical neurons were treated with 0.5 µM of OA in a serum-free medium combination (NB-B27) for 3 h. OA is a PP inhibitor that selectively inhibits different serine/threonine PPs (da Cruz e Silva et al., 1995a; Oliveira et al., 2015; Swingle et al., 2007). Several classes of PPs have been described, including PP1, PP2A, PP2B (calcineurin), PP2C, PP4, PP5, PP6, and PP7. In neurons, PP1, PP2A, and PP2B are the most abundant, accounting for more than 90% of the neuronal phosphoprotein dephosphorylation. OA exhibits much greater activity toward PP2A than PP1 (IC50 PP1>PP2A). It can inhibit PP2B, but at a much higher concentration than PP1 and PP2A. At the concentration used, PP1 and PP2A are inhibited predominantly, although at the same concentration, one can also inhibit PP5 and PP4. After treatment, cell lysates were collected and processed for phosphoprotein enrichment as described below.

Phosphoprotein extraction

Phosphoprotein enrichment was carried out using the TALON® phosphate metal affinity chromatography (PMAC) Phosphoprotein Enrichment Kit (Clontech). PMAC columns used in the kit allowed for the selective binding of proteins that contain a phosphate group on any amino acid, including serine, threonine, or tyrosine. Primary cortical neurons were processed and phosphoproteins extracted as previously described (Henriques et al., 2016). Phosphorylated proteins were eluted using 4 mL of buffer (collected as sequential 1-mL fractions). Protein quantification using bicinchoninic acid assay (Pierce) revealed that the second fraction was the most enriched and the one subsequently lyophilized for mass spectrometry (MS) analysis (Figure II-1).

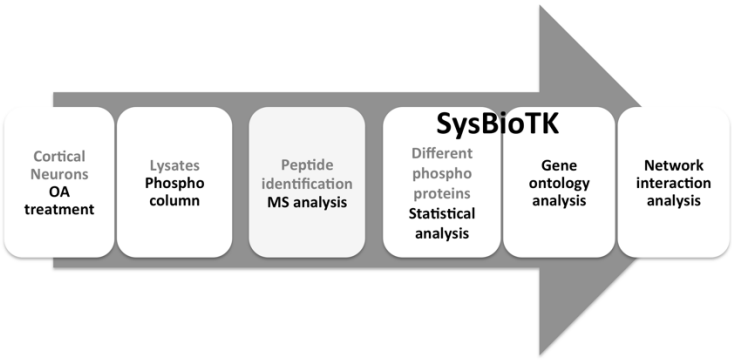

Figure II-1. Overview of workflow. The workflow describes the steps carried out for sample processing and analyses. Neuronal cultures were treated with OA, a PP inhibitor, and the phosphoproteins were recovered from cell lysates using phosphocolumns. The eluted peptides were analyzed by MS analysis. Statistical analysis was carried out to identify the significantly different phosphoproteome, which was subsequently analyzed using SysBioTK and other bioinformatic tools, including STRING and Cytoscape 3.3.0 for interactome analysis. MS, mass spectrometry; OA, okadaic acid; PP, protein phosphatase.

MS/MS analysis

Lyophilized samples dissolved in lithium dodecyl sulfate (LDS) buffer were incubated at 95°C for 10 min. Subsequently, samples were sonicated and loaded onto a one-dimensional polyacrylamide gel system, followed by Coomassie blue staining. Protein bands were excised and prepared for in-gel protein digestion as previously described (Henriques et al., 2016). Peptides were extracted from the gels using trifluoroacetic acid (TFA) 0.1% + acetonitrile/H_2O (ACN/H_2O, 50:50). The resulting supernatant was dried in a SpeedVac, resuspended in TFA 0.1%, and stored at −20°C. Nanoscale liquid chromatography coupled to tandem MS (Nano-LC-MS/MS) was performed on an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific).

Samples were loaded on a trap column (75 µm × 2 cm, particle size 3 µm, pore size 100Å; Dionex) with 0.1% TFA. After washing, the trap column was connected with an analytical C18 column (75 µm × 25 cm, particle size 2 µm, pore size 100Å; Dionex). Peptides were separated with a flow rate of 400 nL/min using the following solvent system: (A) 0.1% formic acid (FA); (B) 84% ACN, 0.1% FA. In a first step, a gradient from 5% B to 40% B (90 min) was used, followed by a second gradient from 40% B to 95% B within 5 min, and finally a gradient from 95% to 5% B within 25 min. Accordingly, the total gradient run time was 2 h.

Electrospray ionization tandem MS was performed on a Q Exactive instrument (Thermo Fisher Scientific), which was directly coupled to the high-performance liquid chromatography system. MS spectra were scanned between 300 and 2000 m/z with a resolution of 30,000 and a maximal acquisition time of 500 ms. The m/z values initiating MS/MS were set on a dynamic exclusion list for 35 s. Lock mass polydimethylcyclosiloxane (m/z 445.120) was used for internal recalibration. The 20 most intensive ions (charge >1) were selected for MS/MS fragmentation in the ion trap. Fragments were generated by low-energy collision-induced dissociation on isolated ions with collision energy of 35% and maximal acquisition time of 50 ms.

Raw files were transformed to *.mgf files imported in ProteinScape™ (version 2.1; Bruker Daltonics, Bremen, Germany) and analyzed using Mascot (Matrixscience, London, United Kingdom) with a peptide mass tolerance of 10 ppm and a fragment mass tolerance of 0.5 Da. Searches for the identification of peptides/phosphopeptides were performed allowing one missed cleavage site after tryptic digestion. All data were searched against a database created by DecoyDatabaseBuilder (Reidegeld et al., 2008) with one additional shuffled decoy for each protein. A false discovery rate of 0.01 was applied to the identified peptides. Qualitative analysis of the data was carried out using the International Protein Index (IPI) database for protein search and *Rattus norvegicus* as the organism model.

The datasets of phosphoproteins obtained in each individual experiment were then statistically analyzed as described below.

Phosphoprotein bioinformatic and statistical analyses using the Systems Biology Tool kit library

Data from each experimental assay were treated as an individual dataset and all datasets were analyzed with a customized script based on the Systems Biology Tool kit (SysBioTK) available at https://bitbucket.org/CrisXed/sysbiotk. In a first step, the protein yield of each dataset was used to identify the outlier datasets applying the modified Thompson tau, τ, test (confidence level of 95%). In both Control and OA conditions, one experimental set was removed, and data from five experiments were considered for the subsequent statistical analysis. Proteins in each dataset were referenced by the corresponding IPI accession number. Subsequently, these were converted to the corresponding UniProt accession numbers, using UniProt resources (The UniProt Consortium, 2017; available at www.uniprot.org) and functionality from the SysBioTK.

In brief, a first step considered the IPI cross-reference information to find the UniProtKB/Swiss-Prot accession numbers. For each of the remaining proteins without an associated UniProt accession number, a blast search was carried out against a subset of the UniProt database (Swiss-Prot accession numbers, organism Rattus), using a similarity parameter of 0.9 for the SysBioTK matching criteria. In the case of no matches, the process was repeated against the UniProtKB/TrEMBL database. This conversion was performed on September 27, 2015.

The list of proteins, identified by their UniProt accession numbers, was thus obtained for each of the experimental conditions, Control and OA exposure. The output contains the detailed information, such as Gene Ontology (GO) annotations, for each gene of the corresponding protein. For ease of handling, the output refers to genes in the GO analysis and to the corresponding protein names for rats in the subsequent analyses.

For each experimental condition (Control and OA), the retrieval rate of each accession number is estimated using a Bayesian approach with a flat prior (Ullrich and Xu, 2007) by taking the assays in which the protein was retrieved and the total number of assays. The recovery rate of each protein in the ensemble of datasets for each experimental condition was employed to identify the proteins with a statistically significant difference between the two experimental conditions, using the Welch's t-test with a confidence level of 95%.

This analysis results in two lists of proteins; one list of phosphoproteins that show a statistically significant decrease in abundance upon the addition of OA (lower and lost phosphoproteins) and another list that shows phosphoproteins with a statistically significant increase in abundance upon the addition of OA (higher and exclusive phosphoproteins).

Phosphoprotein interactions were identified by submitting the protein lists to STRING (Szklarczyk et al., 2017; available at http://string-db.org) on November 10, 2016, and the information was loaded into Cytoscape 3.3.0 (Shannon et al., 2003). The Network Analyzer tool from Cytoscape was used.

RESULTS

OA-induced neuronal phosphoproteome

Primary cortical neurons were exposed to OA at a concentration particularly inhibiting two of the major brain PPs, PP1 and PP2A (da Cruz e Silva et al., 1995a; Oliveira et al., 2015; Swingle et al., 2007). Phosphorylated protein-enriched fractions were obtained using a phosphoprotein enrichment kit. Enriched fractions were then submitted to MS and the data obtained subsequently analyzed using a bioinformatics tool kit (SysBioTK), as described in the Materials and Methods section (Figure II-1). The experiment was repeated six times and each dataset was handled as an individual experiment to identify outliers, using the modified Thompson tau, τ, test, which led to the removal of one experimental set in both Control and OA.

Under these experimental conditions, around 850 and 900 phosphorylated proteins were recovered in Control and OA conditions, respectively (from the sum of all experimental sets; IPIs not yet characterized were not included). A marginal increase in the number of phosphorylated proteins upon addition of OA is readily evident.

GO analysis of the SysBioTK output was used to compare both phosphoproteomes (Figure II-2). In global terms, GO analyses revealed that the distribution of phosphorylated proteins among the different biological processes (BPs) is similar in Control (GpC) and OA (GpOA) conditions. However, in general, the number of phosphoproteins increased in the top 20 categories for OA exposure, with the exception of the small-molecule metabolic process, biosynthetic process, cellular component assembly, and cellular nitrogen compound metabolic processes (Figure II-2). The last two are among the processes that have more hits as consequence of OA treatment, along with cell differentiation, transport, and anatomical structural development. The overall increase in the protein phosphorylation state in response to OA is consistent with its role as a potent PP inhibitor.

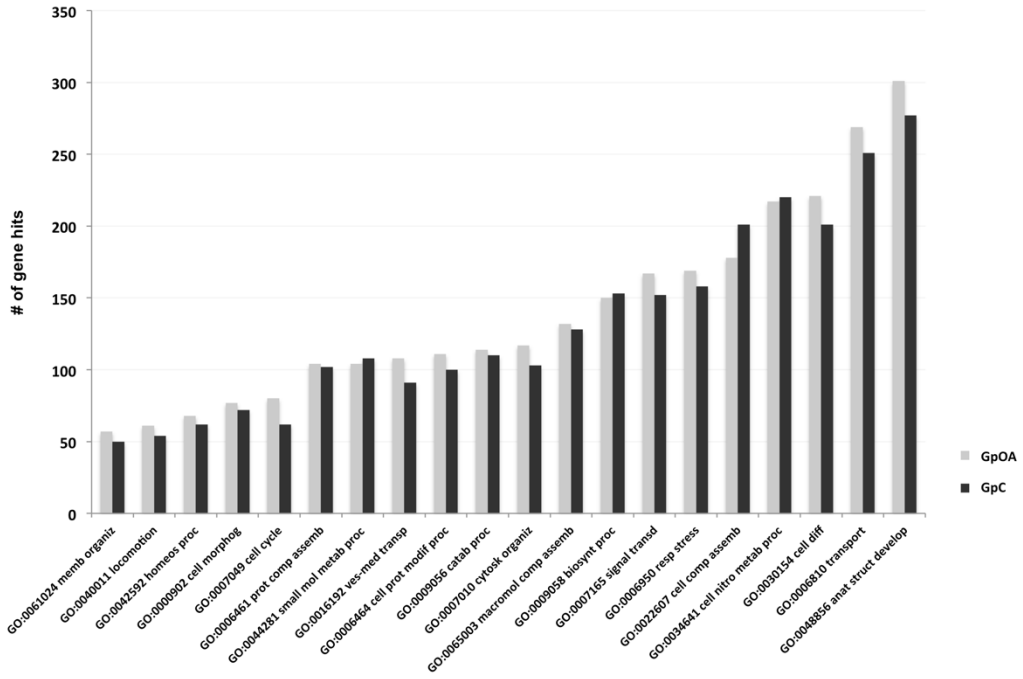

Figure II-2. Biological process ontology of the phosphoproteomes. GO analysis for the BP was carried out for the two lists of Control and OA genes encoding for the phosphorylated proteins, using the SysBioTK. The number of representative genes in Control (GpC, black bars) and OA (GpOA, gray bars) for each process is depicted in the graph. GO terms in the BP: GO:0061024 membrane organization; GO:0040011 locomotion; GO:0042592 homeostatic process; GO:0000902 cell morphogenesis; GO:0007049 cell cycle; GO:0006461 protein complex assembly; GO:0044281 small molecule metabolic process; GO:0016192 vesicle-mediated transport; GO:0006464 cellular protein modification process; GO:0009056 catabolic process; cytoskeleton organization; GO:0007010 macromolecular complex assembly; GO:0009058 biosynthetic process; GO:0007165 signal transduction; GO:0006950 response to stress; GO:0022607 cellular component assembly; GO:0034641 cellular nitrogen compound metabolic process; GO:0030154 cell differentiation; GO:0006810 transport and GO:0048856 anatomical structure development. BPs, biological processes; GO, Gene Ontology.

OA significantly different phosphoproteome

Subsequently, by applying the Welch's t-test, a set of proteins (320 phosphoproteins) were identified, whose recovery rate changed significantly across experimental sets with OA treatment, compared with Control conditions. From those, 245 phosphoproteins exhibited a higher recovery, while 75 exhibited a lower recovery (Table II-1 and II-2).

Table II-1. Phosphoproteins whose recovery rates increased in response to OA. Phosphoproteins recovered that significantly increased ("Higher") or that were detected only upon OA addition ("Exclusive", genes in bold). The genes encoding these significantly different proteins (Welch's t-test) were identified using the SysBioTK. Underlined proteins represent phosphoproteins recovered under both conditions, although different identifiers were involved, see Supplemental Table II-1.

"Higher"				"Exclusive"			
Abi1	Dnajb11	Lmnb1	Psmd7	**Aarsd1**	**Dnajb1**	**Lrrfip2**	**Rab11a**
Acaca	Dnajc6	LOC100911365	Psme2	**Abl2**	**Dnm1l**	**Map2k1**	**Rab6a**
Actn4	Dtd1	Map4k4	Ran	**Abr**	**Dpysl3**	**Mapk1**	**Rab6b**
Actr2	Dync1li1	Mecp2	Rangap1	**Actr1a**	**Drg1**	**Mff**	**rCK1e-3**
Add1	Eci1	Mgea5	Rcn1	**Actr3**	**Dync1li2**	**Mthfd1**	**RGD1559896**
Anp32a	Eef1b2	Msn	Rcn2	**Add2**	**Dynlrb1**	**Myo6**	**Rps10**
Anp32e	Eef1d	Myl12b	Rdx	**Adrbk1**	**Ehd1**	**Nckap1**	**Rps18**
Anxa6	Eef2	Myl6	RGD1307235	**Anxa7**	**Ehd3**	**Ndrg3**	**Rps24**
Ap2a1	Efhd2	Naca	Rtcb	**Arpc1a**	**Eif4a1**	**Ndufa4**	**Sept8**
Ap2m1	Eif3c	Naga	Rtn1	**Asrgl1**	**Eif4g1**	**Nes**	**Slc7a5**
Ap2s1	Eif3j	Nap1l1	Sept6	**Atp6v1d**	**Enah**	**Nudt3**	**Smc3**
Arcn1	Eif5	Ncam1	Sept9	**Atp6v1h**	**Epn1**	**Numbl**	**Snapin**
Arhgap35	Elp3	Ndrg1	Set	**Bag6**	**Ftl1**	**Pabpc1**	**Sorbs1**
Arpc4	Fam129b	Neo1	Sh3gl1	**Banf1**	**Gdi1**	**Pc**	**Spast**
Atg3	Fbl	Nme1	Sh3gl2	**Bcat1**	**Gdi2**	**Pcbp2**	**Sptan1**
Atp1b1	Fsd1	Nt5c2	Shtn1	**Bid**	**Gsk3a**	**Pdcd5**	**Src**
Atp6v1a	Gapdh	Pafah1b2	Slc1a3	**Bin1**	**Gsk3b**	**Pfn2**	**Srcin1**
Calu	Gart	Pak2	Snrpd2	**Bles03**	**Gtpbp1**	**Phgdh**	**Stx12**
Camsap3	Gnai1	Pea15	Srgap2	**Camk2d**	**Hist2h2aa3**	**Phyhipl**	**Stxbp5**
Cbx3	Gnb2l1	Pfdn4	Strap	**Caprin1**	**Hmgcs1**	**Picalm**	**Tln1**
Ccdc50	Gucy1a3	Plxna4a	Stub1	**Carhsp1**	**Hn1**	**Plcg1**	**Tln2**
Cdh2	Hmgb2	Ppp2r2a	Syn2	**Ccdc124**	**Hnrnpa2b1**	**Polr2a**	**Tollip**
Copa	Hnrnph1	Prdx1	Synj1	**Chmp2a**	**Hspa4l**	**Ppm1e**	**Tsg101**
Coro1a	Hnrnph2	Prpsap2	Tmod2	**Chmp3**	**Ikbkap**	**Ppm1h**	**Ube4b**
Csnk2b	Hnrnph3	Prss1	Tpm3	**Clasp1**	**Ist1**	**Ppp1ca**	**Unc119**
Cxadr	Huwe1	Psmb3	Trappc3	**Cltc**	**Klc2**	**Ppp2r5e**	**Vamp4**
Cyfip1	Ina	Psmc1	Ttll12	**Cnpy4**	**Kpna3**	**Ppp3cb**	**Vdac1**
Dclk2	Inpp4a	Psmc5	Ugp2	**Csde1**	**Kpnb1**	**Prkaa1**	**Vps45**
Dctn4	Kif3a	Psmd11	Vamp3	**Ctbp1**	**Krt76**	**Ptbp2**	
Dffb	Kif3b	Psmd3	Vim	**Ctps1**	**Lrrc40**	**Ptk2**	
Dnaja1	Krt6a	Psmd6	Vps29	**Dlg2**	**Lrrc8a**	**Ptpn11**	

Interestingly, from the significantly different phosphoproteome, 121 were OA exclusive (phosphoproteins recovered only after conditions of OA exposure) and 50 were OA lost (phosphoproteins not recovered following OA exposure) (Table II-1 and II-2, respectively). A small subset of these proteins was recovered in both conditions, the response appears to be protein isoform/variant dependent and the results are further detailed in Supplementary Table II-1.

Table II-2. Phosphoproteins whose recovery rates decreased in response to OA. Phosphoproteins recovered that significantly decreased ("Lower") or that were absent upon OA treatment ("Lost", genes in bold). The genes encoding these significantly different proteins (Welch's t-test) were identified using the SysBioTK. Underlined proteins represent phosphoproteins recovered under both conditions, although different identifiers were involved, see Supplemental Table II-1.

"Lower"			"Lost"				
Acbd3	Klc2	Sf3b1	**Aco2**	**Clvs2**	**Mark2**	**Rad21**	**Srsf1**
Alg2	Map4k4	Synj1	**Add2**	**Csnk1e**	**Mical3**	**Rbbp7**	**Stmn3**
Ddx21	Marcks	Tmx1	**Ank3**	**Dlg3**	**Myef2**	**Rhot1**	**Strbp**
Dnm1	Nckap1	Tmx4	**Apc**	**Gapdh**	**Ncam1**	**Sfxn3**	**Taf15**
Dpysl3	Ndufs1	Tpm3	**Arf5**	**Gls**	**Nfic**	**Shtn1**	**Tanc2**
Eef1d	Pgam5		**Arhgap1**	**Grsf1**	**Pabpn1**	**Slain2**	**Tf**
Eif2s3	Prcp		**Brsk1**	**Gtf2i**	**Pcyt1b**	**Slc25a3**	**Trim67**
Fam120b	Prpf6		**Ccdc6**	**Irgq**	**Pfdn2**	**Snap25**	**Uso1**
Farp1	Rpn1		**Ccdc92**	**Magi1**	**Ppp3ca**	**Snrpg**	**Usp47**
Hnrnph1	Rps3		**Chmp2b**	**Magi2**	**Prps1**	**Sptan1**	**Wdr11**

GO analysis for significantly different phosphoproteomes revealed alterations in the top 20 BP categories compared with the entire OA-dependent phosphoproteome. As depicted in Figure II-3, phosphoproteins significantly increased or appeared de novo (red bars) and significantly decreased or were lost (green bars). Particularly noteworthy, signal transduction became one of the top four BPs and cell death newly appeared as the last of the top 20 BP categories (Figure II-3). Top BPs for significantly different phosphoproteins are largely related to alterations in cellular morphology (anatomical structural development and cell differentiation), transport, and signal transduction. As expected, in comparative terms, the number of phosphorylated proteins recovered increased following OA treatment; this is consistent with its phosphatase inhibitory effect.

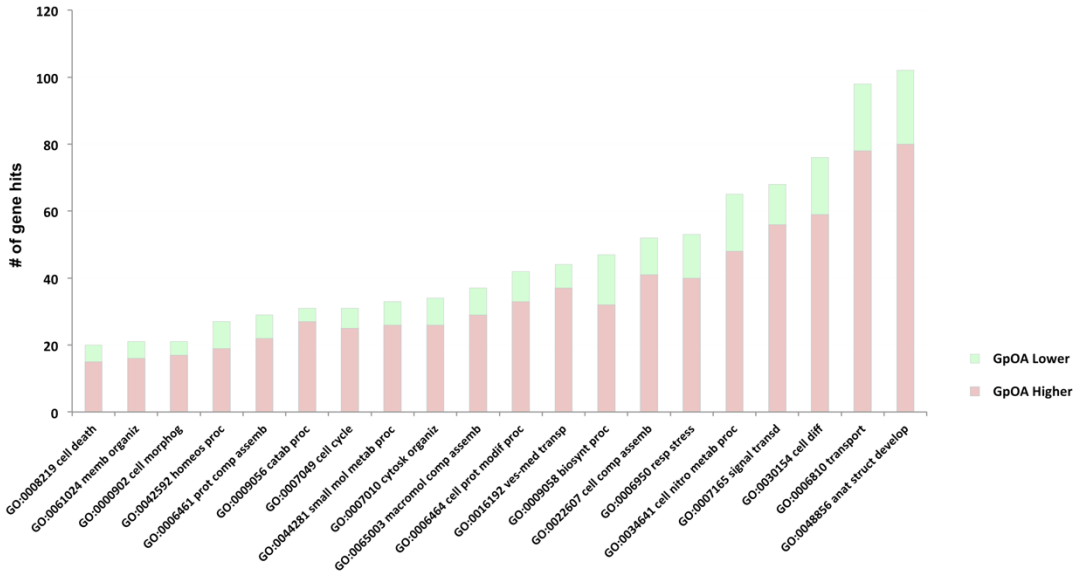

Figure II-3. Biological process ontology of the significantly different OA-induced phosphoproteome. Gene ontology (GO) for the biological process (BP) of the significantly different phosphoproteins induced by OA was achieved using SysBioTK. Green bars, represent the number of genes that significantly decreased (GpOA lower) and red bars represent the number of genes that significantly increased (GpOA higher) in each process, upon OA treatment and comparatively to Control. Abbreviations of GO not in full: GO:0008219 cell death; GO:0061024 membrane organization; GO:0000902 cell morphogenesis; GO:0042592 homeostatic process; GO:0006461 protein complex assembly; GO:0009056 catabolic process; GO:0007049 cell cycle; GO:0044281 small molecule metabolic process; GO:0007010 cytoskeleton organization; GO:0065003 macromolecular complex assembly; GO:0006464 cellular protein modification process; GO:0016192 vesicle-mediated transport; GO:0009058 biosynthetic process; GO:0022607 cellular component assembly; GO:0006950 response to stress; GO:0034641 cellular nitrogen compound metabolic process; GO:0007165 signal transduction; GO:0030154 cell differentiation; GO:0006810 transport and GO:0048856 anatomical structure development.

Analysis of the significantly different networks using STRING and the Network Analyzer tool from Cytoscape (Figure II-4) depicts the interaction of the phosphoproteins as well as those whose recovery rates increased (light red), decreased (light green), appeared exclusively (dark red), or were completely lost (dark green) upon OA treatment. A set of proteins were recovered in both conditions, therefore exhibiting either increased or decreased rates; these are marked as yellow nodes in Figure II-4 and shown in Supplementary Table II-1. Analysis of the interaction network revealed a central node, the proto-oncogene tyrosine-protein kinase Src (Src) (Figure II-4), a phosphoprotein involved in diverse processes, such as cell proliferation and morphology, differentiation, survival, and of course signal transduction (Src mass spectra, Supplementary Figure II-1).

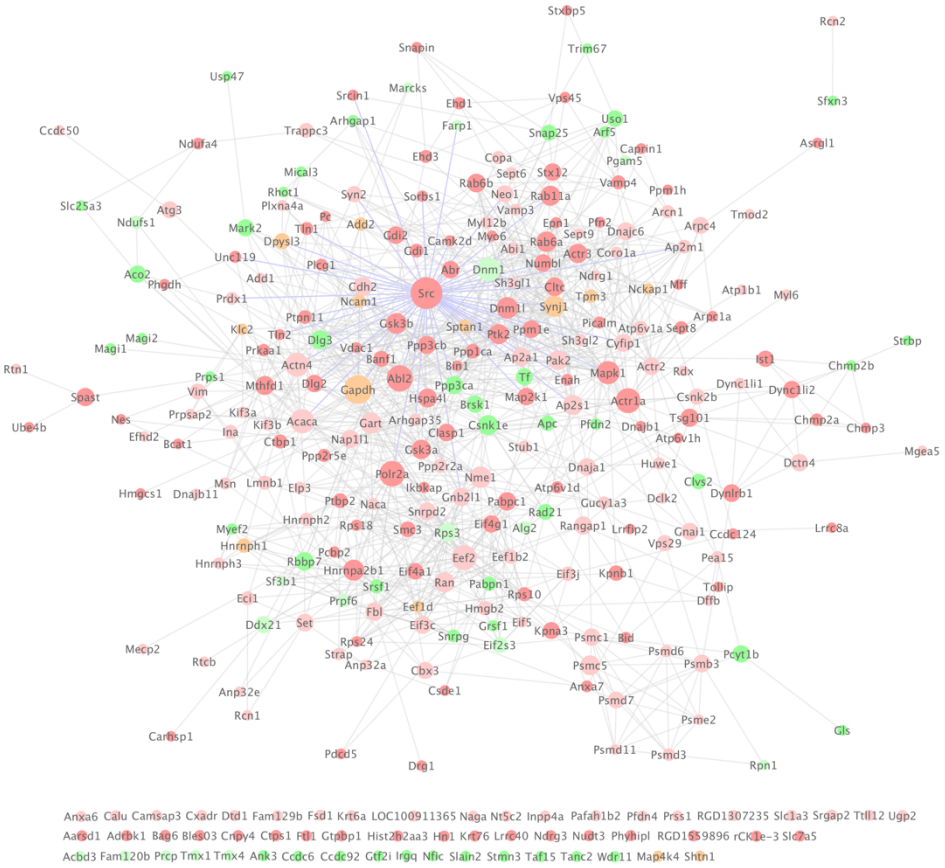

Figure II-4. OA responsive phosphoproteome network. Significantly different phophoproteome recovered upon OA addition was submitted to String and the interactome produced using Cytoscape 3.3.0. and the Network Analyzer tool. Phosphoproteins that increased upon OA addition are represented in light red and dark red represents the phosphoproteins detected only upon OA addition ("exclusive"). The phosphoproteins that decreased in response to OA are represented in light green and the ones completely "lost" upon OA treatment are represented in dark green. In yellow are represented phosphoproteins recovered in both conditions.

Several PPs revealed altered phosphorylation states upon OA exposure. In fact, two phosphatase catalytic subunits were recovered as phosphorylated proteins only in conditions of OA exposure (Figure II-4 and Table II-1); these were serine/threonine PP1 alpha catalytic subunit (Ppp1ca) and serine/threonine PP2B catalytic subunit beta isoform (Ppp3cb). This observation is consistent with OA-targeting PPs. However, of note and in marked contrast, recovery of another PP2B isoform, serine/threonine PP2B catalytic subunit alpha isoform (Ppp3ca), was completely lost upon OA exposure. To our knowledge, this is the first report of PP2B isoforms responding differently to OA-induced molecular events and is a topic that deserves further investigation.

In the significantly different phosphoproteome network, several PP-interacting proteins were evident, among them the serine/threonine PP2A 55 kDa regulatory subunit B alpha isoform (Ppp2r2a) whose recovery increased with OA and serine/threonine PP2A 56 kDa regulatory subunit B epsilon isoform (Ppp2r5e) that was recovered only in the presence of the toxin. This is important as it highlights different effects on distinct PP-interacting proteins and this may be of potential

therapeutic or diagnostic value.

Top BPs affected by OA

As already mentioned, the top BPs most affected by OA are anatomical structure development, transport, cell differentiation, and signal transduction (Figure II-3 and II-5). In all these cases, it is immediately evident that the higher phosphorylated proteins outnumber the lower phosphorylated proteins. In fact, a number of them were only recovered upon exposure to OA (dark red nodes). If one considers anatomical structure development and cell differentiation together (Figure II-5 A, C), as they share a large number of nodes, the central node in both cases is Src, as already discussed for Figure II-4.

Likewise, Abelson tyrosine-protein kinase 2 (Abl2) and disks large homolog 2 (Dlg2) appear *de novo* in both networks and are hub nodes. Ppp3ca, serotransferrin, serine/threonine-protein kinase Mark2 (Mark2), adenomatous polyposis coli protein (Apc), and synaptosomal-associated protein 25 (Snap25) were among the proteins whose recovery of the phosphorylated protein was lost upon OA addition.

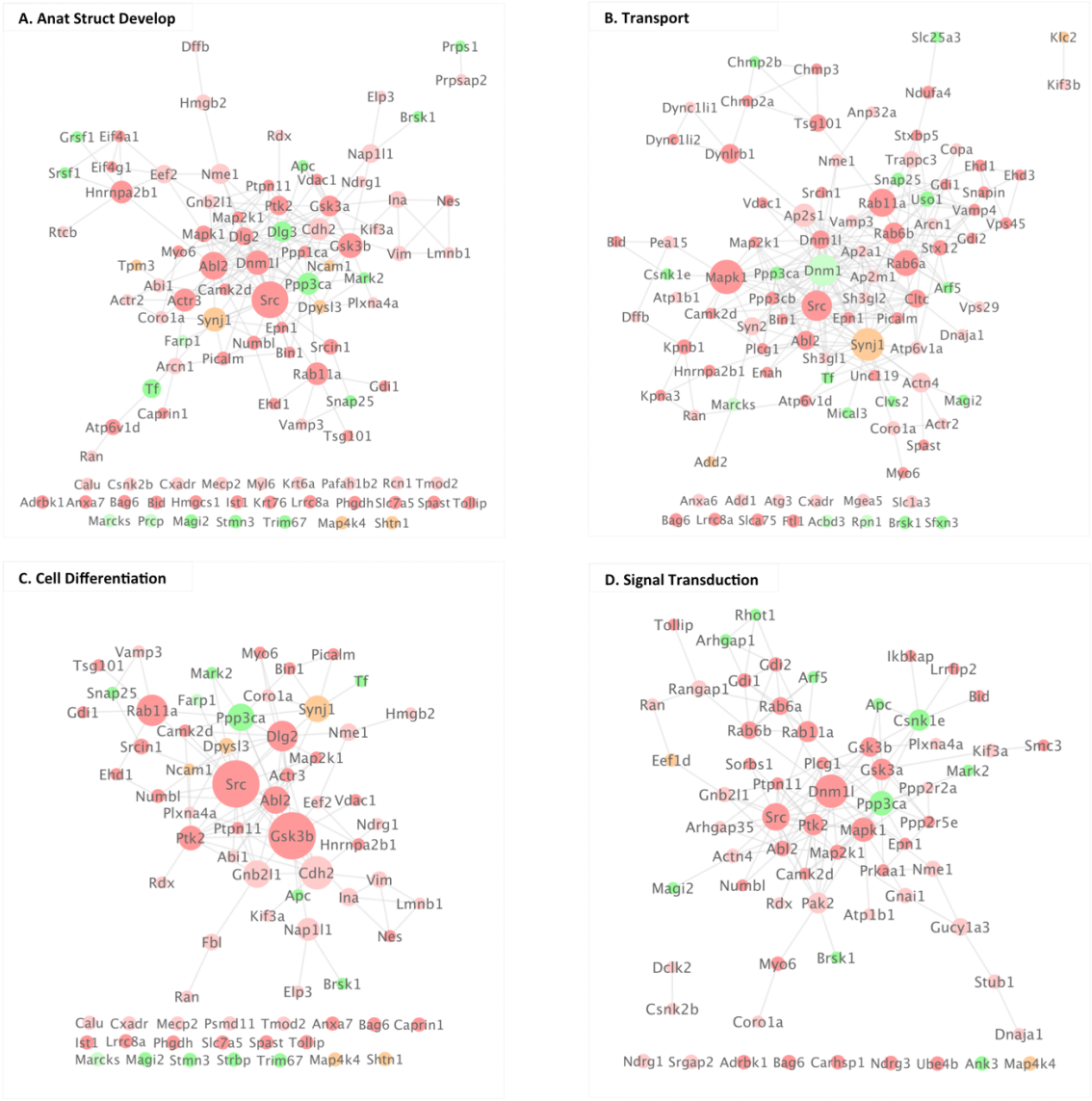

Figure II-5. Phosphoprotein interaction networks of the top biological processes affected by OA. Interaction networks of the significantly different phosphoproteins linked to the 4th top biological processes are presented. **A.** GO:0048856 anatomical structure development **B.** GO:0006810 transport **C.** GO:0007165 signal transduction and **D**. GO:0030154 cell differentiation. Phosphoproteins that increased are represented in light red and the "exclusive" are represented in dark red; phosphoproteins that decreased are represented in light green and the "lost" proteins are represented in dark green. Yellow represents phosphoproteins recovered in both conditions.

The two networks also share a series of proteins that are higher/exclusive or lower/lost, depending on the identifier (Supplementary Table II-1), namely synaptojanin-1 (Synj1), dihydropyrimidinase-related protein 3 (Dpysl3), neural cell adhesion molecule 1 (Ncam1), mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4), and shootin-1 (Shtn1). This is a relevant observation, and as more is learned by applying a systems approach, it may permit modelling and fine-tuning targets to cellular responses in an, for example, isoform-dependent manner.

The transport network (Figure II-5 B) is quite distinct from the two just discussed. Although Src is present, other central hubs appeared, namely mitogen-activated protein kinase 1 (Mapk1), dynamin-1 (Dnm1), Synj1, and ras-related proteins Rab-11A (Rab11a), Rab6a, and Rab6b. Clearly, OA is interfering with intracellular transport and affecting processes that are relevant to diseases such as AD, where APP transport affects proteolytic processing and Aβ production (Rebelo et al., 2007).

The fourth most affected biological function is signal transduction (Figure II-5 D). Two central hubs are dynamin-1-like protein (Dnm1l) and Ppp3ca. The first was exclusive and the second was lost upon OA exposure, thus representing well the dynamic nature of protein phosphorylation systems. Several kinases were also present in this network, such as Mapk1/Erk2 (Mapk1 mass spectra, Supplementary Figure II-1), mitogen-activated protein kinase kinase 1 (Map2k1/Mek1), calcium/calmodulin-dependent protein kinase type 2 subunit delta (Camk2δ, Camk2d in Figure II-5), Gsk3α, and Gsk3β (Gsk3a and b in Figure II-5). All are important in neuronal systems and Gsk3, for instance, is an important kinase phosphorylating Tau.

It is singularly important that signal transduction becomes a more prominent biological function (Figure II-3 and II-5 D) upon OA exposure, and this corroborates that OA is targeting PPs, affecting signaling pathways, and acting as a neurotoxin. The mechanisms and molecular targets underlying OA-induced neurotoxicity have been the focus of attention in different cell models (Kamat et al., 2013, 2014a).

OA-responsive kinases and phosphatases

The interplay between kinases and phosphatases is fundamental not only for the abovementioned processes but also for general intracellular signalling and cellular communication. Therefore, a network was constructed for all the kinases and phosphatases recovered upon OA treatment. To achieve this, the SysBioTK was used, and searches identified the proteins with the term phosphatase and/or kinase in the GO tree (Figure II-6). The phosphoproteins identified were submitted to STRING and the interaction network obtained was analysed using Cytoscape.

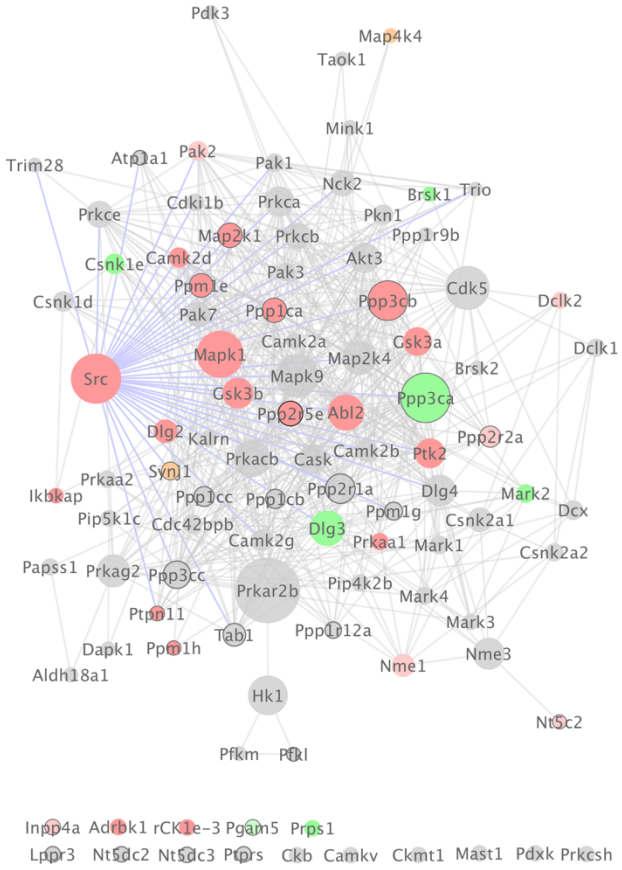

Figure II-6. OA dependent kinase and phosphatase interaction network. Searches were carried out for identifiers with the term kinase and phosphatase in the GO tree using the SysBioTK. The phosphoproteins list was submitted to String and the interaction network produced using Cytoscape 3.3.0 and the Network Analyzer tool. Kinases and phosphatases that increased upon OA addition are represented in light red and dark red represents those "exclusive" detected upon OA addition. The kinases and phosphatases that decreased in response to OA are represented in light green; and the ones completely "lost" are represented in dark green; the phosphoproteins recovered in both conditions are indicated in yellow; and the phosphoproteins recovered in OA condition but not significantly different from Control are represented in gray. Nodes with a circumference correspond to proteins with the phosphatase term in the GO tree.

Under conditions where PPs were inhibited, the phosphoprotein recovery rates of the kinases Src, Gsk3α/β (Gsk3a/b in Figure II-6), Mapk1/Erk2, and the dual-specificity Map2k1/Mek1 are enhanced and exclusive to OA treatment. Src is a central node that interacts with many other kinases and phosphatases (adjacent edges highlighted in blue) and whose recovery rates change in response to OA. The serine/threonine-protein kinase Brsk1 (Brsk1), casein kinase I isoform epsilon (Csnk1e), and Mark2 are kinases whose recovery rate was lost. Other kinases also present, but whose recovery rate did not significantly change in response to OA, such as Cdk5, Camk2α/β/γ (Camk2a/b/g in Figure II-6), mitogen-activated protein kinase 9 (Mapk9), and RAC-gamma serine/threonine-protein kinase (Akt3).

Despite the OA-induced PP1 and PP2A inhibition, and as already mentioned above, the recovery

of phosphorylated Ppp3ca was completely lost, whereas Ppp3cb appeared de novo. As expected, tyrosine-protein phosphatase nonreceptor type 11 (Ptpn11) and PP Mg2+/Mn2+-dependent 1e/h (Ppm1e/h) exclusively increased and Ppp1ca (in Figure II-6) was phosphorylated and thus inhibited (Monick et al., 2006). The Ppp2r2a was also increased upon OA treatment. Taken together, data support an interaction between kinases and phosphatases, involving altered protein phosphorylation levels, and identify the key players, which of course is a major general regulatory mechanism in signaling cascades.

DISCUSSION

Neuroproteomics has impacted research in many health-related fields, including psychiatry/neurology, allowing for the identification of molecular candidates to study psychiatric disease pathophysiology, among them AD. Previous studies have identified that impairment of serine–threonine phosphatases' activity has been linked with various pathologies, including neurodegenerative disorders. As an example, many studies using OA have associated PP1 and PP2A inhibition with Tau hyperphosphorylation, NFT and amyloid-like plaque formation, neurodegeneration, and cognitive dysfunction, suggesting that OA may serve as a good model to address AD pathology (Kamat et al., 2013, 2014a).

From the significantly different OA phosphoproteome, three genes are also associated with the AD KEGG pathway (KEGG map 05010). These are Mapk1, Gsk3β, and BH3-interacting domain death agonist (Bid). The roles of the kinases were discussed above and these have phosphorylation states affected by OA, which in turn influence their activity on AD-relevant substrates, such as Tau. Bid, a proapoptotic member of the Bcl-2 family, is an OA exclusive node capable of inducing apoptosis, a critical event in many neurodegenerative diseases. Previous work has shown not only a strong link between PP inhibition and neuronal death but also cytoskeleton network alterations (Henriques et al., 2015; Opsahl et al., 2013). Cell death is the 20th BP significantly affected by OA exposure and cytoskeleton network alterations the 12th.

Another two proteins, found to be exclusively phosphorylated in response to OA, are also risk genes for AD; bridging integrator 1 (BIN1) and phosphatidylinositol-binding clathrin assembly protein (PICALM) (Karch and Goate, 2015). Risk genes have been proposed as potential targets for AD therapeutic strategies. Having been recovered in the experimental setup presented here reinforces this hypothesis.

GO analysis revealed that the main BPs for the OA entire phosphoproteome and significantly

different proteome did not change significantly; anatomical structural development, transport, and cell differentiation were among the top BPs in both cases from a total list of more than 60 BPs identified. Signal transduction, however, did change from the seventh BP when the entire phosphoproteome is considered to the fourth BP when only the significantly different phosphoproteins were evaluated. One can tentatively deduce that this is the BP most targeted by OA.

Globally, OA led to major alterations in the neuronal phosphoproteome, in particular, 245 phosphoproteins were significantly increased and 75 significantly decreased. Of those, 50 were completely lost and 121 were exclusive. Analysis of the significantly different phosphoproteome revealed one central node, Src. The latter is a nonreceptor protein tyrosine kinase and a key molecular player in OA-mediated neuronal events, whose recovery as a phosphorylated protein was OA exclusive. Src family kinases, including Src, are involved in several processes such as cell morphology, differentiation, proliferation, and survival (Kumar et al., 2015). Src is linked to various signaling transduction pathways, including MAPK, phosphoinositide 3-kinase (PI3K)/Akt, and STAT (for signal transducers and activators of transcription) pathways (Bromann et al., 2004), the latter is intimately tied to inflammatory processes.

Depending on the cellular content, Src can also induce or inhibit apoptosis (Johnson et al., 2000; Lopez et al., 2012) through different mechanisms, including activation of p53. Impaired Src signaling has been observed in AD mouse models (Dhawan and Combs, 2012; Mota et al., 2014), suggesting that targeting Src may be of therapeutic value from a disease perspective. As mentioned, cell death was another BP associated with the OA that induced significantly different phosphoproteome. Cell death is a critical event triggering disease initiation and development. Many PPs have been associated with apoptotic events, and neurotoxic effects mediated by OA have been previously addressed, although the exact mechanisms are not entirely understood.

Various reports have shown that OA promotes the activity of different kinases, including Mapk and Erk1/2, Gsk3β, and Camk2, all PKs associated with AD pathology (Kamat et al., 2013, 2014a). OA was reported to induce the activity of Mapks (Casillas et al., 1993; Pei et al., 2003). Consistently, in the work described here, Mapk1 (also known as Erk2) is a central hub particularly in the transport and signal transduction networks (Figure II-5 B and D), exhibiting higher phosphorylation rates of recovery. Mapk1 regulates several cellular processes and it is a kinase activated by phosphorylation.

The activity of Gsk3β isoform was also reported to be influenced by OA (Bennecib et al., 2000; Lim et al., 2010). This serine/threonine kinase regulates a wide range of cellular processes, therefore it is not surprising that this kinase exhibits both apoptotic and antiapoptotic roles as a

consequence of the cellular context. Depending on the phosphorylated residue, Gsk3 phosphorylation can render either its inactivation (Ser9) or activation (Tyr216). Under our experimental conditions, it would be expected that Gsk3β phosphorylation is increased due to decreased PP activity. Nonetheless, and since Gsk3β is involved in many signaling pathways and its activity depends on specific phosphorylatable residues, one cannot draw specific conclusions in this respect. This is also true for all kinases and phosphatases involved in multiple signaling pathways.

Interestingly, not only Gsk3β was found but also Gsk3α; recovery of the latter in its phosphorylation form was significantly increased in response to OA. Of particular relevance, Gsk3α was shown to be required for Aβ production (Phiel et al., 2003), critical for neuronal toxicity and AD pathogenesis.

Taken together, the OA-induced phosphoproteome networks provide highly relevant correlations, which should be borne in mind in the development of novel phosphorylation mediating drugs, not only for neurodegenerative disorders but also for other pathological conditions, such as cancer drug development (Figueiredo et al., 2014).

PPs may either directly or indirectly affect components of signaling pathways, including PKs and other PPs. For instance, inhibition of Src by direct interaction with PP2A was previously reported (Yokoyama and Miller, 2001), and Src activation can lead to PP2A inhibition (Hu et al., 2009), supporting the interplay between serine–threonine phosphatases and tyrosine kinases. As mentioned, PP2A inhibition by OA can render Erk1/2 activation (Pei et al., 2003) and Tau hyperphosphorylation. Furthermore, other MAP kinases, which are important components of gene regulatory signaling pathways, can be likewise hyperphosphorylated by OA (Kamat et al., 2013, 2014a).

In the work presented here, PP1 and PP2A inhibition led to a general increase in the recovery of several PKs, including Src, Mapk1 (Erk2), and Gsk3α/β, already discussed. Another PK with increased recovery was protein tyrosine kinase 2 beta (Ptk2b), previously described to be involved in MAPK signaling pathway activation (Lev et al., 1995). Additionally, both Ptk2b activation and Src/tyrosine-protein kinase Fyn (Fyn) kinase activation enhances phosphorylation of NMDARs (Heidinger et al., 2002), whose dysregulation is evident in neurological diseases.

Other Mapks, besides Mapk1, are altered in response to OA. Map2k1 (Mek1) is an essential component of the MAPK signaling pathway responsible for Erk1/2 activation (Su and Karin, 1996) and is itself activated by phosphorylation at two adjacent serine residues (218 and 222) by other Mapks (Zheng and Guan, 1994). As already mentioned, Src can act upstream in signaling pathways involving Mek/Mapk (Moon et al., 2002) and the interplay between c-Jun N-terminal kinase 2 (Jnk2,

also known as Mapk9) and Src has been likewise reported in non-neuronal models (Samak et al., 2015). Cdk5 and Camk2α (Camk2a in Figure II-6), although not significantly different with OA treatment, were also recovered following OA exposure conditions. These are key players in AD pathogenesis.

Regarding PPs, the recovery of Ppp1ca was increased, which is consistent with its inhibition by phosphorylation (Monick et al., 2006). PP1 is highly enriched in dendritic spines (da Cruz e Silva et al., 1995b) and plays a critical role in memory and learning processes (Genoux et al., 2002) by modulating various aspects of synaptic signaling. Both PP2A and PP2B play a regulatory role in apoptosis (Sun and Wang, 2012). Intriguingly, the recovery of Ppp3 (PP2B) was differently affected by OA, an aspect that deserves further attention. It is thus evident that under these conditions, several neuronal signaling cascades are compromised, mimicking what happens in AD.

The phosphoprotein recovery of a mitochondrial serine/threonine phosphatase Pgam5 (Pgam5) was decreased, while the recovery of other PPs, such as Ptpn11, Ppm1e, and Ppm1h, was exclusive to OA treatment. Previous data from our group showed that Aβ exerted similar effects on some of the abovementioned proteins (Henriques et al., 2016), but this is not surprising since Aβ itself can inhibit PP1 and PP2A (Vintém et al., 2009).

Conjointly, data suggest that OA impacts different cellular levels and that targeting PPs is a complex approach. Of note, PP activity is regulated by PP-interacting proteins (PIPs); as such, instead of targeting PPs themselves, both PPI and PP regulatory subunits may represent suitable targets for therapeutic strategies directed at phosphatases (Figueiredo et al., 2014). Several PP regulatory subunits were identified in this study, but only Ppp2r5e and Ppp2r2a exhibited a phosphoprotein recovery significantly altered upon OA exposure. Both subunits are important for regulation of PP2A subcellular localization and/or substrate specificity and interestingly Ppp2r2a was reported to mediate Tau dephosphorylation (Yu et al., 2014).

These results reveal novel phosphoproteins involved in signaling events mediated by OA. Clearly, OA exposure does not exclusively mirror AD, but many molecular players relevant to this disease are affected by this neurotoxin. OA represents a powerful experimental tool to address cellular and molecular disease mechanisms linked to PP inhibition. This work highlights the importance of using a systems approach. The fact that various molecular players are involved helps validate the procedure toward understanding the interplay across the different signaling cascades.

In closing, neuroproteomics is an expanding postgenomic biotechnology with applications in preclinical biological psychiatry studies as well as other common complex human diseases (Reddy et al., 2015; Shao et al., 2015). Our data, using neuroproteomics in a preclinical study, resulted in

an array of phosphoproteins as potential key targets for future diagnostic and therapeutic strategies in biological psychiatry, which should be approached with cautious optimism.

Future studies should integrate phosphoproteomics, neuroproteomics, and neurogenomics to unravel new paradigms in biology psychiatry. To exemplify, PPs regulate many signaling cascades, have many interacting proteins, and regulate distinct signaling pathways and the genes encoding them can be alternatively spliced to produce distinct isoforms, including some that are neuronally enriched (da Cruz e Silva et al., 2004). In fact, the work presented here places PPs and their interactors as candidates to be addressed in future research, namely by modulating their expression as well as phosphorylation levels and monitoring the pathophysiology consequences in systems psychiatry.

CONCLUSIONS

In this work, a systems biology approach was used to identify targets and BPs that are responsive to OA treatment. This revealed 320 phosphoproteins whose recovery was significantly altered in response to OA, of which 245 significantly increased and 75 significantly decreased. The top four BPs affected included anatomical structural development, transport, cell differentiation, and signal transduction. The knowledge of these processes is expected to reveal new insights into OA mode of action and contribute to the design of further experiments directed at target biological validation. The phosphoproteins identified not only represent additional molecular targets in OA-mediated events but may also be potential disease-associated phosphobiomarker candidates. Furthermore, the same targets can contribute to the design of novel therapeutic strategies directed at neurodegenerative diseases and other relevant pathologies where PPs play a central role.

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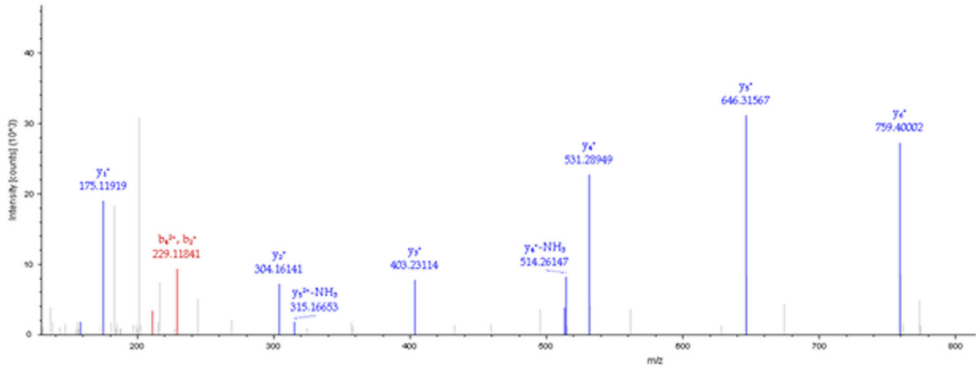
MANUSCRIPT SUPPLEMENTAL MATERIAL

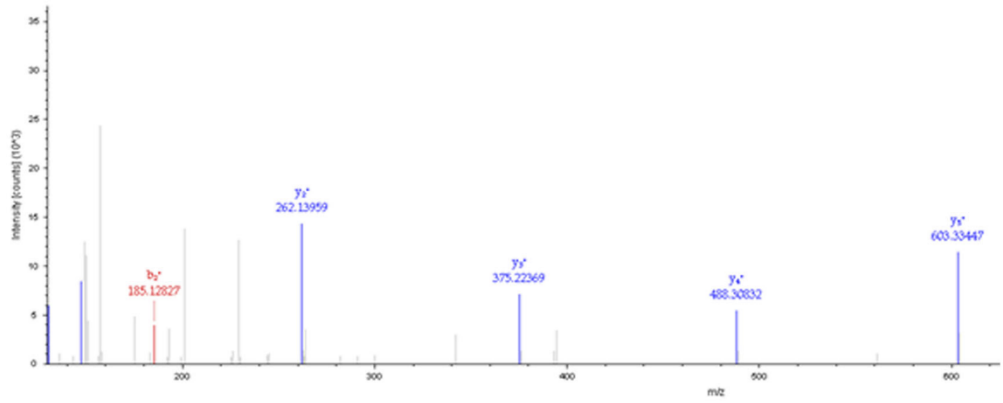
A. Src

Name: Proto-oncogene tyrosine-protein kinase Src
Peptide sequence: IKYPENFFLLR



B. Mapk1

Name: Mitogen-activated protein kinase 1 MAPK1
Peptide sequence: ALDLLDK



Supplementary Figure II-1. Example of phosphopeptides identified by MS/MS analysis. (A) Src and (B**)** Mapk1. Mapk1, mitogen-activated protein kinase 1; MS, mass spectrometry; Src, tyrosine-protein kinase Src.

Supplementary Table II-1. Dual response phosphoproteins. Significantly different phosphoproteins (Sig Dif) recovered in both conditions (“Lower”/“Lost” and “Higher”/“Exclusive") that presented different identifiers corresponding to the same protein. The public databases used did not distinguish the IPIs as different proteins, therefore the corresponding IPI sequences were aligned in SIM Alignment Tool and the differences identified in blue. Differences in the final sequence (marked in bold and italics) are also indicated.

Gene	Sig Dif	Identifiers	Sequence Alignment
Add2	Lost	Q05764 (IPI01027872)	^{522}SVIAEKSRSPSTESQLASKGDADTKDELEETVPNPFSQLT***DQELEEYKKEVERKKLEQEQEGEKDAATEEPGSPVKSTPASPVQSPTRAGTKSPAVSPSKASEDAKKTEVSEANTEPEPEKPEGVVVNGKEEEPSVEEVLSKGPGQMTTNADTDGDSYKDKTESVTSGPLSPEGSPSKSPSKKKKKFRTPSFLKKSKKKEKVES***
	Exclusive	Q05764 (IPI00230818)	^{522}SVIAEKSRSPVQQRLPPTEGEAYQTPGAGQGTPESSGPLT***P*** -
Dpysl3	Lower	Q62952 (IPI00556970)	^{10}PRITSDR .....
	Exclusive	Q62952 (IPI00203250)	^{123}PKDKSDR .....
Eef1d	Lower	Q68FR9 (IPI00197900)	^{35}AGSRQ - SS .....
	Higher	Q68FR9 (IPI00944235)	^{35}AGSRQENGASVILRDIARARENIQKSLAGSS .....
Gapdh	Lost	P04797 (IPI00555252)	^{10}GRIGRLVTR.....^{155}LAPLAKVI.....^{257}KKVVKQAAE .....
	Higher	P04797 (IPI00567177)	^{10}GRIGHLVTR......^{155}LAPLGKVI.....^{257}KKVVKQVAE .....
Hnrnph1	Lower	Q8VHV7 (IPI00203088)	^{369}GGAYEHRYVELFLNSTAGASGGAYGSQ..... ^{432}GYGDQGAVNSSYYS***SGSRASMGVNGMGGMSSMSSMSGGWGM***
	Higher	Q8VHV7 (IPI00958862)	^{369}GGAY - - - - - - - - - - - - - - - - - - - ^{393}GSQ..... ^{412}GYDQVLQENSSDFQS***NIA*** -
Kcl2	Lower	B2GV74 (IPI00764474)	^{593}PVQPG***GTGLSDSRTLSSSSMDLSRRSSLVG***
	Exclusive	B2GV74 (IPI00565164)	^{593}PVQPG***RVFLTAAL*** - - - - - - - - - - - - - - - -
Map4k4	Lower	F1M754 (IPI00948824)	^{216}KKWHLKHSGSIEGCLVK – YMQGPFTEQ..... ^{495}VEDRFRKTNHSSPEAQAKQTGRGLEPPVPSRSESFSNGNSESVHPALQRPAEPQVQW..... ^{743}PAGEVDLTALA.....
	Higher	F1M754 (IPI00764433)	^{301}KKWSKKFFSFIEGCLVKNYMQRPSTEQ..... ^{481}V - QW..... ^{675}PAVRIDLTALA.....
Ncam1	Lost	P13596 (IPI00476991)	^{12}FFLGTAVSLQ.....^{349}ISSEEKASWTRPEKQETLD .....
	Higher	F1LUV9 (IPI00777130)	^{1}FFFSFAVSLQ.....^{338}ISSEEK - - - - - - - - - - TLD .....

Gene	Sig Dif	Identifiers	Sequence Alignment
Nckap1	Lower	P55161 (IPI00951960)	^{241}N - LGFIL.....^{717}LLTSVRAYMTV..... ^{1034} KEFL -
	Exclusive	P55161 (IPI00876636)	^{241}NPAQSDTMPCEYLSLDAMEKWIIFGFIL.....^{739}LLTSVREYMTV .....^{1056} KEFL***ALASSSLLKIGQETDKTTTRNRESVYLLLDMIVQESPFLTMD LLESCFPYVLLRNAYHAVYKQSVTSSA***
Shtn1	Lost	A0MZ67 (IPI00951029)	^{1}LKLLTSGKSQAI.....^{387}PETGSEVHDLARERPEENISRVRKAYIYR PFNQQARQTGKPDS ..... ^{444}LAS***T***
	Higher	A0MZ67 (IPI00855232)	^{10}LQLITSLKEQAI.....^{396}PETAEEVTDLKRQAVEEMMDRIKKGVHLR PVNQTARPKAKPDS ..... ^{453}LAS***Q***
Sptan1	Lost	P16086 (IPI00209258)	^{998}NSTNKARHKAQVND.....^{1050}DNQTRITKEAGSVSLRMKQVEEL YQS..... ^{2229}TYLLDG - SCM .....
	Exclusive	P16086 (IPI01007611)	^{998}NSTNKDWWKVEVND.....^{1050}DNQ - - - - - - - - - - - - - - - - - - - YQS..... ^{2209}TYLLDGIAYRRVIRVYQYEVGDDLSGRSCM .....
Synj1	Lower	Q62910 (IPI00231602)	^{1137}KEFGGVGAPPSPGVTRREMEAPKS .....
	Higher	Q62910 (IPI00231605)	^{1137}KEFG - - - - - - - - - - - - - - - - APKS .....
Tpm3	Lower	Q63610 (IPI00475634)	^{149}LAESKCSELEEELKNVTNNLKSLEAQAEKY..... ^{218}DDLEERLYSQLERNRLLSNELKLTL ..... ^{241} TL***HGLCD -***
	Higher	Q63610 (IPI00372259)	^{149}LAESRCREMDEQIRLMDQNLKCLSAAEEKY..... ^{218}DDLEDKLKCTKEEHLCTQRMLDQTL ..... ^{241}TL***LDLNEM***

CHAPTER III

Aβ Responsive Neuronal Phosphoproteome

Aβ is a key peptide cleaved from the APP protein and is intrinsically associated with AD. This peptide exhibits neurotoxic effects and can trigger several cellular events; from cellular stress to alterations in signal transduction mechanisms, among them protein phosphorylation. In fact, Aβ can induce Tau phosphorylation. Furthermore, previous work from the laboratory showed that Aβ can inhibit protein phosphatase 1 (PP1). Given the central role of PP1 in protein phosphorylation, it follows that Aβ can modulate the phosphorylation state of many proteins and that these alterations can be of diagnostic value in AD. This is relevant given that cells exposed to Aβ have been used as an AD *in vitro* model. Manuscript 3 presents the data obtained when Aβ is used to indirectly inhibit protein phosphatases.

The approach used when exposing cells to OA (manuscript 2) and Aβ (manuscript 3) converged on protein phosphatase inhibition, thus permitting the identification of phosphoproteomes under distinct conditions. In the first case, OA is a general inhibitor but nonetheless a good AD model, whereas Aβ is more specific, given that it not only targets protein phosphatases, but it is the main neurotoxic peptide in AD; its use may identify further AD relevant phosphorylated proteins.

Manuscript 3

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Note: Supplementary material is presented at the end of the manuscript.

Manuscript 3

Altered protein phosphorylation as a resource for potential AD biomarkers

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ABSTRACT

The amyloidogenic peptide, Aβ, provokes a series of events affecting distinct cellular pathways regulated by protein phosphorylation. Aβ inhibits protein phosphatases in a dose-dependent manner, thus it is expected that the phosphorylation state of specific proteins would be altered in response to Aβ. In fact, several Alzheimer's disease related proteins, such as APP and TAU, exhibit pathology associated hyperphosphorylated states. A systems biology approach was adopted and the phosphoproteome, of primary cortical neuronal cells exposed to Aβ, was evaluated. Phosphorylated proteins were recovered and those whose recovery increased or decreased, upon Aβ exposure across experimental sets, were identified. Significant differences were evident for 141 proteins and investigation of their interactors revealed key protein clusters responsive to Aβ treatment. Of these, 73 phosphorylated proteins increased and 68 decreased upon Aβ addition. These phosphorylated proteins represent an important resource of potential AD phospho biomarkers that should be further pursued.

INTRODUCTION

The Aβ peptide, derived by proteolytic cleavage (Lee et al., 2002; Vassar, 2004) of the Alzheimer's Amyloid Precursor Protein (APP), is associated with the onset of Alzheimer's disease (AD). Aβ, typically around 40 amino acids long, is produced under basal conditions but in familial AD (Swedish mutation) the rate can increase by almost 10 fold (Citron et al., 1992). Peptides range in length (Chow et al., 2010; Zhang et al., 2012); the longer species are more toxic. Aβ can be deposited as senile plaques (SPs) in AD patients' brains and is generated via the endosomal/lysosomal degradation pathway, but its production can also be associated to the ER and Golgi/TGN (Sisodia, 1992). However, it is not solemnly a toxic peptide, as it appears to have important physiological functions. Aβ is present in the cerebral spinal fluid of AD patients but also of non-demented individuals and in media from neuronal cell cultures (Haass et al., 1992; Nabers et al., 2016; Tamaoka et al., 1997). Further, Aβ appears to be involved in synaptic activity and protect against excessive glutamate release (Kamenetz et al., 2003; Lesne et al., 2005), thus it is involved in excitability and neuronal survival. Other functions include monitoring cholesterol transport (Igbavboa et al., 2009) and it may even have a role as a transcription factor (Lahiri and Maloney, 2010).

Protein phosphorylation is a key mechanism and regulates many cellular processes. Consequently, abnormal protein phosphorylation has been linked to numerous human diseases including AD. Both APP and TAU phosphorylation have been associated with this dementia (da Cruz e Silva and da Cruz e Silva, 2003; Oliveira et al., 2015). TAU, in a hyperphosphorylated state, forms neurofibrillary tangles (NFTs) which can deposit in the brain (Di Domenico et al., 2011). In AD other brain proteins such as neurofilaments, MAP1B, dynein, CRMP-2, β-tubulin and β-catenin are also hyperphosphorylated. Consistently, in AD, kinase activities and/or expression can be increased (GSK3β, CDK5, ERK1/2, JNK, p38MAPK). Likewise, protein phosphatase (PP) activities and/or expression can be decreased (PP1, PP2, and PTEN - phosphatase and tensin homolog) (Chung, 2009). Taken together, it is evident that protein (de)phosphorylation mechanisms are dysregulated in AD.

Aβ links many AD related anomalies. It can activate Src family protein kinases, activate phosphatidylinositol 3-kinase (Luo et al., 1996) and the cAMP response element-binding protein phosphorylation (Sato et al., 1997). Aβ mediated changes can result in phosphorylation of neuronal proteins, and contribute to the critical early AD pathogenic events, culminating in neuronal death and neurodegeneration (Williamson et al., 2002). Furthermore, Aβ is directly involved in stimulating

kinases (Huang et al., 2010; McDonald et al., 1998; Otth et al., 2002) and inhibiting PP1 and PP2 activities, in a dose-dependent manner (Vintém et al., 2009). It is therefore not surprising that Aβ prompts the production of NFTs via mediating the expression levels and/or activities of TAU protein kinases and phosphatases (Henriques et al., 2010; Huang et al., 2010).

Clearly Aβ affects distinct cellular pathways many of which are regulated by reversible protein phosphorylation. In the work herein described primary neuronal culture lysates were collected upon Aβ exposure, enriched for phosphorylated proteins and the latter identified by mass spectrometry analysis. The approach revealed a series of phosphorylated proteins, whose levels were significantly altered upon Aβ exposure, across experimental sets. The proteins identified are strong biomarker candidates, and the networks presented reveal novel protein relationships and signalling cascades, relevant to unraveling the molecular basis of AD.

METHODS

Neuronal primary cultures

Primary cortical neuronal cultures were prepared from Wistar Hannover rat embryo at 18th day of gestation as previously described (Henriques et al., 2009a). Briefly, cerebral cortex was dissected and dissociated with trypsin (0,23 mg/mL) and desoxyribonuclease I (0,15 mg/mL) in Hanks balanced solution (HBSS). Cells were then plated onto poly-D-lysine coated dishes at 6×10^6 cells/100 mm density in Neurobasal medium (Gibco) supplemented with a serum-free medium combination of B27 (NB-B27), glutamine (0,5 mM) and gentamicin (60 µg/mL). Cells were maintained in an atmosphere of 5% CO_2 at 37 °C and experiments were carried out on the 10th day of primary neuronal cultures *in vitro*.

All experimental procedures followed the European legislation for animal experimentation (2010/63/EU) and no specific ethics approval under EU guidelines was required. This is within the European law (Council Directive 86/609/EEC) and during the procedure all steps were taken to ameliorate animal suffering. Procedures were approved and supervised by the Institutional Animal Care and Use Committee: Comissão Responsável pela Experimentação e Bem-Estar Animal (CREBEA).

Aβ treatment and phosphorylation mimicking conditions

$A\beta_{1-42}$ peptide (American peptide) was aggregated in PBS for 48h at 37 °C (100 µM aggregated stock) as previously described (Henriques et al., 2014). After aggregation, Aβ was added to primary neuronal cultures (Figure III-1), as a 10 µM solution in NB-B27 medium, for 3 h. Cell lysates were collected, and the samples were processed for phosphoprotein enrichment as described below.

Phosphoprotein enrichment

Phosphoprotein enrichment was performed using phosphate metal affinity chromatography (TALON® PMAC Phosphoprotein Enrichment Kit, Clontech) columns, which allows for the selective binding of proteins that contain a phosphate group on any amino acid (including serine, threonine or tyrosine), according to the manufacturer's instructions. Briefly, after the specified treatments, cells were washed with PBS, scrapped, centrifuged at 500 g for 5 min and the resulting pellet was frozen at −80°C. Extraction/loading buffer was added to each sample according to pellet weight (30 µL buffer A/1mg of pellet), supplemented with sodium fluoride (a phosphatase inhibitor) to a final concentration of 10 mM. The samples were then incubated at 4°C for 10 min and centrifuged at 10,000 g, for 20 min at 4°C. In parallel, columns were washed with distilled water and twice with extraction/loading buffer to equilibrate the columns.

The supernatants obtained by centrifugation (total phosphoproteins extracts) were added to the columns and shaken at 4 °C for 20 min, for phosphoprotein binding. After 4 washes, phosphorylated proteins were eluted using 1 ml of buffer B, for 4 times. From the 4 protein fractions obtained, fraction 2 contained the most enriched fraction, as determined by BCA assay (pierce). Samples were stored at −80°C until lyophilization for MS analysis (Figure III-1).

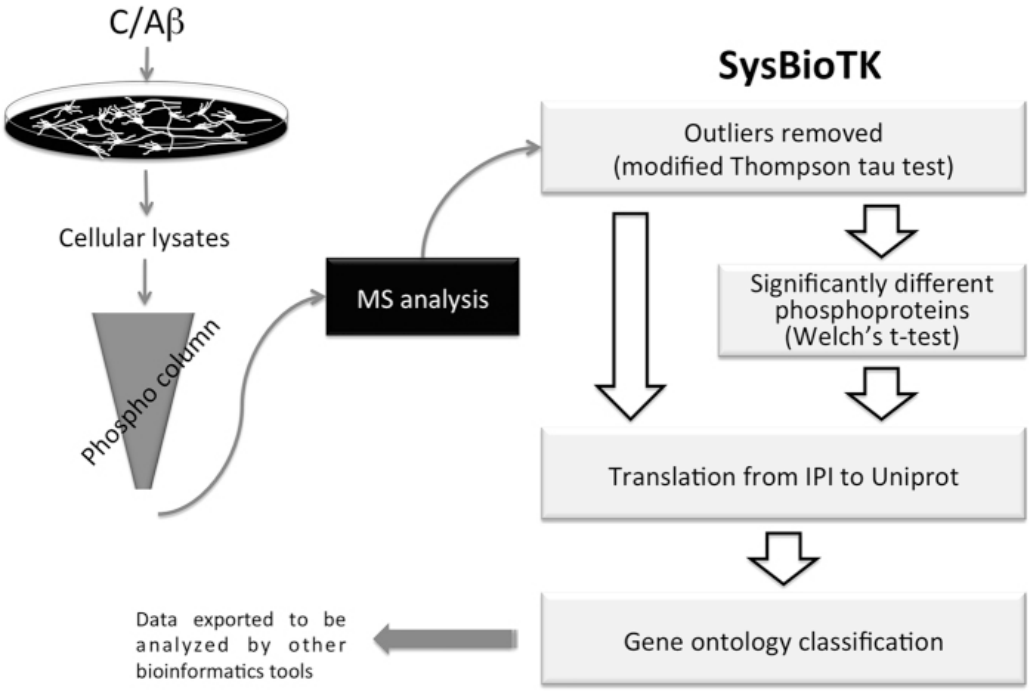

Figure III-1. Workflow. The workflow describes sample processing and analysis. Primary rat neuronal cortical cultures were treated with Aβ, the lysates were collected and the phosphorylated proteins enriched in the phospho column. The eluted peptides were analyzed by mass spectrometry and the resulting data handled by the SysBioTK.

MS/MS Analysis and protein identification

Lyophilized samples were dissolved in lithium dodecyl sulfate (LDS) buffer, incubated at 95°C for 10 min, sonicated and loaded onto a one-dimensional polyacrylamide gel (1DE) system. Trypsin digestion of proteins to peptides occurred after 1DE staining with Coomassie blue and excision of the protein bands. Peptides were extracted from the gels using trifluoroacetic acid (TFA) 0,1% + ACN (50:50), and the resulting supernatant dried in SpeedVac, ressuspended in TFA 0.1% and stored at −20°C. Proteolytic samples were injected in the Q Exactive-Orbitrap LC-MS/MS System (Thermo Scientific) and MS/MS spectra data acquisition followed by phosphoprotein identification (Nensa et al., 2014; Schrotter et al., 2013; Schrötter et al., 2012), using proteome discovery software (Thermo Scientific). Semi-quantitative analysis of the data was carried out using the International Protein Index (IPI) database for protein search and *Rattus novergicus* as the organism model. A false discovery rate of 1% was applied. A single phosphopeptide identification was set to be sufficient for phosphoprotein identification. Further data analysis was done similar to the black-and-white method used before (Nensa et al., 2014; Schrotter et al., 2013). Phosphoproteins resulting from each of the experiments were analysed as described below.

Bioinformatic and Statistical Analysis for phosphoprotein characterization using the SysBioTK library

The Systems Biology Toolkit (SysBioTK) library was employed to analyse the data from each of the experimental conditions. For details see da Cruz e Silva, 2016 (submitted), available at https://bitbucket.org/CrisXed/sysbiotk. For the statistical analysis, each iteration of the experiment is considered a dataset. As a first step, the yield in terms of number of proteins for each dataset was used to identify the outlier datasets, using the modified Thompson tau, τ, test with a confidence level of 95%. Subsequently, the protein IPI accession numbers were converted to the corresponding UniProt accession numbers. For this process, the cross references from the IPI database were used to identify the UniProt/Swiss-Prot accession numbers corresponding to each IPI accession number. For IPI accession numbers not identified, the SysBioTK BLAST+ parser was employed, with a similarity parameter of 0.90. The utility performs a blast search against a subset of the UniProt database (Swiss-Prot accession numbers, organism rattus), effectively identifying UniProt/SwissProt accession numbers corresponding to the IPI accession numbers. The process was then repeated, starting with the cross references, but against the UniProt/TrEMBL databases and only for the IPI accession numbers without a corresponding UniProt/SwissProt accession number. The analysis was carried out on the 27th of September 2015.

The datasets were grouped into two groups, one for each experimental condition (control and exposure to Aβ). The SysBioTK statistical analysis was employed to identify, between the two groups, which accession numbers were significantly different. For this purpose, the tool employed Welch's t-Test with a confidence level of 95%. Consequently, two protein lists were obtained; those where the retrieval of the phosphoprotein showed a significant decrease upon addition of Aβ ("lower" phosphoproteins), and those where the retrieval of the phosphoprotein showed a significant increase upon addition of Aβ ("higher" phosphoproteins). To produce the interacting networks, accession number lists were submitted to IntAct on the 29th of October 2015 and the information was loaded into Cytoscape 3.3.0 (Shannon et al., 2003). Cluster analysis was carried out using the Cytoscape plugin clusterMaker (GLay). Significantly different phosphoproteins were also submitted to STRING, on the 3rd of December 2015, and resulting interactions were plotted using Cytoscape 3.3.0.

RESULTS

Enrichment of the phosphoproteomes

Primary neuronal cultures were exposed to Aβ and phosphorylated proteins enriched using the phospho column, were subjected to mass spectrometry analysis (Figure III-1). For all peptides obtained an accession IPI number was attributed. Although it was not the primary aim to identify specific phosphorylation sites, these were nonetheless readily detected. As examples, for the Tau protein (Mapt gene), three phosphorylated peptides (phosphopeptides) were identified (Supplementary Figure III-1) and one phosphorylated peptide was identified for GAPDH accession number IPI (Supplementary Figure III-2).

The data obtained from the mass spectrometry was subsequently analysed using an informatics library (SysBioTK – Systems Biology Toolkit), specifically developed for this purpose (https://bitbucket.org/CrisXed/sysbiotk). An essential capacity of this platform is to translate accession number IPI to UniProt. The resulting accession numbers and corresponding gene lists were either handled by the SysBioTK (da Cruz e Silva, 2016 submitted), or submitted to other open access analysis tools.

Six experiments were carried out, to evaluate the phosphorylated proteins under basal conditions and upon Aβ exposure. As an initial step, all experimental datasets were analyzed for outliers by the modified Thompson tau, τ, test, (Figure III-1) and one of the experiments from the control condition was removed (Supplementary Table III-1).

Gene Ontology analysis

Under the experimental conditions implemented (Supplementary Table III-1), 986 phosphoproteins were recovered following Aβ exposure (GpAβ–groupAβ) and 870 phosphoproteins under control conditions (GpC–groupControl).

The GpC and GpAβ phosphatomes were analyzed with respect to Gene Ontology (GO) (Figure III-2). The distribution of the phosphorylated proteins across Molecular Functions and Biological Processes, for both GpAβ and GpC is similar. Top Biological Processes with the greater number of phosphorylated proteins are ‘‘anatomical structure development’’, ‘‘transport’’, ‘‘cellular nitrogen compound metabolic’’, ‘‘cell differentiation’’ and ‘‘signal transduction’’. Top Molecular Functions identified are ‘‘ion binding’’, ‘‘RNA binding’’ and ‘‘enzyme binding’’. There are no dramatic shifts or the appearance of novel Biological Processes or Molecular Functions upon Aβ treatment, but in global terms the number of phosphorylated proteins increased marginally across all the GO

categories. This is consistent with Aβ promoting protein phosphorylation and inhibiting protein dephosphorylation.

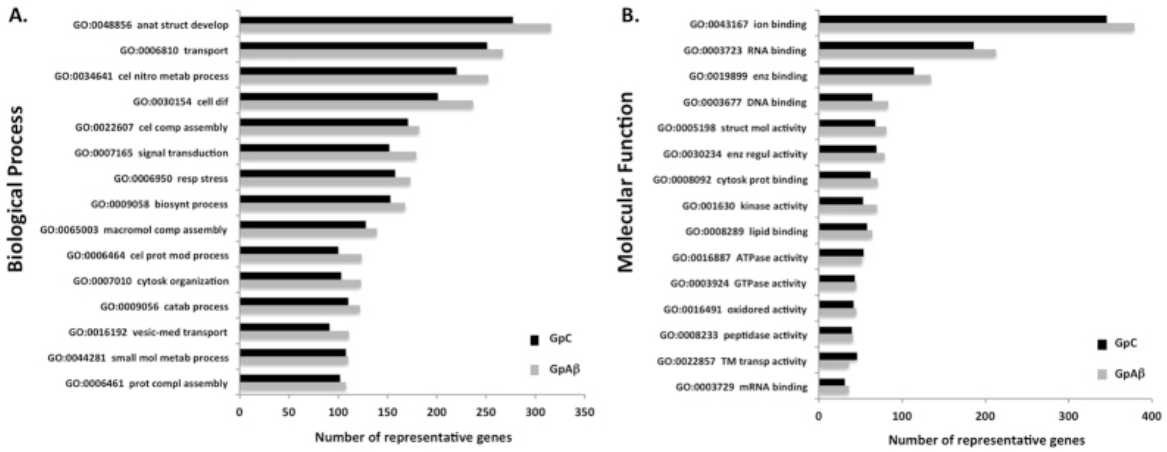

Figure III-2. Gene Ontology of the phosphatomes. Genes encoding the eluted phosphorylated proteins under basal (GpC, black bars) conditions and upon Aβ addition (GpAβ, grey bars) were analyzed with respect to their Gene Ontology (GO) using the SysBioTK for Biological Process and Molecular Function. Abbreviations of GOs not in full: GO:0048856 anatomical structure development; GO:0034641 cellular nitrogen compound metabolic process; GO:0030154 cell differentiation; GO:0022607 cellular component assembly; GO:0007165 signal transduction; GO:0006950 response to stress; GO:0009058 biosynthetic process; GO:0065003 macromolecular complex assembly; GO:0006464 cellular protein modification process; GO:0007010 cytoskeleton organization; GO:0009056 catabolic process; GO:0016192 vesicle-mediated transport; GO:0044281 small molecule metabolic process; GO:0006461 protein complex assembly; GO:0019899 enzyme binding; GO:0005198 structural molecule activity; GO:0030234 enzyme regulator activity; GO:0008092 cytoskeletal protein binding; GO:0016491 oxidoreductase activity; GO:0022857 transmembrane transporter activity.

Deciphering the Aβ induced phosphointeractome

The GpAβ gene list was submitted to IntAct, the interacting proteins identified and the phosphointeractome analyzed using Cytoscape 3.3.0 (Figure III-3 A). Data output from IntAct identifies the gene (represented in italics in the text), even though the experimental procedures yielding the phosphoproteins and the mass spectrometry data are identifying proteins (normal font in the text). In the resulting Aβ induced phosphointeractome (Figure III-3 A) the light grey nodes denote the genes corresponding to the recovered phosphorylated proteins and the dark grey nodes their interactors; identified in IntAct. Of note, phosphorylated TAU (*Mapt*) and phosphorylated APP were recovered (Figure III-3 A). In the phosphointeractome two major clusters are readily evident and have as central nodes *Slc2a4* and *Mapk3*. App connects to *Slc2a4* via the *Tnf* node. The former is involved in glucose transport and the latter encodes a serine/threonine kinase.

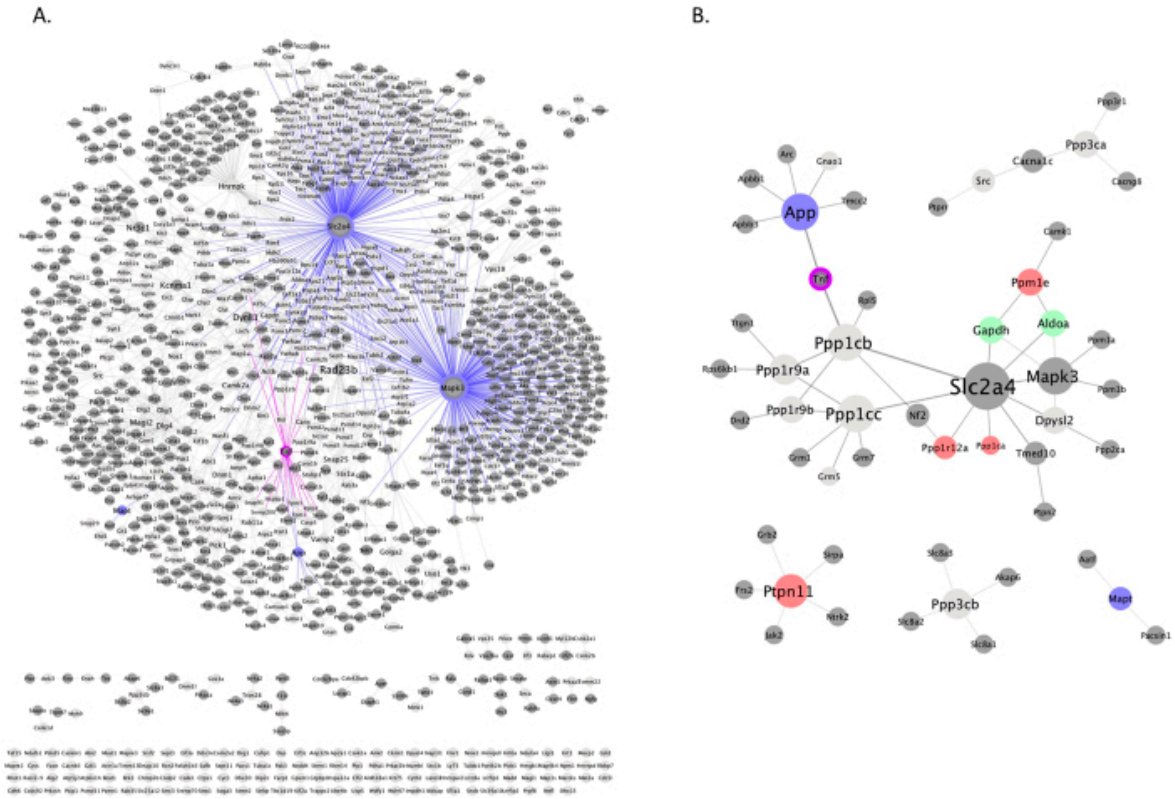

Figure III-3. Aβ phosphointeractome. The Aβ responsive phosphointeractome is depicted in (A). To obtain the phosphointeractome the phosphorylated and significantly different proteins, recovered experimentally upon Aβ addition, were submitted to IntAct and the interactome produced using Cytoscape 3.3.0. Cluster analysis was carried out using the Cytoscape plugin clusterMaker (GLay). The light grey nodes correspond to the phosphorylated proteins recovered in the experimental procedures and the dark grey nodes their interactors, as identified in IntAct. Mapt (encodes the TAU protein) and App are represented as blue nodes and the corresponding edges are colour coded also in blue. Two major clusters are identified; with the central nodes Slc2a4 and Mapk3 with the circumference and edges in blue. App connects to the Slc2a4 cluster via the Tnf node (circumference in purple). **A phosphatase sub network is represented in (B).** (B) was extracted from (A) by selecting phosphatases, App, Mapt and their direct interactors. Bright red nodes were detected only upon Aβ addition (not under basal conditions) and light green nodes represent proteins whose phosphorylation levels decreased significantly upon Aβ addition.

Aβ modulates a phosphatase sub network

Given the Aβ phosphatase inhibitory role and the importance of phosphatases in AD pathology (Braithwaite et al., 2012; da Cruz e Silva and da Cruz e Silva, 2003; Vintém et al., 2009), a sub network with respect to protein phosphatases, TAU and APP was elaborated. From the interactome in Figure III-3 A, the nodes for phosphatases, *App*, *Mapt* and their direct interactors were extracted, and the sub-network was plotted using cytoscape (Figure III-3 B). It is noteworthy that two central nodes, *Slc2a4* and *Mapk3* (dark grey nodes, Figure III-3 B) are sustained in both networks (Figure III-3 A and B) and bind directly to proteins whose levels of phosphorylation alter significantly

following Aβ exposure. In particular, the recovery of phosphorylated GAPDH and ALDOA proteins, decreased upon Aβ addition.

In contrast several phosphorylated proteins appeared "*de novo*" upon Aβ addition. Two experimentally recovered, protein phosphatases, PPM1E and PTPN11 (Figure III-3 B), were found in the GpAβ but not in the GpC (bright red nodes). PTPN11 phosphorylation increased significantly upon Aβ addition and in turn it interacts with another five phosphorylated proteins recovered in the experimental procedures employed.

Furthermore serine/threonine protein phosphatases, and their regulators, appear to be hub nodes in the network depicted in Figure III-3 B. *Ppp1ca*, *Ppp1cb* and *Ppp1cc* code for serine/threonine-protein phosphatase 1 (PP1) catalytic subunits α, β and γ, respectively. Significantly, PP1α (*Ppp1ca*) is recovered only in GpAβ but not in GpC. That is, phosphorylated peptides for PP1α were identified only when Aβ was added to the primary neuronal cultures (Figure III-3 B and Table III-1). Peptides for this protein were absent in the mass spectrometry analysis of the GpC, suggesting that phosphorylated PP1α is preferentially found upon Aβ exposure. Activity of these phosphatases is regulated by regulatory subunits of which three were recovered; PPP1R9A, PPP1R9B and PPP1R12A. As mentioned, the latter is a PP1 regulatory subunit recovered in GpAβ but not in GpC.

Ppp3ca and *Ppp3cb* are serine/threonine-protein phosphatase 3 catalytic subunits α and β (PP2B). PP2B is a phosphatase abundantly expressed in the CNS and implicated in AD pathology. It is interesting to note that both catalytic subunits were recovered in GpAβ and in GpC (Figure III-3).

Of the above-mentioned serine/threonine protein phosphatases, and their regulators, only protein phosphatase PPP1CA and the phosphatase regulatory subunit PPP1R12A increased significantly, precisely by being recovered in their phosphorylated species in response to Aβ exposure. The latter are direct interactors of *Slc2a4*.

Table III-1. Significantly different recovery rates of phosphoproteins upon Aβ addition. Upon Aβ addition the number of phosphorylated proteins recovered decreased significantly ("lower" Phosphoproteins) while others increased ("higher" Phosphoproteins). The genes encoding these significantly different proteins were identified using the SysBioTK (Welch's t-test). Genes underlined correspond to the same protein recovered under both conditions, although different identifiers were involved (see Supplementary Table III-2). Genes in bold and italics correspond to phosphoproteins present under control conditions but absent (Aβ "lost") upon Aβ addition and genes in bold correspond to phosphoproteins detected only upon addition of Aβ (Aβ "exclusive").

'Lower' Phosphoproteins			*'Higher' Phosphoproteins*		
Actn1	Hspa12a	Rbm14	**Abi2**	**Gtpbp1**	Psip1
Aldh18a1	Hspd1	***Rhot1***	Ap2s1	Hexa	Psmb6
Aldoa	Krt14	Rpn1	**Ap3b2**	**Hk1**	**Psmd13**
Alg2	Krt75	Ruvbl2	Atp6v1h	**Idh3B**	**Ptpn11**
Ap2a2	Lancl2	Sfxn1	**Bcat1**	**Ikbkap**	**Rab11a**
Apc	Lasp1	Slc25a12	**Bid**	**Ilf3**	**Rab35**
Atp5f1	Llgl1	***Slc39a10***	**Camk2d**	**Impdh1**	**Rab6a**
Atp5j2	***Ly75***	***Slc3a2***	Cdc5l	Itsn1	Ran
Atp6v1b2	***Magi1***	Soga3	**Cdh6**	**Lrrc8a**	Rtcb
Atp6v1e1	Magi2	Sptbn1	Clip2	**Lrrfip1**	**Sf3a1**
Brk1	Map1s	Stmn2	**Cndp2**	**Lrrfip2**	Slc25a22
Cand1	Map4k4	Strbp	Csde1	**Madd**	**Smc3**
Ccdc92	Marcks	***Tf***	**Ctps1**	Nap1l1	**Snrnp70**
Cdc42bpb	***Mat2a***	Tmx1	**Ddx3y**	*Ncam1*	**Snw1**
Cfl2	Mdh2	Tpm1	**Dhx30**	**Nucb2**	*Sptbn1*
Chmp2b	Ncam1	Uba1	**Dip2c**	**Numbl**	*Strbp*
Cyc1	Ndufs1	Usp5	**Dynlrb1**	**Pabpc1**	**Tbc1d19**
Dbnl	Pfkm	Wdr47	Eci1	**Pc**	**Tceb1**
Dctn1	Prkcsh		Eef1a1	**Pdcd5**	**Timm13**
Erp29	Psmb5		Eef2	Picalm	**Trappc2**
Farp1	Psmd11		**Ehd1**	**Pip5k1c**	**Ube4b**
G3bp2	Psme1		**Ehd3**	**Plcg1**	Wdfy1
Gapdh	***Rab10***		**Enah**	**Ppm1e**	*Wdr47*
Gls	***Rasl2-9***		Epn2	**Ppp1ca**	
Gpsm1	***Rbbp7***		**Fmr1**	**Ppp1r12a**	

Analysis of Aβ induced phosphointeractome

Using the SysBioTK, phosphoproteins recovered in GpAβ were compared with those recovered in the GpC. One hundred forty-one phosphoproteins that significantly change across experimental sets were identified. Phosphorylated proteins whose recovery rate was significantly "higher" (increased) or "lower" (decreased) were identified, herein designated as "higher" or "lower" phosphoproteins. To summarize 73 phosphoproteins were "higher" and 68 "lower", upon Aβ addition (Table III-1). Within the "lower" phosphoproteins, 19 were absent in the GpAβ (Aβ "lost"), and this was significant when compared to the GpC. In contrast 50 phosphoproteins were recovered only in conditions where Aβ was added (Aβ "exclusive"), again these were significantly different across experimental sets, applying the Welch's t-test (Figure III-1).

The GO of significantly different "higher" and "lower" phosphoproteins were analyzed for Biological Process (Figure III-4). The top groups, for Biological Processes, are those where the "higher" phosphoproteins are greater than the "lower" phosphoproteins, the net effect is an increase in phosphorylated proteins. Top functions are consistent with functions described for APP and furthermore, have been associated with AD, for example signal transduction and vesicle-mediated transport.

The middle group of Biological Processes, are those where the "lower" phosphoproteins are greater than the "higher" phosphoproteins, the net effect is a decrease in phosphorylated proteins (Figure III-4). Top functions within this group, include small molecule metabolic processes, cytoskeletal organization and transmembrane transport, these processes have also been associated with AD.

In the bottom group (Figure III-4), the number of "higher" and "lower" phosphoproteins is similar. However, although the number of proteins is sustained, the proteins are different. To better understand the underlying molecular processes, proteins were analysed as described below.

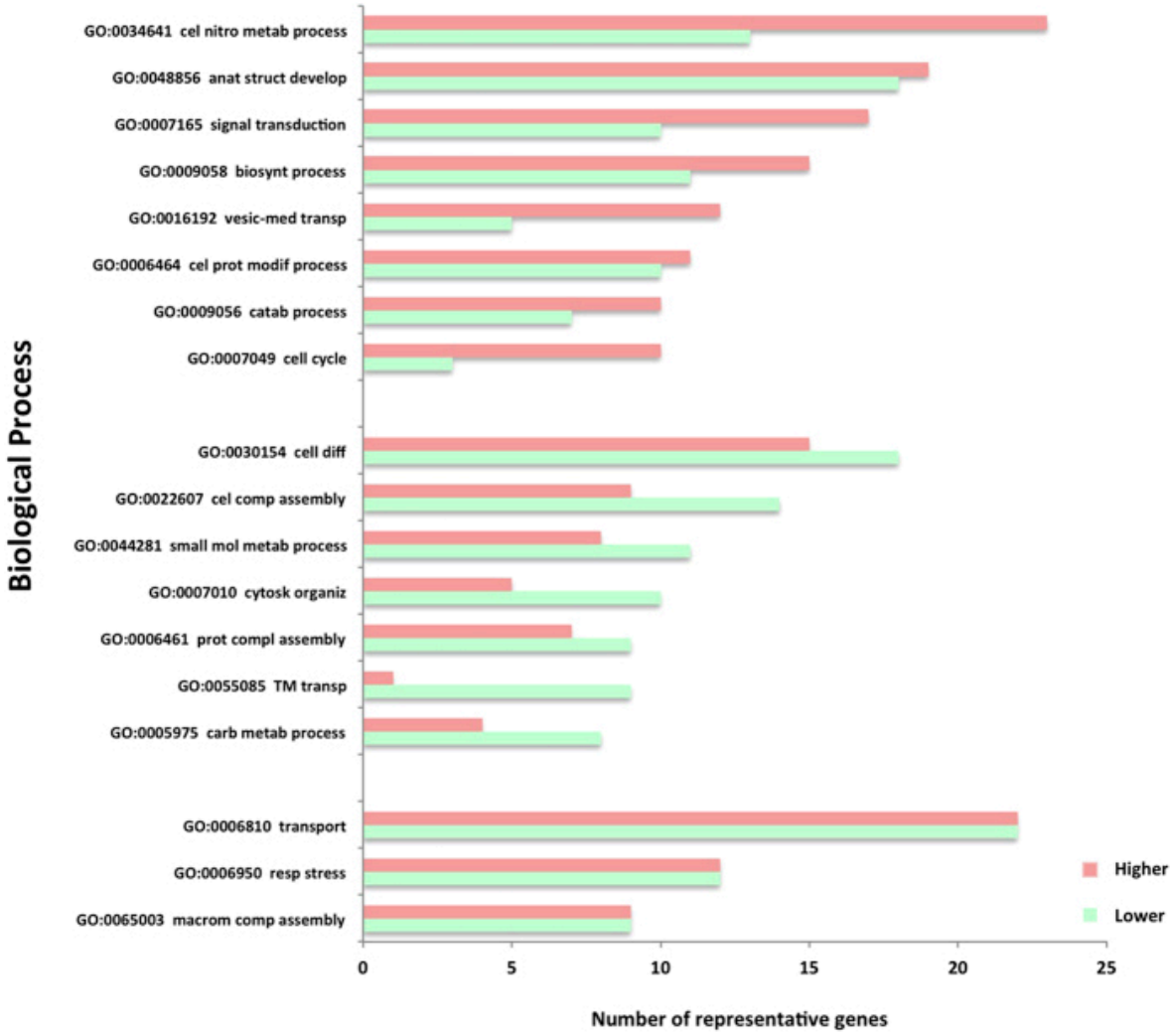

Figure III-4. Gene Ontology of significantly different phosphorylated proteins. The two sets of proteins (GpC and GpAβ) were grouped with respect to their Gene Ontology using the SysBioTK for Biological Process. Red bars represent the phosphorylated proteins whose recovery increased upon Aβ addition "higher" phosphoproteins). Green bars represent the phosphorylated proteins whose recovery decreased upon Aβ addition ("lower" phosphoproteins). Abbreviations of GOs not in full: GO:0034641 cellular nitrogen compound metabolic process; GO:0048856 anatomical structure development; GO:0007165 signal transduction; GO:0009058 biosynthetic process; GO:0016192 vesicle-mediated transport; GO:0006464 vesicle-mediated transport; GO:0006464 cellular protein modification process; GO:0009056 catabolic process; GO:0030154 cell differentiation; GO:0022607 cellular component assembly; GO:0044281 small molecule metabolic process; GO:0007010 cytoskeleton organization; GO:0006461 protein complex assembly; GO:0055085 transmembrane transport; GO:0005975 carbohydrate metabolic process; GO:0006950 response to stress; GO:0065003 macromolecular complex assembly.

Interactome of significantly different phosphoproteins upon Aβ exposure

From the Aβ phosphointeractome (Figure III-3 A), a simplified network was developed using subsets of nodes (Figure III-5). The following classes of nodes and their direct interactors were selected; genes corresponding to significantly "higher" and "lower" phosphoproteins identified across experimental sets (listed in Table III-1), as well as those from the GpAβ with the GO term "protein phosphatase". The nodes *App* and *Mapt* were also included. The interactions were plotted as a network using Cytoscape 3.3.0 (Figure III-5 and Supplementary Table III-2). Bright green nodes correspond to Aβ "lost" phosphoproteins and bright red nodes to Aβ "exclusive" phosphoproteins comparative to Control conditions. Fifteen clusters were identified with the Cytoscape plugin clusterMaker (GLay).

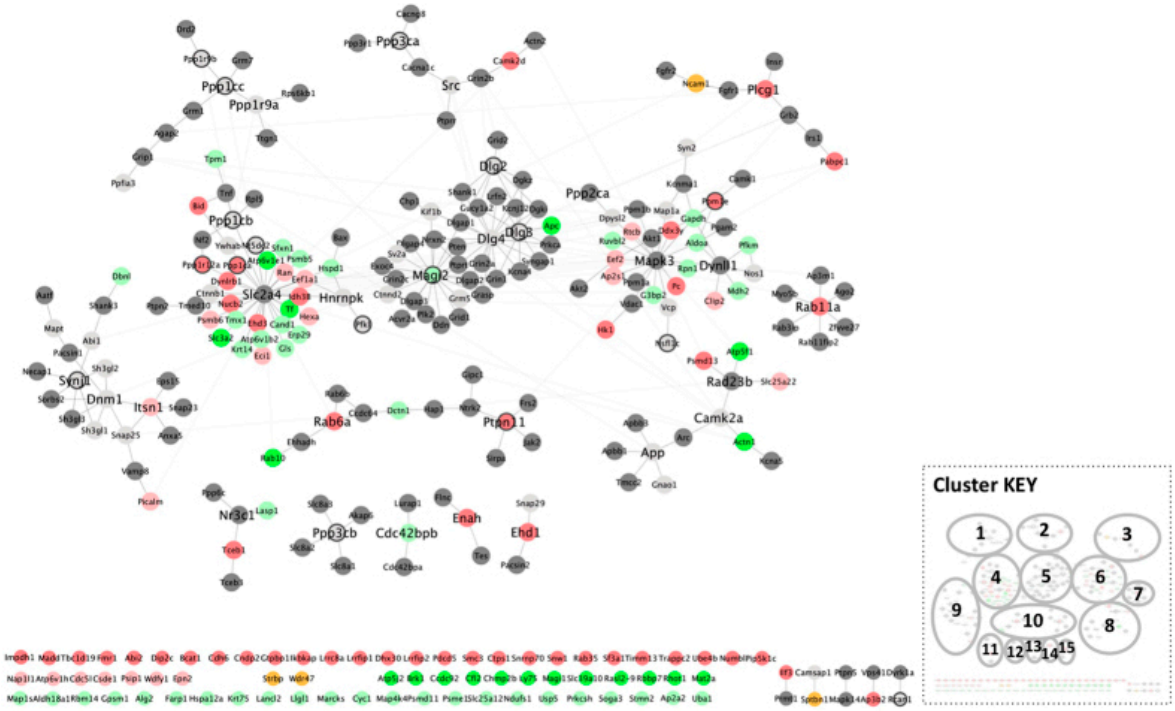

Figure III-5. Aβ induced phospho network. Figure III-5 represents a subset from Figure III-3, retaining the nodes of significantly different phosphoproteins, as well as those with the term "protein phosphatase" in their GO (black contour). Directly interacting nodes of the latter were maintained. The interacting proteins, that were identified in the experimental set up are coloured light grey; other interacting proteins are dark grey. Dark yellow nodes represent proteins that both increased and decreased upon Aβ addition (see Supplementary Table III-3). "Higher" phosphoproteins are light red and phosphoproteins recovered only in conditions where Aβ was added (Aβ "exclusive") are bright red. "Lower" phosphoproteins are light green and phosphoproteins not recovered upon Aβ addition (Aβ "lost") are bright green. The network was produced using Cytoscape 3.3.0 and cluster analysis was carried out using the Cytoscape plugin clusterMaker (GLay).

Unaltered phosphoproteins as central nodes

Clusters 1 (*Ppp1cc*), 2 (*Ppp3ca*) and 12 (*Ppp3cb*) include phosphatases (Figure III-5 and Supplementary Table III-2) that have already been discussed and whose phosphorylation levels do not alter significantly upon Aβ addition. An exception is the *Ppp3ca* cluster (cluster 2) where CAMK2D exhibits "higher" phosphorylation. Cluster 12 includes nodes *Ppp3cb* and *Slc8a3*, evoking putative functional links to long-term memory potentiation, given the biological function of these proteins. These functions are significantly compromised in AD, and it is relevant that the Aβ induced phospho network includes these nodes (Figure III-5).

Clusters 9 (*Synj1*) and 8 (*Camk2a*) include *Mapt* and *App* respectively (Figure III-5 and Supplementary Table III-2). Other nodes in cluster 9, include *Dnm1, Itsn1, Picalm* and *Dbnl*. ITSN1 and PICALM, revealed "higher" phosphorylation levels upon Aβ addition, in contrast DBNL has "lower" levels.

The central node in cluster 8 is *Camk2a*, crucial for plasticity at glutamatergic synapses. This calcium calmodulin-dependent protein kinase is composed of four different chains: alpha, beta, gamma, and delta. CAMK2A interacts directly with ACTN1, but following Aβ addition the phosphorylated form of the latter is no longer recovered. ACTN1 is involved in the regulation of the actin cytoskeleton. *Atp5f1* is another Aβ "lost" phosphoprotein in this cluster, in contrast proteins encoded by *Slc25a22* and *Psmd13* show "higher" phosphorylated levels in response to Aβ, the latter is Aβ "exclusive".

Cluster 5 (*Dlg2/Dlg3/Dlg4*) includes DLG family members (Figure III-5 and Supplementary Table III-2), which are essential for maintaining synaptic architecture and plasticity. These proteins although well represented as phosphoproteins in the primary neuronal cell cultures, did not alter significantly upon Aβ exposure (dark grey nodes).

"Lower" and "Higher" phosphoproteins as central nodes

The *Dlg* cluster 5 has another central node, *Magi2* (Figure III-5 and Supplementary Table III-2). MAGI2 is a "lower" phosphoprotein also involved in development and maintenance of the synapse. Even though cluster 5 does not have a substantial number of nodes that are significantly altered following Aβ exposure, the phosphorylated protein APC is Aβ "lost". *Cdc42bpb* is another central node (cluster 13) exhibiting "lower" phosphorylation across experimental sets.

Clusters with central nodes that represent "higher" phosphoproteins, are in general terms smaller (Figure III-5 and Supplementary Table III-2); these include clusters 14 (*Enah*), 15 (*Ehd1*), 3 (*Plcg1*), 7 (*Rab11a*) and 10 (*Rab6a*). Plcg1 is involved in regulating intracellular signalling cascades

and in this cluster besides Pabpc1, Ncam 1 also exhibited "higher" recovery of the phosphorylated proteins. Two clusters have as central nodes RAB11a (cluster 7) and RAB6a (cluster 10), these proteins are small GTPases involved in intracellular membrane trafficking. Rab11a is Aβ "exclusive" and *Rab6a* represents likewise an Aβ "exclusive" "higher" phosphoprotein. Another "higher" phosphoprotein identified in the latter cluster is PTPN11, already discussed. In contrast RAB10 (cluster 10) is Aβ "lost" and phosphorylated DCTN1 is significantly "lower".

Bioinformatically identified central nodes

Three clusters 11, 4 and 6, have central nodes, which were not experimentally identified but became evident following the bioinformatics analysis. Cluster 11 (*Nr3c1*) is a small cluster and includes PPP6C, LASP1 a "lower" phosphoprotein and TCEB1 that is Aβ "exclusive". Clusters 4 (*Slc2a4*) and 6 (*MapK3*), with central nodes already discussed, contain the greatest number of significantly different phosphoproteins. Taken together they include three Aβ "lost" phosphoproteins and eleven Aβ "exclusive".

Significantly different phosphoprotein network

Many of the "higher" and "lower" phosphoproteins have been reported to interact, thus the identifiers in Table III-1 were submitted to STRING and further interactions identified (Figure III-6). Nine clusters are particularly evident, with the central nodes *Actn1, Atp6v1e1, Gapdh, Hspd1, Rab11a/Numbl, Ran, Ppp1r12a, Eef2/Sfea1* and *Psmb6*. The first four clusters include predominantly "lower" phosphoproteins and the last five, predominantly "higher" phosphoproteins. Clusters were organized by degree such that the central nodes have the greatest number of edges and thus are the most likely key genes with respect to Aβ induced responses. Hence the proteins they encode represent strong AD biomarker candidates, given that the recovery of the phosphorylated protein across experimental sets was significantly decreased or increased upon Aβ addition. In some cases, the recovery of the phosphoprotein was completely "lost" or "exclusive" following conditions of Aβ addition (bright green and bright red nodes respectively, Figure III-6).

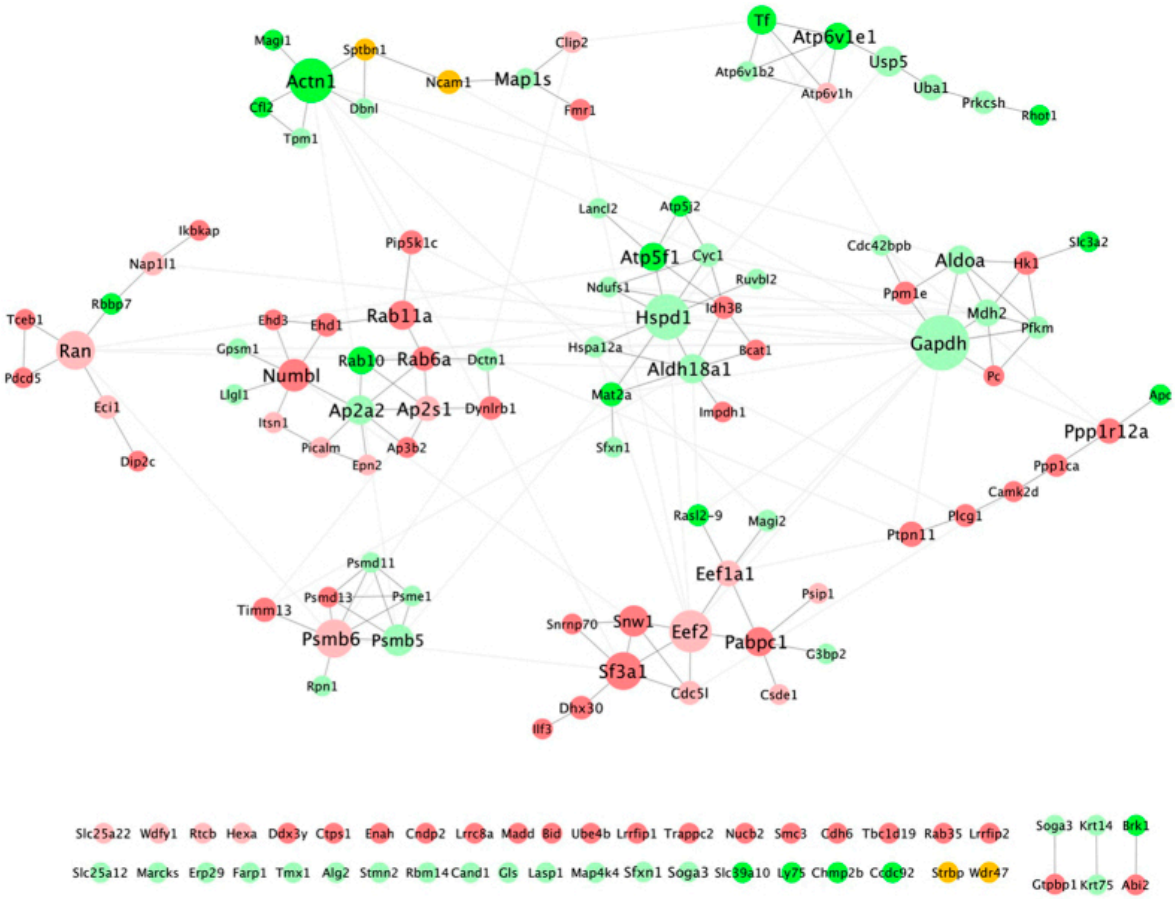

Figure III-6. Aβ induced significantly different phospho network. Protein interactions between phosphorylated proteins, whose recovery significantly increased ("higher") or decreased ("lower") following Aβ addition, were mapped using STRING. "Higher" phosphoproteins are light red and phosphoproteins recovered only in conditions where Aβ was added (Aβ "exclusive") are bright red. "Lower" phosphoproteins are light green and phosphoproteins not recovered upon Aβ addition (Aβ "lost") are bright green. Dark yellow nodes represent the same protein, but distinct identifiers that increased and decreased in response to Aβ addition (Supplementary Table III-3). The network was produced using Cytoscape 3.3.0.

Many of the above-mentioned central nodes have already been discussed, but close analysis of Figure III-6 reveals key cellular processes following Aβ exposure. For example, Aβ induced reduced phosphorylation levels of; the *Actn1* cluster which implies targeting cytoskeletal organization; the *Atp6v1e1* cluster impacting lysosomal, iron transport and ubiquitination processes; and the *Hspd1* cluster which is associated with the heat shock response.

Another cluster includes *Gapdh*, which plays a role in glycolysis and nuclear functions. This cluster has three phosphorylated proteins which are significantly "higher" and five which are significantly "lower" (Figure III-6), among them *Aldoa*; a glycolytic enzyme.

As previously mentioned Aβ causes "higher" *Rab6a* and *Rab11a* phosphorylation, but *Rab10* is Aβ "lost". Another phosphoprotein significantly decreased is AP2A2, a component of the adaptor protein complex 2 (AP2) involved in endocytosis related processes.

The *Ppp1r12a* cluster is particularly interesting from a biomarker standpoint as it includes 5 "higher" Aβ "exclusive" phosphoproteins. This is also the case for the *eEf2* cluster. The latter is a member of the GTP-binding translation elongation factor family and an essential factor for protein synthesis.

DISCUSSION

Identification of significantly "higher" and "lower" phosphoproteins in response to Aβ exposure, proved to be an important approach to providing key biomarker candidates. Analysis of the proteins identified across experimental sets, with respect to Biological Process identified crucial processes that were corroborated, when the functions of the phosphorylated proteins themselves were investigated. Among the most recurring processes are signal transduction, endocytosis, cytoskeletal organization and intracellular transport. Alterations in these cellular events have in turn, all been associated with AD.

The phosphorylation levels of many proteins involved in membrane trafficking changed, as is the case of ITSN1 whose phosphorylation levels increases in response to Aβ. ITSN1 has been implicated in Down's syndrome and AD, possibly via c-JUN N terminal kinase activation (Hunter et al., 2011). It is a cytoplasmic membrane-associated protein involved in endocytic membrane traffic and appears to regulate the formation of clathrin-coated vesicles and to be involved in synaptic vesicle recycling. Furthermore, ITSN1 was shown to interact with AP2 (Pechstein et al., 2010). Interestingly, AP2A2, a component of the AP2 adaptor complex, is a phosphoprotein significantly decreased upon Aβ exposure. This complex is also involved in clathrin-dependent endocytosis and serves as a cargo receptor, selectively sorting the membrane proteins involved in receptor-mediated endocytosis. The AP2A2 gene has been associated with AD (Reitz, 2014). Furthermore, it is noteworthy that the complex AP2/PICALM, interacts with APP directing it to degradation and autophagy (Tian et al., 2013). Given the important roles of endocytosis and Aβ production in AD, genes impacting this process are extremely relevant.

RABs comprise a subfamily of small GTPases also involved in the regulation of several steps during membrane trafficking, including vesicle formation, movement along the cytoskeleton network and fusion at the target membrane. *Rab10* codes for an Aβ "lost" phosphoprotein while

Rab11a, cluster 7 in Figure III-5, is Aβ "exclusive" and is significantly associated with late-onset AD (Udayar et al., 2013). Rab6a is likewise an Aβ "exclusive" "higher" phosphoprotein. RAB6 was shown to regulate intracellular APP processing and trafficking. Furthermore, upregulation of RAB6A in AD is linked to ER stress (Scheper et al., 2007). Aβ can affect the phosphorylation states of many proteins involved in diverse cellular processes and induces stress. As an example, another central cluster includes *Gapdh* and *Aldoa*. GAPDH interacts with APP, and there is significant inhibition of the former in AD (Butterfield et al., 2010). Further, oxidative modification appears to be a relevant neurotoxic pathway in AD cases correlated with GADPH. Alterations of ALDOA have also been associated with AD (Manavalan et al., 2013).

Since APP processing and Aβ production involve intracellular transport and vesicle-mediated transport (Vieira et al., 2009, 2010), one can hypothesize that Aβ may be involved in regulating its own production, via modulating phosphorylation of proteins involved in the above mentioned cellular processes. The peptide was reported to alter APP nuclear signalling (Henriques et al., 2009a) and to impair APP secretion/vesicular anterograde transport and exocytosis, through a mechanism mediated by altered cytoskeleton dynamics of both microtubule and actin networks (Henriques et al., 2009b, 2010).

Indeed, the actin cytoskeleton is also relevant for synaptic remodelling and AD pathogenesis (Henriques et al., 2010). Two cytoskeletal related phosphoproteins whose recovery rate decreased were identified. ACTN1 is a bundling protein and an F-actin cross-linking protein thought to anchor actin to a variety of intracellular structures. It is involved in the regulation of the actin cytoskeleton and is an Aβ "exclusive" phosphoprotein. Reports have associated ACTN1 to AD (Silver et al., 2012). *Cdc42bpb* is a central node (cluster 13) exhibiting "lower" phosphorylation. It regulates actin cytoskeletal organization and is a downstream effector of CDC42, whose activity is increased upon Aβ treatment (Mendoza-Naranjo et al., 2007).

Aβ affected the phosphorylation level of proteins directly involved in synaptic signalling. In particular, MAGI2 is a "lower" phosphoprotein. It is a scaffold molecule at synaptic junctions and assembles neurotransmitter receptors and cell adhesion proteins. It appears to be essential for development and maintenance of the synapse, binding to other scaffold proteins supporting cell junctions (Nagashima et al., 2015). MAGI2 and AD have not been extensively explored but genome-wide association studies placed this gene as a candidate locus in the aetiology of sporadic AD.

Further, several members of the DLG family were also found, even though their phosphorylation did not change significantly in response to Aβ. These proteins can be recruited to the post-synaptic density, where they are essential for maintaining synaptic architecture and plasticity. Both *DLG3*

(encodes SAP-102) and *DLG4* (encodes PSD-95) have been associated with AD; their protein levels are reduced in AD brains (Proctor et al., 2010). Further, given that *DLG3* and *DLG4* encode post-synaptic scaffold proteins, which regulate NMDA receptor synaptic activity and expression, this presents a possible mechanism for aberrant expression in AD. NMDA receptor-evoked excitotoxicity contributes to glutamatergic synapses mediating cognitive decline in AD (Proctor et al., 2010). SYNJ1 is another protein whose phosphorylation levels did not change but its expression is likely to affect synaptic transmission and membrane trafficking. These are also hallmarks of the disease and SYNJ1 was reported to increase in AD (Martin et al., 2014).

Although not found under our experimental conditions, two central genes linking many altered phosphoproteins were identified by the IntAct searches; the *Slc2a4* and *MapK3* nodes. *Slc2a4* encodes a protein that functions as an insulin-regulated facilitative glucose transporter. Altered metabolism of brain glucose has been suggested in diabetes and AD (Shah et al., 2012). Of note, decreased SLC2A4 expression has been observed in adipose tissue from type 2 diabetic patients and diabetes is increasingly associated with AD. *MapK3* encodes a serine/threonine kinase; an essential component of the Map kinase signal transduction pathway. The MAPK/ERK cascade regulates many biological functions, such as cell adhesion, survival, growth and differentiation, regulation of transcription and translation, and cytoskeletal rearrangements. Moreover, this cascade regulates endosomal dynamics, lysosome processing and endosome cycling; these processes, have been associated with APP, TAU and AD. Increased activities and anomalies on MAPK signalling have been closely associated to disease pathology (Kim and Choi, 2010).

The Aβ impact on both kinases and phosphatases is well described. In this work a network of phosphatases whose phosphorylation increases in response to Aβ was recovered. *Ptpn11* (protein tyrosine phosphatase, non-receptor type 11) is a “higher” phosphoprotein that interacts with many genes/proteins likewise altered. Ptpn11 encodes a signalling molecule involved in activation of the RAS/MAPK pathway and STAT signalling pathways. *PTPN11* has been tagged as a hub gene in AD (Liang et al., 2012). One can therefore deduce that phosphorylated PTPN11 should be investigated as a potential AD biomarker. Of note, *Plcg1*, an Aβ “exclusive” protein, can become activated in response to ligand-mediated activation of receptor-type tyrosine kinases and is involved in regulating intracellular signalling cascades. Further, *PLCG1* has been tagged as a hub gene in AD (Liang et al., 2012).

Noticeably, PP1α (*Ppp1ca*) is another phosphatase recovered only in GpAβ. PP1 is an abundant neuronal phosphatase enriched in dendritic spines (da Cruz e Silva et al., 1995; Ouimet et al., 1995) with a key role in synaptic signalling; and it can be inhibited by Aβ. PP1 is required for long-term depression, is involved in memory and learning and has been implicated in TAU dephosphorylation, playing a key role in AD pathogenesis. Interestingly, the activity of phosphatases can be regulated by different regulatory subunits. PPP1R12A is a PP1 regulatory subunit recovered in GpAβ but not in GpC.

Ppm1e encodes a member of the PP2C family of the serine/threonine-protein phosphatases that also exhibits increased phosphorylation. It is brain-specific, involved in synaptic plasticity and dendritic spine morphogenesis and negatively regulates the Ca^{2+}/calmodulin dependent kinases (CaMK) IV and II and the p21-activated kinase (PAK) 1; kinases important in actin cytoskeletal regulation. This phosphoprotein may likewise represent an interesting target in AD pathology.

In conclusion, due to the dynamic nature of protein phosphorylation systems, where the phosphorylation of a given protein can evoke the phosphorylation of further proteins, it is not surprising that a given cluster can have both “higher” and “lower” phosphoproteins. This work clearly showed that Aβ altered the phosphorylation levels of many proteins and this is consistent with the pathophysiological characteristics attributed to Aβ, placing it at the center of AD. From the dataset here presented it was possible to identify 141 putative biomarkers, whose phosphorylated proteins significantly increased (73) or decreased (68) upon Aβ addition across experimental sets. Furthermore 19 phosphoproteins were “lost” upon Aβ exposure and 50 were “exclusive” to Aβ addition. These proteins and their levels of phosphorylation provide a resource as potential phospho biomarker candidates for AD diagnosis and should be pursued in this respect in future studies.

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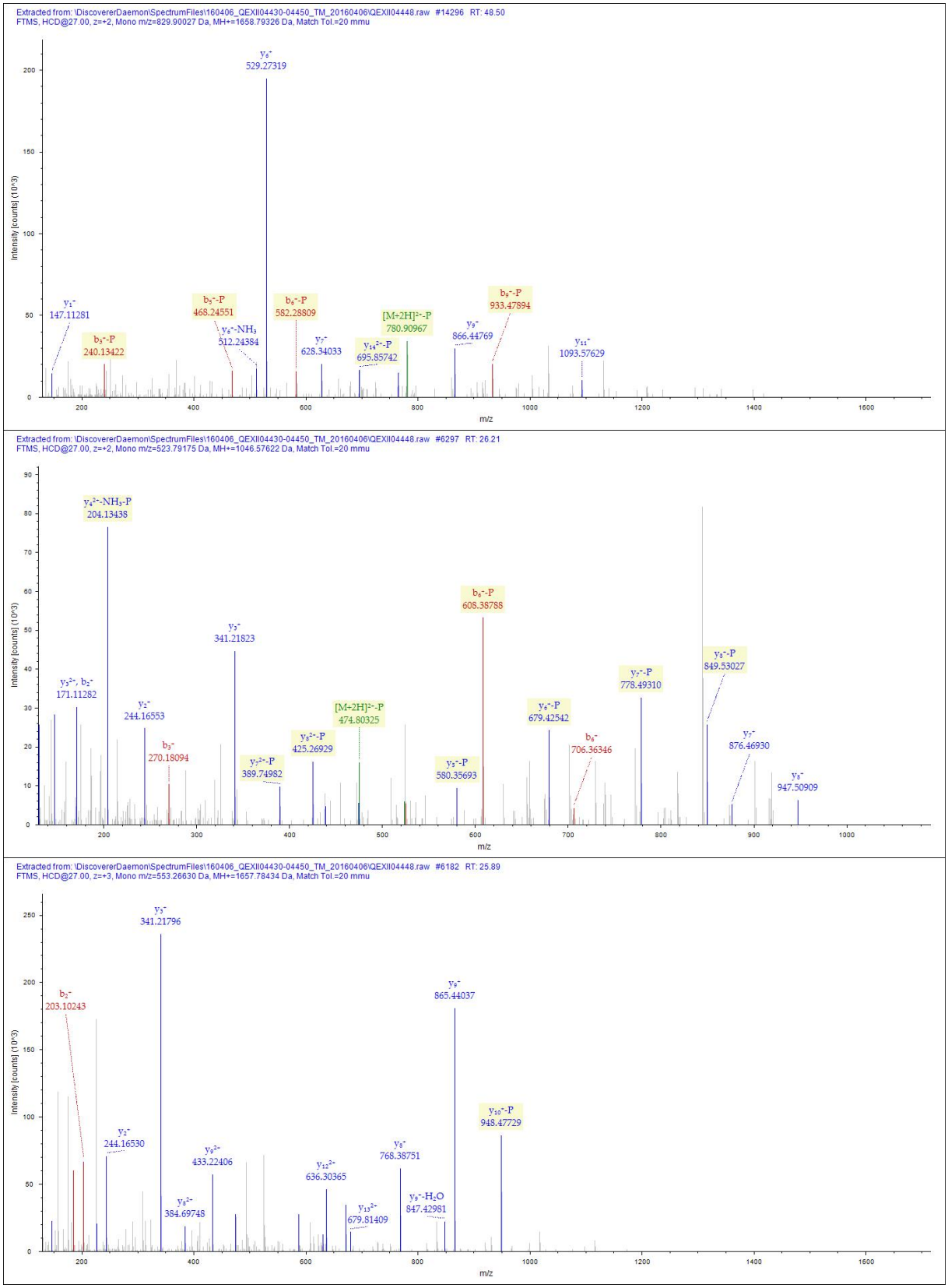
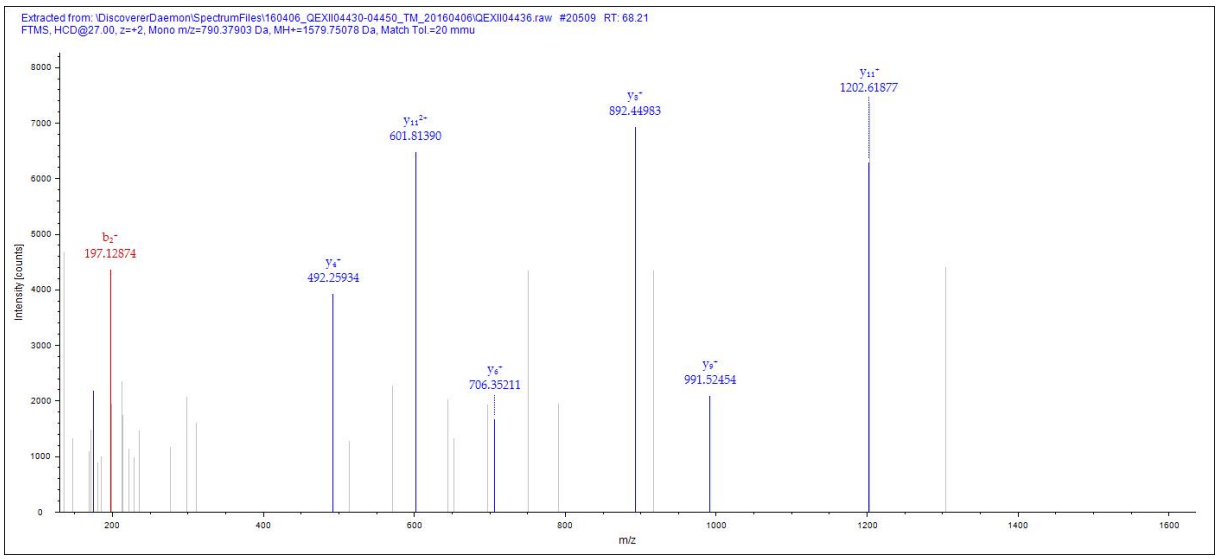
MANUSCRIPT SUPPLEMENTAL MATERIAL



Supplementary Figure III-1. Example of 3 identified phosphopeptides for Mapt. MSMS spectra for peptides IGsLDNITHVPGGGNK, VAVVRtPPK and TTPSPKtPPGSGEPPK are demonstrated.



Supplementary Figure III-2. Example of an identified phosphopeptide for Gapdh. A phosphopeptide VPtPNVSVVDLTCR of GAPDH is shown, where the threonine at residue 3 is phosphorylated (precursor mass m/z=790.37903 Da).

Supplementary Table III-1. Phosphoproteins recovered from phospho columns. The numbers of phosphoproteins (PP) obtained upon addition of Aβ or under basal conditions (PP Control) are indicated for each experimental set (Exp. #). Significantly different results were identified by applying the modified Thompson tau, τ, test and outliers removed (*). The total number of proteins (excluding duplicates) carried forward to subsequent analysis is indicated.

Exp. #	PP Control	PP Abeta
1	505	543
2	1062*	834
3	554	381
4	455	451
5	569	682
6	513	208
Total	870	986

Supplementary Table III-2. Principal clusters in the Aβ-induced phosphoprotein network. Nodes present in the 15 clusters represented in Figure III-5 are detailed. Clusters are numbered and the total and central nodes in each identified. Nodes are subdivided as to whether they were identified as interactors in intAct or in the phospho column. Significantly Higher and 'lower' phosphoproteins, as well as those 'lost' or 'exclusive' to Aβ addition, are also identified. Nodes in bold are the central nodes.

Cluster	1 11 nodes	2 9 nodes	3 8 nodes	4 38 nodes	5 36 nodes	6 32 nodes	7 7 nodes	8 13 nodes	9 20 nodes	10 13 nodes	11 5 nodes	12 5 nodes	13 3 nodes	14 3 nodes	15 3 nodes
Central nodes	Ppp1cc Ppp1r9a	Ppp3ca Src	Plcg1	Hnrnpk Ppp1cb Slc2a4	Dlg2 Dlg3 Dlg4 Magi2	Dynll1 Mapk3 Ppp2ca	Rab11a	App Camk2a Rad23b	Dnm1 Itsn1 Synj1	Ptpn11 Rab6a	Nr3c1	PPP3cb	Cdc42bpb	Enah	Ehd1
IntAct	Agap2 Rps6kb1 Grm1 Grm7 Drd2 Ttgn1 Grip1	Actn2 Cacna1c Cacng8 Grin2b	Fgfr1 Fgfr2 Grb2 Insr Irs1	**Slc2a4** Bax Nf2 Rpl5 Tmed10 Tnf	Acvr2a Chp1 Ddn Dgki Dgkz Dlgap1 Dlgap2 Dlgap3 Dlgap4 Exoc4 Grasp Grid1 Grid2 Grin1 Grin2a Grin2c Gucy1a2 Kcna4 Kcnj12 Lrfn2 Nrxn2 Plk2 Prkca Pten Ptprt Shank1 Syngap1	**Dynll1** **Mapk3** **Ppp2ca** Akt1 Akt2 Camk1 Kcnma1 Pgam2 Vdac1	Rab3ip Ago2 Zfyve27 Ap3m1 Myo5b Rab11fip2	Rad23b Apbb1 Apbb3 Arc Kcna5 Tmcc2	Aatf Anxa5 Eps15 Necap1 Pacsin1 Sh3gl3 Shank3 Snap23 Sorbs2 Vamp8	Ccdc64 Ehhadh Frs2 Gipc1 Hap1 Jak2 Ntrk2 Rab6b Sirpa	**Nr3c1** Tceb3	Akap6 Slc8a1 Slc8a2 Slc8a3	Cdc42bpa Lurap1	Flnc Tes	Pacsin2
Phos Column	**Ppp1cc** **Ppp1r9a** Ppp1r9b Ppfia3	**Ppp3ca** **Src** Ppp3r1 Ptprr	*Ncam1	**Hnrnpk** **Ppp1cb** Nt5dc2 Ywhab Ctnnb1 Ptpn2 Pfkl	**Dlg2** **Dlg3** **Dlg4** Ctnnd2 Grm5 Kif1b Sv2a	Dpysl2 Map1a Nos1 Nsfl1c Ppm1a Ppm1b Syn2 Vcp		**App** **Camk2a** Gnao1	**Dnm1** **Synj1** Abi1 Mapt Sh3gl1 Sh3gl2 Snap25		Ppp6c	**Ppp3cb**			Snap29
Lower				Atp6v1b2 Cand1 Erp29 Gls Hspd1 Krt14 Psmb5 Sfxn1 Tmx1 Tpm1	**Magi2**	Aldoa G3bp2 Gapdh Mdh2 Pfkm Rpn1 Ruvbl2			Dbnl	Dctn1	Lasp1		**Cdc42bpb**		
Aβ lost				Atp6v1e1 Slc3a2 Tf	Apc			Actn1 Atp5f1		Rab10					
Higher				Eci1 Eef1a1 Hexa Psmb6 Ran		Ap2s1 Clip2 Eef2 Rtcb		Slc25a22	**Itsn1** Picalm						
Aβ exclusive		Camk2d	**Plcg1** Pabpc1	Bid Ppp1ca Ppp1r12a Dynlrb1 Nucb2 Ehd3 Idh3B		Ddx3y Hk1 Pc Ppm1e	**Rab11a**	Psmd13		**Ptpn11** **Rab6a**	Tceb1			**Enah**	**Ehd1**

Supplementary Table III-3. Dual response proteins. Different identifiers (UniProt (IPI)) corresponding to the same gene resulted in both 'higher' and 'lower' levels of phosphorylated protein. The public databases used did not distinguish the IPIs as different proteins. Consequently the corresponding IPI sequences were aligned using BLAST and in all cases differences (in red) were identified, which may explain the dual response for 'higher' and 'lower' phosphorylation recoveries for the same protein.

Gene	Sig Dif	Identifiers	Sequence alignment
Ncam1	'Lower'	P13596 (IPI00476991)	^{12}FFLGTAVSL……^{349}ISSEEKASWTRPEKQETLD……^{648}KYRA-LAS
	'Higher'	F1LUV9 (IPI00777130)	^{1}FFFSFAVSL……^{338}ISSEEK - - - - - - - - - - TLD……^{627}KYRAKLAS
Strbp	'Lower'	Q6XD99 (IPI00555287)	^{48}LADER……^{1410}KDLTSV—NILL KKQQ--MLENQMEVRKKEIEELQSQAQALSQEGKSTDEVDSK
	'Higher'	Q5D002 (IPI00952273)	^{1}LTDER……^{1363}KDLTTSAQNKVL DGASVFLLMYSCPPRCWRIRWKRGRAQMR- - - - - - - - - -DSK
Sptbn1	'Lower'	Q9JKU6 (IPI00952472)	^{509}GPILTAWQKS-CDGANEKRR……^{554}GPNKKVAK -- QVQRALEKLF
	'Higher'	Q9JKU6 (IPI00327397)	^{509}GPILTASGKNPVMELNEKRR……^{555}GPNKKVAKASAALAALEKLF
Wdr47	'Lower'	G3V9M3 (IPI00768998)	^{381}VETQQP-AFEPMCQGSGLEK
	'Higher'	Q5BJR0 (IPI00949240)	^{381}VETQQPVSIEPMCQGSGLEK

CHAPTER IV

Aβ, the link between APP and Tau Phosphorylation

From the last chapter it is clearly evident that Aβ modulated phosphorylation levels of several proteins. However, in order to unravel the underlying mechanisms each of the phosphorylation related events have to be specifically and independently addressed. In manuscript 3 the work focused on phosphoproteins whose phosphorylation levels changed in response to Aβ. Further, we know from the literature that protein phosphorylation related changes are not always robust. This may also be the case for Aβ mediated phosphorylation events, in fact even though phospho APP and phospho Tau were recovered upon Aβ exposure (manuscript 3) changes were not significantly different. Nonetheless, given their relevance in AD pathology these were studied in manuscript 4, presented in this chapter.

Manuscript 4

Oliveira J.M., Henriques A.G., Martins F., Rebelo S. and da Cruz e Silva O.A.B. (2015). Aβ modulates both AbetaPP and Tau phosphorylation. J Alzheimers Dis, 45(2), 495-507. doi: 10.3233/JAD-142664

Note: Supplementary material is presented at the end of the manuscript.

Manuscript 4

Amyloid-β Modulates Both APP and Tau Phosphorylation

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ABSTRACT

Two histopathological hallmarks of Alzheimer's disease (AD), the tau rich neurofibrillary tangles and the senile plaques, predominating in amyloid-β (Aβ), have fuelled research in distinct directions. Evidence suggests that Aβ triggers imbalanced activities of protein phosphatases and kinases thus affecting the phosphorylation state of tau in AD. The amyloid-β protein precursor (APP) itself appears to be hyperphosphorylated at different residues in AD brains, including at Thr668. The results reported in this manuscript show, for the first time, that $Aβ_{42}$ can impact upon the APP phosphorylation state at the Thr668 residue. This novel finding supports a putative model, whereby Aβ can modulate the phosphorylation state of APP regulating its processing and consequently its own production. Furthermore, the data presented shows that in primary cortical neurons, GSK3β and Cdk5 are involved in APP phosphorylation at this residue and that PP1 and PP2B participate in APP dephosphorylation. Consistent with other reports, Aβ was capable of increasing tau phosphorylation at the Ser396 and Ser262 residues. This peptide is therefore a strong candidate for promoting the cross talk between signaling pathways, which simultaneously result in APP and tau hyperphosphorylation. In closing, the Aβ effect on protein kinases and protein phosphatases may constitute an alternative mechanism by which the peptide is able to modulate the phosphorylation stat e of both APP and tau in AD.

INTRODUCTION

Alzheimer's disease (AD) is characterized by two histopathological hallmarks, the neurofibrillary tangles (NFTs) and senile plaques, which have distinct compositions of aggregated tau and amyloid-β (Aβ), respectively. NFTs consist of hyperphosphorylated tau protein. The level of Tau phosphorylation is a dynamic and coordinated process that regulates its activity and modulates microtubule assembly and stability *in vitro* (Buée et al., 2000; Gendron and Petrucelli, 2009). Under some pathological conditions, such as AD, tau was found to be hyperphosphorylated, impairing its binding to microtubules and negatively impacting its physiological function, culminating in NFT formation (Johnson and Stoothoff, 2004; Wang et al., 2013). Tau phosphorylation at some sites, such as Ser396 (pSer396 tau), Ser262 (pSer262 tau), and Ser202 and Thr205 (pSer202/Thr205 tau) has been reported to be abnormal in AD and to be highly destructive in terms of decreasing tau biological activity (Amadoro et al., 2011; Augustinack et al., 2002; Bulbarelli et al., 2009; Kopke et al., 1993). In a similar fashion, the amyloid-β protein precursor (APP) is also a phosphoprotein and an integral membrane protein that can be phosphorylated both at the extra- and intracellular domains. Eight putative phosphorylation sites have been described for APP, at the intracellular domain (da Cruz e Silva and da Cruz e Silva, 2003; Oishi et al., 1997). APP phosphorylation events can regulate its normal function, processing, subcellular localization and even protein protein interactions (Ando et al., 2001; Henriques et al., 2005; Lee et al., 2003; Rebelo et al., 2007, 2013, Vieira et al., 2009, 2010). Interestingly, APP was found abnormally phosphorylated in AD brains at several residues, including Thr668 (pThr668 APP) (Lee et al., 2003). APP phosphorylation at this residue appears to dictate APP interactions and its subcellular localization with consequences for AICD (APP intracellular C-terminal domain) nuclear function (Ando et al., 2001; Chang et al., 2006)). Both APP and tau phosphorylation states can be modulated by several protein kinases and these may vary with respect to the residue being considered. For APP Thr668 phosphorylation, the proposed kinases involved are Cdc2, Cdk5, GSK3β, JNK3, and Dyrk1 as demonstrated by other colleagues (Aplin et al., 2002; Iijima et al., 2002; Ryoo et al., 2008; Standen et al., 2009; Suzuki et al., 1994). Tau has several phosphorylatable residues but for the Ser396, Ser262, Ser202, and Thr205 the relevant kinases include Cdk5, GSK3β, GSK3α, Cdc2, MAPK, CaM Kinase II, PKA, and PKC (Billingsley and Kincaid, 1997; Gong et al., 2005; Wang et al., 2013). Of particular interest is the fact that abnormal protein phosphorylation is a key mechanism linked to AD pathogenesis (da Cruz e Silva et al., 2004) and that the Aβ peptide, which derives from the amyloidogenic processing of APP (Haass et al., 2012), plays an important role in many signaling events relevant to AD, including tau

phosphorylation (Bulbarelli et al., 2009; Henriques et al., 2014a; Reifert et al., 2011; Takashima et al., 1993, 1996, 1998; Thornton et al., 2011; Zheng et al., 2002). In fact, Aβ itself can influence the activity of several protein kinases and phosphatases (Alvarez et al., 2001; Koh et al., 2008; Takashima et al., 1993; Vintém et al., 2009). For instance, it was described that Aβ, via GSK3β and Cdk5 activity, can lead to tau hyperphosphorylation (Takashima et al., 1996, 1998; Town et al., 2002), a typical event that precedes NFTs formation in AD. To our knowledge, no such link with Aβ and APP phosphorylation has to date been reported.

Given the reversible dynamic nature of protein phosphorylation systems, the involvement of a protein kinase directly implies the involvement of a protein phosphatase. Using a non-neuronal cell culture model, we recently showed that PP1 is involved in APP Thr668 dephosphorylation (Rebelo et al., 2013). Moreover, it has been reported that APP Thr668 phosphorylation increases APP amyloidogenic cleavage, supporting the notion that APP phosphorylation regulates its traffic and processing (Feyt et al., 2007; Lee et al., 2003). As already mentioned, Aβ appears to be an important signaling peptide but its effect on APP phosphorylation status is unknown. This should be pursued given that Aβ can also modulate tau phosphorylation states. Hence, in this study Aβ effects on APP phosphorylation were evaluated, specifically at theThr668 residue and on tau phosphorylation, in particular at the Ser396 and Ser262 residues. Further, the protein phosphatases that mediate APP Thr668 dephosphorylation in primary neuronal cultures were investigated. The data here reported place Aβ as a central player potentially contributing to the formation of both histological hallmarks characteristic of AD, namely NFTs and senile plaques.

MATERIALS AND METHODS

Antibodies

The polyclonal antibody phospho-APP (Thr668) (Cell Signaling) was used to detect APP and APP C-terminal fragments phosphorylated at this specific residue. The total APP content was detected using the rabbit anti-β-amyloid precursor protein antibody (Invitrogen). APP N-terminal antibody (22C11, Boehringer) was used to detect total APP/secreted APP in the immunofluorescence experiments. The following primary antibodies were used: rabbit polyclonal anti-tau (phospho S396) antibody (Abcam) and rabbit polyclonal p-tau (Ser262) antibody (Santa Cruz Biotechnology, Inc.) directed against the phosphorylated tau at Ser396 and Ser262, respectively; mouse monoclonal anti-tau antibody, clone tau-5 (Millipore) to detect all phosphorylated and nonphosphorylated isoforms of tau. The monoclonal anti-β-tubulin antibody (Invitrogen) was used

as a loading control. For the immunoblotting experiments, the secondary antibodies used were anti-mouse and anti-rabbit horseradish peroxidase-linked antibodies (GE Healthcare). Goat anti-mouse Alexa Fluor 488 (Life Technologies) and goat anti-rabbit Texas Red (Life Technologies) fluorescent conjugated secondary antibodies were employed for immunofluorescence analysis.

Primary Cultures

Pregnant female Wistar rats were obtained from Harlan Interfaune Ibérica, SL. All experimental procedures followed the European legislation for animal experimentation (2010/63/EU). No specific ethics approval under EU guidelines was required for this project, since the rats were only euthanized, by cervical stretching followed by decapitation. This is within the European law (Council Directive 86/609/EEC) and during this procedure all steps to ameliorate animal suffering were taken in consideration and the minimum number of animals was used. The procedures were approved and supervised by our Institutional Animal Care and Use Committee (IACUC): Comissão Responsável pela Experimentação e Bem-Estar Animal (CREBEA).

Primary rat cortical neuronal cultures were established from 18 days rat embryos as previously described (Henriques et al., 2007). Briefly, following brain dissection cortices were dissociated with trypsin (0.23mg/ml) and deoxyribonuclease I (0.15mg/mL) in Hank's balanced salt solution (5–10min at 37ºC), cells were plated onto poly-d-lysine coated dishes at a density of 1.0×10^5 cells/cm^2 in B27-supplemented Neurobasal medium (Gibco, Invitrogen). The medium was further supplemented with glutamine (0.5mM) and gentamicin (60µg/mL). Cultures were maintained in an atmosphere of 5% CO_2 at 37ºC for 10 days, before being used for experimental purposes.

Aβ and Drug treatment

Primary neuronal cultures were incubated with increasing concentrations of aggregated $A\beta_{42}$ (American Peptide Company) for different periods of time and with $A\beta_{25\text{-}25}$ scrambled sequence (American Peptide Company). The peptides were reconstituted in H_2O ultrapure (1mM stock) and aggregated in PBS for 48h at 37ºC (100µM aggregated stock) (Supplementary Figure IV-1) (Henriques et al., 2014b). Cells were incubated with increasing $A\beta_{42}$ concentrations (0.5, 2, 10, and 20µM) or with scrambled Aβ25-25 (20µM), for 30min, 3h, and 24h, in a B27-free Neurobasal medium combination. Neuronal cultures were likewise exposed to increasing concentrations of okadaic acid (OA) (0.1, 0.25, 50, 500, and 5000 nM), cantharidin (100, 250, 500, 1000, 10000 nM) for protein phosphatases inhibition. At different concentrations, OA and cantharidin inhibit different protein phosphatases (da Cruz e Silva et al., 1995; Henriques et al., 2005; Santos et al.,

2013; Swingle et al., 2007). Lithium chloride (2, 5, 10, 20mM) and roscovitine (2, 5, 10 20μM) were used for 30min and 3h in a B27-free Neurobasal medium combination for GSK3β and Cdk5 inhibition, respectively (Goold and Gordon-Weeks, 2001; Monaco and Vallano, 2005; Muñoz-Montaño et al., 1997; Sadleir and Vassar, 2012).

Sample Collection and Immunoblotting Analysis

Following the appropriate treatments, conditioned media were removed and cells were washed with ice-cold phosphate buffered saline (PBS). Prior to collection, radio-immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with NaF (7.5mM), NaOrt (1mM), and a protease inhibitor cocktail (1%, Sigma), were added to the cells and left for 5min at 4◦C. Lysates were collected and protein content determination was carried out using the bicinchoninic acid (BCA) kit (Pierce). Samples were normalized for protein content, and 50μg of lysates loaded and separated on 5–20% gradient SDS-PAGE. Electrophoretic transfer onto a nitrocellulose membrane was performed and immunological detection with specific antibodies was carried out as indicated. Membranes were saturated in 5% bovine serum albumin in TBS-T for 3h and further incubated overnight with primary antibodies. Detection was achieved using horseradish peroxidase-conjugated secondary antibodies and proteins visualized by enhanced chemiluminescence (ECL) detection.

Cell Viability

Cell viability assays were carried out, upon $A\beta_{42}$ exposure, by the MTT method. This procedure is based on the reduction of MTT, a water soluble tetrazolium salt, to an insoluble intracellular purple formazan. The extent of reduction of MTT was measured as previously described (Amador et al., 2004; Mosmann, 1983). Briefly, after cell treatment, the medium was removed and 0.5mg/ml MTT (Sigma) solution (in a B27-free Neurobasal medium combination) was added and incubated for 3h at 37°C. The resulting formazan precipitates were solubilized with 0.04M HCl/Isopropanol and the absorbance was measured spectrophotometrically at 570nm. Cell viability was expressed as mean±SEM, from at least three independent experiments.

Quantification and Statistical Analysis

Quantity One Densitometry software (Bio-Rad) was used to quantify band intensity of the immunoblots. Data are expressed as mean±SEM of at least three independent experiments. Statistical analysis was carried out using one way analysis of variance (ANOVA). When the F values were significant, the Dunnett multiple comparison test was applied to compare the different conditions with respect to control. Statistical differences have been determined at $p<0.05$, $p<0.01$, and $p<0.001$.

Immunofluorescence Analysis

Primary cortical neurons were plated onto coverslips at a confluence of approximately 0.8×10^5 cells/cm^2. After exposure to $A\beta_{42}$ (2μM) for 24h, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2%Triton X-100, and blocked with 3% BSA. Subsequently, cells were immunolabeled with 22C11 and phospho Thr668APP antibodies for 4h and the primary complexes were labelled using either fluorescein or Texas red conjugated secondary antibody, respectively. Cover slips were mounted onto microscope glass slides using antifading reagents containing DAPI for nucleic acids (Vectashield, Vector Laboratories, Burlingame, CA). Preparations were visualized using an LSM510-Meta confocal microscope (Zeiss) and a 63x/1.4 oil immersion objective. The argon laser lines of 405nm, 488nm, and a 561nm DPSS laser were used. Microphotographs were acquired in a sole section in the Z-axis (xy mode) and represent a mean of 16scans. Profiles were acquired and analysed using the Zeiss LSM 5104.0 software as previously described (Rebelo et al., 2008).

RESULTS

Aβ effects on APP phosphorylation

Abnormal protein phosphorylation levels have been associated with several neuropathological disorders including AD (da Cruz e Silva and da Cruz e Silva, 2003; da Cruz e Silva et al., 2004). In fact, hyperphosphorylated tau is intrinsically linked to AD pathology (Amadoro et al., 2011; Augustinack et al., 2002; Bulbarelli et al., 2009; Kopke et al., 1993) and other reports have started to associate APP phosphorylation state with the disease condition. However, the underlying molecular events that trigger the abnormal phosphorylation of these two proteins are not clear. In this study, Aβ effects on APP phosphorylation, specifically at the Thr668 residue were evaluated in rat cortical primary cultures (Figure IV-1) by incubating neurons with increasing concentrations of aggregated $A\beta_{42}$, ranging from 0.5 to 20μM, for different periods of time.

$A\beta_{42}$ exposure for 30min had no effect on total APP levels, as detected using an APP C-terminal antibody (Figure IV-1 A). However, incubation with $A\beta_{42}$ for 24h led to a decrease of around 50% in the total APP levels for the higher peptide concentration (20μM). These observations are in agreement with previous work from our laboratory for primary neuronal cultures (Henriques et al., 2009a, 2009b). In parallel, the same cell lysates were also probed with the phospho Thr668 APP specific antibody. It became evident that the phosphorylation profile of APP at Thr668 varied depending on the Aβ concentration used, as well as with the incubation period. To determine the exact levels of APPThr668 phosphorylation, the ratios of pThr668 APP/total APP were calculated. This permitted excluding differences due to variations in total APP levels. A period of 30min exposure to $A\beta_{42}$, exhibited no differences in the cortical primary cultures, with respect to the phosphorylation state of this residue in response to the peptide. However, upon 3h treatment with $A\beta_{42}$, a general marked increase in the pThr668APP levels was evident for all the concentrations used when compared to control, reaching more than a 1.5 fold increase at the higher $A\beta_{42}$ concentration (Figure IV-1 A). This effect was Aβ dose-dependent. Longer periods of $A\beta_{42}$ incubation (24h) also led to a significant increase in the phosphorylation profile of APP at this residue, particularly at the lower concentrations (0.5 and 2μM). At the higher Aβ concentrations no significant differences were observed in the Thr668 phosphorylation levels (Figure IV-1 A), but cell viability was considerably compromised (more than 50% of cells), as explained below.

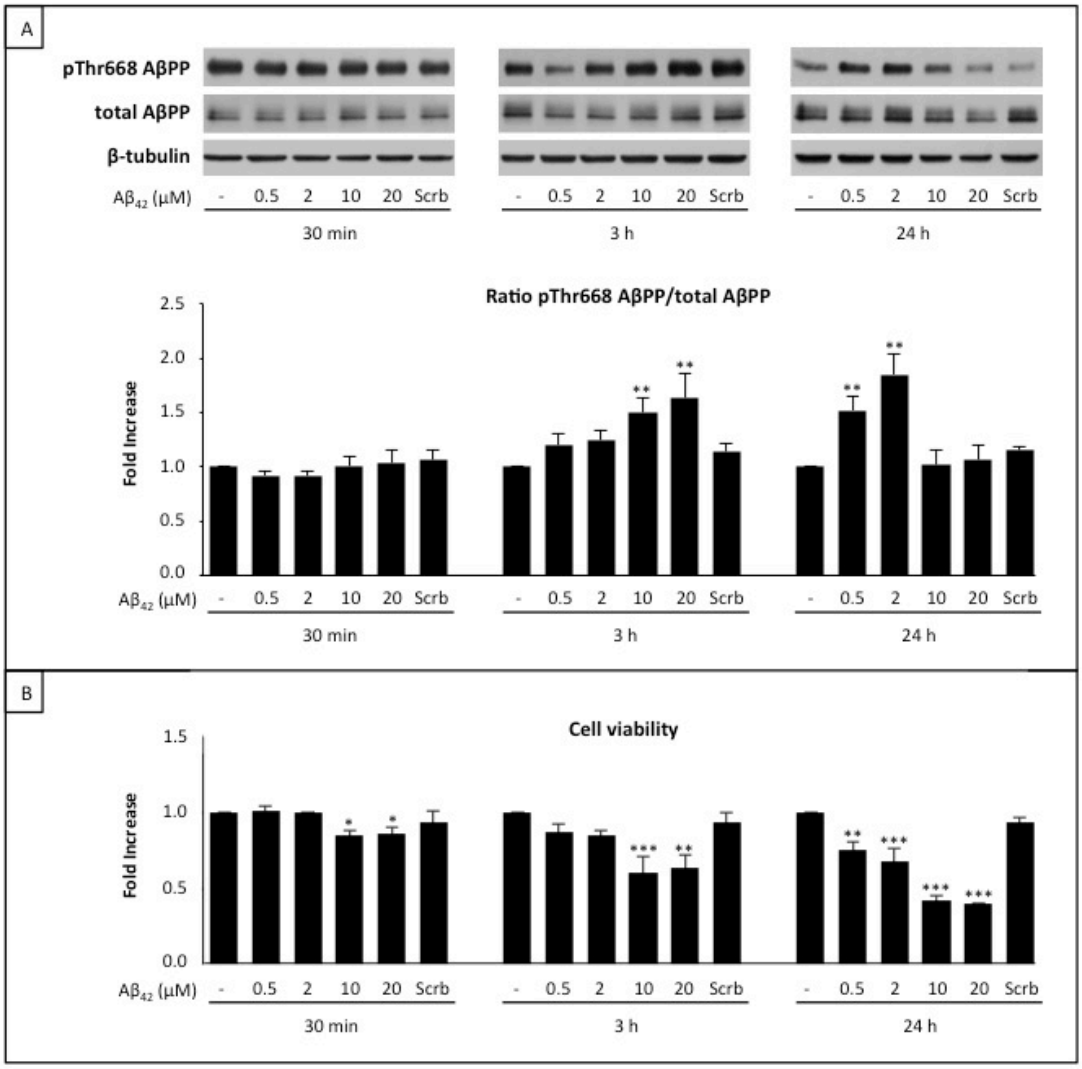

Figure IV-1. Aβ effects on AβPP phosphorylation state at the Thr668 residue. **A.** Rat primary cortical neuronal cultures were incubated for 30 min, 3 h and 24 h with aggregated $A\beta_{42}$ (0.5, 2, 10, and 20 μM) and a scrambled peptide (20 μM). Cell lysates were collected and analyzed by immunoblotting with phospho Thr668 AβPP antibody, C-Terminal antibody for total AβPP detection and β-tubulin antibody; graph depicts AβPP phosphorylation level at the Thr668 residue with values normalized to total AβPP content. **B.** Aβ effects on cell viability. Cell viability measured using MTT reduction test. *p<0.05, ** p<0.01 and ***p<0.001 vs. control, Dunnett *post hoc* test. All values are expressed as mean ± SEM from at least three independent experiments.

The pathophysiological relevance of the Aβ peptide was evaluated by determining how it may affect cell viability. The cell viability studies (Figure IV-1 B) revealed that 30min exposure to $A\beta_{42}$ did not have a marked effect, but exposure periods of 3h and 24h did. In fact a significant decrease was evident in particular at the higher Aβ concentrations (10 and 20μM) for the longer incubation period (24h).

Additionally, immunofluorescence analysis was employed to unravel the effect of Aβ on the subcellular localization of pThr668 APP (Figure IV-2). The APP N-terminal antibody (22C11, detects

total APP and secreted APP) and the phospho-specific Thr668 APP antibody (detects both total APP and APP C-terminal fragments phosphorylated at the Thr668 residue) were used. Co-localization of both antibodies will specifically allow for the intracellular detection of total APP phosphorylated at the Thr668 residue.

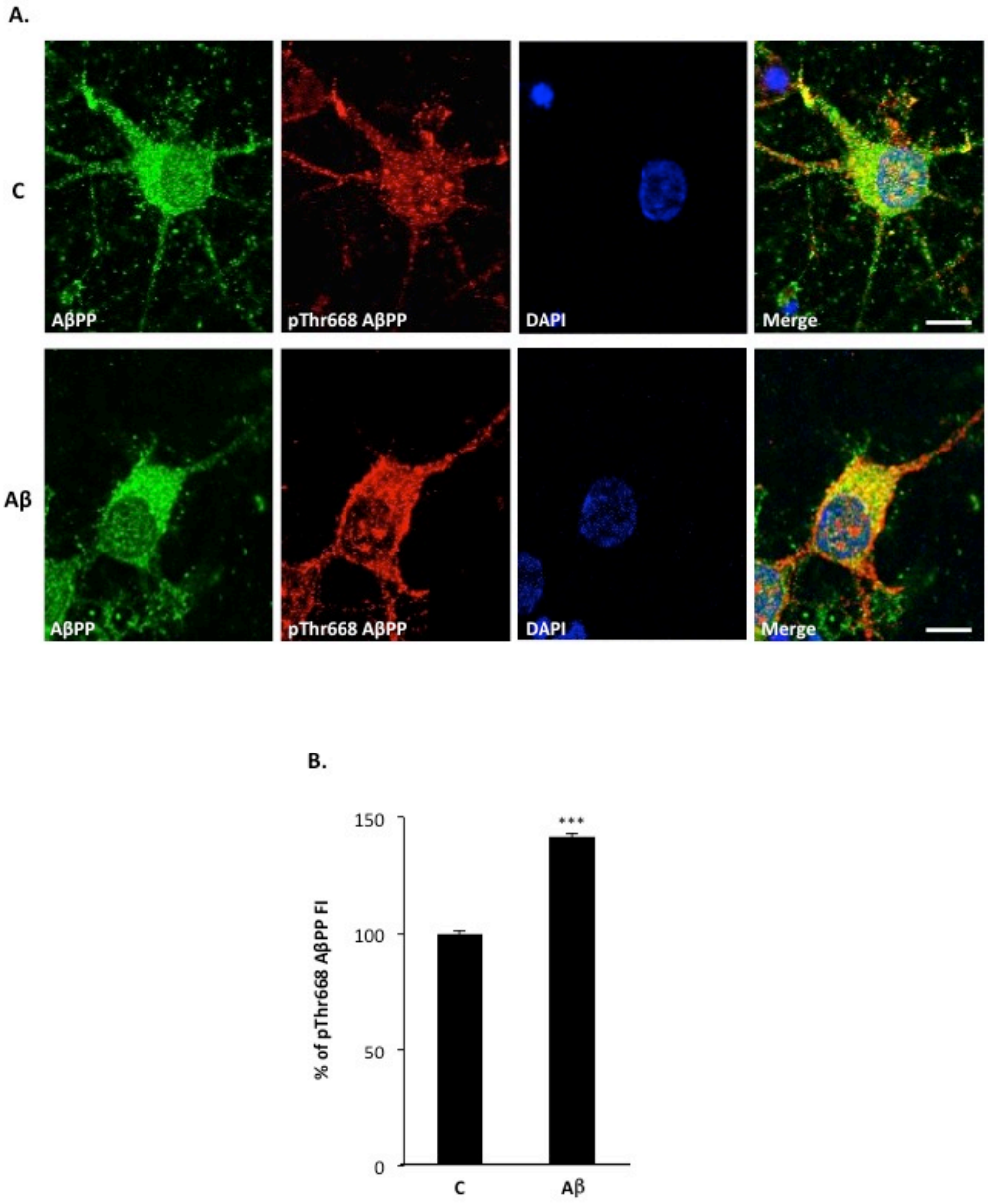

Figure IV-2. Subcellular distribution of pThr668 AβPP in response to Aβ. A. Rat primary cortical neuronal cultures were incubated for 24 h with aggregated Aβ peptide and subject to immunofluorescence analysis using the N-terminal AβPP antibody (22C11, green) and phospho Thr668 AβPP antibody (red). Co-localization of both antibodies (yellow) allows the intracellular detection of total AβPP phosphorylated at the Thr668 residue (pThr668 AβPP). DAPI was used for cell nuclei staining (blue). **B.** Quantification of phospho AβPP Thr668 fluorescence intensity (FI). Values are expressed as mean ± SEM of n= 21 cells, for each condition. ***$p < 0.001$ vs. control, t-test. Photographs were acquired using a LSM 510-Meta confocal microscope. (**C**) control; (**Aβ**) 2 µM $Aβ_{42}$. Bars, 10 µm.

As previously reported, Aβ treatment led to neurite reduction and retraction (Deshpande et al., 2006; Grace et al., 2002). Upon 2µM Aβ incubation for 24h, an increase in the pThr668 APP/APP C-

terminal fragments staining was observed in comparison to control (around 40% above the control levels). This was particularly evident in the cytoplasm and neuritis. Co-localization of both antibodies (merge, yellow staining), which allows for the detection of total APP phosphorylated at Thr668 residue, shows augmented staining of pThr668 APP mostly at the cytoplasm. These observations are in agreement with the increases observed for $A\beta_{42}$ 2μM for 24h, by western blot analysis (Figure IV-1 A). Clearly, Aβ is increasing the APP phosphorylation state and this may be influencing protein processing and targeting. In fact APP phosphorylation *per se* has already been shown to regulate its own metabolism (Rebelo et al., 2007; Vieira et al., 2010).

Neuronal APP Thr668 Phosphorylation

Protein phosphorylation is a dynamic and reversible process controlled by protein kinases and protein phosphatases. In fact, several lines of evidence show that altered activities of protein kinases and protein phosphatases are evident in AD brains, contributing to abnormal phosphorylation of some key proteins such as tau and APP. Therefore, the involvement of GSK3β and Cdk5 in APP phosphorylation was evaluated. In our hands, and consistent with findings from other groups, we were able to show that, also in primary neuronal cultures, Thr668 can be phosphorylated by GSK3β and Cdk5. When primary cortical cultures were incubated with lithium chloride (a GSK3β inhibitor) a dramatic decrease in Thr668 phosphorylation was detected, that was accompanied by a decrease in total APP levels, albeit only at the higher inhibitor concentrations. Nonetheless, the pThr668 APP/total APP ratio was calculated and revealed, that despite the decrease in total APP expression levels, pThr668 APP levels dramatically decrease in a dose-and time-dependent manner (Figure IV-3 A). This was also the case for the shorter exposure period of 30min (Supplementary Figure IV-2).

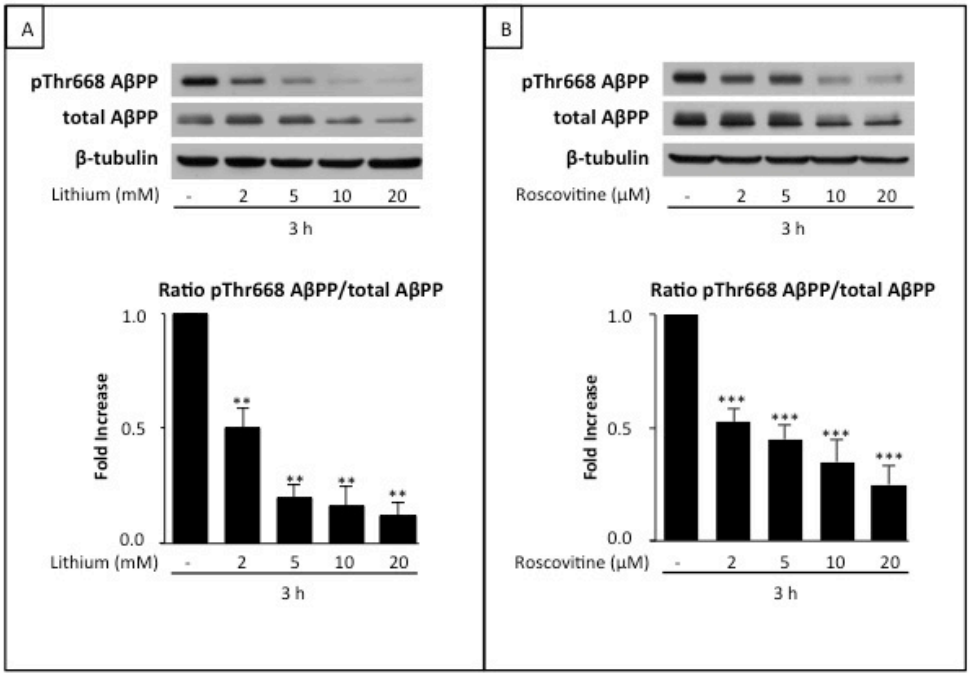

Figure IV-3. GSK3β and Cdk5 involved in AβPP phosphorylation at Thr668 residue. Rat primary cortical neuronal cultures were incubated for 3 h with lithium chloride (2, 5, 10, and 20 mM) (A) or roscovitine (2, 5, 10, and 20 μM) (B). Cell lysates were collected and analyzed by immunoblotting with phospho Thr668 AβPP antibody, C-Terminal antibody for total AβPP detection and β-tubulin antibody; Graphs depict AβPP phosphorylation level at the Thr668 residue with values normalized to total AβPP content. ** p<0.01 and ***p<0.001 vs. control, Dunnett *post hoc* test. All values are expressed as mean ± SEM from at least three independent experiments.

A dramatic decrease in Thr668 phosphorylation could be observed at 2mM and 5mM lithium chloride, although total APP levels were unaffected, and an additional decrease was obtained at the higher GSK3 inhibitor concentrations, respective to control levels. Consistently, roscovitine (a Cdk5 inhibitor) decreased the total APP expression levels at the higher concentrations (10 and 20μM), but did not do so at lower concentrations. Upon determining the pThr668 APP/total APP ratio, it is evident that the APP phosphorylation at the Thr668 residue was significantly decreased (Figure IV-3 B) when Cdk5 was inhibited. Summarizing, the data shows that inhibiting either GSK3β or Cdk5, in primary neuronal cultures, significantly decreases the pThr668 APP levels.

Protein phosphatases involved in APP Thr668 dephosphorylation

Given the dynamic nature of protein phosphorylation, protein phosphatases are likely to be involved in abnormal phosphorylation events mediated by Aβ. To evaluate the protein phosphatases involved in neuronal APP Thr668 dephosphorylation, two protein phosphatase inhibitors were used, namely OA and cantharidin. By varying the concentrations used for these inhibitors (da Cruz e Silva et al., 1995; Rebelo et al., 2013; Santos et al., 2013), protein phosphatases (PP1, PP2A, and PP2B) can be differentially inhibited. For the shorter incubation period and at lower

concentrations, the levels of pThr668 APP/total APP ratios did not change. However, at higher concentrations (500 nM and 5000 nM), when both PP1 and PP2B were inhibited, the levels of this phosphorylatable residue dramatically increased, to 2.0 and 3.5 fold increase, respectively (Figure IV-4 A).

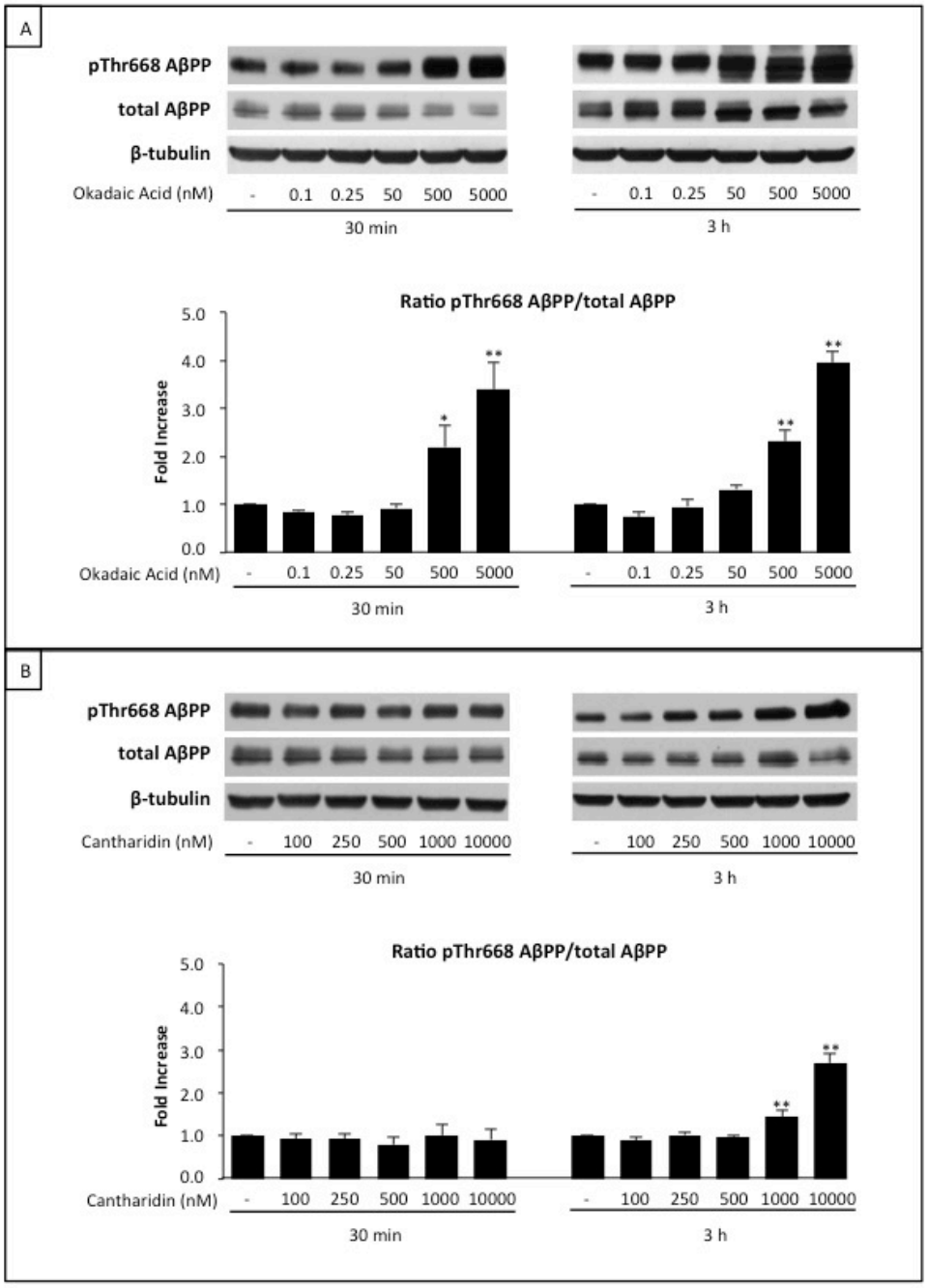

Figure IV-4. Protein phosphatases involved in AβPP dephosphorylation at the Thr668 residue. Rat primary cortical neuronal cultures were incubated for 30 min and 3 h OA (0.1, 0.25, 50, 500 and 5000 nM) (A) or cantharidin (100, 250, 500, 1000, 10000nM) (B). Cell lysates were collected and analyzed by immunoblotting with phospho Thr668 AβPP antibody, C-Terminal antibody for total AβPP detection and β-tubulin antibody; Graphs represent AβPP phosphorylation level at the Thr668 residue with values normalized to total AβPP content. *p<0.05 and ** p<0.01 vs. control, Dunnett *post hoc* test. All values are expressed as mean ± SEM from at least three independent experiments.

An additional increase was observed with 5000 nM OA. These results were more evident after a 3h exposure period, with responses being visible even at lower doses (50 nM). At the latter concentration PP1 is inhibited, but a significant increase was observed at 5000nM OA, suggesting the involvement of another phosphatase, namely PP2B, in the dephosphorylation of this residue. When using cantharidin, no difference in pThr668 APP could be observed, for the shorter incubation period. However, for the 3h timepoint, at concentrations where PP1 and also PP2B (1000 nM and 10000 nM) were inhibited, a significant increase in the ratio pThr668 APP/ total APP was detected, namely to 1.5 and 3.0 fold increase, respectively (Figure IV-4 B). Thus, considering the effects provoked by OA (Figure IV-4 A) and cantharidin (Figure IV-4 B), one can conclude that both PP1 and PP2B are involved in pThr668 APP dephosphorylation in primary cortical neurons.

Aβ mediated effects on tau phosphorylation

As previously mentioned, tau is a phosphoprotein and a relationship between Aβ peptide and the hyperphosphorylation of tau has been proposed as a hypothesis in AD. Thus, in this cell model, the potential for $A\beta_{42}$ to modulate tau phosphorylation at Ser396 and Ser262 was tested. As indicated in Figure IV-5, when cortical neurons were incubated with increasing concentrations of aggregated $A\beta_{42}$, the levels of total tau protein detected with the tau5 antibody did not fluctuate markedly for the lowest peptide concentrations and for the shorter incubation periods. However, for the 24h timepoint, a marked decrease in total tau protein level was detected, particularly with the two higher $A\beta_{42}$ concentrations (10 and 20μM). In order to have a comparable term of the tau phosphorylation state, following $A\beta_{42}$ treatment, the ratio of pSer396 tau or pSer262 tau versus total tau was calculated (Figure IV-5).

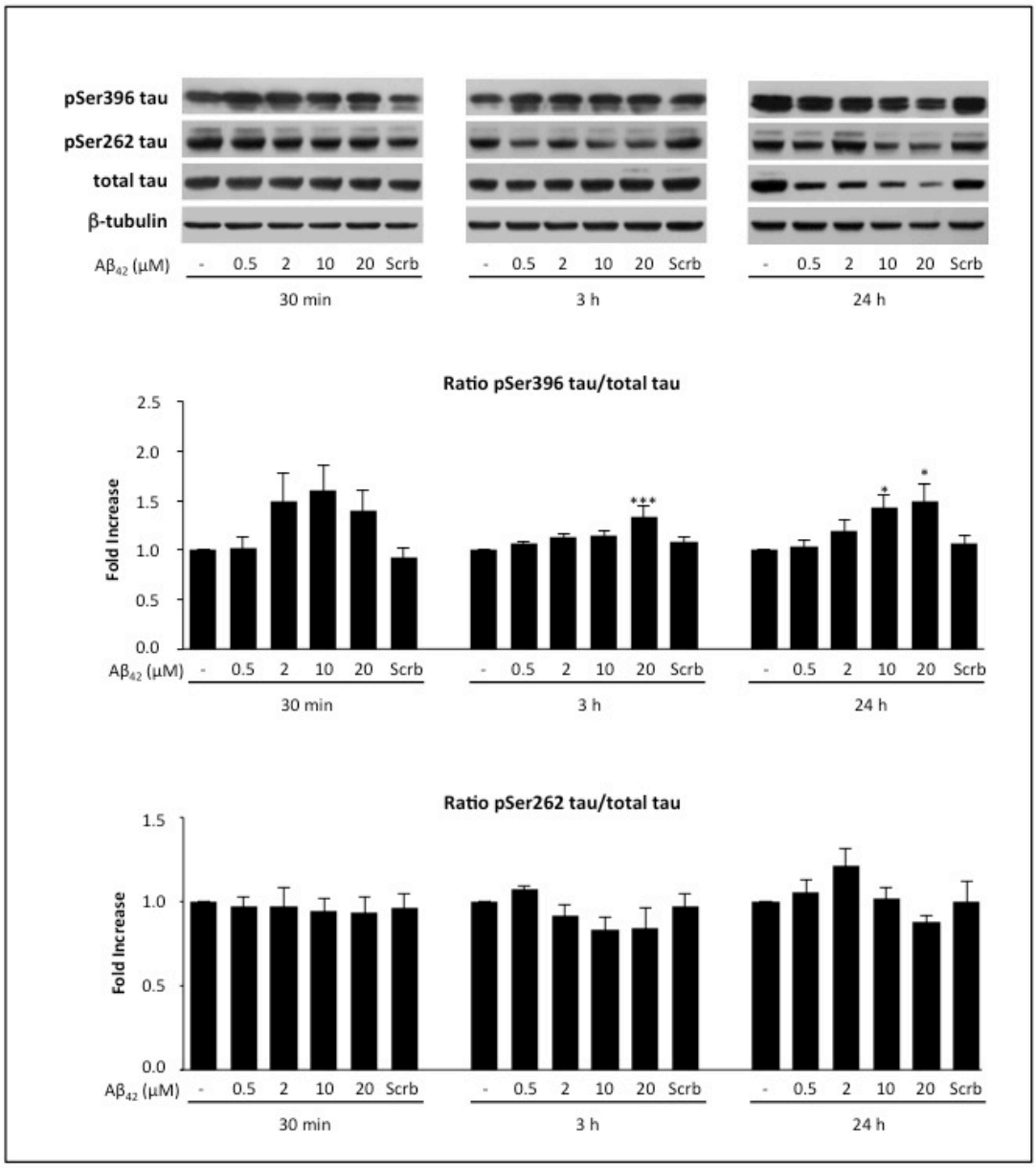

Figure IV-5. Aβ effects on tau phosphorylation state at Ser396 and Ser262 residues. Rat primary cortical neuronal cultures were incubated for 30 min, 3 h and 24 h with aggregated $A\beta_{42}$ (0.5, 2, 10, and 20 μM) and scrambled peptide. Cell lysates were collected and analyzed by immunoblotting with phospho-tau Ser396 antibody, phospho-tau Ser262 antibody, tau5 antibody for detection of total tau and β-tubulin antibody; Graphs show tau phosphorylation levels at the Ser396 and Ser262 residues with values normalized to total tau content. All values are expressed as mean ± SEM from at least three independent experiments.

An increase of the pSer396 residue could be observed upon Aβ exposure for the 30min incubation period. The increases observed at this timepoint are less marked than some previously reported (Thornton et al., 2011), but the observations are consistent. At the 3h timepoint, a significant increase was evident for the higher concentration (around 1.3 fold increase). In agreement with previous observations for the 24h period, decreases in total tau and in pSer396

levels were observed (Bulbarelli et al., 2009; Reifert et al., 2011). Nonetheless, when the ratio pSer396/total tau was determined, a significant increase on the phosphorylation levels at this residue could be detected. Thus, one can deduce that at all timepoints tested $A\beta_{42}$ promotes Ser396 phosphorylation, as previously observed for $A\beta_{25-35}$ (Takashima et al., 1998).

With respect to the effect of $A\beta_{42}$ on pSer262 tau, this remained relatively unaltered for the 30min and 3h timepoints. In contrast, for the 24h period of exposure, a tendency to increase tau phosphorylation at this residue could be detected for 2μM $A\beta_{42}$ by approximately 1.2 fold, which was not sustained for the higher concentrations (10 and 20μM) of $A\beta_{42}$. In relation to pSer202/Thr205 tau (detected by AT8 antibody), we did not observe an increase in the phosphorylation state with increasing $A\beta_{42}$ concentrations and incubation periods tested (data not shown). This is consistent with the results reported by Davis and colleagues, although in the latter $A\beta_{25-35}$ peptide was used (Davis et al., 1995).

DISCUSSION

Protein phosphorylation is the major posttranslational modification mechanism, involved in a number of regulatory processes and signaling pathways. Alterations in this mechanism are sometimes related to pathological conditions such as AD. In fact, protein kinases and phosphatases expression and/or activity are altered in AD brains (Chung, 2009). The neurotoxic Aβ peptide has been proposed to be related to these alterations, contributing to impaired protein phosphorylation in AD, as is the case for tau hyperphosphorylation and NFTs formation (Bulbarelli et al., 2009; Reifert et al., 2011; Takashima et al., 1993, 1996, 1998; Thornton et al., 2011; Zheng et al.,2002).

Abnormal APP phosphorylation was also reported in AD. In particular, APP phosphorylation at Thr668 residue appears to be neuron-specific and plays a role on APP function and metabolism, consequently impacting on protein processing and targeting (Ando et al., 2001; Chang et al., 2006). Lee et al. showed that pThr668 APP and pThr668 CTFs are upregulated in AD brains and that pThr668 APP co-accumulates with BACE1 in endocytic vesicles, suggesting that APP phosphorylated at this residue is preferentially cleaved by β-secretases (Lee et al., 2003). In fact, when APP phosphorylation at Thr668 is reduced or even abolished the levels of extracellular Aβ decreased suggesting that Thr668 phosphorylation modulates peptide production. The work here showed a significant increase in pThr668 APP levels upon exposure of primary cortical neurons to the synthetic $A\beta_{42}$.

Hence, by impacting APP phosphorylation levels at Thr668 residue, Aβ can potentially promote its own production via stimulation of the amyloidogenic processing pathway (Figure IV-6).

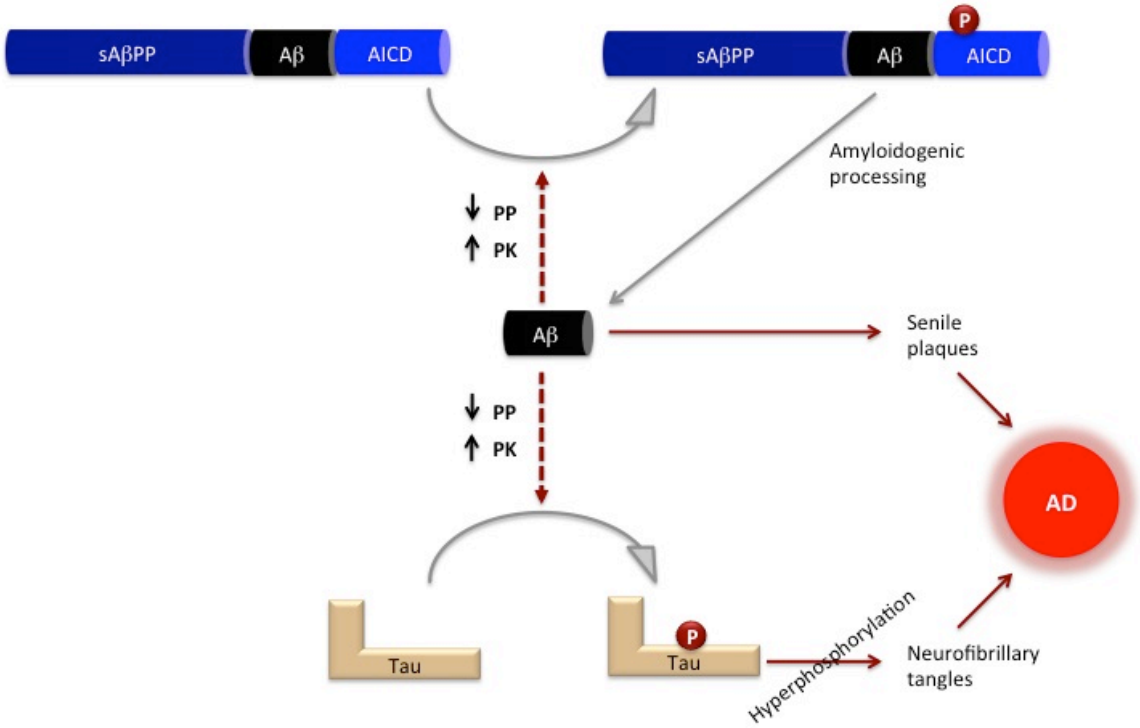

Figure IV-6. Aβ as a pivotal player in AD related phosphorylation events. Aβ is derived from the amyloidogenic processing of its precursor protein (AβPP) and is the major constituent of senile plaques in AD. Neurofibrillary tangles, enriched in hyperphosphorylated tau are also typical of AD. Aβ can imbalance protein kinases and phosphatases activity. Several protein kinases (PK) have been associated with Aβ mediated tau hyperphosphorylation, such as GSK3β and Cdk5. These two protein kinases are also involved in APP phosphorylation at Thr668 and abnormal phosphorylation of AβPP can lead to increased Aβ production. Protein phosphatases (PP), as PP1 dephosphorylate both AβPP and tau, and Aβ is able to inhibit protein phosphatase activity. Putatively Aβ can impact on both tau and AβPP phosphorylation states rendering in senile plaques and neurofibrillary tangles formation.

The protein kinases GSK3β and Cdk5 have been proposed to be involved in Thr668 APP phosphorylation. When inhibiting both GSK3β and Cdk5 activities a significant decrease in the phosphorylation state of APP is evident. The Aβ effects on these two protein kinases are well documented and in fact, several reports demonstrated that tau hyperphosphorylation could be due to an increase in GSK3β activity as well as to an increase in Cdk5 activity. Of note, exposure of rat hippocampal neurons to different Aβ peptides led to an increase in tau phosphorylation through activation of GSK3β, an event that may be related to PI3K inactivation (Takashima et al., 1996). In agreement, a tendency for an increase in GSK3β activity could be observed for the longer periods, when pThr668 phosphorylation was augmented (Supplementary Figure IV-3).

As already mentioned, Cdk5 may also be implicated in Aβ induced tau phosphorylation. In fact, the latter correlated with an increase in both p25 to p35 ratio and Cdk5 activity in an N2a cell line model treated with soluble $A\beta_{42}$ peptide. Thus, activation of both GSK3β and Cdk5 may constitute a mechanism by which the toxic peptide, Aβ, is modulating tau and APP phosphorylation.

Less effort has been devoted to elucidating the protein phosphatases involved in pThr668 APP dephosphorylation. Recently, our group showed the involvement of PP1 in pThr668 APP in COS-7 cells (Rebelo et al., 2013). Here, the protein phosphatases involved in APP dephosphorylation in primary cortical neurons were evaluated. Both PP1 and PP2B inhibition render in increased pThr668 APP levels. The involvement of PP2B in pThr668 APP in neurons appears to be particularly relevant, since PP2B is a serine/threonine phosphatase physiologically activated by Ca^{2+} and calmodulin that are present at higher levels in neurons and, Aβ was reported to increase Ca^{2+} levels (Kuchibhotla et al., 2008). Moreover, previous studies from our group showed that Aβ can directly inhibit the protein phosphatases activity both *in vitro* and *in vivo*, in particular PP1 and PP2A (Amador et al., 2004; Vintém et al., 2009). Therefore, PP1 inhibition by Aβ may constitute a pathway that leads to increased neuronal APP hyperphosphorylation at Thr668.

Considering that the same protein phosphatases dephosphorylate tau and APP, one could argue that the system is highly unspecific. However, protein phosphatases have a unique mode of action and are highly dependent on the binding to interacting proteins (PIPs). This means that the same protein phosphatase can dephosphorylate different proteins, such as tau and APP and that the specificity can be conveyed by the different PIPs. For example, pThr668 APP is determinant in defining APP binding to other proteins, such as Fe65. Indeed, we have recently shown that a tri-complex APP:Fe65:PP1 exists *in vivo* and is the functionally active complex (Rebelo et al., 2013).

The effects of Aβ on tau phosphorylation at Ser262 and Ser396 were addressed and a general increase in the pSer396 tau that was significant after 3h and 24h exposure was observed (Takashima et al., 1998; Thornton et al., 2011). However, at Ser262 only a slight tendency to increase could be observed at 24h and at the lowest Aβ concentrations. The phosphorylation pattern of tau is sequential, meaning that some residues have to be phosphorylated first for the phosphorylation of others to occur. In this particular case, pSer396 tau is related to early tau pathological events in AD followed by phosphorylation at other residues (Mondragón-Rodríguez et al., 2014). Given that the majority of the protein kinases and phosphatases involved in the phosphorylation state of these two residues are the same, the differences in the phosphorylation levels observed in these tau residues may be explained in the light of sequential phosphorylation steps. Putatively, Aβ may promote the early tau phosphorylation at Ser396 and the phosphorylation of Ser262 may occur

later. At the microtubule dynamics level, tau hyperphosphorylation results in tau dysfunction and loss of its biological capacity of promoting microtubule assembly and stabilization. Aβ can induce microtubule instability by promoting abnormal tau hyperphosphorylation at these residues. Future studies should address the simultaneous sequential phosphorylation of tau and APP and take advantage of transgenic animal models. The level of protein phosphorylation may provide an interesting biomarker with respect to disease stage and development.

In closing, the data here presented supports a model whereby Aβ modulates the signaling pathways that regulate the phosphorylation state of tau and APP (Figure IV-6), involving multiple protein kinases and protein phosphatases. APP phosphorylated on Thr668 is preferentially directed to amyloidogenic processing, which, in turn, can result in increased Aβ production. Given that Aβ influences both kinase and phosphatase activities, further consequences may occur, via other signaling events involving the interplay between protein phosphatases and/or protein kinases. Although several phosphorylation dependent processes have been studied, the crosstalk between signaling cascades and the protein phosphatases and protein kinases involved has not been comprehensively described. It follows that monitoring phosphorylation levels on APP and tau may provide a reliable biomarker for AD. The work here presented places Aβ as a critical player in this crosstalk. Clarification of these aspects would undoubtedly be an important step towards developing novel effective signal transduction based therapeutic strategies for AD and possibly for other neuropathologies.

Acknowledgments

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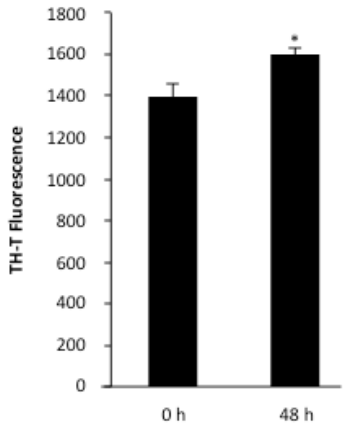
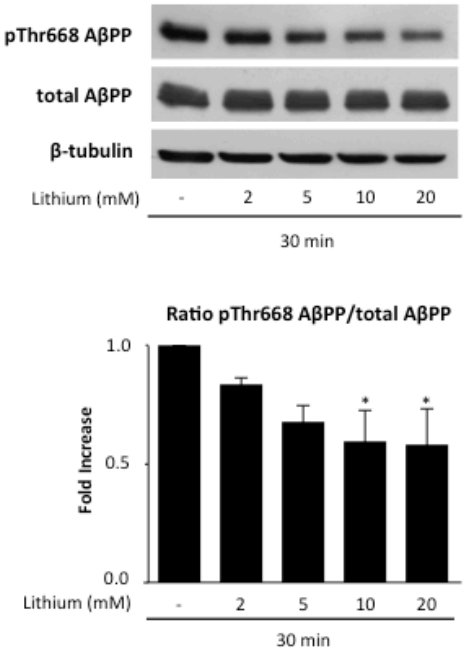
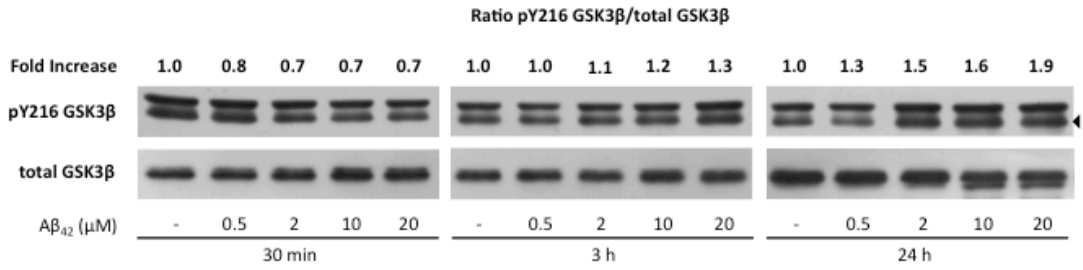
MANUSCRIPT SUPPLEMENTAL MATERIAL



Supplemental Figure IV-1. Evaluation of Aβ aggregation by Thioflavin-T. Thioflavin (TH-T) assay was prepared by mixing 25 µl of $A\beta_{42}$ 100 µM stock with 225 µl of TH-T 15 µM solution in phosphate buffer pH=7.4. The fluorescence measurements were recorded in a 96-well black micro plate using a micro plate reader (Infinite M200-Tecan and I-control TM software). The fluorescence intensity of Th-T was measured at λex = 450 nm and λem = 482 nm and the values recorded at time-point 0 h and 48 h after $A\beta_{42}$ incubation at 37ºC. Results are expressed as mean ± SEM of at least three independent experiments. * $p < 0.1$, significantly different from Aβ 0 h; unpaired t-test.



Supplemental Figure IV-2. GSK3β and AβPP phosphorylation at Thr668 residue. Rat primary cortical neuronal cultures were incubated for 30 min with lithium chloride (2, 5, 10, and 20 mM). Cell lysates were collected and analyzed by immunoblotting with phospho Thr668 AβPP antibody, C-Terminal antibody for Total AβPP detection and β-tubulin antibody; Graphs depict AβPP phosphorylation level at the Thr668 residue with values normalized to total AβPP content. *$p<0.05$ vs. control, Dunnett *post hoc* test. All values are expressed as mean ± SEM from at least three independent experiments.



Supplemental Figure IV-3. Aβ effects on GSK3β activity. Rat primary cortical neuronal cultures were incubated for 30 min, 3 h and 24 h with aggregated $A\beta_{42}$ (0.5, 2, 10, and 20 μM). Cell lysates were collected and analyzed by immunoblotting with GSK3β (pTyr216) phospho-specific antibody (GSK3β active form) (BD Biosciences) and GSK3β antibody (Abcam) for detection of total GSK3β. The ratio pY216-GSK3β/total GSK3β and the fold increase, for each condition respective to control, was calculated considering the pY216 GSK3β (≈46kDa band, ◂). All values are expressed as mean of duplicate independent experiments.

CHAPTER V

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

CONCLUDING REMARKS

Protein phosphorylation is a key event in AD pathogenesis, responsible for many of the protein changes that lead to neurodegeneration. Noticeably, phosphorylated proteins can constitute biomarkers candidates for AD, and Tau phosphorylation is indeed such an example. AD diagnosis is difficult, particular in the early disease stages and discovery of novel biomarkers candidates can be of clinical value. Hence, the main objective of this thesis was to identify phosphorylation related biomarkers for AD diagnosis. To fulfil this goal, we evaluated phosphorylation states of different proteins in AD mimicking conditions (okadaic acid and Aβ exposure) in a neuronal cell model (**Chapter II**, **Chapter III** and **Chapter IV**). Particular attention was given to two distinct proteins, APP and Tau, in **Chapter IV**. Both proteins were shown to be involved in AD pathogenesis as reviewed in **Chapter I**.

In **Chapter II** we used OA, a protein phosphatase (PP) inhibitor, to mimic the hyperphosphorylation state observed in AD pathology. Upon neuronal cells exposure to OA, cell lysates were submitted to a phosphoprotein enrichment process with further analysis by mass spectrometry. The top four biological processes affected included anatomical structural development, transport, cell differentiation, and signal transduction. Results revealed that the recovery rate of 245 phosphoproteins significantly increased (121 were exclusive of OA condition), while that of 75 significantly decreased (50 were completely lost upon OA exposure).

The phosphoprotein recovery rates of several kinases, such Src, Gsk3α/β, Mapk1/Erk2, and the dual-specificity Map2k1/Mek1 are enhanced or exclusive to OA treatment, some of which have already been reported to be altered in AD. Analysis of the significantly different phosphoproteome revealed Src as a one central node. This kinase is linked to various signaling transduction pathways, including MAPK, phosphoinositide 3-kinase (PI3K)/ Akt, and STAT, the latter is intimately tied to inflammatory processes, a disease associated event. Impaired Src signaling has been observed in AD mouse models suggesting that targeting Src may be of therapeutic value. As such, impairment of Src phospho state can potential contribute to neurodegeneration typical of AD.

Likewise, the phosphorylated form of Gsk3α was significantly increased in response to OA. This was shown to be required for Aβ production and also involved in Tau hyperphosphorylation. Additionally, oligomeric Aβ was demonstrated to bind to and to antagonize components of the insulin signaling pathway resulting in increased activity of GSK3β. This is an attractive hypothesis involving phosphorylation mediated responses and insulin resistance. Other protein kinases recovered when we consider the OA-dependent kinase and phosphatase interaction network was

Mapk1/ERK2. Mapk1 regulates several cellular processes and is a central hub particularly in the transport and signal transduction networks.

Regarding PPs, many of those were also found to be altered under this condition. The recovery of Ppp1ca (PP1α) was increased, which is consistent with its inhibition by phosphorylation, while the phosphoprotein recovery of a mitochondrial serine/ threonine phosphatase Pgam5 (Pgam5) was decreased. The recovery of other PPs, such as Ptpn11, Ppm1e, and Ppm1h, was exclusive to OA treatment. Of note, PP activity can also be regulated by PP-interacting proteins (PIPs); as such, instead of targeting PPs themselves, both PIP and PP regulatory subunits may represent suitable targets for therapeutic strategies directed to PPs. Several PP regulatory subunits were identified in this study, but only Ppp2r5e and Ppp2r2a exhibited a phosphoprotein recovery significantly altered upon OA exposure. Both subunits are important for regulation of PP2A subcellular localization and/or substrate specificity and Ppp2r2a was reported to mediate Tau dephosphorylation.

Conjointly, data suggest that OA impacts at different cellular levels, supporting that this neurotoxin represents a powerful experimental tool to address cellular and molecular disease mechanisms linked to PP inhibition. The fact that various molecular players are involved helps to validate the procedure toward understanding the interplay across the different signaling cascades. The phosphoproteins identified represent additional molecular targets in OA-mediated events and may constitute potential disease-associated phospho biomarker candidates.

In **Chapter III** a systems biology approach was adopted and the phosphoproteome, of primary cortical neuronal cells exposed to Aβ, was evaluated. Aβ is the core component of SPs, one of the hallmarks of AD pathology. The amyloidogenic peptide have been described to provoke a series of events affecting distinct cellular pathways regulated by protein phosphorylation and leading to neuronal death, as such this represents a more specific AD mimicking model. Primary neuronal culture lysates were collected upon Aβ exposure, enriched for phosphorylated proteins and the latter identified by mass spectrometry analysis. Under the experimental conditions implemented, 986 phosphoproteins were recovered following Aβ exposure and 870 phosphoproteins under Control conditions. It was possible to identify 141 putative biomarkers, whose phosphorylated proteins significantly increased (73) or decreased (68) upon Aβ addition. Furthermore 19 phosphoproteins were 'lost' upon Aβ exposure and 50 were Aβ 'exclusive'. In the Aβ 'exclusive' proteins we find PPs as Ppm1e, Ptpn11 and Pp1α. Ptpn11 is reported to be involved in activation of the RAS/MAPK pathway and STAT signalling pathways. PP1 is an abundant neuronal phosphatase enriched in dendritic spines, with a key role in synaptic signalling, that can be inhibited by Aβ. This phosphatase is required for long-term depression, is involved in memory and learning and has been

implicated in Tau dephosphorylation, playing a key role in AD pathogenesis. Pppm1E is a member of the Pp2C family of the serine/threonine-protein phosphatases that is brain-specific, involved in synaptic plasticity and dendritic spine morphogenesis. It is interesting to note that the results relative to PP1α, Ptpn11 and Ppm1E are similar when using Aβ (**Chapter III**) or OA (**Chapter II**) as model to mimic AD conditions. These results highlight the relevance of the PPs in the signalling events underlying AD.

Aβ affected not only the phosphorylation level of PPs but also the phosphorylation levels of proteins involved in several biological processes such as synaptic signalling (MAGI2) and membrane trafficking (ITSN1, AP2A2, Rab10, Rab11a, Rab6a). This work clearly showed that Aβ altered the phosphorylation levels of many proteins and this is consistent with the pathophysiological characteristics attributed to Aβ, placing it at the centre of AD.

The recovery levels of phosphorylated proteins described in **Chapter II** and **Chapter III** provide a resource as potential phospho biomarker candidates for AD diagnosis and should be pursued in this respect in future studies. One approach could be the validation of some of these candidates in human samples. The current gold standard molecular based diagnostics for AD, centres in the monitoring of the biomarker triplet (Aβ, Tau and p181-Tau) in the cerebrospinal fluid (CSF) of AD patients. CSF collection requires an invasive procedure and therefore validation of biomarkers candidates in peripheral biofluids, such as blood, would be an interesting approach.

In **Chapter IV** we looked to the phosphorylation levels of two proteins, APP and Tau, both particular relevant in AD pathology. Aggregated Tau is the major component of NFTs and Aβ peptide is the major component of SPs. Upon exposure of rat primary neuronal cultures to Aβ we observed a significant increase in phosphorylation level of APP at Thr668 residue which can potentially promote its own production via stimulation of the amyloidogenic processing pathway. We also looked for the PPs involved in APP dephosphorylation at Thr668 using OA which revealed that, under this experimental condition, PP1 and PP2B are two PP candidates involved in APP dephosphorylation. As described in **Chapter III**, Aβ can affect the phosphorylation state of PP1 which may result in its inhibition constituting a possible mechanism by which Aβ can also lead to an increase in the phosphorylation level of APP at this specific residue, and thus contribute to its own production. Relative to Tau phosphorylation, we observed an increase in the phosphorylation level at Ser396. This specific residue is related to early Tau pathological events in AD followed by phosphorylation at other residues. Tau hyperphosphorylation results in Tau dysfunction and loss of its biological capacity of promoting microtubule assembly and stabilization. These results support a model whereby Aβ can modulate signaling pathways that regulate the phosphorylation state of

both Tau and APP, playing a critical player in this crosstalk. Phosphorylation levels of both APP and Tau, at this particular phospho residue, may constitute reliable AD biomarkers that can be further validated. In addition, understanding the molecular events triggering abnormal signalling pathways may also open avenues for development of future therapeutic strategies.

FUTURE PERSPECTIVES

The pathophysiological process of AD is thought to begins years, if not decades, before dementia diagnosis. In this "preclinical" phase, brain changes, including Aβ accumulation may already be in progress, but significant clinical symptoms are not yet evident. The preclinical phase provides an opportunity for potential intervention with disease-modifying therapy. At this stage, we should be able to elucidate the link between the pathophysiological process of AD and the emergence of the clinical syndrome. Therefore, it is of extreme importance to develop new diagnostic strategies that allows for an early diagnostic, in order to have a timely therapeutic intervention to avoid disease progression and irreversible brain lesions. The systems biology approach used in this work, as well as the APP and Tau targeted approach, holds promising results for the development of novel, clinically useful phospho biomarker candidates for AD diagnosis. Of note, an interesting approach would be the comparison of both OA and Aβ proteomes. The OA exclusive phosphoprotein pool 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 can be relevant for other Aβ associated events. Finally, the common phosphoproteins between OA and Aβ exclusive pools may reveal biomarkers to distinguish AD from other dementias since it represents a merge between two models that mimic AD.

Considering the nature and the localization of the disease lesions at brain level, it is important that the new diagnostic tools be in peripheric and accessible body fluids such as blood, urine or saliva. Henceforth, the most promising candidates should be selected for validation in human blood samples (plasma or serum from de Aveiro Cohort already stablished in our laboratory). Validation includes the establishment of thresholds (basal versus pathological levels) for each candidate biomarker and address the design and cost-effectiveness of an array of biomarkers to AD diagnosis. Peripheral biomarker candidates will certainly be of clinical value since these can represent a cost-effective and widely available screening tool.

CHAPTER VI

ANNEXES

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

Cell Culture and experimental models

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 175eionized H_2O. Final Composition:

- 137 mM NaCl
- 5.36 mM KCl
- 0.44 mM KH2PO4
- 0.34 mM Na2HPO42H2O
- 4.16 mM NaHCO3
- 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 175eionized 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 176ml deionized H_2O 9.28 g of boric acid (Sigma-Aldrich). Adjust to pH 8.2, sterilize by filtering through a 0.2 µM filter, and store at 4°C.

- PBS (1x)

For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in 176ml deionized H_2O. Final composition:

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

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

- RIPA buffer (Sigma-Aldrich)
  - To 6.5 ml of RIPA buffer add:
  - 40.3 µL NaF
  - 65 µL NaOrt
  - 65 µL Protease inhibitor cocktail (Sigma-Aldrich)

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

- Okadaic Acid (Calbiochem)

Equipment

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

Protein Content Determination

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.

Reagent B: 4% cupric sulfate.

Equipment

- Spectrophotometer Cary 50 (Varian)

SDS-PAGE

Reagents

- Acrylamide stock mixture (30% acrylamid, 0.8% Bisacrylamide)

To 70 ml of deionised H_2O add:

- 29.2 g Acrylamide
- 0.8 g Bisacrylamide

Mix until the solute has dissolved. Adjust the volume to 100 ml with deionised water. Filter through a 0.2 µm filter and store at 4°C.

Stacking gel and resolving gel

	Stacking Gel	**Resolving gel**	
	3,5%	**5%**	**20%**
H_2O	13.2 ml	17.4 ml	2,2 ml
Acrylamide stock mixture	2.4 ml	5 ml	20 ml
UGB (5x)	4.0 ml	--	--
LGB (4X)	--	7.5 ml	7.5 ml
10% APS	200 µl	150 µl	150 µl
10% SDS	200 µl	--	--
TEMED	20 µl	15 µl	15 µl

- UGB (Upper gel buffer) (5x)

To 900 ml of deionised H_2O add:

- 75.69 g Tris

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

- LGB (Lower gel buffer) (4x)

To 900 ml of deionised H_2O add:

- 181.65 g Tris
- 4 g SDS

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

- APS (Ammonium Persulfate) 10%

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

- SDS (Sodium Dodecilsulfate) 10%

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

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

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

- Tris 1 M (pH 6.8) solution

To 150 ml of deionised H_2O add 30.3 g Tris base. Adjust the pH to 6.8 and adjust the final volume to 250 ml.

- 10x Running Buffer
  - 30.3 g (250 mM) Tris
  - 144.2 g (2.5 M) Glycine

- 10 g (1%) SDS

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

Equipment

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

Western-Blotting

Reagents

- 1x Transfer Buffer
  - 3.03 g (25 mM) Tris
  - 14.41 g (192 mM) Glycine

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

Immunoblotting

- 10x TBS (Tris buffered saline)
  - 12.11 g (10 mM) Tris
  - 87.66 g (150 mM) NaCl

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

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

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

- Blocking Solution

5% of nonfat dried milk or BSA (Bovine Serum Albumin, Merck) in 1x TBS-T.

- ECL or ECL Plus Kits (Amersham Biosciences)

- Developer and fixer Solution (Sigma)

- Membranes Stripping Solution
  - 3.76 g (62.5 mM) Tris-HCl (pH 6.7)
  - 10 g (2%) SDS
  - 3.5 ml (100 mM) Beta-mercaptoetanol

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

Equipment

- Transphor Electrophoresis unit (Hofer TE 42)
- Electrophoresis power supply EPS 1000 (Amersham Pharmacia Biotec)

Phosphoprotein extraction

Reagent

- Phosphoprotein Enrichment Kit Talon PMAC (Clontech)

Quantitative Analysis

Equipment

- GS-710 calibrated imaging densitometer (Bio-Rad)