Universidade do Minho
Escola de Medicina

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A positive modulator of AMPA receptors against AD pathology: *in vivo* studies

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DECLARAÇÃO

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ABSTRACT

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder among the elderly, estimated to affect approximately 47 million people worldwide, a number that is expected to triple in just 3 decades. While the currently available drugs only temporarily improve memory and cognitive function or delay the progression of dementia, there is no effective cure at the moment with AD remaining a devastating disorder with unclear initiators. Clinical evidence suggests that stressful life events can be a risk factor for AD while recently, depression, another stress-related disorder, is also shown to predispose to AD. Experimental evidence supports stress as the pathological link between depression and AD, as stress and main stress hormones, glucocorticoids, trigger the two AD pathomechanisms, Aβ overproduction and Tau hyperphosphorylation; both trigger neuronal malfunction and loss with glutamate excitotoxicity having an essential role in AD neuropathology. Based on the above, this Master thesis investigates the potential therapeutic action of S 47445, a positive allosteric modulator of AMPA receptors, against Ab-driven AD pathology using two experimental set-ups: i) Aβ oligomers hippocampal injection followed by acute administration of the S47445 compound and, ii) combined chronic stress/$A\beta_{1-40}$-infusion model. Our findings demonstrate that S 47445 compound reverted different short- and long-term memory deficits found in our AD model, suggesting a beneficial effect of hippocampus- and prefrontal-cortex-dependent function. Indeed, S 47445 was able to ameliorate the spatial and reference memory deficits found upon hippocampal injection of Aβ oligomers and to revert the recognition and spatial reference memory impairments in the stress/$A\beta_{1-40}$-infusion model. Notably, S 47445 protective action was specific to the modulation of the cognitive dimension of behavioral deficits in of this AD model, as it exhibited no positive impact on mood deficits (lack of anxiolytic or anti-depressive effects). These studies constitute the first *in vivo* evidence of the potential therapeutic benefit of S 47445, targeting AMPA receptors and their signaling against AD pathology.

RESUMO

A doença de Alzheimer é a doença neurodegenerativa mais prevalente entre a população idosa, e estima-se que afete cerca de 47 milhões de pessoas mundialmente, um número que se espera triplicar nas próximas 3 décadas. Enquanto que as terapias disponíveis apenas melhoram temporariamente a memória e a função cognitiva ou atenuam a progressão da doença, ainda não existe uma cura efetiva para esta patologia, pelo que a doença de Alzheimer continua uma condição devastadora, cujos fatores iniciantes permanecem ainda pouco claros. Evidências clínicas sugerem que eventos de vida stressantes podem constituir um fator de risco para a doença de Alzheimer, enquanto que também já foi demonstrado que a depressão, outra patologia associada ao stress, pode predispor para a doença de Alzheimer. Evidências experimentais suportam um papel do stress na conexão entre a depressão e a doença de Alzheimer, uma vez que o stress e as hormonas do stress, os glucocorticoides, despoletam dois mecanismos patológicos da doença de Alzheimer, a produção excessiva de Aβ e a hiperfosforilação da proteína Tau; ambos levam á má função e perda neuronal, sendo que a excitotoxicidade glutamatérgica exerce também um papel essencial na patologia da doença de Alzheimer. Tendo isto em consideração, a presente tese de mestrado investiga o potencial terapêutico do S47445, um modulador alostérico positivo dos recetores AMPA, contra a patologia de Alzheimer, usando duas experiências principais: i) injeção da forma oligomérica da Aβ no hipocampo seguida de administração aguda do S47445 e, ii) modelo combinatório de stress e infusão de $A\beta_{1\text{-}40}$. Os nossos resultados demonstram que o composto S47445 consegue reverter tanto os déficits de memória a curto prazo como de memória a longo prazo do nosso modelo de Alzheimer, sugerindo um efeito benéfico nas ações dependentes da função do hipocampo e córtex pré-frontal. De facto, o composto S47445 foi capaz de atenuar os déficits da memória espacial e de referência resultantes da injeção da forma oligomérica da Aβ no hipocampo, e de reverter os déficits de memória no modelo combinado de stress e Aβ. É de salientar que a ação benéfica do composto foi específica na modulação da dimensão cognitiva dos déficits comportamentais do modelo, uma vez que não exibiu nenhum impacto positivo nos déficits humorais (falta de efeitos ansiolíticos e antidepressivos). Estes estudos constituem as primeiras provas in vivo do potencial terapêutico do S47445, tendo como alvo os recetores AMPA e a sua sinalização contra a patologia de Alzheimer.

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LIST OF ABREVIATIONS

Aβ – Amyloid-β

ACTH – Adrenocorticotropic hormone

AD – Alzheimer's Disease

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AICD – Amyloid precursor protein intracellular domain

APP – Amyloid Precursor Protein

BACE1 – β-secretase

Cdk5 – Cyclin-dependent kinase 5

CRH – Corticotropin-releasing hormone

EPM – Elevated Plus Maze

FST – Forced Swimming Test

GC – Glucocorticoids

GR – Glucocorticoid receptor

GSK3β – Glycogen synthetase 3β

HPA – Hypothalamic-pituitary-adrenal axis

LTD – Long-term depression

LTP – Long-term potentiation

MARK – Microtubule affinity-regulating kinase

MAPT – Microtubule associated Protein Tau

MR – Mineralocorticoid receptor

MWM - Morris Water Maze

NFTs – Neurofibrillary tangles

NPR – Novel Preference Recognition

NOR – Novel Object Recognition

NMDA – N-methyl-D-aspartate

OF – Open Field

PSD – Post-synaptic Density

PSD95 – Post-synaptic Density 95

PFC – Pre-frontal cortex

PKA – AMP-dependent protein kinase

PP1 – Protein phosphatase 1

PP2A – Protein phosphatase 2A

PP2B – Protein phosphatase 2B

PP5 – Protein phosphatase 5

PSEN1 – Presenilin 1

PSEN2 – Presenilin 2

SAPK/JNF – Stress-activated protein kinase/Jun amino-terminal kinases

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CHAPTER 1

INTRODUCTION

NTRODUCTION

1. Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia and the most prevalent neurodegenerative disorder worldwide, recognized by the Health World Organization as a global health priority. Current estimates suggest that around 47 million people worldwide suffer from dementia, a number that is predicted to triple by 2050 as the population ages (Prince et al., 2016; Wortmann, 2015), rising AD as a major public health problem. Clinical symptoms include a progressive decline in language and learning abilities, followed by severe apathy and mood deficits. The commonest AD presentation focuses on progressive memory problems centered in episodic memory while as disease progresses, cognitive impairments become more pronounced, interfering with activities of daily living (Khan and Bloom, 2016; Mota et al., 2014).

Based on the pathologic and genetic features of the disease, AD can be classified in two major types: familial and sporadic (Sadigh-Eteghad et al., 2012). Early-onset familial AD accounts for less than 5% of the AD cases, resulting from one or more mutations in genes related to the disease; these genes include amyloid precursor protein (APP), presenilin (PSEN1) and presenilin 2 (PSEN2) (Tanzi, 2012), Note that symptoms of these patients develop earlier than in sporadic AD patients, typically between 30 and 50 years of age (Bateman et al., 2011). In contrast, late onset sporadic AD, which accounts for more than 95% of the AD cases, appears later in life (usually after 65 years old, roughly doubling its prevalence every 5 years after this age) (Wortmann, 2015) and has no clear genetic basis, likely resulting from the combined complex action of multiple genetic susceptibilities and environmental risk factors (Lambert and Amouyel, 2011). Genome-wide association studies using thousands of samples have identified more than 20 genetic risk factors, involved in inflammatory, cholesterol metabolism and endosomal vesicle recycling pathways (Karch and Goate, 2015). These relatively common genes confer only a very small increased risk, although when combined can almost double the prediction of developing the disease.(Escott-Price et al., 2015).

From an histological point of view, AD is linked to two cardinal neuropathological signatures: i) accumulation of extracellular senile plaques, resulting from the accumulation of amyloid β peptide (Aβ) and ii) intracellular neurofibrillary tangles, formed by the aggregation of abnormally hyperphosphorylated

Tau protein (Butterfield and Boyd-Kimball, 2004; Wang et al., 2017).These accumulations mainly occur in the neocortex, hippocampus and other subcortical regions, the vulnerable areas involved in memory and proper cognitive function (Moreira et al., 2010; Zhang et al., 2016). The appearance of these histopathological features has been shown to occur many years prior to the appearance of the clinical signs and symptoms of the disease. In addition to these 2 markers, AD brains also present neuropil threats, dystrophic neurites, associated astrogliosis and microglial activation, whereas cerebral amyloid angiopathy frequently co-exists (Serrano-Pozo et al., 2011). The downstream consequences of these pathological processes embrace neurodegeneration and neuronal loss, leading to severe macroscopic atrophy in later stages of the disease.

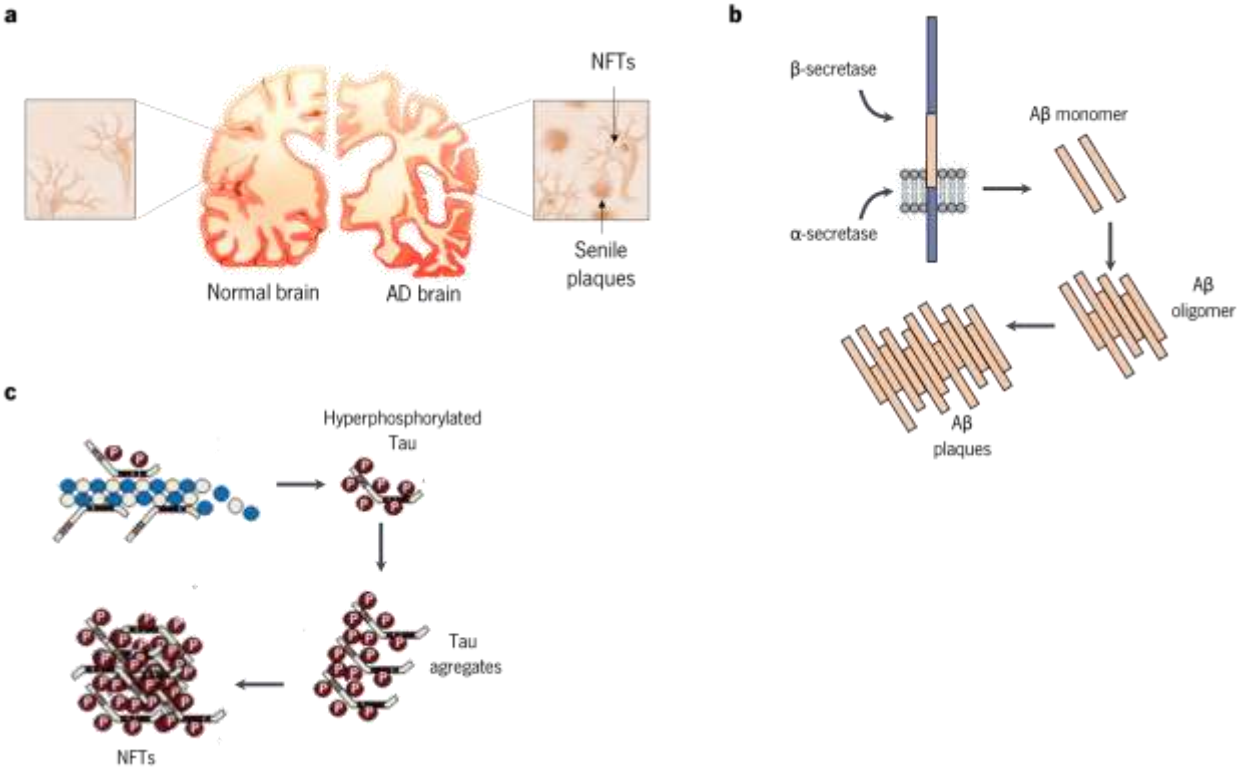

Figure 1. Alzheimer's disease brain pathology. A*) A severe atrophy of several regions characterizes AD brain, along with the presence of amyloid plaques and neurofibrillary tangles.* ***B****) Senile plaques are deposits of amyloid-beta peptides, generated by the proteolytic cleavage of APP by β-secretase and α-secretase.* ***C****) Neurofibrillary tangles are composed of hyperphosphorylated Tau protein, that detaches from microtubules, aggregates, and gives rise to insoluble fibrils that accumulate into NFTs.*

1.1. Amyloid beta

Amyloid precursor protein (APP) is a single-pass transmembrane protein type I which is expressed at elevated levels ubiquitously in the brain and metabolized in a rapid and highly complex manner (O'Brien and Wong, 2011). It is synthetized in the endoplasmic reticulum, transported to Golgi network, and later to the cell membrane through the secretory pathway (Mattson, 2004). APP is the precursor of the Aβ protein (Dawkins and Small, 2014; Nalivaeva and Turner, 2013), the main component of amyloid plaques (Masters et al., 1985). Although its metabolic products have been linked to the etiology of AD, APP itself seems to play an important role in cell biology and in a variety of developmental processes. Indeed, it has been implicated as a regulator of synaptic formation and repair (Priller et al., 2006), neuronal stem cell proliferation and differentiation (Dawkins and Small, 2014), anterograde neuronal transport (Turner et al., 2003) and iron export (Duce et al., 2010). It is located in the chromosome 21, much likely explaining why individuals with Down syndrome develop AD-like pathology early in life (Patterson et al., 1988). Full-length APP can be post-translationally cleaved by several proteases by two main pathways, either yielding or not Aβ as a final product. In turn, Aβ, in physiological concentrations, is a normal product of physiological cellular/neuronal metabolism, also necessary for synaptic plasticity and memory (Puzzo et al., 2015) (Walsh et al., 2000). In the non-amyloidogenic pathway, APP is cleaved sequentially by α-secretase and ϒ-secretase. After cleavage by the α-secretase, a large amino acid N-terminal ectodomain is produced (sAPPα) and secreted to the extracellular medium (Kojro and Fahrenholz, 2005). The resulting 83-amino-acid C-terminal fragment (C83) is retained in the membrane and subsequently cleaved by ϒ-secretase, producing a short fragment termed p3 (Haass et al., 1993). Importantly, cleavage by the α-secretase occurs within the Aβ region, thereby precluding generation of Aβ. The amyloidogenic pathway is the alternative cleavage pathway for APP that culminates in Aβ production. Cleavage of APP initially by BACE-1 (β-secretase), generates a

large ectodomain (sAPPβ) and the C-terminal peptide (C99); proteolytic cleavage of the C99 peptide by ϒ-secretase releases the APP intracellular domain (AICD) and Aβ (Golde et al., 2000).

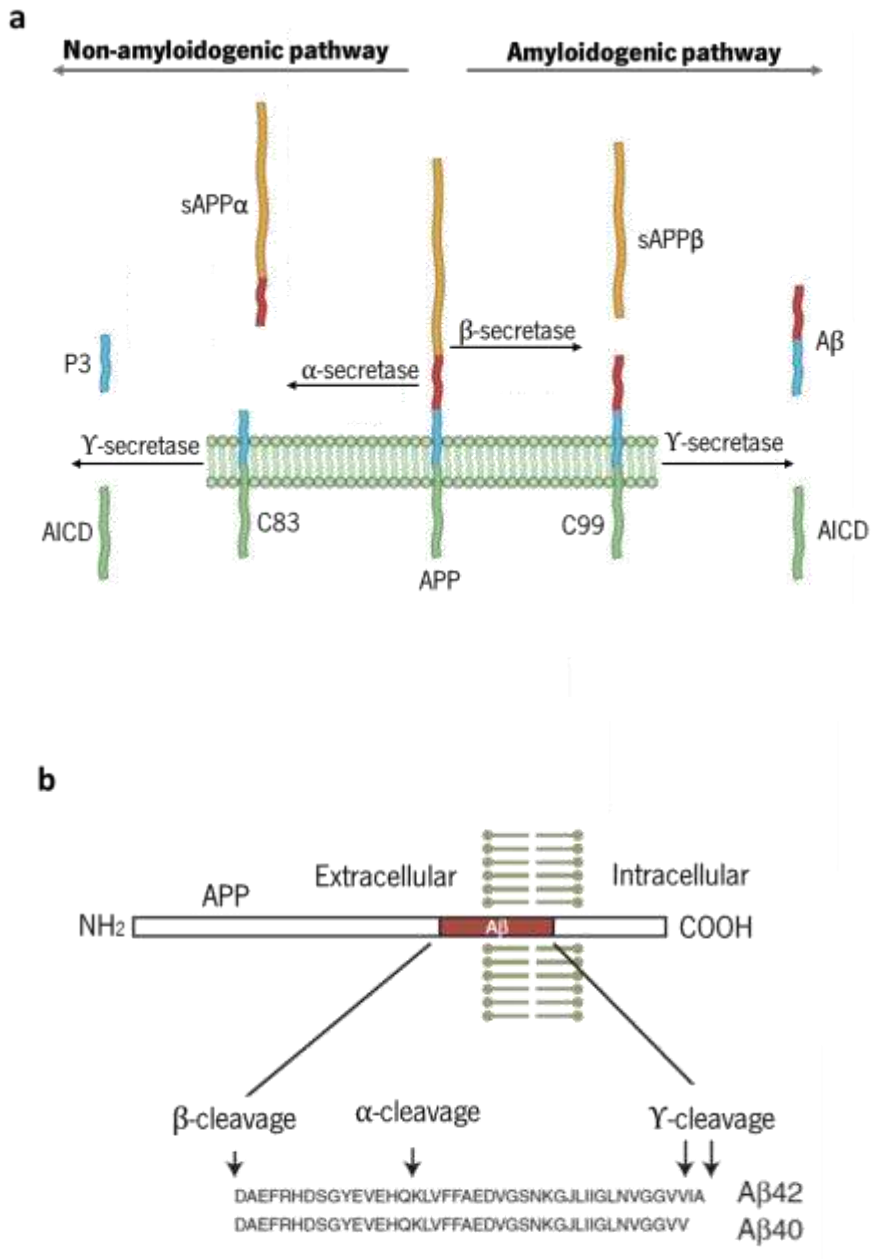

Figure 2: APP processing and Aβ peptide. **A**) The amyloid precursor protein (APP) is cleaved by two main pathways. In the amyloidogenic pathway, sequential cleavage by β-secretase and ϒ-secretase yields the formation of the Aβ peptide. Cleavage by α-secretase (non-amyloidogenic pathway) prevents the formation of amyloid beta, resulting in the liberation of the sAPPα and p3 peptide. **B**) Diagram of the APP peptide and sequence of $A\beta_{40}$ and $A\beta_{42,}$ the two major Aβ peptide forms involved in AD, with proteolytic cleavage sites indicated (adapted from Sheng et al., 2017).

1.2. Senile plaques

Amyloid plaques are extracellular accumulations mainly composed of abnormally folded Aβ with 40 or 42 amino acids, two by-products of APP metabolism. $A\beta_{42}$ is more abundant inside the plaques core that $A\beta_{42}$ due to its higher rate of fibrilization and insolubility. Amyloid deposition does not always follow a stereotypical pattern of progression but broadly develops in the neocortex and progresses through the allocortex, then diencephalon, striatum and basal forebrain cholinergic nuclei, followed by progression to brainstem nuclei and finally the cerebellum (Thal et al., 2002). Unlike NFTs, amyloid plaques involve the entorhinal cortex and hippocampal formation to a less extent (Serrano-Pozo et al., 2011). Neuronal and synapse loss typically parallel tangle formation, and so, NFTs pathology is a better correlation of the clinical features and severity of AD (Serrano-Pozo et al., 2011), whilst β-amyloid pathology reaches a plateau early in the asymptomatic phase of the disease (Ingelsson et al., 2004).

1.3. Tau protein

Tau protein is encoded by the *microtubule-associated protein Tau gene*, *MAPT*, which lies on the human chromosome 17q21 (Poorkaj et al., 1998) and comprises 16 exons. Alternatively splicing of exons E2, E3 and E10 drives the expression of 6 Tau isoforms in the adult human brain (Cox et al., 2016; Goedert et al., 1989), ranging from 352 to 441 amino acids. These different isoforms differ between them in the number of N-terminal inserts, encoded by E2 and E3, resulting in Tau isoforms with none, one or two 29 residue near-amino terminal inserts (0N, 1N and 2N, respectively) (Pittman et al., 2006). Alternative splicing of E10 generates isoforms with three (exon 10 exclusion, 3R) or four (exon 10 inclusion, 4R) microtubule binding repeats (MTBRs) (Trabzuni et al., 2012). These six isoforms have specific physiological roles and so, their expression is tissue specific and differently expressed during brain development, which is likely attributed to its function in cytoskeletal plasticity during neurite outgrowth and synaptogenesis (Goedert et al., 1989). Indeed, during early stages of mammalian development (fetal), the shortest form of Tau (0N,3R) is the most prominent and is highly phosphorylated (Goedert et al., 1989). During brain development, Tau expression patterns change in a way that fetal Tau expression is reduced, and the amount of 3R/4R roughly equals a ratio of 1:1.

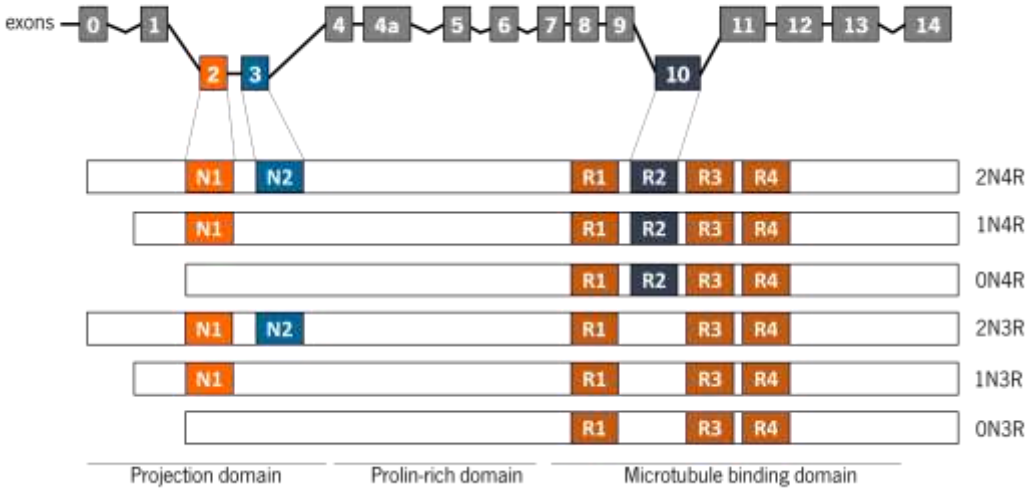

Figure 3: Tau gene and the 6 isoforms expressed in the human brain. Alternative splicing of the gene results in the expression of 6 different Tau isoforms in the human brain, varying among them by the presence of 0,1 or 2 29-residue N inserts and by the insertion of 3 or 4 microtubule-binding repeats (R). Tau protein include 3 main domains: i) projection domain, ii) proline-rich domain and iii) microtubule-binding domain (adapted from Y.Wang and Mandelkow 2015).

Tau is an intrinsically disorder protein (Ittner et al., 2010) (Skrabana et al., 2006), highly soluble, that plays a critical role in tubulin assembly and microtubule stabilization, thereby promoting neuronal normal function (Duan et al., 2017). Tau protein can be divided in 3 major domains accordingly to its interaction with microtubules: i) the C-terminal assembly domain; ii) the projection domain and iii) the N-terminal projection domain. Tau interacts with microtubules through its microtubule-binding repeats, whereas its proline-rich domain mediates interactions with other partners, such as tyrosine protein kinase FYN (Ittner et al., 2010; Lee et al., 1998). Indeed, both phosphorylation of Tau and expression of pathogenic mutations in MAPT result in a stronger interaction of Tau with Fyn in vitro (Bhaskar et al., 2005), suggesting that it may be relevant for disease. Indeed, the Tau-Fyn interaction facilitates the target of Fyn to the post-synaptic compartment. At post-synaptic sites, Fyn phosphorylates the NMDA receptor subunit 2b (NMDA2B), thereby mediating the coupling of the receptors with the postsynaptic density protein 95 (PSD95) (Salter and Kalia, 2004). This interaction is required for excitotoxic downstream signaling (Gendron and Petrucelli, 2009); overactivation of NMDA receptors results in neuronal damage and death, thereby providing a possible mechanism for the role of Tau protein in neurodegeneration (Roberson et al., 2011).

Tau is mainly found in axons due to an incompletely understood sorting mechanism (Konzack et al., 2007; Utton et al.). Indeed, Tau is ubiquitous expressed in young neurons, but it becomes enriched in axons during the maturation process; it can also be found in dendrites and nuclei

(Papasozomenos and Binder, 1987; Sultan et al., 2011), although in a much minor extent (Ittner et al., 2010). Besides its well-known function in stabilizing microtubules, Tau also plays an important role in influencing the motor function of dynein and kinesin, thereby influencing the cargo transport (Stamer et al., 2002). At the synapse, Tau is likely involved in the regulation of synaptic plasticity (Frandemiche et al., 2014), and in a context of pathology, is thought to contribute to synaptic dysfunction, by suppressing AMPA-receptor mediated synaptic response, possibly through the disruption of postsynaptic targeting and anchoring glutamate receptors, as well as impaired axonal trafficking (Hoover et al., 2010)

Tau has at least 84 putative phosphorylation sites, most of them located in the proline-rich regions (residues 172-251) and the C-terminal tail region (residues 368-441) (Liu Fei et al., 2007). Out of these 84 sites, 45 are serines, 33 threonines and 4 tyrosines (Liu Fei et al., 2007). How phosphorylation of Tau affects Tau function it's not entirely understood, but it negatively regulates its binding to the microtubules. In disease brain, Tau protein undergoes numerous alterations, at both physiological and pathological sites, leading to aberrant conformational alterations, which causes its detachment from the microtubules (Ballatore et al., 2007; Martin et al., 2011). Thereby, functions involving microtubules, such as microtubule stabilization and regulation of axonal transport (Götz et al., 2006), may be compromised, possibly contributing to the disease. Numerous protein kinases which can phosphorylate Tau have been identified, including glycogen synthase kinase 3b (GSK3b) (Lucas et al., 2001), protein kinase A (PKA) (Liu et al., 2004), cyclin dependent kinase 5 (cdk5) (Yamaguchi et al., 1996), microtubule-associated regulatory kinase (MARK) (Drewes et al., 1997) and stress activated kinase/c-jun N-terminal kinase (SAPK/JNK) (Johnson and Nakamura, 2007) Phosphatases which can dephosphorylate Tau in the human brain include PP1, PP2A, PP2B, PP5 (Liu et al., 2005). Among them, PP2A is considered a crucial one since its activity has been shown to be downregulated in AD brains, providing an extra clue for Tau hyperphosphorylation in AD (Martin et al., 2013). Indeed, phosphorylation is one of the post-translational modifications of Tau protein in AD brains, at least in 19aa residues ((Augustinack et al., 2002; Ihara et al., 1986). Numerous studies have described Tau phosphorylation in the binding domain, which hinders Tau binding to microtubules, namely in Ser202 (Biernat et al., 1993), Thr231 (Goedert et al., 1994), Thr212 and Ser204 (Zheng-Fischhöfer et al., 1998).

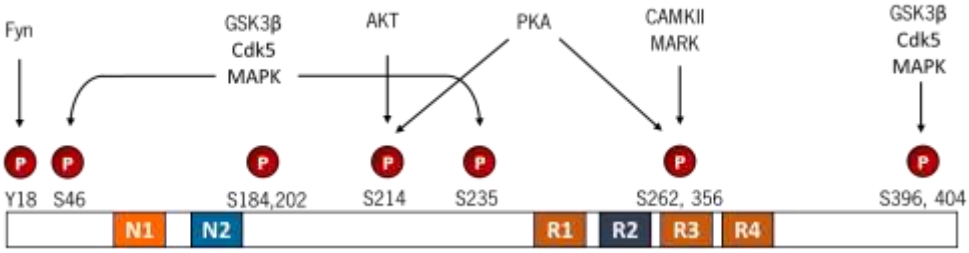

Figure 4: Tau protein phosphorylation epitopes. Tau protein is subjected to a complex array of post-translational modifications, with being phosphorylation being one of the most important. Tau as at least 84 putative phosphorylation sites, some of which are represented in the figure, as well as the kinases responsible for Tau phosphorylation.

1.4. Neurofibrillary tangles

Accumulation of Tau occur in a wide spectrum of neurodegenerative diseases such as supranuclear palsy, corticobasal degeneration, Pick's disease and AD, among others (Goedert and Spillantini, 2006). The formation of NFTs is directly associated with neuronal dysfunction and the number of NFTs is related to the degree of dementia in AD.

During the course of AD, Tau becomes abnormally phosphorylated, which prompts its detachment from microtubules, accumulation in the somatodendritic compartment and formation of neurofibrillary tangles (Goedert and Spillantini, 2006). In AD, Tau pathology initially appears at very circumscribed brain regions and subsequently progresses throughout the brain in a fairly predictive stereotypical pattern. This hierarchical pattern of progressive involvement of regions provides the basis for a neuropathological staining system (Braak and Braak, 1991) that classifies the topography of neurofibrillary degeneration throughout the cortical mantle into 6 stages, progressing from the transentorhinal region of the hippocampus (transentorhinal stages I and II), then to the temporal, frontal and parietal neocortex (limbic stages III and IV) and finally to unimodal and primary sensory and motor areas of the neocortex (isocortical stages V and VI).

2. Environmental stress as a risk factor for Alzheimer's disease onset

Alzheimer's disease is a multifactorial disorder, with a highly complex pathophysiology and several undefined causes. Several risk factors have been associated to the onset of the disease, with aging being the most prominent and preponderant factor. In addition, mutation in several genes, such as ApoE and APP-processing related genes, gender and cardiovascular problems, have been found to increase the risk to develop this disorder. Recently, strong evidence supports an etiopathogenic role for environmental chronic stress and elevated stress hormones, glucocorticoids (GCs), in the development of AD pathology. Indeed, human studies suggest that exposure to chronic stress advances the onset of familial AD (Simard et al., 2009), while several AD patients exhibit high circulating glucocorticoids levels (Hartmann et al., 1997).

2.1. Stress response

All organisms must maintain a complex dynamic equilibrium, termed homeostasis, which is constantly challenged by internal and external stressors. Stress occurs when homeostasis is threatened or perceived to be so; this begins a multistep process that induces the activation of certain physiological brain systems that orchestrate a repertoire of adequate behavioral and neuroendocrine stress responses to ensure an appropriated response to the received stimulus. Adequate intensity and duration of the response is essential to enable the organism to cope with external and internal factors threatening homeostasis (Dhabhar and McEwen, 1997). During the stress response, two main neuroendocrine systems are activated in response to stressors, the sympathetic nervous system and the hypothalamic-pituitary-adrenal system (HPA). The rapid activation of the sympathetic nervous system leads to the release of norepinephrine and epinephrine from the adrenal gland, leading predominantly to the redistribution of the blood flow to vital organs, enabling them to produce an appropriated physical reaction to the stressor (Chrousos, 2009). In the second part of the response, the activation of the HPA axis leads to the release of corticosteroids (corticosterone in rodents and cortisol in humans) which exert an inhibitory feedback signal back to the hypothalamus and pituitary. Corticosteroid receptors function as transcriptional regulators through the modification of expression levels of responsive genes, related to aspects of cell metabolism, structure and synaptic transmission.

In this way, stress and GCs evoke alterations in dendritic structure of neurons and impact on neuronal plasticity; however, prolonged and sustained corticosteroid response can have detrimental impact on neurons, for example, resulting in decreased LTP and facilitating LTD (Kim and Diamond, 2002). Indeed, exposure to chronic levels of stress/GCs is strongly associated with neuronal atrophy and dysfunction as well as impaired cognition and mood and affective disorders, such as depression (Cerqueira et al., 2008; de Kloet et al., 2005; Rajkowska et al., 1999; Sheline et al., 2003).

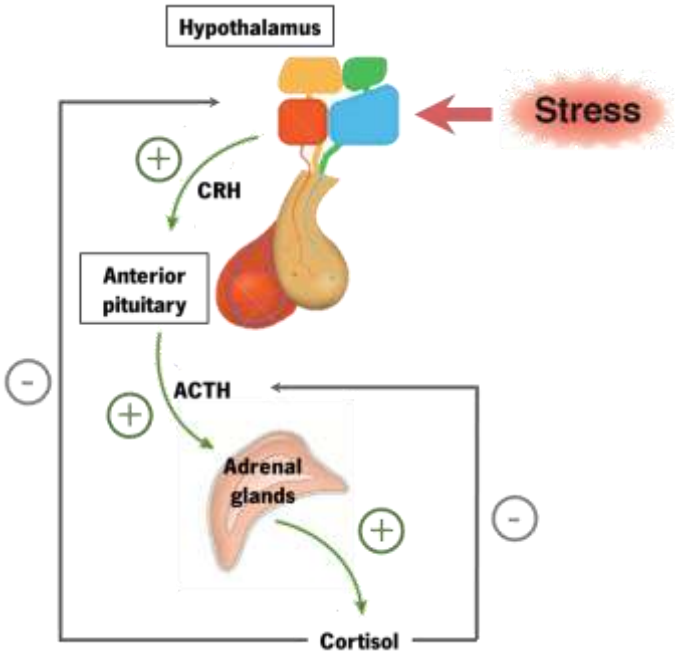

Figure 5: Stress and hypothalamic-adrenal-pituitary axis (HPA). Upon a stressful stimulus, the secretion of the CRF and consequently ACTH, leads to the production of corticosteroids from the adrenal glands. Once in circulation, they reach the pituitary gland and the hypothalamus, exerting a feedback inhibition of the HPA axis, thereby regulating HPA response to the threat. An inadequate/prolonged stress response may disrupt physiological HPA axis response, resulting in damages of brain structure and function.

2.2. Chronic stress, Aβ and Tau – a link to neurodegeneration?

While stress and stress response are adaptive and important for the a proper response to stressful situations, exposure to prolonged stress can become maladaptive and possibly result in brain damage (Sapolsky et al., 1985). Indeed, excess GC secretion is strongly associated with neuronal atrophy and dysfunction as well as impaired cognition and mood disorders. Human and animal studies demonstrated that exposure to chronic stress/elevated GCs resulted in smaller hippocampal volumes and dendritic atrophy, while similar volumetric changes were found in the PFC (Cerqueira et al., 2005;

Rothman and Mattson, 2010). Once high levels of GCs are causally linked to impaired cognitive function and underlie several structural and synaptic alterations, it is likely that GCs contribute to the progressive cognitive decline observed in AD patients. Indeed, the potential link between stress/GCs and AD is strengthened by studies demonstrating that either high GC levels and/or prolong exposure to stress increase the production of Aβ and exacerbate memory deficits in transgenic mouse models of AD (Green et al., 2006; Jeong et al., 2006). Treating transgenic AD mice with the synthetic GC, dexamethasone, accelerated the formation of extracellular Aβ deposits and worsen the memory deficits (Green et al., 2006); similar finding were found in vitro, where dexamethasone treatment triggered APP misprocessing (Green et al., 2006). Moreover, GCs exposure has been linked to upregulation of transcription of APP and β-secretase (Green et al., 2006). In agreement with the above, other studies demonstrated that stress and chronic stress drive APP processing towards the amyloidogenic pathway, leading to an increase in the production of Aβ, known to have neurotoxic and cognition-impairing properties (Catania et al., 2009). In addition to the amyloidogenic pathway, high levels of stress are also reported to trigger Tau protein hyperphosphorylation, the other major hallmark of AD brain. Indeed, previous studies from our team have demonstrated that chronic stress/GCs treatment trigger Tau hyperphosphorylation in several AD animal models, specifically in several epitopes strongly implicated in cytoskeletal dysfunction and synaptic loss (eg., Ser262) as well as hippocampal atrophy (eg., pThr231) in AD patients (Handoko et al., 2013); importantly, the degree of phosphorylation of these Tau epitopes correlates strongly with the severity of cognitive impairments in AD affected individuals (Augustinack et al., 2002). Moreover, previous studies from our team have also demonstrated that prolonged GC administration to wild-type animals drives cytosolic and dendritic Tau accumulation and triggers its phosphorylation, while leading to the accumulation of Tau in the synaptic compartment, related to synaptic loss (Sotiropoulos and Sousa, 2016), thereby providing a possible role for Tau missorting in stress-driven synaptic atrophy observed in AD. However, the mechanistic evidence underling these chronic stress/GC-driven Tau/amyloid-beta alterations is only beginning to emerge.

3. Oligomeric amyloid-beta and Tau – the toxic players in Alzheimer's disease

The amyloid hypothesis, the most prevalent theory for AD pathogenesis, suggest that accumulation into senile plaques of pathological forms of Aβ produced by APP cleavage in the brain is the primary pathological process, driven through an imbalance between Aβ production and clearance. The formation of NFTs and subsequent neuronal dysfunction and neurodegeneration that are observed in AD brains are thought to be downstream processes of Aβ (Hardy and Selkoe, 2002). Indeed, a robust evidence for the fundamental role of Aβ in the pathogenesis of the disease comes from the familial forms of AD: all familial AD mutations are involved in Aβ generation and/or production and result in relative overproduction of toxic forms of amyloid-β. However, accumulating data suggest that small soluble Aβ (oligomers) are the main contributors to neuronal toxicity (Busche et al., 2012; Selkoe, 2008; Shankar et al., 2008; Viola et al., 2008; Wu et al., 2010), accumulating in the vicinity of the senile plaques and at the synapse (Koffie et al., 2009). In fact, the accumulation of plaques in the brain does not correlate well with the cognitive impairment observed in AD patients (Giannakopoulos et al., 2003; Ingelsson et al., 2004), and the reduction of plaque load by immunotherapy does not result in a cognitive improvement for the patients (Holmes et al., 2008). Indeed, several studies suggest that senile plaques could appear in the absence of neurodegeneration, synaptic loss and cognitive impairments; on the contrary, the previous pathological alterations could be induced by soluble Aβ oligomers in the absence of senile plaques (Jin et al., 2011).

On the other hand, it is still unclear whether, or to what extent, Tau pathology in AD might be independent of amyloid-beta action. However, it is widely accepted that AD pathology is inseparably linked to neuronal death mediated by Tau oligomers/aggregates. Indeed, Tau is an essential mediator of Aβ-induced toxic effects (Ittner et al., 2010) since Tau-lacking neurons are not susceptible to neurodegeneration and cell death upon treatment with soluble amyloid beta (Rapoport et al., 2002). Also, amyloid beta formation in APP transgenic mice causes hyperphosphorylation of Tau protein, whereas there is no overt amyloid beta plaque pathology in Tau transgenic mice (Götz et al., 2004). For example, Aβ pathology may facilitate Tau pathology in a mouse model of AD by accelerating Tau fibrillization and conducting a reduction of density of dendritic spines and cognitive impairment (Chabrier et al., 2014). Also, reduction in endogenous Tau levels prevent behavioral deficits in an AD model that overexpress human APP with familial AD mutation, with no alteration in plaque load

(Roberson et al., 2007). According to other studies, Aβ oligomers can stimulate Tau hyperphosphorylation in hippocampal neurons (De Felice et al., 2008). Altogether, the above findings support the importance of amyloidogenesis in AD and suggest a mutual relationship between Tau and amyloid beta underlying the neuropathology of the disease.

4. Glutamatergic signaling and its role in Alzheimer's disease

The concept of synaptic plasticity and its role in learning was put forward by Ramon y Cajal, who noted that the number of neurons in the brain did not appear to change significantly over our lifespan, making it unlikely that new memories were the result of new neurons being born and integrated into the brain. Instead, changes in the strength of connections between existing neurons could constitute the mechanism for memory formation (Cajal, 1894).

The description of long-term potentiation (LTP) and long-term depression LTD provided the molecular understanding of the phenomenon of synapse strengthening or weakening. LTP corresponds to a long-lasting increase in the strength of synaptic transmission; there are early and late phases of LTP, with the early phase dependent upon kinase activation causing several changes to synaptic AMPA receptors, including phosphorylation, increased activity and insertion of new receptors into the postsynaptic density. On the other hand, LTD is a weakening of synaptic strength following a stimulus. LTD can occur via several mechanisms, which have opposite effects to those seen in LTP, including internalization of AMPA receptors (Collingridge et al., 2010; Dudek and Bear, 1992; Massey and Bashir, 2007). Interestingly, LTD may be hijacked during AD as very similar molecular mechanisms involved in LTD are also present in AD-related synapse degeneration. Along with depotentiation of synaptic strength, structural changes occur in response to brain plasticity (Bastrikova et al., 2008; Matsuzaki et al., 2004; Zhou et al., 2004). In AD, the normal function of synapses is impaired, synapses are eliminated, and pathological proteins are transported through synapses. Indeed, among all the neuropathological features of the disease, synapse loss correlates more strongly with dementia (Koffie et al., 2009). Moreover, animal models suggest that dysfunction of synapses and impaired synaptic plasticity are key components of the neurodegenerative process of AD and that both Aβ and Tau contribute to this degeneration (Crimins et al., 2013).

But what are the cellular and molecular mechanisms underlying synapse loss? Several studies point out glutamate signaling and related excitotoxicity as a major player in AD pathogenesis. Indeed, glutamate is involved in a diverse array of physiological processes, namely in coordinating higher cognitive functions, such as learning and memory, and so, the disruption of these signaling could be underlying the early cognitive deficits detected in AD. Besides its involvement in the neuro-circuit of memory and cognition, glutamate signaling also underlies circuits related to emotions, and is implicated in a myriad of psychiatric conditions, including depression and anxiety, that are often found in AD patients (Sanacora et al., 2012). Although the mechanisms though are still not well understood, recent experimental studies have shown that glutamatergic signaling is compromised by amyloid-beta/Tau-induced modulation of glutamatergic receptors in several brain regions, paralleling early cognitive deficits (Parameshwaran et al., 2008).

5. Alzheimer's disease current therapies -what do we miss?

Despite the first description of the disease more than 100 years ago, the etiopathology and pathophysiology of AD remains complex and poorly clarified. Although many of the mechanisms that contribute/underlie the onset of disease are beginning to be understood, and many risk factors have now been pointed out to be important for AD development, there is still a lack of disease-modifying treatments, and no drug has proven to be fully efficient in slowing down/stopping the progression of the disease. In light of the high number (47 millions) of AD patients and considering the expected increase in the number of individuals that will be affected in the near future, finding new ways to fight the disease is of greater importance. Indeed, there is an urgency to rapidly come up with new therapies for this increasingly affected or at-risk of developing AD population, that either prevent/delay the onset of AD or that are capable of alleviate/slow down the progression of patient's symptoms, such as memory and cognitive decline. Despite new therapies are being assessed in clinical trials, the success rate of AD drug development is still very poor (Cummings et al., 2014).

As the synaptic loss has been suggested to be a better predictor both of AD clinical symptoms and of disease progression (Arendt, 2009), it now suggested that AD could be a disease of synaptic failure with glutamatergic signaling impairment being widely implicated in AD synaptic dysfunction and neurodegeneration (Esposito et al., 2013) . Thus, pharmacological targeting of synaptic mechanisms

underlying memory and cognitive function could prevent the initial symptoms of AD as well as prevent the progression from an impairment in synaptic plasticity to an irreversible synaptic and neuronal loss observed in later-stages of AD. Indeed, the production of drugs which address changes in the glutamatergic system is recently becoming more attractive in the fight against cognitive and mood impairment. For example, ketamine, an NMDA antagonist, that also affects AMPA receptor signaling has been shown to improve mood deficits (Aleksandrova et al., 2017). Furthermore, memantine, a NMDA receptor antagonist has been widely used in patients for the treatment of AD (Parsons et al., 2013); however, the overall effectiveness of this agent in delaying the progression of the disease is quite modest (Doraiswamy and Xiong, 2006).

In the light of the suggested detrimental role of glutamate signaling in AD, targeting AD synaptic dysfunction, namely the glutamatergic signaling, during the early symptoms of AD, could constitute a potential therapeutic action to prevent the development/advance of AD pathology. Based on the suggested link between stress and AD and the potential role of glutamatergic malfunction in the above disorders (Sotiropoulos et al., 2008a) the current Master thesis monitors the effect on a novel modulator of AMPA Receptor modulator against Aβ & stress-driven AD pathology monitoring both cognitive and mood behavior.

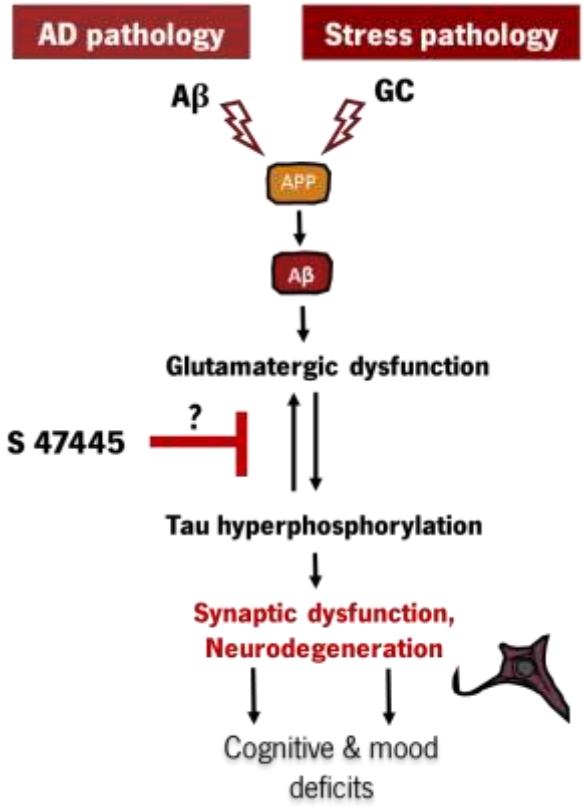

Figure 6. Glutamatergic signaling in AD pathologies. *Schematic representation of the hypothetical model suggesting commonalities between chronic stress and AD brain pathologies as both of them exhibit neuronal atrophy and synaptic loss followed by cognitive and mood deficits possibly though Aβ and Tau key molecules. As glutamatergic signaling is thought to exhibit an essential role in Aβ- and Tau-driven neuropathology, pharmacological modulation of glutamate receptors dysfunction may prevent AD neuropathology, and possible help towards novel treatment of other stress-related disorders such as depression.*

CHAPTER 2

RESEARCH OBJECTIVES

RESEARCH OBJECTIVES

Clinical and experimental evidence suggests that chronic stress maybe a risk factor for AD while preclinical studies suggest stress as the pathological link between depression and AD. As glutamate signaling and synaptic malfunction are suggested to essentially contribute to the above pathologies, the current Master thesis will:

I. Test whether a novel modulating compound of AMPA receptors, S 47445, can bock the acute phase of $A\beta_{1\text{-}40}$ oligomers-driven AD brain malfunction.

II. Monitor whether prolonged treatment with S 47445 can attenuate the combined model of stress/ Aβ-driven AD brain pathology and related memory loss.

III. Understand whether this novel compound can revert anxious and depressive behavior that may accompany the AD memory impairment.

CHAPTER 3

MATERIALS & METHODS

MATERIAL & METHODS

Animals

Male Wistar rats (Charles River Laboratories, France) were used in two animal studies of the current thesis. Animals were maintained under standard laboratory environmental conditions (12h light:12h dark cycles, room temperature 22°C, relative humidity 55%, *ad libitum* assess to food and water). All experiments were conducted in accordance with the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457) and the Directive 2010/63/UE of the Portuguese Parliament and Council.

1rst experiment

Experimental design

5 months old rats (n=6 per group) were randomly assigned to the following five experimental groups: saline stereotaxic injection + vehicle administration (control group), $A\beta_{1-40}$ stereotaxic injection + vehicle administration, $A\beta_{1-40}$ stereotaxic injection + 1mg kg^{-1} S 47445 dose, $A\beta_{1-40}$ stereotaxic injection + 3mg kg^{-1} S 47445 dose and $A\beta_{1-40}$ stereotaxic injection + 10mg kg^{-1} S 47445 dose. Animals start receiving drug/vehicle 7 days following the surgical procedure, over a period of 7 days. The behavioral assessment was conduct during the 7 last days of treatment.

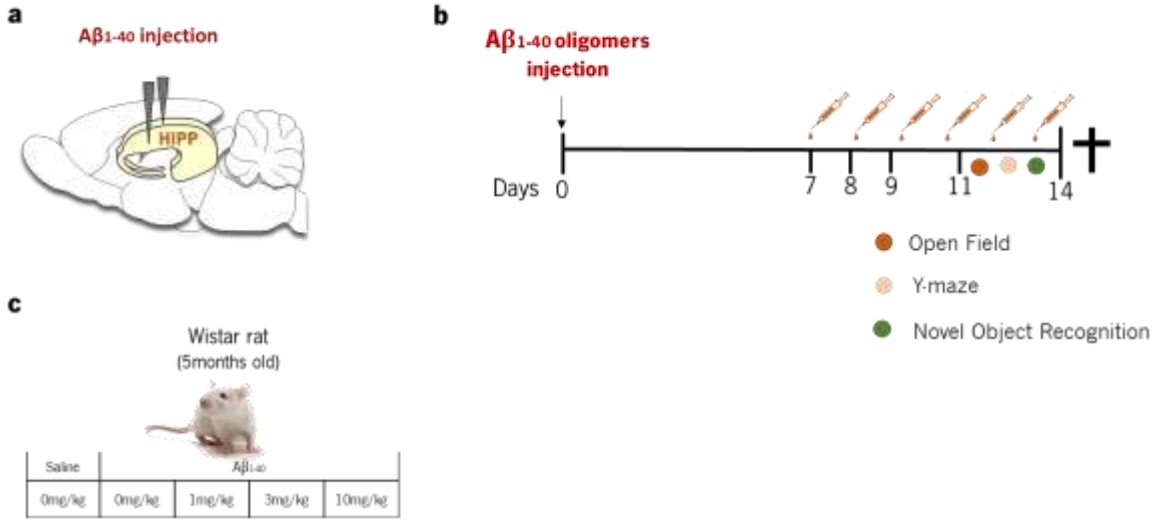

Figure 7. Experimental design of 1st experiment. **A**) Schematic representation of the site of $A\beta_{1-40}$ oligomers injection. **B**) Seven days after the surgery, the animals start receiving the treatment with S 47445, administered daily by intraperitoneal injection, at three different doses; behavioral analysis was conducted in the last 6 days of treatment. **C**) Animal groups.

Surgery

Pre-treatment of $A\beta_{1-40}$

$A\beta_{1-40}$ (No. 1655719. Eurogentec) was diluted to 2.5 μg/μl with sterile normal saline and incubated at 37°C for 1 week for induction of $A\beta_{1-40}$ aggregates.

Surgical procedure

Animals were anaesthetized with 75 mg kg^{-1} ketamine (Imalgene, Merial) and 1mg kg^{-1} medatomidine (Dorbene, Cymedica) administered intraperitoneally. Adequate anesthesia was confirmed by absence of withdrawal responses to hindlimb pinching. The aggregated $A\beta_{1-40}$ [4μl (600nl/min)] was stereotaxically injected bilaterally into the hippocampus (coordinates from Bregma, according to Paxinos and Franklin: -3.0mm anteroposterior, +2.2mm mediolateral, -2.8mm dorsoventral) with a Hamilton syringe. The needle was left in place for 5 minutes after the injection to prevent withdraw of the solution. The animals were removed from the stereotaxic frame, suture and let to recover for one day before starting the drug treatment. Sterile saline was injected into the bilateral hippocampus of control group rats using the same procedure as above.

Drug treatment

S 47445 substance was diluted in 1% (w/v) hydroxyethylcellulose (Sigma-Aldrich) and 1% (v/v) polysorbate 80 in distilled water, which serves as vehicle. The compound was administered daily intraperitonially at the 3 different dosages: 1mg kg^{-1}, 3mg kg^{-1} and 10mg kg^{-1} over a period of 7 days.

2nd Experiment

Experimental design

10 months old animals were randomly assigned to the following eight groups: three control groups without stress exposure, treated with saline, 3mg kg^{-1} or 10mg kg^{-1} dose of S 47445 compound, and 4 groups exposure to CUS stress protocol, receiving infusions of either saline of $A\beta_{1\text{-}40}$. These last groups were further treated as follows: the CUS + saline infusion group received saline, and the CUS + $A\beta_{1\text{-}40}$ infused groups were treated with saline, 3mg kg^{-1} or 10mg kg^{-1} dose of S 47445 compound.

After a 4 weeks stress protocol, the animals were implanted with a pump to deliver either saline or $A\beta_{1\text{-}40}$ and start drug treatment one day after the surgical procedure over a period of 4 weeks. After a period of 2 weeks the pumps were surgically removed, and the animals were let to recover for 2 days before the beginning of the behavioral testing.

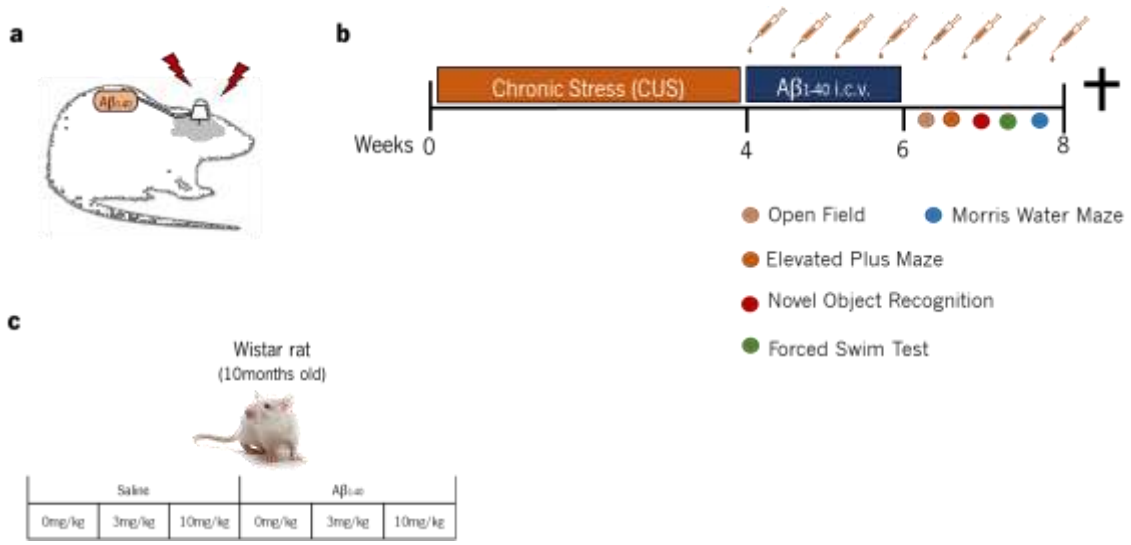

Figure 8. Schematic representation of the experimental design of 2nd experiment. A) *Schematic representation of the AD model.* ***B)*** *After a period of 4 weeks of chronic unpredictable stress, animals start receiving saline or $A\beta_{1\text{-}40}$ delivered through osmotic pumps. After 2 weeks of S 47445 treatment, animal's behavioral performance was evaluated.* ***C)*** *Animal groups.*

Stress protocol

Animals were subjected to a chronic unpredictable stress protocol over 4 weeks before the surgical intervention. The protocol consists of different stressors, namely rocking platform, air dryer, cold water and overcrowding (one stressor per day during 2hours) applied in a random order to prevent habituation. Stressors were also applied during the behavioral testing period (two hours after the completing of the behavioral task).

Intracerebral infusions

$A\beta_{1-40}$ and osmotic pump preparation

$A\beta_{1-40}$ (No. 1655719. Eurogentec) was diluted to 0.125 µg/µl with sterilized normal saline. After osmotic pump assembly (Alzet® Osmotic Pumps, DURECT, 2002 model) the pumps were filled either with saline or $A\beta_{1-40}$ (25µg/200µl) and incubated in sterile saline (0.9%) at 37°C overnight before pump implantation. Pumping of saline or $A\beta_{1-40}$ starts 24 hours after the implantation surgery. This allows animal to recover from the surgery prior to the potential stress of Aβ infusion.

Surgical procedure

Animals were anaesthetized with 75 mg kg^{-1} ketamine (Imalgene, Merial) and 1mg kg^{-1} medatomidine (Dorbene, Cymedica) administered intraperitoneally. Adequate anesthesia was confirmed by absence of withdrawl responses to hindlimb pinching. Pump catheter was stereotaxically implanted in the lateral ventricule (-0.6mm anteroposterior, -1.4mm mediolateral, -3.5mm dorsoventral) and pump inserted into a subcutaneous pocket located in the mid-scapular area of the back of the animal. The pocket is created by opening and closing a hemostat to blunt dissect a short subcutaneous tunnel from the scalp incision to the mid-scapular area. The catheter was held in place with dental cement (C&B kit, Sun Medical). Rats were removed from the stereotaxic apparatus, sutured and let to recover for one day before drug treatment. Two weeks after surgery, osmotic pumps were removed by making a small injection (approximately 1cm) in the mid-scapular arear of the back of the animal and cutting the connection with the cannula.

Drug treatment

AMPAD substance was diluted in 1% (w/v) hydroxyethylcellulose (Sigma-Aldrich) and 1% (v/v) polysorbate 80 in distilled water, which serves as vehicle. The compound was administered daily intraperitonially at the 2 different dosages: 3mg kg^{-1} and 10mg kg^{-1} (chosen based on the previous study) over a period of 4 weeks.

Behavioral analysis

Open Field (OF) test was used as a measure of locomotor performance and exploratory activity as well as anxious-like behavior. The test apparatus consists of a brightly illuminated square arena (42.3x42.3x30.5cm) with transparent acrylic walls and white floor (Med Associates Inc., St Albans, VT, USA). Rats were placed individually in the center of the arena and their movement tracked using a two 16-beam infrared system for 5 minutes. The resulting data was analyzed using the Activity Monitor software (Med Associates, Inc.) considering two pre-defined areas of the arena: a central an outer area. Distance traveled as well as time spent by the animals in each of the two areas was analyzed and the time spent in the center used as an indicative of anxious-like behavior. Total distance traveled was used to assess locomotive activity.

Y-maze (YM) consists of a Y-shaped maze with three identical arms at 120°angle from each other. Briefly, animals were placed in the center of the maze with assess to two arms of the maze and allowed to move freely through the maze for 15min. Three hours later, the animals were again exposed to the maze, but this time with assess to the three arms of the maze. Again, the animals were let to explore the maze freely for 5 minutes. Ratio between distance travelled in the novel arm and the total distance traveled in the maze was used as a measure of short-term recognition memory.

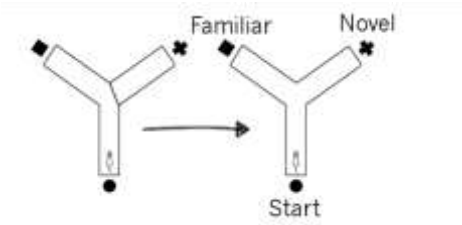

Figure 9. Schematic representation of Y-maze behavioral task. The animals were allowed to explore the maze with assess to only two arms of the apparatus. Three hours later, the animals return to the

maze, this time with assess to the three arms of the apparatus. The distance travelled in the new arm in comparison to the total distance travelled was used as a measure of short-term memory.

Novel Object Recognition (NOR) was used to assess cognitive function. Briefly, rats were first habituated to the test arena consisting of a black acrylic box (50x50x150cm) with an open field space for 10 minutes. On the following day, each animal was allowed to freely explore two identical objects placed in the arena for 10 minutes. One hour later, rats explored the same arena for 10 minutes, with one of the objects placed in a novel position. Twenty-four hours later, the animals returned to the arena for 10 minutes, with one of the previous objects replaced by a new one. The familiar and the novel object differ among them in shape, color, size and texture. All sessions were videotaped and the time spending exploring each one of the objects was determined manually. Short and long-term memory performance were measure by the time exploring the displaced and the novel objects, respectively, and represented as discrimination index. The discrimination index for short-term memory was determined as follows: time of exploration of new location object-time exploration old location object/total time exploration; discrimination index for long-term memory was defined as: time exploration novel object-time exploration familiar object/total time exploration.

Figure 10. Schematic representation of Novel Object Recognition task. Short-term memory and long-term memory test paradigms. The animals were exposure to two identical objects and allowed to freely explore them. One hour later, one of the objects was assigned to a new location; again, the animals were allowed to freely explore, and the time spent in the new location of the object was used as a measure for short-term memory. Twenty-four hours later, one of the objects was replaced by a different novel object, and the time spent exploring the new objected versus the time exploring the old object was taken as a measure of long-term memory.

Elevated Plus Maze (EPM) was used to evaluate anxious-like behavior. The apparatus consists in two opposite open arms (50.8x102cm) and two enclosed arms (50.8x10.2x40.6cm) elevated 72.4cm above the flour, made of black plolypropylene (ENV-560; Med Associates). The junction area between the four arms measured 10x10cm. rats were placed individually in the center of the apparatus facing a closed arm and were allowed to freely explore over a period of 5 minutes. Data was collected using a

CCD camera by the use of NIH Image program (http://rsb.info.nih.gov/nih-image/) and were analyzed using EthoVision®XT software (Noldus).

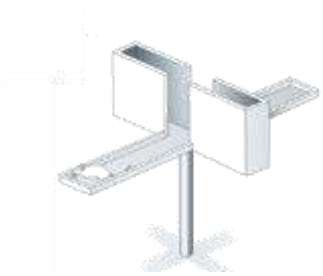

Figure 11. Elevated Plus Maze apparatus. Briefly, the animals were allowed to explore the maze for 5 minutes with assess to two close and two open arms. The time and the entries in the open arms were taken as a measure of anxious-like behavior.

Forced Swim Test (FST) was used to assess depressive-like behavior. The test was conducted 24h after a 5 minutes pre-training session, by placing the animals in transparent cylinders filled with water (22°C, 50cm of depth) for 5 minutes. Sessions were video recorded, and the immobility time was used as a measure of depressive-like behavior.

Morris Water Maze (MWM) - Reference memory task. This test was conducted in a circular black pool (170cm diameter) filled with water at 22°C to a depth of 34cm in a room with extrinsic clues (triangle, square, cross and horizontal stripes) and dim light. An invisible platform (12x12cm, invisible for rats) was placed in one of the four quadrants at a height of 30cm and maintained in the same position across the test. In the spatial learning task, animals were tested for 4 consecutive days (four trials per day). In each of the daily trials animals were positioned in a different starting point (north, south, west and east) and the trial was considered as concluded when the platform was reached. The trials had a maximum duration of 2 minutes. When the platform was not reach within this time window, the animals were guided to the platform and allowed to stay in it for 30 seconds. The test was video-taped and analyzed using EthoVision®XT software (Noldus). The time and distance traveled to reach the platform was used as a measure of spatial reference memory. In the last day, a fifth trial was conducted, were the platform was removed, and the time spent by the animals in the platform quadrant was evaluated.

Figure 12. Morris Water Maze protocol. During the four trials of each day, the animals started the test in a different quadrant of the pool, chosen in a clockwise fashion, and had a maximum of 2 minutes to find the platform. The distance traveled to find the platform was taken as a measure of cognitive function. In the 4th day, a 5th trial was conducted, were the platform was removed, and the time spent in the platform quadrant recorded.

Morris Water Maze (MWM)-Swimming strategies analysis. The pattern of swimming used by each animal in each trial to reach the platform was classified as previously described. Briefly, the pattern was attributed to one of the following categories: *thigmotaxis (T)* were the animals swims exclusively in the periphery, occasionally encountering the platform accidentally; *circling (C)*, when the animal moves away from the wall to explore the pool, usually drawing circular trajectories; *random searching (RS)*, were the trajectories become jagged with sudden changes of direction and velocity; *scanning (S)*, when the search becomes more focused in the center of the pool; *Self-orienting (O)*, when after a missing attempt to find the platform, the animal re-orients himself and finds the platform in the second attempt; *approaching target (A)*, where the animals adjusts its trajectory while approaching the platform, *direct finding (DF),* when the animal swims straight to the platform. These categories are the grouped in non-directed strategies (comprising strategy F, C, RS and S) and in directed strategies (strategies O, DF and A), and the percentage of direct strategies is calculated by the number of times the animals chooses a direct strategy to encounter the platform within the 16 trials.

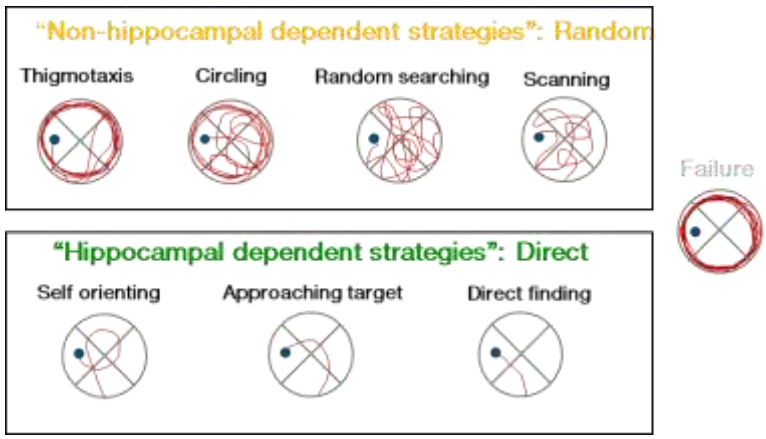

Figure 13. Morris Water Maze strategies classification. The pattern of swimming of the animals in each trial across the 4 days was classified accordingly to the 7 categories presented in the figure. These strategies where then clustered into 2 bocks: random, comprising the "non-hippocampal dependent" strategies or direct, comprising the "hippocampal dependent strategies", as a measure of cognitive performance. (adapted from Rudiger, 2013).

CSF collection

After behavioral tests completed, animals were anesthetized, CSF collected and stored at -80ºC until mass spectrometry analysis performance.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics ver.22 (IBM Co., USA) and graphic representation using GraphPad Prisma ver.6 (GraphPad Software, La Jolla, USA). For 1st experiment analysis, a one-way ANOVA was applied to compare the means of the five groups. Normality was measured using the Kolmogorov-Smirnov and Shapiro-Wilks statistical tests and taking into account the respective histograms and measures of skewness and kurtosis. Equality of variances and Sphericity were measured using the Levene's and Mauchly tests, respectively, and was assumed when $p>0.05$. Multiple comparisons between groups were accomplished through the Bonferroni statistical test.

For the 2nd experiment, Statistical evaluation for animal behavioral assessments was done performing a two-way ANOVA, to compare the interaction between two variables. For Morris Water Maze analysis, a repeated-measurement 2 way-ANOVA. Normality was measured using the Kolmogorov-Smirnov and Shapiro-Wilks statistical tests and taking into account the respective histograms and measures of skewness and kurtosis. Equality of variances and Sphericity were measured using the Levene's and

Mauchly tests, respectively, and was assumed when $p>0.05$. Multiple comparisons between groups were accomplished through the Bonferroni statistical test. Values were accepted as significant if $p<0.05$ and all the results were expressed as mean±SEM (standard error of mean).

CHAPTER 4

RESULTS

RESULTS

Short-term treatment with S47445 compound blocks cognitive deficits driven my Aβ oligomers

Previous clinical and experimental studies indicate that the neuronal malfunction and related memory deficits in AD do not correlate well with the amyloid plaque burden (Yu and Lu, 2012) highlighting a central role of soluble oligomeric forms of Aβ that are shown to exert synapto- and neuro-toxic effects (Arendt, 2009). Hereby, we evaluate the action of a positive allosteric modulator of AMPA receptors, both administered in a short- and long-term way, in two different experimental set-ups that mimic the early phases of Aβ-driven AD neuropathology.

Our first experimental set-up focused on accessing the *in vivo* efficacy of short treatment with S47445, evaluating 3 different dosages of the compound against the detrimental effects of Aβ oligomers bilateral injection in rat hippocampus. Administration of $A\beta_{1-40}$ oligomers in the hippocampus have been shown to induced neuronal malfunction and related cognitive deficits few days after its delivery, proving to be a reliable and fast experimental platform for monitoring dose-dependent protective effects of compounds of interest (Li et al., 2004; Xuan et al., 2012). After 7 days of $A\beta_{1-40}$ oligomers injections, intraperitoneal (i.p.) compound administration was performed daily for 7 days.

Behavioral analysis of the animals started with Open Field (OF) test for monitoring locomotion. As shown in Figure 14, no differences were found in the distance traveled in the OF task [1-way ANOVA; $F_{(4,23)}$ =0.155, p=0.145], indicative that drug administration exerted no effects on animal's locomotive activity (**Figure 13b**).

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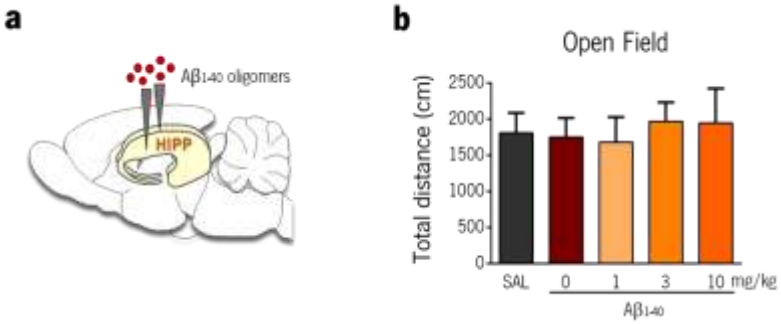


Figure 13: Treatment with S 47445 didn't alter animal's locomotor activity. A*) Schematic representation of the in vivo model used were Aβ oligomers were bilaterally injected into the dorsal part of rat hippocampus.* ***B****) No significant differences of total distance travelled in the OF arena were found*

among all groups suggesting no effect of any treatment on locomotion. All numeric data are represented as mean ± SEM.

Next, we monitored cognitive performance using two different behavioral tests, Y-maze and Novel Object Recognition (NOR) for assessing short- and long-term memory, respectively. In Y-maze test (**Figure 14a**), animals injected with $A\beta_{1-40}$ oligomers exhibited a tendency for reduced preference of travelled distance in the novel arm of Y-maze task (monitored by preference index) in comparison to the control group but this effect didn't reach significance. However, Aβ-injected animals treated with 1mg/kg and 10mg/kg S47445 exhibited a significant increase of their preference index ($p_{1mg/kg}$=0.040, $p_{10mg/kg}$ =0.044) which is suggestive of a clear cognitive improvement in comparison to Aβ-injected animals [1-way ANOVA, $F_{(4,22)}$ =4.098 p=0.012] (**Figure 14b**). Note that the 3mg/kg S47445 dose seems to exhibit a beneficial effect too but this is not significant. Furthermore, the Aβ-injected animals presented severely reduced discrimination index indicating clear deficits of recognition memory (p=0.009), as accessed by the Novel Object Recognition test [$F_{(4,23)}$ =4.550, p=0.007] (**Figure 14d**). The above Aβ-driven cognitive impairment was reverted upon S47445 treatment at the two higher dosages of the compound as the discrimination index of Aβ+3mg/kg and Aβ+10mg/kg groups were significantly higher compared to Aβ group ($p_{3mg/kg}$ =0.048; $p_{10mg/kg}$ =0.035). Note that, in contrast to Y-maze test, hereby, the lower dose exhibit no clearly beneficial effect on Aβ group. Altogether, the above behavioral findings suggest a cognitive-enhancing role of a short-term (7 days) administration of S 47445 against Aβ-driven cognitive impairment which was more profound in the two higher doses.

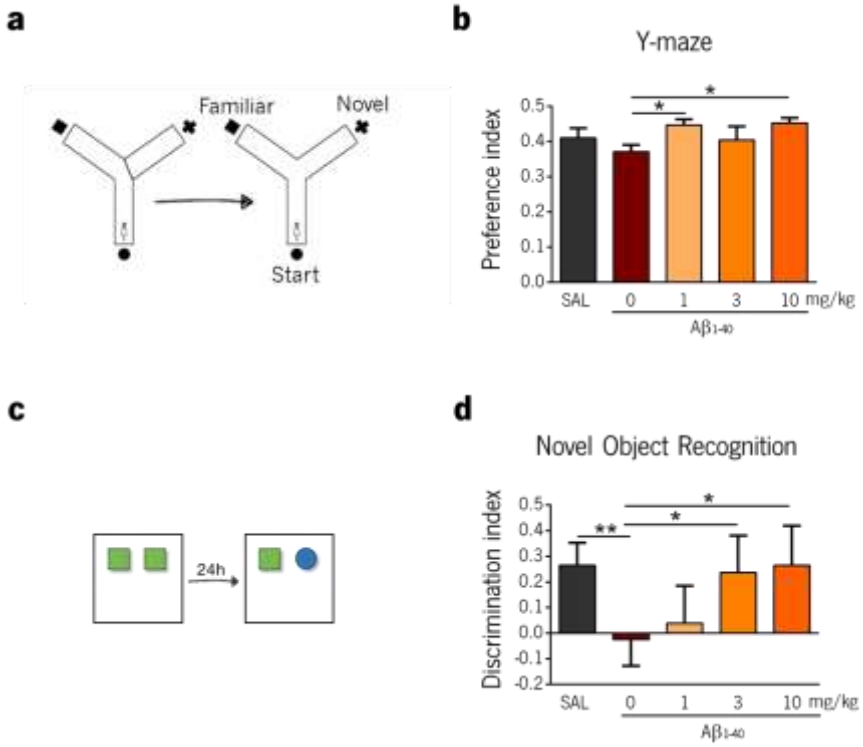


Figure 14: Aβ1-40 oligomers injection in the hippocampus triggers cognitive deficits that are reverted upon treatment with S 47445 compound. **A***) Schematic representation of the Y-maze testing protocol monitoring short-term memory where the animal explores to 2 arms of the Y-maze apparatus and, 3 hours later, the same animal is free to explore all 3 arms including the novel one.* ***B****) Aβ group exhibited a tendency towards a decreased preference index compared to the control (vehicle-treated) group while treatment with 1mg/kg and 10mg/kg of S47445 significantly increase their preference index indicating cognition-enhancing effects.* ***C****) For NOR long-term recognition memory test, the animals were exposed to two identical (familial) objects while, 24 hours later, they are presented to a novel and a familial object.* ***D****) Aβ group presented a decrease in the discrimination index in comparison to the controls while 3 and 10, but not 1mg/kg dose of S47445 ameliorated this reduction suggesting that the two higher doses of the compound blocked Aβ-driven memory deficits. All numeric data are represented as mean ± SEM, *p<0.05.*

Long treatment S47445 reverts cognitive but not mood, deficits against a combined stress/Aβ-driven AD model

Clinical studies suggest depression as a risk and maybe prodromal state to AD with common neurological basis between depressive and AD pathologies. Moreover, as stress is causally related to both depression and AD, previous works support chronic stress as the connecting parameter between both disorders (Sotiropoulos et al., 2008). For monitoring the potentially beneficial effect of S47445 compound against both depressive and early stages of AD neuropathology, we have used a previously

described model where chronic stress is combined with $A\beta_{1-40}$ i.c.v. infusion followed by chronic treatment (30 days) with two higher dosages of the S47445 compound (3mg/kg and 10mg/kg) based on the above findings – for more info, see materials and methods.

We have performed an extended battery of behavioral tests monitoring anxiety, depression and cognitive performance in these animals.

Using the OF test for monitoring locomotion activity, we found that there were no overall differences among all groups in the total distance that animals travelled in the OF arena suggesting the absence of significant effect on locomotor activity (**Figure 15b**).

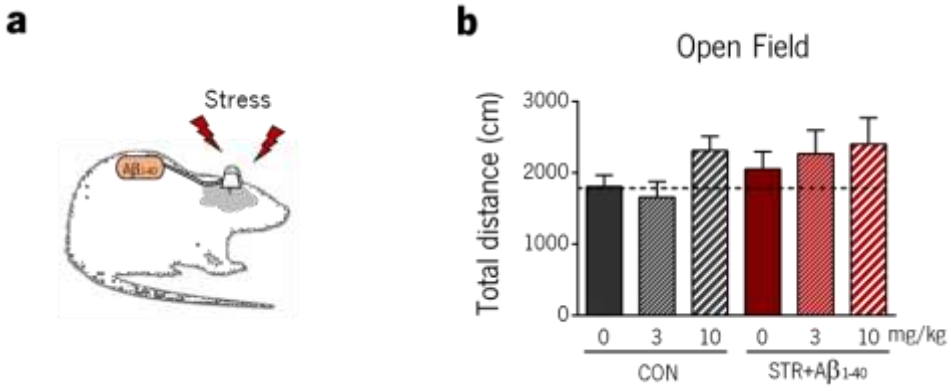


Figure 15. Long treatment with S47445 does not impair locomotor activity. **A**) Schematic representation of the combined stress/Aβ AD model (Catania et al., 2009) where animals were exposed to a chronic unpredictable stress protocol along with $A\beta_{1-40}$ infusion for 2 weeks while S47445 compound were administered daily for 4 weeks. **B**) No differences of total distance travelled in OF arena among all experimental groups suggesting no locomotor deficits. All numeric data are represented as mean ± SEM.

For measuring anxiety levels, we have next used Elevated Plus Maze (EPM) test (Figure 17). 2-way ANOVA analysis revealed an overall *Stress/Aβ* effect [$F_{(1,45)}$=12.668, p=0.0009] on time that animals spent in open arms of EPM while further analysis showed that the stress/Aβ group exhibited decreased time in the open arms compared to control group (p=0.021) suggesting increased anxiety levels (**Figure 16b**). Similar significant differences were found between control and Stress/Aβ that receive 3 or 10 mg/kg of S47445 compound ($p_{3mg/kg}$=0.048, $p_{10mg/kg}$=0.036). No overall effect of *Compound* on time in open arms was found ($F_{(2,45)}$ =0.567, p=0.571). Similar effects were found regarding the entries in the open arms of the EPM with an overall *Stress/Aβ* effect [$F_{(1,45)}$ =14.73, p=0.0004]. Posthoc analysis showed that the Stress/Aβ group, Stress/Aβ+3mg/kg and Stress/Aβ+10mg/kg presented elevated levels of anxiety compared to the control group as monitored

by decreased number of entries in the open arms ($p_{Stress/A\beta}$ =0.039, $p_{3mg/kg}$=0.016, $p_{10mg/kg}$=0.049); no overall effect of *Compound* was also found for entries in the open arms (**Figure 16c**).

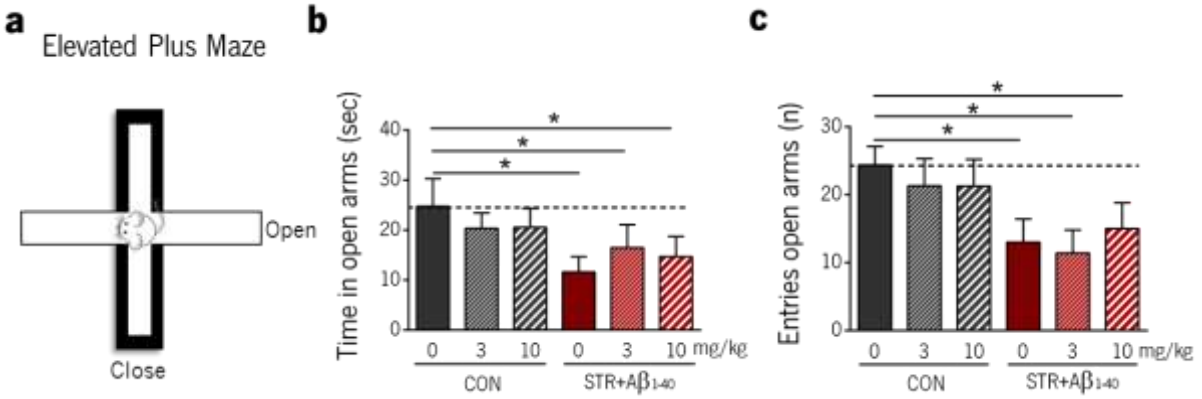


Figure 16. Long treatment with S47445 does not exhibit an anxiolytic effect on stress/Aβ1-40 AD model. A*) Schematic representation of the Elevated Plus Maze task paradigm where animal is freely exploring both open and closed arm of the apparatus.* ***B-C****) The* stress/Aβ group *exhibited reduced time (****B****) and entries (****C****) in the open arms of EPM apparatus in comparison to the control group suggesting increased anxiety levels. Furthermore, no effect of the S47445 compound was observed in both the time and entries in the open arms suggesting that the compound lacks anxiolytic action. All numeric data are represented as mean ± SEM, *p<0.05.*

Next, we evaluated the learned helplessness parameter of the depressive symptomatology using the Forced Swim Test (FST). Statistical analysis revealed an overall effect of *Stress/Aβ* [$F_{(1,45)}$ =35.504, p<0.0001], as Stress/Aβ group presented an increase in the time of immobility (p=0.0001), indicative of a depressive-like behavior. Similar changes were observed in Stress/Aβ+3mg/kg and Stress/Aβ+10mg/kg when compared to control group ($p_{3mg/kg}$ <0.0001, $p_{10mg/kg}$ =0.01). No overall effects of *Compound* were observed [$F_{(2,45)}$ =1.616, p=0.21] (**Figure 17b**). These results indicate that the S47445 had no effect in anxious behavior in the Stress/Aβ groups or the control ones.

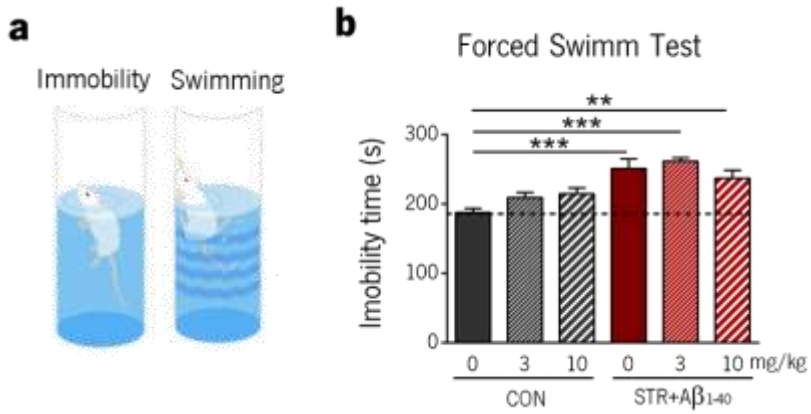


Figure 17. S47445 treatment exerted no anti-depressive action against stress/Aβ$_{1-40}$ exposure. A*) Schematic representation of immobile and swimming behavior during the Forced Swim test.* ***B****) The stress/Aβ, Stress/Aβ+3mg/Kg and Stress/Aβ+10mg/Kg groups exhibit increased immobility time when each compared to the control group indicating learned helplessness, an essential parameter of depressive-like behavior. No overall effect of S47445 compound was found on depressive-like behavior. All numeric data are represented as mean ± SEM, **p<0.01; ***p<0.001.*

We evaluated the cognitive performance of the animals using different behavioral tests. For assessing short-term memory, we have used the Novel Place Recognition (NPR) test where statistical analysis revealed an interaction between *Stress/Aβ* and *Compound* on discrimination index [$F_{(2,45)}$ =6.081, p=0.005]. Posthoc analysis showed that stress/Aβ group exhibited decreased discrimination index compared to control (p<0.0001), indicative of memory loss as this index expresses the ability of the animal to positively distinguish between the new and old location of the object (time of exploration of new location object-time exploration old location object/total time exploration) (**Figure 18b**). In addition, the discrimination index of Stress/Aβ+3mg/kg and Stress/Aβ+10mg/kg groups were significantly different when compared with the index of the (vehicle-treated) Stress/Aβ group ($p_{3mg/kg}$=0.001, $p_{10mg/kg}$=0.0004) suggesting that 3mg/kg and 10mg/kg reverted the Stress/Aβ-driven cognitive deficits. A similar pattern was observed in the preference index reflecting the preference of the animal to the new location of the object (time of exploration of new location/total time exploration). We found *Stress/Aβ* x *Compound* interaction [$F_{(2,45)}$ =6.081, p=0.005] with stress/Aβ group exhibiting decreased preference index compared to control (p<0.0001). Moreover, 3mg/kg and 10mg/kg treatment increased the preference index compared to the (vehicle-treated) stress/Aβ group ($p_{3mg/kg}$ =0.001, $p_{10mg/kg}$=0.0004). No effect of the S47445 compound was found in the control animals in both indexes monitored.

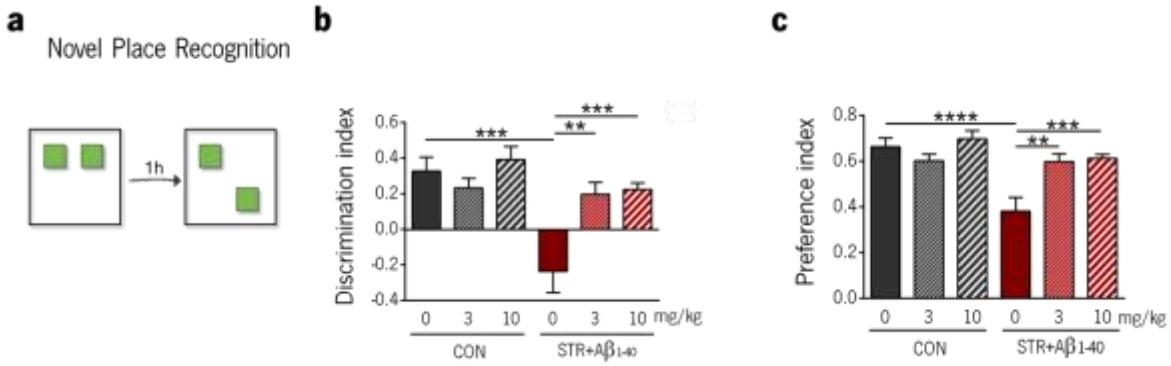


Figure 18. Long S47445 administration blocked the stress/Aβ1-40-induced deficits in short-term recognition memory. **A**) *Schematic representation of the Novel Place Recognition (NPR) test where animals where presented with two identical objects while 1 hour later, the place of one of the objects was changed.* ***B-C****) Exposure to combined stress/Aβ decreased both the discrimination and the preference index when compared to control conditions in the NPR (discrimination index: p<0.0001, preference index: p<0.0001) indicating deficits in short-term recognition memory. Both treatment with 3mg/kg and 10mg/kg were able to revert the stress/Aβ-driven cognitive deficits increasing the discrimination and the preference indexes up to the control levels (discrimination index: $p_{3mg/kg}$=0.001; $p_{10mg/kg}$=0.0004) (preference index: $p_{3mg/kg}$ =0.001; $p_{10mg/kg}$=0.0004). All numeric data are represented as mean ± SEM, **p<0.01; ***p<0.001.*

In addition, we also performed the Novel Object Recognition (NOR) for monitoring long-term memory. 2-way ANOVA statistical analysis revealed an *Stress/Aβ* x *Compound* interaction [discrimination index: $F_{(2,45)}$ =4.232, p=0.021] [Preference index: $F_{(2,45)}$=4.232, p=0.021]. Posthoc analysis showed that stress/Aβ group showed decreased discrimination and preference indexes (discrimination index: p=0.002, preference index: p=0.002) (**Figure 19b**) when compared to control animals. Furthermore, only the higher, 10mg/kg, dosage of the S47445 compound were significantly different from the (vehicle-treated) Stress/Aβ group indicating that the higher dose reverted the stress/Aβ-driven cognitive deficits (p=0.002, for both discrimination and preference index). No effect of the S47445 compound was found in the control animals in both indexes monitored.

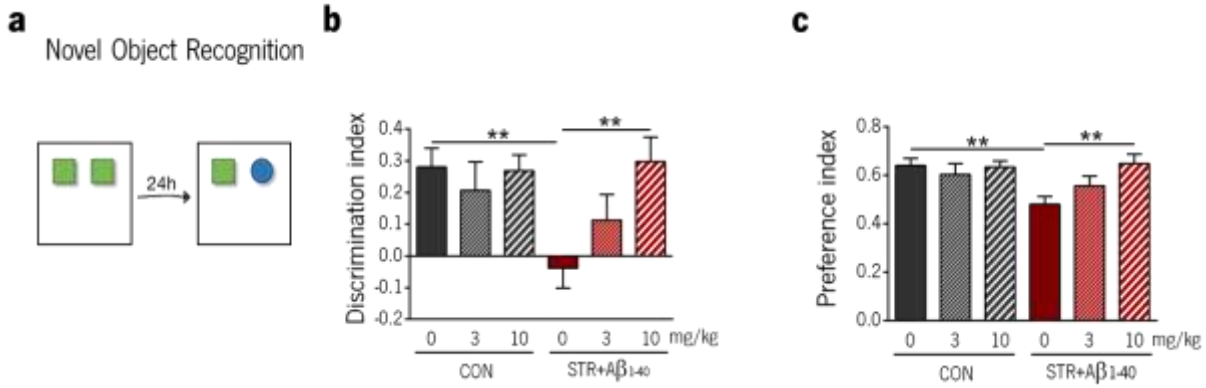


Figure 19. Long-term memory deficits in the stress/Aβ1-40 animals were reverted by the higher dose of S47445. **A***) Experimental process of Novel Object Recognition (NOR) test were animals are allowed to freely explore two identical objects while 24 hours later, one of object is changed with a novel one.* ***B-C****) stress/ Aβ group showed reduced discrimination and preference indexes when compared to control group suggesting impairment of long-term recognition memory (discrimination index: p=0.002, preference index: p=0.002). These cognitive deficits were only reverted upon treatment with 10mg/kg of S47445 compound (discrimination index: p=0.002, preference index: p=0.002). All numeric data are represented as mean ± SEM, **p<0.01; ***p<0.001.*

Furthermore, we also monitor animal's spatial reference memory using the Morris Water Maze (MWM) test. Repeated-measurement 2-way ANOVA statistical analysis showed an overall *Compound* effect [$F_{(3,116)}$=3.954, p=0.01]. Further analysis revealed that Stress/Aβ+3mg/kg and Stress/Aβ+10mg/kg groups exhibited reduced distance swum to reach the escaping platform in Day 2 when compared to Stress/Aβ indicating improvement of spatial reference memory ($p_{3mg/kg}$=0.04; $p_{10mg/kg}$=0.027) (**Figure 20b**). However, no differences were found between control groups treated with different doses of the S47445 compound (**Figure 20a**). Moreover, no differences in time swum in the target quadrant were found among groups during the Probe test ($F_{(2,45)}$ = 1.091, p=0.345), with all groups spending similar time (>25%) in the target quadrant; this is indicative of a learning process indicating that, in the end of the test, all groups learned the position of the platform (**Figure 20c**).

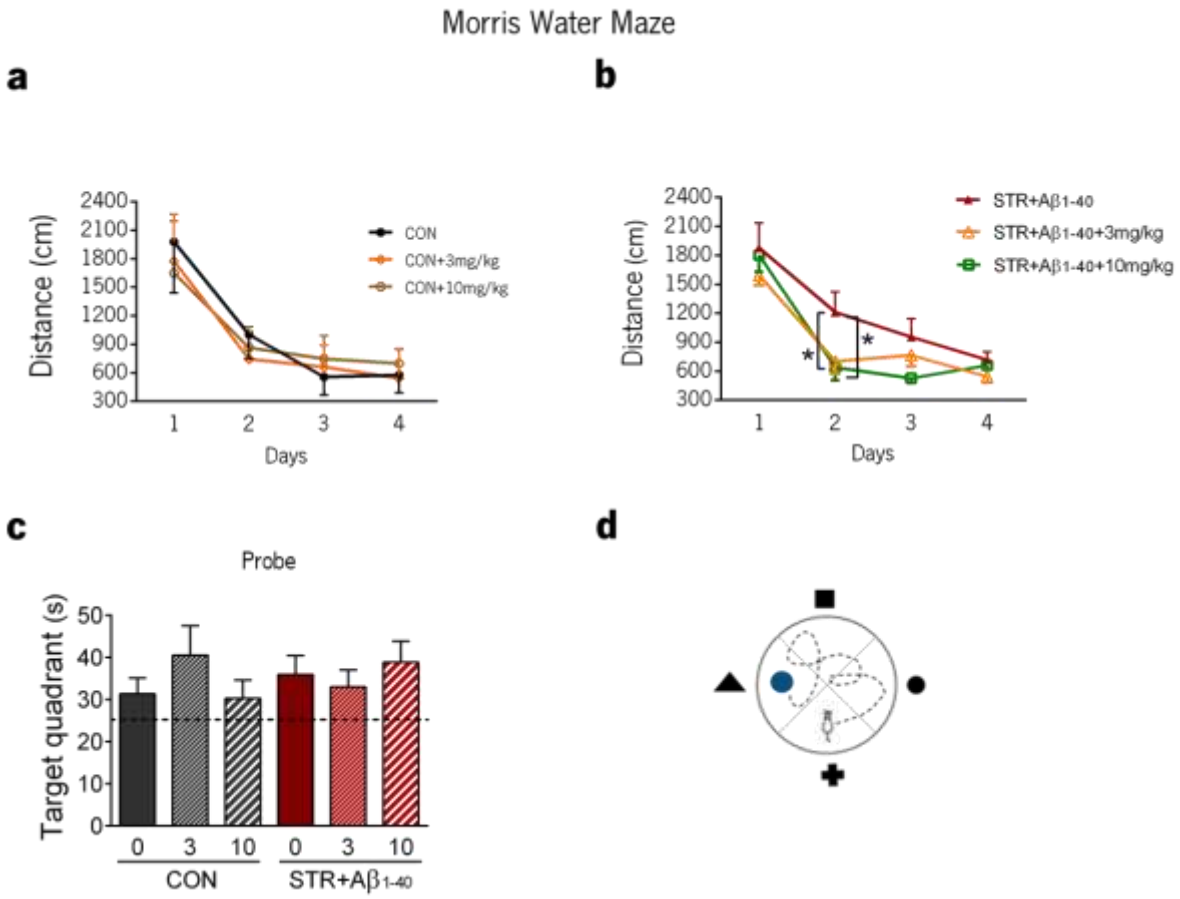


Figure 20. S47445 long treatment significantly improves spatial reference memory of stress/Aβ1-40 animals. A-B*) While no effect of the S47445 compound was found in the control groups (**A**), the stress/Aβ group treated with 3mg/kg or 10mg/kg of S47445 exhibited reduced distanced swum in Day 2 than the (vehicle-treated) Stress/Aβ group (3mg/kg: p=0.0447, 10mg/kg: p=0.0270) (**B**). **C**) In the probe test trial, all animal groups swum more than 25% in the target quadrant while no significant differences were found among groups. **D**) Scheme showing the Morris Water Maze swimming platform and the target quadrant. All numeric data are represented as mean ± SEM, *p<0.05.*

Further analysis of MWM data includes the division of the spatial navigation strategies which were followed by the animals during the MWM learning phase into three categories: 1) Failure, where animal never found the platform 2) Random strategies, where animals exhibit non-hippocampal-dependent, random searching or circling strategies and 3) Direct, where the animal has a hippocampal-dependent, very spatially directed swimming path towards the escaping platform. ((Graziano et al., 2003); see also Figure 22a). 2-way ANOVA statistical analysis revealed an interaction between *Stress/Aβ* and *Compound* on the percentage of direct strategies [$F_{(2,45)}$ =6.963, p=0.002] followed by overall *Compound* effect [$F_{(2,45)}$ =5.133, p=0.009]. Posthoc analysis showed that Stress/Aβ group adopted reduced percentage of direct (hippocampus-dependent) strategies to reach the escaping platform suggesting hippocampal malfunction (p=0.035) (**Figure 21b**). Furthermore, 3 or 10mg/kg

treatment in Stress/Aβ animals attenuated the above reduction in direct strategies ($p_{3mg/kg}$ =0.005; $p_{10mg/kg}$ =0.001). In addition, an overview of the group division into the three categories of swimming strategies (stacked area charts; **Figure 21c**) can also provide obvious differences between Stress/Aβ+10mg/kg animals that exhibit higher direct strategies, as well as less random strategies and failures when compared to Stress/Aβ or Con+10mg/kg animals.

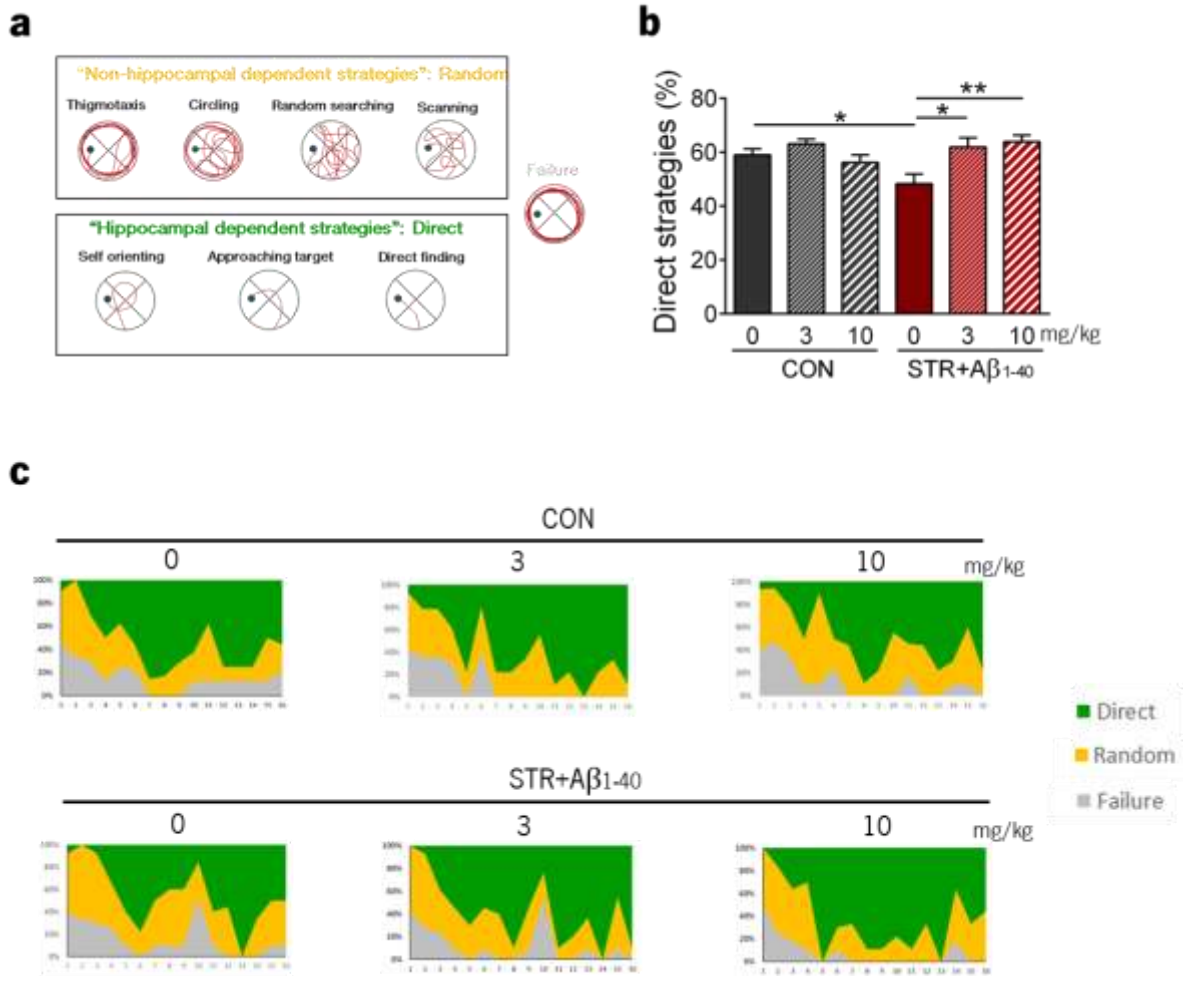


Figure 21. Long treatment with S47445 switched the strategy choices towards the hippocampal-dependent ones. A*) Schematic representation of the division of swimming strategies into three categories: 1) Failure 2) Random strategies and 3) Direct strategies.* ***B****) The Stress/Aβ animals followed significantly less direct strategies to reach the platform across the 4 days of the trial than the control group (p=0.035).* ***C****) The stress/Aβ-driven decrease in the percentage of directed strategies was reverted upon treatment with 3mg/kg or 10mg/kg.* ***D****) Stacked area charts displaying the percentage of failures (grey), random (yellow) and direct (green) strategies across the 16 trials of the learning period of MWM for all groups. Note that the Stress/Aβ+10mg/kg animals exhibit higher direct strategies, and less random strategies and failures when compared to Stress/Aβ or Con+10mg/kg animals.*

Altogether, the above behavioral data provide the first *in vivo* evidence of the cognition-enhancing properties of long-term S47445 treatment against the combined Stress/Aβ AD model. However, the S47445 had no anxiolytic or antidepressive action in this animal model.

Molecular underpinning of S47445 beneficial effects: insights by CSF

Loss of synaptic structure and function is thought to mark early stages of AD (Selkoe, 2002) while soluble Aβ oligomers are thought to strongly damage synaptic plasticity, dendritic spines, and pre- and postsynaptic composition (Shankar et al., 2007; Walsh et al., 2002). Many clinical and experimental studies suggest the usefulness of cerebrospinal fluid (CSF) analysis for the diagnosis of AD as many biochemical markers related to neuronal and synaptic pathology in AD brain (e.g. APP, Aβ, Tau) can be also measured in CSF reflecting the state and progression of AD (Portelius et al., 2017).

Thus, we performed Mass Spectroscopy analysis of CSF samples of the long S47445 treatment experiment focused on different proteins related to synaptic structure and plasticity. Our trypsin-based MS analysis detected transmembrane APP, known to be concentrated at the neuronal synapses regulating their formation (Priller et al., 2006) and plasticity (Turner et al., 2003). Although no statistically significant differences were found among the groups, the stress/Aβ group exhibited a tendency of decrease of APP levels in CSF whereas this decrease is obviously reverted upon treatment with both 3 and 10mg/kg of S47445 compound, with the higher dose having the clearer effect (**Figure 22a**). We also detected the levels of Rab3, known to play a regulatory role in exocytosis process during neurotransmitter release (Roselli et al., 2009). Here, our preliminary findings suggested a decrease in the Rab3 levels of the Stress/Aβ group; importantly, this decrease seems to be reverted upon treatment with S47445 (**Figure 22b**). Furthermore, the CSF MS Spec analysis also revealed that exposure to combined stress/Aβ seems to slightly decrease the levels of HOMER1, an essential postsynaptic scaffold protein, whose levels alterations could indicate an impairment/reduction in dendritic spine.s Moreover, the S47445 treated animal groups exhibit an elevation of HOMER1 levels to the control group levels, possibly indicating a synaptogenic action of the compound (**Figure 22c**). In addition, we also detected neuroplastin, a protein that has recently been identify to mediate the contact between pre- and postsynaptic neurons implicated into the synaptic plasticity in hippocampus (Bernstein et al., 2007). Despite that they are no significant changes, our preliminary data suggested a

decrease of neuroplastin in CSF of Stress/Aβ animals in comparison to the controls, and that this decrease is partially reverted upon treatment with the compound (**Figure 22d**). Note that the CSF levels of SHANK2 did not change in Stress/Aβ group. Although these CSF findings are preliminary, they support a synaptogenic effect of S47445 compound against the synaptic malfunction and loss driven by Stress/Aβ.

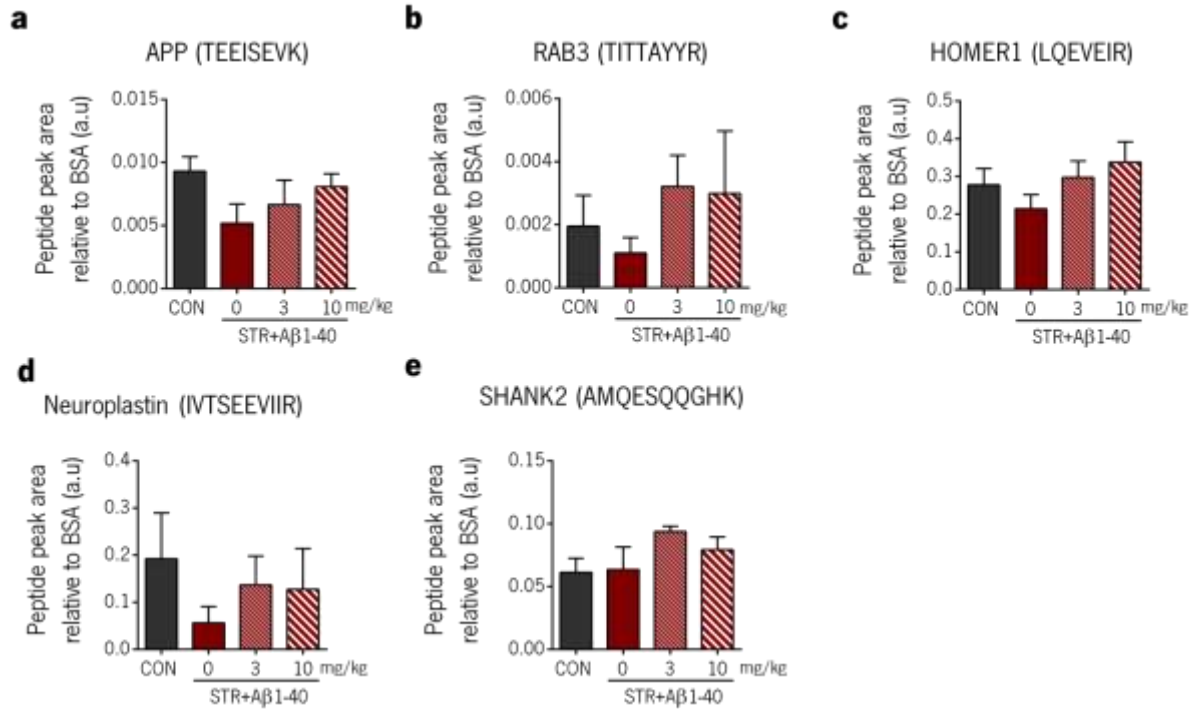
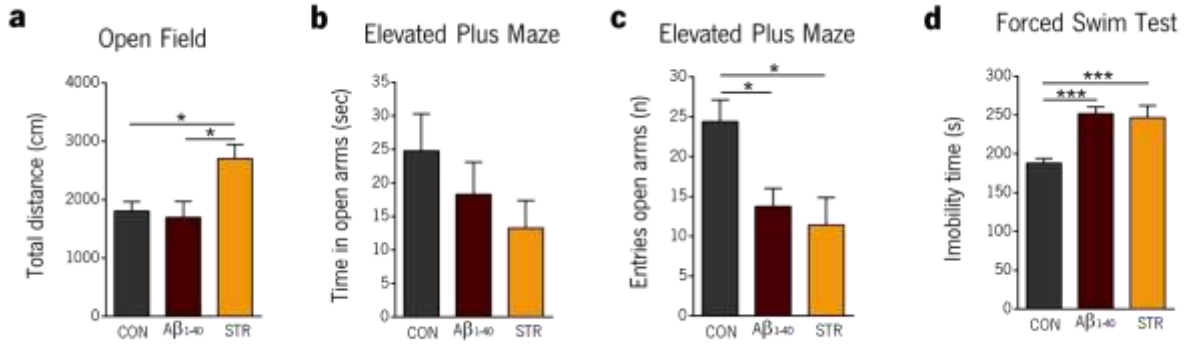


Figure 22: Mass spectrometry analysis of CSF suggested an overall enhancement of synaptic structure and function against the synaptotoxicity driven by Stress/Aβ. *Despite preliminary, the first MS data suggest that the stress/Aβ animals exhibit decrease in the levels of different synaptic proteins such as APP levels (**A**), Rab3 (**B**), Homer 1 (**C**) and Neuroplastin (**D**) while S47445 treatment seems to exhibit a synaptogenic action in all above proteins. However, the levels of Shank2, a scaffold protein, are not altered among all groups. All numeric data are represented as mean ± SEM.*

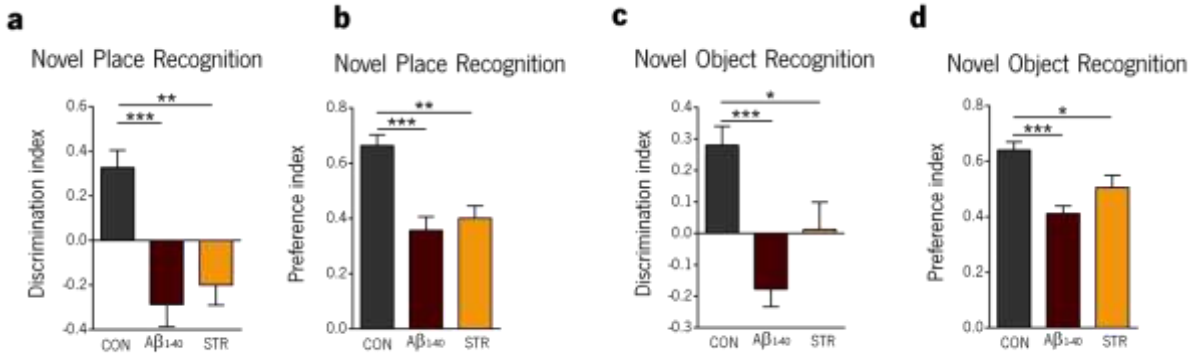
Supplementary Figure 1

Supplementary Figure 1. Both Aβ1-40 administration as well as exposure to chronic



stress alone leads to an anxious and depressive-like behavior. A*) In contrast to stress, Aβ infusion didn't induce locomotor deficits as assessed by no changes in the total distance traveled in the Open Field (OF) arena [1-way ANOVA; $F_{(2,24)}$ =0.531, p=0.59).; **B-C**) Exposure to stress or Aβ infusion evoked no changes in the time spent in the open arms of the EPM apparatus; however, entries in the open arms were reduced by stress (p=0.013) and Aβ (p=0.043)[1-way ANOVA; $F_{(2,24)}$=5.552, p=0.01]. **D**) In Forced Swim test, both stressed and Aβ-infused groups presented increased immobility time (p_{Stress}=0.003; $p_{Aβ}$=0.007) [1-way ANOVA; $F_{(2,24)}$=7.817, p=0.002].*

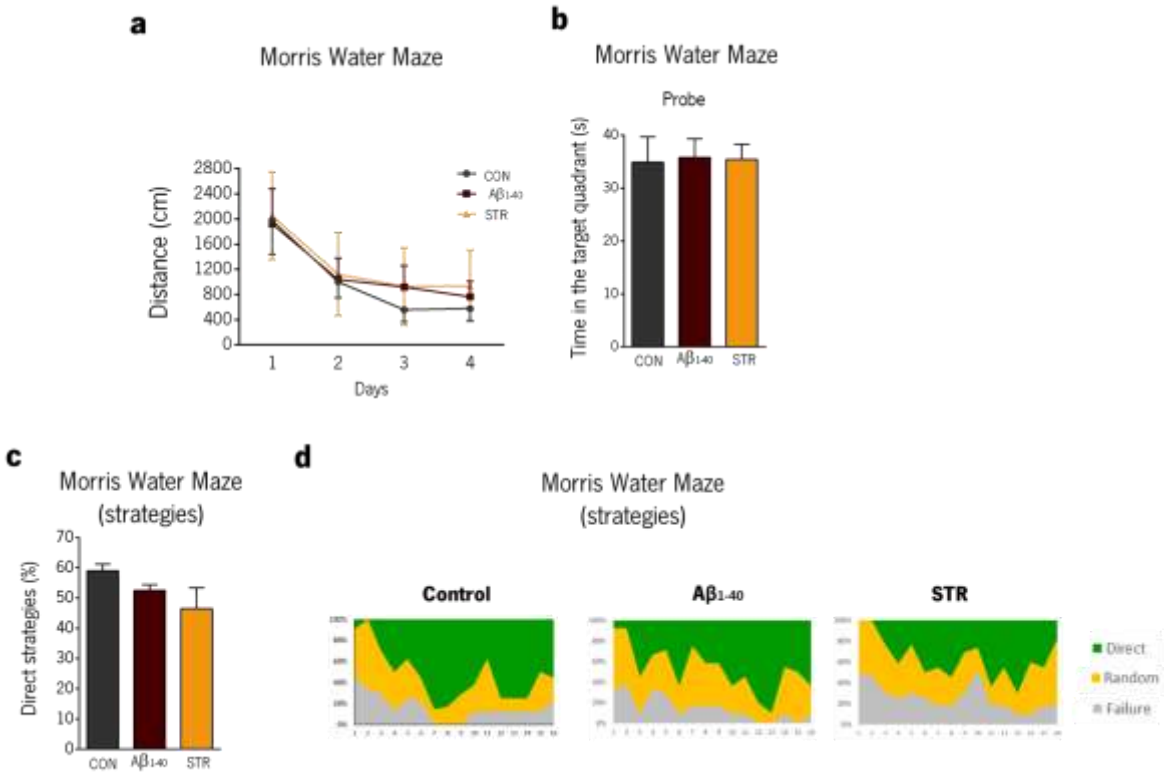
Supplementary Figure 2



Supplementary Figure 2. Exposure to chronic stress or Aβ1-40 infusion leads to impairments of recognition memory. A-B*) In Novel Place Recognition (NPR) test, chronic stress or Aβ infusion led to reduced discrimination index when compared to control animals (p_{Stress}=0.001; $p_{Aβ}$ =0.0003) [1-way ANOVA; $F_{(2,24)}$=12.36, p=0.0002) while a similar pattern is observed in the preference index (p_{Stress}=0.001; $p_{Aβ}$ =0.0003) [1-way ANOVA; $F_{(2,24)}$=12.34, p=0.0002] indicating deficits in short-term recognition memory. Similarly, long-term recognition memory as assessed by Novel Object Recognition (NOR) was also damaged by chronic stress or Aβ infusion; for discrimination index, $F_{(2,24)}$=10.77, p=0.0005 & p_{Stress}=0.041; $p_{Aβ}$ =0.0003; for preference index, $F_{(2,24)}$=10.77, p=0.0005)& p_{Stress} =0.041, $p_{Aβ}$ =0.0003).*

Supplementary Figure 3

Supplementary Figure 3. No significant changes were found in stress or Aβ groups



in the Morris Water Maze. **A-B**) No differences were found in distance swum during the learning period as well as probe test of MWM among the above groups [for learning period, $F_{(6,69)}$=0.447, p=0.844]; [the probe test $F_{(2,24)}$=0.014, p=0.986). **C-D**) No significant differences were found among groups in the direct swimming strategies [$F_{(2,24)}$= 1.206, p=0.317); however, stacked area charts displaying the percentage of failures (grey), random (yellow) and direct (green) strategies across the 16 trials of the learning period of MWM suggest that stressed animals exhibited more random, non-directed strategies and less direct, hippocampus-dependent strategies (**D**).

CHAPTER 5

DISCUSSION

DISCUSSION

Alzheimer's disease and glutamate signaling

The increasing awareness that dementia and particularly Alzheimer's disease, represents one of the major challenges to health systems in upcoming years, has prompt an unprecedent emphasis on the need for effective therapies, that could change the heading of the disease (Winblad et al., 2016). However, although the disease has been described more than 100 years ago and our knowledge about the disease pathology has tremendously increased, there is still no effective treatment capable of blocking AD neuropathology. Accumulating evidence identifies AD as a primarily synaptic disorder promoting a change of the focus of our preclinical and clinical studies during the recent years towards the synaptic protection and/or recovery from AD synaptotoxicity (Reuillon et al., 2016). Since synaptic dysfunction seems to be one of the earliest pathological mechanisms underlying Alzheimer's disease, and a clear dysfunction of the glutamatergic pathways is found in several dementias such as AD (Henley and Wilkinson, 2013; Rudy et al., 2015), targeting glutamate-related pathways could constitute a promising therapeutic approach against AD. Moreover, recent evidences have shifted the researchers focus from the amyloid plaques to soluble oligomeric Aβ as a more likely triggering molecule of AD pathology (Barghorn et al., 2005; Christensen et al., 2008). These Aβ soluble oligomers have been tied to disruptions in glutamate synaptic transmission (Mucke et al., 2000; Shankar et al., 2007; Walsh et al., 2002) and can result in excitotoxicity through several different mechanisms, including stimulation of glutamate release, inhibition of glutamate uptake and alteration of signaling pathways related to activation of glutamatergic receptors. This dysregulation of excitatory glutamatergic neurotransmission by (soluble) Aβ may be underlying the early cognitive decline in AD, by promoting synaptic alterations and Tau hyperphosphorylation (Crimins et al., 2013). Two main receptors are involved in the response to glutamate, NMDA and AMPA receptors, which are involved in regulating synaptic plasticity and play a major role in learning and memory (Eccles, 1983). Several drugs have been developed so far targeting NMDA receptors function; however, results are inconsistent, and memantine, the most promising NMDA-targeting drug that mainly inhibit extrasynaptic NMDA receptors (Xia et al., 2010), exhibits limited magnitude effect (Rampa et al., 2013). Thus, pharmacological regulation of other glutamate-related target should be tested (Partin, 2015; Reuillon et al., 2016; Tayeb et al., 2012). For instance,

the modulation of AMPA receptors offer a promising therapeutic possibility, due to their crucial role in long-term potentiation (LTP), known to underlie memory and learning, a mechanism that is highly dysfunctional in AD (Brown and Banks, 2015; Gruart et al., 2015; Huganir and Nicoll, 2013). Several AMPA-targeting compounds have been recently development, such as AMPA positive allosteric modulators. These modulators act either by attenuating desensitization of the receptor, a process by which the receptor ion channel closes although glutamate remains tightly bound, and/or by slowing receptors deactivation, i.e slowing the rate at which the ion channel closes after removal of glutamate (Morrow et al., 2006; Partin, 2015). In general, they stabilize AMPA receptor in its active conformation following glutamate binding and enhance synaptic currents, thereby promoting synaptic transmission and plasticity (Hanada, 2014; O'Neill and Dix, 2007). These modulators have been reported to facilitate the LTP induction (Sobolevsky, 2015; Whitlock et al., 2006), improve episodic and spatial working memory (Bernard et al., 2010; Black, 2005; Partin, 2015) and thus, may constitute promising targets for treatment of AD synaptic pathology.

The beneficial effect of treatment with S47445 compound against Aβ-driven brain pathology

The current Master studies report for the first time the use of S47445 compound, a positive and selective AMPA modulator against the cognitive deficits induced by Aβ oligomers as well as by chronic stress combined with Aβ. S47445 has been shown to exert no deleterious effects even in prolonged administration, and to enhance glutamate's action at AMPA receptors (Bretin et al., 2017). This action occurs both via decreasing receptor response decay time and by increasing the sensitivity of the receptor to glutamate. Moreover, S47445 did not demonstrate neurotoxic effects against glutamate-mediated excitotoxicity in vitro while exhibits a protective effect against this excitotoxicity in rat cortical neurons (Bretin et al., 2017). We hereby evaluated the efficacy of short S47445 treatment in a non-transgenic AD model consisting of bilateral hippocampal injection of aggregated $A\beta_{1-40}$ peptide that may resembles the early phases of AD (Medeiros et al., 2007; Prediger et al., 2007). Although this model has been shown to be unable to reproduce all the pathological AD hallmarks, it is useful for studying Aβ oligomeric neurotoxicity and related spatial learning and memory impairments (Colle et al., 2013; Haass and Selkoe, 2007; Prediger et al., 2007). Indeed, in line with the above findings, Aβ

oligomers injection in rat hippocampus presented both short-term and long-term memory impairments. Interestingly, treatment with the 3 doses of S 47445 ameliorated these cognitive deficits, with the higher doses being the most effective ones.

In fact, Giralt and colleagues reported that administration of S47445 displays a beneficial effect on synaptic plasticity and connectivity both in hippocampus and pre-frontal cortex of aged mice, supporting the memory-enhancing properties of S47445 (Giralt et al., 2017a). Administration of S47445 was shown to improve episodic-like memory as well as hippocampal-dependent contextual discrimination tasks. Moreover, Louis and colleagues also showed that S47445 is able to improve working memory performance in several cognitive behavioral tests, supporting the reversion of the cognitive deficits that we also observed with 3 and 10mg/kg of S47445 compound. Moreover, Giralt group has also shown that S47445 exerts positive long-lasting effects on LTP without disturbing basic synaptic physiology in the hippocampus, in line with previous data showing no noticeable CNS side-effects of the compound that are in line with the absence of S47445 behavioral impairments that we found in our behavioral analysis.

The above findings are in line with the current Master Mass Spect findings. Although not reaching statistical significance due to limited numbers of samples per group that are analyzed by the time that this Master thesis was written, the stress/Aβ group exhibited a decrease in several proteins related to synaptic plasticity and structure, namely Rab3, Homer1 and neuroplastin; however, no changes in the levels of Shank2 were found. These data goes along with previous data demonstrating a decreased levels of Rab3 in brain of AD patients (Reddy et al., 2005), indicating that reduction of synaptic vesicle proteins may be related to clinical dementia, given the important role these proteins play in synaptic transmission (Südhof et al., 1993). Additionally, Homer1 is known for acting as a modulator of glutamatergic signaling, highly implicated in AD and stress pathologies; and so, its downregulation could be implied in depression/Aβ pathology (Rietschel et al., 2010). Furthermore, previous studies have shown that neuroplastin depletion in glutamatergic neurons impaired specific behaviors, namely cognition, implying dysfunction of this protein in AD. In contrast, no changes were found in Shank2 levels, probably indicating that in these early stages of the disease, we are only facing a mild synaptic impairment. Moreover, we also found a decrease in the CSF levels of APP in stress/Aβ animals, indicating that stress and Aβ trigger the APP misprocessing towards the amyloidogenic pathway (Catania et al., 2009). Indeed, previous studies have demonstrated that chronic stress promotes APP misprocessing, while similar findings were reported when the Aβ was i.c.v administered

to rats (Catania et al., 2009). Though, is likely that the combination of these two factors are exerting the same effect as when employed alone, providing and explanation for the decreased APP levels in this animal group. In the other hand, we observed an increase in the levels of all these synaptic proteins upon treatment with both dosages (3 and 10mg/kg) of the compound; these findings can be related to the neurotrophic and synaptogenic action of S47445 (Calabrese et al., 2017); indeed, previous studies have already demonstrated that this compound is able to preserve the synaptic cytoarchitecture of hippocampal synapses in old mice (Giralt et al., 2017b).

Long-term treatment of S47445 improves damaged cognitive performance without exhibiting anxiolytic and antidepressant properties

The vast majority of AD cases has a sporadic nature without marked genetic basis, which strongly suggests that the variation in susceptibility to and the time course of the disease arises from a complex interplay between genetic and environmental parameters. Recent studies have implied exposure to chronic stress as an important precipitating factor of AD. Moreover, another stress-related disorder, depression, is shown to predisposes to AD (Sotiropoulos et al., 2008a) while chronic stress is suggested as the connecting parameter between depression and AD. Stress and AD pathology seem to share common neurobiological mechanisms that lead both to synaptic atrophy and loss resulting to cognitive impairments (Lopes et al., 2016; Sotiropoulos and Sousa, 2016; Sotiropoulos et al., 2008a) Moreover, both *in vitro* and *in vivo* studies have pointed out that exposure to stress or stress hormones, glucocorticoids (GC), increase Aβ production and exacerbate Tau pathology, two hallmarks of Alzheimer's disease (Green et al., 2006; Sotiropoulos et al., 2008b, 2011) Previous studies have already demonstrated that the combination of chronic stress and Aβ infusion impairs learning and memory as well as decrease LTP in hippocampus (Catania et al., 2009; Sotiropoulos et al., 2011; Tran et al., 2011; Tran Trinh T. et al., 2011). Thus, we have also tested S47445 compound against the combined model of chronic stress and Aβ infusion that exhibits both stress-driven depressive symptomatology and cognitive impairment. Our behavioral analysis showed anxious and depressive-like behavior in this model, which goes along with reports of frequently neuropsychiatric symptoms in AD (Jost and Grossberg, 1996). Indeed, although not central to the dementia symptomatology, anxiety is a very common feature in demented individuals (Porter, 2013); in fact, some studies suggest that Aβ may play a central role in the manifestation of anxious behavior as intraneuronal Aβ overproduction in

amygdala of 3xTg mice and exogenous amyloid-beta administration resulted in symptoms of anxiety (Catania et al., 2009; España et al., 2010). In addition, chronic stress has been shown to play a major role triggering episodes of depression, while it has also been suggested as a risk factor for AD. Importantly, neuronal and synaptic malfunction are key underlying mechanisms in both AD and depression, while stress is also casually associated with neuronal and synaptic atrophy resulting in impaired mood and/or cognition. Moreover, the pathological mechanism of AD, APP misprocessing and Tau hyperphosphorylation have also been reported to be affected in depression, possibly explaining why exposure to stress combined with amyloid-beta infusion may result in an increased depressive-like behavior. Indeed, our data goes along with data showing that soluble Aβ can affect synaptic function and cognition, as well as emotional behavior (Olariu et al., 2001). In fact, other groups have shown that i.c.v. administration of soluble Aβ induces a depressive-like phenotype (Catania et al., 2009; Colaianna et al., 2010). However, S47445 treatment didn't exhibit anxiolytic and antidepressant action in the combined Stress/Aβ model. Thus, targeting AMPA receptor function under these specific conditions may not be enough to revert these anxious and depressive-like phenotypes.

In regards of what has been described above, it is of major importance to understand the signaling cascades are and molecular targets that underlie S47445 action against Aβ and Stress/Aβ AD pathology. Understanding the molecular mechanisms regulated by S47445 could also offer an explanation to the differential regulation of the different dimensions of the behavior (anxiety, depression and cognition). Overall, this work provides new insights into the potential therapeutic effect of S47445 and opens new window for glutamate-related drug discovery against AD pathology. Thus, S47445 may be considered as potential candidate for the treatment of diseases associated with glutamatergic disfunction, such as Alzheimer's disease.

CHAPTER 6

CONCLUDING REMARKS

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During the past decades, a large effort has been made to unreveal the complex molecular pathways that underlie the AD neuronal and synaptic pathology. However, there is a lack of disease-modifying therapy for AD that stops AD neurodegeneration as the current treatments can only ameliorate temporarily the symptoms of the disease or slow the progression of the cognitive decline. Thus, the search for novel therapies is a pivotal gateway to fight these devastating disorders that affects more than 47 million people worldwide. In this Master thesis, we report a novel modulator of AMPA receptors as a promising therapeutic compound against cognitive impairment of AD. Future studies should clarify the molecular underpinning of the cognitive-enhancing properties of S47445 compound focusing on the key AD pathomechanisms, namely APP misprocessing and Tau hyperphosphorylation related to synaptic malfunction.

Taken together, these Master studies provide the first *in vivo* evidence of the therapeutic efficacy of AMPA regulation against Aβ-driven AD pathology.

CHAPTER 7

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REFERENCES

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