Universidade de Aveiro Departamento de Ciências Médicas
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Margarida Isabel Nascimento Vaz

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Chemokines effect on kinases and phosphatases involved in Alzheimer's disease pathology

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Dra. Ana Gabriela Henriques, Investigador Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

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Dedico esta dissertação aos meus pais, irmã, restante família e amigos por serem as pessoas mais importantes da minha vida.

o júri

presidente

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palavras-chave

Doença de Alzheimer, Neuroinflamação, Quimiocinas, IL-8, MCP-1, Tau, Fosforilação, Cinases e Fosfatases.

resumo

A doença de Alzheimer (DA) é uma doença devastadora e a principal causa de demência a nível mundial. Na sua patologia estão incluídas duas principais características: a deposição extracelular de péptidos Aβ, formados durante o processamento amiloidogénico da APP, que agregam em placas senis (SP) e a formação de traças neurofibrilares (NFT), agregados de proteína tau hiperfosforilada. A hiperfosforilação da tau ocorre devido ao aumento da atividade de proteínas cinases (incluindo a GSK3β e a CDK5) e à diminuição da atividade de proteínas fosfatases (PP2A, PP1 e PP2B).

A neuroinflamação é um evento crucial na patogénese da DA, durante o qual, a microglia e os astrócitos são constitutivamente ativados, o que leva a uma produção massiva de citocinas. Estas moléculas podem alterar o processamento amiloidogénico da APP, a agregação do Aβ ou a fosforilação da tau. Entre as citocinas, as quimiocinas parecem ter um papel importante na progressão da DA.

No presente trabalho foi avaliado o impacto das quimiocinas, interleucina 8 (IL-8) e proteína quimiotática de monócitos 1 (MCP-1), na fosforilação da tau. Para além disso, o efeito destas quimiocinas na atividade de cinases (GSK3β e CDK5) e nas fosfatases (PP1 e PP2A) importantes neste processo também foi avaliado. Para as duas quimiocinas, foi observado um aumento significativo nos níveis de pTau S396 para os períodos de incubação mais longos e nas concentrações de quimiocinas mais elevadas. Além disso, os resultados em relação à GSK3β indicam que esta não é a cinase maioritariamente envolvida neste processo. No entanto, resultados preliminares sugerem que a CDK5, a PP1 e a PP2A podem desempenhar um papel no aumento da fosforilação da tau, promovido pelas quimiocinas.

Este trabalho suporta o envolvimento das quimiocinas no estado de hiperfosforilação da tau e potencialmente na formação de NFT que, consequentemente resultam em neurodegeneração na DA.

Do ponto de vista da doença, esta tese permite uma melhor compreensão dos mecanismos moleculares que estão na base da patologia, evidenciando o papel da inflamação neste processo.

keywords

Alzheimer's Disease, Neuroinflammation, Chemokines, IL-8, MCP-1, Tau, Phosphorylation, Kinases and Phosphatases.

abstract

Alzheimer's disease (AD) is a devastating worldwide disease and the main cause of dementia. In its pathology, are include two main hallmarks: the extracellular deposition of Aβ peptides, formed in amyloidogenic processing of APP, that aggregate into senile plaques (SP) and the intracellular formation of neurofibrillary tangles (NFT), aggregates of hyperphosphorylated tau protein. Tau hyperphosphorylation occurs due to increased kinase activity (including GSK3β and CDK5) and impaired protein phosphatase activity (PP2A, PP1 and PP2B).

Neuroinflammation is a crucial event in AD pathogenesis, during which microglia and astrocytes are constitutively activated, leading to massive production of cytokines. These molecules may alter APP amyloidogenic processing, Aβ aggregation or tau phosphorylation. Among cytokines, chemokines seem to have an important role in AD progression.

In the present work, chemokines interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) impact on tau phosphorylation was addressed. Additionally, the effect of these chemokines on kinases (GSK3β and CDK5) and phosphatase (PP1 and PP2A), important in this process, was evaluated. For both chemokines, a significant increase in ptau S396 levels was observed at longer incubation periods and higher chemokines concentration. Further, results regarding GSK3β support that this is not the main involved kinase in this process. However, preliminary results suggest that CDK5, PP1 and PP2A can have a role in tau increased phosphorylation, promoted by chemokines.

This work supports the involvement of chemokines on tau hyperphosphorylation and potentially in NFT formation, that consequently result in AD neurodegeneration.

At a disease view, this thesis allowed a better understanding of molecular mechanisms that underling the disease pathology, highlighting the role of inflammation in this process.

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List of abbreviations

A	AD	Alzheimer's Disease
	AD-EOAD	Autosomal-dominant Early-onset Alzheimer's Disease
	AICD	β-Amyloid Precursor Protein Intracelular Domain
	APOE	Apolipoprotein E
	APP	β-Amyloid Precursor Protein
	Aβ	β-Amyloid peptide
B	BCA	Bicinchoninic Acid
	BSA	Bovine Serum Albumin
C	C83	C-terminal fragment with 83 amino acids
	C99	C-terminal fragment with 99 amino acids
	CDK	Cyclin-Dependent Kinase
	C_f	Final Concentration
	CNS	Central nervous system
	CTF	C-terminal fragment
	CSF	Cerebrospinal Fluid
D	dH_2O	Deionized water
	DMSO	Dimethyl sulfoxide
	DS	Down Syndrome
	DTT	Dithiothreitol
E	ECL	Enhanced Chemiluminescence
	EOAD	Early-onset Alzheimer's Disease
	EO-FAD	Early-onset Familiar Alzheimer's Disease
F	FAD	Familiar Alzheimer's Disease
G	GSK3β	Glycogen Synthase Kinase 3 β
H	h	Hours

I	IP-10	Interferon-inducible Protein 10
	IL	Interleukin
J	IFN-γ	Interferon γ
L	LB	Loading Buffer
	LDLR	Low Density Lipoprotein Receptor
	LOAD	Late-onset Alzheimer's Disease
	LO-FAD	Late-onset Familiar Alzheimer's Disease
M	MAP	Microtubule-Associated Protein
	MAPK	Mitogen Activated Protein Kinase
	MCP-1	Monocyte Chemoattractant Protein 1
	min	Minutes
	MIP-1α	Macrophage Inflammatory Protein 1-α
	mm	Millimetres
	MT	Microtubule
N	NaF	Sodium Fluoride
	Na_3VO_4	Sodium Orthovanadate
	NFT	Neurofibrillary Tangles
	NFM	Non-Fat Milk
	NP40	Nonidet™ P40
P	PDPK	Proline-Directed Protein Kinase
	PHF	Paired Helical Filaments
	PP	Protein Phosphatase
	PSEN	Presenilin
R	RA	Retinoic Acid
	RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
	ROS	Reactive oxygen species
	RT	Room Temperature

S	S (or Ser)	Serine
	SAD	Sporadic Alzheimer's Disease
	sAPP	Soluble β-Amyloid Precursor Protein
	SD	Standard Deviation
	SDS	Sodium Dodecyl Sulfate
	SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
	SF	Straight Filament
	SORL1	Sortilin-Related Receptor 1
	SP	Senile plaques
T	T (or Thr)	Threonine
	TBS	Tris-Buffered Saline
	TBS-T	Tris-Buffered Saline – Tween 20
	tg	Transgenic
	TGF-β1	Transforming Growth Factor β1
	TNF-α	Tumour Necrosis Factor α
V	VPS10	Vacuolar Protein Sorting 10
W	WB	Western Blotting
Y	Y (or Ty)	Tyrosine

1 – Introduction

1. Introduction

1.1. Neurodegenerative diseases

Neurodegenerative diseases are a worldwide epidemic. This term refers to a variety of conditions, that primarily cause progressive degeneration and/ or death of nerve cells (1). Although neurodegenerative diseases have relatable causal pathways (including protein misfolding and aggregation, proteostasis dysregulation, mitochondrial dysfunction and chronic inflammation)(2) the clinical outcomes and symptoms are very distinct, ranging from memory and cognitive impairment (3,4) to loss of motor functions like moving, speaking and breathing (5–7).

The most common neurodegenerative diseases are dementias such as Alzheimer's disease (AD), frontotemporal dementia, vascular dementia, dementia with Lewy bodies; Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (1,2).

1.2. Alzheimer's Disease

In the last decades, dementia has been one of the biggest global public health challenges. According to Alzheimer's disease International report, it is estimated that 47.47 million people or nearly 5% of the world's elderly population were affected by dementia in 2015, reaching 75.62 million in 2030 and 135.46 million in 2050 (8). AD alone represents about 60-70% of all dementia cases (9).

In Portugal it was estimated that there were 19,9 cases of dementia per 1000 habitants, in 2017, representing about 200 thousand people living with dementia in the country. In 2037 the prediction is that this will increase to over 300 thousand cases. These numbers placed Portugal as the fourth country with the highest dementia prevalence, in OCDE (10).

AD was first described by Alois Alzheimer, a German psychiatrist and neuropathologist, in 1907. In this report, Alzheimer described two histological alterations (later known as senile plaques (SP) and neurofibrillary tangles (NFT)) in the brain tissue of Auguste D., a 51-year-old woman that showed rapid loss of memory and completely disorientation in time and space (11,12).

AD is characterized by progressive cognitive decline, initially affecting mainly memory and learning function and later on all other intellectual functions such as language, complex attention, praxis, executive and visuospatial function and social-behaviour (13). This pathology can be divided into several clinical stages, including mild cognitive impairment, mild AD, moderate AD, moderate-severe AD and severe AD (stage 3 to 5), according to Global Deterioration Scale (14).

Memory decline in AD is related to the impairment of recent memory (with progressive loss of episodic memory). Patients have difficulty in remembering quotidian events and in retaining new information (learning difficulties). However, comparatively, old memories are relatively spared at the beginning of the disease. After memory, the other main affected function is language, where patients may present problems in finding specifics words, following and stablish complex conversations, due to aphasia (since their vocabulary is restricted), or in handwriting (15).

Patients also present visuospatial deficits, causing disorientation and difficulties in, for instance, following a routine trajectory to home. Impairment in praxis is exhibited by difficulties in carrying out a complicated request or in autonomously do basic daily living activities such as dressing or

bathing. Behavioural changes also occur in the forms of inappropriate social behaviour, obsessive-compulsive behaviours or changes in food preference, for example. (15).

AD is a devastating disease not only for the patients but also for their family since the progressive cognitive decline causes memory impairment and behaviour changes, that severely compromises the quotidian of all involved individuals. These causes emotional and physiological stress among the patients' relatives as they saw their loved one "becoming less than a shadow" of what they were before the disease. Additionally, AD causes huge economic burdens due to high expenses associated with hospitalization, nursing home services or specialised residential communities stay (16,17).

1.2.1. AD forms

Alois Alzheimer described for the first time AD, based on the characteristics of Augustine D.' case, a 51-years-old woman. At this time, according to A. Alzheimer definition, AD was defined as an early-onset dementia, which distinguishes it from senile dementia of Alzheimer´s type that occurs in older patients (18).

However, later studies showed that the two conditions had the same neurological and clinical features, with age being the only differentiating criteria. So the two conditions should be the same entity, Alzheimer's disease, with two variants, late-onset AD (LOAD) and early-onset AD (EOAD) (19). These two forms of AD differ not only on the age of onset but also on some clinical outcomes since EOAD display less memory loss and major cognitive dysfunction when compared with LOAD (20).

LOAD is the most common form of the disease, representing approximately 95% of all AD cases, with the incidence increasing with age. AD cases are classified as LOAD if the diagnose occurs after the age of 65 years. These patients present greater memory impairment (21,22), specifically in visual memory (23), recent events (21,22) and orientation (23) than EOAD patients.

EOAD occurs in patients under 65 years and represents only 3-6% of all cases of AD (24). However, it is the most common among early-onset neurodegenerative dementias (25) and several studies indicate that this form presents a faster cognitive decline (26) and high mortality than LOAD (27). This form of AD is often diagnosed later than LOAD, with an average delay of approximately 1.2 years between symptoms onset and disease diagnose (28). This seems to occur because EOAD patients present more atypical symptoms of AD, like impairment in praxis, attention, learning tests and executive functions (23,29,30) and better visual and semantic memory (23,30).

On the other hand, these patients present specific psychosocial problems, like anxiety and depression (31), that are related with the unexpected loss of independence (at an early age) and with the difficulty in dealing with active responsibilities (familial and professional) (17).

Nonetheless, AD can also be classified according to its etiology as sporadic AD (SAD) or familial AD (FAD) (32). SAD represents about 90% of all AD cases, mostly LOAD patients. Although the cause of this AD form is unknown (and the main risk of this form is age), it has been proposed that it is caused by the combination of rare genetic variants (70%) and environmental factors (30%) (32).

Some genetic risk factors have been associated with SAD, like polymorphism in the apolipoprotein E (*APOE*) gene, in particular, the *ApoE* allele ε4 (recently reviewed in (33)).

Familial Alzheimer's disease (FAD) represents the cases that have a positive familiar history for dementia (at least three generations) (34). Patients with FAD can have early-onset – familial Alzheimer's disease (EO-FAD) or late-onset – familial Alzheimer's disease (LO-FAD). Among EO-FAD cases, about 10-15% are classified as the autosomal dominant form of early-onset AD (AD-EOAD) since they exhibit an autosomal dominant mode of inheritance (35–37).

So far, mutations in three genes were been recognized as the main cause of EO-FAD cases: amyloid beta precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*)(38). However, mutations on these genes have also been reported in families with late-onset (LO-FAD) (38,39).

1.2.2. Genetics and other risk factors for AD

Over decades, several risk factors have been associated with AD (40). These can be divided as unmodifiable risks, including genetics, age or sex and family history; or as acquired /modifiable risks that are related to lifestyle and medical conditions.

Genetics

According to Ballard et al. genetics is responsible for 70% of the risk of developing AD. These include established genetics causes like mutations of *APP*, *PSEN1* and *PSEN2* and other potential risk genes of AD, namely *APOE* (41).

➔ APP, PSEN1, PSEN2 and SORL1

APP (21q21.3), *PSEN1* (14q24.2) and *PSEN2* (1q42.13) mutations are established causes of AD-EOAD. However, some publications also associate rare variants of these genes with LOAD (38,39). Mutations on *PSEN1* are the most common causes of EOAD, with 268 pathogenic mutations reported (according to Alzforum database). For *APP* 27 mutations has been linked with AD and PSEN2 mutations rarely cause AD, with only 11 linked with this pathology (42).

APP is a transmembrane precursor protein that is cleaved by secretases (α-, β- and γ- secretase complex) in two distinct pathways: amyloidogenic or non-amyloidogenic. If APP processing occurs via the amyloidogenic pathway, β-Amyloid (Aβ) peptides are formed, aggregate and can trigger a cascade of events that lead to AD (for more details consult section 1.2.3) (43). Mutations and duplications of *APP* usually result in increased $A\beta_{42}$ production, since the amyloidogenic pathway became preferential, and increased $A\beta_{42}/A\beta_{40}$ ratio, promoting Aβ deposition and aggregation (considering that $A\beta_{42}$ is more willing to aggregate than $A\beta_{40}$) (44,45).

Presenilins (PSEN) are catalytic subunits of the γ-secretase complex. Mutations of *PSEN1* causes the most severe forms of AD, with an onset age as early as 30 years. On the other hand, *PSEN2* mutations are a rare cause of EO-FAD (45). Mutations in these two genes have been linked with increased $A\beta_{42}/A\beta_{40}$ ratios as a result of increased $A\beta_{42}$ production, decreased $A\beta_{40}$ production, or a combination of both (45).

Sortilin-related receptor 1 (*SORL1*) (11q24.1) is another gene that possesses rare variants that recently emerged as a cause of FAD (early or late-onset) (46–48). Originally identified as a risk gene for AD (46), *SORL1* encodes a mosaic protein that belongs to at least two families: the vacuolar protein sorting 10 (VPS10) domain-containing receptor family, and the low-density lipoprotein receptor (LDLR) family (47). SORL1 is responsible for redirecting APP to Golgi complex, through the

recycling pathway, reducing amyloidogenic processing and decreasing Aβ production. Therefore, mutations in this gene, that cause loss of protein function, impaired SORL1 protective role (46).

➔ *APOE* and other risk genes

Although FAD causes can be attributed to mutations in the above mentioned genes, SAD genetics causes are more complexes and relatable to risk gene.

Along with age and sex, polymorphism in the *APOE* (19q13.32) gene is the other major risk factor of SAD. APOE is a glycoprotein (or apolipoprotein) whose function is the transport of lipoproteins (triglycerides and cholesterol) between organs by interacting with lipoprotein cell-surface receptors (49). In the central nervous system (CNS) APOE is expressed in astrocytes, microglia and stressed neurons (in less extent) and is critical for maintenance of myelin and neuronal membranes (33,50).

The major allelic variants of *APOE* gene (19q13.32) are ε2, ε3 and ε4. Individuals that carry *APOE-ε4* allele have an increased risk for AD relative to *APOE-ε3* allele, whereas *APOE-ε2* allele seems to confer protection against AD (33). The association between *APOE* allelic variants and the risk of AD are synthesized in Table 1.

Table 1: *APOE* allelic variants in AD. Adapted from (33,51)

APOE allelic variant	Frequency in patients with AD (%)	Odds ratios for AD development	Findings
APOE-ε2	4	0.621	→ **Reduced risk of AD** → Reduced Aβ deposition and neuritic plaques → Delayed age of onset in individuals carrying *PSEN1* mutations (EOAD)
APOE-ε3	58	1.00	→ **Reference allelic variant is most studies**
APOE-ε4	38	3.680	→ **Increased risk of AD** → *APOE-ε4/ε4* genotype is associated with an even increased risk (OR=14.49) → Earlier age of onset (2-5 or 5-10 years depending on number of alleles) → Greater risk of AD in women carrying *APOE-ε4* allele → Greater Aβ deposition and aggregation → Related with tau pathology in AD → Modulation of neuroinflammation → Synaptic degeneration

The impact of APOE isoforms in AD pathology is strongly correlated with their differential effects on Aβ aggregation and clearance in the brain. However, recent evidences suggests that the

role of APOE isoforms in AD may also involve tau pathology, neuroinflammation, lipid transport, glucose metabolism and synaptic function (33).

Other genes that have been recognized as risk genes of AD include *ADAM10, BIN1, CLU, CR1, PICALM, CD33, ABCA7, CD2AP, EPHA1, FERMT2, CASS4, PTK2B, INPP5D, MEF2C, DSG2, MS4A6A/MS4A4E, HLA-DRB5/HLA-DRB1, ZCWPW1, SLC24A4/RIN3, NME8, CELF1, TREM2, PLD3, UNC5C, AKAP9, TM2D3, COBL* and *SLC10A2*. Most of their pathological pathways in AD are related with Aβ clearance and aggregation, immune response or tau metabolism (52).

Age

Age is by far the greatest risk for AD. This is sustained by the fact that the disease affects more persons above 65 years (LOAD), and that the incidence increases with age, reaching 13.8/1000 (cases per person-years) for patients aged 75-84 years and 35.7/1000 (cases per person-years) for patients aged 85 years and older (53,54). This fact is very concerning when global ageing is taken into account. According to United Nations report, in 2050 the number of older people (≥ 60 years) will exceed the number of younger (10-24 years) globally and the proportion of persons aged 80 years or above in the older people group will be more than 20% (55). So, with an even older population, AD incidence may increase dramatically.

Gender

Gender, specifically feminine, could also be considered as a risk factor for the disease. This is supported by the fact that the prevalence of AD in women is greater than in man, proportionally more than two-thirds of all cases occur in women on the United Kingdom, for example (53,56). However, this greater prevalence is often attributed to women larger life span relative to man, on average 4.5 years more (55). Also, various studies fail to detect an association between sex and risk of AD (40).

On the other hand, some investigators believe that the difference in AD prevalence between women and men may be linked with sex-related characteristics. Muller et al. described an association between sex hormone-biding globulin and increased risk for AD (57) and Chu et al. reported that higher testosterone levels were associated with a lower risk of AD (58). It was also reported differences, between males and females with the same *APOE* genotype, on AD risk and incidence (50).

Family history

The existence of family history cases with AD is another risk factor of the disease. These cases are classified as FAD, as previously described. In these cases, the increased risk for AD are influenced by two factors: the presence of rare fully penetrant mutations in *APP*, *PSEN1* and *PSEN2* that are passed through generations that are associated with; or the presence of other genetic variants that when combined with environmental factors, that all family members are exposed, can cause SAD (32).

As mentioned, besides the unmodified risk factors, certain modifiable habits, like lifestyle, may increase the risk to develop AD.

Lifestyle

Diet is one of the most important unmodified risk factors for AD, related to lifestyle. Certain nutrients like saturated fat and cholesterol seem to increase AD incidence, whereas the consumption of fish, fruits, vegetables, antioxidants (vitamin E and C), vitamins B may confer

neurocognition protection, decreasing AD risk (59,60). So a diet with these characteristics, particularly Mediterranean diet, is associated with a lower risk for AD (40). Other interesting data is that wine or coffee (3-5 cups/ day) drinking may also confer a protective effect against AD (40,60).

Smoking is another habit that can increase the risk of AD, specifically current smokers (40). This association may be linked with the formation of free radicals, oxidative stress, inflammatory response or cerebrovascular diseases that are caused by smoking (59).

Finally, regular physical activity has been described as a habit that has a protective effect on AD development. Hamer and Chida reported that physical activity reduces the risk of AD by 45% (61). Other studies even suggest that exercise can be used as a treatment for AD since it improves cognitive function (62,63). Cognitive activities also appear to reduce the risk of AD. Interestingly, higher educational attainment seems to be associated with faster cognitive decline in AD (40).

Medical conditions

Among acquired risk factors of AD, cerebrovascular disease, such as haemorrhagic infarct or vasculopathies, are the most commonly reported. Other diseases that are associated with increased risk of AD are hypertension, type 2 diabetes, obesity and dyslipidaemia (59).

Down Syndrome (DS) or 21 trisomy is a major disorder associated with EOAD, since there is a high incidence of EOAD among DS patients, who present AD clinical manifestation at a very early age (40 years). This has been attributed to the triplication of chromosome 21 that directly leads to APP overexpression which ultimately causes Aβ overproduction (64).

1.2.3. Pathogeneses of AD

The pathogeneses of AD can be characterized at a macroscopic level by the progressive loss of brain tissue (Figure 1). This atrophy occurs on the hippocampus (a structure essential for the formation of long-term memory) and on association cortices (areas that are responsible for cognition, i.e. the processes of CNS to attend, identify and act on complex stimuli) (65,66).

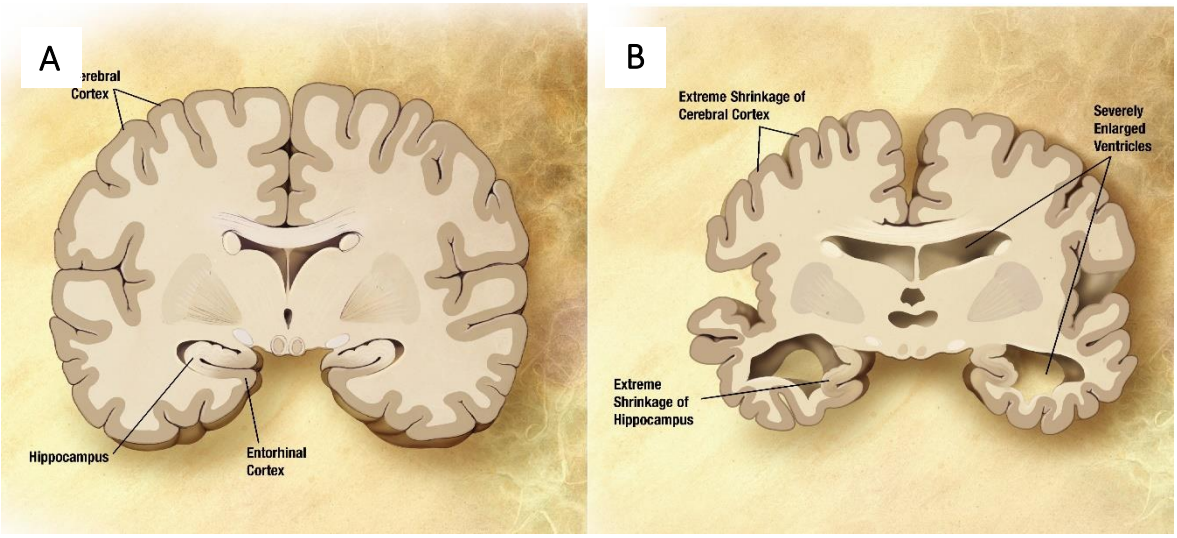

Figure 1: Macroscopic hallmarks of AD. Schematic representation of hippocampal and cortical atrophy between pre-clinical AD **(A)** and severe AD **(B)**. (Taken from: National Institute on Aging; Copyright© 2002, Christy Krames).

At a microscopic level, AD is characterized by two hallmarks: extracellular deposition of Aβ peptides that aggregate and form senile or neuritic plaques (SP); and intraneural presence of neurofibrillary tangles (NFT) that are aggregates of hyperphosphorylated tau protein (Figure 2).

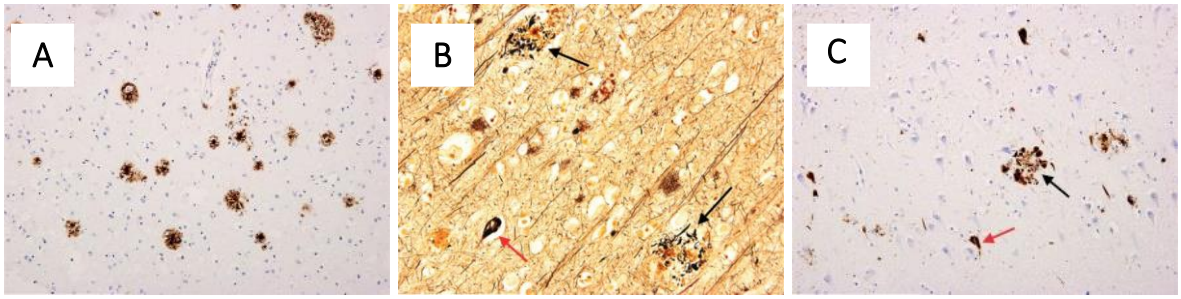

Figure 2: Microscopic hallmarks of AD. (A) Immunohistochemical stain of temporal cortex of a patient with AD, showing SP accumulation (4G8 antibody, against Aβ amino acids 17-24; 100x); **(B)** Photomicrograph of the temporal cortex of a patient with AD, showing SP (black arrows) and NFT (red arrow) (modified Bielschowski stain; 100x); **(C)** Immunohistochemical stain of temporal cortex of a patient with AD, showing SP (black arrows) and NFT (red arrow) (TG-3 antibody; 100x) (adapted from [67])

APP, Aβ peptides and neuritic plaques

APP is one member of a protein family that includes amyloid precursor like proteins 1 (APLP1) and 2 (APLP2), with only APP containing the Aβ domain (68). It is a cell surface protein ubiquitously expressed, with high expression in the brain. The principal isoforms are APP_{965} (predominantly expressed in neuronal tissue), APP_{751} and APP_{770} (both widely expressed in non-neuronal cells) (69).

After maturation, APP is processed proteolytic by two major pathways: the amyloidogenic pathway and the non-amyloidogenic pathway, as briefly referred above. In the non-amyloidogenic pathway, APP is sequentially cleaved by α-secretase within Aβ region, and γ-secretase complex. The α-secretase cleavage precludes Aβ formation, and results in the releasing of soluble APP peptide alfa (sAPPα) and the retention of a membrane bound C-terminal with 83 peptides (C83). C83 is further cleaved by γ-secretase producing C-terminal fragment (CTF), also known as APP intracellular domain (AICD) and P3 peptide (Figure 3) (68).

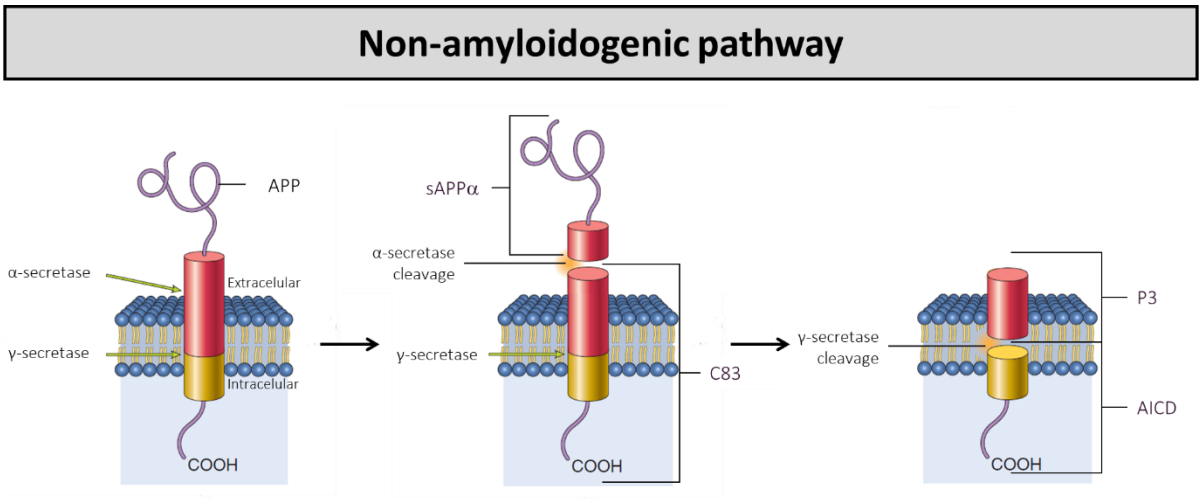

Figure 3: Non-amyloidogenic processing of APP. First α-secreatase cleaved APP, realising sAPPα. Then γ-secretase cleavage forms AICD and P3. (Adapted from (43)).

In the amyloidogenic pathway, APP is cleaved firstly by β-secretase at the N terminus of Aβ sequence, releasing soluble APP peptide beta (sAPPβ) and producing a membrane bound C-terminal fragment with 99 amino acids (C99). C99 is then cleaved by γ-secretase releasing AICD (identical to non-amyloidogenic pathway) and Aβ peptid, that is sequentially cleaved (also by γ-secretase) into shorter peptides ($Aβ_{42}$, $Aβ_{40}$ and $Aβ_{38}$) (Figure 4) (68).

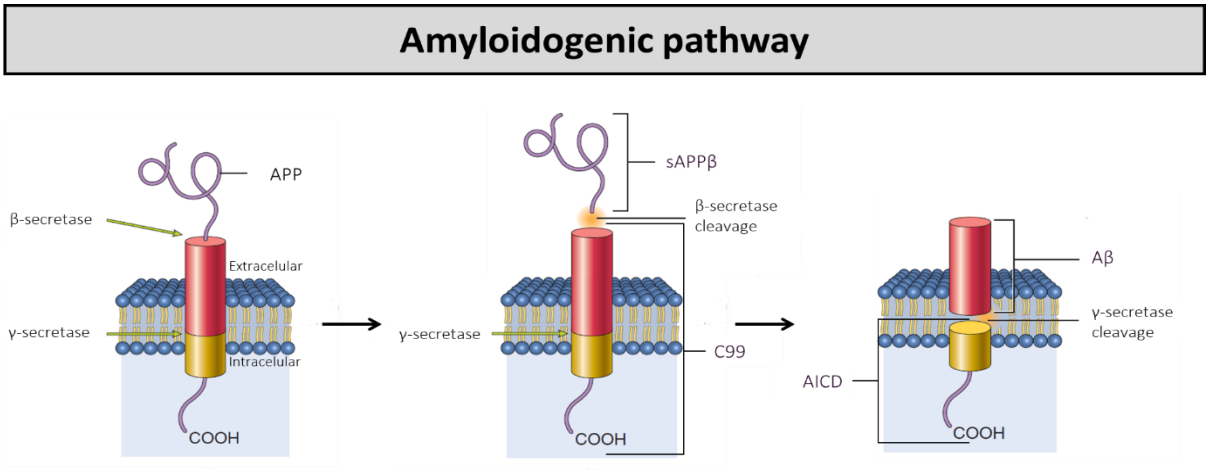

Figure 4: Amyloidogenic processing of APP. The sequential cleavage of APP by β-secretase and γ-secretase leads to the production of Aβ peptides.(Adapted from (43))

After release, Aβ peptides tend to deposit and aggregate on the extracellular space into higher forms like oligomers and fibrils and later in SP, pathological structures found in AD. $Aβ_{42}$ is the most presented peptide among Aβ, in SP due to this greater propensity for aggregation (caused by having a more hydrophobic C-terminal) (13,44).

Aβ oligomers or fibrils are very toxic to the cell in several ways such as promoting cell apoptosis through destabilizing cell membrane; disrupting calcium ions homeostasis, leading to loss of membrane potential; causing synaptic impairment and loss; promoting neuroinflammation, mitochondria failure and oxidative damage (70). SP can also cause synaptic and neuritic loss, nevertheless, some studies also support that Aβ oligomers and fibrils are the main agents of Aβ toxicity (70,71).

Tau and neurofibrillary tangles

The other hallmark of AD is the intracellular formation of NFT. These structures have been associated with AD progression and severity (72). NTF contains hyperphosphorylated tau (13).

Tau is a microtubule-associated protein (MAP) expressed in neurons, mostly found in axons, whose functions include microtubule polymerization and stabilization, processes closely involved with neuronal cytoskeleton architecture, and axonal polarity (along other MAPs) (73). This protein can suffer multiple post-translational modifications, such as phosphorylation.

In AD and other tauopathies, tau phosphorylation becomes abnormal, resulting in a hyperphosphorylated tau form, that detaches from microtubules and aggregates into straight filament (SF) or paired helical filaments (PHF), building up NFT (Figure 5) (74,75). Due to tau detachment from microtubules and NFT formation, neuronal cytoskeletal becomes dysfunctional and axonal transport is impaired, affecting neurons access to nutrients and other essential components leading to energy depletion, mitochondria dysfunction, synaptic loss and lastly neuronal degeneration (74).

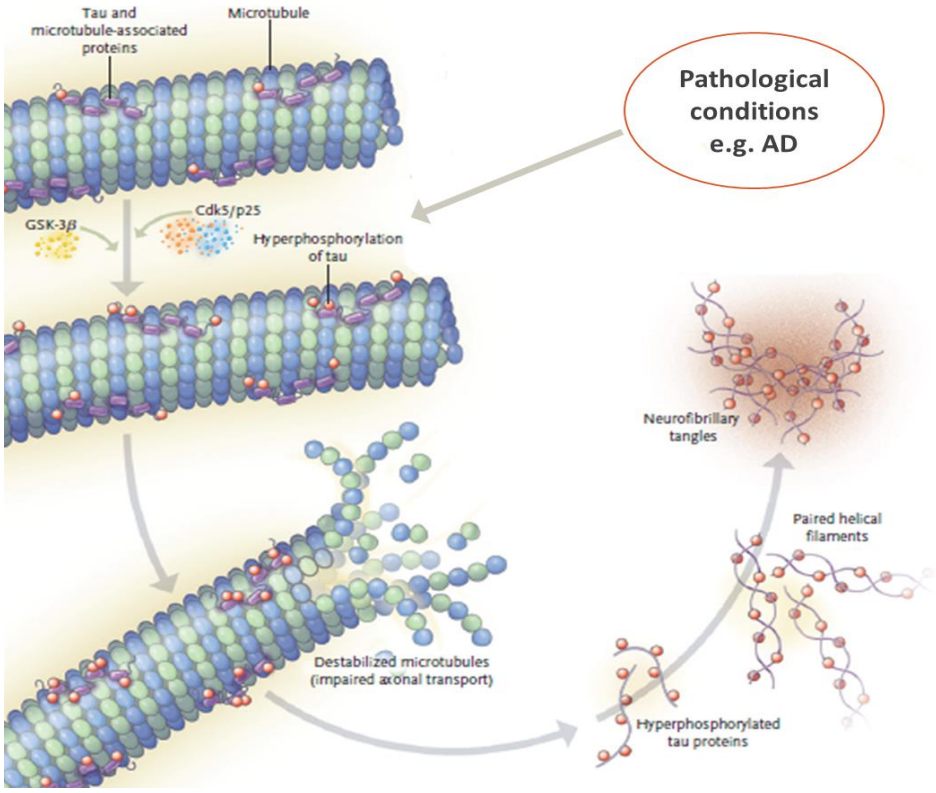

Figure 5: NFT formation from hyperphosphorylated tau. Tau present in neurons microtubules can became hyperphosphorylated in pathological conditions (by the action of GSK3β and CDK5 kinases). This destabilizes microtubules and leads to tau aggregation into PF and NFT, as the highest structure. (Adapted from (76)).

Tau longest isoform possesses 85 potential phosphorylation sites at serine, threonine, and tyrosine residues (77). Of these sites, 45 has been linked with AD, with 29 of them being exclusively found in AD brain (78). The main protein kinases responsible for tau hyperphosphorylation in AD are proline-directed protein kinases (PDPK), that are kinases that target serine and threonine preceding a proline residue. These include glycogen synthase kinase 3 β (GSK3β), cyclin-dependent kinases (CDK) such as CDK2 and CDK5 and mitogen activated protein kinase (MAPK) (Figure 6) (79,80).

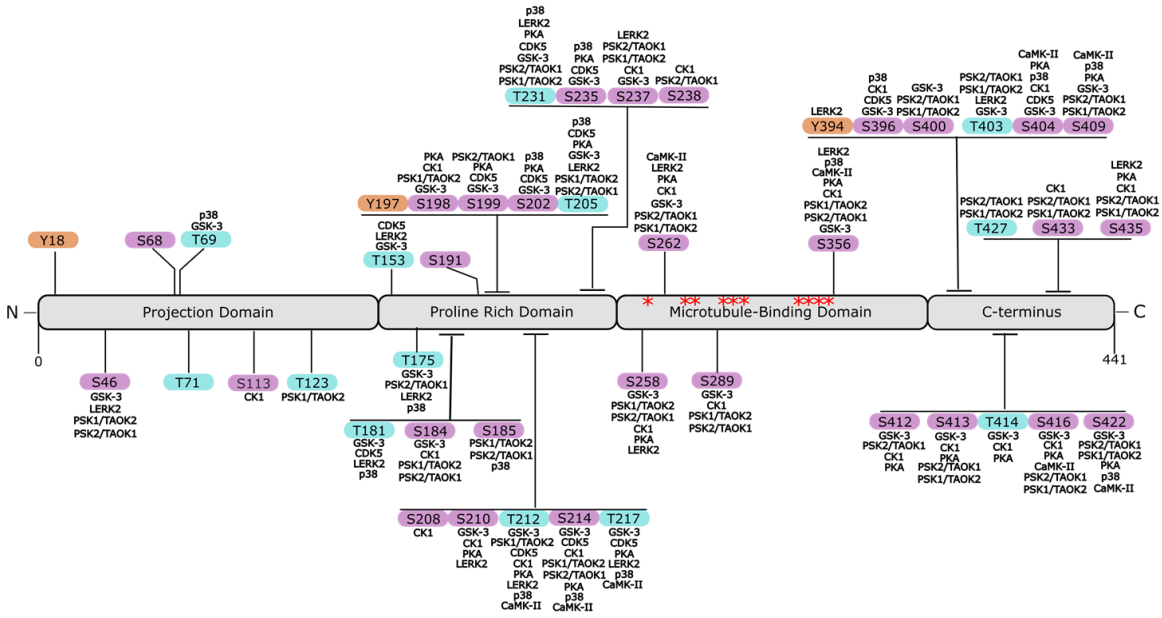

Figure 6: Tau phosphorylation sites identified in brains of AD patients. Diagrammatic representation of the most common tau residues (tyrosines (Y) in orange; serines (S) in purple and threonines (T) in blue) shown to be phosphorylated in the AD brain (Taken from (78))

GSK3β involvement on tau hyperphosphorylation in AD has been widely studied, either in cellular or animal models (79,81). Although the exact mechanism by which GSK3β is anomaly activated and causes tau hyperphosphorylation is not kwon, it may involve one or more of the established GSK3β regulatory mechanisms. These include the association of GSK3β in protein complexes, substrate priming (i.e. pre-phosphorylation of the substrate, on a residue approximately 4 residues C-terminal to GSK3β target residue) and post-translational phosphorylation at specific residues (e.g. serine-9 or tyrosine-216) (82,83).

The other main kinase involved in tau abnormal phosphorylation in AD is CDK5. This kinase possesses two regulatory subunits, p35 and p39. In neurotoxic situations, like AD, p35 and p39 are cleaved into p25 and p29 that forms a more stable complex with CDK5, leading to a constitutively activated kinase. In AD the most predominant CDK5 complex is with p35 or with p25 (74).

On the other hand, decreased activity of several protein phosphatases (PPs) has been associated with tau hyperphosphorylation. Tau can be dephosphorylated by several PPs, like protein phosphatase 2A (PP2A), PP1 or PP2B, with PP2A being the most important (about 71% of total tau phosphatase activity). These phosphatases seem to dephosphorylate the same tau residues but with different efficiencies (84). The relation between PPs decreased activity and AD was established by using, for example, okadaic acid (OA), a PP2A and PP1 inhibitor. In this work, the OA treatment induced the accumulation of NFT (85).

1.2.4. Hypothesis underlying AD pathogenesis

Although neuropathological features and hallmarks of AD are well identified and established, the exact causes underlying these changes are not completely understood.

Originally, Aβ deposition was considered the initial pathological event of AD that would lead to tau hyperphosphorylation and to SP and NFT formation. This hypothesis is known as the "Amyloid Cascade Hypothesis". However, as anti-Aβ therapies have failed in different therapeutic strategies tests and clinical trials, other hypotheses have gained strength (86). Among them are Dual pathway, Mitochondrial cascade, Metabolism cascade, Inflammatory cascade, Cell cycle re-entry hypotheses, where most of them place Aβ deposition and tau hyperphosphorylation at the same level of importance in AD pathogenesis.

In this section, two hypotheses will be analysed in detail: the Amyloid cascade hypothesis, because it's the original theory, and the Inflammatory cascade hypothesis, since it is the most relevant hypothesis for this thesis.

Amyloid cascade hypothesis

Initially proposed in 1992 by Hardy and Higgins, the amyloid cascade hypothesis placed the deposition of Aβ peptide as the first and main causal pathological event in AD. The increased deposition would be caused by abnormal amyloidogenic processing of APP and directly lead to tau phosphorylation, NFT formation, synaptic loss and neuronal death (87). There is evidence that Aβ promotes tau phosphorylation and modulates APP phosphorylation, regulating this processing, thought GSK3β, CDK5, PP2A, PP1 and PP2B (Figure 7) (80,88).

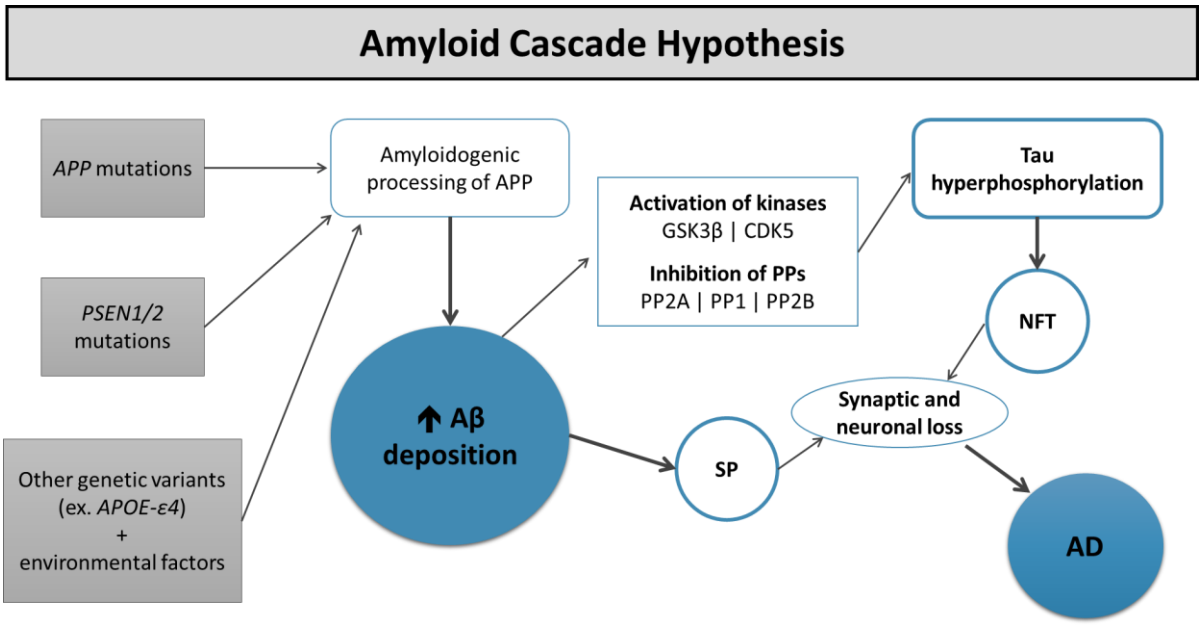

Figure 7: Amyloid Cascade Hypothesis. Aβ deposition is considered the trigger event of AD pathogenesis, and the promoter of tau hyperphosphorylation.

This hypothesis is largely supported by two facts: the occurrence of autosomal-dominant mutations in *APP, PSEN1* and *PSEN2* since these genes encode proteins directly involved in increased $A\beta_{42}$ production (38); and the large incidence of EOAD among Down Syndrome (DS)

patients, previously described, which leads to APP overexpression and potential increased Aβ production (64).

However, many anti-Aβ therapies have failed over the years. These include anti-Aβ vaccines and antibodies directly against Aβ peptides and oligomers (including bapineuzumab, solanezumab, aducanumab and crenezumab), with the last being scientists greatest hope for AD treatment and cure, recently discussed in AD/PD conference 2019 in Lisbon, Portugal (86,89).

Inflammatory cascade hypothesis

One of the emerging theories underlying AD pathology, in the Post- Amyloid Cascade Hypothesis Era, is the inflammatory cascade hypothesis. In this theory, the neuroinflammatory process is proposed as the other main event that occurs in AD, along with Aβ deposition and aggregation into SP and tau hyperphosphorylation forming NFT (90).

During neuroinflammation, microglia activation is the main event. Contrarily to what happens in normal brain, where microglia do not produce large amounts of inflammatory molecules and mediators, under pathological situations, microglia produces several inflammatory mediators: proinflammatory, anti-inflammatory and chemotactic cytokines. These cytokines seem to be involved in AD pathology through affecting APP expression and abnormal amyloidogenic processing, Aβ aggregation and accumulation into SPs and tau phosphorylation (93).

Some studies support that microglia activation in AD is caused by Aβ oligomers and SP (94), while others propose that it could be the leading event of AD etiology rendering in increased cytokines production that can cause even more microglia activation (Figure 8) (95).

Neuroinflammation and microglia activation are discussed in detail below.

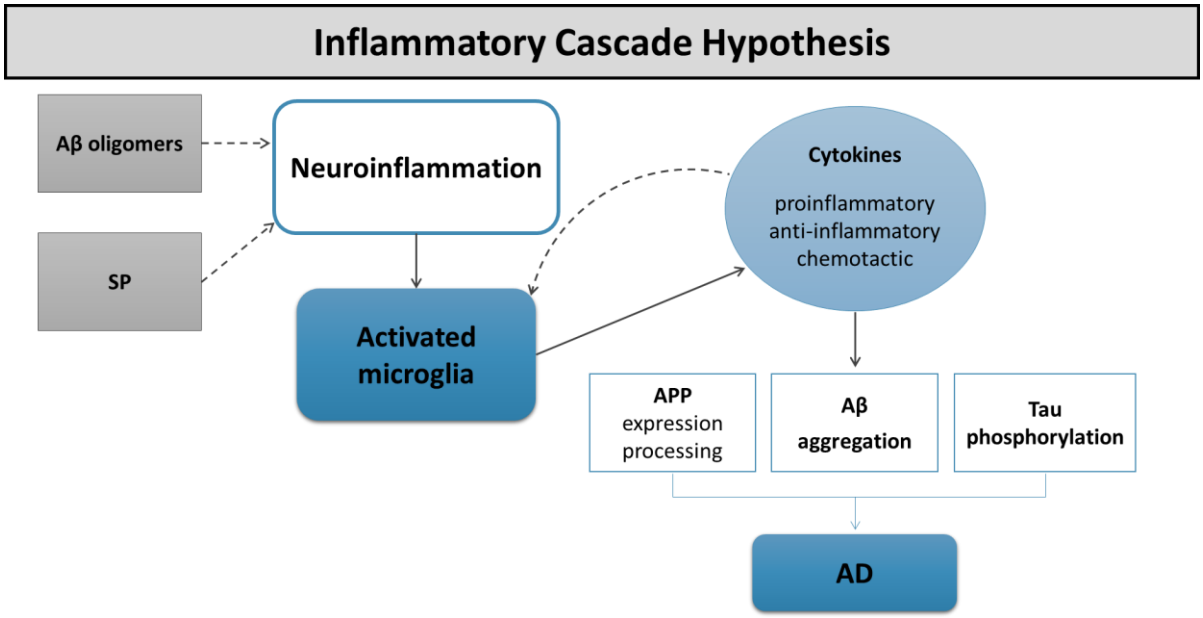

Figure 8: Inflammatory Cascade Hypothesis. Microglia activition is the main causing event of AD pathogenesis, leading to cytokines production that alter APP expression and processing; Aβ aggregation and tau phosphorilation. Microglia activation may be caused by Aβ oligomer or SP or be a independent event.

1.3. Neuroinflammation in AD

Inflammation is the response of the immune system to abnormal or damaging situations, such as pathogens, damaged or apoptotic cells and toxic substances. In the brain, inflammation, that can be referred as neuroinflammation, occurs in pathological situations, like AD (96). The cells responsible for neuroinflammation are microglia and astrocytes. Microglia are specialised macrophages in CNS and are the second most important cells in the brain, after neurons, while astrocytes are the most abundant glial cell in the brain (90).

In AD, neuroinflammation is a complex process and the exact involvement of immune system in AD pathology is not fully known (Figure 9). The involvement of neuroinflammation with the disease pathology has been strongly sustained by the observation of altered levels of several proinflammatory, anti-inflammatory and chemotactic cytokines in the blood and CSF from AD patients (91,92). These cytokines, that are produced by microglia and astrocytes, include proinflammatory cytokines: interleukin (IL) 1β, IL-1α, IL-6, IL-18, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ); anti-inflammatory cytokines: IL-4, IL-10 and transforming growth factor-β1 (TGF-β1); chemotactic cytokines: CCL2 or monocyte chemoattractant protein 1 (MCP-1), CXXL8 or IL-8, CXCL10 or interferon-inducible protein 10 (IP-10), CCL5 or Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES), CCL3 or macrophage inflammatory protein 1-α (MIP-1α), and CX3CR1 or fractalkine (93).

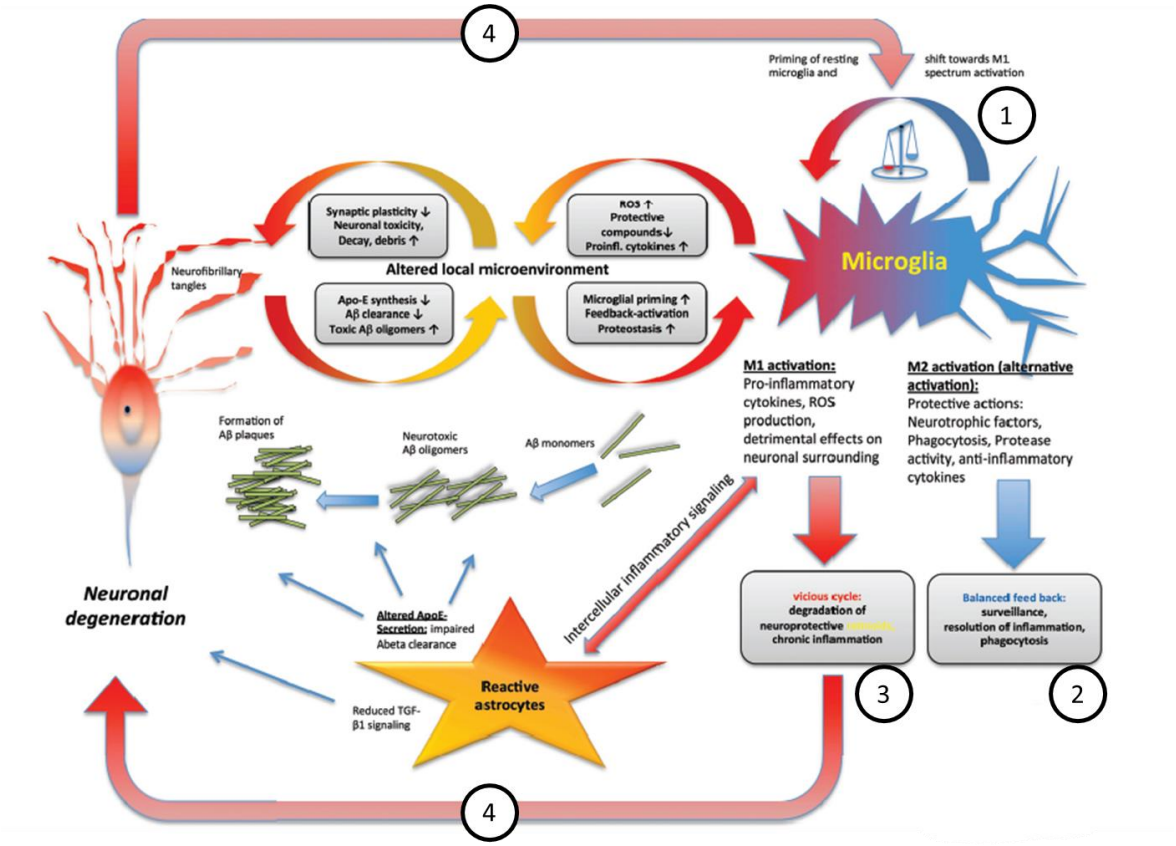

Figure 9: Neuroinflammation in AD. (1) Microglia activation on AD is caused by SP (Aβ aggregates) and NFT. This can lead to beneficial effects **(2)**, resulting in reduced inflammation, or to harmful effect **(3)** whereas astrocytes become reactive, more Aβ peptides are produced and tau phosphorylation is increased. Therefore a vicious circle occurs resulting in chronic neuroinflammation **(4)** (Adapted from (90))

In addition, the accumulation of microglia and astrocytes on Aβ deposition and SP sites corroborates with the fact that these cells may play a role in AD neuroinflammation (97). Another fact that supports the role of the immune system in AD pathogenesis was the identification of risk genes associated with immune response, namely important microglial genes *CD33, TREM2* and *CLU.* This discovery also contributes to the theory that neuroinflammation may be the first event of AD etiology (52).

The role of neuroinflammation on AD appears to be a brief protective response that with prolonged activation, becomes harmful to neurons. If microglia is moderately activated (namely M2 form), it triggers Aβ and SP clearance (by phagocytosis) and anti-inflammatory cytokines production (98). However, as the activation of microglia continues constantly (namely M1 form), pro-inflammatory, chemotactic cytokines, complement factors and harmful substances (like reactive oxygen species (ROS) and C-reactive protein) are produced. These cytokines may affect APP expression and amyloidogenic processing, Aβ aggregation and tau phosphorylation (by activating kinases) (93). On the other hand, NFT, Aβ fibrils and oligomers *per se* can increase proinflammatory cytokines production, that activates microglia, becoming a vicious cycle (Figure 9) (90,93,94).

1.3.1. Chemokines role in AD

Chemokines are chemotactic cytokines whose main function is to mediate immune cells migration to inflammation sites, through the binding of these small molecules to their specific receptor. On the other hand, chemokines and their receptors are also expressed in other cells to allow cells communication, contributing to physiologic processes. In the brain, chemokines can be produced by microglia and astrocytes to mediate neuronal migration or synaptic activity in physiologic conditions or as a response to abnormal or pathological situations (99,100).

These molecules are classified according to number and spacing of cysteine residues in the N-terminal as CXC (or α-chemokines), CC (or β-chemokines), CX3C (or γ-chemokines) and C (or δ-chemokines) (101).

In several neuroinflammatory conditions, upon damaged areas, chemokines can have a beneficial or a prejudicial effect, as proposed by Le et al, illustrated in (Figure 10) (99).

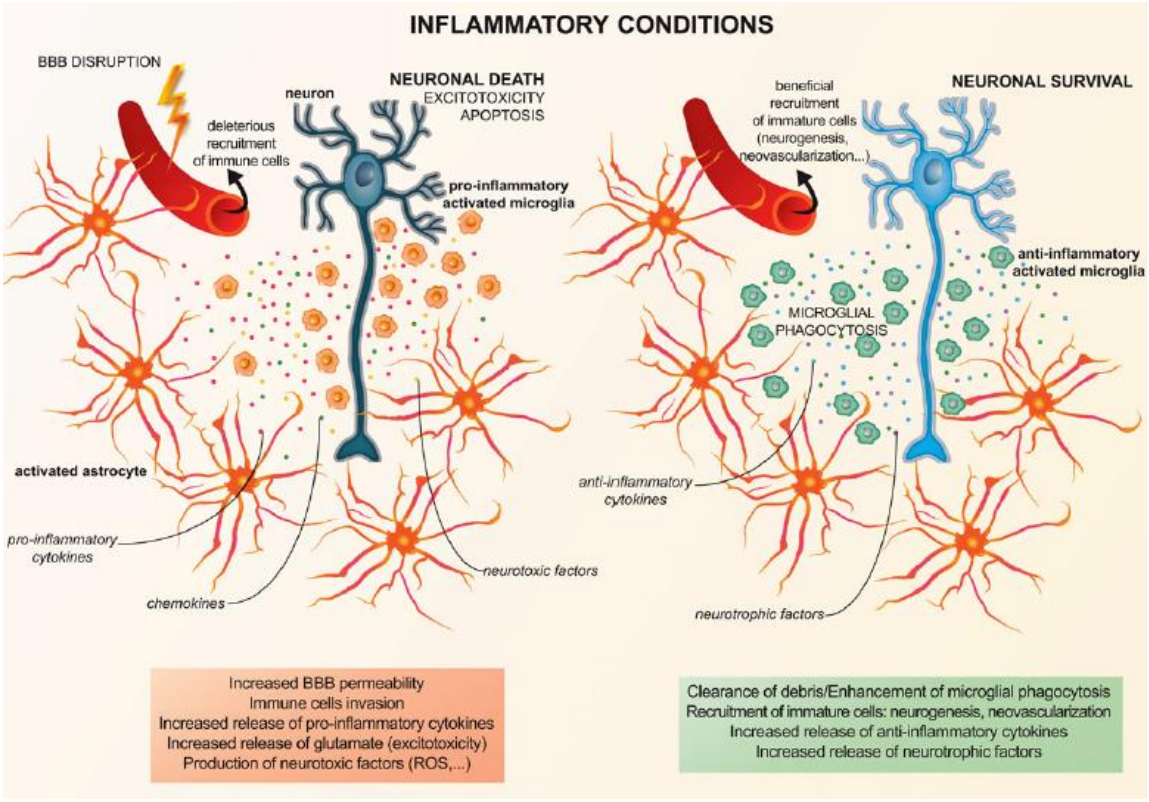

Figure 10: Chemokine role in neuroinflamation. Prejudical (left) and beneficial (right) efect of chemoquines in inflammation conditions. (Taken from (99))

In AD, several chemokines like CCL2 or MCP-1, CXXL8 or IL-8, CXCL10 or IP-10, CCL5 or RANTES, CCL3 or MIP-1α, and CX3CR1 or fractalkine and/ or their receptors were found at altered levels on patients' serum, CSF and brain tissue (Table 2) (93,100).

Table 2: Chemokines levels in AD serum, plasma, blood, CSF and brain. (adapted from (100))

Chemokines	Serum, plasma or blood	CSF	AD Brain	References
MCP-1 (CCL2)	↑	↑	↑	(102–107)
IL-8 (CXXL8)	↑	↑	↑	(105,106,108)
IP-10 (CXCL10)	=	= ↑	–	(105,106,109)
RANTES (CCL5)	↓	=	↑	(105,110,111)
MIP-1α (CCL3)	–	=	↑	(103,105)
Fractalkine (CX3CR1)	↑ (↓ along AD progression)	–	–	(112)

Guedes et al propose that chemokines role in AD may be related to the mediation of peripheral monocytes infiltration to the damaged areas, similar to what happens in non-pathological situations. At the damaged area monocytes differentiate into macrophages, similar to microglia cells, that accumulate on Aβ deposition sites (113). This occurs due to the production of chemokines by activated local microglia, astrocytes and neurons. On the site, microglia produces even more chemokines, leading to both beneficial (most probably in an initial phase) and prejudicial effects (due to sustained microglia activation) (99).

Also regarding to chemokines role on AD pathogenesis, RANTES (or CCL5) and MIP-1α can recruit peripheric monocytes and microglia to Aβ deposition site and have been linked with a protective role on AD, since their (or their receptors) depletion or knock-out (KO) has associated with increased Aβ deposition and β- and γ-secretase activity. Instead, some chemokines like IP-10 (or CXCL10) and fractalkine (or CXCL1) may have a prejudicial effect on AD pathogenesis because their receptors impairment caused decreased Aβ levels and deposition, therefore promoting Aβ clearance. However, fractalkine deficiency can likewise cause increased phosphorylated tau levels and, considering the anterior effect, it seems to have conflicting contributions to AD pathogenesis since this chemokine may either promote Aβ production and accumulation or inhibit tau phosphorylation (93,105,113).

MCP-1 and IL-8

MCP-1 or CCL2 is produced by microglia and astrocytes and it seems that this chemokine has a dual role in AD. In some reports, MCP-1 is associated with AD pathogenesis, for instance, Aβ levels, deposition and aggregation were increased in APP/CCL2 tg mice (that overexpress APP and MCP-1) compared to APP tg mice, an effect that may be caused APOE (since increased levels of this protein were also observed) (114). Moreover, another study reports that APP/CCL2 tg mice lead to microglia accumulation on Aβ oligomers also showing an association of MCP-1 with Aβ aggregation. Further, accelerated cognitive dysfunction was associated with MCP-1 overexpression (115).

On the other hand, the experimental inhibition of MCP-1 receptor (CCR2) caused Aβ accumulation and premature death in APP/CCR2$^{-/-}$ tg mice (that overexpress APP and do not express CCR2) (116). Additionally, an increase in Aβ oligomers levels, impaired cognitive and worsened memory deficits were reported in APP/PS1/CCR2$^{-/-}$ tg mice (that overexpress APP and PS1 and do not express CCR2) (117,118). All of these finds indicate that MCP-1 may have a protective role in AD, since MCP-1 (or its receptor) depletion lead to a worst pathological situation.

IL-8 or CXCL8, is also produced by microglia and astrocytes. This was the first confirmed chemokine in human brain and reported to be significantly increased in serum, CSF and brain from AD patients, comparing to controls (105,106,108). However, and contrary to MCP-1, IL-8 has not been so extensively studied on its role on AD pathology. Even so, Bakshi et al. observed a relation between IL-8 and increased Aβ production, thought modulating γ-secretase activity. In their work, the depletion or the impairment of IL-8 receptor (CXCR2) caused decreased $A\beta_{42}$ and $A\beta_{40}$ production, concomitantly with an accumulation of C99 and C83, γ-secretase substrates, suggesting that this secretase activity was impaired (119,120). Taken together, data support that neuroinflammation plays a key role in AD pathogenesis.

2 — Aims

2. Aims

AD is the most prevalent neurodegenerative disorder worldwide and the main cause of dementia. It is characterized by impaired memory, cognition and behaviour and its pathogenic processes involve the damage and progressive neuronal loss related with two main AD hallmarks: the extracellular presence of SP, mainly composed of Aβ peptides and the intracellular formation of NFT, resulting as aggregates of hyperphosphorylated tau.

Specifically, tau hyperphosphorylation and NFTs formation can imbalance microtubule dynamics, axonal transport and thus contribute to neurodegeneration. The proteins whose expression/ activity has been associated with tau hyperphosphorylation in AD include kinases activation, like GSK3β and CDK5, and phosphatases inhibition, namely PP2A and PP1.

Although the exact mechanisms leading to AD are not fully understood, neuroinflammation seems to have a key role in the disease etiology and/or progression, with microglia and astrocytes as the main involved cells. In an initial stage, inflammation on AD appears to have a brief protective response, limiting Aβ deposition and other pathogenic processes. However, sustained microglia and astrocytes activation, lead to harmful neuronal effects.

Cytokines are the main molecular mediators of this process and several cytokines have been found altered in the blood, CSF or brain of AD patients. Among these are chemokines that have been associated with a beneficial and/or prejudicial role on AD pathogenesis. However, the impact of these molecules on tau related AD pathogenesis has not received much attention.

Additionally, previous work done by the group suggested that chemokines IL-8 and MCP-1 can affect tau phosphorylation (121).

Therefore, in the present work, the influence of IL-8 and MCP-1 on tau phosphorylation was further investigated. Hence, the specific aims of this thesis were to::

1. Evaluate IL-8 and MCP-1 impact on tau phosphorylation;
2. Analyse GSK3β and CKD5 levels and activity after chemokines treatment;
3. Determine the effect of IL-8 and MCP-1 on PP1 and PP2A levels and activity.

3 — Material and Methods

3. Materials and Methods

3.1. Cell culture

For all assays, SH-SY5Y neuroblastoma cell line (ATCC® CRL-2266™) was used. This cell line is a thrice cloned subline of parental line SH-N-SH, which was established in cell culture from a bone marrow biopsy of a metastatic neuroblastoma patient in 1970 (122).

SH-SY5Y cell line was chosen due to their wide use in neurodegenerative research, in particular in Alzheimer's disease (AD), since these cells express human-specific proteins and exhibit several functional and biochemical features of neurons when differentiated (123–125).

SH-SY5Y cells were cultured in complete culture medium: Minimal Essential Medium (MEM)/F12 (1:1) supplemented with 10% fetal bovine serum (FBS), 0,45 mM sodium pyruvate and 1% of antibiotic-antimycotic mix (100 U/mL penicillin, 100 µg/mL streptomycin and 0,25 µg/mL Amphotericin B). Cells were maintained in an incubator, at 37°C with 5% CO_2 and 95% humidity. Cell culture medium was changed every two days and cells were subcultured whenever 80-90% confluence was reached.

3.1.1. Cell differentiation

Differentiated SH-SY5Y cells were used since these cells present more mature neuron-*like* features including neuronal morphology, cell division arrest and some neuron specific proteins, including microtubule-associated proteins (MAP) (particularly MAP2 (126) and β-III tubulin(123); reviewed in (127)) and synapse protein Sv2 (123), among others (128).

For SH-SY5Y differentiation, cells were detached from an 80-90% confluent plate and plated as $5x10^5$ cells/well into 6-well plates (30 millimetres (mm) diameter wells). To promote differentiation, cells were grown in 1% FBS culture medium supplemented with 10 µM retinoic acid (RA; diluted in DMSO) for two days, as previously described (123). In all assays, an undifferentiated control was grown in the same media without the addition of RA but with DMSO the solvent of RA solution.

3.1.2. Chemokine treatment

To assess the impact of Interleukin 8 (IL-8) and Monocyte Chemoattractant Protein 1 (MCP-1) on tau phosphorylation, differentiated SH-SY5Y were treated with human purified IL-8 and MCP-1 (BD Biosciences). Lyophilized IL-8 was reconstituted in ultrapure-sterile water (pH 6,3) containing 1 mg/mL Bovine Serum Albumin (BSA). Both chemokines were aliquoted and stored at -80°C.

For the experimental procedure, work solutions of IL-8 and MCP-1 were prepared by successively diluting each chemokine stock solution in serum-free culture medium, to achieve the final concentrations of 25, 50, 75 and 100 ng/mL for IL-8; or 10, 50 and 100 ng/mL for MCP-1 (Figure 11). Treatment mediums were freshly prepared on the assay day.

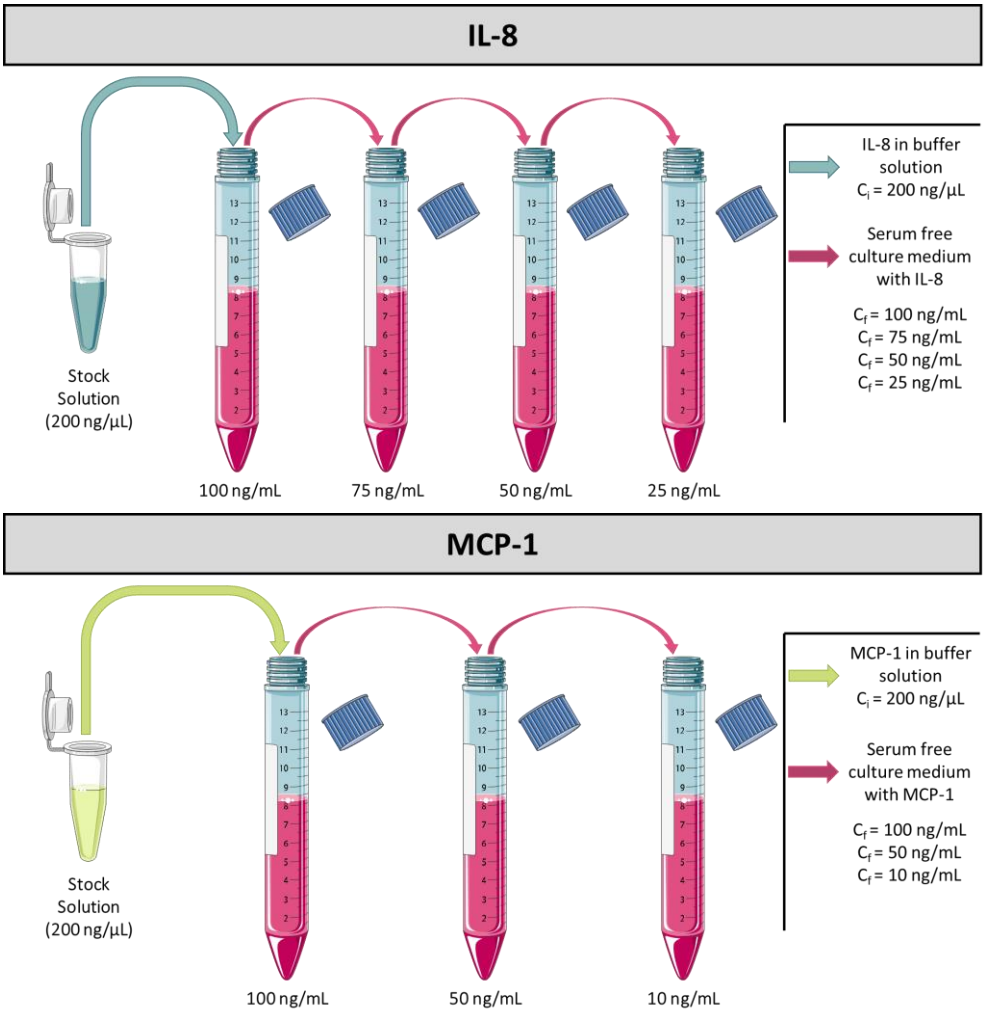

Figure 11: Representative scheme of serial dilution for IL-8 and MCP-1. Adapted using images from Servier Medical Art by Servier.

Cells were treated for a maximum of 48 hours (h) or 24h, with IL-8 and MCP-1 respectively, and collected at different time points: 6h, 24h and 48h for IL-8 or 3h, 6h and 24h for MCP-1. Experimental timeframe and plates display are illustrated on (Figure 12).

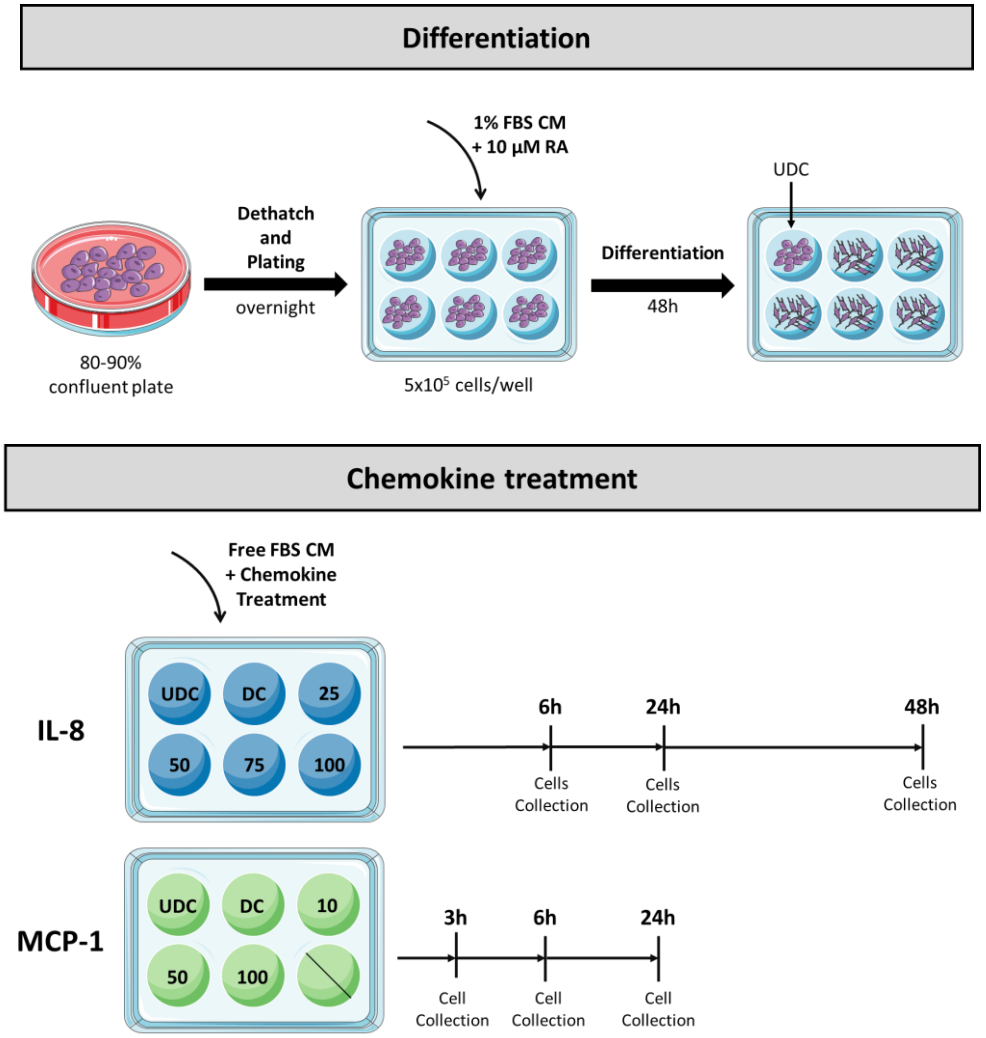

Figure 12: Differentiation and chemokine treatment. Adapted using images from Servier Medical Art by Servier.

3.1.3. Cells morphology evaluation

SH-SY5Y morphology was evaluated after differentiation or chemokine treatment by acquiring contrast-phase images of cells using Olympus IX81.

3.2. Western Blotting

3.2.1. Cells collection

After the respective treatments, cells were washed with Tris-buffered saline (TBS), pH 8 and collected with RIPA lysis buffer (25mM Tris-HCl, 150mM NaCl, 1% Nonidet™ P40 (NP40), 1% sodium deoxycholate, 0.1% SDS; pH 7.6) supplemented with Sodium Fluoride (NaF; to inhibit Serine/Threonine and acidic phosphatases), Sodium Orthovanadate (Na_3VO_4; to inhibit Tyrosine and alkaline phosphatases) and Protease Inhibitor Cocktail, using a cell scraper. After that, cell

lysate was sonicated for 10s. All samples were kept on ice during the collection process and stored at -20°C at the end.

3.2.2. Protein concentration determination

Bicinchoninic Acid (BCA) Protein Assay (Pierce - Thermo Scientific) was used to determinate samples protein concentration. This assay consists of a colorimetric detection and quantitation of total protein content based on the reduction of Cu^{2+} to Cu^{+1} by proteins in an alkaline medium. The production of Cu^{+1} is detected by the formation of a purple-colored chelate between two molecules of BCA and one Cu^{+1} ion. This purple-colored product exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations (129).

For sample analysis, 5 µL of collected lysates and 20 µL of 1% Sodium Dodecyl Sulfate (SDS) was added to each well. A standard curve was prepared using BSA and 1% SDS according to Table 3. Samples and standards were then incubated with 200 µL of working reagent (mix of reagent A and reagent B in 50:1 proportion) for 30 minutes (min) at 37°C. After some minutes, for cool down at RT, absorbance was measured at 552 nm on a plate reader.

Table 3: BCA standards composition.

Standard	BSA (µL) stock 2 mg/mL	1% SDS (µL)	Protein mass (µg)
P0	–	25	0
P1	1	24	2
P2	2	23	4
P3	5	20	10
P4	10	15	20
P5	20	2	40

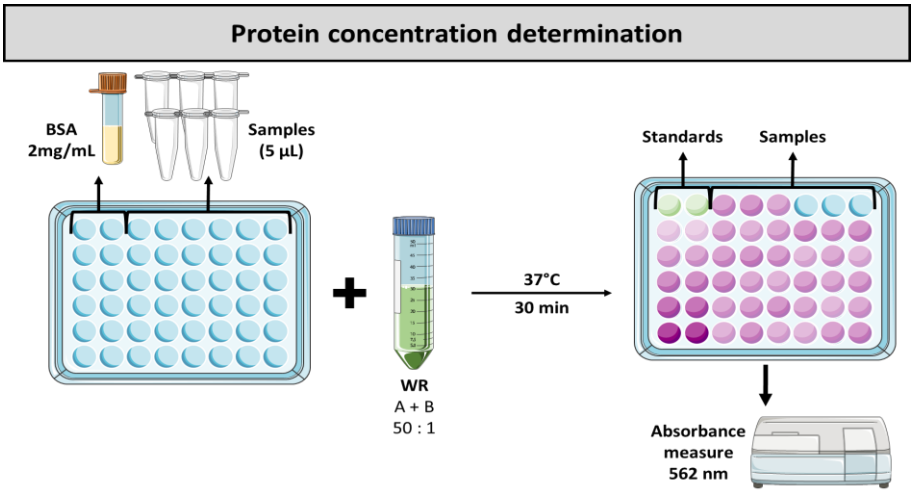

Figure 13: BCA protocol. Adapted using images from Servier Medical Art by Servier.

3.2.3. Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated according to their molecular weight and negative charge by gel electrophoresis. In this technic, molecules are separated through a porous matrix when a voltage is applied. This happens because molecules with negative charge (e.g. proteins), when exposed to an electrical field, migrate towards the positive end or cathode. In electrophoresis, proteins migrate towards the cathode in a porous matrix, with decreasing size pores that restrict proteins according to their sizes. Thus, large proteins are restricted on top and small proteins, that keep migrating towards the cathode, are restricted on the bottom.

In the present work, SDS-Polyacrylamide Gel Electrophoresis was utilised since Polyacrylamide Gel (PAG) is a matrix that present pore with a size similar to proteins size. On the other way, when SDS is added to PAG and samples, SDS binds to proteins and confer the same negative charge to all. This allows performing a separation based only on protein size and independently of native charge (130).

The first step of SDS-PAGE is gel preparation. SDS-PAGE gel comprised two phases: at the bottom a resolving or separating gel, with small pores for proteins restriction and separation and at the top a stacking or spacer gel, where samples are loaded, to stack all samples proteins in one band so that all proteins come into resolving gel at the same time. Resolving gel was prepared as a 5 to 20% gradient gel and left to polymerize. After that, staking gel was prepared and loaded on top and left to polymerize for approximately 1h. Resolving and stacking compositions are described in Table 4.

Table 4: SDS-PAGE – Gels constitution

Reagents	Resolving		Stacking
	5%	20%	3,5%
LGB	3,75 mL	3,75 mL	–
UGB	–	–	2 mL
Acrylamide (29:1)	1,875 mL	7,5 mL	0,88 mL
10% SDS	–	–	100 µL
APS	75 µL	75 µL	100 µL
TEMED	7,7 µL	7,5 µL	10 µL
dH_2O	9,29 mL	3,67 mL	6,92 mL

The second step of SDS-PAGE is sample preparation. For each sample, the volume corresponding to the desired protein mass (25 µg or 50 µg; calculated from protein concentration whose determination is described in section 0) was mixed with loading buffer 6x (LB) containing glycerol (to increase samples' density and enable loading and sample anchor in the wells until electric field is applied), SDS (to confer the same negative charge to all samples), dithiothreitol (DTT; as reducing agent to disrupt quaternary and tertiary protein structures) and bromophenol blue (to dye gel leading front) (130). Finally, samples were boiled for 5 min at 95°C (for protein denaturation) and spin down.

Before loading, running buffer was added to the electrophoresis system. Samples were gently loaded on the gel, as well as Precision Plus Protein Dual Color Standards (a molecular weight marker; BioRad). Proteins were separated at 90 mA for 1 system (two gels) or 45 mA for ½ system (one gel) for approximately 3h (Figure 14).

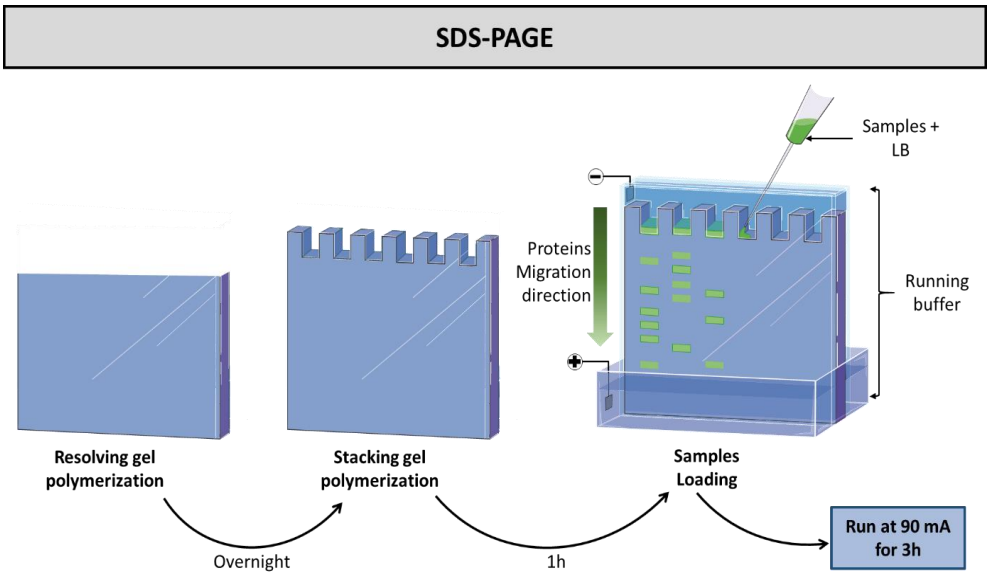

Figure 14: SDS-PAGE protocol. Adapted using images from Servier Medical Art by Servier.

3.2.4. Western Blotting analysis

Western blot (WB) or immunoblot is a technique used to detect specific proteins in complex samples, for example, cells or tissue extract, or in samples of purified protein (130).

Firstly, proteins separated by SDS-PAGE were electrotransferred (specifically wet transferred) to a nitrocellulose membrane. A sandwich was assembled with the resolving gel containing separated proteins, one nitrocellulose membrane, two blotting papers and two sponges, and placed between two plastic supports (called cassette) as illustrated in Figure 15.

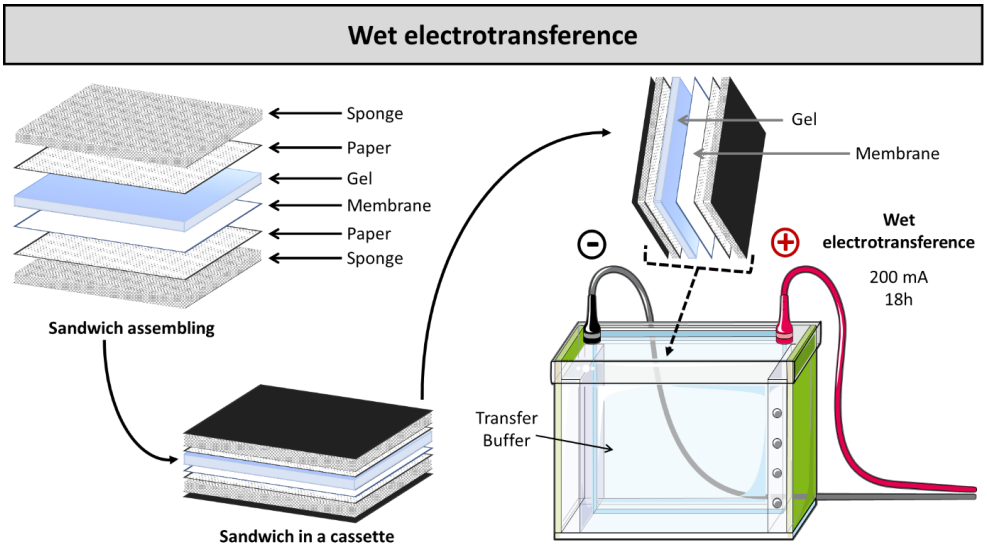

Figure 15: Wet electrotransference protocol. Adapted using images from Servier Medical Art by Servier.

After transference, all proteins are immobilized in the membrane, on the respective migration site in the gel. At this point membrane is ready for protein immunodetection. Before proceeding with the immunodetection protocol, transfer efficiency was evaluated. To do that, membranes were stained with Ponceau S, which is an easy, quick and reversible technique to visualize proteins transferred to membranes (130). Membranes were incubated with Ponceau S solution for 5 min, washed with deionized water (to remove the excess and to make bands visible) and photographed in ChemiDoc™ (BioRad). Membranes were then washed with TBS-T to remove Ponceau staining.

To immunodetect the target proteins, membranes need to be blocked to ensure that antibodies do not bind to non-specific binding sites. This was carried out by incubating membranes with 5% non-fat milk (NFM) in TBS-T for 3h. After, membranes were incubated with unlabelled primary antibodies, for different time periods (depending on primary antibody used; described in Table 5). Membranes were then washed three times with TBS-T (10 min each) and incubated with secondary antibodies, conjugated with horseradish peroxidase (HRP) or with IRDye© (depending on primary antibody used; described in Table 5) for 1h, with agitation, at RT. Before detection, membranes were washed again with TBS-T.

Table 5: Antibodies used to detect protein of interest.

Primary antibody				Secondary antibody		
Target protein	Molecular Weight (kDa)	Dilution	Brand	Target specie/ Type	Dilution	Brand
Tau	50	1:500	Invitrogen	Rabbit-HRP	1:10 000	Cell Signalling Technology
p-Tau S396	50	1:20 000	Abcam	Rabbit-HRP	1:10 000	
GSK3-β	46 (2 bands)	1:10 000	Abcam	Mouse-IR	1:10 000	LI-COR
p-GSK3-β S9	47 (2 bands)	1:1 000	Santa Cruz Biotechnology	Rabbit-HRP	1:5 000	Cell Signalling Technology
p-GSK3-β Y216	47 (2 bands)	1:1 000	BD Biosciences	Mouse-HRP	1:5 000	
PP1 α	37	1:2500	Affinity purified rabbit RU34 (131)	Rabbit-HRP	1:5 000	
PP2A	37	1:2 000	Millipore	Mouse-HRP	1:10 000	
CDK5	35	1:100	Santa Cruz Biotechnology	Mouse-HRP	1:5 000	

The detection (or revelation) of proteins in membranes incubated with HRP-linked secondary antibodies was done by chemiluminescence. In this reaction (illustrated on Figure 16), the HRP enzyme linked to secondary antibodies catalyzes the oxidation of luminol in presence of hydrogen peroxide, resulting in light emission that can be detected by film or sensitive camera. This light can also be enhanced by certain chemicals, in a reaction called enhanced chemiluminescence (ECL). The emitted light was detected using a ChemiDoc™ imaging system (BioRad). Two types of chemiluminescence solution were used, Luminata™ Crescendo (Millipore) or Amersham ECL Select™ (EG Healthcare Life Sciences). Quantification analysis was done using ImageLab™ software 6.0 (BioRad).

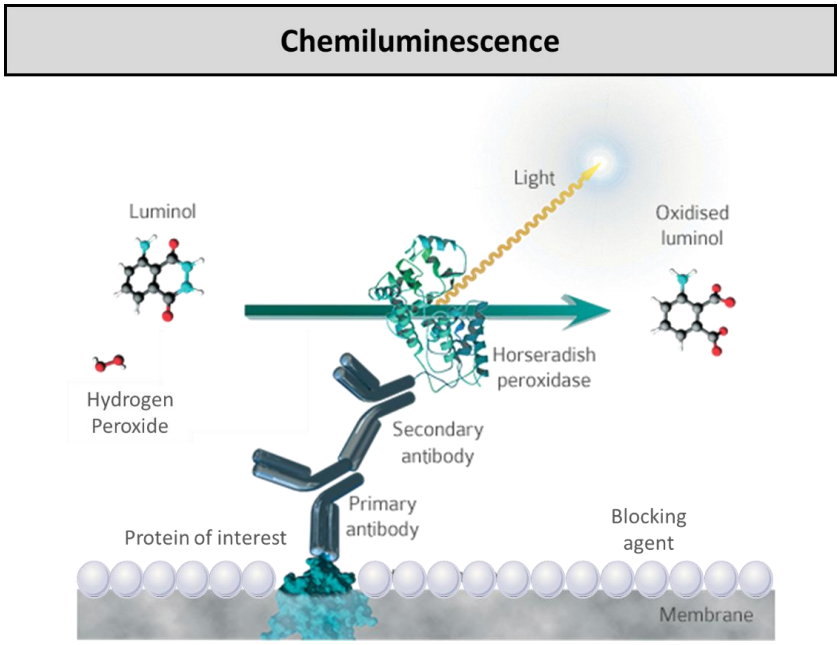

Figure 16: Chemiluminescence reaction on detection of HRP-linked antibodies. Adapter from (132).

For membranes incubated with IRDye® -linked secondary antibodies, proteins detection was carried out by fluorescence, a direct method of detection since no additional reagent is necessary for detection. In this method, fluorophores (conjugated to secondary antibodies) are subjected to excitation light, increasing their energy levels to an unstable excited state. As fluorophores cannot stay in this excited state, they return to their ground state, releasing emission light. This phenomenon is illustrated in Figure 17. The emitted light was detected using an Odyssey® 9120 imaging system (LI-COR). Quantification analysis was done using ImageJ 1.52a

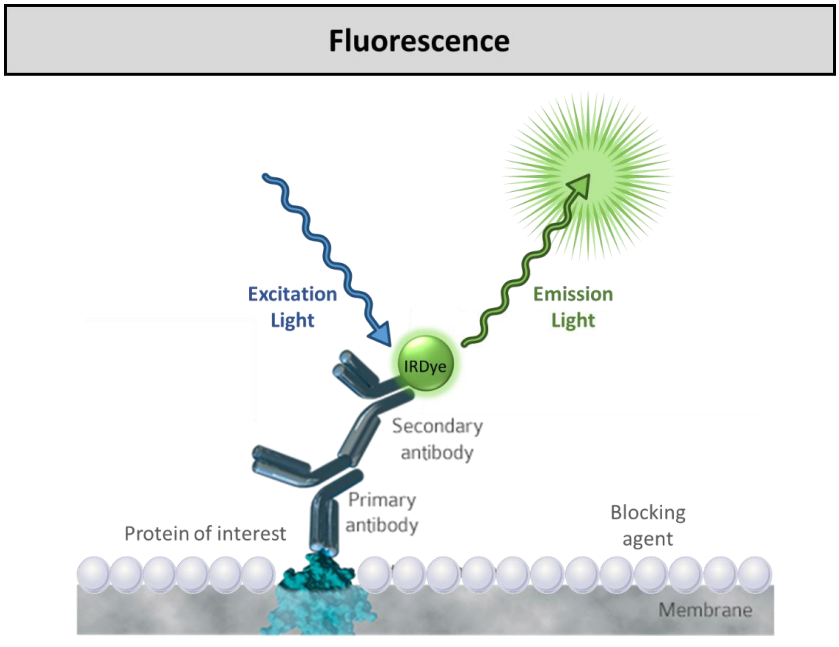

Figure 17: Fluorescence reaction on detection of HRP-linked antibodies. Adapter from (132).

After revelation, membranes could be dried and stored for future uses. However, when it was necessary to detect different proteins with similar molecular weight in the same membrane, primary and secondary antibodies were removed from membrane, on a process called stripping. To do that, a mild stripping protocol was employed. Membranes were incubated with mild stripping solution (pH 2.2), containing glycine, SDS and Tween 20, twice during 10 min. each. Membrane was washed with PBS (2 times for 10 min each) and then with TBS-T (2 times for 5 min each). After this procedure, membrane was ready to be reprobed.

3.3. Serine/threonine (Ser/Thr) phosphatase activity assay

SH-SY5Y were detached from an 80-90% confluent plate and plated as 1,2 x 10^6 cells into 66 mm diameter plates. Cells were differentiated (with the same protocol described in section 3.1.1) and then treated with IL-8 (75 µM or 100 µM) or MCP-1 (50 µM or 100 µM).

After 24h and 48h for IL-8 or 6h and 24h, for MCP-1, cells were washed and collected, in TBS using a cell scraper. After that, samples were centrifugated at 5000 g for 10 s and cells pellet were resuspended in lysis buffer and kept agitated for 30 min, at 4 °C, in a rotary agitator. Following this incubation period, samples were centrifuged at 13000 g for 10 min, at 4 °C, and transferred to new tubes. Finally, samples protein concentration was determined (using the previous method; section 3.2.2) and kept on ice until the assay.

To keep PP native structure and activity, a different lysis buffer was used in this assay since denaturing detergents (e.g. SDS) and PP inhibitors (e.g. NaF or NA_3VO_4) could not be used. The lysis buffer was composed by a NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 250mM NaCl, 1% NP-40) supplemented with phenylmethylsulfonyl fluoride (PMSF; to inhibit serine proteases) and Protease Inhibitor Cocktail.

For measuring serine/ threonine phosphatase activity, the RediPlate™ 96 EnzChek® serine/ threonine phosphatase (Ser/Thr PP) assay kit from Molecular Probes was used. This kit uses 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP), that is a specific PP substrate. DiFMUP, after being dephosphorylated generates DiFMU that exhibits excitation/emission maxima of 358/452 nm. Thus, PP activity can be quantified by measuring the DiFMU generated fluorescence. The kit also contains inhibitors to ensure that the assay is only specific to Ser/Thr PPs.

The assay was carried out accordingly to manufacture protocol. First, reaction buffer (RB) was prepared. There were used two different RB, one specific for measuring PP1 activity (that contained DTT and manganese (II) chloride or $MnCl_2$) and other for PP2A (that contained nickel (II) chloride or $NiCl_2$). RB was added to wells (of the 96-microplate provided by the manufacturer), followed by the addition of the freshly prepared samples (a total of 25 µg was added/well; each sample was measured in duplicate, for PP1 activity and for PP2A activity determination). The plate was incubated at 37°C and fluorescence was measured (excitation = 355 nm; emission = 460 nm) after 15 min and 45 min.

In this assay, a negative control was added, consisting of differentiated SH-SY5Y cells, without IL-8 or MCP-1 treatment, collected in the same lysis buffer but supplemented with NaF and Na_3VO_4 to inhibit PPs. The assay kit also contained fluorescent reference standards, allowing phosphate quantification, since the dephosphorylation of 1 M of DiFMUP releases 1 M of phosphate.

3.4. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.0.2 software. All data were expressed as means ± standard deviation (SD) of at least three independent experiments (n). Kruskal-Wallis tests with Dunn's pairwise comparisons were used (since data didn't follow a Normal distribution). $p<0.05$ was considered significant.

4 – Results

4. Results

4.1. Analysis of SH-SY5Y morphology after differentiation and chemokines treatment

The neuronal cell model chosen for all experiments was differentiated SH-SY5Y using RA treatment. Therefore, the efficiency of SH-SY5Y differentiation treatment was evaluated, through the analysis of cells morphological alteration. SH-SY5Y were photographed before (T0), after one (T24h) and two (T48h) days of differentiation treatment, with 1% FBS culture medium supplemented with 10 µM RA.

Morphological alterations were evident between differentiated cells and undifferentiated control (SH-SY5Y grown in 1% FBS culture medium only).

Initially, SH-SY5Y cells presented none or few neurites but after just 24h both control and treated cells displays neuritic grown, although these are more pronounced in differentiated cells. At the last stage of RA treatment (T48h) cells presented even long neurites, whereas control cells, that seem to had neurites with the same length as before (Figure 18). This fact occurs because it is expected that 1% FBS culture medium also induces some cell differentiation but at a slower rate than when this medium is supplemented with RA.

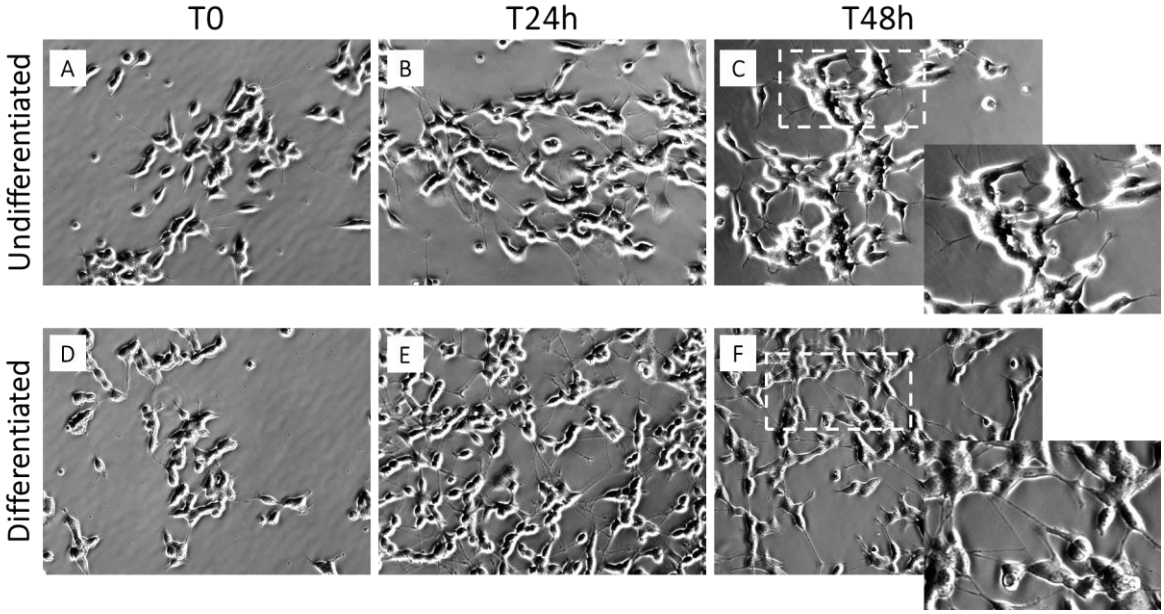

Figure 18: Morphological alteration of SH-SY5Y upon RA treatment. Untreated control cells and cell treated with RA; before treatment **(A; D)** and after 24h **(B; E)** and 48h **(C; F),** respectivly**.** In **C** and **F** is shown a zoomed image where is possible to visualise the differences between undifferentiated and differentiated cells' neurites. Ampliation 200x

A closer analysis at the T48h showed that RA-differentiated SH-SY5Y formed a larger neuritic network than undifferentiated (Figure 18 – C and F). Data supports that RA treatment during 48h is a suitable model to induce SH-SY5Y differentiation. This is also supported by previous results done by the group that showed increased expression of the differentiation marker MAP2 and βIII-tubulin under these conditions (133).

SH-SY5Y morphology was also evaluated after IL-8 and MCP-1 treatment. No differences were observed, even for the highest concentration and longer incubation period (Figure 19).

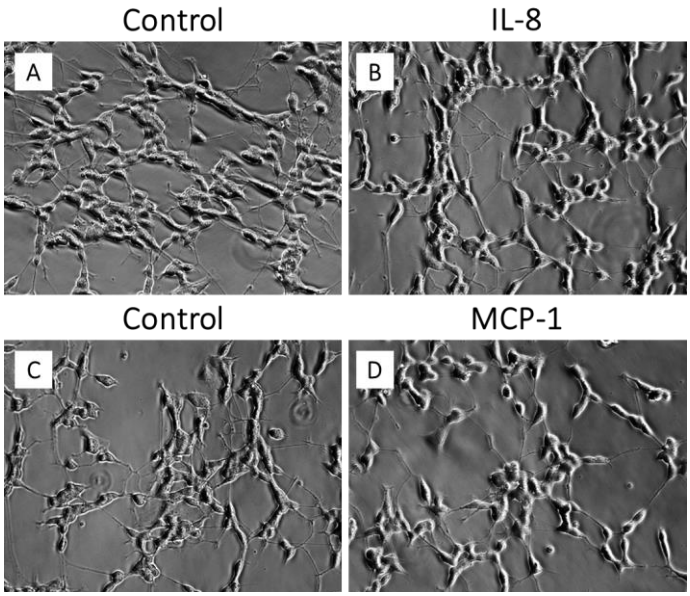

Figure 19: Morphologic analysis of SH-SY5Y upon chemokines treatment. SH-SY5Y were treated with 100 ng/mL IL-8 (B) or with MCP-1 (D) for 48h and 24h, respectively. Controls for both incubation periods (48h and 24h) are also displayed (A; C). Ampliation 200x

4.2. Evaluation of IL-8 and MCP-1 impact on tau phosphorylation

Abnormal tau phosphorylation is a key event in AD pathogenesis. Preliminary results done by the group suggested that chemokines can impact tau phosphorylation. Hence, in order to further investigate this effect, a neuronal cell model, differentiated SH-SY5Y was exposed to IL-8 and MCP-1 at several concentrations and for different time periods (IL-8: 25, 50, 75 and 100 ng/mL for 6, 24 or 48h; MCP-1: 10, 50 or 100 ng/mL for 3, 6 or 24h).

After treatments, cells were collected and protein levels of total tau (T-tau) and phosphorylated tau at Ser396 (ptau S396) residue was accessed by WB. This particular residue was addressed because of previous work developed by the group (121) but also because this is one AD pathological relevant residue (78,134,135).

As show in Figure 20 IL-8 treatment for 6h and 24h didn't affect total tau levels. However, after 48h a significant increase could be detected at 75 and 100 ng/mL when compared with control.

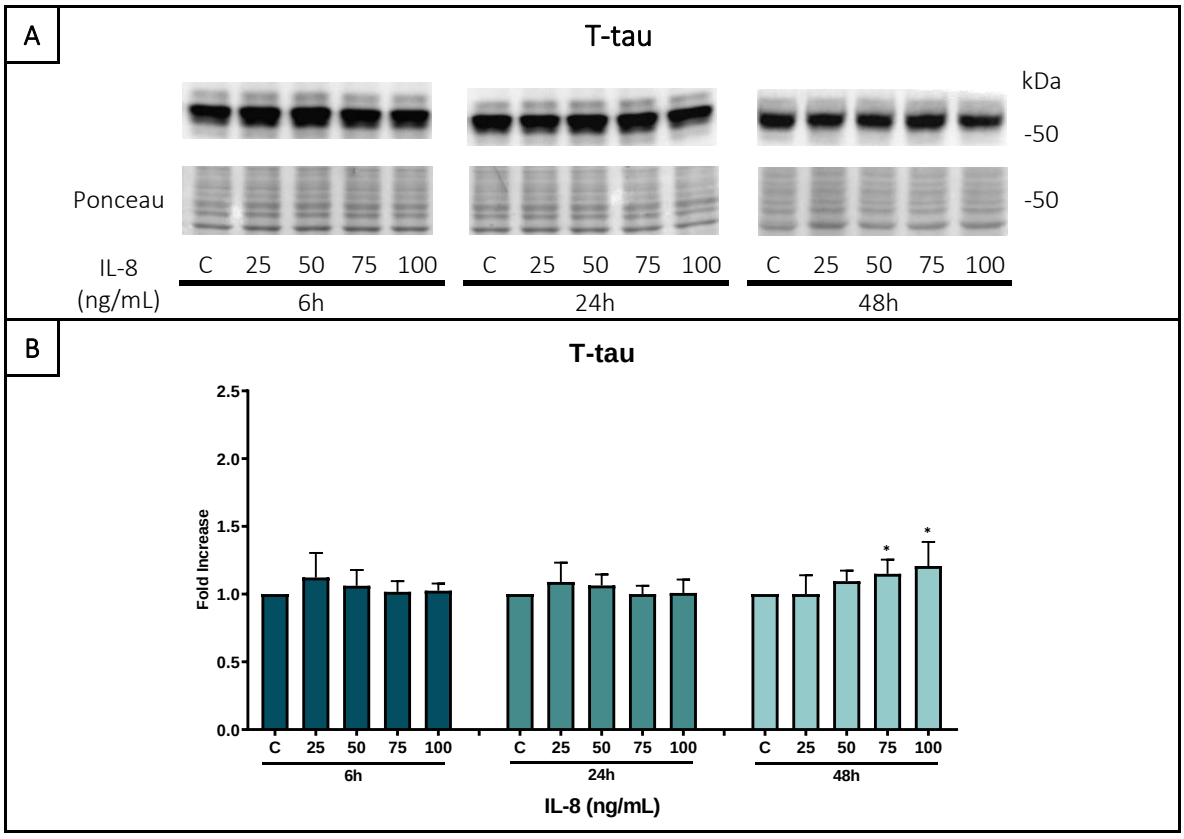

Figure 20: tau levels after IL-8 treatment. (A) Levels of total tau levels on differentiated SH-SY5Y treated with IL-8. Ponceau S was used to normalise values. **(B)** Means of total tau levels ± SD. * $p<0.05$ by Dunn's test. $n \geq 3$.

Regarding tau phosphorylation at S396 residue (Figure 21 – B), a tendency to an increase in response to IL-8 longer treatments was observed. Precisely, the levels of ptau S396 were significantly increased at 24h and 48h for almost all IL-8 concentrations.

Additionally, ratio ptau S396/T-tau was calculated, since there were significant differences on total tau level after IL-8 exposer. pTau S396 significant differences were maintained for 100 ng/mL treatment at 24h and 48h periods (Figure 21 – C).

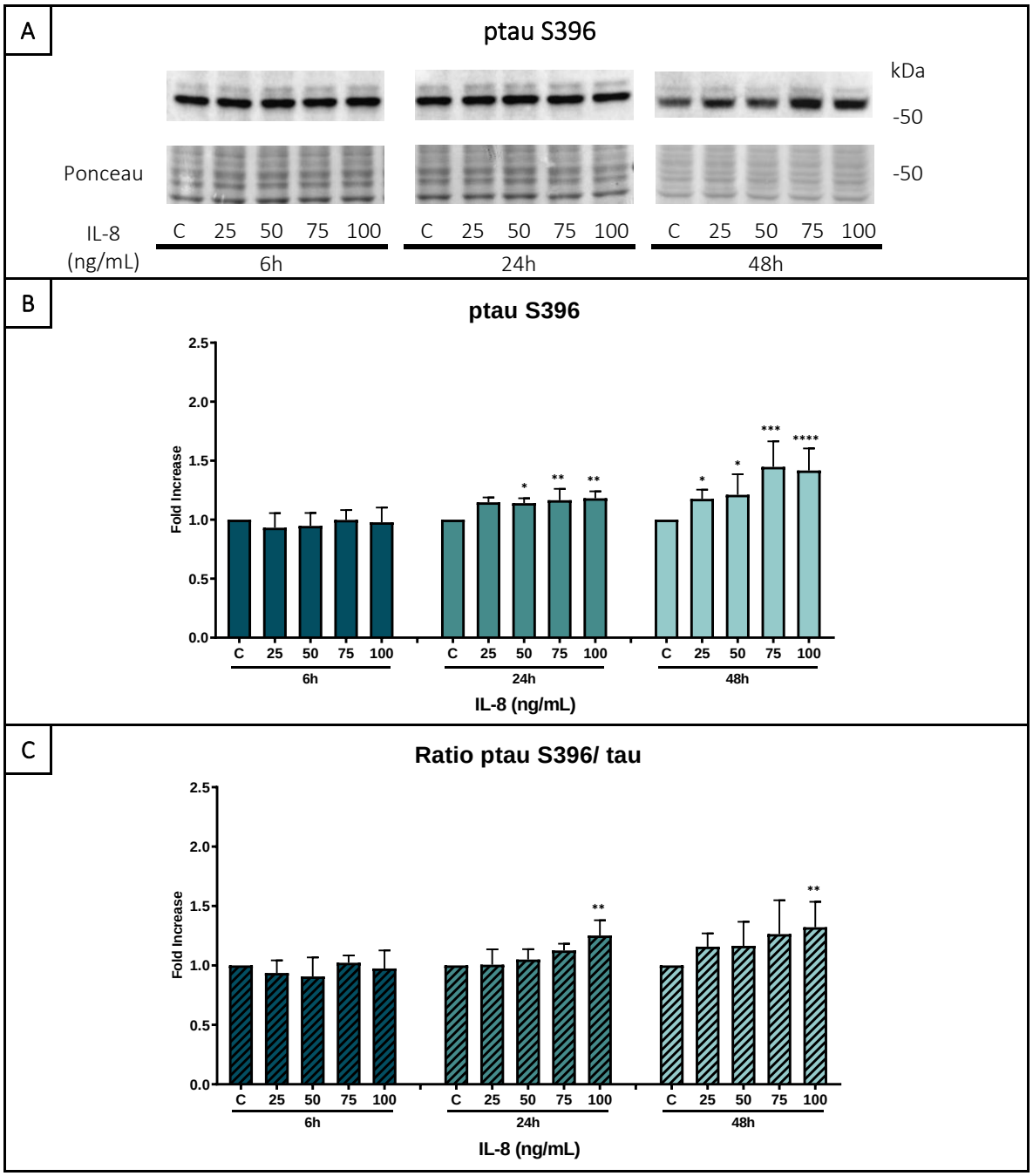

Figure 21: ptau S396 levels after IL-8 treatment. (A) ptau at S396 residue levels on differentiated SH-SY5Y treated with IL-8. Ponceau S was used to normalise values. **(B)** Means of ptau S396 levels ± SD. **(C)** Ratio of ptau S396/ T-tau levels ± SD. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 by Dunn's test. n ≥ 3.

MCP-1 exposure did not significantly change total tau levels (Figure 22), at least for the incubation periods tested.

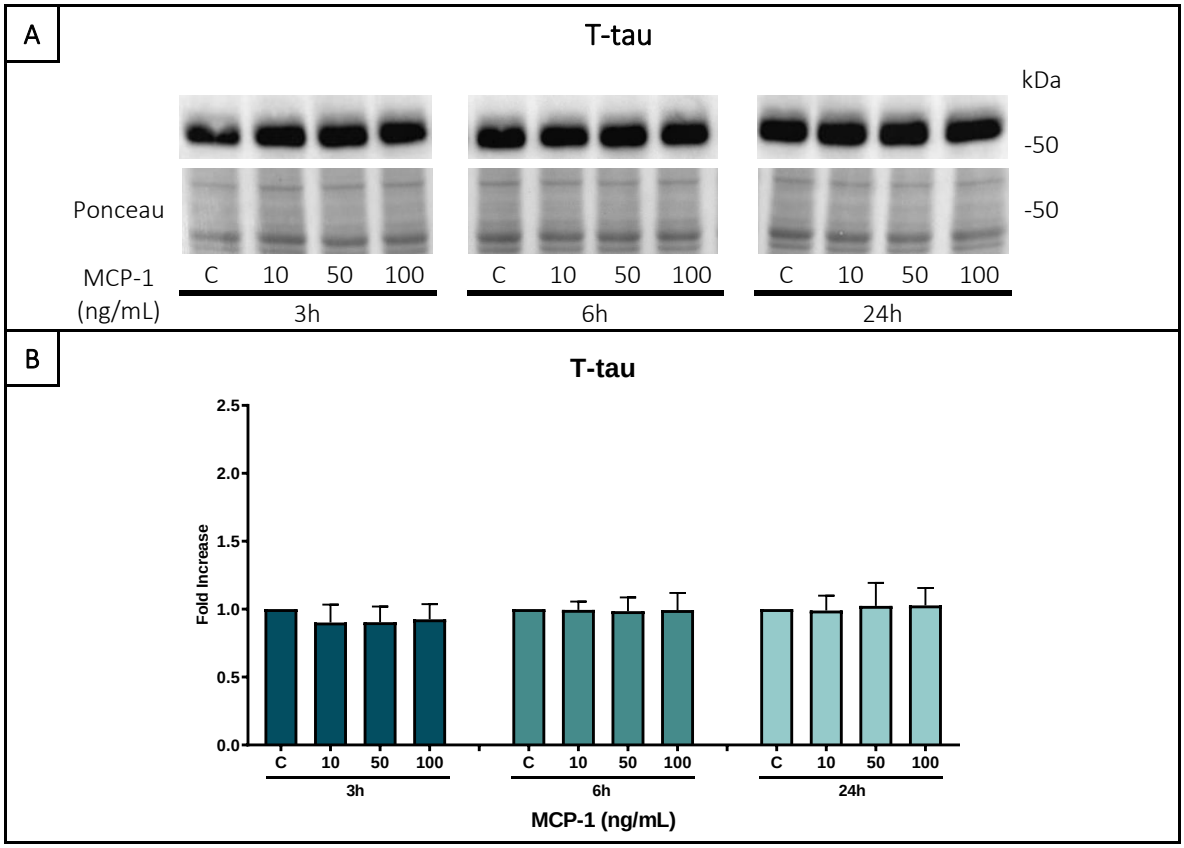

Figure 22: tau levels after MCP-1 treatment. **(A)** Total tau levels on differentiated SH-SY5Y treated with MCP-1. Ponceau S was used to normalise values. **(B)** Means of total tau levels ± SD. n ≥ 3.

Similar to what was observed for IL-8, tau phosphorylation at ptau S396 residue was found significantly increased for the higher concentrations, 50 and 100 ng/mL, upon 6h and 24h treatment (Figure 23 – B). Additionally, the ratio between ptau S396 and T-tau levels was significantly increased at highest concentration (100 ng/ mL) for the longer incubation periods (Figure 23 – C).

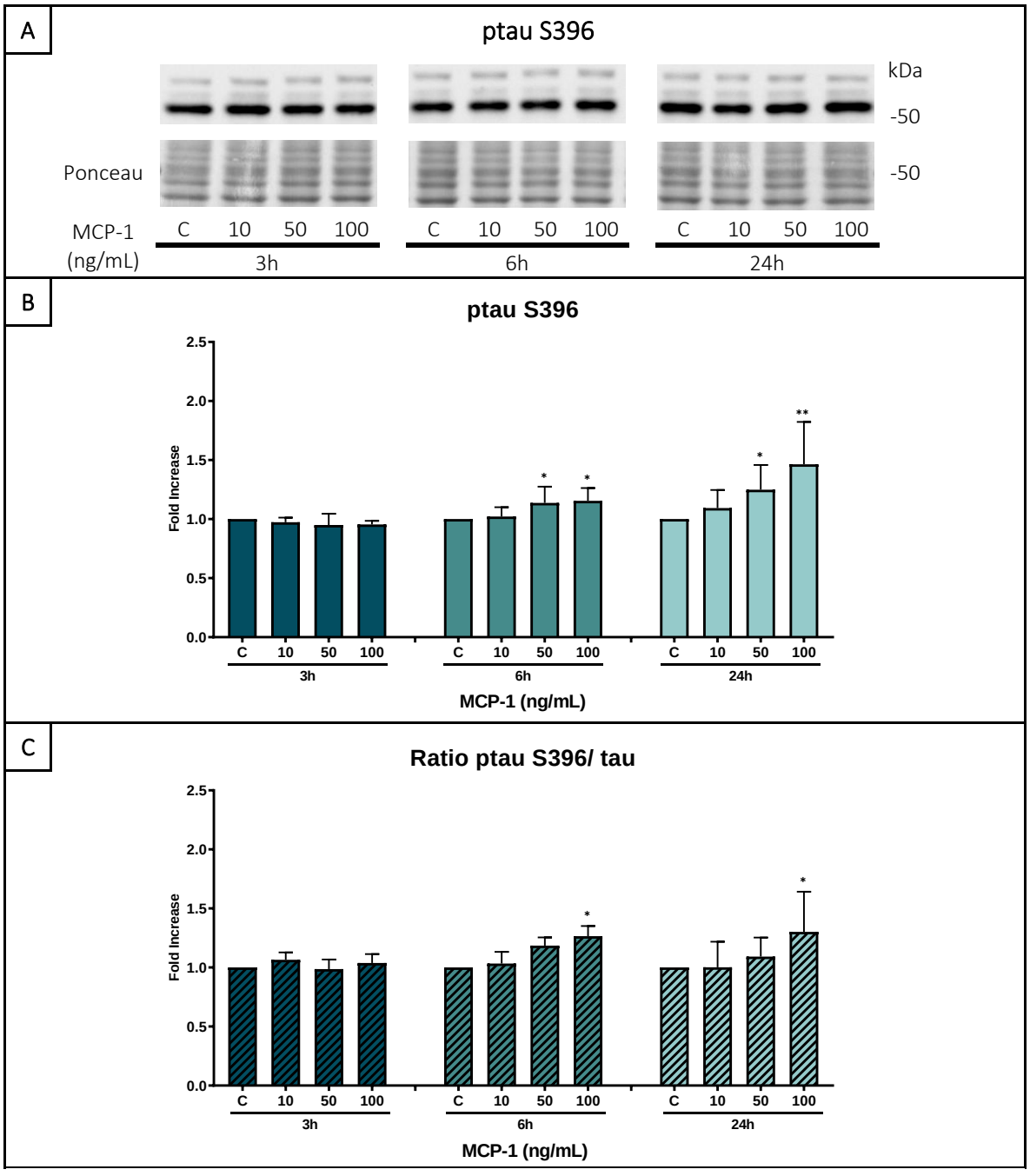

Figure 23: ptau S396 levels after MCP-1 treatment. (A) ptau at S396 residue levels on differentiated SH-SY5Y treated with MCP-1 Ponceau S was used to normalise values. **(B)** Means of ptau S396 levels ± SD. **(C)** Ratio of ptau S396/ T-tau levels ± SD. *p<0.05; **p<0.01 by Dunn's test. n ≥ 3.

Taken together, these results suggest that both chemokines had a substantial impact on tau phosphorylation, in particular at the ptau S396 residue.

A significant increase in T-tau levels was also observed at 48h for IL-8 treatment. However, when the ratio between phosphorylated and total tau was calculated, significant differences were maintained for for the highest concentration and longer incubation periods. Therefore, this result supports that IL-8 still had an effect on tau phosphorylation.

4.3. Analysis of kinases levels and activity upon chemokines exposure

Considering that phosphorylated tau levels were significantly increased with chemokines treatment, identifying the putative kinase proteins involved in this process was the next objective.

As stated before, tau can be phosphorylated by several kinase proteins, being GSK3β and CDK5 the most relevant in AD pathology. Therefore, GSK3β levels and activity were analysed by assessing total GSK3β, phosphorylated GSK3β on serine 9 residue (pGSK3β S9) and phosphorylated GSK3β on tyrosine 216 residue (pGSK3β Y216) levels. GSK3β phosphorylation at S9 residue causes kinase inactivation whereas phosphorylation at Y216 residue its necessary of maximal kinase activity.

Total GSK3β levels were not significantly altered with response to IL-8 treatment (Figure 24).

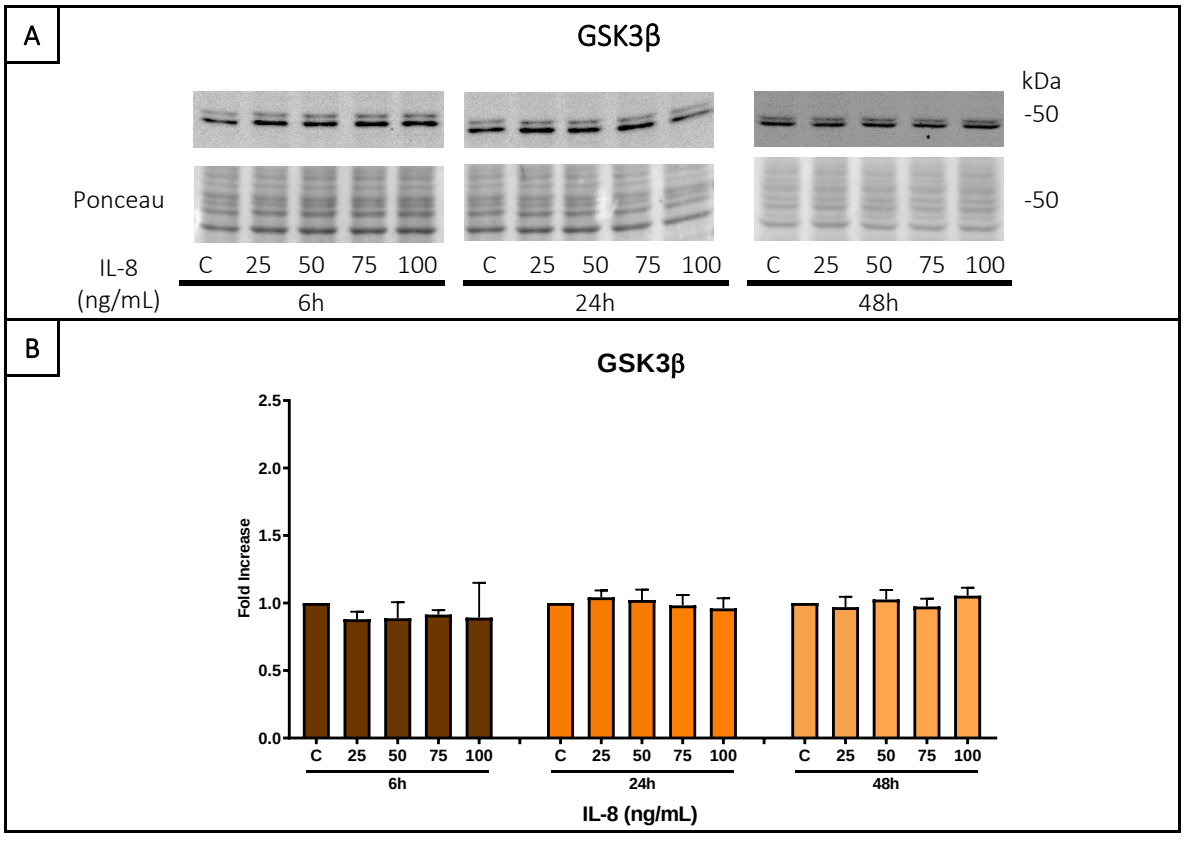

Figure 24 GSK3β levels after IL-8 treatment. (A) Total GSK3β levels on differentiated SH-SY5Y treated with IL-8. Ponceau S was used to normalise values. **(B)** Means of GSK3β levels ± SD. n ≥ 3.

Regarding phosphorylation of GSK3β at S9 (pGSK3β S9), which correlates with its inactivation state, a general tendency to increase was observed for shortest and longest incubation periods, with significant differences at 6h for 100 ng/mL. However, IL-8 treatment for 24h lead to a tendency of decreasing levels of pGSK3β (Figure 25).

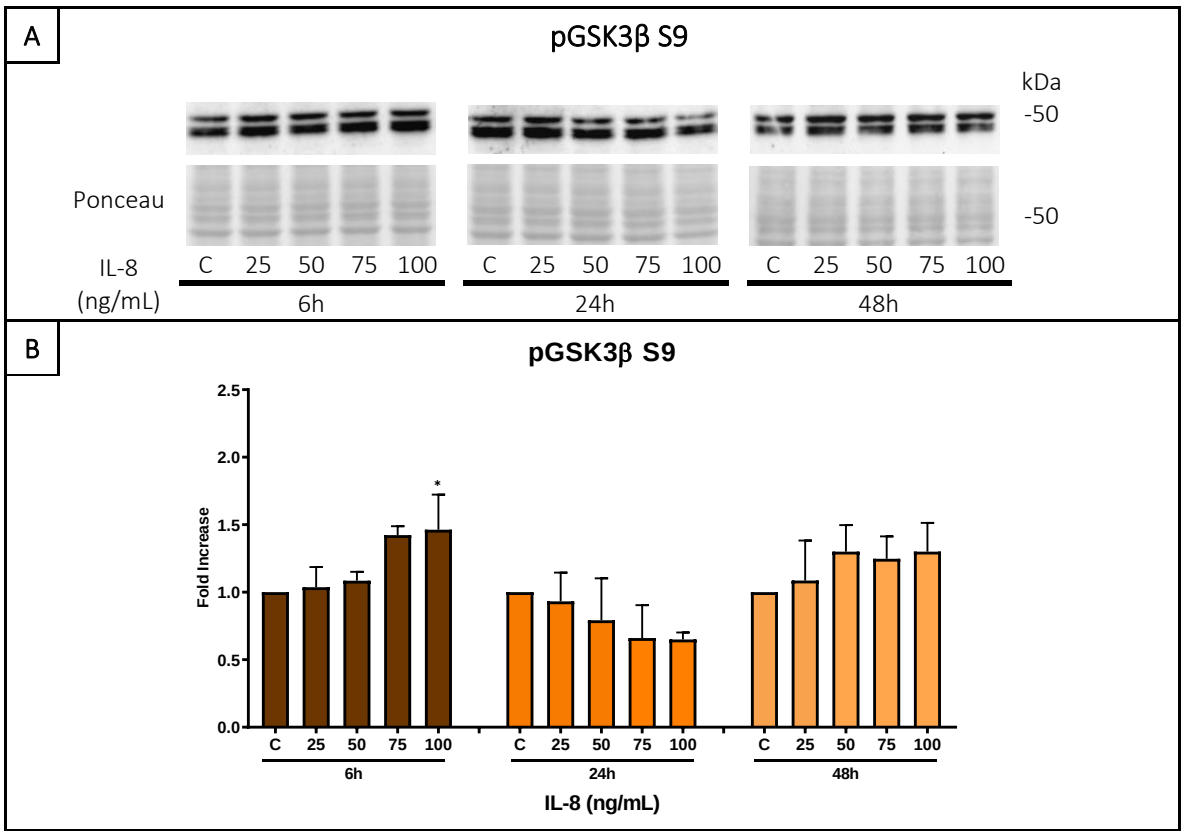

Figure 25: pGSK3β S9 levels after IL-8 treatment. (A) pGSK3β at S9 residue levels on differentiated SH-SY5Y treated with IL-8. Ponceau S was used to normalise values. **(B)** Means of pGSK3β S9 levels ± SD. n ≥ 3.

Relatively to GSK3β phosphorylation at Y216 residue, which correlates with kinase activation state, significant decrease levels were observed at 6h for 75 ng/mL treatment. Also a small tendency to increase were detected at some IL-8 concentrations, for the 24h and 48h periods (Figure 26).

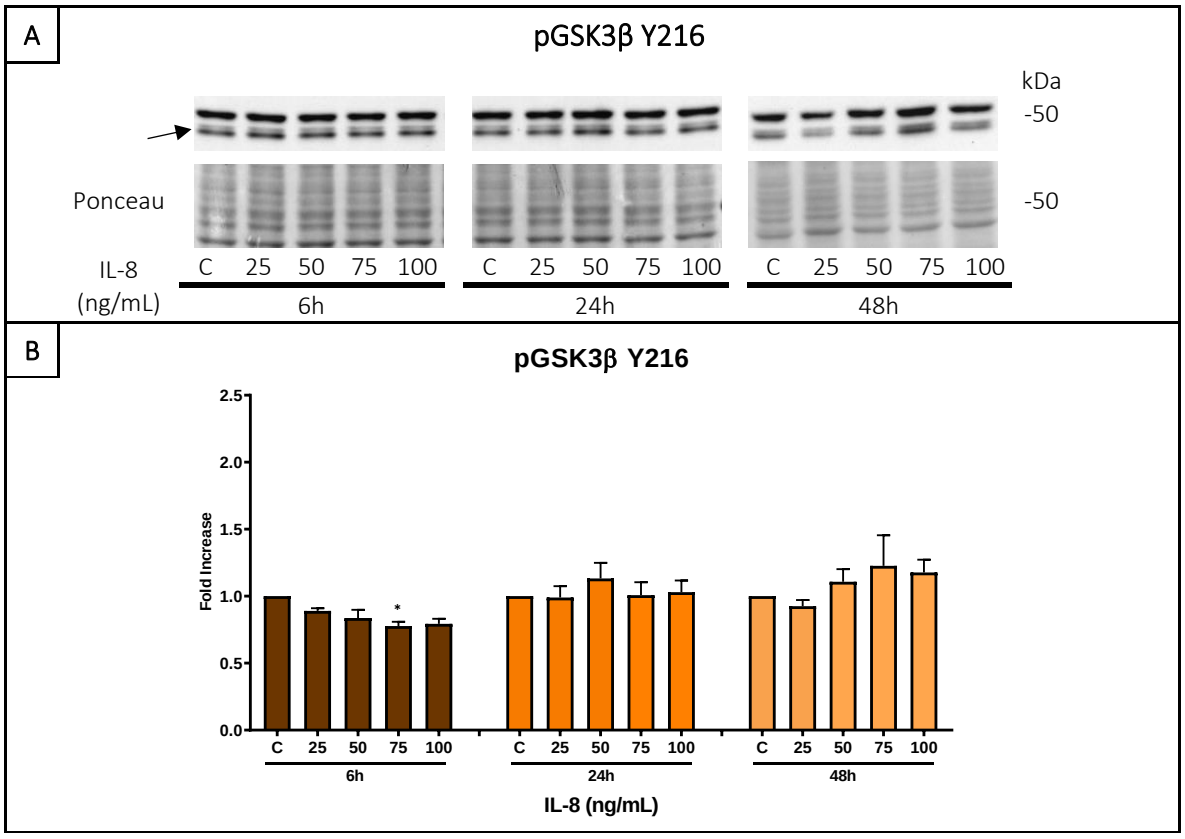

Figure 26: pGSK3β Y216 levels after IL-8 treatment. (A) pGSK3β at Y216 residue levels on differentiated SH-SY5Y treated with IL-8. pGSK3β Y216 band are indicated by the arrow. Ponceau S was used to normalise values. **(B)** Means of pGSK3β Y216 levels ± SD. n ≥ 3.

To enable the analysis of GSK3β results, a graph with means of all explored GSK3β forms are presented in Figure 27.

Taken together, these results indicate that IL-8 induced an inhibition of GSK3β at the lowest incubation period (6h), since in this timepoint pGSK3β S9 levels were increased whereas pGSK3β Y216 were decreased. For 24h treatment, a small tendency to decreased inhibition was observed although results were not statistically significant, and no differences were detected for pGSK3β Y216 levels. For 48h treatment none significant differences were observed.

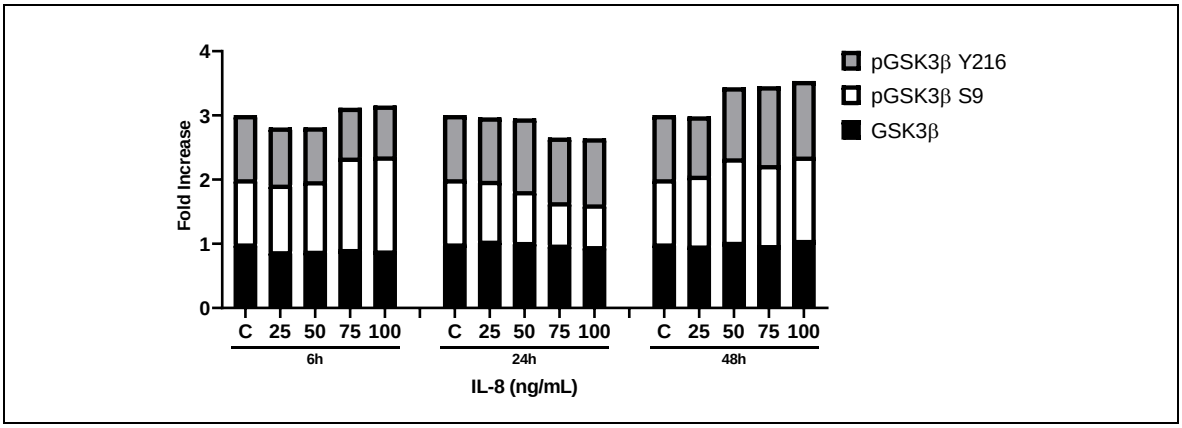

Figure 27: GSK3β total and phosphorylated levels after IL-8 treatment. Means levels of GSK3β (black), pGSK3β S9 (white) and pGSK3β Y216 (grey) on differentiated SH-SY5Y treated with IL-8. n ≥ 3.

MCP-1 treatment did not alter total GSK3β level (Figure 28), similarly to what was observed for IL-8.

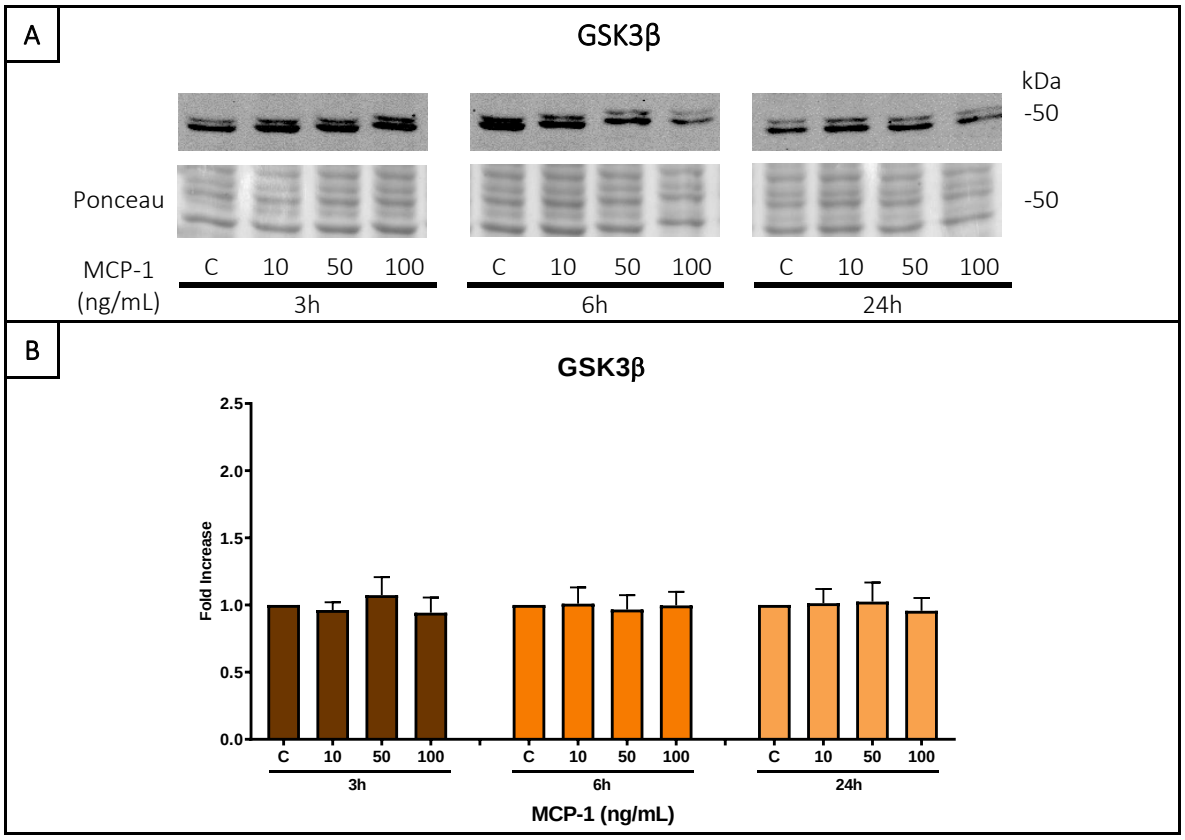

Figure 28: GSK3β levels after MCP-1 treatment. (A) Total GSK3β levels on differentiated SH-SY5Y treated with MCP-1. Ponceau S was used to normalise values. **(B)** Means of GSK3β levels ± SD. n ≥ 3.

Regarding phosphorylation of GSK3β at S9 residue, a tendency to increase was observed after 3h treatment with MCP-1, with significant differences at highest concentration. For 6h and 24h periods no substantial differences were observed (Figure 29).

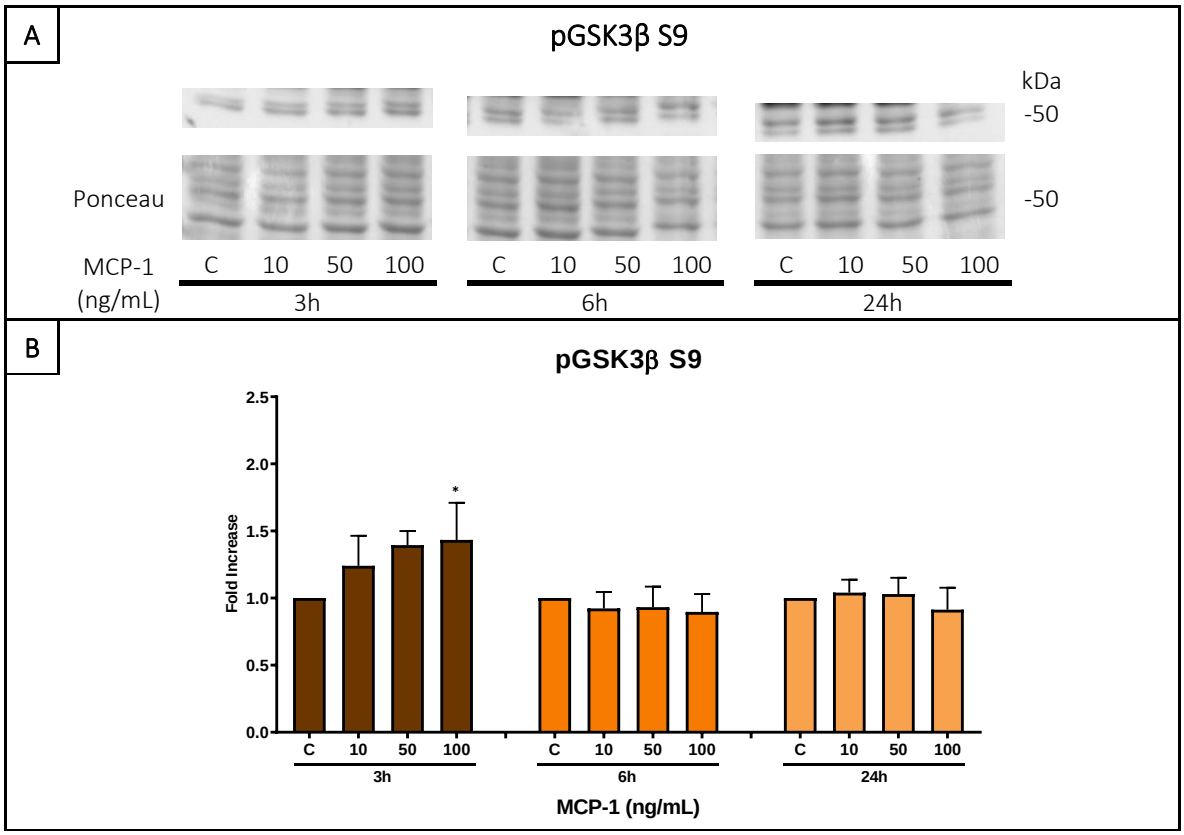

Figure 29: pGSK3β S9 levels after MCP-1 treatment. (A) pGSK3β at S9 residue levels on differentiated SH-SY5Y treated with MCP-1. Ponceau S was used to normalise values. **(B)** Means of pGSK3β S9 levels ± SD. *p<0.05 by Dunn's test. n ≥ 3.

Relatively to pGSK3β Y216 levels (Figure 30) an opposite tendency, comparatively to pGSK3β S9, was observed, with a significant decrease obtained for MCP-1 exposure with 50 and 100 ng/mL concentrations, at the 3h treatment. A tendency for increase was also observed at 6h for MCP-1 higher concentrations although not significant.

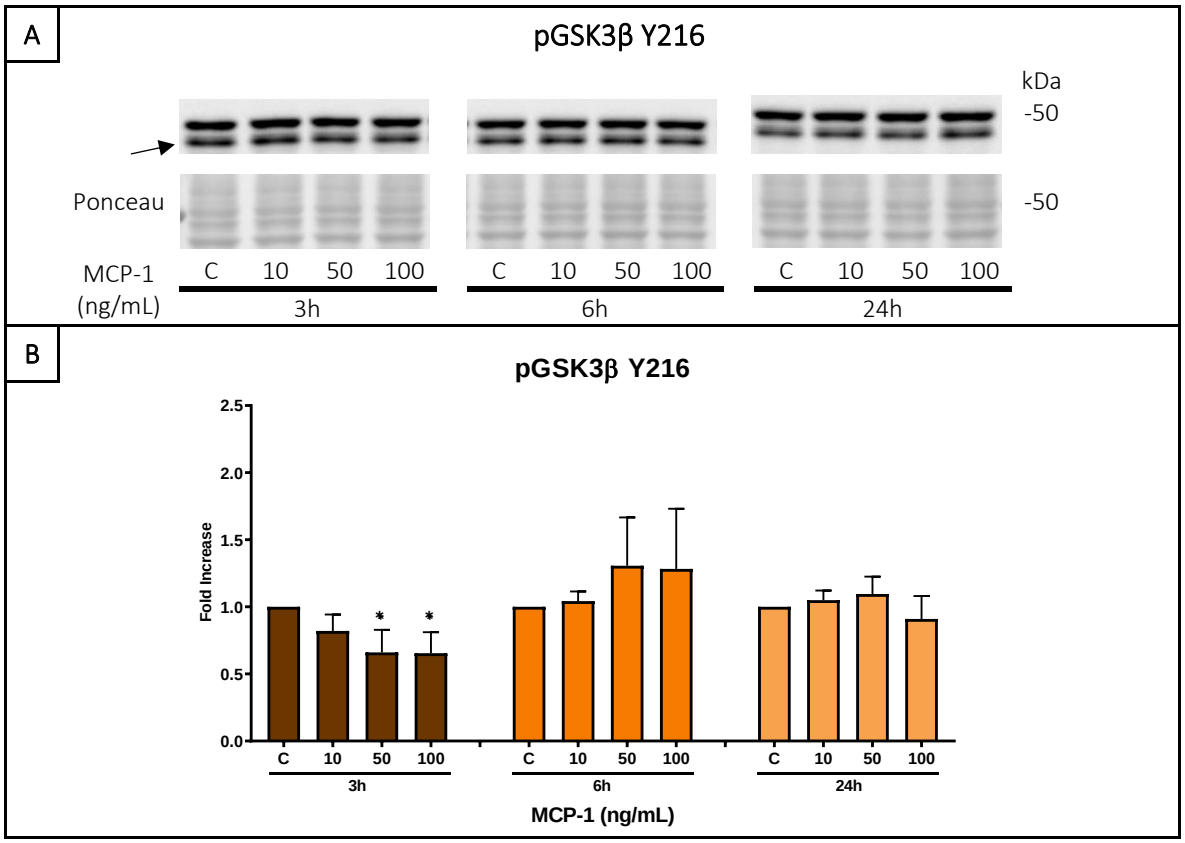

Figure 30: pGSK3β Y216 levels after MCP-1 treatment. (A) pGSK3β at Y216 residue levels on differentiated SH-SY5Y treated with MCP-1. pGSK3β Y216 band are indicated by the arrow Ponceau S was used to normalise values. **(B)** Means of pGSK3β Y216 levels ± SD. *p<0.05 by Dunn's test. n ≥ 3.

All results regarding GSK3β levels after MCP-1 exposer are resumed at Figure 31. The increased levels of pGSK3β S9 and decreased levels of pGSK3β Y216 at 3h incubation leads to the conclusion that this kinase is inhibit by MCP-1 at this time period. For 6h and 24h treatments no differences where observed.

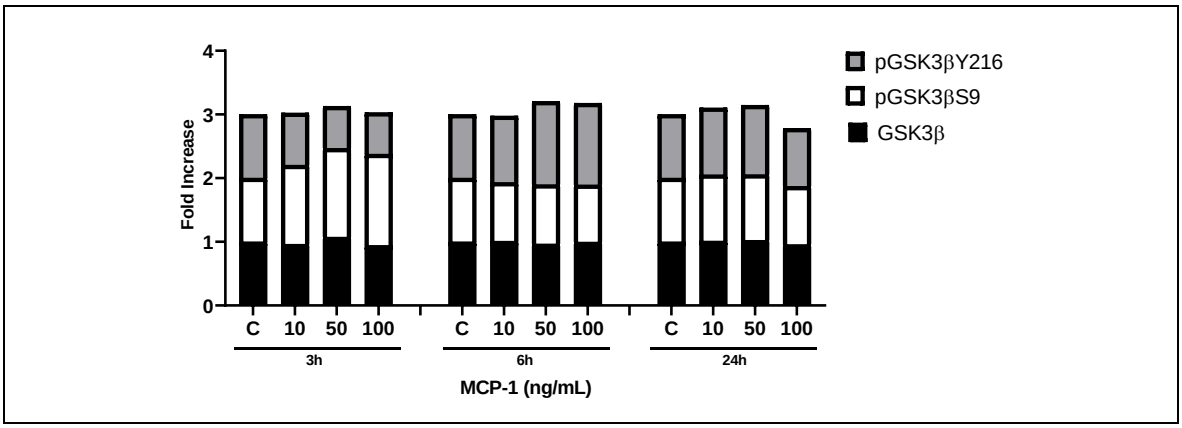

Figure 31: GSK3β total and phosphorylated levels after MCP-1 treatment. Means levels of GSK3β (black), pGSK3β S9 (white) and pGSK3β Y216 (grey) on differentiated SH-SY5Y treated with MCP-1. n ≥ 3.

Altogether, data suggest that GSK3β is not the kinase involved in tau phosphorylation at S396 observed upon IL-8 or MCP-1 treatment since GSK3β activity seemed to not be augmented after incubation for 24h or 48h. Indeed, for both chemokines, at the short incubation a decrease on GSK3β activity could be observed.

Therefore, chemokines effect on CDK5, another kinase involved in tau phosphorylation, was evaluated.

IL-8 treatment significantly decreased CDK5 levels at the lowest incubation period (6h). For the remaining periods no significant differences or tendencies where found (Figure 32).

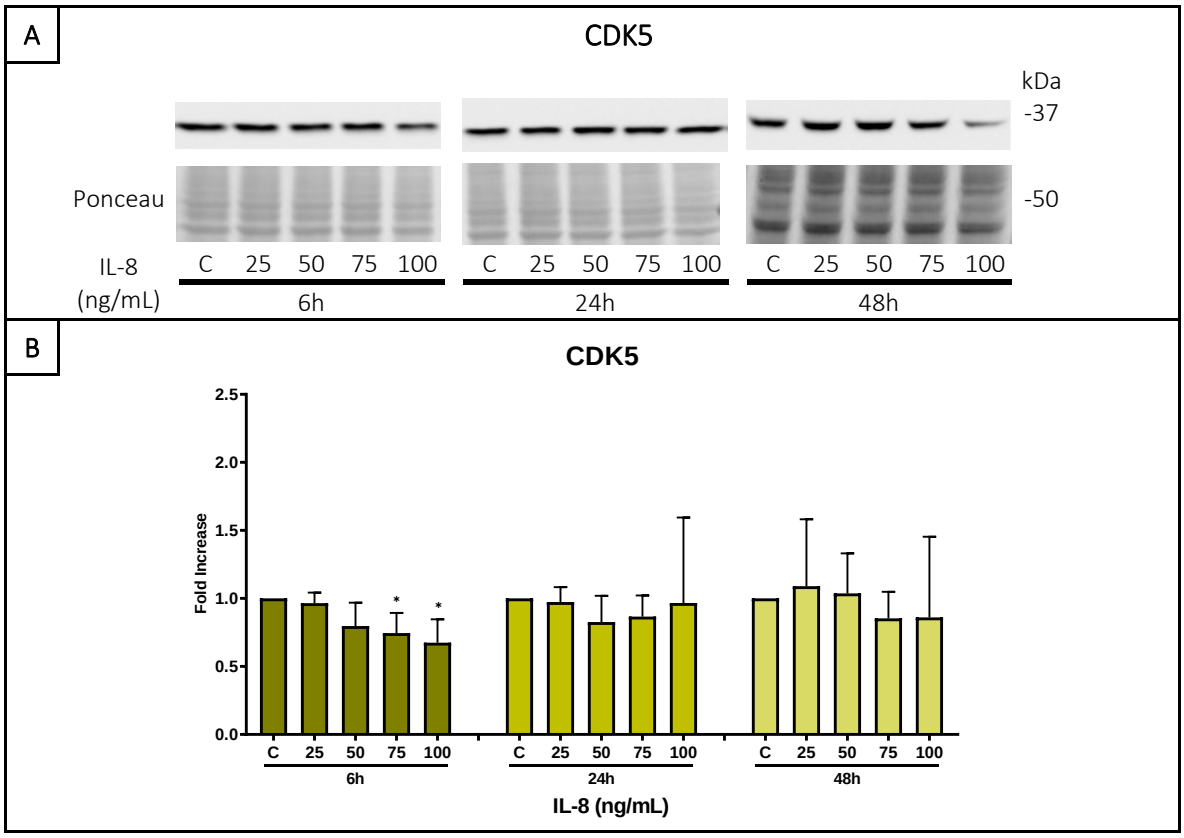

Figure 32: CDK5 levels on differentiated SH-SY5Y treated with IL.8. (A) CDK5 levels on differentiated SH-SY5Y treated with IL-8. Ponceau S was used to normalise values. **(B)** Means of CDK5 levels ± SD. *p<0.05 by Dunn's test. n ≥ 3.

Relatively to MCP-1 treatment, a tendency to increase was observed at 3h and CDK5 levels were significantly increased at 24h for the higher concentrations (50 and 100 ng/mL) (Figure 33).

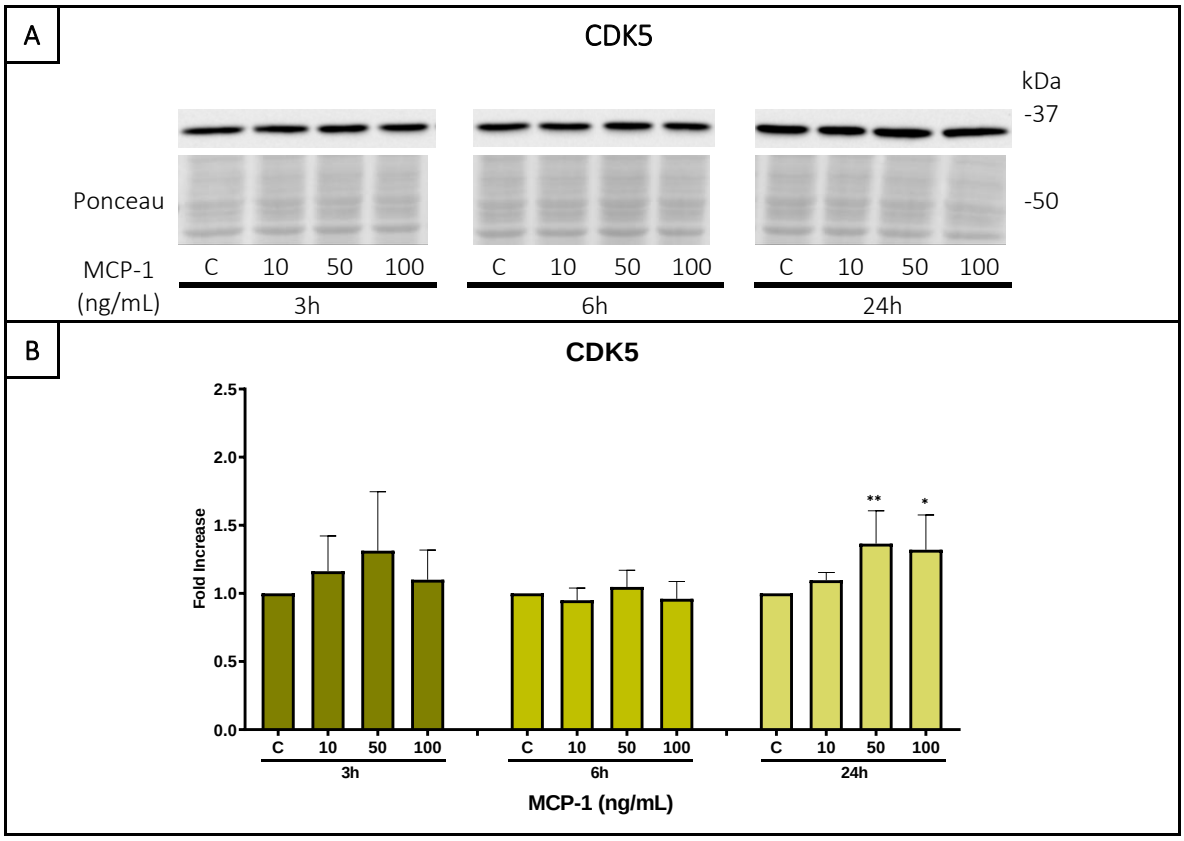

Figure 33: CDK5 levels on differentiated SH-SY5Y treated with MCP-1. (A) CDK5 levels on differentiated SH-SY5Y treated with MCP-1. Ponceau S was used to normalise values. **(B)** Means of CDK5 levels ± SD. *p<0.05; **p<0.01 by Dunn's test. n ≥ 3.

Both results suggest that chemokines have an effect on CDK5 levels, whereas IL-8 decreases it levels at shorter term exposer and MCP-1 longer treatment leads to increased levels. Although this increase can be correlated with ptau S396 increased levels upon MCP-1 treatment, further testes regarding CDK5 activity (e.g. monitoring p35 or p25 fragments) should be conducted to determine if this is the kinase responsible of tau increased phosphorylation caused by MCP-1 treatment.

4.4. Determination of IL-8 and MCP-1 influence on PP levels and activity

PPs play a key role on tau phosphorylation in AD since, while kinases add phosphate groups to tau, PP remove it and thus PP inactivation can contribute to underbalanced protein phosphorylation events. Therefore, as chemokines may mediate tau phosphorylation via PP inhibition, the levels and activities of PP2A and PP1 were assessed. PP1 and PP2A are the two major human brain enzymes involve in tau dephosphorylation.

Some variations were detected for PP1 levels after IL-8 exposure for 6h and 24h however with no clear tendency. At 48h incubation period, a substantial tendency for increase was observed, although not statistically significant (Figure 34).

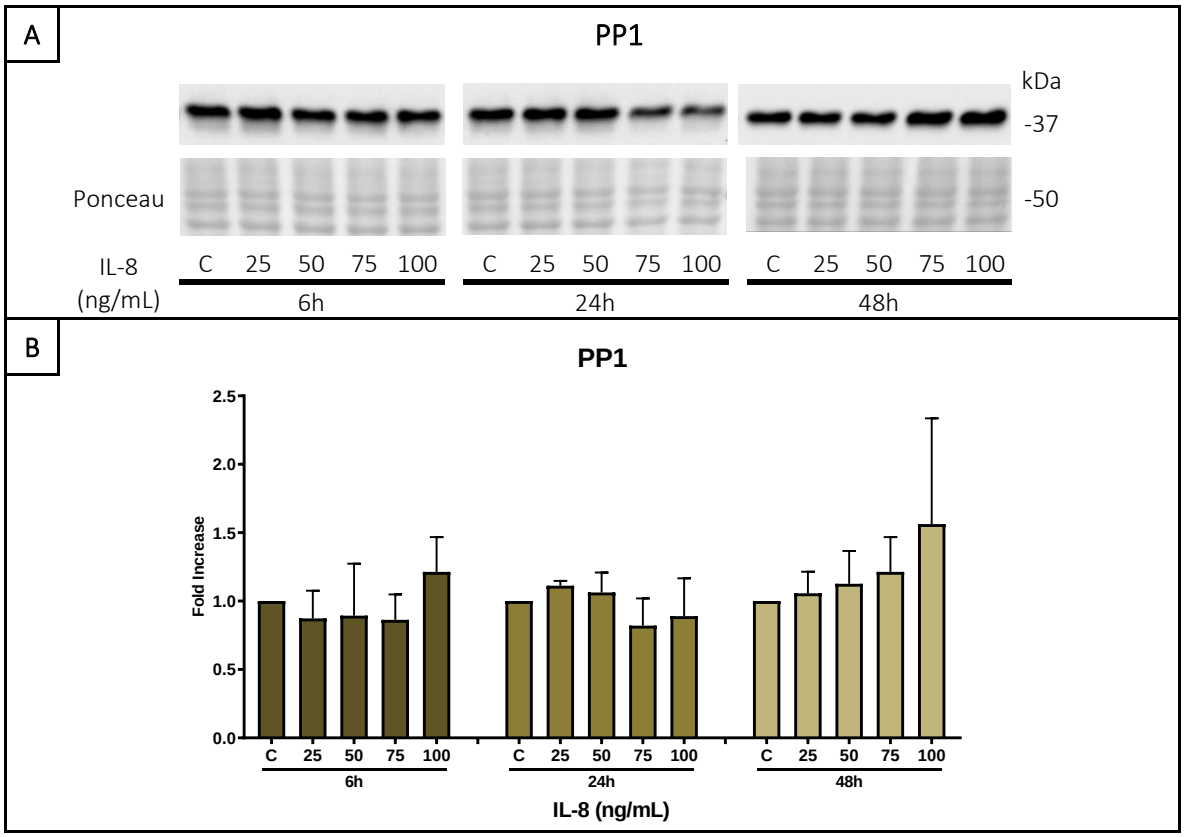

Figure 34: PP1 levels after IL-8 treatment. (A) PP1 levels on differentiated SH-SY5Y treated with IL-8. Ponceau S was used to normalise values. **(B)** Means of PP1 levels ± SD, normalise for control. n ≥ 3

For MCP-1 treatment, small tendency for increase in PP1 levels was observed at 6h (Figure 35).

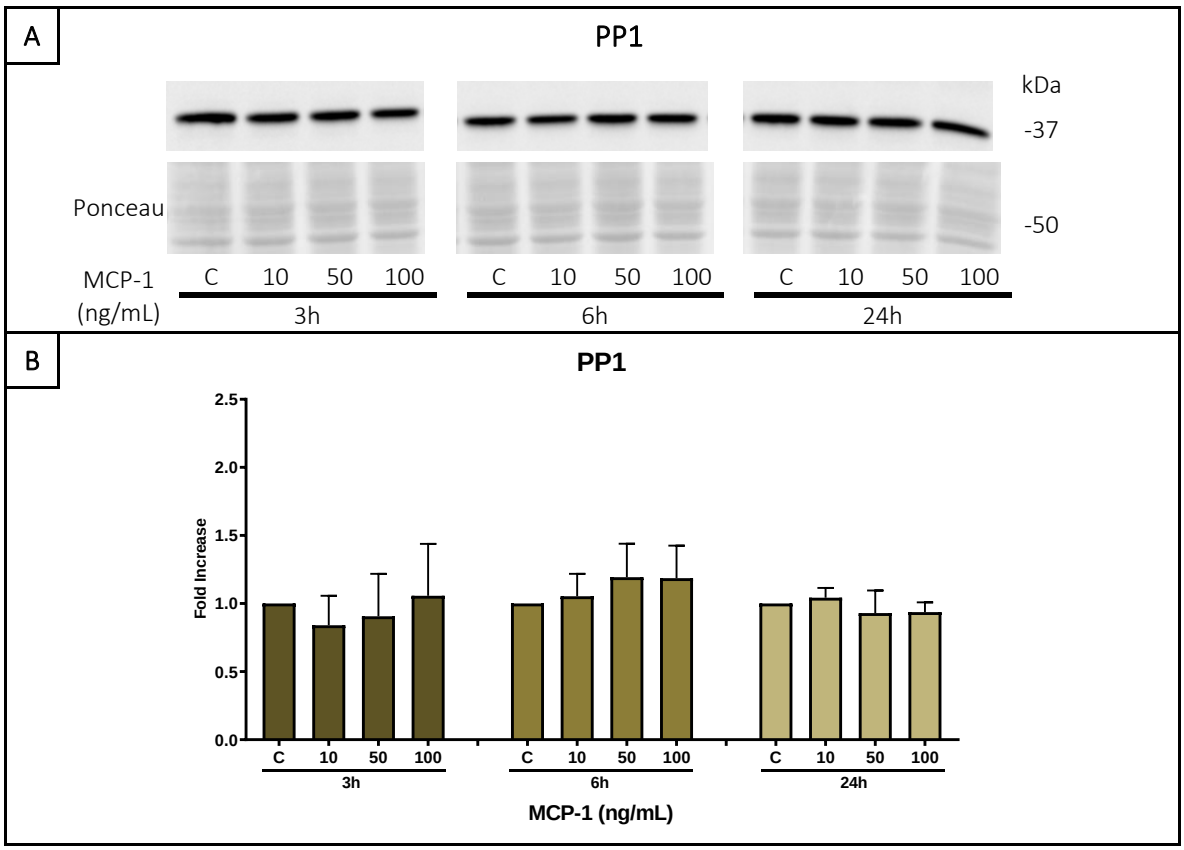

Figure 35: PP1 levels after MCP-1 treatment. (A) PP1 levels on differentiated SH-SY5Y treated with MCP-1. Ponceau S was used to normalise values. **(B)** Means of PP1 levels ± SD, normalise for control. n ≥ 3

Regarding PP2A, upon chemokines treatment its levels were stable and no significant differences were observed for IL-8 or MCP-1 treatment (Figure 36 and Figure 37).

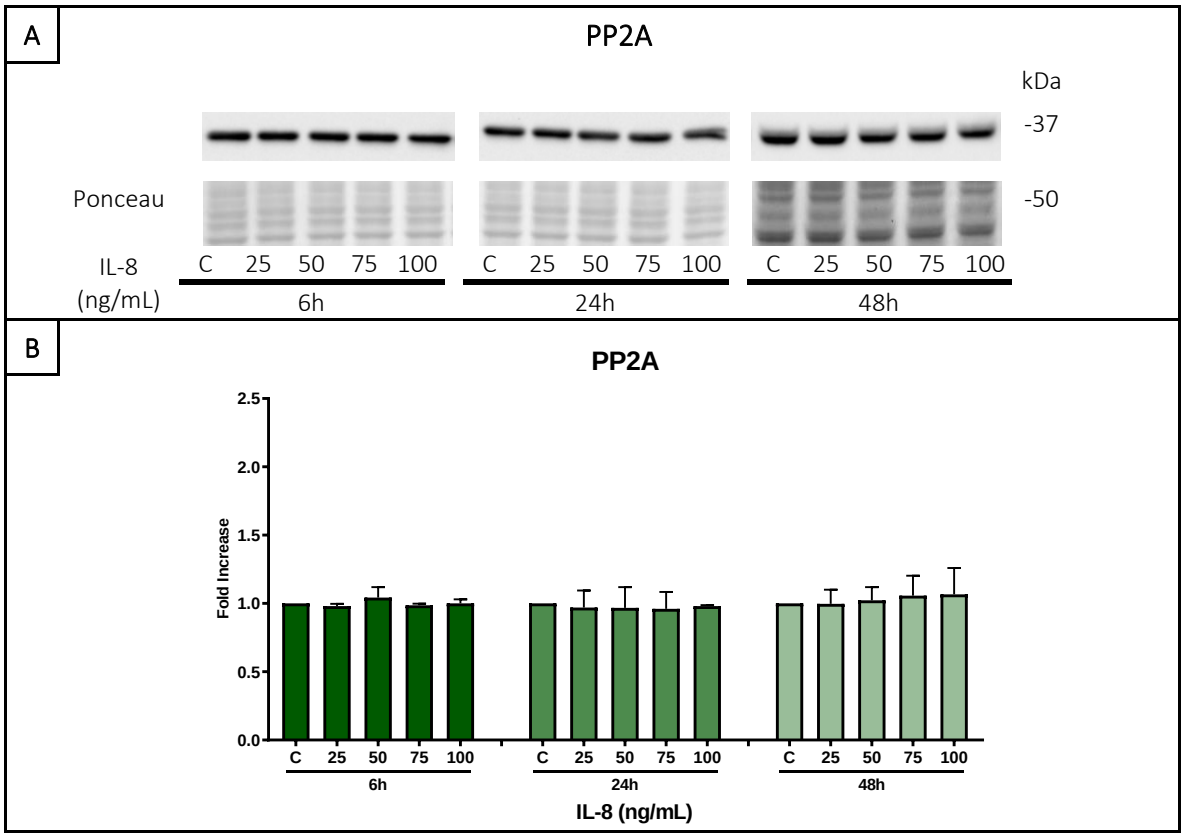

Figure 36: PP2A levels after IL-8 treatment. (A) PP2A levels on differentiated SH-SY5Y treated with IL-8.Ponceau S was used to normalise values. **(B)** Means of PP2A levels ± SD, normalise for control. n ≥ 3

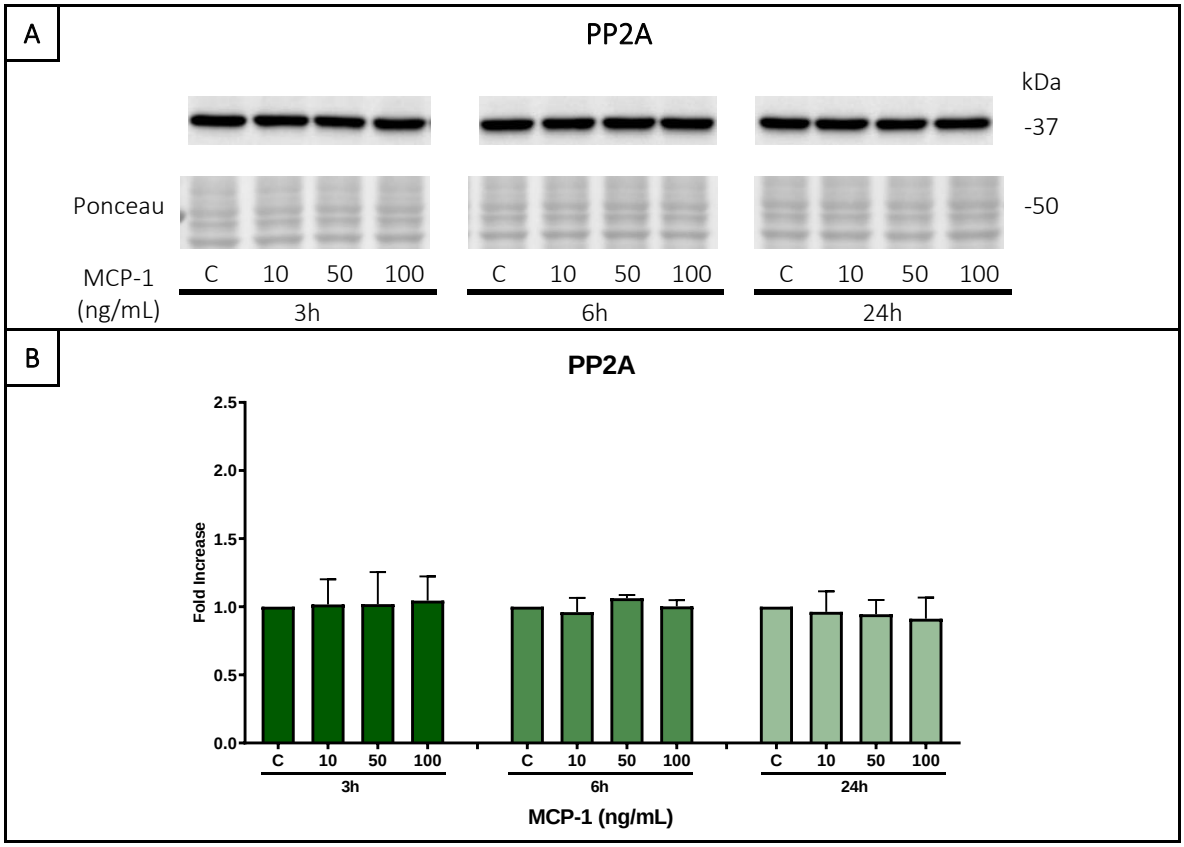

Figure 37: PP2A levels after MCP-1 treatment. (A) PP2A levels on differentiated SH-SY5Y treated with MCP-1. Ponceau S was used to normalise values. **(B)** Means of PP2A levels ± SD, normalise for control. n ≥ 3

Therefore, both chemokines affected only PP1 levels, whereas PP2A levels were not altered. IL-8 longest exposer increased PP1 levels and its levels after MCP-1 treatment for 24h also increased, but in a small variation.

Finally, protein phosphatase activities were measured for both PP2A and PP1, using a Ser/ Thr PP assay kit, in differentiated SH-SY5Y treated with chemokines. IL-8 and MCP-1 concentrations and incubation periods selected were those were ptau at S396 residue showed a higher increment (75 and 100 or 50 and 100 ng/ mL for 24h and 48h or 6h and 24h; respectively). Under these experimental conditions, both PP2A and PP1 activities changed.

For IL-8, both PP2A and PP1 activities decreased, with a more pronounced variation for PP1 (Figure 38).

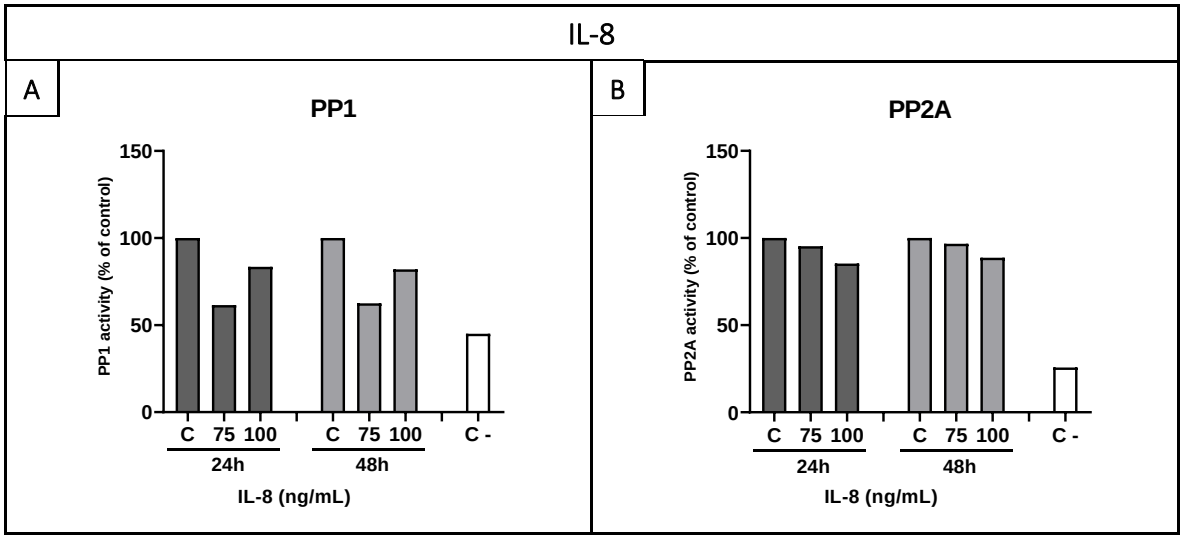

Figure 38: PP1 and PP2A activity after IL-8 treatment. PP1 **(A)** and PP2A **(B)** mean activity in differentiated SH-SY5Y treated with IL-8, measured as pM of phosphate (PO_4) per minute per µg of protein, between 15 and 45 min of incubation. n=2

For MCP-1, a tendency for decrease was also observed, being more evident after exposure for 24h. (Figure 39).

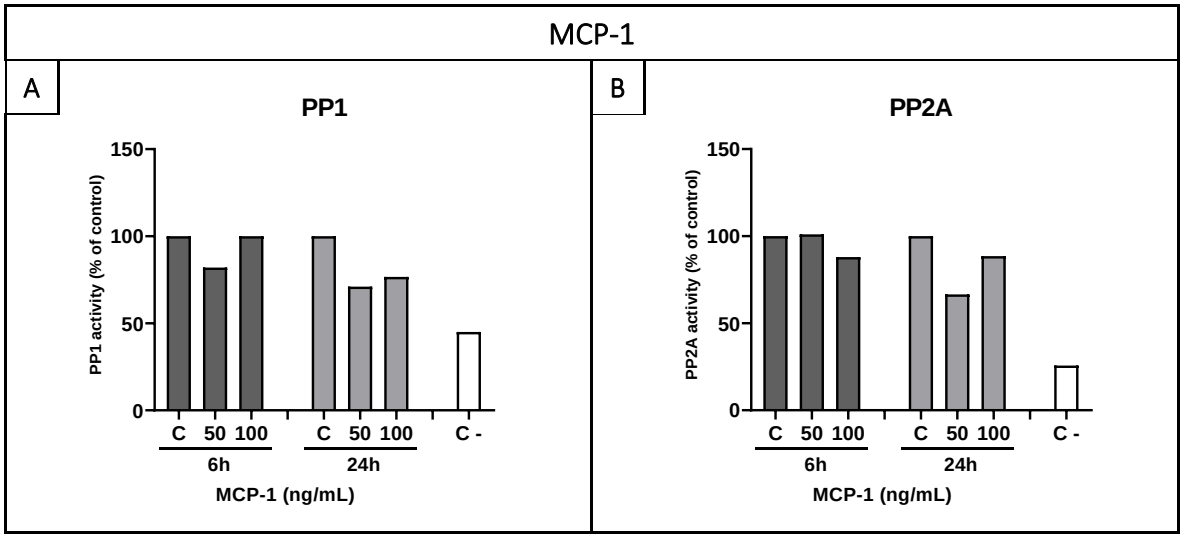

Figure 39: PP1 and PP2A activity after MCP-1 treatment. PP1 **(A)** and PP2A **(B)** mean activity in differentiated SH-SY5Y treated with MCP-1, measured as pM of PO_4 / min / µg of protein, between 15 and 45 min of incubation. n=2

Although only preliminary results, this results suggest that chemokines can impact PP1 and PP2A activity, by inhibiting these PPs. This could mean that IL-8 and MCP-1 increases tau phosphorylation by inhibiting PP. However, more experiments should be done to confirm this theory.

Finally, all results are resumed in Table 6 and Table 7.

Table 6: Resume of data obtained for IL-8 exposer. ⇡ or ⇣ tendency to increased or decreased levels or activity; ↑ or ↓ significantly increase or decrease levels or activity; **=** no difference in levels or activity.

	IL-8											
	6h				24h				48h			
	25 ng/mL	50 ng/mL	75 ng/mL	100 ng/mL	25 ng/mL	50 ng/mL	75 ng/mL	100 ng/mL	25 ng/mL	50 ng/mL	75 ng/mL	100 ng/mL
ptau S396	=	=	=	=	⇡	↑	↑	↑	↑	↑	↑↑	↑↑
ptau S396 ratio	=	=	=	=	=	=	⇡	↑	⇡	⇡	⇡	↑
GSK3β	=	=	=	=	=	=	=	=	=	=	=	=
pGSK3β S9	=	=	↑	↑↑	⇣	⇣	⇣	⇣	↑	↑	↑	↑
pGSK3β Y216	⇣	⇣	↓	⇣	=	↑	=	=	=	=	↑	↑
CDK5	=	⇣	↓	↓	=	⇣	⇣	=	=	=	⇣	⇣
PP1	⇣	⇣	⇣	⇡	=	=	⇣	⇣	=	⇡	⇡	⇡
PP2A	=	=	=	=	=	=	=	=	=	=	=	=
PP1 Activity							⇣	⇣			⇣	⇣
PP2A Activity							=	⇣			=	⇣

Table 7: Resume of data obtained for MCP-1 exposer. ⇡ or ⇣ tendency to increased or decreased levels or activity; ↑ or ↓ significantly increase or decrease levels or activity; = no difference in levels or activity.

	MCP-1								
	3h			6h			24h		
	10 ng/mL	50 ng/mL	100 ng/mL	10 ng/mL	50 ng/mL	100 ng/mL	10 ng/mL	50 ng/mL	100 ng/mL
ptau S396	=	=	=	=	↑	↑	⇡	↑	↑↑
ptau S396/ tau	=	=	=	=	⇡	↑	=	⇡	↑↑
GSK3β	=	=	=	=	=	=	=	=	=
pGSK3β S9	⇡	⇡	↑	=	=	=	=	=	⇣
pGSK3β Y216	⇣	↓	↓	=	⇡	⇡	=	=	⇣
CDK5	⇡	⇡	⇡	=	=	=	=	↑	↑
PP1	⇣	=	=	=	⇡	⇡	=	=	=
PP2A	=	=	=	=	=	=	=	=	=
PP1 Activity					⇣	=		⇣	⇣
PP2A Activity					=	⇣		⇣	⇣

5 – Discussion

5. Discussion

AD is the most prevalent neurodegenerative disorder worldwide. Clinically, this pathology is characterized by impaired memory, cognition and behaviour and its pathogenesis is characterized, at a macroscopic level, by progressive loss of brain tissue and at a microscopic level, by the extracellular presence of SP and the occurrence of NFT inside neurons. SP results from the aggregation of Aβ peptides, produced on amyloidogenic processing of APP whereas NFT are aggregates of hyperphosphorylated tau (13).

Although the molecular mechanism underling AD etiology are not fully understood, inflammation seems to play a key role (90). This is sustained by the presence of immune response mediator molecules, cytokines, found in high levels on blood, CSF or brain of AD patients (102–111). Among cytokines, chemokines are one of the main mediators of inflammation and they seem to play a dual role on neuroinflammation (99). While the influence of several chemokines on AD pathogenesis, related with Aβ deposition and APP processing, has been more extensively evaluated, the impact of these molecules on tau phosphorylation and aggregation has been less explored (93,113).

Therefore, this wok aimed to understand the effect of chemokines, namely IL-8 and MCP-1, on AD pathogenesis, in particular focussing on tau phosphorylation and on the proteins that could be involved in the mechanism behind this effect.

5.1. IL-8 and MCP-1 increased tau phosphorylation

IL-8 or CXCL8, is produced by microglia and astrocytes. Although this chemokine was found increased in AD patients' blood, CSF and brain (105,106,108), its role on disease pathology has not been extendedly study. The few data available associated IL-8 with increased Aβ production (119,120).

MCP-1 or CCL2 are also produced by microglia and astrocytes and contrarily to IL-8, its role on AD pathogenesis has been extendedly studied. This chemokine was described as having a protective role on AD pathogenesis since it was associated with Aβ clearance (116–118). However, conflicting data were also reported (114,115). So, it seems that MCP-1 may have a dual role on AD pathogenesis.

To date, there are no information regarding the direct influence of both chemokines on tau phosphorylation in an AD pathological context. Only few studies have tried to correlate IL-8 or MCP-1 and ptau levels.

Bettcher et al associated high levels of IL-8 with high levels of ptau T181 on plasma and CSF samples of asymptomatic and positive family history AD individuals (136). However, this correlation only highlights the potential use of chemokines as AD biomarkers, when combined with ptau high levels.

For MCP-1, the depletion of its receptor, CCR2, lead to increased levels of ptau. Nevertheless, these results were obtained on a traumatic brain injury animal model and therefore cannot be translated to AD pathological context.

Therefore, in this work, the phosphorylation of tau at S396 was assessed on a neuronal cell model (differentiated SH-SY5Y) after IL8 or MCP-1 treatment. ptau S396 phosphorylation is one of the residues known to be altered in AD cases (78,134,135). Tau phosphorylation at S396–S404 sites has been considered one of the earliest events in AD pathogenesis (137), also present in brains of patients who suffered from mild cognitive impairment (75). Additionally, phosphorylation at these sites may contribute to filaments assembling process and to NFT formation (138).

IL-8 and MCP-1 exposure significantly augmented ptau levels at S396 residue, for the higher chemokines' concentration and incubation periods. This data suggests that these chemokines, in a chronic neuroinflammation scenario, can affect tau phosphorylation and potential contribute to NFT formation, an AD typical hallmark. On the other hand, unpublish data from the group support that Aβ can lead to alterations in MCP-1 levels. Therefore, we can hypothesize that in AD pathogenesis, chemokines alterations caused by Aβ accumulation, would lead to tau hyperphosphorylation and later to NFT formation, in a feedback mechanism.

Nonetheless, it would be interesting to address other ptau phophospecific residues, also known to be altered in AD brains, as Y18, T231 and S262, since chemokines might have a different impact on tau phosphorylation at different residues.

5.2. GSK3β is not the main kinase involved in chemokines-mediated tau phosphorylation

Tau is a MAP expressed in neurons, mainly on axons, that in AD becomes hyperphosphorylated, detaches from microtubules and aggregates into NFT, impacting on neuronal normal function. GSK3β is a kinase known to be involved in abnormal tau phosphorylation in AD (79).

Although the exact mechanism by which GSK3β is anomaly activated and causes tau hyperphosphorylation is unclear, it may involve post-translation phosphorylation events at different residues. GSK3β phosphorylation at S9 causes its inactivation because this leads to the bound of GSK3β N-terminal tail to the primed substrate binging domain. The bound between this domain and the primed substrate (i.e. pre-phosphorylated substrate) is necessary for GSK3β activation. Therefore, GSK3β phosphorylation at S9 preclude substrate bound to the primed substrate binging domain, causing GSK3β inhibition (83).

On the other hand, phosphorylation at Y216 seems to be necessary for GSK3β maximal activity (83), although some studies refer that this kinase can be active even without this residue phosphorylation (139,140).

Considering that ptau levels were significantly increased with IL-8 and MCP-1 treatment, we hypothesised that this could be mediated via GSK3β.

For IL-8, no significant differences were found in total GSK3β levels. Regarding this kinase activity, a significant increase in pGSK3β S9, which correlates with GSK3β inactivation, was found at 6h for the highest concentrations, as well as a general tendency for increase at 48h, although not significant. For the pGSK3β at the Y216 residue, a general decreasing tendency was observed at 6h. Therefore, IL-8 shortest exposer period seems to be inactivating GSK3β. This can be correlated with ptau stable levels at this time point. However, at major ptau S396 levels' differences no significant difference regarding GSK3β activity was observed. Therefore, GSK3β doesn't appear to be associated with tau phosphorylation at this specific residue upon IL-8 exposure.

Like for IL-8, MCP-1 treatment did not change GSK3β total levels. However, this chemokine significantly increases pGSK3β S9 levels and decreases pGSK3β Y216 levels at 3h treatment period. This robustly indicates that GSK3β is inactivated by MCP-1 treatment at this time point. This could be associated with ptau results since at this incubation period there were no alterations on ptau S396 levels. However, since for longer incubation periods no alterations were observed, the association between ptau levels and GSK3β inactivation could be just a coincidence.

Hence, these results suggest that IL-8 and MCP-1 may have an initial impact on GSK3β activity, decreasing it, but over time this is no longer evident, probably due to other cells response mechanisms to IL-8 and MCP-1 effect. Of note, we need to take into consideration that phosphorylation is a dynamic process, that can involve different players at different time points. Besides, different kinases can be involved in the phosphorylation of distinct residues, which can turn difficult data interpretation. Also, other mechanisms regarding GSK3β activity, that weren't assessed, might be affected by chemokines. Therefore, in the future additional analysis should be carried out to verify GSK3β activity.

Since GSK3β does not appear to be a key kinase involved in tau phosphorylation at this residue under these chemokines' exposure conditions, other kinases activities should be addressed. Data relatively to CDK5 levels showed that IL-8 exposer decreased it, in the shortest period. Contrarily, MCP-1 treatment seems to increase CDK5 levels at 3h and 24h. Although this increase at 24h can be correlated with ptau increased levels at this time point, complementary studies that can assess CDK5 activity, like measuring p35/p25 levels, should be carried out in the future to test this hypothesis.

5.3. Phosphatase decreased activity may be associated with increased tau phosphorylation upon chemokines exposure

Tau hyperphosphorylation in AD can also be due to PP impairment or decreased activity, with PP1 and PP2A being the most important phosphatases in the brain (84).

For IL-8 a trend to increase was observed for PP1 levels at the longest incubation period. MCP-1 treatment only caused a small decrease in PP1 levels at 3h. For PP2A levels no differences were observed for both chemokines.

Regarding PP activity, PP1 and PP2A seem to decrease its activity upon IL-8 and MCP-1 treatment, with this decrease being more pronounced for PP1. Although these are only preliminary results, we can hypothesise that chemokines may decrease PP activity, which can lead to increased tau phosphorylation, since this dynamic process became imbalanced. On the other hand, the increased levels of PP1 with IL-8 treatment could be a cell-mechanism to increases the PP1 activity by augmenting its levels. However, it is necessary to carry out an additional set of experiments to confirm that PP activity is increased with chemokines treatment.

5.4. Conclusion and future perspectives

Although the exact levels of chemokines in AD brain are unknown, sustained neuroinflammation is a disease hallmark. In this work, IL-8 and MCP-1 treatment increased tau phosphorylation at S396 residue, at higher concentrations and for longer incubation periods, in a neuronal cellular model. In a disease perspective, this result suggests that long term exposure to high IL-8 and MCP-1 amounts can potentially correlate with abnormal tau phosphorylation, leading to NFT formation, typical of AD brains.

Data also support that GSK3β is not the main kinase underlying IL-8 and MCP-1 induced tau phosphorylation at S396. Also, CDK5 levels were affected by chemokines treatment. Therefore, in the future CDK5 activity should be addressed.

In addition, preliminary data support that PP1 and PP2A can have a role in tau phosphorylation induced by chemokines since the IL-8 and MCP-1 exposure render in decreased PPs activities. However, a further set of experiments are necessary for conclusive results.

A putative model for chemokines action on tau phosphorylation, based on the obtained results, is presented (Figure 40).

Finally, in the future it would also be interesting to test the effect of IL-8 and MCP-1 on a more conventional neuronal model, for instance, SH-SY5Y differentiated with RA and Brain-derived neurotrophic factor (BDNF) or primary cortical neurons, or even in an AD animal model.

It would be likewise relevant to assess tau phosphorylation at other ptau residues also relevant for AD pathology. Also, inner-cell aggregation should be evaluated to verify if chemokines treatment induces NFT formation. Additionally, since in physiologic conditions several chemokines are simultaneously involved in the inflammatory response, it would also be interesting to analyse the combined treatment of chemokines affects tau phosphorylation.

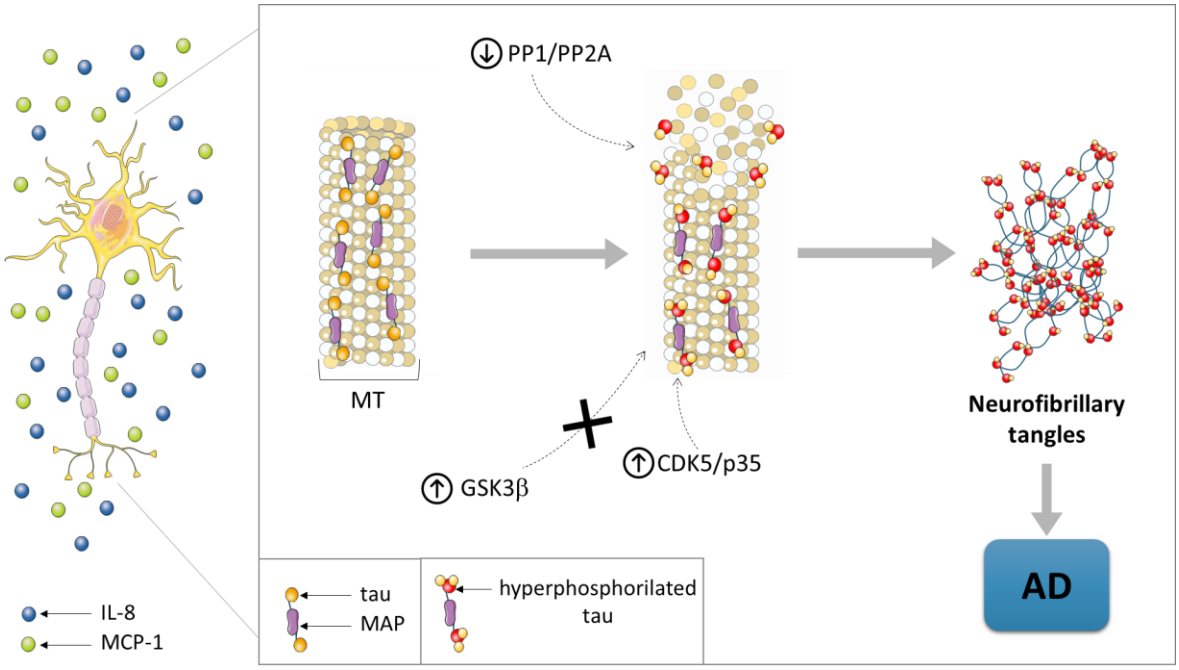

Figure 40: Putative model of IL-8 and MCP-1 action on tau phosphorylation. The long-term exposure to IL-8 induces tau hyperphosphorylation on neurons which can lead to NFT formation and thus to AD. This process is not mediated by GSK3β but rather it may involve CDK5 and/or PP1 and/or PP2A. (MT – microtubule; MAP – microtubule-associated protein; ⓓ decreased activity; ⓤ increased activity). Adapted using images from Servier Medical Art by Servier.

6 — References

6. References

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7 – Annexes

7. Annexes

7.1. Reagents and equipment

➔ Cell culture

SH-SY5Y neuroblastoma cell line (ATCC® CRL-2266™)

Reagents and solutions

Complete Culture medium – MEM/F12 (1:1) 10% FBS

For 1L of culture medium

MEM	Gibco	440 mL
F12	Gibco	440 mL
Sodium Pyruvate (45mM)	Sigma-Aldrich	10 mL
Antibiotic-Antimycotic mix (100x)	Gibco	10 mL
FBS	Gibco	100 mL

Differentiation Culture medium – MEM/F12 (1:1) 1% FBS 10 µM RA

For 1L of culture medium

MEM	Gibco	440 mL
F12	Gibco	440 mL
Sodium Pyruvate (45mM)	Sigma-Aldrich	10 mL
Antibiotic-Antimycotic mix (100x)	Gibco	10 mL
FBS	Gibco	10 mL
Sterile dH_2O	–	90 mL

For 10 mL of culture medium

Retinoic Acid (in DMSO; stock 20 mM)	Sigma-Aldrich	5 µL

Chemokine treatment Culture medium – MEM/F12 (1:1) without FBS

For 1L of culture medium

MEM		Gibco	440 mL
F12		Gibco	440 mL
Sodium Pyruvate (45mM)		Sigma-Aldrich	10 mL
Antibiotic-Antimycotic mix (100x)		Gibco	10 mL
Sterile dH_2O		–	100 mL
Recombinant Human IL-8 554609	(stock solution; 200 ng/µL)	BD Biosciences	–
Recombinant Human MCP-1 554620	(stock solution; 200 ng/µL)	BD Biosciences	–

Typsin-EDTA	Gibco
PBS	Thermo Fisher Scientific
Dimethyl Sulfoxide (DMSO)	Fisher Scientific

Equipment

Hera cell CO_2 incubator	Heraceus
Safety cabinet Hera safe	Heraceus
Inverted optical microscope	Leica
Motorized inverted widefield microscope	Olympus IX-81
Mr. Frosty – freezing container	Thermo Scientific
Hemocytometer	Thermo Fisher Scientific

➔ Cell collection and protein concentration determination

Reagents and solutions

Washing solution – TBS	For 50 mL	
Tris	0,121 g	
NaCl	0,438 g	
	in dH2O	pH 7,4

Collection buffer – RIPA lysis buffer

For 1 mL

RIPA buffer	Sigma-Aldrich	905 µL
Sodium Fluoride (NaF; 100 mM)	–	75 µL
Sodium Orthovanadate (Na_3VO_4; 100 mM)	–	10 µL
Protease Inhibitor Cocktail (25x)	Sigma-Aldrich	10 µL

BCA assay kit		Pierce™ - Thermo Scientific
SDS 1%	For 10 mL; in dH2O	0,1 g

Equipment

Sonicator	IKA
Heated Incubator	–
Infinite M200 – Plate reader	Tecan
i-Control – software	Tecan

➔ SDS-PAGE and WB

Reagents and solutions

Resolving and Stacking gels

(recipes on section 3 – Materials and Methods)

LGB	For 500 mL; in dH_2O pH 8,9	Tris	90,825 g
		SDS	2 g
UGB	For 500 mL; in dH_2O pH 6,8	Tris	37,845 g
Acrylamide (29:1)		Fisher Scientific	
SDS 10%	For 10 mL; in dH2O	1 g	
APS	For 1 mL; in dH_2O	0,1 g	
TEMED		–	

TBS	For 1 L	
Tris	12,11 g	
NaCl	87,66 g	
	in dH_2O	pH 8,0

TBS-T	For 1 L	
Tris	12,11 g	
NaCl	87,66 g	
Tween 20	5 mL	
	in dH_2O	pH 8,0

Ponceau S solution	For 50 mL
Ponceau S	12,11 g
Acetic Acid	2,5 mL
dH_2O	47,5 mL

Mild stripping solution	For 1 L	
Glycine	15 g	
SDS	1 g	
Tween 20	10 mL	
	in dH2O	pH 8,0

Luminata Crescendo	Millipore
Amersham ECL Select	EG Healthcare Life Sciences

Equipment

Electrophoresis System	Hoefer SE600 vertical protein unit
Power supply system	–
Transfer tank	Hoefer TE42
Power supply system – EPS 100	Amersham Pharmacia Biotec
Orbital shaker	–
ChemiDoc Touch Imaging System	BioRad
Odyssey 9120	LI-COR

➔ Ser/Thr phosphatase activity assay

Washing solution – TBS	For 50 mL	
Tris	0,121 g	
NaCl	0,438 g	
	in dH_2O	pH 7,4

Collection buffer – NP40 lysis buffer

For 50 mL

Tris	0,303 g
NaCl	0,7305 g
NP40 (100%)	0,5 mL
PMSF (40 mM)	1,25 mL
Protease Inhibitor Cocktail (25x)	2 mL
For negative control:	
+ Sodium Fluoride (NaF; 1 M)	375 µL
+ Sodium Orthovanadate (Na_3VO_4; 100 mM)	500 µL

RediPlate™ 96 EnzChek® Serine/ Threonine Phosphatase Assay Kit	Molecular Probes

Equipment

Centrifuge	–
Rotational plate	–
Heated Incubator	–
Infinite M200 – Plate reader	Tecan
i-Control – software	Tecan

7.2. Scientific communications

Poster presented in the congress SINAL 2019 – 10th meeting in signal transduction, that occurred between 18 and 19 of October in Olhão, Portugal.

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IL-8 and MCP-1 impact on kinases and phosphatases involved in Tau phosphorylation

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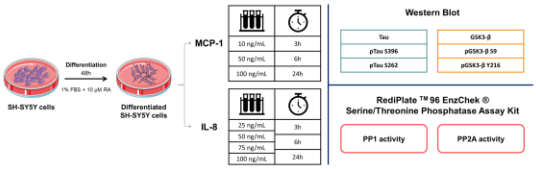
Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder worldwide, and the main cause of dementia, characterized by impaired cognition and behaviour. Although the exact mechanisms leading to AD are not fully understood, its pathogenesis involves the damage and progressive neuronal loss related with two main AD hallmarks: the presence of extracellular senile plaques mainly composed of β-amyloid (Aβ) peptides and the formation of intracellular neurofibrillary tangles of hyperphosphorylated Tau [1]. Tau is a microtubule-associated protein participating in microtubule polymerization and stabilization, processes closely involved with neuronal cytoskeleton architecture and axonal polarity. In AD, Tau hyperphosphorylation and NFTs formation imbalance microtubule dynamics and thus contribute to neurodegeneration [2]. Chronic neuroinflammation is another event occuring in AD, mainly as a result of microglia and astrocytes activation in response to Aβ peptides [3]. In this process, chemokines are released and recruit astrocytes and microglia to the affected area, extending local inflammation [4], [5]. In this work, the effect of two chemokines, MCP-1 and IL-8, in Tau phosphorylation was evaluated and the protein kinases and phosphatases involved in this event addressed. Tau-based therapeutic approaches in neurodegeneration are being pursed and thus understanding the molecular mechanisms involved in Tau phosphorylation may be of particular interest in AD.

Objectives

- Address the effect of the chemokines MCP-1 and IL-8 on Tau phosphorylation.
- Evaluate the impact of MCP-1 and IL-8 on GSK3β protein kinase activity.
- Test the effect of MCP-1 and IL-8 on the activity of the protein phosphatases 1 (PP1) and 2A (PP2A).

Methods



Results

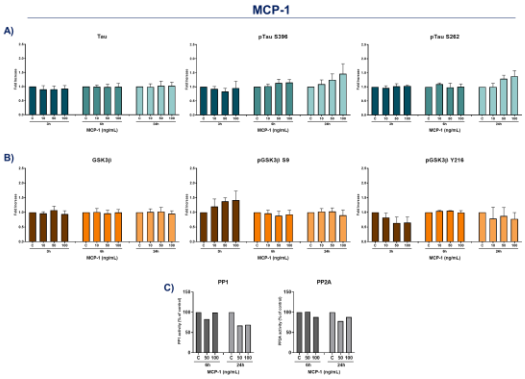


Figure 1 – Phosphorylation of Tau in differentiated SH-SY5Y cells exposed to MCP-1.

SH-SY5Y cells were differentiated for two days with 10 μM retinoic acid, and then treated with different MCP-1 concentrations. Cells were collected at indicated time points, and the levels of Tau, pTau S396 and pTau S262 in (A), and the levels of GSK3β, GSK3β S9 and GSK3β Y216 in (B), were evaluated by western-blot (n≥3). PP1 and PP2A activity in (C) was determined (n=2), using RediPlateTM 96 EnzChek Ser/Thr Phosphatase Assay Kit

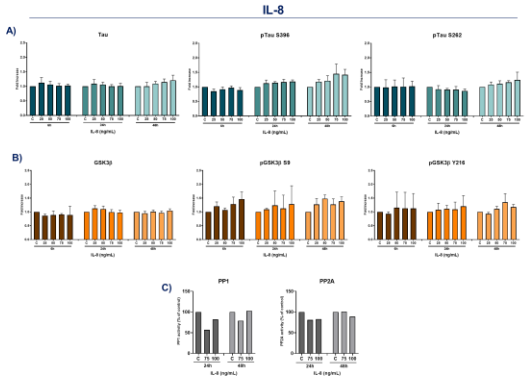


Figure 2 – Phosphorylation of Tau in differentiated SH-SY5Y cells after exposure to IL-8.

SH-SY5Y cells were differentiated for two days with 10 μM retinoic acid, and then treated with different concentration IL-8. Cells were collected at indicated time points, and the levels of Tau, pTau S396 and pTau S262 in (A), and the levels of GSK3β, GSK3β S9 and GSK3β Y216 in (B), were evaluated by western-blot (n≥3). PP1 and PP2A activity in (C) was determined (n=2), using RediPlate™ 96 EnzChek Ser/Thr Phosphatase Assay Kit.

Conclusions

Exposure of differentiated SH-SY5Y to MCP-1 or IL-8 promotes Tau phosphorylation, particularly at serine 396.

Under these conditions, GSK3-β activity does not seem to be directly associated to Tau phosphorylation at S396 and S262, suggesting that other kinases may be involved in this process.

MCP-1 and IL-8 treatment modulates PP1 and PP2A activity, lowering it, supporting its involvement on Tau phosphorylation upon chemokines exposure.

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