Universidade de Aveiro Secção Autónoma de Ciências da Saúde
2012

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A função da tau nuclear é mediada pela proteína fosfatase 1 gama

Nuclear tau function is mediated by protein phosphatase 1 gamma

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Odete da Cruz e Silva, Professora Auxiliar com agregação da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro

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o júri

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palavras-chave

Células SHSY5Y, tauopatias, proteina tau associada a microtúbulos, tau nuclear, agregados da proteína tau, proteína fosfatase 1 gama, proteína fosfatase 1 alfa, proteína fosfatase 2A, ácido ocadaico, cloreto de lítio, roscovitina, tau nuclear.

resumo

As tranças neurofibrilhares constituídas pela tau hiperfosforilada são os principais marcadores das tauopatias. Contudo, sabe-se que os dímeros da proteína tau (primeira fase de formação das tranças neurofibrilhares) podem ser fisiológicos e controlam uma das funções da tau nuclear, a sua interação com o ADN. Esta interação leva a uma mudança conformacional no ADN que por sua vez conduz a uma alteração da expressão genética.

No trabalho aqui descrito foram avaliados fatores que afetam a agregação da tau, em particular a função das proteínas fosfatases. Duas técnicas foram usadas, o ensaio de membrana de filtro e o "*western blotting*" para medir a agregação da tau e os níveis de expressão das fosfatases e da tau, respetivamente.

Os resultados sugerem que a proteína fosfatase 1 gama (PP1γ) está envolvida na formação dos agregados e esta parece ser regulada pela PP2A e PP1α. Por outro lado, a diminuição da formação dos agregados após inibição da GSK3 e CDKs parece envolver dois mecanismos diferentes. Assim, o mecanismo desencadeado pela inibição da GSK3, provavelmente, é dependente da PP1γ, PP1α e PP2A, contudo, a via desencadeado pela inibição das CDKs parece ser menos dependente destas PPs.

keywords

SHSY5Y cells, tauopathies, microtubule-associated tau protein, nuclear tau, tau aggregates, protein phosphatase 1 gamma, protein phosphatase 1 alpha, protein phosphatase 2A, okadaic acid, lithium chloride, roscovitine.

abstract

Neurofibrillary tangles constituted by hyperphosphorylated tau are a principle hallmark of tauopathies. However, it is known that tau dimmers (first phase of tangle formation) are physiological and control one of the functions of nuclear tau, DNA-tau interaction. This interaction leads to a DNA conformational change that in turn impact upon in gene expression.

In the work here described factors affecting nuclear tau aggregation were addressed, in particular the role of protein phosphatases. Two techniques were used, a membrane filter assay and a western blotting to measure tau aggregates formation and the expression levels of phosphatases and tau, respectively.

The results suggest that protein phosphatase 1 gamma (PP1γ) is involved in aggregate formation and in turn this PP appears to be regulated by PP2A and PP1α. On the other hand, decrease in aggregate formation upon inhibiting GSK3 and CDKs appear to involve different mechanisms. Thus, the mechanism triggered by GSK3 inhibition, probably, is dependent of PP1γ, PP1α and PP2A, however the pathways triggered by CDKs inhibition appears to be less dependent of these PPs.

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Abbreviations

AD	Alzheimer´s Disease
APP	Amyloid Precursor Protein
APS	Ammonium persulfate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CBD	Corticobasak Degradation
cdc2	cyclin-dependent kinase 2
cdk	Cyclin-dependent kinase
cdk5	Cyclin-dependent kinase 5
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
FBS	Fetal Bovine Serum
FTDP	Frontotemporal Dementia with Parkinsonism-17
GSK3	Glycogen Synthase Kinase-3
KD	Knockdown
LB	Loading buffer
LiCl	Lithium Chloride
MAPs	Microtubule Associated Proteins
MAPT gene	Microtubule Associated Protein *tau* gene
MBD	Microtubule binding domain
MD	Myotonic dystrophy
MEM	Minimum Essential Medium

NaCl	Sodium Chloride
NFT	Neurofibrillary Tangles
OA	Okadaic Acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PHFs	Paired Helical Filaments
PiD	Pick´s Disease
PLC-γ	Phospholipase C gamma
PKs	Protein Kinases
PPs	Protein Phosphatases
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PP2B	Protein Phosphatase 2B
PP4	Protein Phosphatase 4
PP5	Protein Phosphatase 5
PP7	Protein Phosphatase 7
PSP	Progressive Supranuclear Palsy
shRNA	short hairpin RNAs
siRNA	small interfering RNA
SDS	Sodium Dodecyl Sulfate
tau	Microtubule-associated protein tau
TBS	Tris buffered saline solution
TBS-T	Tris buffered saline-Tween 20
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane

UPS	Ubiquitin-Proteosome-System
WR	Working Reagent

1. Introduction

1.1. Tau biology

Microtubule-associated protein tau was first isolated in 1975 by Weingarten and colleagues. Presently it is know that tau is a microtubule-associated protein, exclusively found in higher eukaryotes namely *Caenorhabditis elegans (Goedert et al., 1996, McDermott et al., 1996)*, *Drosophila (Irminger-Finger et al., 1990, Cambiazo et al., 1995)*, goldfish (Liu et al., 1997), bullfrog (Yin et al., 1995), rodents (Kosik and Finch, 1987, Lee et al., 1988), bovines (Himmler, 1989, Himmler et al., 1989), goat, monkeys (Nelson et al., 1996) and human (Goedert et al., 1989a, Goedert et al., 1989b).

In human, tau is highly expressed in neurons, although non-neuronal cells usually have trace amounts (reviewed in Buee et al., 2000). Thus, this protein can also be expressed in glial cells, although mainly in pathological conditions (Chin and Goldman, 1996), and it is possible to detect tau mRNA in several peripherical tissues such as heart, kidney, lung, muscle, pancreas, testis, as well as in fibroblasts (Gu et al., 1996, Ingelson et al., 1996, Vanier et al., 1998)

1.1.1. Microtubule associated protein tau

The human microtubule associated protein *tau* gene (MAPT) is located on the long arm of chromosome 17 at position 17q21.3, spanning for 100kb and contains 16 exons (Andreadis et al., 1992). The processing of their mRNA, result in six different tau isoforms (Kosik et al., 1989, Goedert and Jakes, 1990).

Tau isoforms result from the tau primary transcript with fourteen exons of which exon 1, 4, 5, 7, 9, 11, 12 and 13 are constitutive, 4A, 6 and 8 exons are not translated, and exons 2, 3, and 10 undergo alternative splicing, giving rise to six different mRNAs, translated in the six tau isoforms. Therefore, these differ by the absence or presence of one or two 29 amino acids inserts encoded by exon 2 and 3 in the amino-terminal part, in combination with either three (R1, R3 and R4) or four (R1-R4) repeat-regions in the carboxy-terminal part, respectively. The last exon implicated in different tau isoforms is

the exon 10, that is responsible by 4R or 3R tau isoform when exon 10 is present or absence, respectively, Fig.2 (Cleveland et al., 1977, Fulga et al., 2007).

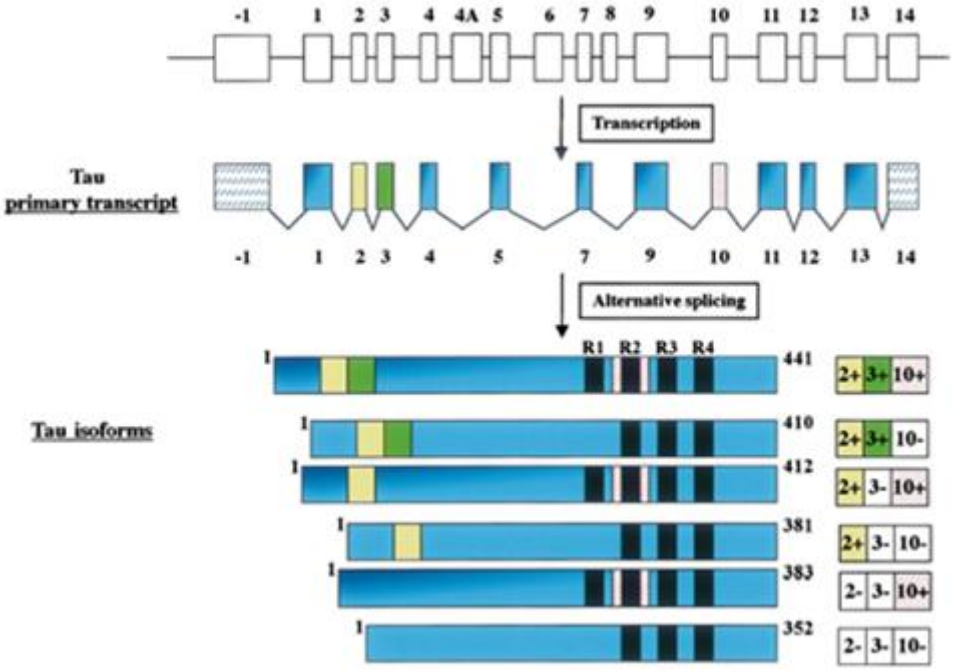

Fig. 2 – Schematic representation of the human *tau* gene, the human tau primary transcript and the six human CNS tau isoforms (adapted from Buee et al., 2000).

Tau isoforms are differentially expressed during development, thus in the foetal human central nervous system only the shortest tau isoform is expressed, on the other hand, all six alternatively spliced isoforms are found in the adult human brain (Goedert et al., 1989a), located in both the nucleus and the cytosol. However, little is known with respect to the functions and the regulation of the nuclear form.

1.1.2. Functions of tau

1.1.2.1. Cytosolic tau

Tau is constituted by a projection domain which encompasses a proline-rich region and an acidic region and a C-terminal microtubule-binding domain composed by repeats of highly conserved tubulin-binding motifs, Fig. 3 (Andreadis et al., 1992).

Projection domains of tau, determine the axonal diameter and spacing between microtubules in axons (Buee et al., 2000), interact with a neural plasma membrane (Brandt et al., 1995), cytoskeletal elements (by the bind with actin filaments and spectrin) (Knowles et al., 1994) and cytoplasmic organelles (Ebneth et al., 1998, Terwel et al., 2002). This domain is also involved in signal transduction pathways involving phospholipase C gamma (PLC-γ) (Hwang et al., 1996). On the other hand, the microtubule binding domain interacts with microtubules and permits their stabilization, bundling, (Lee and Rook, 1992, Knowles et al., 1994) and regulates the dynamic stability of microtubules, thus being implicated in their normal function (Lee et al., 1988, Goedert and Jakes, 1990, Knowles et al., 1994, Stam et al., 2006).

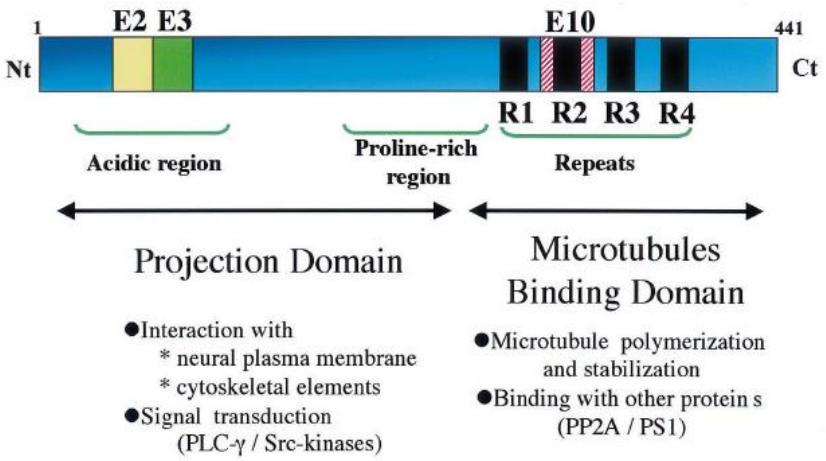

Fig. 3 – Schematic representation of the functional domains of the longest tau isoform (2+ 3+ 10+) (Buee et al. 2000)

In this way, tau-microtubule interaction is very important for axonal transport defined as the movement of protein and organelle cargoes through axons. In

physiological conditions microtubule associated proteins (MAPs), including tau, bind to microtubules and deliver the organelles from the soma to the nerve terminal and back to the soma by retrograde transport for recycling or re-energizing the organelles for subsequent anterograde transport. However, in pathological conditions, the tau hyperphosphorylation leads to dissociation from microtubules and this inhibits kinesin-dependent transport of peroxisomes, mitochondria, neurofilaments, and Golgi-derived vesicles to the neuritis, Fig. 4. Moreover the transport of amyloid precursor protein (APP) inside axons and dendrites is impaired, leading to the accumulation of APP in the cell body (Stamer et al., 2002).

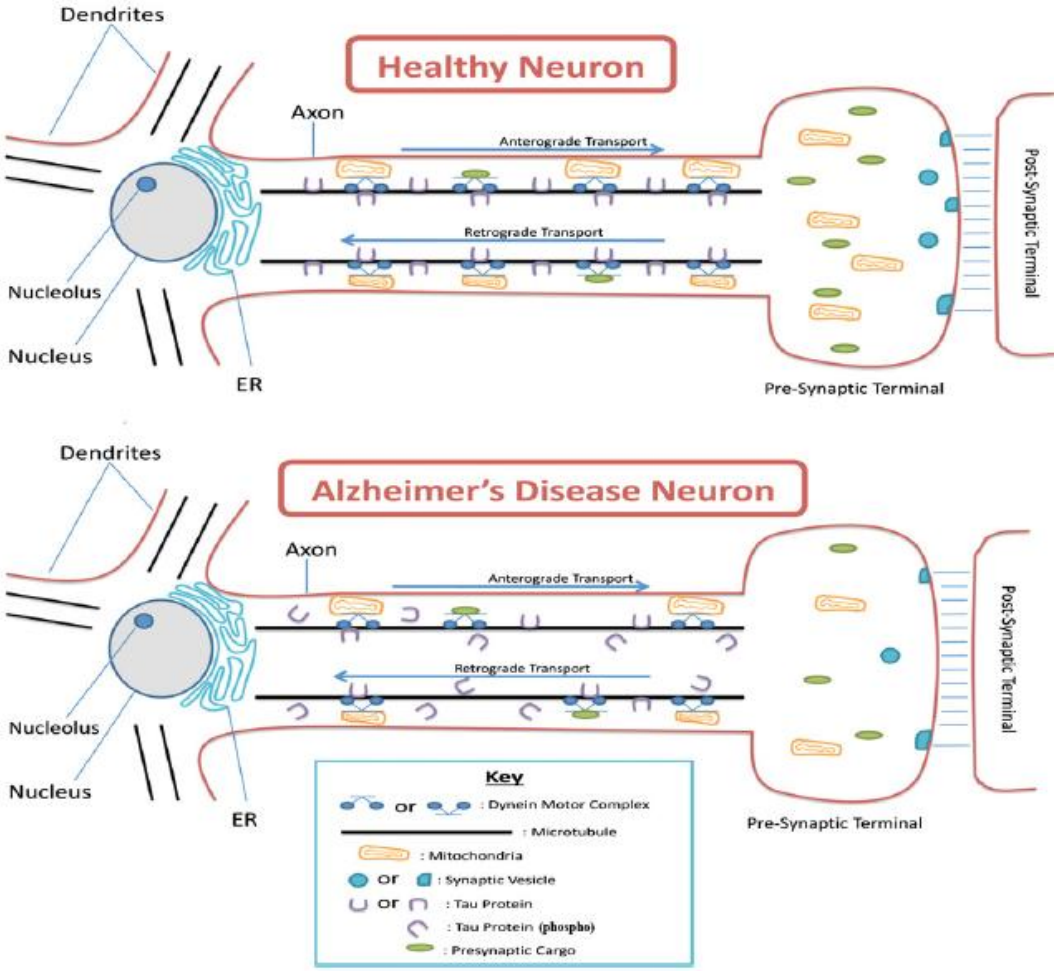

Fig. 4 – Schematic representation of axonal transport of organelles in neurons. Upper panel represents neuron from control subject and lower panel represents a neuron from Alzheimer's disease patient. In healthy neuron, tau protein binds a tightly to microtubule and allows normal axonal transport of organelles, including mitochondria. Axonal transport of organelles is impaired in AD neuron due to destabilization of hyperphosphorylated tau (Reddy, 2011).

1.1.2.2. Nuclear tau

Nuclear tau has likewise been described in both neuronal and nonneuronal cells, namely in neuroblastoma cells and in mouse and human brain; in non-neuronal cells it has been described in human cervical carcinoma, human macrophages and monkey kidney (Loomis et al., 1990, Lu and Wood, 1993, Lambert et al., 1995, Thurston et al., 1997, Cross et al., 2000).

The possible physiological function of nuclear tau is unknown as well as its role in tauopathies and neurodegeneration. Presently, it is known that nuclear tau can be found associated with nuclear organizer region (NOR) and binding to DNA (Corces et al., 1980, Sullivan et al., 2001, Sjoberg et al., 2006). So, when tau is associated with NORs, present in short arms of acrocentric chromosomes (Rudd and Willard, 2004) it is involved in the formation of the nucleolus (Sullivan et al., 2001). Additionally, when nuclear tau is bound to DNA it can induce conformational changes in the DNA from normal B-conformation to B-C-A mixed conformation consequently leading to gene expression changes (Padmaraju et al., 2010). This tau-DNA interaction is dependent on tau aggregation, but is independent of phosphorylation. In this way, aggregated tau loses its capacity to interact with DNA (Hua and He, 2002).

Moreover, *in vitro*, tau-DNA interaction, protects the DNA against denaturation (Hua and He, 2003) and in primary embryonic neuronal cultures protects the cell against both oxidative and heat stress (Sultan et al., 2011), although residues or phosphorylation states that control this interaction are not yet known.

However, it is believed that the phosphorylation is the mechanism that control the transport of tau from the cytosol to the nucleus and back to the cytosol (Hua et al., 2003). This possibility results from observation that cytosolic tau dephosphorylation was correlated with nuclear accumulation of tau, on the other hand, the increase of cytosolic tau phosphorylation results in decreased levels of nuclear tau (Hua et al., 2003).

1.1.3. Post-translational modifications and implications in tau aggregation

Like many proteins, the tau protein undergoes post-translational modifications, such as phosphorylation, glycosylation, truncation, nitration, and ubiquitination. All of these modifications are implicated in tau aggregate formation and stabilization (reviewed in Martin et al., 2011).

Glycosylation (addition of a sugar on the amino radical of aspargine on the hydroxyl radical of serine or threonine) promotes tau polymerization and stabilization of tau aggregates. In pathological conditions it is responsible for the production of free radicals, blocks the degradation of tau, and promotes tau accumulation and neuronal cell death (Ledesma et al., 1996, Smith et al., 1996, Nacharaju et al., 1997, Deng et al., 2009, Liu et al., 2009a, Liu et al., 2009b) because the glycated protein cannot be degraded or released from cell (Yan et al., 1994),

Truncation, especially found in PHFs (Mena et al., 1996), suggesting that it might contribute to tau aggregation (Gamblin et al., 2003) and also to apoptosis, (Horowitz et al., 2004, Basurto-Islas et al., 2008).

Nitration of tau (addition of nitrogen dioxide on tyrosine of an organic molecule (Horiguchi et al., 2003) occurs at four sites: Y18, Y29, Y197 and Y394 and this is involved in aggregation and oligomerization (Zhang et al., 2005b) of the latter decreasing its ability to promote tubulin assembly (Reynolds et al., 2006).

Ubiquitination is the specific binding of one or more molecule(s), of a small protein, ubiquitin, on proteins that signal for their degradation in the cytosol by the ubiquitin-proteosome-system (UPS). In nonpahological conditions, the tau is ubiquitinated and proteolytically processed by UPS (David et al., 2002, Zhang et al., 2005a, Arnaud et al., 2009, Liu et al., 2009c) and the residues that undergo this post-translational modifications are localized in the C-terminus, at K254, K311 and K353 (residues found in the MBD region) (Morishima-Kawashima et al., 1993, Cripps et al., 2006). On the other hand, in pathological conditions this process is impaired, consequently, higher levels of ubiquitinated tau protein can be found in PHFs and cerebrospinal fluid of AD patients (Iqbal and Grundke-Iqbal, 1991, Iqbal et al., 1998).

Finally, phosphorylation is the post-translational modification most studied, and is involved in tau function and aggregation (Khatoon et al., 1992, 1994). This aspect will be addressed below.

1.1.3.1. Tau phosphorylated residues

Reversible protein phosphorylation is controlled by protein kinases and phosphatases, and involves either the addition of phosphate groups via the transfer of the terminal phosphate from ATP to an amino acid residue by protein kinases or its removal by protein phosphatases, Fig. 5 (Cohen, 1989).

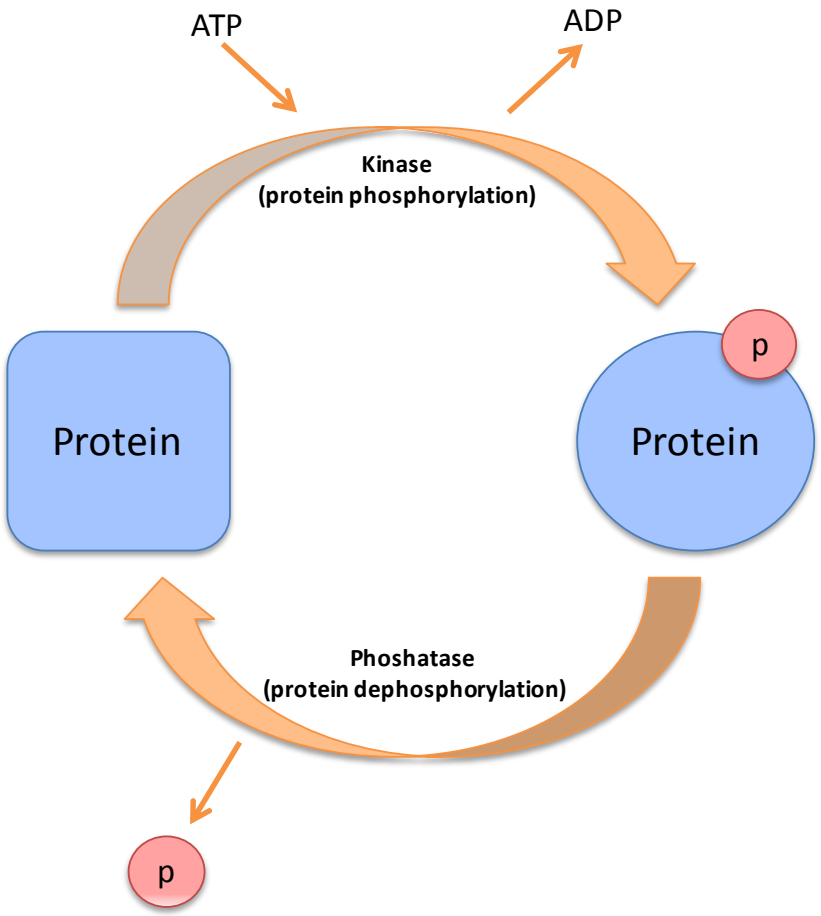

Fig. 5 - Schematic representation of reversible protein phosphorylation. Protein kinases transfer a phosphate group from ATP to a target protein (protein phosphorylation), while protein phosphatases catalyze the hydrolysis of the phosphate group from the target protein (protein dephosphorylation) (Cohen, 1989).

In physiological conditions, tau phosphorylated is involved in multiples cellular function such as cell morphology, retards cell growth and alters the localization of several organelles (Wang and Liu, 2008), induction of neuronal cell cycle (Andorfer et al., 2005, Khurana et al., 2006, Zhu et al., 2007), and protects neurons from oxidative stress (Nunomura et al., 2001) and from apoptosis (Lassmann et al., 1995, Guo et al., 2004). On the other hand, in pathological conditions, tau hyperphosphorylated is responsible for microtubule disassembly and have deleterious effects on cellular processes, for example disruption of microtubule dynamics leads to impaired function of the neurons and promotes neuronal death, blocks intracellular trafficking of neurons which is essential for normal metabolism and inhibits the proteosomal activity (Hasegawa et al., 1998, Salehi et al., 2003, Vandebroek et al., 2006).

Tau phosphorylation and hyperphosphorylation (considered as the increase in the number of sites phosphorylated in the same tau molecule and/or as an increase in the number of tau molecule phosphorylated at a given site) occurs because tau has 85 putative phosphorylation sites (45 of them are serines, 35 are threonines and only 5 are tyrosines), Fig. 6, that are phosphorylated and/or dephosphorylated by multiple proteins kinase (PKs) and phosphatase (PPs).

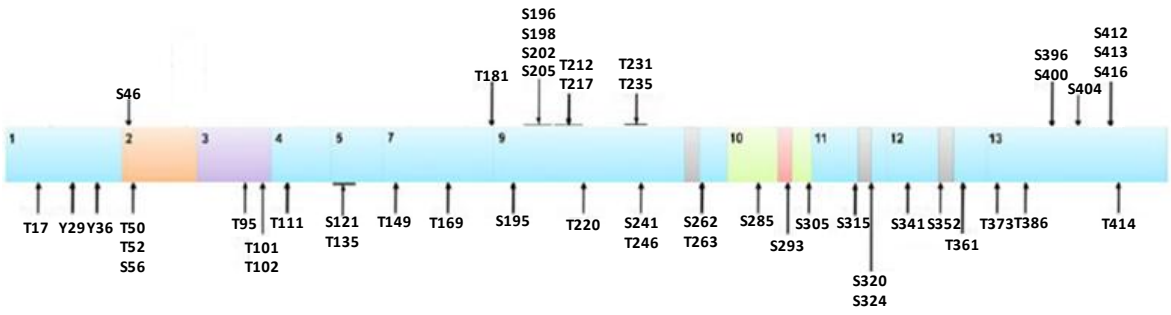

Fig. 6 – Representation of tau phosphorylation sites in normal brain. T - threonine, S - serine, Y - tyrosine (adapted from Martin et al., 2011).

In this way, multiple studies have been performed to understand which major protein kinases and phosphatases are involved in tau phosphorylation/ dephosphorylation. These have reported that the main kinases involved in tau

phosphorylation are GSK3 followed by CDK5; On the other hand, the PPs more implicated in tau dephosphorylation are protein phosphatase 2A (PP2A) followed by protein phosphatase 1 (PP1), table 1.

Table 1 – Tau phosphorylated residues by protein kinases (PKs) and protein phosphatases (PPs). S – serine, T – Threonine

		tau residues
PK	GSK3	S_{46}, T_{50}, T_{149}, T_{181}, S_{195}, S_{199}, S_{202}, T_{205}, T_{212}, T_{217}, T_{220}, T_{231}, S_{235}, S_{241}, T_{245}, S_{262}, S_{285}, S_{324}, S_{352}, S_{396}, S_{400}, S_{404}, S_{413}
	CDK5	S_{195}, S_{202}, T_{205}, T_{212}, T_{217}, T_{231}, S_{235}, S_{396}, S_{404}
PP	PP2A	S_{46}, S_{199}, S_{202}, T_{205}, S_{214}, S_{235}, S_{262}, S_{396}, S_{404}
	PP1	S_{199}, S_{202}, T_{231}, S_{235}, S_{262}, S_{393}, S_{404}

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase widely expressed and highly conserved. In mammals, this is encoded by two genes located on chromosome 19 and 3 generate GSK-3α and GSK-3β, respectively. This kinase (GSK-3) is largely considered as a cytoplasmic protein, but it can also be detected in the nucleus and mitochondria and it is involved in multiples cellular functions such as signaling pathways, metabolic control, apoptosis/cell survival, oncogenesis and memory impairment. The GSK-3 activity appears to be positively dependent on tyrosine phosphorylation at residue 279 for GSK-3α and 216 for GSK-3β via PP1, and negatively regulated by N-terminal phosphorylation of serine residues, Ser21 for GSK-3α and Ser9 for GSK-3β via Akt (reviewed in Kaidanovich-Beilin and Woodgett, 2011).

Cyclin-dependent kinase 5 (CDK5) is a member of the Cdk family found in both cytosol and nucleus. This kinase is controlled by specific activators p35 and p39 and does not require any additional phosphorylation in order to become active, although the phosphorylation at Tyr15 by Scr-related tyrosine kinases can increase the activity of this protein. In pathological conditions, p35 is proteolytic cleaved by calpain to generate p25, which causes aberrant Cdk5 activation and leads to abnormal phosphorylation of its substrates, such as tau. Several studies support that in pathological condition, Cdk5 can phosphorylate tau on sites that are found in paired helical filaments (a form of tau

aggregation associated with different pathologies) (reviewed in Lopes and Agostinho, 2011).

Relatively to PPs, protein phoshatase 1 is highly conserved among all eukaryotes (Lin et al., 1999) and of all mammalian tissues, the brain expresses the highest levels of protein kinases and phosphatases, and PP1 is highly expressed in both neurons and glia (da Cruz e Silva et al., 1995, Ouimet et al., 1995). This PP1 is responsible for regulating a variety of cellular events through the dephosphorylation of multiple substrates and its multifunctionality is due to its association with different regulators and/or targeting subunits (Bollen, 2001, Cohen, 2002, Ceulemans and Bollen, 2004, Fardilha et al., 2010) known as PP1 Interacting Proteins (PIPs) (Esteves et al., 2012a, Esteves et al., 2012b).

Finally, PP2A is one of the most abundant enzymes in some tissues, structurally it is a trimeric holoenzyme, constituted by a structural subunit (also known as the A or PR65 subunit), a catalytic subunit (C subunit) and a regulatory subunit (B subunit) (Martin et al., 2010). PP2A in all eukaryotic cells can be found in the nucleus and the cytoplasm and is involved in a large number of cellular processes, such as cell proliferation and death, cell mobility, cytoskeleton dynamics, the control of the cell cycle, and the regulation of numerous signaling pathways (reviewed in Janssens and Goris, 2001) it is also likely to be an important tumor suppressor (Janssens et al., 2005, Mumby, 2007).

1.1.4. Neurofibrillary tangles formation

As previously described, neurofibrillary tangles (NFTs) are formed from hyperphosphorylated tau, the main hallmark of tauopathies. NFTs are nonmembrane-bound bundles of abnormal fibers localized in the cytoplasm of neurons, and electron microscopy showed that fibers consist of pairs of approximately 10nm filaments, wound into helices (paired helical filaments, PHFs), with helical periods of approximately 160nm (Selkoe, 2001).

The formation of PHFs and consequently NFTs can be due to truncation (Binder et al., 2005), site-specific phosphorylation (Liu et al., 2007) or aggregation inducers (Chirita et al., 2005), leading to conformational changes and β-sheet enrichment of tau that in

turn promote the adoption for an aggregation-competent conformation (von Bergen et al., 2000). This conformation change may form side chain/ side chain interactions ending in the shaping of the tau-tau dimmers (Andronesi et al., 2008). These dimmers adopt a stable structure and with the help of a tau-membrane interaction can begin a process of nucleation (Gray et al., 1987, Kuret et al., 2005, Lira-De León et al., 2009). After, the elongation of dimmers is reached forming oligomers, this aggregation process can continue and begin forming subunits of filaments, termed promoters. Mature tau filaments, called PHFs have two protofilaments around each other (Congdon et al., 2008), this process ends with NFTs formation, Fig. 7.

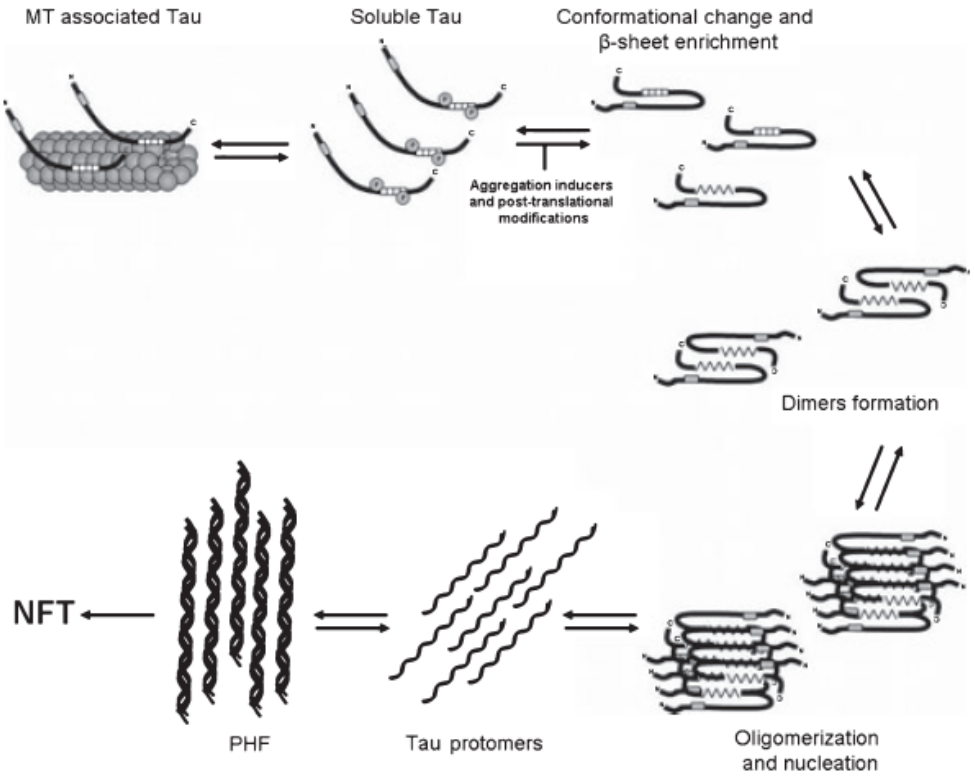

Fig. 7 - Schematic representation of a possible pathway of tau aggregation and consequently NFT formation (Meraz-Rios et al., 2010).

1.2. Tauopathies

The term ”Tauopathy” is used to define a group of neurodegenerative disorders in which tau hyperphosphorylation and aggregation of neurofibrillary tangles (NFTs) is believed to be directly associated with neuronal death and disease progression (Susanne and Ratan, 2004).

In this way, NFTs are consistently found in multiple disorders, such as Alzheimer’s disease (AD), postencephalitic parkinsonism, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick’s disease, frontotemporal dementia with parkinsonism 17 (FTD-17), Down’s syndrome and myotonic dystrophy (DM), among others. However NFTs also occur in normal aging and contain the hyperphosphorylated microtubule-associated protein tau (reviewed in Buee et al., 2000). These disorders are characterized by different types of NFTs according to the tau isoform involved in their formation, in this way the tauopathies can be subdivided in five classes (0, I, II, III and IV), Fig 1.

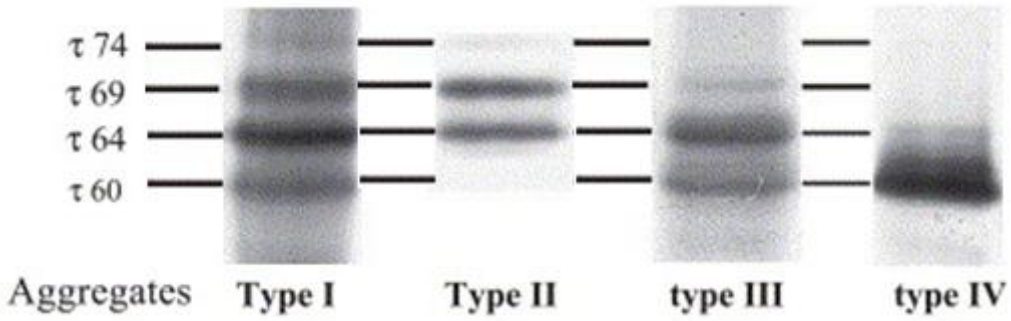

Fig.1 – Electrophoretic profiles of pathological tau proteins with their molecular masses (kDa), and classification of several aggregate types (adapted from Sergeant et al., 2005).

1.2.1. Class 0: Frontal lobe degeneration non-Alzheimer non-Pick

In frontal lobe degeneration there is a loss of tau protein expression, thus the tau aggregates are absent. However, this pathology can be identified by several morphological changes, such as neuronal cell loss and gliosis mainly in the superficial cortical layers of the frontal and temporal cortex (Delacourte and Buee, 1997, Zhukareva et al., 2001, Zhukareva et al., 2003).

1.2.2. Class 1: A major tau triplet at 60, 64 and 69 kDa

The pathological tau triplet corresponds to the aggregation of the six tau isoforms (Goedert et al., 1992, Sergeant et al., 1997). This class includes ten neurological disorders including postencephalitic parkinsonism, Alzheimer´s disease, and Down's syndrome, (reviewed in Sergeant et al., 2005).

Postencephatilic Parkinsonism results from patients that previously had influenza. Such patients do not exhibit cognitive changes, aphasia or apraxia. However, the brain does present NFTs in variable density in the hippocampal and cerebral cortex (reviewed in Buee et al., 2000).

Alzheimer´s disease is a progressive neurodegenerative disorder that leads to dementia. The first symptoms, one typically, memory loss followed by aphasia, agnosia, apraxia and behavioral disturbances. This neuropathology has two main hallmarks, senile plaques (extracellular accumulation of amyloid-β peptide) and neurofibrillary tangles (intracellular hyperphosphorylated tau accumulation) that can be found in hippocampus, cortex and subcortical areas (reviewed in Buee et al., 2000).

Down´s syndrome is due to trisomy of chromosome 21, these patients have numerous somatic dysfunctions, such as deficient growth, delayed brain maturation and cognitive impairment, usually leading to dementia after 50 years of age. Additionally, their brain has NFTs and amyloid deposits that occur prior to neuronal loss, principally in hippocampal, cortex and subcortical areas (Hof et al., 1995).

1.2.3. Class 2: A major tau doublet at 64 and 69kDa

Class 2 is characterized essentially by the aggregation of 4R-tau isoforms (tau isoforms with exon 10, more detail in 1.2.1). This pathological tau profile is observed in progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Argyrophylic gain and FTDP-17 (reviewed in Sergeant et al., 2005).

Neuropathologically, PSP is characterized by neuronal loss, gliosis and NFT formation. Initially, the neurofibrillary tangles and neurodegeneration are found in basal ganglia, brain stem, and cerebellum (Steele et al., 1964). This is followed by degeneration in the perirhinal, inferior temporal and prefrontal cortex, with the same features as subcortical NFT (Hauw et al., 1990, Hof et al., 1992) and in glial fibrillary tangles (Hauw et al., 1990, Hof et al., 1992, Komori et al., 1998).

Corticobasal degeneration (CBD) is a rare, sporadic and slowly progressive late-onset neurodegenerative disorder. It is clinically characterized by cognitive disturbances and extrapyramidal motor dysfunction. Neuropathological examination reveals NFTs and severe glial and neuronal abnormalities in cortex, brainstem and subcortical structures. The glial pathology is marked by astrocytic plaques and numerous tau-immunoreactive inclusions in the white matter (Rebeiz et al., 1967, Rinne et al., 1994).

FTDP-17 consists of autosomal-dominantly inherited neurodegenerative disease with diverse clinical and neuropathological features (Foster et al., 1997, Crowther and Goedert, 2000). Neuropathologically, abundant filamentous tau aggregates one present in nerve cells and also in glial cells; clinically it is characterized by marked neuronal loss in affected brain regions, with extensive neuronal or neuronal and glial fibrillary pathology composed of tau aggregates (reviewed in Spillantini et al., 1998). FTDP-17 is associated with multiple mutations in the *tau* gene, such as, K257T, G272V, 1280K, P301L, P301S, V337M, and R406W, promoting heparin- or arachidonic acid–induced tau filament formation *in vitro* relative to WT tau (Arrasate et al., 1999, Goedert et al., 1999, Nacharaju et al., 1999, Barghorn et al., 2000, Gamblin et al., 2000, Rizzini et al., 2000).

1.2.4. Class 3: A major tau doublet at 60 and 64kDa

The pathological profile is essentially characterized by 3R-tau isoforms (tau isoforms without exon 10, more detail in 1.2.1.) and the Pick´s disease is an example of this class of tauopathy (reviewed in Delacourte, 2005).

Pick's disease is characterized neuropathologically by prominent frontotemporal lobar atrophy, gliosis, severe neuronal loss, tau-immunoreactive intraneuronal inclusions known as Pick bodies and by insoluble tau proteins with predominantly three microtubule-binding repeat tau isoforms (Buee Scherrer et al., 1996, Delacourte et al., 1996, Zhukareva et al., 2002). Therefore, Pick's disease is characterized by an accumulations of Pick bodies in the hippocampal region and cortex as well as the presence of NFTs in both cortical gray and white matter, that distinguish this tauopathy from other neurodegenerative disorders (Zhukareva et al., 2002).

1.2.5. Class 4: A major tau 60kDa

Class 4 is characterized by a strong pathological tau band at 60 kDa and, to a lesser extent, by a pathological tau component at 64 and 69 kDa. This typical pathological tau profile is reflected by a reduced number of tau isoforms expressed in the brain of individuals (Delacourte, 2005).

Myotonic dystrophy (MD) is an example of this class. MD is an inherited autosomal dominant disorder caused by a single gene mutation consisting of expansion of a CTG trinucleotide motif in the 3´ untranslated region of the myotonic dystrophy protein kinase gene (dmpk), located on chromosome 19q (Brook et al., 1992). Characteristically multiples systems are affected, such as the central nervous system (cognitive and neuropsychiatric impairments), the heart (cardiac conduction defects), the genital tract (testicular atrophy), the eyes (cataracts), the ears (deafness), gastrointestinal tract (smooth muscle), endocrine system (insulin resistance), thus leading to a wide and variable complex panel of symptoms (reviewed in Sergeant et al., 2005). In central

nervous system (CNS) the brain regions more affected are the temporal lobe, the hippocampus and the entorhinal lobe (Jaspert et al., 1995, Buee et al., 2000).

2. Objectives

Tauopathies are a group of diseases characterized by NFTs formation. However, presently, it is known that dimmers (first stage of NFTs formation) of tau can be physiological and they are a possible form of tau function regulation, namely they bind to DNA.

Moreover, Liu et al in 2005, established that the phosphatase activities more relevant to tau cytosolic phosphorylation in human brain are PP2A (71%), PP1 (11%), PP5 (10%) and PP2B (7%). On the other hand, more recently (Padmaraju et al., 2010) described that nuclear tau can have an important role in neurodegeneration. However, the possible link between cytosolic and nuclear tau is unknown; though it is known that this connection, probably, is tau phosphorylation dependent.

In this way, it is crucial to understand if the phosphatases with major expression/activity involved in nuclear tau phosphorylation and consequent aggregation are the same as those implicated in cytosolic tau phosphorylation.

Thus the specific aims of this dissertation are:

- ✓ Validation of the cell model to study nuclear tau;
- ✓ Study of tau aggregation and protein phosphatases expression upon exposure to drugs modulating phosphorylation events (roscovitine, lithium chloride and okadaic acid);
- ✓ Determination tau aggregates formation as a consequence of modulating protein phosphatases expression (PP1α, PP1γ and PP2A knockdown).

3. Study of tau aggregation and protein phosphatases expression upon exposure to drugs modulating phosphorylation events (roscovitine, lithium chloride and okadaic acid)

3.1. Introduction

As previously described both nuclear and cytosolic tau may be implicated in neurodegeneration, but the relationship and possible interdependence between them are unknown.

However, the transport of nuclear tau from the cytosol to the nucleus, and this back to the cytosol is supposed to be regulated by phosphorylation (Hua et al., 2003). It is also known that the PP more implicated in tau cytosolic phosphorylation and aggregation is PP2A (Liu et al., 2005), however the protein more implicated in phosphorylation and aggregation of nuclear tau is unknown. Thus, to evaluate these two stages it was necessary to identify a cell type for this study, with respect to tau expression and aggregation.

The measurement of tau aggregates was realized using the membrane filter assay, Chang and Kuret in 2008 tested this method and concluded that it is a rapid and viable form to quantify tau aggregation stage (Chang and Kuret, 2008).

We used a Bio Dot SF blotting apparatus with 48 wells arranged in 8 rows and 6 columns; all with dimensions of 7mm x 0.75mm, this makes it easy to compare between samples. This apparatus can be repeatedly autoclaved, and is resistant to many chemicals, including acids, bases and ethanol and can be used to quantify DNA, RNA and proteins.

In this chapter the results obtained from incubation of cells with okadaic acid, chloride lithium (LiCl) and roscovitine are described. These chemicals are responsible for PPs, GSK3 and CDKs inhibition, respectively. Subsequently, tau expression and aggregation were measured.

3.2. Methods

3.2.1. Cell culture

The cell line used in this study was undifferentiated SH-SY5Y cells; this is a neuroblastoma human cell line. SH-SY5Y cells were grown in MEM with Earle's salts and L-alanine and L-glutamine, supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, 100μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

3.2.2. SH-SY5Y treatment with kinases inhibitors

In order to establish the kinases involved on tau phosphorylation and consequently, affecting tau aggregation, SH-SY5Y cells were incubated with GSK3 and CDK inhibitors: lithium chloride and roscovitine, respectively.

Stock solutions of LiCl (sigma) and roscovitine (Calbiochem) were prepared and used for the following incubation. SH-SY5Y cells were plated and let be confluent. Cells were washed twice with PBS before LiCl (0, 2, 5, 10 or 20mM) and roscovitine (0, 2, 5, 10 or 20μM) treatment in SH-SY5Y serum and antibiotics medium free for 30 minutes or 3 hours.

3.2.3. SH-SY5Y treatment with phosphatase inhibitor

In order to establish the protein phosphatases (PPs) involved on tau phosphorylation and consequently tau aggregation, SH-SY5Y cells were incubated with protein phosphatase inhibitor: okadaic acid (OA).

As stock solution of okadaic acid was prepared and used for the following incubation. SH-SY5Y cells were plated and let be confluent. Cells were washed twice with PBS before OA (0, 0.1, 0.25, 50, 500 or 5000nM) treatment in order to inhibit different PPs, table 2, in SH-SY5Y serum and antibiotics medium free for 30 minutes or 3 hours.

Table 2 – Range of IC50 values of protein phosphatase inhibition. All values expressed as nanomolar (nM). PP – protein phosphatase; IC50 – 50% inhibition concentration. Adapted from Swingle et al 2007 (Swingle et al., 2007).

Inhibition of Ser/Thr protein phosphatases activity (IC_{50}*)						
Compound	**PP1**	**PP2A**	**PP2B (calcienurin)**	**PP4**	**PP5**	**PP7**
Okadaic Acid (nM)	15-50	0.1-0.3	~4000	0.1	3.5	>1000

3.2.4. Membrane Filter Assay

Aggregated tau was evaluated using a vacuum-based 48-well format filter assay. Cells were lysed in a buffer containing 0.5% Nonidet P-40, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, and protease inhibitors mixture. After brief sonication, cell lysates were submitted to membrane filter assay or western blotting because the tau aggregation levels result from the ratio between membrane filter assay and SDS-PAGE band intensity relative values. The membrane obtained from membrane filter assay was washed with 1%SDS for 10 min. The membranes acquired from both methods were developed with total tau 5 antibody (Zhang et al., 2006, Chang and Kuret, 2008).

3.2.5. BCA assay

Measurements of total protein concentration were carried out using Pierce's BCA protein assay kit, following the manufacturer's instructions. This method combines the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium (the biuret reaction), with a sensitive colorimetric detection of the Cu^{+} cation using a reagent containing bicinchoninic acid (BCA). The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one Cu^{+} ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration over a working range of 20 g/mL to 2000 g/mL. At least duplicates of all samples were assayed by this method, as well as the appropriate protein standards as described below, table 3.

Table 3 - Standard curve used in the BCA protein assay. WR. working reagent; BSA. Bovin serum albumin; SDS. Sodium dodecyl sulfate

Standard	BSA (µL)	10%SDS (µL)	H_2O (µL)	Protein mass (µg)	W.R (mL)
P0	0	5	45	0	1
P1	1	5	44	2	1
P2	2	5	43	4	1
P3	5	5	40	10	1
P4	10	5	35	20	1
P5	20	5	25	40	1
P6	40	5	5	80	1

The Working Reagent was prepared by mixing BCA reagent A with BCA reagent B in the proportion of 50:1. Then, 1 mL of WR was added to each microtube (standards and samples) and the microtubes were incubated at 37°C for 30 min. Once the tubes cooled to room temperature the absorbance was measured at 562 nm. A standard curve was obtained by plotting BSA standard absorbance vs BSA concentration, and it was then used to determine the total protein concentration of each sample.

3.2.6. SDS-PAGE

Samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE the migration of the proteins was determined by their molecular weight. For the time course analysis and to visualize the full-length tau protein, with a molecular weight around 100 kDa, a 12% polyacrylamide gel was used, table 4.

Table 4 - Composition of the running and stacking gels for SDS-PAGE. APS – ammonium persulfate; LGB – lower gel buffer; SDS – sodium dodecyl sulphate; UGB – upper gel buffer

Components	Running Gel (12%)	Stacking gel (3.5%)
Water	24.73mL	13.2mL
30%Acryl/8%Bisacryl.	19.96mL	2.4mL
6xLGB	15mL	--
5xUGB	--	4.0mL
SDS 10%	--	200 µL
10% APS	300µL	200 µL
TEMED	30 µL	20 µL

The running and the stacking gels were prepared as indicated in table 4. The samples were prepared by the addition of ¼ volume of loading gel (LB) buffer and run at 90 V for approximately 4 hours.

3.2.7. Immunoblotting

In our experimental system, after electrophoresis, proteins were transferred to nitrocellulose membranes for 17 hours (hr) at 200 mA and then visualized with specific antibodies.

Membranes from filter assay or SDS-PAGE were visualized with enhanced chemiluminescence immunodetection.

Immunodetection by enhanced chemiluminescence (ECL)

ECL™ is a light emitting non-radioactive method for the detection of immobilized antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. In order to visualize the proteins the membranes were soaked in 1x TBS (5 min). Non-specific binding sites were blocked by incubating the membrane in 3% BSA in 1x TBST (2 hr). The membrane was further incubated with the primary antibody, table 5, diluted in 3% BSA in 1x TBST. After three washes of 10 min each in 1x TBST the membrane was incubated with an horseradish peroxidase conjugated secondary antibody, table 3, in 3% low fat milk in 1x TBST (2 hr with shaking). The membrane was then washed 3 times with 1x TBST for 10 min. Subsequently, the membrane was incubated for 5 min with the

ECL detection solution. After exposure to X-ray film (Kodak), immunoblots were scanned and quantified using Quantity One densitometry software (Bio-Rad).

Antibodies

The following primary antibodies were used: mouse monoclonal anti-tau antibody, clone Tau-5 (Millipore); polyclonal anti-PP1γ antibody (CBC3C); polyclonal anti-PP1α antibody (CBC2C); monoclonal anti-PP2A antibody and mouse monoclonal anti-β-tubulin antibody (Invitrogen) (table 5).

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

Table 5 – Summary of the antibodies used to detect target proteins and specific dilution used for the different assays. PP1α, protein phosphatase 1 alpha; PP1γ, protein phosphatase 1 gamma; PP2A, protein phosphatase 2A; IB, Immnunoblotting; ECL-IM, Immunodetection by enhanced chemiluminescence – developed "in house"

Target Protein/ Epitope	Primary Antibody	Secondary Antibody	Detection Method	Expected bands sites (kDa)
total Tau	Tau-5 (1:500)	Anti-mouse (1:5000)	Luminata™ crescendo Western HRP Substrate	100
total PP1α	PP1α (1:2500)	Anti-rabbit (1:5000)	ECL-IM	37
total PP1γ	PP1γ (1:5000)	Anti-rabbit (1:5000)	ECL-IM	37
Total PP2A	PP2A (1:1000)	Anti-mouse (1:5000)	ECL-IM	37
Tubulin	Anti β-tubulin (1:1000)	Anti-mouse (1:5000)	ECL-IM	50

3.3. Results

3.3.1. Nuclear and total tau expression in SH-SY5Y cells

As described above, one of the aims of this work was to develop a model which would permit identifying the phosphatases involved in dephosphorylation of nuclear tau. Thus, a suitable cell type for this is a neuronal or “neuronal like” cells with high nuclear tau levels. In the last few years multiple cell types with nuclear tau expression have been reported. One of these cell type is the SH-SY5Y neuroblastoma undifferentiated cells (Uberti et al., 1997) which was the cell type used in the assays reported in this dissertation.

3.3.2. Protein phosphatases involved in tau nuclear aggregation after inhibition of protein kinases or phosphatases

Tau aggregation in SH-SY5Y cells was measured following incubation with different concentrations of roscovitine that inhibit CDKs, and for the same experimental conditions PP expression levels were also measured.

In this way, when CDKs were inhibited, Fig. 8. Tau aggregation levels decreased for both time points; 30 minutes and 3 hours, moreover a greater inhibition of CDKs was correlated with diminished tau aggregation. For PPs expression, at 30 minutes, it was verified that PP1γ and PP2A expression increased comparative to control, by 4 and 2 fold respectively. However, it was also observed that the PP1γ and PP2A expression decreased with increasing concentrations of roscovitine. Regarding the levels of PP1α, there is a slight increase for 2 and 5μM, and further decrease to 10 and 20μM. At 3 hours, it was observed that PP1γ and PP2A expression decreased comparative to control, though intracellular levels increased with higher concentrations of roscovitine. On the other hand, the PP1α levels increased relative to control.

The SH-SY5Y cells were incubated with different LiCl concentrations, which inhibit GSK3 activity via Akt (Pan et al., 2011), Fig. 9, it was demonstrated that the aggregation levels decreased, but the higher GSK3 inhibition lead to an increase in tau aggregates. The expression levels of PPs were also obtained from the same cells which were used to measure tau aggregation. These results demonstrated that PP1γ, PP1α and PP2A levels increase at 30 minutes for all concentrations tested in comparison to control, but decreased with increasing LiCl concentrations. On the other hand, at 3 hours, the PPs more expressed were PP1γ and PP2A, in turn PP1α levels were approximately half of the others PPs, and the levels of all PPs analyzed also decreased when GSK3 inhibition increase.

After PPs inhibition with OA, Fig. 10, it was verified that the tau aggregation levels increased for all concentrations and times tested. It was also evident that for 0.25nM (PP4 and PP2A inhibition) and 50nM (PP4, PP2A, PP5 and PP1 inhibition), the aggregation levels were higher at 30 minutes compared to 3 hours. The results for PPs inhibition also

demonstrated that PP4 inhibition alone (0.1nM) was sufficient to increase tau aggregates at both 30 minutes and 3 hours.

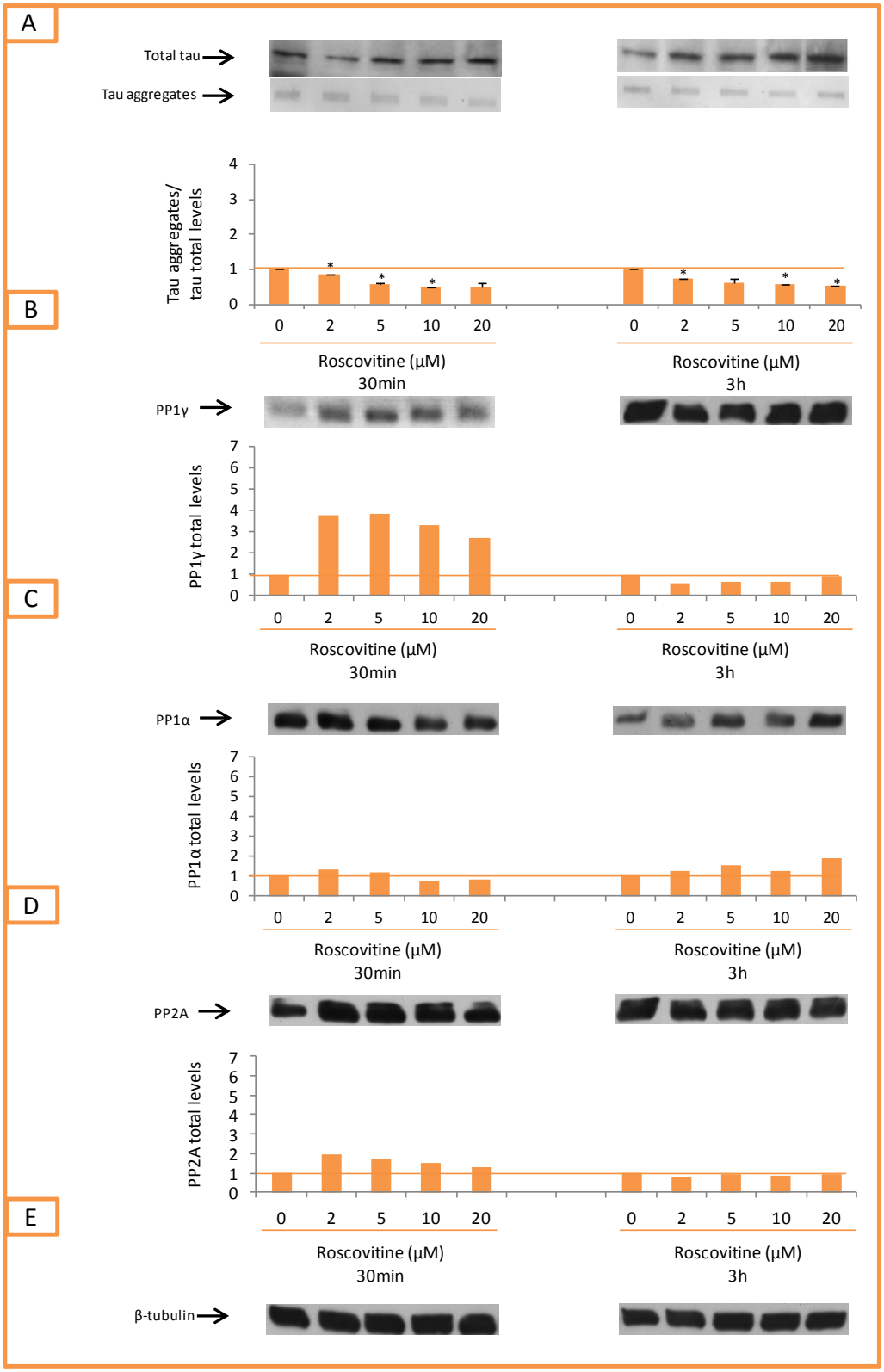

Fig. 8 – Roscovitine (CDKs inhibition) effects on both tau aggregation and protein phophatases expression (PP1γ, PP1α and PP2A). SH-SY5Y neuroblastoma cells were incubated at 37ºC in SH-SY5Y medium without serum or antibiotics for 30 minutes or 3 hours with roscovitine (0, 2, 5 10 or 20μM). Cells lysates were collected and analyzed by immunoblotting and filter assay. Both were blotted with Tau5 antibody which recognizes total tau. (A) total tau aggregates formed; (B) PP1γ expression; (C) PP1α expression; (B) PP2A expression; (E) tubulin expression was used as a loading control detected with β-Tubulin antibody. *p<0.05 (experimental vs control data).

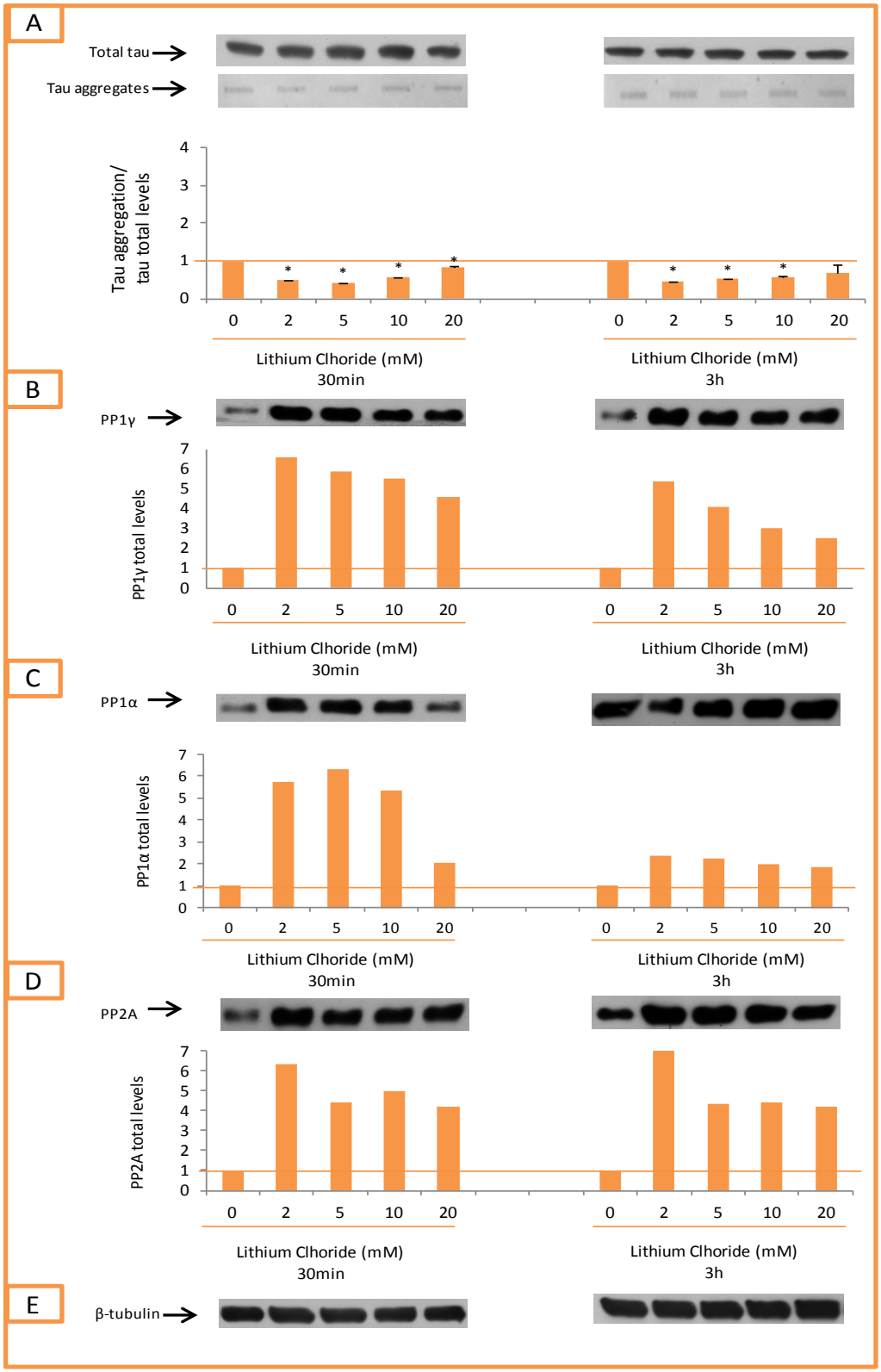

Fig. 9 – Lithium chloride (GSK3s inhibition) effects on both tau aggregation and protein phophatases expression (PP1γ, PP1α and PP2A). SH-SY5Y neuroblastoma cells were incubated at 37ºC in SH-SY5Y medium without serum or antibiotics for 30 minutes or 3 hours with lithium chloride (0, 2, 5 10 or 20mM). Cells lysates were collected and analyzed by immunoblotting and filter assay. Both were blotted with Tau5 antibody which recognizes total tau. (A) total tau aggregates formed; (B) PP1γ expression; (C) PP1α expression; (B) PP2A expression; (E) tubulin expression was used as a loading control. *p<0.05 (experimental vs control data).

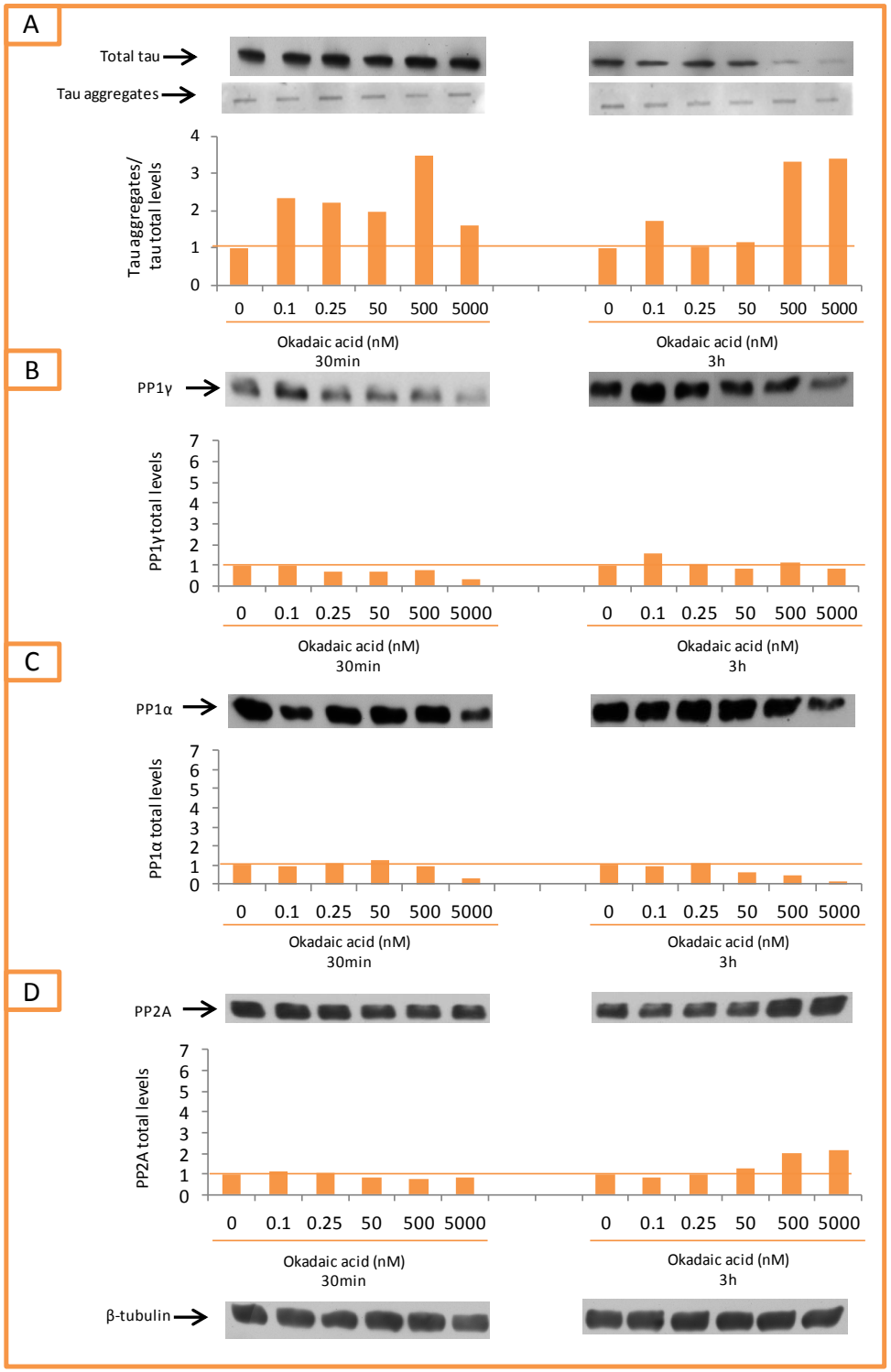

Fig. 10 – Okadaic acid (PPs inhibition) effects on both tau aggregation and protein phophatases expression (PP1γ, PP1α and PP2A). SH-SY5Y neuroblastoma cells were incubated at 37ºC in SY-SY5Y medium without serum or antibiotics for 30 minutes or 3 hours with okadaic acid (0, 0.1, 0.25, 50, 500 and 5000nM). Cells lysates were collected and analyzed by immunoblotting and filter assay. Both were blotted with Tau5 antibody which recognizes total tau. (A) total tau aggregates formed; (B) PP1γ expression; (C) PP1α expression; (B) PP2A expression; (E) tubulin expression was used as loading control. n=2

3.4. Discussion

Several studies have concluded that the most important protein kinases that are involved in tau phosphorylation are GSK3 and CDK5, leading to tau aggregates formation.

Our results suggest that when CDKs are inhibited (roscovitine incubation), tau aggregates decreased for both 30 minutes and 3 hours incubation periods, and these decreases are progressive with increasing roscovitine concentration. PPs expression was verified and it is evident that PP1γ is substantially more expressed than the others PPs analyzed that when CDKs are inhibited, GSK3 is activated by PP1 (Morfini et al., 2004), in this way, our results suggest that this activation can be via PP1γ. Nevertheless, at 3 hours, it seems that a mechanism is involved that also contributes to tau aggregation decrease, but this is independent of PP1γ, since their expression levels decreased relative to control.

Regarding the results obtained from GSK3 inhibition (LiCL incubation) suggested that the mechanism that leads to a decrease in tau aggregates at 30 minutes is equally dependent on PP1γ, PP1α and PP2A. However, at 3 hours, it appears to be primarily dependent of PP1γ and PP2A, for which the expression levels remain very high. Moreover, it has been found that increasing of GSK3 inhibition led to a slight increase tau aggregate, which is not an expected result. However, we can perhaps explain this based on several previous studies. Other authors have reported that the PP1 and PP2A can dephosphorylate tau directly, moreover PP1 activate GSK3 and CDKs can in turn phosphorylate tau (Bennecib et al., 2000). Our results demonstrated that the PP1γ, PP1α and PP2A expression decreased with increasing the GSK3 inhibition. Thus, the progressive decrease in the PPs expression can be related to tau aggregates increase.

Nonetheless, the results obtained from GSK3 and CDKs inhibition, lead us to suppose that there are at least two efficient mechanisms that control the tau aggregation levels.

However one cannot dismiss that increase in PP levels may be due to lateral effects in other pathways related to, for example, ubiquitination or proteosome targeting. This aspect would have to be further addressed.

Finally, after PPs inhibition (OA incubation) it was verified that tau aggregates increase for all concentration and times tested. However, after PP2A (0.25nM) and PP1 (50nM) inhibition, it was observed that the tau aggregation levels are higher at 30 minutes compared to 3 hours. Nonetheless, the OA concentration is not specific for just one PP, thus the real influence of isoform specific PPs in tau aggregation could not be determined. Thus this was addressed in the following chapter.

4. Determination of tau aggregate formation as a consequence of modulating protein phosphatases expression (PP1α, PP1γ and PP2A knockdown)

4.1. Introduction

In order to evaluate the role of specific protein phosphatases on nuclear tau aggregation, small interfering RNA (siRNA) methodology for PP1γ, PP1α, and PP2A was used.

siRNA are99 small fragments of double-stranded (ds) RNA, usually with about 21 nucleotides long, with 3' overhangs (2 nucleotides) at each end that can be used to "interfere" with the translation of proteins by binding to and promoting the degradation of messenger RNA (mRNA) at specific sequences. In doing so, they prevent the production of specific proteins based on the corresponding mRNA nucleotide sequences. The process is called interference RNA (RNAi), but may also be referred to as siRNA silencing or siRNA knockdown.

4.1.1. Assembly of interference RNA

The oligonucleotide structure inserted in the vector is constituted by a target sense sequence, hairpin loop, and target antisense and terminal sequences, Fig. 11. The hairpin loop sequence shown is one of many functional loop sequences used to generate siRNAs. Termination is signaled using a poly(T) tract (terminal sequence). Including a unique restriction site which allows for confirmation of the cloned insert after the ligation and transformation reactions. The 5′ *BamH I* and 3′ *EcoR I* overhangs are necessary for directional cloning into RNAi-Ready pSIREN vectors (Liang et al., 2008).

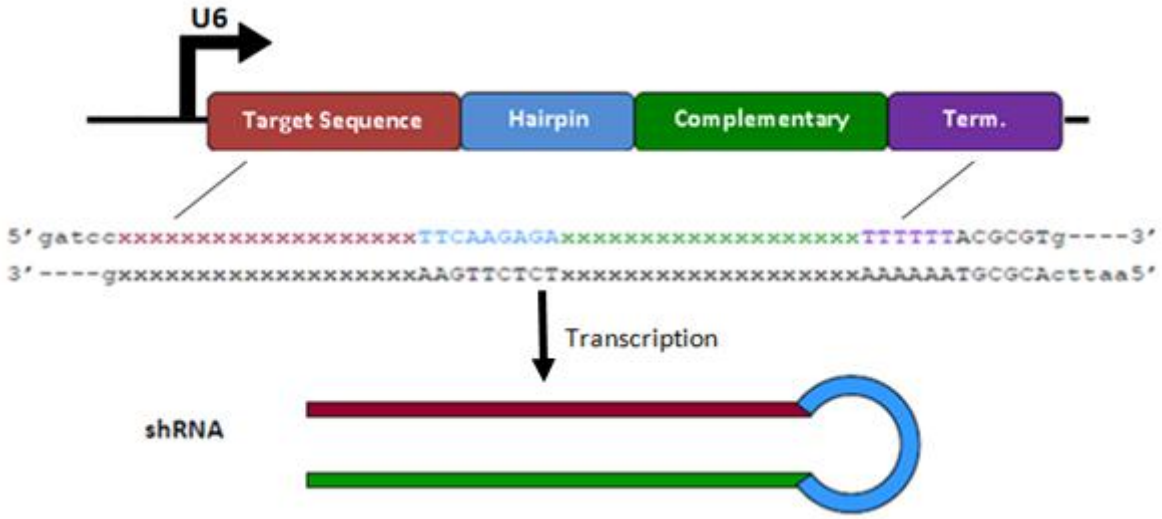

Fig. 11 – Schematic representation of siRNA oligonucleotide sequence design. shRNA expression is dependent by promoter U6 (*BamHI* and *EcoRI* sites) (adapted from Liang et al., 2008).

The target sequences used in the vector construction to shRNA expression were selected using bioinformatic tools (http://bioinfo.clontech.com/rnaidesigner/sirnaSequenceDesignInit.do) that allow for the choice of sequences according to criteria established by Elbashir (Elbashir et al., 2001). These criteria include the localization of sequences downstream of adenine dimmers; sequences constituted by 19 nucleotides and that do not contain the same basic nitrogen successively repeated four or more times. Moreover, the sequences should not have a %GC greater than 70 or less than 30 or have a GC region with more than 7 base pairs of extension.

Thus, the target sequences used in this work were PP1α, 5´-GAGACGCTACAACATCAAA-3´; PP1γ, 5´-AGAGGCAGTTGGTCACTCT-3´; PP2A, 5´-GGATATTACTCAGTTGAA-3´.

4.1.2. Construction of the pSERIN-RetroQ/protein phosphatase vector

The system used to activate the interference RNA mechanism to silence PP1γ, PP1α and PP2A is based on the utilization of pSIREN RetroQ vector, Fig 12.

pSIREN RetroQ vector was previously cut by *BamHI* and *EcoRI* enzymes, to permit cloning of a double chain DNA sequence, this sequence has the information to shRNA be produced. The oligonucleotides have cohesive extremities resulting from being previously cut by *BamHI* and *EcoRI*, which in turn permit a direct insertion in *BamHI/EcoRI* of pSIREN-RetroQ vector. To efficiently annealing of nucleotides, the single complementary chains were mixed in a ratio of 1:1 in annealing buffer (10mM Tris-HCl pH 7.5, 50mM NaCl, 1mM EDTA) to permit a correct annealing of complementary chains.

Finally, the double chain fragment resulting was introduced in pSIREN-RetroQ with the ligase enzyme of DNA of Fago T4, previously prepared according to the manufacture's recommendations. The ligation mix was incubated for 1 hour at room temperature, and then used to transform competent *E. coli* DH5α cells, according to the calcium chloride method (Maniatis et al., 1989). Another plasmid (pSIREN-RetroQ/) was also constructed called missense control, this also permits the expression of shRNA to mRNA, however this mRNA does not recognize anything in human, in this way it can be used as negative control in the assays here performed targeted of silencing protein phosphatases.

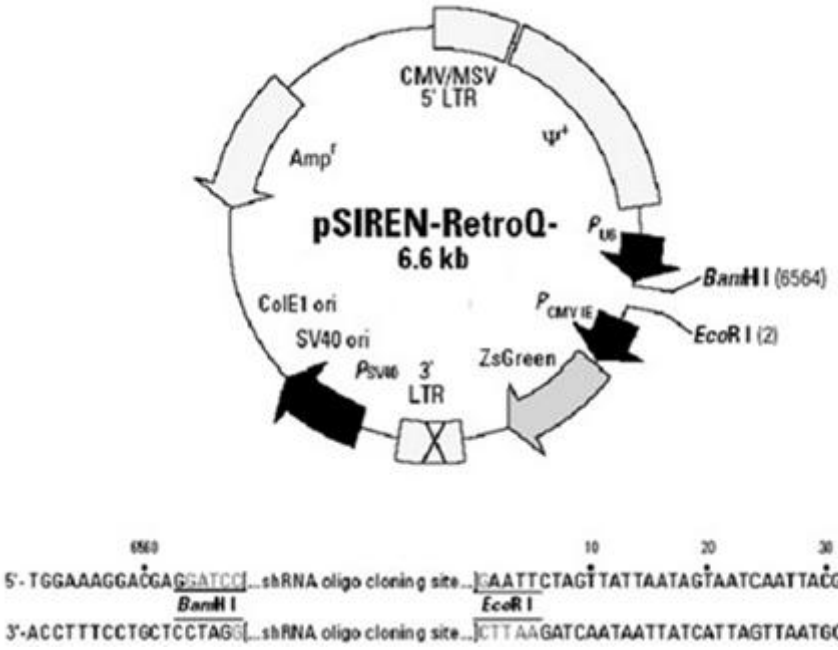

Fig. 12 – Schematic representation of a vector expressing siRNA. Restriction map and cloning site of RNAi sSIREN-RetroQ is a self-inactivating retroviral expression vector designed to express a small hairpin RNA (shRNA) using the human U6 promoter (RNA Pol III-dependent). It is provided as a linearized vector digested with *BamH I* and *EcoR I*. (Liang et al., 2008).

4.2. Material and Methods

4.2.1. Cell culture

The cell line used was undifferentiated SH-SY5Y cells and the medium and growth condition used were as previously described in 3.2.1.

4.2.2. Transfection with Lipotectamine

SH-SY5Y cells were grown in complete SH-SY5Y medium until 60-70% confluency was reached and on the transfection day the culture medium was replaced with serum and antibiotic/antimycotic-free medium. The quantity of DNA added to each plate was 2µg for PP1α and 5µg to PP1γ and PP2A, table 6, and this was diluted in serum and antibiotic/antimycotic-free. In turn, the lipofectamine 2000 reagent (7µL) was diluted in 243µL of the same medium, and the tubes were left for 5 min at room temperature. The DNA solution was added to the lipofectamine solution drop by drop, and the solution was mixed by gentle bubbling with the pipette. In order to form the DNA-lipid complexes, the tube was allowed to rest for 25-30 min at room temperature. Then, the solution was directly added into the cell medium, drop by drop and with gentle rocking of the plate. The cells were then incubated at 37°C/5% CO_2 for 48 h. After this period cells were collected for further analyzed.

Table 6 – Quantity of DNA used for proteins phosphatase knockdown. PP1α: protein phosphatase 1 alpha; PP1γ: protein phosphatase 1 gamma; PP2A- Protein phosphatase 2A.

Sample	DNA concentration (µg)	Volume Lipofectamine (µL)
Control missense	2 and 5	7
PP1α	2	7
PP1γ	5	7
PP2A	5	7

Following quantification by the BCA assay, membrane filter assay, SDS-PAGE and Immunoblotting were performed as described previously in chapter 3.

4.3. Results

4.3.1. Pilot Experiment – determining concentration of DNA for PP1α, PP1γ and PP2A knockdown

A pilot experiment was delineated in order to optimize the concentration of DNA for PP1α, PP1γ and PP2A knockdown. For that, SH-SY5Y cells were plated at 70-80% confluence and transfected with 1, 2 and 5µg of each PP2A, PP1α and PP1γ and control missense (CMS) siRNA. After, cells were collected for western blot analysis and then, the membrane was analyzed with PP2A, PP1α and PP1γ antibody to detect the amount of DNA that was better for PP2A, PP1α and PP1γ knockdown, respectively.

The amount of DNA was chosen according to that which induced a greater reduction in the expression of the corresponding PP. Moreover, the corresponding control missense (CMS) was included to verify if their levels were near to the non-transfected, which demonstrated that the siRNA missense does not affect the PP expression. In this way, the amount of DNA chosen for PP2A was 5µg, for PP1α was 2µg and for PP1γ was 5µg, Fig.13.

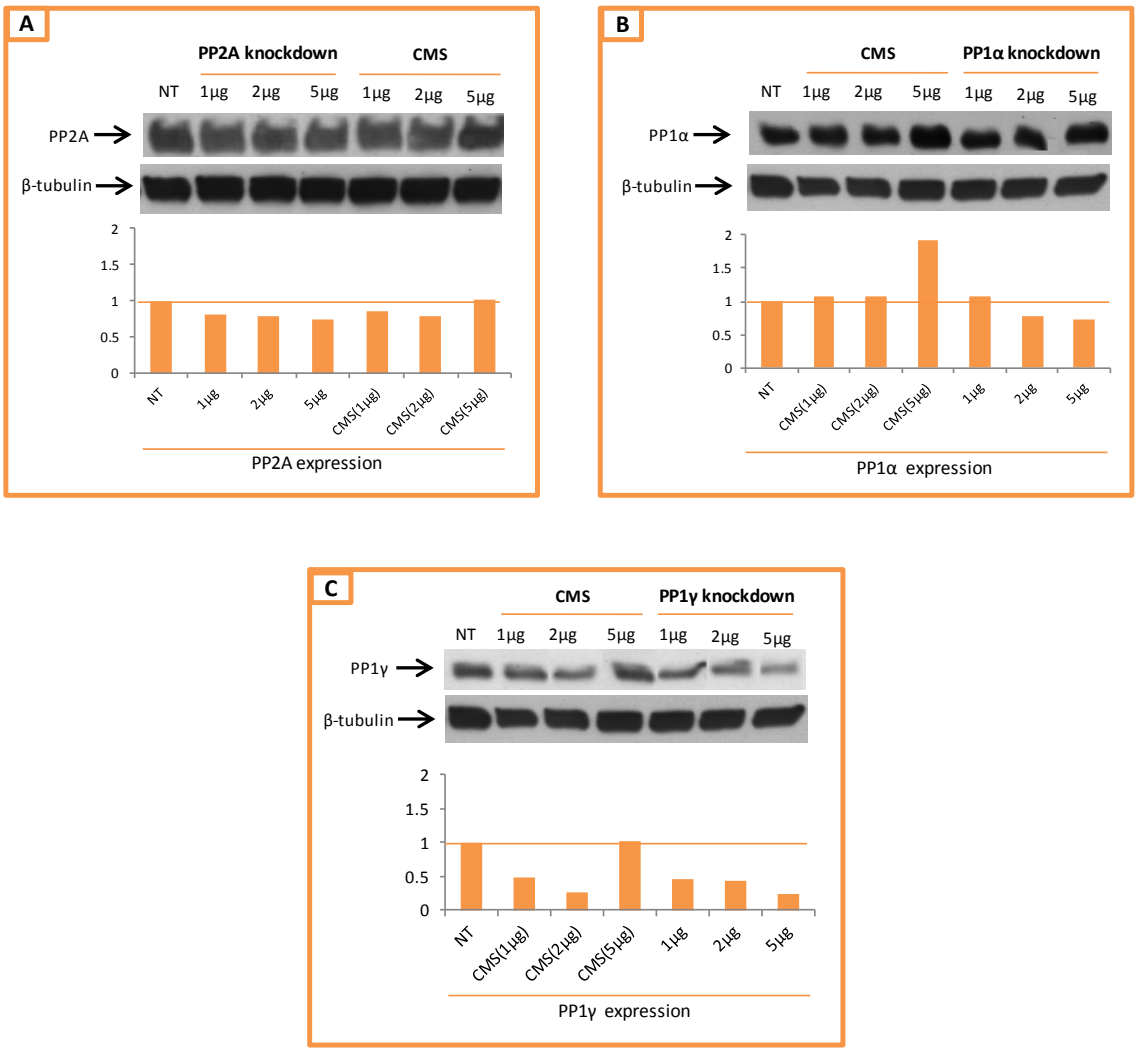

Fig. 13 - Evaluation of the amount of DNA needed to induce PP2A, PPα and PP1γ knockdown. (A) PP2A knockdown with 1, 2 or 5µg of DNA. Representation of western blotting and quantitative analysis. (B) PP1α knockdown with 1, 2 or 5µg of DNA. Representation of western blotting and quantitative analysis. (C) PP1γ knockdown with 1, 2 or 5µg of DNA. Representation of western blotting and quantitative analysis. For all knockdowns the tubulin was used as a loading control and was detected with β-tubulin antibody.

4.3.2. Effects of protein phosphatases knockdown in protein phosphatases and in tau aggregation

Observing the effects of PP2A, PP1α and PP1γ knockdown (KD) in tau aggregation, we can realize that all KD led to an increase in tau aggregation. However, the levels of PPs are different according to the protein that was inhibited.

In this way, after PP1α knockdown (by28%) it was verified that the PP1γ and PP2A levels also decreased, 11 and 26%, respectively, and the tau aggregates increased by 22%. In turn, the PP2A knockdown (by23%) led to a PP1α and PP1γ decrease, by 24 and 45%, respectively, and the tau aggregates increased, 110%. In contrast, the PP1γ knockdown (36%) led to an increase of PP1α and PP2A expression (by 2% and 22%), and also an increase in tau aggregation (by 83%), Fig. 14 and 15.

The statistical analysis with ANOVA to p-value<0,05 (STATISTICS 7.0) revealed that the homogeneity of variances (*Levene Test,* table in annexes) between PP1α and PP2A knockdown were significant to PP1γ and tau expression and tau aggregates, so the PP1α and PP2A knockdown have the same effect in these three parameters. On the other hand, when PP1γ was compared with PP1α and PP2A knockdown, the differences (*ANOVA,* table in annexes) between all parameters were statistically significant; in this way the PP1α and PP2A knockdown have different consequences on PPs and also on tau aggregates formation comparative to PP1γ knockdown.

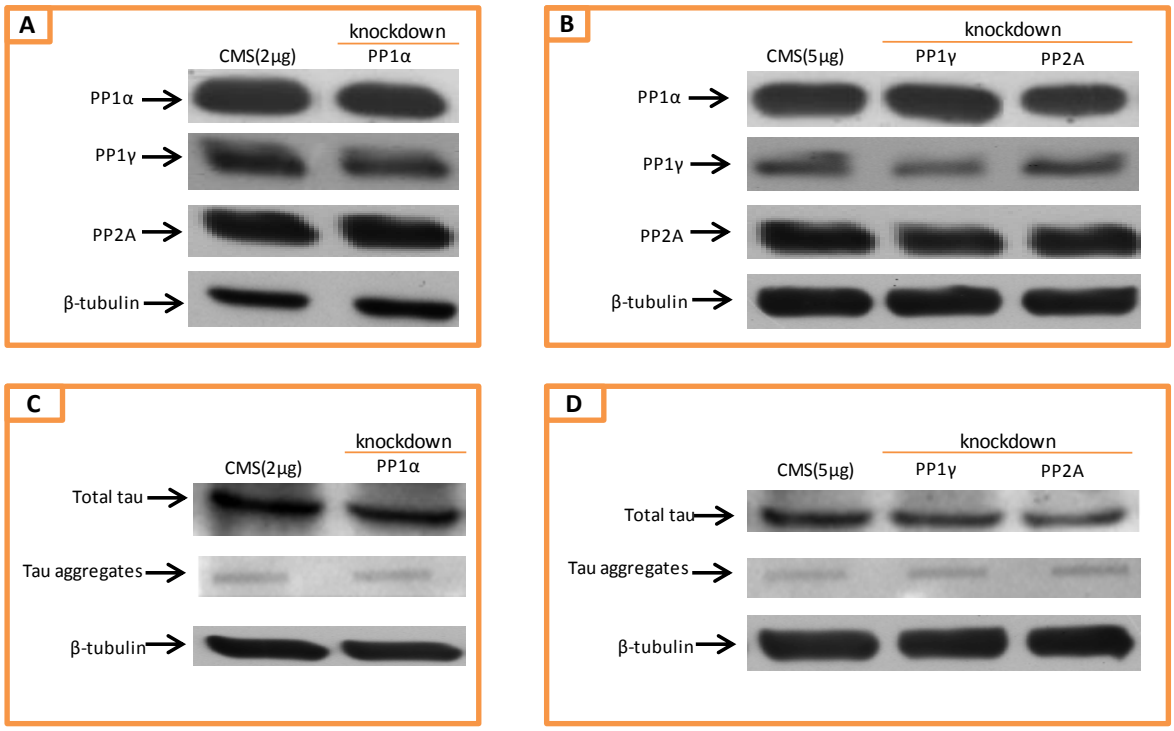

Fig. 14 – Effects of protein phosphatases knockdown on PP expression and tau aggregate formation. (A) Representative western blot analysis using PP1α, PP1γ or PP2A antibody after PP1α knockdown. (B) Representative filter assay using PP1α, PP1γ or PP2A antibody after PP1γ or PP2A knockdown. (C) Representative western blot and membrane filter assay analysis using Tau5 antibody after PP1α knockdown. (D) Representative western blot and membrane filter assay analysis using Tau5 antibody after PP1α knockdown. Tubulin was used as loading control and was detected with β-tubulin antibody for all knockdowns. CMS: control missense. n=3

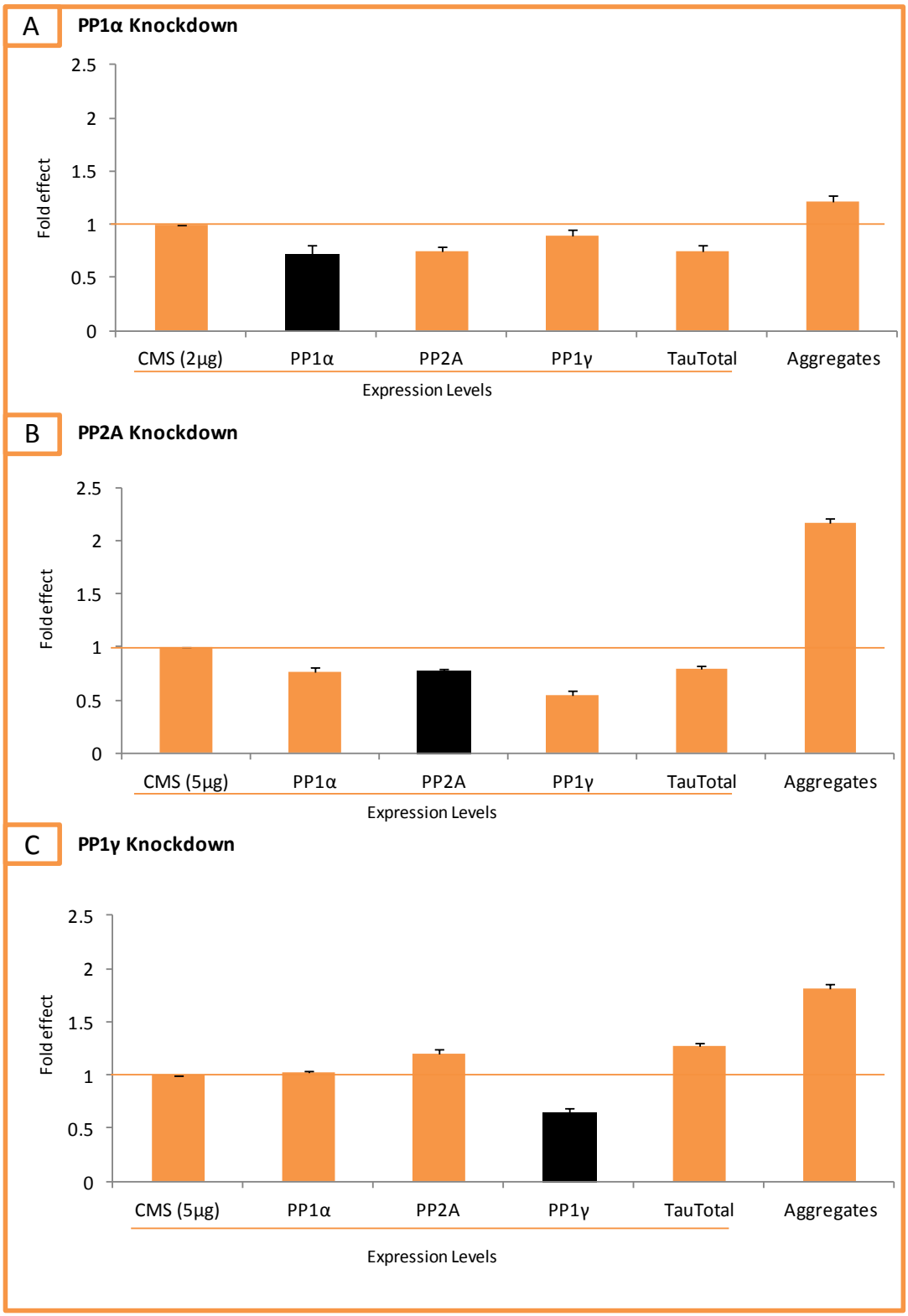

Fig. 15 – Effects of isoform specific protein phosphatase knockdown in its expression and tau aggregation. SH-SY5Y cells were transfected with siRNA using lipofectamine to siRNA inserted in cell (A), protein phosphatase 1 alpha (PP1α) knockdown and their effected in PP1γ, PP2A or tau expression and in tau aggregation. (B), protein phosphatase 1 gamma (PP1γ) knockdown and their effected in PP1α, PP2A or tau expression and in tau aggregation. (C), protein phosphatase 2A (PP2A) knockdown and their effected in PP1α, PP1γ or tau expression and in tau aggregation. The black boxes represent the PP knockdown. CMS: control missense.*p-value < 0.05 (experimental vs control missense data). n=3.

4.4. Discussion

After PPs knockdown it was verified that the nuclear tau aggregates increased for all PPs KD. Moreover it was observed that isoform specific PP KD had different effects on the expression levels of the others PPs analyzed as well as tau expression levels.

The KD of PP1γ, reduced the expression level of the latter by 36%, but led to increases in PP1α (2%) and PP2A (21%). This indicates that by decreasing PP1γ expression levels a compensatory mechanism is triggered resulted in increases of the others PPs. Moreover, these results lead us to deduce that PP1γ is highly relevant for tau dephosphorylation and consequently aggregate formation.

In contrast, for both the PP2A and PP1α KD decrease of all others PPs analyzed were also detected. Moreover, our results demonstrated that the PP2A KD had an effect more significant than PP1α KD in the PP1γ expression decrease, 45% and 11%, respectively. This can indicate that, probably, PP2A is the PP involved preferentially in this process. However the PP1α cannot be dismissed, as the latter also decrease when PP2A is downregulated.

Protein knockdown is defined as efficient when the protein expression level for which it was designed, decreases at least 60% comparatively to control. In this way, our PPs knockdowns were not efficient, thus the results and conclusions described above just are suggestive and speculative. However, due to lack of time, it was impossible the repeat and/or perform additional experiments to obtain results that support the conclusions reported.

5. Discussion

Neurofibrillary tangles are the principles hallmark in multiple neurodegenerative disorders and their formation is dependent on several phases. The process implicated in their formation starts with hyperphosphorylated tau that leads to dimmer formation and these in turn undergo multiples processes that permit the NFTs formation.

On the other hand, recent studies have indicated that the dimmers of tau protein are implicated in regulation of nuclear tau function (Padmaraju et al., 2010), namely they bind to DNA, it has been reported that this process is independent of phosphorylation (Hua and He, 2002). Moreover, it is also known that this interaction (DNA-tau) could lead to a DNA conformational change that in turn alters the gene expression, however the specific genes remains undefined (Padmaraju et al., 2010).

Thus, for a first analysis it is essential to understand if nuclear tau aggregation (dimmers) is equally controlled by PPs and PKs involved in phosphorylation and dephosphorylation of cytosolic tau and consequent tau aggregates formation.

For that, we incubated the SH-SY5Y undifferentiated cells with different concentrations of roscovitine and lithium chloride that inhibits CDKs and GSK3 activity, respectively, and then PP1γ, PP1α and PP2A expression and tau aggregation was measure.

In this way, the results for GSK3 inhibition demonstrated that PP1γ, PP1α and PP2A had approximately the same expression levels at 30 minutes, but at 3 hours the levels of PP1α decreased to approximately half of the other PPs although the tau aggregation decreased for both times points and for all concentrations tested.

In turn, for CDKs inhibition a decrease in tau aggregation at 30 minutes and at 3 hours was verified. Regarding PPs expression, it was detected that, at 30 minutes, there was an increase in PP1γ (three times more than control) and also in PP2A. Moreover, these decreases were more accentuated for higher concentrations of roscovitine. On the other hand, at 3 hours the PP1γ and PP2A decreased for all concentrations comparative to control.

It is clear that there is a correlation between PPs and PKs with phosphorylation and aggregation of tau protein. Our results suggested that there are at least two different mechanisms that lead to tau aggregate formation. One appears to be triggered following

GSK3 inhibition, Fig. 16, and it is dependent on PP1γ, PP1α and PP2A, and the other appears to be independent of these proteins phosphatases and it is triggered following CDKs inhibition, Fig. 17.

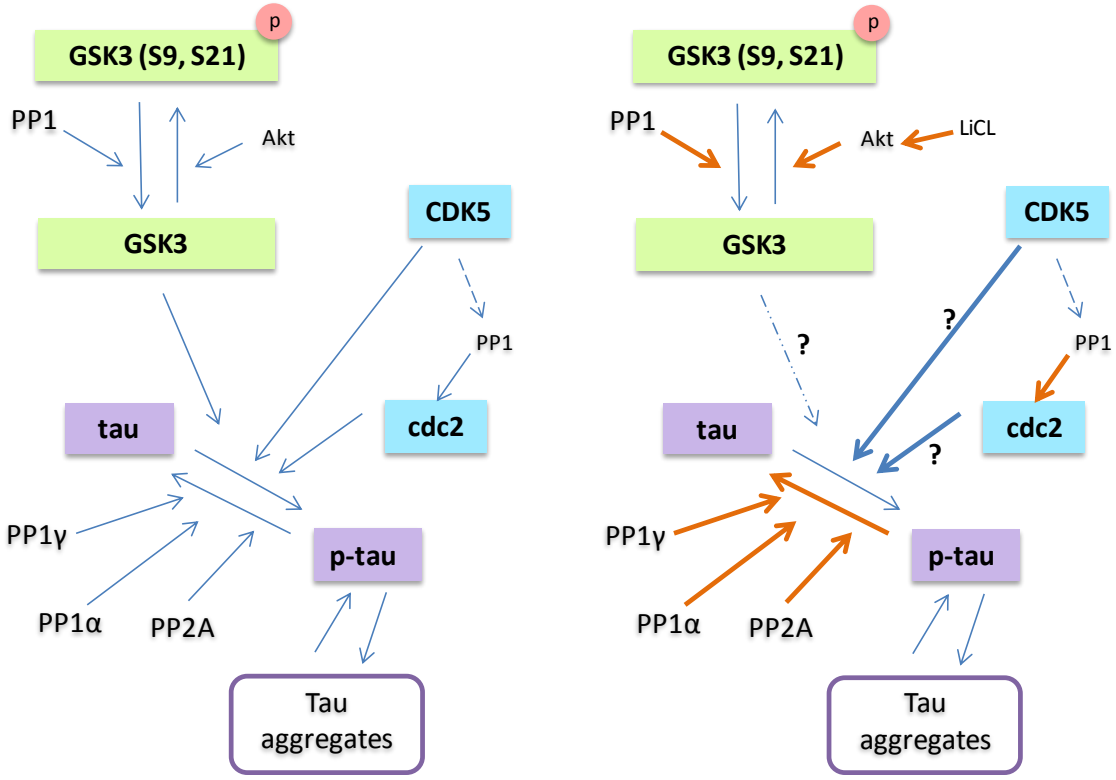

Fig. 16- Schematic representation of pathways implicated in increased and decreased tau aggregates formation. Left panel represents the pathways involved in tau aggregates formation in physiological conditions. Right panel represents the main changes in protein phosphatases expression after LiCl incubation. Dashed arrow represents protein activity inhibition. Orange arrows represent the proteins with higher expression. LiCl: lithium chloride; CDK5: cyclin-dependent kinase 5; GSK3: glycogen synthase kinase-3; cdc2: cyclin-dependent kinase 1; PP1: protein phosphatase 1; PP1γ: protein phosphatase 1 gamma; PP1α: protein phosphatase 1 alpha; PP2A: protein phosphatase 2A; tau: microtubule associated protein tau; p-tau: phosphorylated tau protein.

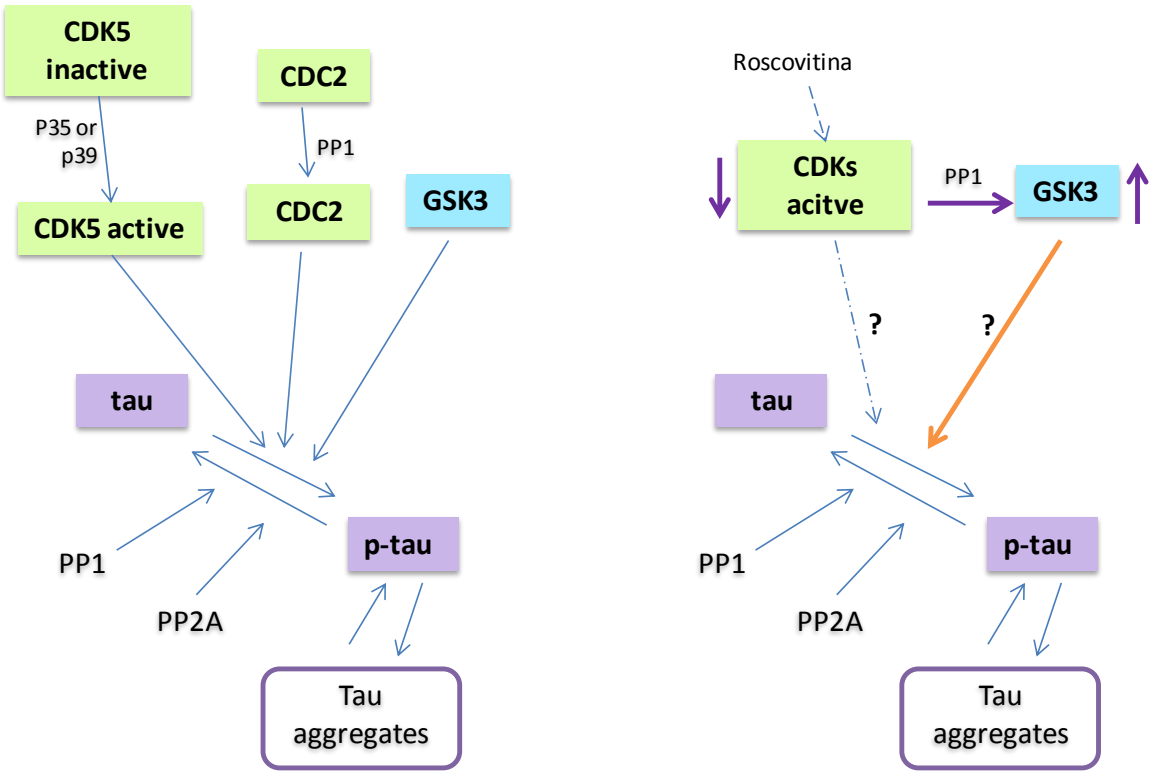

Fig. 27- Schematic representation of pathways implicated in increased and decreased tau aggregate formation. Left panel represents the pathways involved in tau aggregates formation in physiological conditions. Right panel represents the main changes in protein phosphatases expression after roscovitine incubation. Dashed arrows represent protein activity inhibition. Orange arrow represents the proteins with higher activity. Purple arrows represent the increase or decrease in kinases and phosphatases activity. CDK5: cyclin-dependent kinase 5; GSK3: glycogen synthase kinase-3; cdc2: cyclin-dependent kinase 1; PP1: protein phosphatase 1; PP1γ: protein phosphatase 1 gamma; PP1α: protein phosphatase 1 alpha; PP2A: protein phosphatase 2A.

The results described above suggest that the PP1γ, PP1α and PP2A have an important role in decreasing tau aggregation. Thus, in order to obtain more information with respect to the role of these PPs in tau aggregation, PP1γ, PP1α and PP2A were downregulated and then the expression of each was measured as well as the effect on tau aggregation.

As a result, after PP2A, PP1γ and PP1α knockdown an increase in tau aggregation was verified. Moreover, our results also indicate that the PP1γ has an important role in tau aggregation, because the inhibition of this PP alone was sufficiently for tau aggregation increase, even though under these experimental conditions there was an increase of the other proteins, PP1α (2%) and PP2A (21%). Furthermore, PP2A KD also suggested that this PP affects PP1γ expression levels and consequently, its activity.

Taken together the results here presented suggest that the main protein phosphatases involved in dephosphorylation of nuclear tau are different when compared to cytosolic tau. Liu et al in 2005 reported that the cytosolic tau is mainly regulated by PP2A (72%) followed PP1 (11%), however our results suggested for nuclear tau the PP more implicated is PP1γ, that in turn can be regulated by PP2A. Moreover, our results demonstrated that these two PPs are also implicated in tau aggregation decreases, namely after GSK3 inhibition. In fact, after LiCl incubation it was verified that the expression of PP1γ and PP2A increased significantly. In this way, although GSK3 has an essential role in tau phosphorylation, our results appears to suggest that the decrease in tau aggregation after LiCl incubation is not only due GSK3 inhibition. Additionally, the results obtained from CDKs inhibition appear suggest that there is other mechanism controls tau aggregation, this is dependent on PP1γ to a quickly answer. However, to a long term response this mechanism appears too independent of this PP as well as PP1α and PP2A.

In summary, and even though it is known that cytosolic tau is preferentially dephosphorylated by PP2A (Liu et al., 2005) it is reasonable to deduce that PP1γ is essential for modulating tau aggregates, particularly with respect to nuclear tau.

The differences in PP more relevant for nuclear and cytosolic tau dephosphorylation are highly relevant from a physiological perspective and may help to explain the functional differences between nuclear and cytosolic tau.

6. Future perspectives

- Evaluate the expression and activity of all proteins phosphatases and GSK3 and CDK5 after incubation with LiCl, roscovitine and okadaic acid;

- Knockdown of PP4, PP2B, PP7, GSK3 and CDK5;

- Assess the tau localization after overexpression and knockdown of proteins phosphatases and kinases.

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8. Annexes

CELL CULTURE

SH-SY5Y medium (1L)

In deionised H_2O (600mL) dissolve:

-MEM 4.805 g

-F12 5.315 g

-NaHCO3 1.5 g

-Sodium pyruvate 0.055 g

Adjust pH 7.3 with hydrogen chloride (HCl)

Next, add:

-Antibiotic (AAs) 10 mL

-Fetal Bovine Serum (FBS) 100 mL

-L-glutamine 2,5 mL

Adjust the volume to 1 liter. Sterilize by filtering through a 0.2 µm filter and store at 4^{o}C.

PBS (1x)

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

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

Adjust the volume to 1000 mL. Filter through a 0.2 µm filter and store at 4^{o}C.

PROTEINS MANIPULATION

MEMBRANE FILTER ASSAY:

1M Tris (pH 6.8) solution (250 ml)

Dissolve 30.3 g of Tris base in deionised H_2O, adjust pH to 6.8 and adjust final volume to 250 mL.

0.5M EDTA (1L)

- EDTA (MW 372.24) 186,12 g

Dissolve 800mL of deionised H_2O (Note: To dissolve the EDTA completely, solution pH 8.0 is required).
Adjust the volume to 1000 mL.

0.1M NaCl (1L)

- NaCl 5.884 g

Dissolve in deionised H_2O, and then adjust the volume to 1000 mL.

SDS-PAGE:

Loading buffer (10mL)

- Tris (1M) 2,5 mL

Dissolve in deionised H_2O, adjust the pH to 6.8 with HCl

- SDS 0,8 g
- Glycerol 4 mL
- Blue bromophenol 1 mg
- B-mercaptoetanol 2 mL

Adjust the volume to 10 mL.

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

- Tris 181.65 g
- SDS 4 g

Dissolve in deionised H_2O, adjust the pH to 8.9 with HCl and adjust the volume to 1 liter.

UGB (Upper gel buffer) (5x) (1 L)

Dissolve 75.7 g of Tris base in deionised H_2O, adjust the pH to 6.8 with HCl and adjust the volume to 1 liter.

30% Acrylamide/0.8% Bisacrylamide solution (100 ml)

- Acrylamide 29.2 g
- Bisacrylamide 0.8 g

Dissolve in deionised H_2O and adjust the volume to 100 mL. Filter through a 0.2 µm filter and store at 4^oC.

10% APS (ammonium persulfate) (10 ml)

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

10% SDS (sodium dodecilsulfate) (10 ml)

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

Running buffer (10x) (1 L)

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

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

15 cm gels:

Resolving (lower) gel solution (60 ml) **10%**

- H_2O 24.73 mL
- 30% Acryl/0.8% Bisacryl solution 19.96 mL
- LGB (4x) 15 mL
- 10% APS 300 µL
- TEMED 30 µL

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

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

Immunoblotting solutions:

Electrotransfer buffer (1x) (1 L)

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

Dissolve in deionised H_2O, adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with deionised H_2O. Just prior to use add 200 ml of methanol (20%).

TBS (Tris Buffered Saline) (10x) (1 L)

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

Dissolve in deionised H_2O, adjust the pH to 8.0 with HCl and adjust the volume to 1 liter.

TBS-T (Tris Buffered Saline + Tween) (10x) (1 L)

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

Dissolve in deionised H_2O, adjust the pH to 8.0 with HCl and adjust the volume to 1 liter.

Blocking solution (100 ml)

- TBS-T stock solution (10x) 10 mL
- Bovine Serum Albumin (BSA) 5 g

Dissolve in deionised H_2O and adjust volume to 100 mL.

Primary Antibody solution (25 ml)

- TBS-T stock solution (10x) 2.5 mL
- Bovine Serum Albumin (BSA) 3% 0.75 g

Dissolve in deionised H_2O and adjust volume to 25 mL. Add antibody, mix gently without vortex, and store at -20^{o}C.

Secondary Antibody solution (25 ml)

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

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

Tables of ANOVA

Table 7 – Levene's test of homogeneity of variances, p-value<0,05.

		Sum of squares	df	F	p-value
PP1α and PP2A	**tau**	0.069282	4	12.92518	0.022856
	tau aggregates	0.330753	4	9.03724	0.039699
	PP1γ	0.114497	4	8.34916	0.044586
	PP1α	0.001260	4	0.16315	0.706934
	PP2A	0.000713	4	0.05622	0.824227
		Sum of squares	**df**	**F**	**p-value**
PP1α and PP1γ	**tau**	0.134146	4	13.74096	0.051710
	tau aggregates	0.081536	4	4.08094	0.113480
	PP1γ	0.007091	4	0.34143	0.590375
	PP1α	0.057252	4	0.37879	0.571546
	PP2A	0.040578	4	0.39705	0.562821
		Sum of squares	**df**	**F**	**p-value**
PP1γ and PP2A	**tau**	0.010618	4	0.705645	0.448171
	tau aggregates	0.083848	4	1.619133	0.272148
	PP1γ	0.064599	4	7.334589	0.053635
	PP1α	0.072800	4	0.079877	0.791486
	PP2A	0.056924	4	0.571325	0.491804

Table 8 – Results obtained from ANOVA, p-value<0,05.

		Sum of squares	df	F	p-value
PP1α and PP2A	**PP1α**	3.577990	1	76.12224	0.000951
	PP2A	17.08188	1	62.52181	0.001384
		Sum of squares	**df effect**	**F**	**p-value**
PP1α and PP1γ	**tau**	6.185643	1	71.63741	0.001068
	tau aggregates	13.77012	1	135.4708	0.000311
	PP1γ	3.512678	1	20.64770	0.010465
	PP1α	4.565563	1	75.68415	0.000961
	PP2A	5.684748	1	66.17923	0.001242
		Sum of squares	**df effect**	**F**	**p-value**
PP1γ and PP2A	**tau**	6.410571	1	48.31542	0.002251
	tau Aggregates	23.68528	1	65.95928	0.001250
	PP1γ	2.137730	1	30.03578	0.005397
	PP1α	4.747961	1	66.83820	0.001219
	PP2A	5.889659	1	70.20787	0.001110