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DOENÇA DE ALZHEIMER PRECOCE: EIXO HPA E MEMÓRIA EPISÓDICA

HPA AXIS FUNCTION AND EPISODIC MEMORY LOSS IN EARLY ALZHEIMER DISEASE

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica especialização em Bioquímica Clínica, realizada sob orientação da doutora Hélène Marie, Chefe de equipa no Institut de Pharmacologie Moléculaire et Cellulaire e do Doutor Pedro Miguel Dimas Neves Domingues, Professor Auxiliar do Departamento de Química da Universidade de Aveiro

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palavras- Chave

Doença de Alzheimer, hipocampo, plasticidade sináptica, memória episódica, stress, eixo hipotálamo-pituitária-adrenal, Corticosterona.

Resumo

A Doença de Alzheimer (DA) é uma doença neurodegenerativa do tecido cerebral que leva à perda da memória e das propriedades intelectuais. É caracterizada pelo aparecimento de oligómeros de amilóide-β (Aβ) que depois se agregam em placas, aparecimento progressivo de agregados neurofibrilares constituídos por proteína tau hiperfosforilada, alterações sinápticas e morte neuronal. O hipocampo, uma estrutura chave responsável pela codificação da memória, é a primeira região cerebral afectada na DA levando numa fase precoce, à perda da memória episódica.

A acumulação de Aβ parece ter uma função importante no desencadeamento de stress crónico na DA levando ao comprometimento da função do eixo HPA e das várias estruturas envolvidas na sua regulação, nomeadamente o hipocampo.

Neste estudo pretendeu-se estudar a função do eixo HPA e avaliar a memória episódica usando um modelo transgénico da DA, o ratinho Tg2576, numa fase precoce da doença de Alzheimer, definida neste modelo por volta dos 4 meses.

Os estudos relativos à função do eixo HPA foram feitos através da quantificação de corticosterona, a hormona principal no stress, por teste ELISA na fase de repouso, na fase activa e após teste de supressão pela dexametasona. Quantificaram-se ainda os receptores aos glucocorticoides (RGs) no hipocampo por western blot. Os ratinhos Tg2576 mostraram um comprometimento do eixo HPA, caracterizada pelo aumento de corticosterona no ínicio da fase activa e ausência de regulação negativa induzida pela dexametasona. Ainda, os RGs estão aumentados e mostram comprometimento na regulação negativa induzida no eixo HPA.

Para avaliar a memória episódica foi efectuado um teste de reconhecimento de objectos que combina a capacidade de recordar o 'quê, quando e onde' de um evento. Os ratinhos Tg2576 apresentaram um deficit na componente 'onde' deste tipo de memória. Foi em seguida aplicado um tratamento *in vivo* com um antagonista dos RGs (RU486) para avaliar se bloqueando a função dos RGs se poderia reverter o deficit observado.

Os nossos primeiros resultados revelam que o bloqueia dos RGs pode prevenir o deficit na memória episódica.

Assim este trabalho mostrou que os ratinhos Tg2576 apresentam uma perturbação ao nível do eixo HPA e da sua regulação pelos RG do hipocampo, traduzidos por um nível de stress aumentado, e perturbação ao nível da memória episódica.

Este trabalho mostra que o nível de stress está aumentado numa fase muito precoce da DA neste ratinho devido à disfunção do eixo HPA. Para além disso, a alteração nesta sinalização mediada pelos RGs, contribui provavelmente para os deficits precoces na memória episódica observados neste ratinho. Estes resultados suportam a nossa hipótese de que o stress é um factor de risco muito importante no desenvolvimento precoce da neuropatologia na doença de Alzheimer.

Key-words Alzheimer's disease, hippocampus, synaptic plasticity, memory, stress, hypothalamus-pituitary-adrenal axis, Glucocorticoids, Corticosterone.

Abstract

Alzheimer's disease (AD) is a brain neurodegenerative disease leading to progressive loss of memory and intellectual abilities. It is characterized by the appearance of amyloid-β oligomers (Aβ), which then aggregate into plaques, progressive appearance of neurofibrillary tangles composed of hyperphosphorylated tau, synaptic impairment and neuronal death. The hippocampus, a key structure responsible for memory encoding, is the first brain region affected in AD leading to early episodic memory loss.

Aβ accumulation seems to have an important role in triggering chronic stress in AD, compromising the hypothalamic-pituitary-adrenal (HPA) axis function and the structures involved in its regulation, notably the hippocampus.

The purpose of the present study was to evaluate the HPA axis function and episodic-like memory in a model of AD, the Tg2576 mice, in the early phase of the pathology, which was defined in these mice at about 4 months of age.

To study the HPA axis function, corticosterone, the main stress hormone, was quantified by ELISA at the onset of light phase, at the onset of dark phase and after inducing the negative feedback with a dexamethasone supression test. Hippocampal glucorticoid receptors (GRs) were also quantified by Western blot. Tg2576 mice showed impairment in HPA axis, characterized by an increase in corticosterone at the onset of active phase and an absence in the negative feedback response induced by dexamethasone. Also, hippocampal GRs are increased and seems to fail in the downregulation of the stress response mediated by the HPA axis.

To evaluate episodic-like memory, an object recognition task was conducted, which combines the ability to remember the 'what, when and where' components of an event. A deficit in the 'where' component of this type of memory was observed in Tg2576 mice. An in vivo treatment with the GR antagonist RU486 was then applied to evaluate if blocking GR function could reverse this deficit. Our first results suggest that blocking GR function can prevent this memory deficit in Tg2576 mice.

These data demonstrate that corticostesterone levels, and thus stress signaling, are increased in the early phase of AD in these mice, due to dysfunction of the HPA axis. Furthermore, this altered signaling, via GRs, probably contributes to the early episodic memory deficits observed in these mice. These data strongly support our hypothesis that elevated stress is an environmental factor contributing to the onset of AD neuropathology.

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LIST OF ABBREVIATIONS

Aβ - amyloid-β

ACTH - adrenocorticotropic hormone

ADAM - A Desintegrin And Metalloproteases

AD - Alzheimer's Disease

AICD - amyloid precursor protein intracellular domain

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

APP - β-amyloid precursor protein

AVP- arginine vasopressin

BACE - ß-site APP-cleaving enzyme

BST – Bed nucleus of stria terminallis

CRH - corticotropin-releasing hormone

CSF- Cerebrospinal fluid

DEX – Dexamethasone

DG – Dentate gyrus

EC – Entorhinal cortex

FAD – Familiar Alzheimer 's disease

GABA- γ−aminobutyric acid

GC - Glucocorticoid

GR – Glucocorticoid Receptor

HPA – hypothalamic-pituitary-adrenal

MR – Mineralocorticoid receptor

LHPA – limbic- hypothalamic-pituitary-adrenal

LTD – Long term depression

LTP – Long term potentiation

NFTs- Neurofibrillary tangles

NMDA - N-methyl-d-aspartate

PEN – Presenilin

PVN - paraventricular nucleus

Tg – Transgenic

wt- Wild-type

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1. INTRODUCTION

1.1. ALZHEIMER DISEASE

Alzheimer's disease (AD) is a neurodegenerative disease of the brain tissue that leads to progressive and irreversible loss of intellectual abilities. It is the most common form of dementia and cognitive decline in the world and has a progressive incidence. AD has become the most important neurodegenerative disease and the number of people affected rises every day. Nearly 36 million people are believed to be living with Alzheimer's disease or other dementias. By 2030, if breakthroughs are not discovered, we will see an increase to nearly 66 millions. By 2050, rates could exceed 115 millions. The projected rate of rise is even greater in the developing world than in the high income countries (figure 1).

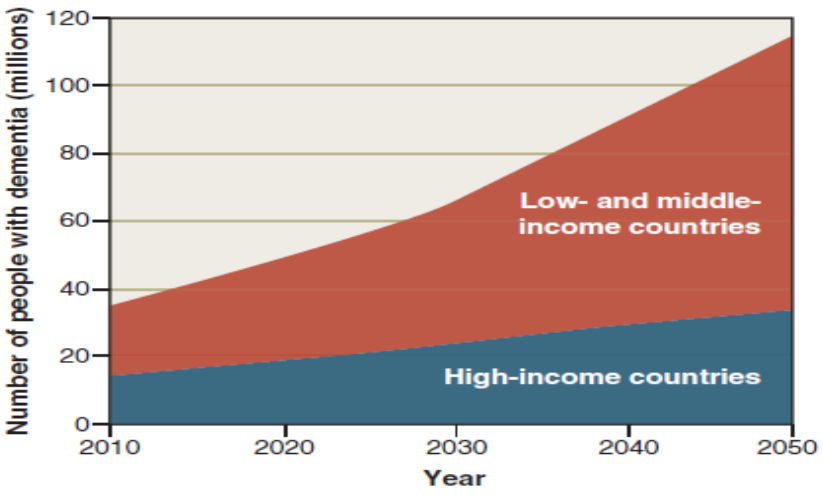

Figure 1: Projected increase in the numbers of people with dementia in high-income countries and in low- and middle-income countries. Adapted from (Wimo and Prince, 2010)

Losing our most human qualities, like memories, reasoning, emotional control, social and occupational functions is feared by everyone. The scientific community currently focuses its attention on understanding early steps in the pathogenesis and find biomarkers that could help to slow down the progression of the disease (Selkoe, 2012). There is evidence that the pathological characteristics of AD, like amyloid deposits in brain, increased levels of tau in cerebrospinal fluid (CSF) and progressive brain atrophy appears roughly 15 years before expected clinical symptom onset (Selkoe, 2012). AD was first described in the 1900's as detailed in the next section.

1.1.1. Neuropathology

AD leads to progressive and irreversible loss of mental functions such as memory, in elderly. It was first described by Alois Alzheimer in 1906 as a rare dementia occurring in the "presenile" period and a characteristic disease of the cerebral cortex (Selkoe, 2001). The following studies allowed to characterize AD by neuronal atrophy, synapse loss (cholinergic neurons) and the abnormal accumulation of amyloid-β protein (Aβ) as senile plaques and hyperphosphorylated tau protein as neurofibrillary tangles (NFTs) (Karantzoulis and Galvin, 2011).

Aβ plays an important role in the development of Alzheimer disease. Amyloid was characterized in 1960 as tissue deposits of specific proteins that are visible in various organs in several seemingly unrelated diseases (Haass and Selkoe, 2007). Then in 1984, amyloid deposits were associated with neurodegenerative diseases based on studies on the cerebrovascular fluid of Alzheimer's patients. Nowadays, we know that Aβ is a 4 k-Da protein that is the primary component of the senile plaques of AD patient brain tissue (Hardy and Selkoe, 2002; Haass and Selkoe, 2007). Aβ is produced from the cleavage of β-amyloid precursor protein (APP). APP is a transmembrane receptor-like protein expressed in neural and non-neural cells that is encoded by a gene located on chromosome 21(Hardy, 2002). The biological function of APP and Aβ are not well established, but we know much about it processing (Müller, Pietrzik et al., 2012). There are two pathways leading to APP cleavage: the amyloidogenic pathway which produces Aβ and the non-amyloidogenic pathway which prevents Aβ formation. In the non-amyloidogenic pathway (that normally occurs preferentially), an α-secretase complex composed of metaloproteases of the ADAM (A Desintegrin And Methaloproteases) family, cleaves APP in two fragments: sAPPα and C83. The amyloidogenic cleavage is done in two steps by the enzymes β- and γ- secretases. The β-secretase complex or BACE-1 (*ß-site APP-cleaving enzyme*) cleaves APP in the extra membrane space and releases an extracellular fragment sAPPβ called C99. In the next step, γ-secretase complex composed by Presenilin 1 and 2, nicastrin, PEN2 and APH-1 cleaves C99 in the intra membrane space and there is releasing of Aβ and AICD amyloid precursor protein intracellular domain (AICD) (figure 2).

The presenilins are transmembrane domain proteins which form part of the γ-secretase complex. Mutations on these proteins may alter the conformation of APP

resulting in a different position of cleavage by γ-secretase, and therefore generation of more amyloidogenic forms of Aβ (Thinakaran and Koo, 2008). The released Aβ has different sizes in which the main forms are Aβ42 and Aβ40. Aβ42 is more toxic and more pathogenic because of its lesser solubility and higher propensity for aggregation (Small, Mok et al., 2001).

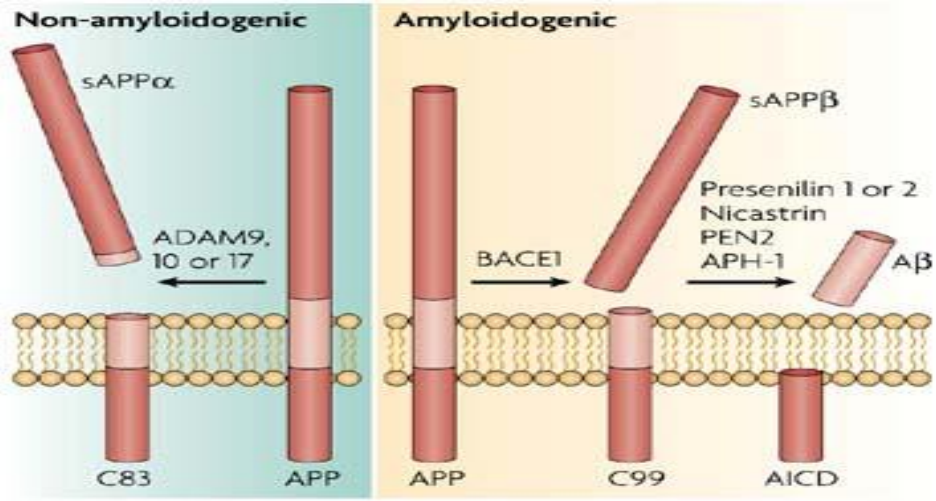

Figure 2: Pathways of APP protein process of cleavage. The process is done in two steps:
In non-amyloidogenic pathway (left), APP is cleaved by the α-secretase complex realeasing C83 and sAPPα.
In amyloidogenic pathway (right), a β-secretase complex (BACE1) cleaves APP and leads to the formation of sAPPβ and C99. A second cleavage the occurs by a γ-secretase complex (presenilin 1 and 2, nicastrin, PEN2 and APH-1) where C99 is cleaved into AICD and Aβ (LaFerla and Oddo, 2005).

The first specific genetic cause of AD to be identified, called familiar AD (FAD) and corresponding to about 1% of AD patients, was the occurrence of missense mutations in APP or in its processing enzymes (PEN1 or PEN2 genes). The mutations in APP are strategically located either before β-secretase cleavage site and shortly COOH-terminal to the γ-secretase cleavage site (Thinakaran and Koo, 2008). A mutation in PEN1 and PEN 2 genes alters the presenilins proteases and therefore γ-secretase activity which affects APP cleavage and Aβ release. In genetic disorders, there is a higher production of Aβ-42, enhancement of the Aβ-42/ Aβ-40 ratio and less clearance which enhances pathogenesis. Insoluble Aβ-42 accumulates and promotes the formation of senile plaques also called neuritic plaques (figure 3). These plaques were once considered the toxic element in AD, but it is now believed that is the more soluble oligomeric forms of Aβ that are toxic and correlate with memory loss (Westerman, Cooper-Blacketer et al., 2002; Haass and Selkoe, 2007; Gimbel, Nygaard et al., 2010; Ferreira and Klein, 2011). Also, recent data have implicated β-C-terminal fragments as

C99 or AICD as other possible causative factors for neurodegenerative processes and memory deficits in mice models of AD (Lauritzen, Pardossi-Piquard et al., 2012; Tamayev, Matsuda et al., 2012; Melnikova, Fromholt et al., 2013). The most studied current hypothesis the 'Aβ hypothesis', stipulates that Aβ accumulation initiates a cascade of cellular and molecular events that leads to synaptic alterations, modification of soluble tau protein into oligomers and then into insoluble paired filaments, progressive neuronal loss and cognitive failure (Haass and Selkoe, 2007).

Tau is a highly soluble cytoplasmic protein that normally binds to tubulin and promotes microtubules assembly and stabilization. Studies suggest that Aβ accumulation perturbs tau protein function which leads to aggravation of the disease. Tau normally exists in phosphorylated and unphosphorylated states (Swerdlow, 2007). In AD, tau protein exhibits altered solubility properties, forming filamentous structures and is the main constituent of neurofibrillary tangles (figure 3) accumulating inside neurons (LaFerla and Oddo, 2005). In tau-containing neurons, tau exists in a hyperphosphorylated state (Swerdlow, 2007). The pathogenesis of tau protein can be dependent or independent of Aβ, so neuritic plaques and neurofibrillary tangles can occur independently of each other (Selkoe, 2001).

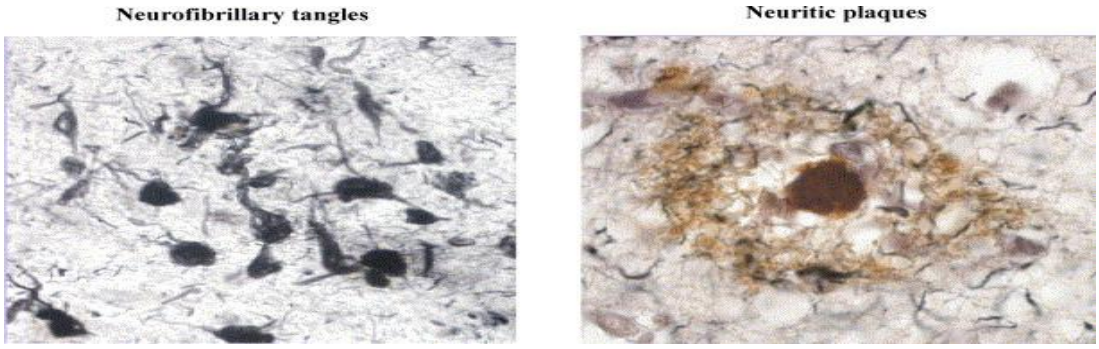

Figure 3: Neuropathological hallmarks of AD. Neurofibrillary tangles, consisting of non-membrane-bound bundles of paired helicoidal filaments (PHF), whose main component is the hyperphosphorylated form of Tau, and neuritic plaques, which consist of extracellular deposits of aggregated and fibrilar Aβ peptide immediately surrounded by dystrophic neuritis.(Sastre, Klockgether et al., 2006)

The expression of Apolipoprotein E4 was recognized to be a risk factor for AD, however, the mechanism by which it is processed is not easy to pinpoint. It seems to be involved in clearance and stability of Aβ and was seen in a high percentage in Aβ deposits in brain tissue of Alzheimer's patients (Selkoe, 2001; Selkoe, 2011). Having

the apoE4 allele is not deterministic to have AD but tends to forward the age of onset in those perhaps destined to develop the disease anyways (Swerdlow, 2007).

Although many risk factors were found, such as hypertension, high levels of cholesterol, diabetes or lack of exercise, their mechanism of action remains unclear. On the other hand, familiar AD represents a little percentage of cases, so the pathological cause of the development of sporadic cases (the majority) is unknown.

Another problem generating controversies to the Aβ hypothesis is that the amount of Aβ (number of senile plaques) present in brain does not correspond to the severity of memory deficits (Arriagada, Growdon et al., 1992). Tangle counts correlate more closely with the degree of clinical dementia than plaque counts (Swerdlow, 2007). Other studies show that loss of synapses can better explain cognitive deficit than the amount of amyloid plaques (Small, Mok et al., 2001; Selkoe, 2002) and that before their deposition, neuron degenerescence occurs and synaptic plasticity is altered (Koo, 2002). Mechanisms by which Aβ affects synaptic transmission are not established, but studies showed that oligomers contribute to changes in receptors expression and dendritic spines morphology (Lacor, Buniel et al., 2007; Renner, Lacor et al., 2010). It causes lesions in key brain areas involved in learning and memory, which become irreversible. Memory deficits appear quite early in AD and could be a good marker of synaptic transmission alteration.

1.1.2. Memory Deficits

Characterization of cellular and molecular properties involved in memory functions becomes crucial for a more extensive knowledge of cognitive deficits expressed in AD. Modern neuroscience has made many advances in understanding synaptic plasticity mechanisms and their possible role in memory (Eichenbaum, 2002). These advances are important to understand neurodegenerative pathologies like AD. Memory loss is the predominant feature of AD. In the early stage, AD patients exhibit episodic memory impairment, expressed as a poor memory for autobiographical events, current affairs, names and faces, familial routes, associated with cognitive decline. With the progression of the disease, there is progressive dysfunction of other cognitive domains and at a final stage there is a severe global impairment of cognitive function (Walker, 2007).

1.1.2.1. Memory systems in the human brain

Human long term memory can be classified in explicit/declarative and implicit/non-declarative memories (figure 4). Declarative memory is a conscious memory that can be verbally transmitted to others and can be divided into episodic memory and semantic memory (Squire, 1992). Non-declarative memory is dissociated in procedural memory, priming memory and conditioning memory which represent the ‘’knowing how’’. Episodic memory characterizes episodes of personal life and is integrated in a spatial and temporal context (Tulving, 2002). Semantic memories are those that we acquire by learning, like factual information and general knowledge (Tulving, 1972). To distinguish between these two memories it can be said that episodic memory require recollection of a prior experience and semantic memory does not (Tulving, 2002). Implicit memory is a collection of nonconscious memory abilities, and is called ‘’procedural memory’’ (Tulving, 1985). Non-declarative memory includes information that is acquired during skill learning, habit formation, emotional learning, and other knowledge that is expressed through performance rather than recollection. The two kinds of knowledge can arise independently, however some learning episodes may be difficult to classify (Squire, 1992).

Memory retrieval is a highly complex cognitive process and this way is not confined to discrete brain region. Encoding memories is mediated by the interaction among a number of functionally related neural areas (Burianova, McIntosh et al., 2010). In other words, cognitive function is due to the interaction of neurons among numerous brain areas that comprise a neural network (Mcintosh, 1999). The medial temporal lobe (entorhinal cortex, hippocampus, and amygdala) in interaction with the prefrontal cortex (PFC) and other structures, plays an important role in declarative memory (Burianova, McIntosh et al., 2010). The hippocampus is associated with spatial contexts and supports episodic memory (Tulving, 2002). A large body of evidence supports the hypothesis of a specific role of the striatum and related basal ganglia structures in non-declarative memory, as associative learning based on stimulus response associations (Packard and White, 1991).

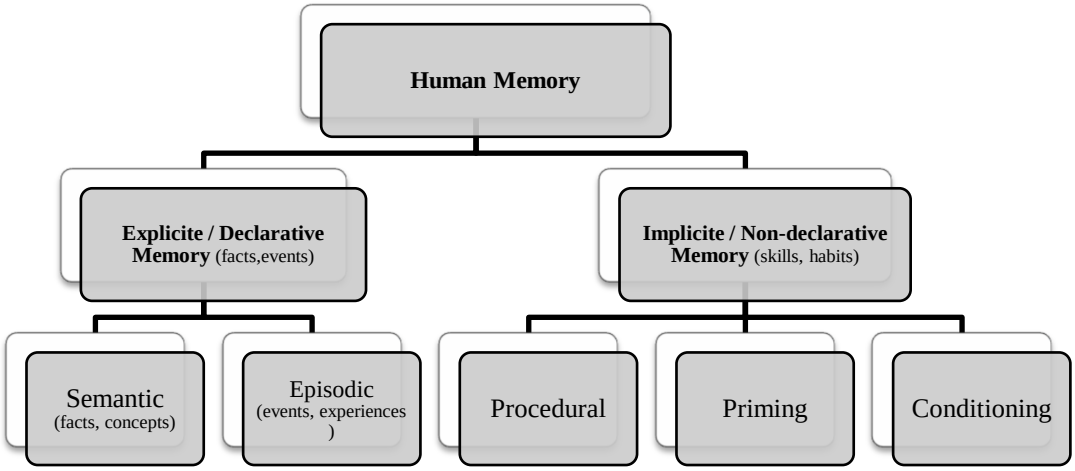

Figure 4: Classification of human long term memory. Declarative memory represents what can be declared and can be divided in episodic and semantic memories. Non declarative memory is the memory used in tasks or skills and can be divided in procedural, priming and conditioning memories (Squire and Zola-Morgan, 1988).

In late stage of AD both declarative and non-declarative memories become impaired (Selkoe, 2002), however in the early phase of the disease only episodic memory seems to be affected. This is the most pronounced and consistent cognitive deficits in pre-clinical AD (Bäckman, Small et al., 2001). One of the new challenges in research of AD is to find early biomarkers associated with this early episodic memory impairment.

1.1.3. Episodic Memory in AD and neurodegenerescence

Episodic memory was first described by Tulving as ‘’ temporally dated episodes or events, and the temporal-spatial relations’’ among them (Tulving and Thomson, 1973). This memory has three specific components: a particular object or person (memory for ‘’what’’ happened), the context or environment in which the experience occurred (memory for ‘’where’’ it happened) and the time at which the event occurred (memory for ‘’when’’ it happened) (Tulving and Markowitsch, 1998). The ability to describe the ‘’what’’, ‘’where’’ and ‘’when’’ of an episode is determinant to classify the experience of human episodic memory. Episodic memory is a memory oriented towards the past that makes possible remembering of previous experience (Tulving and Markowitsch 1998). The integration of this hippocampus-dependent memory is decreased in Alzheimer’s patients, in the early stages of the disease and is usually most prominent throughout the disease course (Walker, 2007). Patients are unable to learn

new material, have difficulty recalling recent events and are incapable of consciously recollecting past experiences (Tulving and Markowitsch, 1998).

To explore the pathophysiology and neurobiology of AD, and better correlate the hallmarks of the disease with memory deficits, it is important to use animal models, even the presence of episodic-memory in animals is discussed. The ability to test animal model of AD's behaviour enabled scientists to know about its memory decline progression correlated with synaptic impairment, histopathological biomarkers or changes in physiological mechanisms during the course of the disease.

1.1.4. Alzheimer's disease mice models

Transgenic (Tg) mice models are used for *in vivo* study of the pathophysiology of a gene of interest. AD models are important to understand the relation between amyloid-β, tau protein, synaptic dysfunction and cognitive decline in AD. Most Tg AD models are based on insertion of one or more human mutations (identified in FAD) into the mouse genome (Marchetti and Marie, 2011).

Several mouse models have been studied, as single/multiple APP-mutation, single presenilins (PS) mutation, APPxPS1 mutation and APPxPS1xTau mutation (3xTg). These mice seem to be the most promising in reproducing some of the hallmarks of disease and although they do not replicate all aspects of the disease they seem to represent faithfully early cognitive decline and progressive AD-like neuropathology (Janus and Westaway, 2001).

APP-derived mice over-express the human form of APP, mutated in one or more sites. The single mutations inserted in the APP gene represent mutations characterized in FAD, which are named the Swedish (swe), the Indiana (ind), the London (Ld) or arc mutations. Another set of mice harbour the swe mutation together with either the ind or arc mutation. With aging, these models exhibit Aβ accumulation and plaques, modest levels of hyperphosphorylated Tau, and hippocampus-dependent memory deficits reminiscent of AD pathology, but do not display NFTs, cholinergic deficits or neuronal loss (Morrissette, Parachikova et al., 2009).

PS1-derived mice models over-express the human presenilin gene (PS1). The mutations are named by the mutated amino acid (for example PS1M146L) (Borchelt, Thinakaran et al., 1996; Duff, Eckman et al., 1996; Citron, Westaway et al., 1997). These presenilin FAD mutant mice consistently show an age-dependent elevation of

Aβ42 with little effect on Aβ40, but they generally do not develop plaques, tau pathology, cholinergic deficits, neuronal loss and have little cognitive deficits (Games, Buttini et al., 2006).

APP/PS1 models were created to increase the brain Aβ accumulation and plaque load. Most double transgenic models studied harboured the human APPswe transgene together with a PEN transgene. APP/PS1 double transgenic mice generally develop early and extensive Aβ plaque formation and exhibit tau hyperphosphorylation and cognitive deficits, but still lack cholinergic deficits, neuronal loss and NFTs (Borchelt, Thinakaran et al., 1996; Duff, Eckman et al., 1996; Hsiao, Chapman et al., 1996).

The 3xTg model over expressing human APPswe and tau MAPTP301L and harbouring a knock-in of PS1M146V was engineered by Oddo et al. (2003). With aging, these mice display both Aβ plaques and tangle pathology, including NFTs, and exhibit hippocampal-dependent memory deficits. They also exhibit cholinergic alterations and cortex-specific neuronal loss (Oddo, Caccamo et al., 2003; Perez, He et al., 2011).

It has recently emerged that impaired synaptic function is an early detectable pathological alteration, even before the neurological markers. Since then, many studies have been done to clarify the hippocampal synaptic changes in these mice models [for review see (Marchetti and Marie, 2011)]. The next paragraph will focus on our model Tg2576 mice which is an APP-derived model. These mice mimic Aβ accumulation in Alzheimer patients with a good correlation between Aβ deposition, age and cognitive deficits (Stewart, Cacucci et al., 2011). Moreover, Tg2576 is a relatively simple model (with only one mutation), it has been well documented and so has become a good model to study *in vivo* AD.

1.1.4.1. Tg2576 and Aβ accumulation

The Tg2576 transgenic model developed by Hsiao et al. (1996), overexpresses the 695-amino acid isoform of human Alzheimer APP containing a Swedish mutation, K670N-M671L. Although this mouse displayed only modest hyperphosphorylation of Tau late in the disease process and did not develop neurofibrilary tangles, it accumulates Aβ into oligomers and then into plaques in the brain and displays age-dependent and hippocampus-dependent memory deficits. In table 1, Aβ (40 and 42) brain levels are shown for these mice, increasing from 2 - 4 months of age which plaques appearing at

about 11-12 months (Hsiao, Chapman et al., 1996). Control mice, which do not have the APP mutation have only traces of Aβ. Other authors described Tg2576 mice by showing soluble Aβ42 and Aβ40 elevation levels at 5 months of age, insoluble Aβ at 7 months-old and amyloid plaques in the brain at 8-9 months of age (Apelt, Bigl et al., 2004; Kawarabayashi, Shoji et al., 2004).

Table 1: Concentrations of Aβ in transgenic (Tg2576) (+) and control mouse (-) brains at 2 to12 months old. Brain tissues was stained with monoclonal antibody which recognizes both human and mouse Aβ. ++, 2 to 5 plaques per section, +++, 6 to 10 plaques per section; ++++, > 10 plaques per section; -, no staining; +/- traces of plaques. Adapted from (Hsiao, Chapman et al., 1996).

Transgene	Age when killed (months)	Aβ (1-40)(pmol/g)	Aβ (1-42/43)(pmol/g)	Amyloid plaques
		Mice killed at 2 to 5 months		
-	2	< 2	< 2	-
-	4	< 2	< 2	-
-	4	< 2	< 2	-
-	4	< 2	< 2	-
+	2	32	2	-
+	2	45	10	-
+	4	71	21	-
		Mice killed at 11 to 13 months		
-	11	< 2	< 2	+/-
-	11	< 2	< 2	+/-
-	11	< 2	< 2	-
-	12	< 2	< 2	-
+	11	192	129	++
+	12	273	177	++++
+	12	325	219	+++

1.1.4.2. Mnesic Deficits in Tg2576

Onset of memory deficits in this model of AD is incertain and question of debate. In recent years, evidence has accumulated demonstrating that synaptic loss, rather than Aβ plaques or neuronal loss, is the best pathological correlate of cognitive impairement (Selkoe, 2002; D'Amelio, Cavallucci et al., 2011). In their publication *D'Amelio et al.* showed early deficits at 3 months of age in hippocampus-dependent memory using the contextual fear conditioning task. As said before, this early memory deficit is interesting because it appears even before amyloid plaques deposition is detectable. Finding biomarkers that are associated with these memory deficits may help to prevent or slow down the progression of the disease. The earliest memory which declines in AD patients is the episodic memory which is highly hippocampus-dependent.

Humans are conscious of the process of retrieving events from the past, and also can declare if they remember but it is not so easy to study episodic memory in animals. One approach to episodic memory in non-humans postulates that memory for ‘’what’’ occurred, ‘’where’’ it occurred and ‘’when’’ it occurred constitutes memory for a unique episode called episode-like memory (Morris, 2001; DeVito and Eichenbaum, 2010). The first who have described this type of memory based on the food-storing ability of birds are Clayton and Dickinson (1998). Since this study, episodic memory-like tests have been developed in different animals. One behavioral paradigm used in rodents is a test based on object recognition (Ennaceur and Delacour, 1988; Eacott and Norman, 2004). The three trial what, where, when object exploration task is a combination of different versions of the novelty preference paradigm. This is the association of object recognition memory (what), with the memory for location (where) and temporal order memory (when) (Dere, Huston et al., 2005). This complex episodic-like memory has already been described in rats (Eacott and Norman, 2004) and mice (DeVito and Eichenbaum, 2010) and is highly hippocampus-dependent (Langston and Wood, 2010). In this last paper, the authors showed that animals with hippocampal lesions were impaired only on the object-place-context task (What, where, when) and not in object-memory (what), object-place memory (where) or object-time memory (when), when tested independently. These data confirm that not all forms of context-dependent associative recognition are mediated by the hippocampus but the integration of all information requires the hippocampus (Langston and Wood, 2010). In the early stage of AD, impaired synaptic function of the hippocampus appears to be particularly affected, leading to defective hippocampus-dependent memory processing, well before the advanced stage of amyloid plaque accumulation and general cell death.

1.2. HIPPOCAMPUS AND MNESIC DEFICITS IN ALZHEIMER'S DISEASE

The neuropathological changes in AD are thought to be manifested initially in the entorhinal cortex (EC), progressing from there to the hippocampus, with increasing involvement of the neocortex as the disease progresses (Braak and Braak, 1991; Walker, 2007). Early studies on humans demonstrated that the hippocampal formation is strongly involved in memory formation (Scoville and Milner, 1957) and that hippocampal lesions produce devastating impairments in memory (Scoville and Milner, 1957; Squire and Zola-Morgan, 1988). Further studies demonstrated that hippocampus is not involved in all types of memories. It acts as a temporary store in declarative memory but it is not required in non-declarative memory (Redish, 1999). The hippocampus is affected in early stages of AD and, corroborating this finding, a number of studies demonstrated that AD is associated with significant hippocampal volume loss and hypometabolism (Jack, Petersen et al., 1998).

1.2.1. Hippocampal neuronal circuits

The hippocampus is a complex structure of the brain. It belongs to the limbic system, closely associated with the cerebral cortex and it is located in the medial temporal lobe (figure 5). Humans and other mammals have two hippocampi, one in each side of the brain. The hippocampus contains two parts: the Ammons horn, which contains CA1, CA2, CA3 and CA4, and the dentate gyrus (Renner, Lacor et al., 2010) (figure 5).

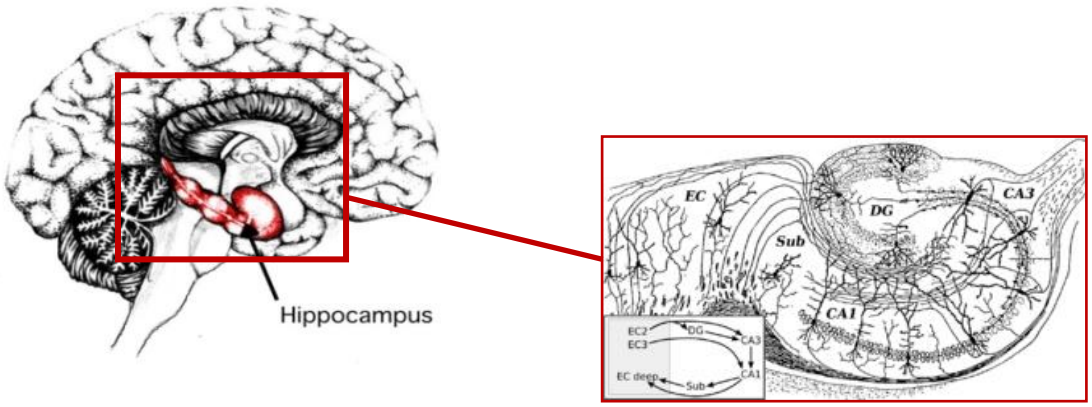

Figure 5: Anatomic location of hippocampus and schema of his internal structure proposed by Santiago Ramon y Cajal. DG-Dentate Gyrus. Sub: Subinculum. EC: Entorhinal cortex (y Cajal, 1968).

The Ammons horn contains a dense layer of pyramidal cells with variable lengths. The entorhinal cortex (EC), located in the parahippocampal gyrus, represents the main input to the hippocampus because of its anatomical connections. The hippocampus has a lamellar organization and a perforant path of three glutamatergic synapses forming the dentate-gyrus-to-CA3-to-CA1connections, which represents a trisynaptic circuit (Andersen, Bliss et al., 1971). The axons of layer II neurons in the EC project to the dentate gyrus through the perforant pathway (PP). The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibres. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back projections into deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons trough the PP. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway (TA) (figure 6 (Deng, Aimone et al., 2010)).

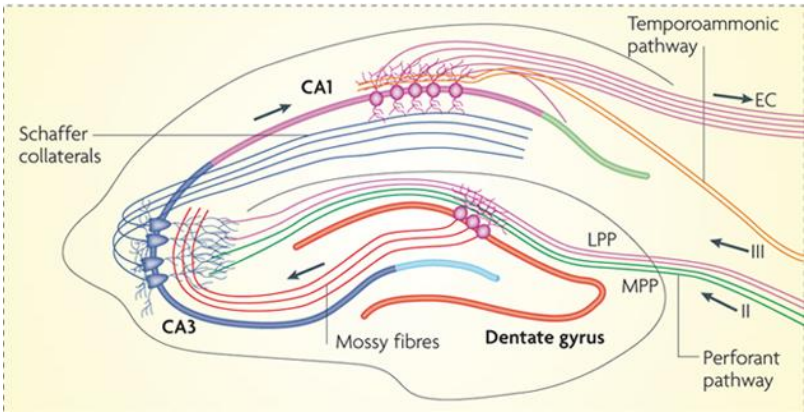

Figure 6: The flow of information from the EC is largely unidirectional, with signals propagation trough a series of tightly packed cell layers, first to the dentate gyrus, then to the CA3 layer, then to the CA1 layer, then to the subinculum and then out of the hippocampus to the EC (Deng, Aimone et al., 2010).

1.2.2. Hippocampal synaptic plasticity and dependent memories

Synaptic plasticity plays an important role in neurochemical foundations of learning and memory. Hebb (1949) first said that memory formation is due to changes in the synaptic efficiency and that storage is done by cellular junctions that associate among them. In other words, synaptic plasticity is the ability of the interconnection, or synapse, between two neurons to change in strength, in response to the transmission over synaptic pathways (two neurons active at the same time strengthen it connection to ensure that its future connection is easier) (Purves, 2005). This change in synaptic efficiency in the mammalian brain can be expressed by a strengthening called long-term potentiation (LTP) or a weakening called long-term depression (LTD) (Hebb, 1949). The biochemical mechanisms of hippocampal synaptic transmission are principally mediated by NMDA (N-methyl-d-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) glutamate receptors. LTD is an LTP's complementary form of synaptic plasticity that leads to selective weakening of some synapses and reverse the potentiation process. Evidence suggests that phosphorylation of AMPA receptors culminate in their insertion in the synapse during LTP, while successive desphosphorylation results in the removal of synaptic AMPA receptors as a reversal of the LTP mechanism leading to LTD (Carroll, Lissin et al., 1999) (figure 7).

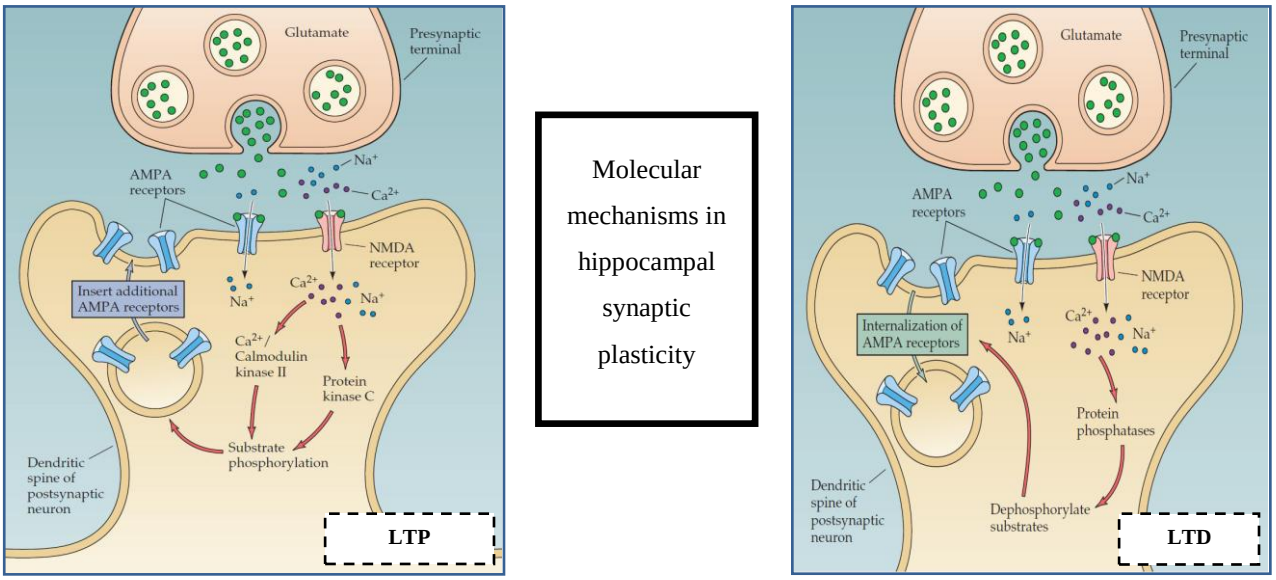

Figure 7: Molecular mechanisms in long-term potentiation (LTP) and long-term depression (LTD) (Purves, 2005).

LTP and LTD therefore regulate the AMPA receptors movements into and out of the synaptic membrane, and this molecular plasticity seems to be intimately linked to learning and behavior organization (Malenka, 2002). In fact, recent studies confirmed that hippocampal LTP sustains long-term memory (Gruart, Muñoz et al., 2006; Whitlock, Heynen et al., 2006). Understanding these physiological mechanisms is crucial to understand memory dysfunction, such as in AD.

1.2.2.1. Impairment of synaptic plasticity in Alzheimer's disease

Studies suggest that synapses are the first element to be affected by the toxicity of β-amyloid peptide oligomers. Aβ has a toxic action in functional and synaptic plasticity and leads to a deficit in encoding memories (Small, Mok et al., 2001; Selkoe, 2002). Some early studies have demonstrated that the Tg2576 mice have impaired *in vivo* and *in vitro* long-term potentiation in CA1 and DG regions of the hippocampus (Chapman, White et al., 1999). Others showed that a mice model with APP and PS1 gene mutation had inhibitory glutamate synaptic currents that decrease AMPARs post-synaptic density (Chang, Savage et al., 2006). These molecular mechanisms could be correlated with LTD as seen before. Aβ could be involved in NMDARs expression, enhancing LTD (Snyder, Nong et al., 2005). Other evidence relating LTD with AD, is that post-mortem brain of Alzheimer's patients shows dendritic spines loss, which occurs during LTD in physiological conditions. *D'Amelio et al.* (2011) show that 3-

months-old Tg2576 mice, has an increased LTD compared with wt (wild-type) mice. Other studies have been done to explain how Aβ affects synaptic transmission but it remains unclear. It is known that Aβ affects the glutamate receptors expression in synapses by different mechanisms like changes in membrane or intracellular traffic of these receptors (Snyder, Nong et al., 2005).

Changes in synaptic efficiency occurs in early stage of the disease, even before Aβ deposition and general cell death and can thus constitute a good target for research and therapeutic. However, it is important to know the cause of all changes occurring in AD pathology. As mentioned above, a majority of AD cases are sporadic and do not have any known genetic component. Consequently the identification of risk factors that increase vulnerability to AD is critical for the development of preventing strategies. There is now strong clinical evidence suggesting that stress is an important risk factor in the development of the pathology (Rothman and Mattson, 2010) and it is perhaps related with increasing AD incidence (Wilson, 2007). Stress enhances the production of glucocorticoids into the circulation by HPA axis activation. These glucocorticoids then coordinate neural, immune and endocrine response to stressors and could matter in triggering the disease (Hebda-Bauer, Simmons et al., 2012).

1.3. STRESS, HIPPOCAMPAL SYNAPTIC FUNCTION AND MEMORY

Exposure to physical and psychological stressors is a natural process, which can be regulated by physiological responses in animals. Stress can be defined as ‘’the non-specific response of the body to any demand upon it’’(Selye, 1974) or a ‘’sensed threat to homeostasis’’ (McEwen and Stellar, 1993). Imposition or perception of environmental changes can trigger a stress, and it needs to be countered to promote adaptive mechanism to re-establish homeostasis (Herman and Cullinan, 1997). The mechanism of dealing with a stressor comprises three phases: an initial ‘’alarm reaction’’, a stage of adaptation with resistance and a stage of exhaustion (Selye, 1974). Chronic stress is associated with the stage of exhaustion and more related with triggering pathogenesis. Chronic stress exacerbates the release of glucocorticoids (GCs) by the hypothalamus-pituitary-adrenal (HPA) axis pathway, impairs limbic-hypothalamus-pituitary-adrenal (LHPA) axis and in particular the hippocampus. This seems to be related with ageing and AD (Miller and O’Callaghan, 2005; Elgh, Lindqvist Åstot et al., 2006).

1.3.1. Physiological responses to Stress

The physiological response to stress is mediated by an efficient and highly conserved set of interlocking systems in the most demanding of circumstances (Ulrich-Lai and Herman, 2009). Behavioural, autonomic, endocrine and immune systems are activated and interact to produce an integrated stress response (Myers, McKlveen et al., 2012). Two main systems are involved in this stress response: the sympathetic nervous system, which is part of the autonomic nervous system (a neural mechanism) and the HPA system (an endocrine mechanism) (figure 8). The autonomic nervous system provides the most immediate response, ‘’*fight or flight’’* or the alarm response, to stressors by release of catecholamines, specifically norepinephrine and epinephrine (Ulrich-Lai and Herman, 2009). This neural response leads to a quick increase in blood glucose levels and blood pressure, rise of the respiration level and metabolism acceleration – resulting in short-term responses. In the stress adaptive stage, the HPA axis is activated to produce GCs by an endocrine pathway. Released GCs induce blood

pressure rise, increase in blood glucose (stimulation of gluconeogenese and reduction of utilization), mobilization of fatty acids and suppression of the immune system in a so-called long-term response (Hall, 2006).

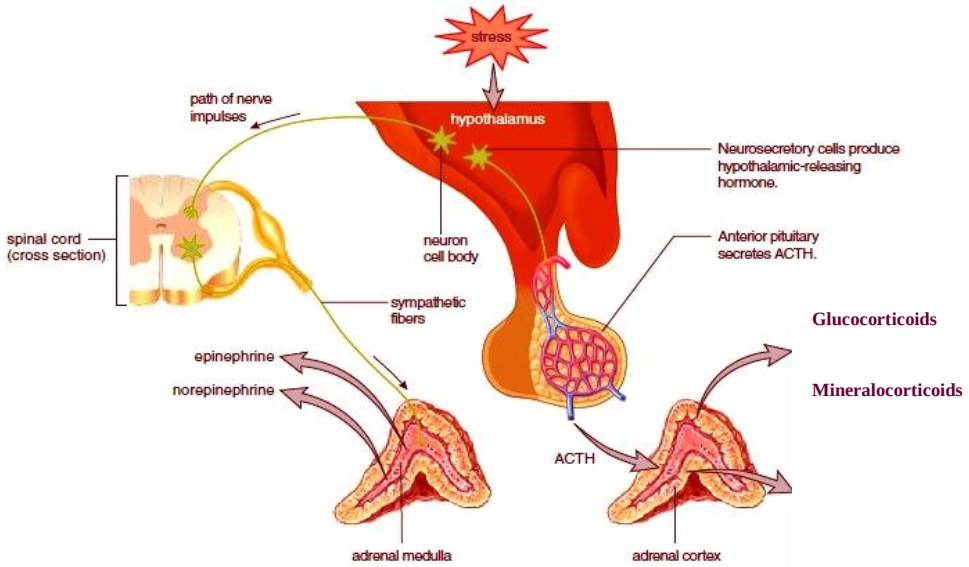

Figure 8: Short term and long-term responses to stress. The stress response is mediated by the sympathetic pathway in a short term response and leads to epinephrine and norepinephrine release. It is also mediated by hypothalamus-pituitary-adrenal axis in a long-term response with the release of glucocorticoids and mineracorticoids. Adapted from (Hall, 2006).

Catecholamines and GCs, which mediate the first response to stress, trigger an adaptive response to restore homeostasis by the engagement of a series of negative feedback loops (Sotiropoulos, Cerqueira et al., 2008). As seen before, GCs have many biological functions. However, they are also involved in long-term responses to stress and could be synthesized at high levels in a chronic stress situation. As they accumulate, they can trigger dysfunction in some metabolic pathways.

1.3.2. Glucorticoids and Hypothalamic-Pituitary-Adrenal response to stress

The HPA axis activation occurs in response to circadian signaling pathways (Schibler and Sassone-Corsi, 2002) and to a stressor or anticipation of a stress situation. In normal conditions, HPA axis activity respects a circadian cycle because of the pulsatile secretion of GCs, of higher amplitude in the awake phase for humans, which corresponds to nigh time in rodents (Lightman, Wiles et al., 2008).

The hypothalamic paraventricular nucleus (PVN) is the first actor in the HPA axis activation. Neurosecretory neurons localized in the medial parvocellular portion of the PVN are stimulated to produce corticotropin-releasing hormone (CRH) and vasopressin (AVP). Both peptides are released into the pituitary portal circulation and stimulate the production and secretion of adrenocorticotropic hormone (ACTH) through activation of type 1 CRH receptors and type V1b VP receptors in the pituitary gland. ACTH stimulates type 2 mineralocorticoids receptors and promotes the release and synthesis of GCs, mineracorticoids and androgens from the adrenal cortex (Sandi, 2004). The released GCs are dispatched throughout the body via blood circulation and act on specific receptors to induce a negative feedback loop onto the hypothalamus and pituitary gland. This inhibits CRH and ACTH release and results in reduction of glucocorticoid synthesis by adrenal glands (figure 9) (Dallman and Jones, 1973). Activation and inhibition of GC release is a temporally regulated process involving rapid neuronal activation and efficient inhibition (Myers, McKlveen et al., 2012).

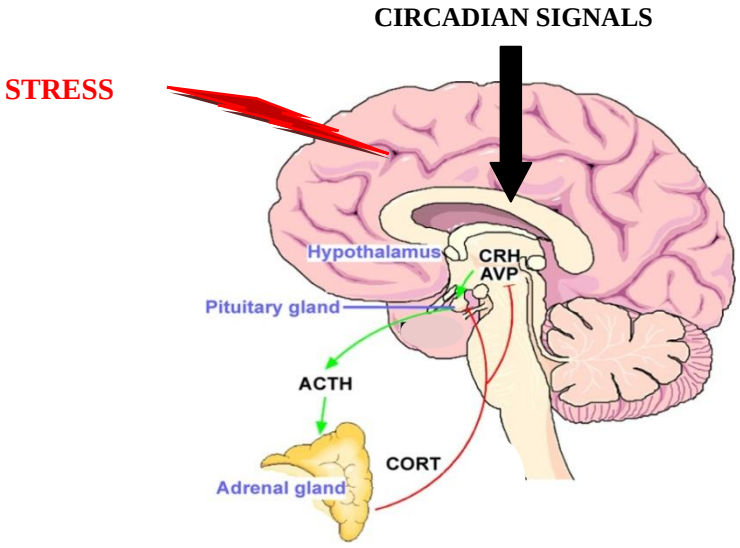

Figure 9: When certain neurochemical signals related to either stress or circadian signaling pathways are received by the hypothalamus, a subset of neurosecretory cells in the paraventricular nucleus (PVN) releases corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) to stimulate adrenocorticotropic hormone (ACTH) synthesis and secretion from the pituitary. ACTH then induces adrenocortical cells to produce and secrete glucocorticoids (GC) in blood circulation (in green). GCs turns off the HPA axis activity by a negative feedback (in red). Adapted from (Murgatroyd and Spengler, 2011).

GCs are adrenocortical hormones which have gained their name because of their important effects in increasing blood glucose concentration. GCs bind to receptors and mediate pathways in lipid, protein, carbohydrate, muscle and bone metabolisms, as well as in inflammation, immune system response and in the CNS (Hall, 2006).

All corticosteroids secreted by the adrenal cortex are synthesized from cholesterol (essentially in mitochondria and endoplasmic reticulum) by steps catalyzed by a specific enzyme system (showed in figure 10). In humans, cortisol is the principal GC, while corticosterone is the main GC in rodents. They are synthesized from 11-deoxycortisol or 11-deoxycorticosterone, respectively, through the 11-β-hydroxilase enzyme and both can be converted in their inactive form cortisone, by the 2 11-β-hydroxystreroid dehydrogenase (11-β-HSD2) enzyme. This reaction is reversible and is catalyzed by 11-β-HSD1 in a NADPH mediated process (Hall, 2006; Schnackenberg, 2008). All these reactions occur in the mitochondria and in the endoplasmic reticulum (Hall 2006).

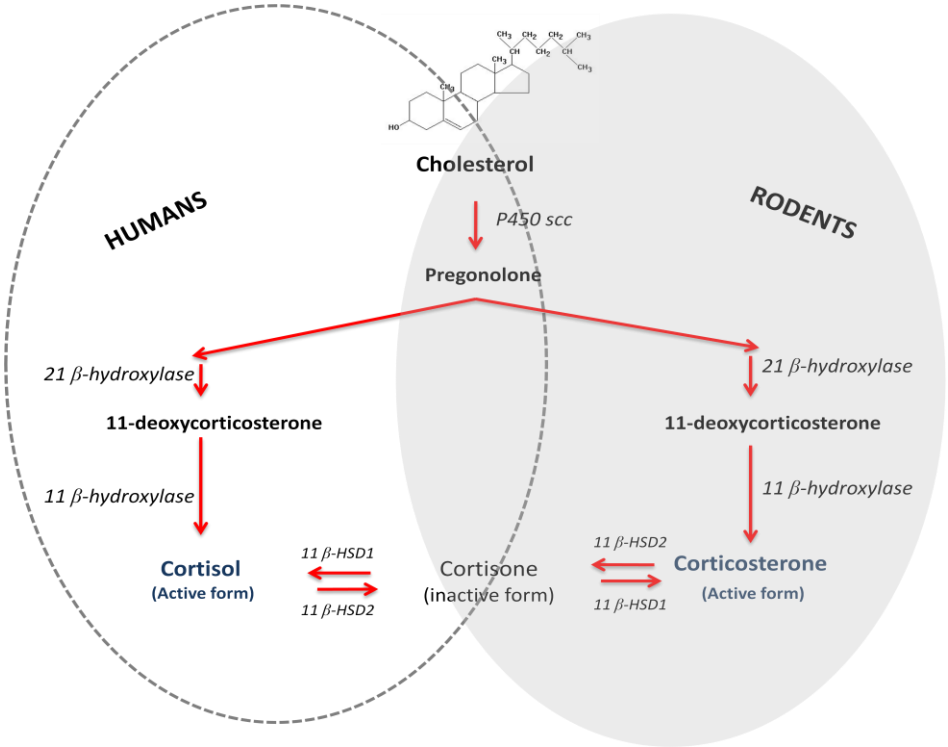

Figure 10: Biosynthetic pathway of cortisol and corticosterone in humans and rodents respectively. Cholesterol is converted (in adrenal cortex) into cortisol and corticosterone. Cortisol and corticosterone may be converted in its inactive form – cortisone. Specific enzymes of each step are noted in italic. Adapted from (Kostadinova, Hostettler et al., 2012) .

Cortisol and corticosterone readily cross the blood-brain barrier, diffuse through the cell membrane and bind to intracellular receptors. There, they can either be

irreversibly degraded into the cytosol by the type 2 11-β-HSD enzyme or bind to one of the two types of intracellular receptors: the mineralocorticoids receptor (MR) representing the type I receptors and the glucocorticoid receptor (GR) representing the type II receptors (figure 11) (Sandi, 2004). It also important to say that MRs are only present in the brain and GR have an ubiquitous distribution in the body (De Kloet, Vreugdenhil et al., 1998). GCs binds with higher affinity to the MR than to the GR in the cytoplasm (Funder, 1997), but can trigger both MR and GR activities. In the absence of hormone, GR resides in the cytoplasmic compartment as heterocomplex with heat shock proteins (Sanchez, Hirst et al., 1990). After activation with ligand binding, GR dissociates from the heterocomplex, translocates to the nucleus, and regulates neuronal target gene expression, including the downregulation of the GR itself (Gustafsson, Carlstedt-Duke et al., 1987; Sapolsky, Romero et al., 2000). Recent publications have reported a new family of membrane-bound MR and GR (mMR and mGR respectively), which can exert rapid actions by a non-genomic mechanism (G-coupled protein) (de Kloet, Karst et al., 2008; Groeneweg, Karst et al., 2012), but less is known about this mechanism. Intracellular MRs are considered regulator of basal, diurnal tone of the HPA axis (Akil and Morano, 1996) and are always occupied, while GRs are considered a sensor of stress (occupied only during periods of to high glucocorticoid secretion, active phase of circadian cycle and under stress conditions) and as key players in the negative feedback response (Reul and De Kloet, 1985).

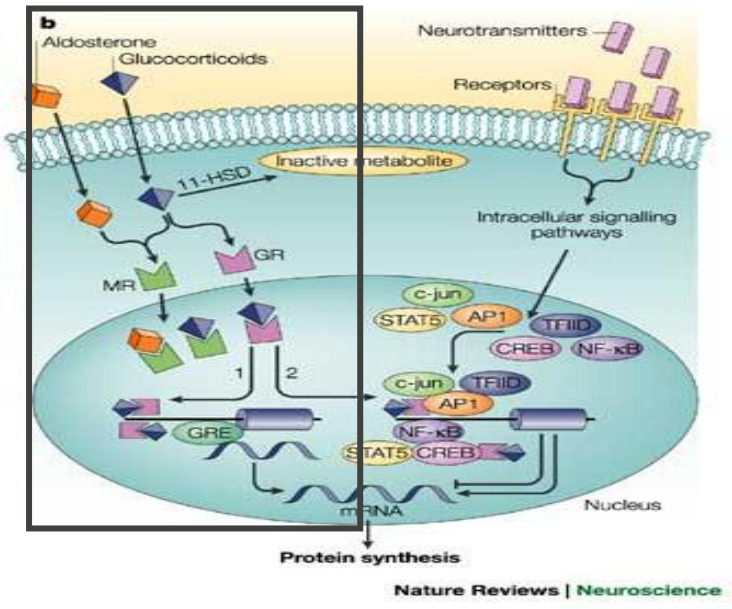

Figure 11: Glucocorticoids enter the brain and enter cells by passing cell membranes. In the cytosol they can be irreversibly degraded by the enzyme 2 11-β HSD or bind to one of two types of intracellular corticosteroid receptors: Mineralocorticoid receptor (MR) or Glucocorticoid receptor (GR). Activated corticosteroid receptors promote their effect via transcription and modulation of transcription factors in the nucleus. Adapted from (Sandi, 2004)

Stress also induces transient activation of the HPA axis at PVN neurons by a transient increases in CRH transcription (Aguilera, 1998). Consequently, CRH stimulates adrenal cortex to produce GCs. The mechanisms that contribute to limit the stress response and restore homeostasis in HPA axis regulation are intracellular feed-back of the CRH neuron, changes in stimulatory and inhibitory circuitry and glucocorticoid negative feed-back. GCs plays the most important role in the negative feed-back response of HPA axis (Aguilera, 2011) and the hippocampus, which expresses GRs in high levels, has an important function to turn off the stress response and inhibit GC synthesis (Reul and De Kloet, 1986; Herman, Patel et al., 1989).

1.3.3. Hippocampal function in stress

Early studies indicate that limbic forebrain regions including the hippocampus, amygdala and prefrontal cortex are strongly implicated in HPA axis regulation, forming a limbic-hypothalamus-pituitary-adrenal (LHPA) axis. Increased GCs may induce a negative feed-back in hippocampus, hypothalamus and pituitary (figure 12). The hippocampus and prefrontal cortex inhibit HPA axis regulated secretion, whereas amygdala is an activator promoting GC release (figure 12) (Jacobson and Sapolsky, 1991; Feldman, Conforti et al., 1995; Herman and Cullinan, 1997). Hippocampal stimulation has been implicated in decreasing GC secretion in rat and human (Rubin and Mandell, 1966; Dunn and Orr, 1984), suggesting that it could inhibit HPA axis activation.

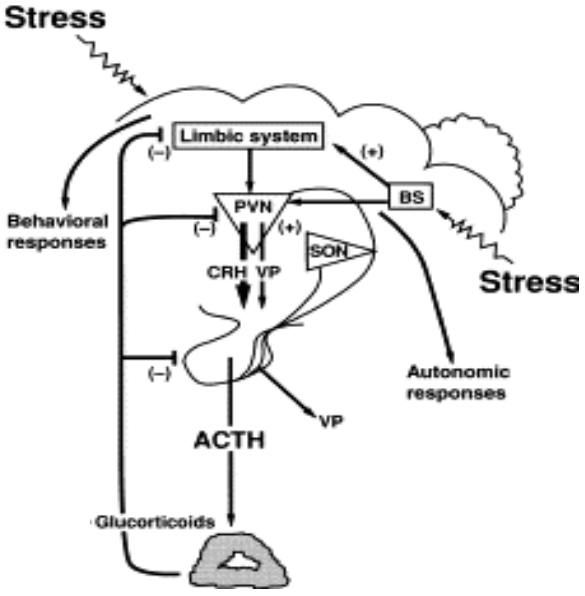

Figure 12: Integrated response to stress emphasizing the activation of hypothalamic-pituitary-adrenal axis and the feed-back and regulatory effects mediated by corticosteroids (Aguilera, 1998).

To confirm this hypothesis, other studies have demonstrated that complete hippocampectomy increase corticosterone or/and ACTH release (Fendler, Karmos et al., 1961; Knigge, 1961). The previous studies, in conjunction with others and the fact that the hippocampus is a region with high levels of GRs (Reul and De Kloet, 1986; Herman, Patel et al., 1989) lead some to posit a critical role of the hippocampus in the inhibition of the HPA axis in order to turn off the stress response (Herman, Ostrander et al., 2005). MRs are predominantly expressed in hippocampus and GRs although expressed in all the brain, are more concentrated in the CA1 region of the hippocampus (Nishi and Kawata, 2007).

The involvement of the hippocampus and the other limbic structures in the regulation of the HPA axis represents a complex issue and it is still not well understood. There is evidence that it is mediated by indirect innervations of the PVN. Information arrives from hippocampus to stress effector neurons by an intermediary synapse: the bed nucleus of stria terminallis (BST) mediated by its excitatory neurons (Herman, 2003). It seems that hippocampal regulation of the HPA axis is likely mediated by glutamatergic neurons in the ventral subiculum and GABAergic neurons in the BST and hypothalamus (figure 13). In a stress situation, the hippocampus sends an excitatory glutamatergic input to the BST mediated and activates GABAergic neurons to release the inhibitory neurotransmitter GABA (figure 14). These activated GABAergic neurons, in turn shut down the HPA axis response mediated by stress (Herman and Seroogy, 2006).

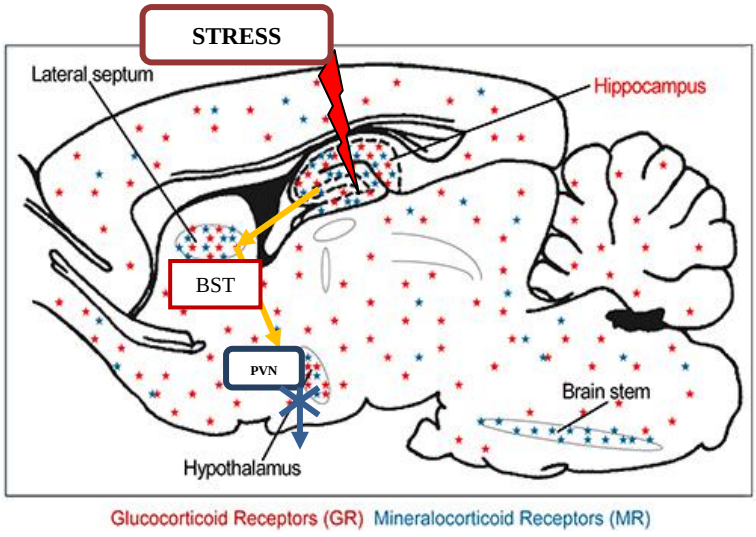

Figure 13: Schematic representation of GR and MR levels in brain. LHPA axis pathway is mediated by hippocampus, bed nucleus of stria terminallis and PVN in hypothalamus. Adapted from (Almeida).

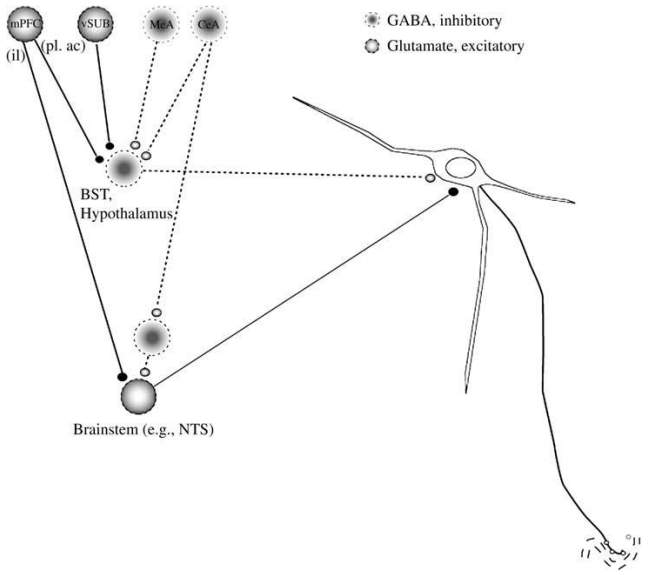

Figure 14: PVN neurons receive direct inhibitory (GABA-containing) input from neurons in the BST and many hypothalamic nuclei and excitatory input from central amygdaloid nucleus (CeA) and other regions. By hippocampal-BST signaling, a negative feedback signal is sent to the PVN to turn off the stress response of the HPA axis (Herman and Seroogy, 2006).

Chronic stress, high level of corticosteroids and aging are associated with hippocampal impairment. Maintained stimulation of the hippocampus in a chronic stress situation causes damage to it, and consequently the negative feed-back in hypothalamus does not occur, which leads to increase in glucocorticoid concentration. Evidence from human and rodent studies point to chronic stress or exposure to excess GCs as increasing vulnerability to AD or accelerating cognitive and neural decline (Hebda-Bauer, Simmons et al., 2012).

1.3.4. Chronic stress exposure in Alzheimer disease

The HPA axis regulation and the negative feed-back mediated by stress hormones may fail when stress is maintained for a long period, leaving the individual exposed to elevated levels of GCs for prolonged periods of time (Sotiropoulos, Cerqueira et al., 2008). GC accumulation in the body is mediated by several factors including the intensity, chronicity of the stressful stimulus and the efficiency of GC-receptor-mediated negative feed-back and enzymes that metabolizes GCs (Aguilera, 2011). Differences in stress reactivity may be associated to genetic predisposition and environmental exposure (Lupien, 1998) and can increase vulnerability to neurodegenerative disorders.

Clinical studies have implicated GCs in the pathogenesis and progression of AD, because AD patients present significantly high levels of circulating cortisol and impaired LHPA axis regulation (Csernansky, Dong et al., 2006; Elgh, Lindqvist Åstot et al., 2006; Wilson, 2007; Rothman and Mattson, 2010). In addition, the occurrence of a major stressful event seems to lower the age of onset of familial AD (Mejia, Giraldo et al., 2003). In animals studies, increased Aβ and enhanced tau-pathology have been reported in triple-transgenic (3XTg-AD) mice following GC administration (Green, Billings et al., 2006).

Chronic stress exposure may induce enhancement in basal HPA axis tone and stress reactivity. Hypersecretion of GCs causes changes in HPA axis feed-back inhibition. This leads to baseline corticosteroids hypersecretion, adrenals hypertrophy and/or thymic atrophy (Herman, Adams et al., 1995; Ulrich-Lai, Figueiredo et al., 2006), down regulation of GRs in key feed-back regions (Herman, Adams et al., 1995; Ulrich-Lai, Figueiredo et al., 2006), hippocampal damage and memory deficits (Herman and Seroogy, 2006).

1.3.4.1. Adaptive changes in Hypothalamus-pituitary-adrenal axis and brain corticoid receptors

HPA axis is overactivated in a chronic stress situation and GC concentration in body remains high for an indeterminate period (Hebda-Bauer, Simmons et al., 2012). The ability to turn the stress off, in which high concentration of GCs exert a negative feed-back in HPA axis is impaired in elderly. Evidences suggest that AD patients have a decrease in the negative feed-back using dexamethasone suppression test (Näsman, Olsson et al., 1995). Dexamethasone (DEX) is a synthetic GC, 30 times as potent as cortisol and with zero mineralocorticoid activity that can be useful for mimic cortisol activity in the HPA axis. DEX acts on hypothalamus and pituitary GRs to decrease GC production by a negative feedback (Hall, 2006). In addition, baseline levels of GCs (resting period) may not be different in young and elderly, but a delay in turn off of the HPA axis in response to an acute stressor is commonly reported in the elderly (Morano, Vazquez et al., 1994). Recently, studies demonstrated that baseline levels of corticosterone in a mouse model of AD (3xTg-AD) are not different from wild-type mice at 4 months of age, a presymptomatic age in these mice, but these mice show HPA

axis-dependent gene expression in several brain areas (Hebda-Bauer, Simmons et al., 2012).

Hippocampal MRs and GRs are involved in basal neuronal function and in negative feed-back regulation of the HPA axis (Sousa, Cerqueira et al., 2008). As said before, MRs are activated at low corticosterone concentration and GRs are activated during the circadian peak or under stress conditions, when MR become saturated and GR becoming more manifest (Reul and De Kloet, 1985). The imbalance between GR/MR can damage nervous tissues; such damage can impair the negative feed-back in stress responses and be responsible for psychopathologies. GRs play the most important effect on neurotoxicity. Some studies associate deficits in HPA axis regulation with deficits in GC signaling (via GRs) in the hippocampus (Herman, Adams et al., 1995; Mizoguchi, Ishige et al., 2003). All these changes in stress hormones regulation after a chronic stress exposure are associated with a deficient response in negative feed-back mediated by GCs in hippocampus, hypothalamus and pituitary (Herman and Seroogy, 2006).

In 1980, the GC cascade hypothesis describes the relation between GCs and hippocampus with aging. This hypothesis showed that GCs secreted during periods of stress desensitize the hippocampus to further GCs exposure by downregulating GRs, a reversible effect (Sapolsky, 1992; Sapolsky, Krey et al., 2002). Confirming this studies, some authors showed that in chronic stress situation, there is a downregulation in GR proteins (Herman, Adams et al., 2008; Zhou, Zhu et al., 2011). GRs downregulation with GC elevation leads to permanent cell death in hippocampus (Conrad, 2011). When these two events coincide hippocampal damage occurs, and HPA regulation by the hippocampus become less effective, leading to pathological increase in GC levels.

Recently this GC cascade hypothesis has been challenged. Some studies report results in the opposite direction as chronic isolation of stress in a transgenic model of AD, the Tg2576 mice, caused an increase in GR expression in the hippocampus (Dong, Yuede et al., 2008). Genetic expression of stress molecules seems to be increased in early phase of AD mice models. Hebda-Bauer and her colleagues demonstrated that 4-months old 3xTg-AD mice presented activated central HPA axis with altered mRNA levels of MRs and GRs in the hippocampus, GR and CRH in the PVN of hypothalamus and CRH in the BST (Hebda-Bauer, Simmons et al., 2012). GR and MR expression seems to be increased during prolonged stress stimulus or during prolonged GC exposition and this is related with damage to neuronal tissues (Sousa, Cerqueira et al.,

2008). However, other data, in a novel direction, suggest that forebrain GRs might not be involved in chronic stress situations (Furay, Bruestle et al., 2008).

These controversial results need to be clarified, however, and the correct mechanism by which pathological GR activation induces hippocampal impairment remains unclear. It is not known yet which hormones and transmitters play the pathogenic role. For instance, there is evidence that the ratio of MR relative to GR is going down after prolonged exposure to elevated GC levels and that the administration of GR antagonists may restore the functional balance between activated MRs and GRs (Bachmann, Linthorst et al., 2003; De Kloet, Joëls et al., 2005).

1.3.4.2.Hippocampal dysfunction and neuronal loss

As mentioned before, the hippocampus plays an important role in the stress response. In addition, it is the brain region which harbors the highest levels of GRs and MRs (Reul and De Kloet, 1985). A large body of evidence demonstrated that the action of GCs on these two receptors contributes to the regulation of basal hippocampal synaptic function, synaptic plasticity and memory (Kim and Diamond, 2002; Sousa, Cerqueira et al., 2008; Chaouloff and Groc, 2011; Maggio and Segal, 2012).

Some authors demonstrated that LTP is observed when GCs are kept within basal levels (DM Diamond, 1992), but it is impaired when corticosteroids rise (e.g. during stress). Other studies show that hypercortisolemia facilitates long-term depression (LTD) in the hippocampus (DM Diamond, 1992; McEwen, 1999). Results from our laboratory show an increase of LTD in CA1 hippocampus region at 4-months of age in Tg2576 mice related to higher level of corticosterone in Tg2576 than in wt mice. We further showed that this LTD increase can be reversed by the GR antagonist (RU486) (results to be published). These data suggest that the adverse effects of stress on the hippocampus are largely mediated by GRs and that AD synaptic dysfunction could be intimately linked to GC levels.

Studies in the middle 1980s indicate that prolonged and intense stress exposure can result in hippocampal neuronal death, with higher vulnerability of the CA3 subfield (a region particularly sensitive to stress) (Sapolsky, Krey et al., 2002). Others authors also demonstrated that prolonged GC treatment causes retraction on apical dendrites in the same region (Magariños, Orchinik et al., 1998). Hippocampal damage strongly perturbs learning and memory functions. As early impairment in hippocampus-

dependent memory as been reported in AD, as discussed above, GR activation via pathological chronic stress could be an important environmental risk factor associated with the incidence of this pathogenesis.

1.3.4.3.Memory impairment

Coordinated actions mediated by MRs and GRs in higher brain areas represent the main pathway by which GCs influence emotional and cognitive behaviours (Sousa, Cerqueira et al., 2008). Acute release of stress hormones has a positive impact in learning and memory, but elevation of these hormones trigger memory deficits (Jacobson and Sapolsky, 1991). This correlation is mediated by a U-shaped function, in which the ratio of occupation of GRs and MRs is associated with behavior performance (figure 15). In acute stress, GCs are released and activate MRs and GRs in the hippocampus and PFC to turn off the stress response. This situation is also associated with an improvement in information processing and cognitive function, in order to facilitate behavior adaptation to stress and restore HPA axis function. When stress persists, GRs become saturated and this results in deficits in spatial reference memory (McEwen and Sapolsky, 1995; Sousa, Lukoyanov et al., 2000).

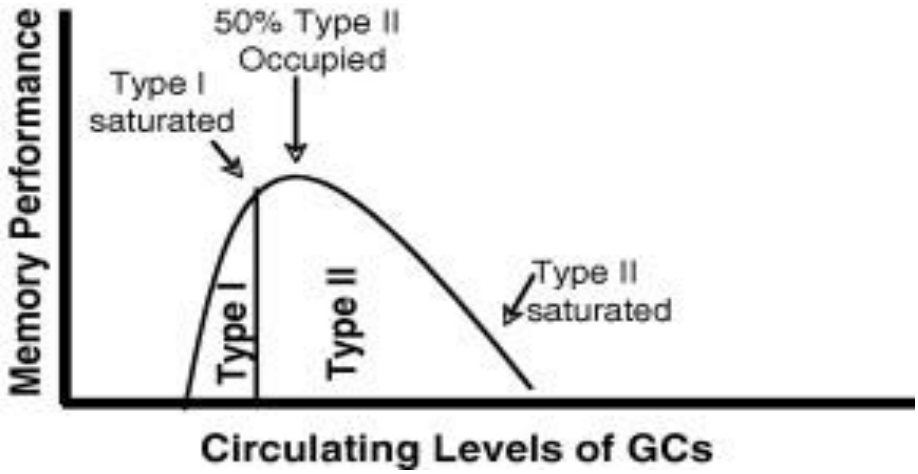

Figure 15: The Type I/Type II glucocorticoid ratio hypothesis of the association between circulating levels of glucocorticoids, and memory performance. The figure shows occupancy of GC receptors as a function of circulating levels of GCs and resulting modulation of memory. When Type I receptors (mineralocorticoid receptors) are saturated and there is partial occupancy of Type II receptors (glucocorticoid receptors), there is maximization of memory, while when both Type I and Type II receptors to glucocorticoids are not occupied or are saturated (right side of the inverted-U shape function), there is an impairment in memory performance. (Lupien, Maheu et al., 2007)

A recent study shows that 4 months-old Tg2576 mice exposed to chronic mild stress display an increase in Aβ and phosphorylated Tau levels and a worsening of

cognitive dysfunction (Cuadrado-Tejedor, Ricobaraza et al., 2012). These findings support the hypothesis that chronic stress accelerates the onset of cognitive impairment and produces an increase in pathological markers levels increasing AD susceptibility. The correct mechanism by which stress affects AD neuropathology remains unclear. Many aspects of this hypothesis need to be clarified, notably the extent of the HPA axis dysfunction in AD and how a chronic rise in GC levels in AD might contribute to episodic memory loss.

2. OBJECTIVES

Stress disrupts homeostasis and normal metabolic functions in the body. The hippocampus, which presents high levels of GRs, has an important role in turning the stress response off inducing a negative feedback in the HPA axis. In a chronic stress situation, GRs become saturated and hippocampal function fails; hippocampal neuronal function becomes impaired affecting the formation of hippocampus-dependent memories. Also, hippocampus dysfunction alters the negative feedback of the HPA axis to turn of the stress response, giving rise to overproduction of GCs, which can induce damage in many tissues.

Early AD pathology is characterized by Aβ accumulation, an event which could favor a chronic stress situation within the brain. Indeed, GC levels in Alzheimer's patients are increased early in the pathology, associated to impairment in the negative feedback of the HPA axis. Early AD pathology is also characterized by episodic memory loss. The relationship between increased Aβ brain levels, increased GC levels and early impairment of episodic memories is still not understood.

The objective of this project was to evaluate HPA axis integrity and the contribution of GC signaling to AD-like memory loss using a mouse model (the Tg2576 mice) at an early symptomatic stage (4 months-old) when Aβ starts to accumulate but does not yet aggregate.

Using these Tg2576 mice and wild-type (wt) littermates, we:

- Quantify corticosterone levels in resting and awake conditions;
- Evaluate HPA axis regulation by the dexamethasone suppression test;
- Evaluate hippocampal glucocorticoid receptors expression (GRs);
- Evaluate impairment in episodic-like memory in Tg2576 using a memory task;
- Reverse this memory deficit by blocking GRs with an antagonist (RU486);

3. MATERIALS AND METHODS

3.1. Animals

Tg2576 males [K670N-M671L] (APPswe positive) (Taconic Farms Inc., Germantown, USA) were generated as previously described (Hsiao, Chapman et al., 1996) and were bred with B6SJLF1 females (Janvier farms, St Berthevin, France). The transgenic and non-transgenic (wt) littermates were used for this study. At the time of weaning, littermates were identified through ear punching and genotyped to differentiate wt and Tg2576 [K670N-M671L] (APPswe positive) mice. 100 Tg2576 and 106 wt inbreeding males were used for this study. For experiments mice were housed in individual in cages, under controlled laboratory conditions with a 12h light/ 12h dark cycle, a temperature of 21± 2ºC, a light density of 26 lux and a humidity of 60-70%. Mice had free access to standard rodent diet and tap water.

3.2. Genotyping

Genotyping for transgenic screening in the offspring was performed using DNA obtained from post-weaning tail biopsies (±0,8 mm). Tail biopsies were digested overnight at 56ºC and gentled shaking with lysis buffer (50 mM Tris-HCl pH 8, 100 mM EDTA pH 8, 100 mM NaCl 1% SDS) and proteinase K (10 mg/ML). After overnight incubation, samples were centrifuged for 10 minutes at 15000 g to get rid of undigested tissues and supernatants were transferred into new eppendorf tubes. To lyse cells and access genomic DNA, saturated NaCl was added to samples and incubated for 10 minutes on a rocking platform. Samples were then centrifuged for 10 minutes at 15000g at 4ºC to get rid of cell debris. Supernatants were transferred into new eppendorf tubes and DNA was precipitated with 2-propanol. After centrifugation for 10 minutes at 15000g, the pellet was washed with 70% Ethanol. After air drying the pellet, DNA was resuspended in TE (100 mM Tris-HCl pH 7,5 and 1 mM EDTA pThe DNA of each sample was quantified by spectrophotometry (A260 and A280) stored at -20ºC. For PCR analysis, DNA was placed in PCR tubes and used as template for amplification by adding the Master Mix solution (each primer (presented on table 2), ddH_2O, Buffer

5X, dNTPS and Taq Polymerase). 1502 and 1503 APP primers amplify mutated Swedish APP DNA, while Myosine SENS and Myosine AntiSENS amplify an internal DNA fragment, as a positive control for the PCR reaction. PCR was performed by denaturing the DNA at 96ºC for 15 min, followed by 30 cycles of amplification: 95ºC for 45 sec, 55ºC for 1 min, 72ºC for 1 min and a final extension step at 72ºC for 5 min. The PCR-specific primers used are presented in table 2.

Table 2: Protocol primers for transgenic mice screening.

Primer	Sequence 5'→3'	Primer type
1503 APP	CTG ACC ACT CGA CCA GGT TCT GGG T	APP transgene
1502 APP	GTG GAT AAC CCC TCC CCC AGC CTA GAC CA	APP transgene
Myosine SENS	CCA AGT TGG TGT CAA AAG CC	Internal Positive control Forward
Myosine AntiSENS	CTC TCT GCT TTA AGG AGT CAG	Internal Positive control Forward

PCR products were visualized on a 2% agarose gel containing ethidium bromide to confirm the presence of DNA of human APP in offspring (Tg+). To allow band size estimation, 100 bp DNA ladder was added. Gel was run for 40 minutes at 100 volts in an electrophoresis bath containing 1x Tris-Acetate-EDTA (TAE) buffer and then placed under UV light for band visualization.

3.3. Biochemical Experiment

3.3.1. Blood sampling

For corticosterone quantification, blood was collected from the submandibular vein without anaesthesia (Fernández, Peña et al., 2010). The mouse was restrained with the non-dominant hand by grasping the loose skin over the shoulders and behind the ears. The submandibular vein was punctured with a 25 gauge needle slightly behind the mandible, but in front of the ear canal. Blood flowed immediately. The blood sample was collected in a heparin-coated tube to avoid coagulation (Sarsedt, France) (Figure 16).

Figure 16: Blood collection from the submandibular vein in heparin-coated tubes.

3.3.1.1.Assessment of Corticosterone concentration

Sixteen Tg2576 mice and twenty-seven wt mice of 4 months of age were used to measure circadian plasma corticosterone levels. Blood was collected from the submandibular vein at light (08:00 h) phase and dark (20:00 h) phase. After 15 minutes of centrifugation at 2000 x g, at 4 C, plasma samples was stored at – 80ºC (Cota, Steiner et al., 2007). Plasma concentrations of corticosterone were measured using an Enzyme Immunoassay (EIA) kit from Enzo Life Science following the manufacturer's instructions (Enzo Life Science, Villeurbanne, France). The sensitivity of this assay was 27.0 pg/ml.

3.3.1.2. Dexamethasone suppression test

Dexamethasone 21-phosphate disodium salt (Sigma-Aldrich, St- Quentin Fallavier, France) dissolved in saline (NaCl 0,9%) or vehicle (saline) were injected intraperitoneally in wt mice at concentrations of 0,01, 0,1 and 0,2 mg/kg at 12h00 (Cota, Steiner et al., 2007) (6 wt-NaCl, 8 wt-DEX 0,01 mg/kg, 6 wt-DEX 0,1 mg/kg and 8 wt-DEX 0,2 mg/kg). Blood was collected from the submandibular vein at 18h00 and plasma corticosterone levels were assessed by ELISA. After completion of this dose-response test, the half maximal effective concentration was chosen for testing on wt and Tg2576 mice. DEX at a concentration of 0,05 mg/kg or vehicle (saline NaCl 0,9%) were injected in Tg2576 and wt mice (8 wt NaCl, 11 wt-DEX 0,05 mg/kg, 17 Tg2576-NaCl and 22 Tg2576-DEX 0,05 mg/kg). At 18h00, blood was collected. After 15 minutes of centrifugation at 2000 x g, at 4ºC, plasma samples was stored at – 80ºC. Plasma concentrations of corticosterone were measured by ELISA.

Additionally, an assay was run in order to determine the levels of cross reactivity of this ELISA assay with DEX to ensure that the assay was appropriate for DEX suppression testing. This was done by using DEX concentrations ranging from 0,8 ng/mL to 20 ng/mL in the ELISA protocol (Koerner, 1997). With these results, it was possible to calculate the relative cross-reactivity (CR, specificity) in % as follows: CR (%) = CCORT / CDEX * 100, where CCORT is concentration of corticosterone needed for 50% Antibody Bound and CDEX is concentration of DEX needed for 50% antibody bound.

3.3.2. Glucocorticoid Receptor quantification

3.3.2.1. Protein extraction from hippocampal total homogenate

Brains from 3 Tg2576 and 4 wt mice of $4^{1/2}$ months of age were removed and dissected to obtain the hippocampus. Mice were taken one by one from the stabulation room to the dissection room to minimize the stress. After cervical dislocation, hippocampi were rapidly dissected out of the brain and immediately placed on ice in 100 µL of lysis buffer (88% RIPA (1X) (Tris 10mM, NaCl 150 nm, EDTA 5 mM, SDS 0,1%, DOC 0,5%, NP40 1% *pH=8*), and protease and phosphatise inhibitors). Tissue was homogenized with a tissue homogenizer. After 30 minutes on ice, the hippocampus homogenates was centrifuged during 5 minutes, at 15000g at 4ºC. Supernatants are transferred into new tubes that were placed on ice. Protein fraction was then quantified using the standard BRADFORD method. For absorbance quantification, 1 mL of 20% Bradford (1part Bradford: 4 parts water) was added to 2 µL of sample (diluted 3 times in lysis buffer) and the assay was conducted in triplicate. The measurement was done after 5 minutes of colorimetric reaction at the wavelength of 585nm. Homogenates were stored at -80ºC until used for Western blotting.

3.3.2.2. Western Blot

- *Antibodies*

Primary rabbit polyclonal Anti-GR (Santa Cruz, Biotechnology, UK) and mouse Anti-β-Actin (Sigma-Aldrich, France) antibodies, secondary horseradish-peroxidase-

conjugated goat anti-rabbit (Beckman, France) and horseradish goat anti-mouse (Jackson immuno Research Europe, UK) antibodies (table 3) were used for this experiment.

Table 3: Primary antibodies used in GR's quantification in total hippocampus. Characterization by secondary antibody, dilution and molecular weight (kDA).

SAMPLE	ANTIBODY	SECONDARY ANTIBODY	DILUTION	MOLECULAR WEIGHT (kDa)
Total HPC homogenate	Anti-GR	Anti-Rabbit	1/1000	82
	Anti-β_Actin (internal positive control)	Anti-Mouse	1/5000	42

- *Protein separation in a TG-SDS gel of electrophoresis*

Lysates were resuspended in loading buffer (Tris 0,5 M, 10% Glycerol, 10% SDS, 0,05 % bromophenol blue at pH= 6,8 and 2,5% β-Mercaptoethanol) at a concentration of 1:1. TG-SDS gel was prepared with stacking 4% (stacking 2x (Tris 0,75M, SDS 0,2% pH=8,8), Acryl bis 37,5%, H_2O, TEMED and APS 10%) and resolving 10% (resolving 2x (Tris 0,25M, SDS 0,2% pH=6,8), Acryl bis 37,5%, Glycerol 58%, H_2O, TEMED and APS 10%) solutions and samples were added to the walls (~20µL). Samples were loaded under denaturing and reducing conditions with TG-SDS (Tris 125 mM, Glycine 1M, SDS 0,5% at pH=8,3) running buffer during 2-2,5 hours.

- *Proteins transfer to membrane and blotting*

Proteins were transferred by placing the TG-SDS gel in direct contact with the nitrocellulose membrane, protein binding support and ''sandwiching'' between two electrodes submerged in transfer buffer (Tris 155mM, Glycine 1.2M) at 65 V for 2 h. As the membranes have high affinity for proteins, the remaining surface of membranes after transfer were blocked with milk in PBS1X (50g/L) during 15 minutes, to prevent non-specific binding of the detection antibodies. Total hippocampus homogenate membrane were then exposed to primary antibody anti-GR (1/1000 in 2,5% PBS-tween20) and the protein loading control anti-β-actine (1/5000 in 2,5% PBS-tween20) at 4ºC overnight. The next morning, membranes were washed 3 times for 15 minutes in

5% PBS1x tween20 and then incubated with the secondary antibodies horseradish-peroxidase-conjugated goat anti-rabbit and Goat-anti-mouse for 2 hours at room temperature. Finally, blots were washed three times with 5% PBS1x tween20 (Adzic, Djordjevic et al., 2009). The specific antibody–antigen complex was detected by an enhanced chemiluminescence detection system Lumilight (Roche Applied Science). The intensity of bands was analysed using the LAS-3000 imaging system (Fuji, Japan). This intensity of bands was quantified by densitometry using Multi Gauge v3.1 software (Fujifilm, Japan), and was expressed as the ratio relative to β-actin protein and normalized to the mean of GR content in wt mice.

3.4. Behavioural Experiment

3.4.1. Object Recognition (what-when-where)

This object recognition task, used to assess episodic-like memory in mice and integration of ‘’What, When and Where an event occurred’’, was adapted from (Dere, Huston et al., 2005). The object recognition task measures spontaneous exploration behaviour (Ennaceur and Delacour 1988). Animals approach and explore objects. They preferentially explore novel objects, presented at novel places, and objects it as not explored most recently (Eacott and Norman 2004).

3.4.1.1. Apparatus (Object Recognition box)

Object exploration was assessed in a plexiglass box (30x30x30). The box was covered with a 30x30 plexiglass cover with a centre hole for camera recordings to prevent mice from escaping from the box. Three of the inside walls contained different spatial cues, the first wall had 2 yellow stripes on a dark blue background, the second one had a black triangle on a white background and the third wall had black circles (6cm) on a white background (figure 17). A video camera, connected to a video recorder, was mounted 40 cm above the box to record samples and test trials on video-tapes for off-line analysis. Diffuse light provided an illumination density of approximately 3.0 lux at the centre of the box. After each trial, the apparatus was thoroughly cleaned with a 70 % ethanol solution and rinsed with water.

Figure 17: Spatial cues in the tree walls of the object recognition box

3.4.1.2. Objects

Two different objects, a black charger plug and a pink and green lego (in quadruplicate) made of plastic that differed in terms of height, colour, shape and surface texture were used. The objects were fixed to the floor to ensure that mice could not displace them. After each trial the objects were cleaned with 70% ethanol solution in order to remove odor cues and rinsed with water. Objects were placed according to the schema depicted on figure 18 with enough distance between each object allowing animals to circulate around.

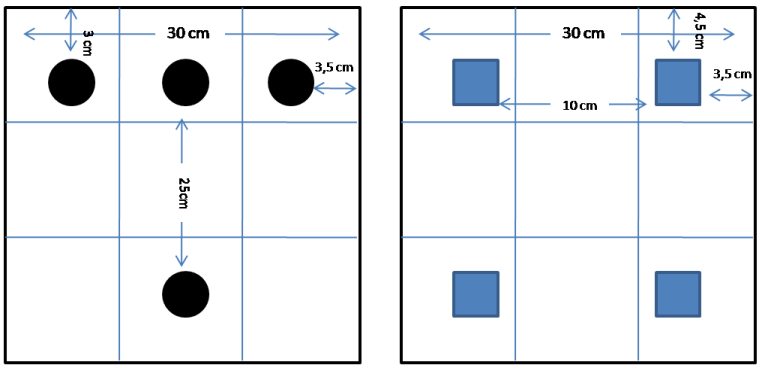

Figure 18: Distance between object-object and between object-wall.

3.4.1.3. Behavioural procedure

Four experiments with different animals were run: **1)** object recognition task (what-when-where) (25 wt, 13 Tg2576 mice), **2)** object recognition task what-when-where with RU486 dissolved in DMSO injection (4 Tg2576-DMSO, 8 Tg2576-RU486

(DMSO)) and **3)** object recognition task (what-when-where) with RU486 dissolved in H_20/tween20 injection (5 Tg2576- H_20/tween20, 7 Tg2576 RU486 (H_20/tween20)).

3.4.1.3.1. Handling and Acclimation

Before behavioural testing the mice were habituated to the handling procedure for three days (3 minutes each mouse). All testing was conducted during the light phase of the circadian cycle in cohorts of 12 mice, balanced by genotype and treatment. Mice were transferred from the storage room to a small room near the one in which the behavioural procedure occurred for 2 days before beginning the experiment and stayed there until the end of behavioural procedure. The temperature and illumination density was the same of that in the previous storage room.

3.4.1.3.2. Habituation to the box

After handling, the mice were further familiarized with the test apparatus devoid of containing objects for 2 consecutive days, 5 minutes exploration for each mouse. On the following 2 days, in order to habituate the animals to the presence of objects, the mice received 3 more daily sessions of 10 minutes duration, with at least 50 minutes inter-trial interval, and two objects placed into the corners of the open field, which were not reused in the (what –where-when) object exploration task. A full diagram of the procedure is depicted in figure 19.

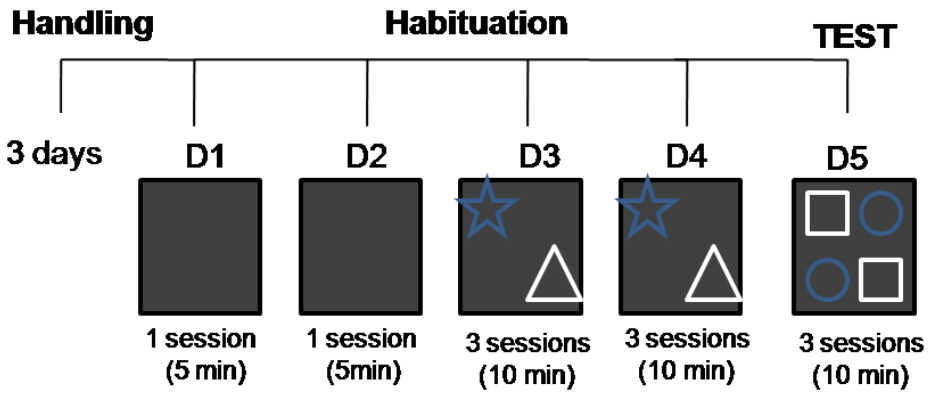

Figure 19: Object Recognition protocol. Adapted from (Dere, Huston et al., 2005)

3.4.1.3.3. Simultaneous assessment to what-when-where

On the final day of the procedure, each mouse received two sample trials and a test trial of 10 min duration per trial, with 50 min inter-trial intervals. The mouse was placed in the centre of the box containing novel objects and allowed to explore. In sample 1, four identical novel objects were placed in a triangle-shaped spatial configuration, one object in north-west (NW) corner, one in the centre of northern wall (NC), one in north-east (NE) corner and one in the centre of southern wall (SC) as seen in figure 20. In sample 2, the second set of four identical novel objects was placed in a square conformation, one in NW corner, one in NE corner, one in south-east (SE) corner and the other in south-west (SW) corner. In the final test (discrimination trial), two objects of sample 1, called old objects and two objects of sample 2, called recent objects were presented. Recent objects were placed at the same previous locations: NW and SE corners (figure 20). One old object was placed at the same previous position and named ‘’old stationary’’, whereas the other old object was displaced to a new position and called ‘’old displaced’’ (as represented in figure 20). In the inter-trial intervals mice were placed in the holding cages. The set of objects used in samples was counterbalanced to remove any preference for objects. According to recency discrimination, and placement discrimination, mice should explore the a greater extent the old objects than recent objects and in particular the old displaced object should be more explored than the old stationary object.

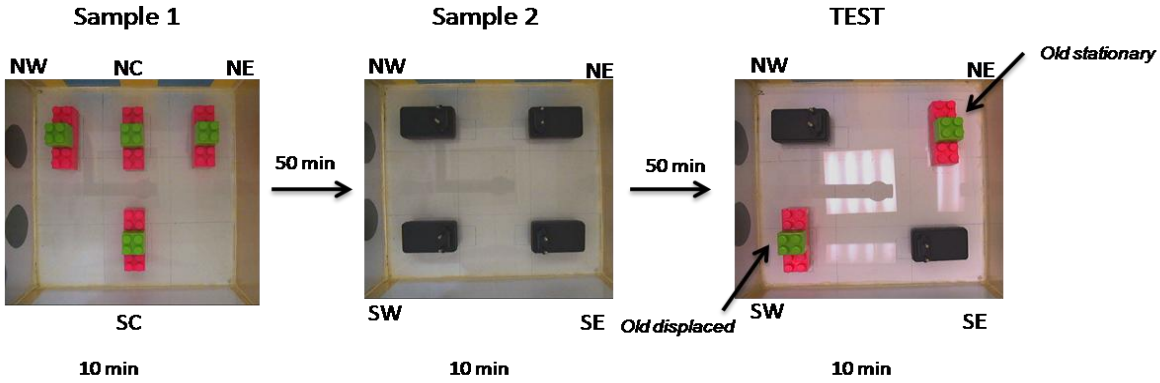

Figure 20: Schematic drawing of the (what-when-where) object exploration task on the last day of the behavioural procedure. The mice received three 10-minutes trials with a 50-minutes inter-trial interval. On sample 1, 4 novel objects were presented in a triangle spatial conformation. On sample 2, another 4 novel objects were presented in a square conformation. During the test trial, two ‘’old objects’’ and two ‘’recent objects’’ were presented as depicted. Object locations: NC=north-centre, SC=South-centre, NW=north-west, NE=north-east, SW=south-west, SE=south-east.

3.4.1.4. In vivo pharmacological treatment

The GR antagonist RU486 was administered in animals before the (what-when-where) assessment protocol of episodic-like memory. In a primary assay glucocorticoid receptor antagonist RU486, also called mifepristone (17b-hydroxy-11a (4-dymethylaminophenyl)-17a(1-propynyl)-estra-4,9-dien-3-one) (abcamBiochemicals, Cambridge, UK) was dissolved in DMSO (Dimethyl sulfoxide) according to the manufactorer's protocol at a concentration of 40 mg/kg. Tg2576 mice were injected subcutaneously (2mL/kg) with RU486/DMSO or vehicle (DMSO) two times per day (09:00 and 18:00), during the four days of habituation and a last injection on the morning of the test day. Considering the secondary effect observed with DMSO on behaviour of rodents (Castro, Hogan et al., 1995), further experiments were conducted with RU486 dissolved in water containing a droplet of tween 20 (~7,2%) at a concentration of 40 mg/kg (Chaouloff, Hémar et al., 2007). The injection protocol was identical to the RU486/DMSO above.

3.4.1.5. Behaviour analysis

For each mouse, the time spent exploring the different objects was scored off-line from video tapes using first stop-watches count and confirmed with the AnyMaze® behavioural software while the experimenter was blind for genotype condition. Each object was associated to a key of the keyboard, and the time of exploration was counted as the time of key pressing for each object. Exploration of an object was assumed when the mouse approached an object and had physical contact with it, either with its vibrissae, snout or forepaws at a distance less than 2 cm.

The configuration used in test trial allows for testing the three components of episodic-like memory: (what-when-where). The combination of these three components is required to define an episodic-like memory. Impairment in only one aspect of these three is sufficient to disrupt this type of memory.

The ‘’what’’ component cannot be dissociated from where and when components. The discrimination ratio for recognition of the distinct objects (“What” memory) was calculated as the difference between the average exploration times for both “old” objects minus that for the “recent” objects, divided by the sum of those times. The discrimination ratio for “when” memory was calculated as the difference

between the exploration time for the ‘’old stationary” object and that for the average exploration times for “recent familiar” objects, divided by the sum of those times. The discrimination ratio for “where” memory was calculated as the difference between the exploration time for ‘’old displaced” and that for ‘’old stationary”, divided by the total time exploring both objects (Dere, Huston et al., 2005; DeVito and Eichenbaum, 2010). A positive discrimination ratio represented a good memory whereas a negative ratio manifested memory impairment.

3.5. Statistical analysis

All data are presented as mean ± standard error mean (SEM) in each group, with n corresponding to the number of animals used in each experiment. Statistical significance of differences between means was determined through SPSS software. Corticosterone and GR quantification data were analyzed using the two-tailed Student’s t test for independent groups (wt, Tg2576). For behavioural data analysis, all statistical analyses were conducted only on the test trial and data were analyzed with one or two factors analysis of variance (ANOVA). The normality of the data distribution was verified using the Kolmogorov-Smirnov test. As there were differences in means further analysis were performed to explore where differences were. All ANOVAs post-hoc tests and interactions analysis consisted of independent two-tailed student’s t test because groups presented a normal distribution and equally of variances:

- For the first analysis of the four objects exploration in the test trial, two-way ANOVA was applied (genotype x object) in which genotype has two independent variables (wt and Tg2576) and objects present four dependent variables (recent 1, old stationary, old displaced and recent 2). Differences within each dependent group were performed by two-tailed student’s t test between independent groups.
- For the integration of what-when-where analysis in the test trial, differences between groups (wt, Tg2576) for each component ‘what’, ‘where’ and ‘when’ were analyzed by the two two-tailed student’s t test for independent groups (wt, Tg2576).
- For drug treatment analysis, each component of episodic-memory integration what-when-where was analyzed with one factor ANOVA, for each component of episodic memory and the four independent groups (wt, Tg2576, Tg2576-vehicle;Tg2576-Ru486). Because the normality of data distribution was found, future

analyses were performed comparing pairs of independent groups of interest by two tailed student's t test. The level of significance was $p<0,05$ for all tests.

4. RESULTS

Previous studies suggest that stress could be an important risk factor contributing to the development of AD. This is supported by strong clinical evidence that AD patients show an increase in circulating cortisol (Csernansky, Dong et al., 2006; Elgh, Lindqvist Åstot et al., 2006; Wilson, 2007; Rothman and Mattson, 2010). Also a dysregulation of HPA axis in these patients is suggested, because they show an impaired response to dexamethasone induced negative feedback (Greenwald, Mathe et al., 1986; Näsman, Olsson et al., 1995; Swanwick, Kirby et al., 1998). Associated with these changes, episodic memory is the first type of memory impaired in AD patients (Bäckman, Small et al., 2001). In addition, data from our laboratory showed that deficits in hippocampal long-term depression (LTD) present in Tg2576 mice at 4 months of age, could be reversed by blocking GRs with RU486 (unpublished results). This evidence prompted us to study the integrity of the HPA axis and glucocorticoid receptors and also episodic-like memory in Tg2576 mice at this early phase of the disease (when Aβ begins to accumulate, but before it aggregates into senile plaques).

4.1 Discrimination between transgenic and non-transgenic mice

Before experiments, mice were genotyped to differentiate between non-transgenic (wt) and transgenic animals (Tg2576). Figure 21 shows a typical progeny genotyping containing bands for Tg2576 and wt mice. Wt mice presents one single band of 150 bp that corresponds to Myosin control (lanes 1, 3, 4, 8, 9, 10), while Tg2576 mice exhibits two bands, one of 150 bp (Myosin) and a band of 466 bp that corresponds to the transgene (lanes 2, 5, 6, 7). Transgene-negative (Tg -) littermates were used as controls (wt mice).

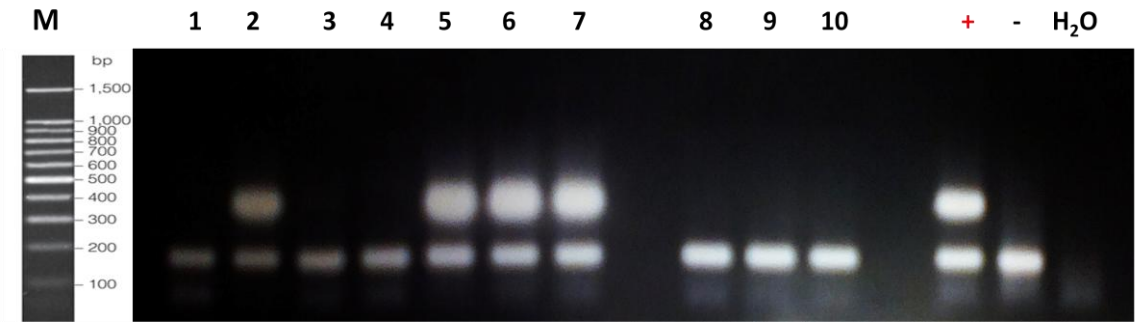

Figure 21: Identification of Tg2576 and wt mice in the 2% agarose gel. On the right side, there are positive control (+), negative control (-) and H_2O (to demonstrates that master mix solution have no contaminating DNA). On the left side, there is a 100 DNA bp ladder marker (M) separated equally in the gel. WT mice harbour only the control PCR product represented at 150 bp (eg. lane 1), whereas Tg2576 mice habour both the control and the APPswe transgene products at 150 and 466 bp, respectively (eg. lane 2). Mice 2, 5, 6 and 7 are Tg2576 (+) and mice 1,3,4,8, 9 and 10 are controls (-).

4.2. Increased corticosterone level in Tg2576 mice during the active phase

To evaluate the stress level in Tg2576 mice at the early phase of AD pathology, measured as the plasma corticosterone concentration, blood was collected during the resting and awake phases in 4 months of age wt and Tg2576 mice. Free corticosterone levels in plasma displayed a clear diurnal rhythm in wt and Tg2576 mice, with low levels during the morning hours and a significant peak in the evening (Figure 22, $p<0,001$, student's t test). At the onset of the light (resting) phase, the mean level of basal plasma corticosterone in Tg2576 mice (36,84 ng/ml±9,21, n=16) was approximately the same as in wt mice (26,25 ng/ml±2,67, n=27) (Figure 22, $p>0,05$, student's t test), showing no differences by genotype. By contrast, at the onset of the dark (awake) phase, when corticosterone levels are normally higher (respecting the circadian cycle), Tg2576 mice showed significantly higher plasma corticosterone concentration (178 ng/ml±17,96 ,n=17) than wt mice (106 ng/ml±6,8,n=27) (Figure 22, $p<0,01$, student's t test).

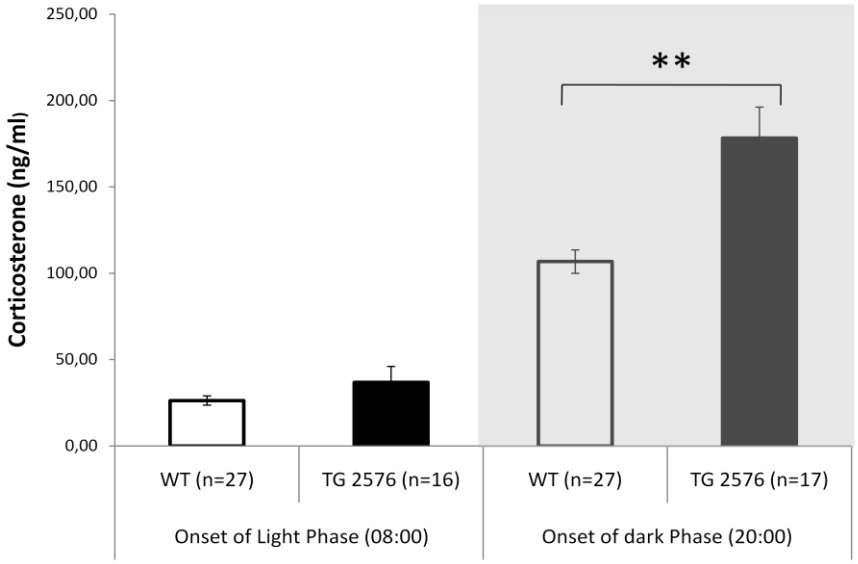

Figure 22: Plasma corticosterone levels were measured by ELISA at the onset of the light/resting (08h00) (with white background) and dark/awake (20h00) (with grey background) phases in 4 months old male Tg2576 mice and wt mice. Students t-test **p<0,01, bars represent mean±sem and n=number of mice.

4.3. Decrease in negative HPA axis feedback function in Tg2576 revealed by the dexamethasone supression test

As corticosterone was increased in Tg2576 mice at the onset of the dark phase, it was important to know if the regulation of corticosterone via the HPA axis activation and notably the negative feedback control of the HPA axis were perturbed. To test this, we applied the DEX supression test in Tg2576 mice and wt littermates. DEX is a synthetic glucocorticoid which can be injected in vivo to rapidly suppress the levels of GCs in the body via activation of this feedback loop. Before performing the experiment, we had to ensure that the ELISA kit did not cross-react with DEX to any significant extent, a technical issue that would have prevented data interpretation. We therefore first tested the cross-reactivity of the anti-corticosterone antibody used in the ELISA kit against dexamethasone by analyzing standard curves of corticosterone and dexamethasone (0,8, 4 and 20 ng/mL). The antibody used in the corticosterone assay showed very low cross-reactivity with DEX compared to corticosterone, CR=0,001% (figure 23), ensuring suitability of this ELISA kit for the DEX suppression test.

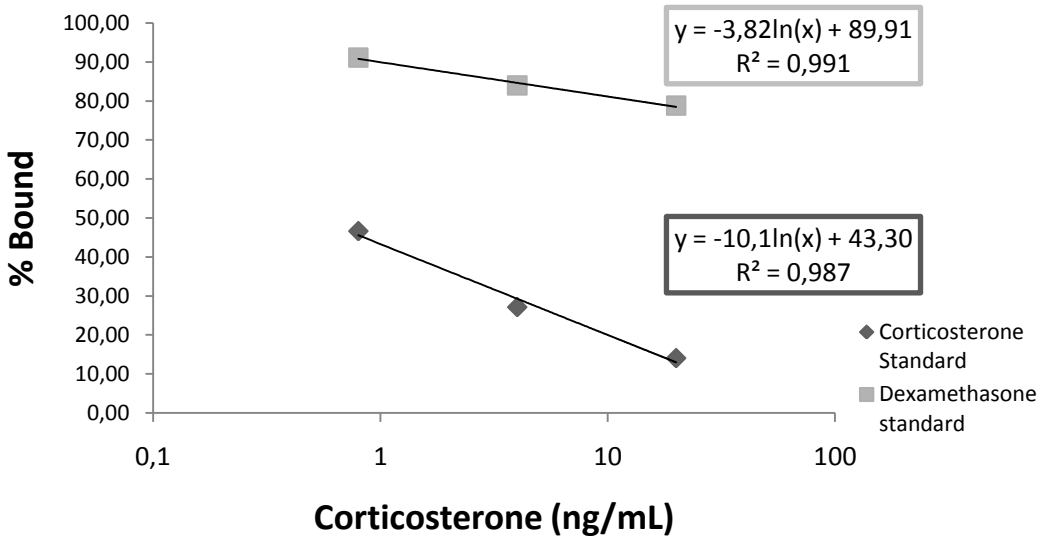

Figure 23: Comparison of the cross reactivity of the anti-corticosterone polyclonal antibody with DEX and corticosterone used in ELISA kit (Enzo Life Sciences) at a range of 0,8 to 20 ng/mL. The x axis is a logarithmic scale of corticosterone concentrations, (ng/mL).

Then, in order to find the dexamethasone concentration in which the negative feedback response is optimal in mice resulting in significant reduction in plasma corticosterone levels, different DEX concentrations (0,01, 0,1 and 0,2 mg/kg) were injected intraperitoneally in wt mice.

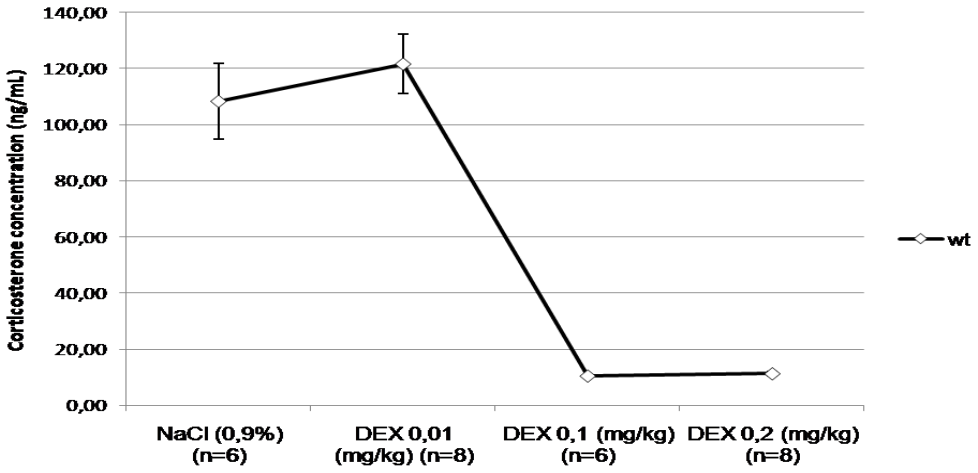

Figure 24: Plasma corticosterone levels measured in wt mice six hours after DEX (0,01, 0,1 or 0,2 mg/kg) or saline in vivo injection. n= number of mice.

Figure 24 shows that, at 18h00, saline (NaCl) wt mice have levels of corticosterone (108 ng/ml±13,37, n=6) similar to those measured at the onset of the dark awake period (20h00) for wt mice (106 ng/ml±6,8,n=27). With the injection of DEX at 0,01 mg/kg, the concentration of corticosterone did not decrease, demonstrating that at

this low concentration of DEX, we did not observe any effect in the HPA axis regulation. At concentrations of 0,1 and 0,2 mg/kg, the HPA axis feedback effect was fully engaged resulting in a plateau effect of very low levels of corticosterone (10,48 ng/ml±0,35, n=6 and 11,29 ng/ml±0,38, n=8). In light of these results, we chose a more suitable concentration between these tested doses to work in the dynamic range of the HPA axis feedback effect. For the next experiment, DEX was therefore injected at a concentration of 0,05 mg/kg in Tg2576 and wt mice (Figure 25).

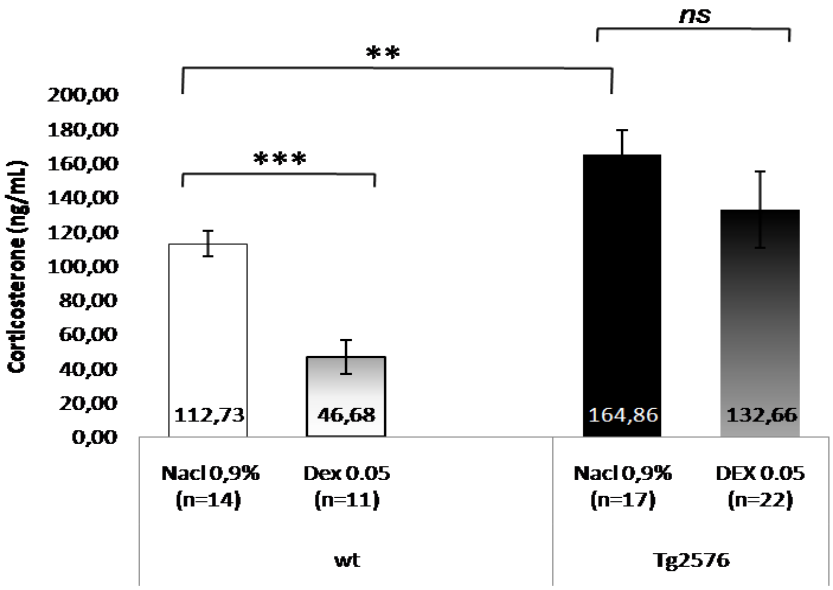

Figure 25: Plasma corticosterone levels six hours after in vivo injection of DEX at 0,05 mg/kg or saline was measured by ELISA in 4 months old wt and Tg2576 mice. Saline was injected in wt (white bar) and in Tg2576 mice (black bar). DEX (0,05 mg/kg) was injected in wt (white-grey bar) and Tg2576 mice (black-grey bar). Students t-test *ns*-non-significant, **p<0,01, ***p<0,001 bars represent mean±sem and n=number of mice.

First, we confirm that saline controls Tg2576 mice have higher corticosterone level than wt mice ($p<0,01$, student's t test) close to the onset of the dark awake phase, as seen previously in figure 22. Also, corticosterone concentration significantly decreased in wt mice after DEX injection (0,05mg/kg) when comparing to saline controls ($p<0,001$, student's t test), confirming good engagement of the HPA axis negative feedback mechanism in these control mice. By contrast, Tg2576 mice did not show a significant decrease in corticosterone levels after DEX injection, as seen in figure 25 ($p>0,05$), demonstrating that this negative feedback function is compromised in these transgenic mice.

4.4. Increased Glucocorticoid receptors levels in hippocampus of Tg2576 mice.

As hippocampal GRs are involved in HPA axis regulation, it was important to quantify them to better correlate their action with the HPA axis dysregulation previously demonstrated. GR expression was quantified in total hippocampus homogenate from 4 wt and 3 Tg2576 mice of four months of age by western blot. The protein of interest was identified using a specific anti-GR antibody. Actin was used as a protein loading control using a specific anti-actin antibody. In the western blot membrane fractions (figure 26, left), we observed that actin (42 kDa) is equally distributed in all samples (Tg2576 and wt) as expected. The band that migrated at the position expected for GRs (82kDa) is increased in intensity for Tg2576 mice compared to wt mice. The quantitative analysis of GR content, normalized to actin and to the average of wt, revealed that Tg2576 mice have significantly higher GRs (1,51±0,09, n=3) than wt mice (1±0,09, n=3) in total hippocampus homogenates (Figure 26 right, $p<0,05$, student's t test).

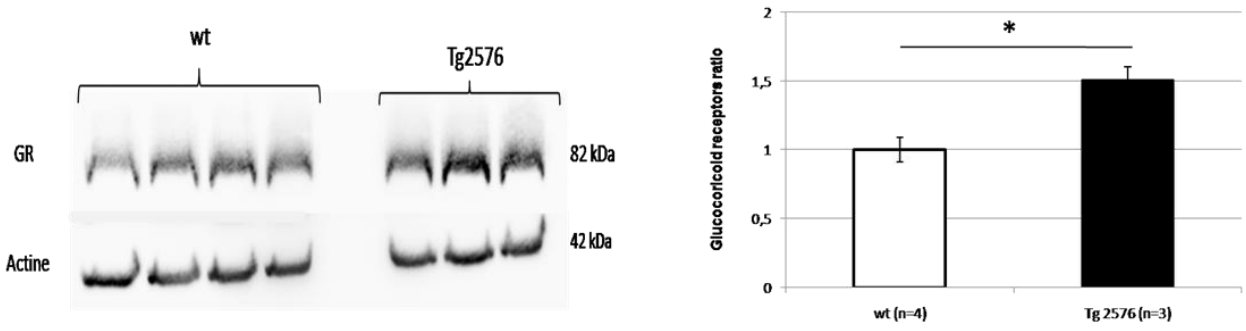

Figure 26: Glucocorticoid receptors protein levels in total hippocampus homogenate were measured by western blot in 4 months old wt and Tg2576 mice. On the left side, immunoblots of fractions with anti-GR and anti-actin antibodies (actin was used as a protein loading control) are shown. On the right side bars represent the GR quantification normalized to β-actin and to the mean of GR content in wt. Students t-test *p<0,05, bars represent mean±sem and n=number of mice.

4.5. Impaired episodic-like memory in Tg2576 mice

To assess episodic-like memory in the early stage of AD, Tg2576 mice and wt controls were allowed to explore objects presented in a variable temporal order and spatial conformation in a what-when-where task. The quantitative analysis was

conducted only during the test trial. Figure 27 represents the exploration ratio (specific object/all objects) for the four objects in the two groups (wt mice, Tg2576 mice). The first observation was that wt and Tg2576 mice explored each object more than 7 seconds, which corresponded to a discrimination ratio higher than 0,16. Second, according to the recency discrimination, the two groups explored to a greater extent the old objects (presented earlier in the sequence) than the recent objects as expected. The exploration ratio (specific object/ all objects) is similar for both recent objects (recent 1 and recent 2) between wt and Tg2576 mice. According to our hypothesis, wt mice spent more time exploring the old displaced object, which was presented both earlier in the sequence of trials and in a different location in comparison to the other objects (old stationary and both recent objects). In contrast, Tg2576 mice showed no preference between the two old objects (old stationary, old displaced). An analysis of variance revealed a significant main effect of objects $F(3,108)=30.57$ $p<0.001$ and a significant interaction between groups (wt and Tg2576) and objects, $F(3,108)=4.80$ $p<0.01$. Post hoc analysis revealed that the exploration ratio of the old displaced object differed between the groups (pair-wise comparisons: Bonferroni, $p< 0.01$). For the wt group, post hoc analysis revealed differences in exploration among old stationary object and old displaced object ($p<0.01$), a value which was not significant for the Tg2576 group. These data demonstrated that temporal order and object discrimination are preserved in Tg2576 mice, whereas spatial placement discrimination is impaired.

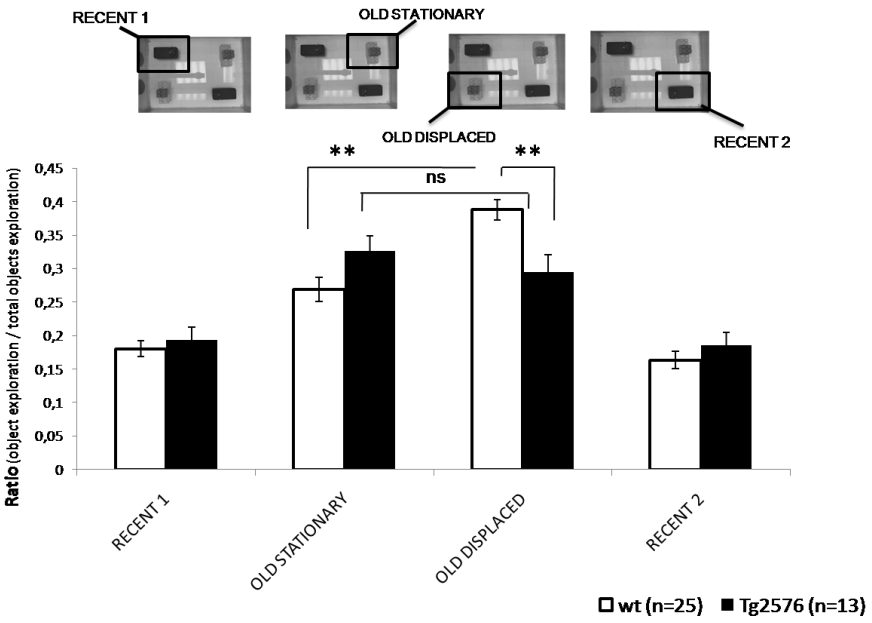

Figure 27: Exploration ratio of each object presented in the test trial by 4 months old wt and Tg2576 mice. ns - non-significant, ANOVA, Bonferrini post-hocs **p<0,01, n= number of animals

Exploration times of the two groups of animals were then integrated in the three episodic-like memory components (what-when-where) as described in the methods section. Figure 28 represents the discrimination ratio for these three components. The data show a positive discrimination in the wt mice for the three components, ‘what’ ‘when’ and ‘where’, confirming the existence of a good episodic memory in these mice. By contrast, at 4 months of age, Tg2576 mice showed impairment in the spatial component ‘where’ of the episodic-like memory.

The statistics were applied on each component separately. Indeed ‘what’, ‘where’, ‘when’ were calculated on the basis of the exploration ratio for the four objects, which means that each component is intimately related to the others. For this reason, we could perform two-tailed tests between groups (wt/Tg2576) and each component (what-where-when). The Tg2576 mice showed a lightly decrease in the component ’what’, but it was not significant compared to wt ($t(36)=1.46$, $p>0,05$, ns). Also, the difference in the ‘when’ component between Tg2576 and wt mice was not significant ($t(36)= -0,45$, $p>0,05$, ns). Only the impairment observed in the component ‘where’ for the Tg2576 mice was significantly different from wt ($t(36)=2.91$, $p<0.01$).

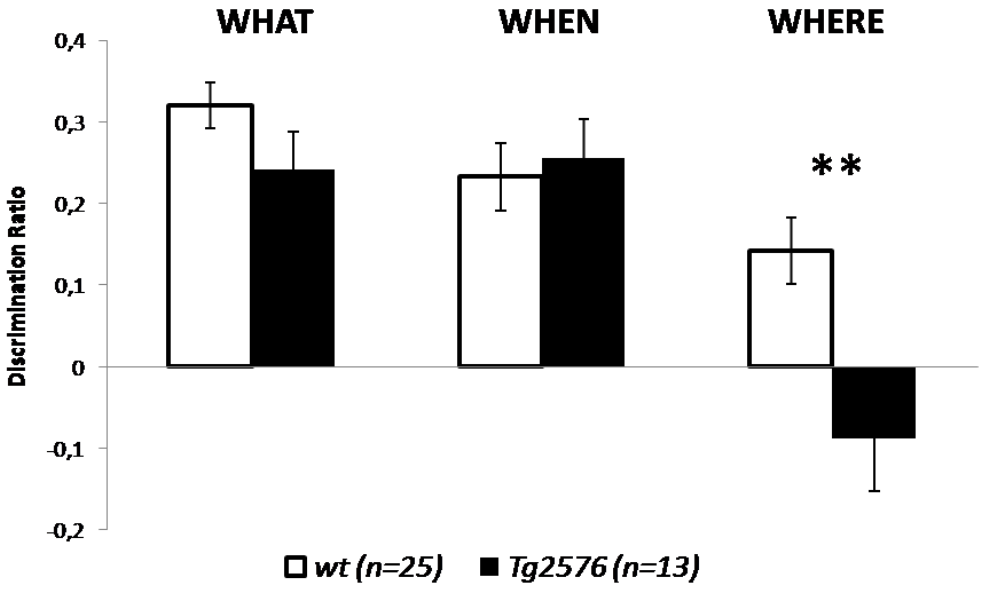

Figure 28: Episodic-like memory in 4 months old wt and Tg2576 mice. Discrimination ratio for each component of episodic-like memory. Student’s t-test**p<0,01, n= number of animals

4.6. Rescue of episodic-like memory deficit in Tg2576 with a glucocorticoid receptor blocker

To try to reverse the deficit presented in the 'where' component of episodic-like memory at 4 months of age by Tg2576 mice, a chronic treatment with the glucocorticoid receptor blocker (RU486) was administered in a new batch of Tg2576 mice before submitting these animals to the episodic-like memory behavioural protocol. RU486 was first dissolved in DMSO and then, because of the observed negative effects of DMSO on behaviour during the task, we performed new experiments using 7,2% H_2O/tween20 as vehicle.

- **Treatment with RU486 in DMSO vehicle**

The object recognition (what-when-where) task was conducted after sub-chronic in vivo RU486 treatment in Tg2576 mice. Figure 29 represents the discrimination ratio for each component of the episodic memory test for the two new groups of tested Tg2576 mice (Tg2576(DMSO),Tg2576(RU486)). To compare the DMSO and RU data to our previous experiment, this graph also includes our previous results obtained in untreated Tg2576 and wt mice. Injections of RU486 or DMSO had no effect in the 'what' ($F(3,49)=0,829, p>0,05$,ns) and 'when' ($F(3,49)=0,448, p>0,05$,ns) components of episodic-like memory. The analysis of variance for the component 'where' revealed a difference ($F(3,49)=3.53$, $p<0.05$). Further analysis showed a significant difference between Tg2576 and Tg2576 (RU486) mice ($t(19)=-2.37$, $p<0.05$), which indicates a rescue of episodic-like memory in Tg2576 with RU486. In addition, the wt group is not different from Tg2576 (RU486) ($t(31)=-0.38$, $p>0.05$,ns).

However, the Tg2576-vehicle (DMSO) group also presented a positive discrimination ratio for the 'where' component that is not significantly different from Tg2576 mice ($t(15)=-1,41$, ns), but also not significantly different from the wt mice ($t(27)=0,26$, ns). We attribute this unexpected inconclusive effect to the influence of in vivo DMSO treatment on behaviour, as reported previously by other researchers (Castro, Hogan et al., 1995).

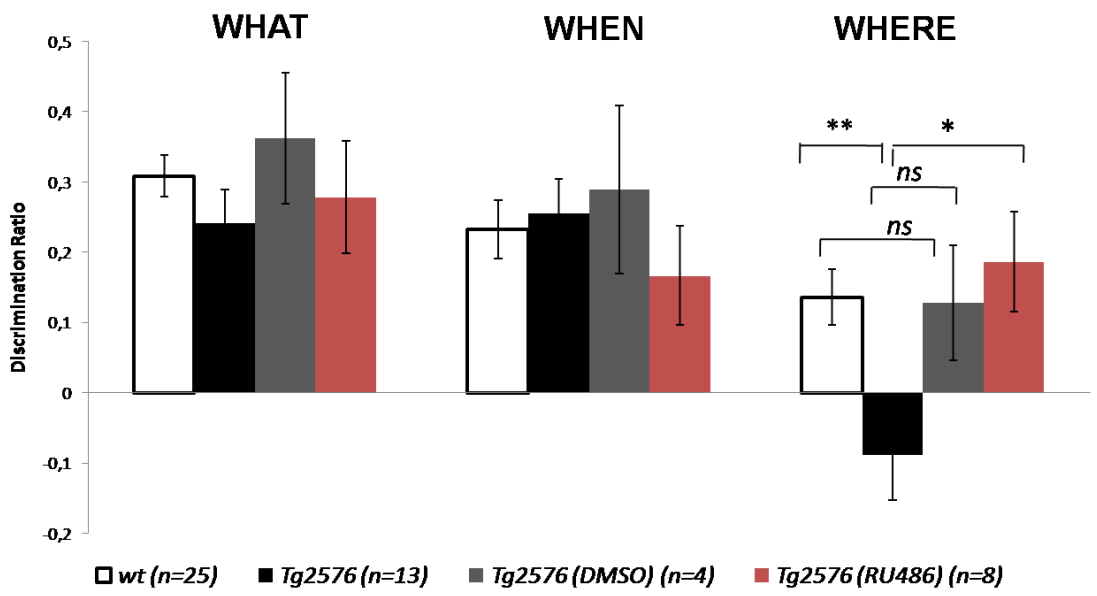

Figure 29: Episodic-like memory in 4 months of age Tg2576 mice after RU486 injection or vehicle (DMSO). Discrimination ratio for each component of episodic-like memory. ANOVA and post-hoc students-t-test, ns-non-significant, *p<0,05, **p<0,01, n= number of animals.

- **Treatment with RU486 in H_2O/tween 20 vehicle**

Due to the inconclusive nature of the data obtained with DMSO as vehicle and the previously reported unsuitability of DMSO for behavioural experiments, we tested a newbatch of Tg2576 mice with another vehicle. RU486 was dissolved in H2O/tween20 vehicle. Figure 30 showed that there were no significant changes in the 'what' and 'when' components after RU486 and vehicle (H_2O/tween20) injections (F(3,49)=1,38,ns) and (F(3,49)=1,21,ns) in Tg2576 mice, respectively. Analysis of variances revealed a significant difference in 'where' (F(3,49)=3,04,p<0,05) between the different groups of mice. As expected, Tg2576-H_2Otween20 mice had a negative discrimination ratio for the 'where' component significantly different from wt (t(28)= 2,051, p<0,05) and no different from Tg2576 mice (t(18)=-1,32,ns), demonstrating that use of this vehicle did not significantly modify the behaviour of these Tg2576 mice. We observed that Tg2576-RU486 displayed a positive discrimination ratio for this component, suggesting potential rescue of this episodic memory deficit by RU486, However, the difference between Tg2576 and Tg2576-H_2Otween20 or Tg2576-H_2Otween20 and Tg2576-RU486 did not reach yet statistical significance (t(18)=-1,32,ns, t(10)=-0,909,ns, respectively). The data using this new vehicle were obtained

from a few mice, and although these preliminary data are encouraging, more groups need to be tested to validate this finding.

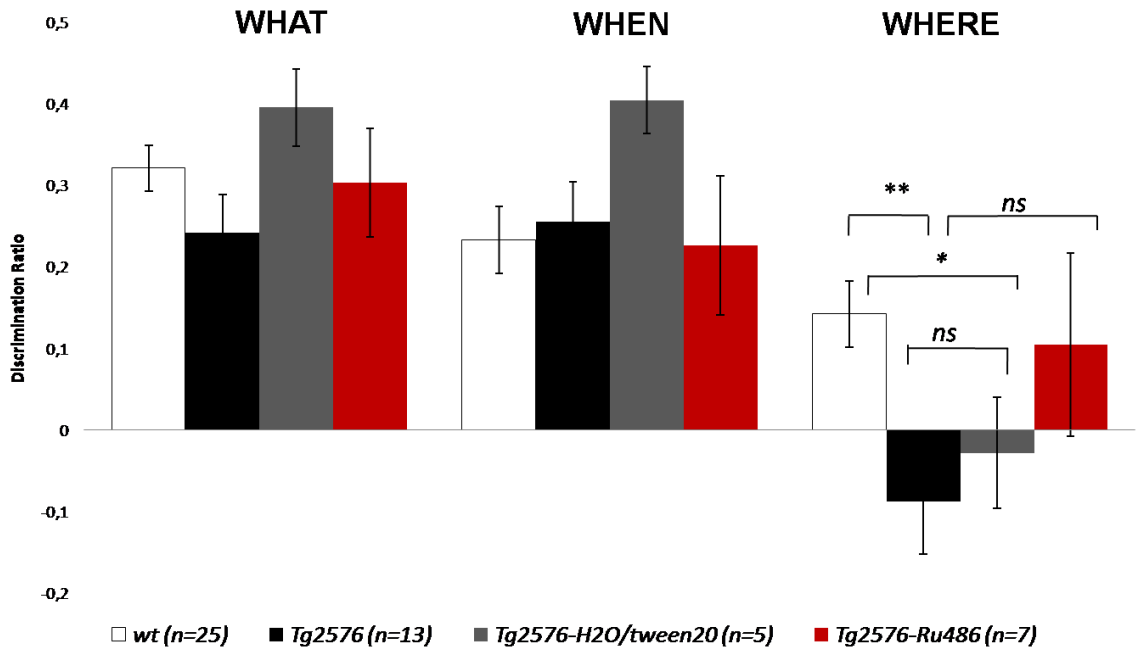

Figure 30: Episodic-like memory in 4 months old Tg2576 mice after RU486 injection or vehicle (H_2O/tween20). Discrimination ratio for each component of episodic-like memory. ANOVA and post-hoc students-t-test, n= number of animals, ns-non-significant, *p<0,05, **p<0,01, n= number of animals.

DISCUSSION

The goal of the present study was to investigate the influence of the early co-accumulation of APP and stress metabolites to trigger the onset of memory deficits in AD. To address this issue, we used a transgenic model of AD, the Tg2576 mice at 4 months of age, an early symptomatic age at which Aβ begins to accumulate, but does not yet aggregate into plaques. The results reported here clearly show that at this early symptomatic age, Tg2576 mice display a perturbed HPA axis regulation and episodic-like memory deficits. Indeed, we demonstrated that Tg2576 mice present enhanced HPA axis activation characterized by increased plasma corticosterone levels at the onset of the dark awake phase and cannot activate the HPA axis negative feedback loop. They also display enhanced GR protein levels in the hippocampus. Also, Tg2576 mice present early deficits in episodic-like memory, specifically in the 'where' component of this type of memory and we have preliminary data suggesting that this memory deficit could be rescued by blocking GRs .

- **Dysregulated HPA axis in 4 months old Tg2576 mice**

For the assessment of the status of the HPA axis, plasma levels of total corticosterone were measured at the onset of the resting and awake phases. Although Tg2576 mice presented a circadian rhythm of corticorterone levels as wt mice, awake phase corticosterone levels were abnormally high in these mice. These data suggest that Tg2576 display HPA axis hyperactivity and this enhances plasmatic corticosterone levels. Elevated corticosterone levels have also been reported in this and other transgenic AD mouse models during the early and later phases of the pathology (Dong, Yuede et al., 2008; Cuadrado-Tejedor, Ricobaraza et al., 2012), although only one time point was estimated and circadian levels were not assessed in these studies. At the onset of the resting phase, corticosterone levels were not different in wt and Tg2576 mice. Some studies reported that the dip of the corticosterone rhythm (observed during the resting phase) is most susceptible to stress and also highly sensitive to negative feedback (Hebda-Bauer, Simmons et al., 2012). This hypothesis is based on studies suggesting that aged animals have typically elevated GC levels in the resting period

inducing then an impaired HPA axis response to further stimulus (Sapolsky, Krey et al., 2002). By contrast, another study showed that baseline levels of GCs (resting period) are not different between young and old subjects, but it was the delay in turning off of the HPA axis in response to an acute stressor that was significantly different (Morano, Vazquez et al., 1994). Differences in awake phase, but not in resting conditions, as we observed in these transgenic mice, may suggest that HPA axis maintains a normal function when GC levels are low in wt and Tg2576 mice but that when GC levels rises, the HPA axis cannot adequately cope with turning off GC secretion in these transgenic mice.

This hypothesis is supported by our other data obtained with the DEX suppression test. Wt mice show a decrease in plasma corticosterone levels after DEX injection, demonstrating a good ability to decrease HPA axis activation by inducing the negative feedback response. By contrast, Tg2576 show no significantly differences in plasma corticosterone concentration after DEX injection. This provides strong evidence that the HPA axis negative feedback regulation is severely impaired in this mice model at this early stage of the pathology. This in line with early clinical analysis in humans, demonstrating that AD patients fail to show cortisol supression after a DEX challenge (Greenwald, Mathe et al., 1986; Näsman, Olsson et al., 1995; Swanwick, Kirby et al., 1998; Magri, Cravello et al., 2006). Surprisingly, little was published about HPA axis dysregulation in AD mice models using DEX supression test. Only *Brureau and his colleagues* recently showed that rats injected with $A\beta_{25-35}$ had an impairment in HPA axis negative feedback response after dexamethasone injection (Brureau, Zussy et al., 2013). Our data therefore demonstrate that this HPA axis dysregulation is also present in the Tg2576 mice and suggest that early Aβ accumulation is intimately linked with this pathological phenotype.

As explained before, feedback response in the extrinsic HPA axis structures, like the hippocampus, is regulated by GRs (Reul and De Kloet, 1985; Myers, McKlveen et al., 2012). GR were quantified in the hippocampus, because this area is rich in those receptors (Herman, Ostrander et al., 2005). In the present study, we observed that Tg2576 mice have significantly increased expression of GR in the hippocampus as compared to wt mice. This data is not in line with some previous publications regarding the relationship between GC and GR levels, as it was demonstrated that an increase in GCs, and insufficient response by hippocampal GRs, leads to GRs downregulation and decreased expression (Herman, Adams et al., 2008; Zhou, Zhu et al., 2011). This was

explained by the GC cascade hypothesis, perhaps as an adaptation to prevent GC-induced damage (Sapolsky, Krey et al., 2002). Some recent studies in AD mouse models, however, associated GR or MR increase with a stress situation and are in line with our data. *Hebda-Bauer and her colleagues* showed that 3xTg-AD mice presents early activated HPA axis characterized by elevated mRNA levels of GR and MR in the hippocampus (Hebda-Bauer, Simmons et al., 2012). Also, Tg2576 mice exposed to a chronic stress situation displayed increased corticosterone levels and increased GR expression, associated to an increase in Aβ levels and Aβ deposition (Dong, Yuede et al., 2008). In a rat model of AD, injections of $A\beta_{25-35}$ caused an increase in GR expression in amygdala and hippocampus brain areas (Brureau, Zussy et al., 2013). Interestingly, a study using rats exposed to a chronic stress situation showed increased GRs in the hippocampus, but decreased GR in PFC and no changes in hypothalamic GRs and also decreased response to DEX in the hippocampus (Mizoguchi, Ishige et al., 2003). This study posits that the GC effects on GRs response are attenuated by repeated exposure to excessive GC levels in chronic stress situations. The mechanism by which increased corticosterone levels possibly increase GR is still unknown. But strong evidence suggests that excessive GRs activation appears to be detrimental for neuroplasticity that is required for coping with the demands imposed by stress upon an individual (Sousa, Cerqueira et al., 2008). Associated to that, Tg2576 mice used in the present study has increased GR levels and could be more susceptible to synaptic plasticity changes than control wt mice (unpublished results). However, little is known about how components of HPA axis and its regulator structures, upstream of GCs, impact an individual's vulnerability to AD.

- **Impaired episodic-like memory in 4 months old Tg2576 mice**

The assessment of episodic-like memory clearly showed that wt mice have a good episodic-like memory confirmed by the ability to recreate spatial and temporal order of the different objects presentation. In contrast, Tg2576 mice showed a deficit in encoding the spatial 'where' placement of the object, suggesting that episodic-like memory is affected early (at 4 months of age) in these mice. The results showed that Tg2576 mice and wt mice can use recency discrimination of objects presentation ('when' component), but Tg2576 mice did not remember the spatial placement of objects('where' component). According to our episodic-like protocol, an impairment in

only one component (what, when, or where) is sufficient to disrupt episodic-like memory (Dere, Kart-Teke et al., 2006). Indeed the definition of episodic-like memory is the integrative capacity of the hippocampus to combine a multi-dimensional memory in order to build a flexible trace of an event or personal experience containing information about the spatial and temporal context of these events (Clayton, Yu et al., 2001; Eacott and Norman, 2004; Langston and Wood, 2010). Animals with hippocampal lesions fail to form memory of spatial-temporal properties of objects (Clayton, Yu et al., 2001; Morris, 2001).

Consistent with the amyloid hypothesis, mice harbouring human APP mutations develop impairments in hippocampus-dependent spatial memory tasks with age (Hsiao, Chapman et al., 1996). Episodic memory is the first memory affected in the progression of AD (Bäckman, Small et al., 2001). The finding that episodic memory deficits in early AD are common is consistent with evidence that some of the earliest brain changes in this disease occur in the hippocampus and related structures (Braak and Braak, 1991). Episodic-like memory deficits have also been reported in fully symptomatic Tg2576 mice (10-12 months old) with a slightly different protocol of the object recognition task (Good, Hale et al., 2007) . Also, deficits were recently reported in another type of episodic-like memory during the early symptomatic phase in the 3xTgAD model (Davis, Easton et al., 2013). In our present study, our results showed that the deficits presented in episodic-like memory at 4 months of age in Tg2576 mice might be prevented by four days of subcutaneous injection of the GR antagonist, RU486.

When dissolved in DMSO, according to the manufacturer's protocol, it was showed that the RU486 rescue the deficits. However, vehicle solution, expected to have no effect in Tg2576 mice, also induced a positive discrimination ratio for the 'where' component. Although this ratio was not significantly different from Tg2576, suggesting that there is no effect, it was no significantly different from wt mice either, suggesting a kind of rescue with DMSO alone. Because of negative effects caused by DMSO on animal's behavior (Castro, Hogan et al., 1995), we changed vehicle and RU486 was then dissolved in 7,2% tween20/H_2O. Tg2576 mice injected with this vehicle had the expected profile showing a persistent deficit not significantly different from untreated Tg2576 mice and different from untreated wt mice. When dissolved in Tween20/H_2O, Tg2576 mice injected with RU486 show a positive discrimination ratio for the 'where' component, suggesting a possible tendency for the rescue, but these data did not yet reach statistical significance due to the few animals tested. These data however are

encouraging and suggest that blocking GRs might be sufficient to rescue this episodic memory deficit. Supporting this result, results of electrophysiological analysis of hippocampal function from the laboratory showed that RU486 treatment normalized CA1 NMDA-induced LTD alterations presents in these Tg2576 mice (unpublished data). Recently, other authors showed that RU486 treatment rescues the cognitive impairments and markedly reduces Aβ levels, as well as tau pathology, in 12 months old fully symptomatic 3xTg-AD (Baglietto-Vargas, Medeiros et al., 2012), again supporting our hypothesis that blocking GR function could have a good therapeutic potential for AD. A small clinical trial in AD patients treated with RU486 for just 6 weeks reported an increase in AD assessment Scale-Cognitive Subscale (Pomara, Doraiswamy et al., 2002). Together, these results strongly support the hypothesis that stress signalling, via GRs, could be implicated in the early memory deficits observed in AD.

- **Relationship between dysregulated HPA axis and episodic memory loss**

The link between HPA axis dysregulation and memory deficits have been reported before (Kim and Diamond, 2002; Wolf, 2003; Oitzl, Champagne et al., 2010). It has previously been reported that the activation of hippocampal GRs is relevant for memory consolidation (Oitzl, Fluttert et al., 1998). Stress effects on memory depend on the extent to which stress also affects consolidation and retrieval plasticity, in addition to encoding processes. Depending on the intensity of the stressor, stress can improve or impair memory (Schwabe, Joëls et al., 2012). Changes in the HPA axis, notably its hyperactivation, causes an increase in CRH and ACTH and in turn, GC levels, and changes in these components of the HPA axis have been suggested to have deleterious effects on the structure and function of various brain areas (Sapolsky, 1992; Swaab, Bao et al., 2005). Long-term exposure to stress or increased GC levels produce numerous alterations in hippocampal structure, including altered neurochemistry, excitability, neurogenesis, neuronal morphology and even cell death (Sousa and Almeida, 2002; Herman and Seroogy, 2006; Conrad, 2008; Joëls, 2008). As the hippocampus has a determinant role in memory encoding and termination of the stress response, it is reasonable to speculate that prolonged GC exposure and HPA axis dysregulation could significantly contribute the initiation and progression of AD pathology. In rodents, atrophy of neuronal dendrites within the hippocampus (Donohue et al. 2006) and

deficits in spatial memory (Coburn-Litvak, Pothakos et al., 2003; Wright, Lightner et al., 2006) have been reported after exposure to chronic stress or administration of the GC, corticosterone. Several theories of hippocampal functions have highlighted the important contribution that spatial information plays in episodic memory (Burgess, Maguire et al., 2002; Smith and Mizumori, 2006). Also, GRs could be implicated in the memory dysfunction observed. Overexpressing GRs, even in absence of chronic stress exposure, causes an ‘’aging-like’’ phenotype characterized by altered HPA axis responsiveness and a hippocampal memory impairment in the Morris water maze task in young animals (Wei, Hebda-Bauer et al., 2007). So, hippocampal neuronal damage induced by stress and elevated GCs seem to initiate a repetitive cycle of HPA axis dysregulation and further neuronal injury (Sapolsky, Krey et al., 1985; Sapolsky, Krey et al., 2002) leading to learning and memory deficits (Newcomer, Craft et al., 1994; Rothman and Mattson, 2010). In our Tg2576 mice model, dysregulated HPA axis associated to high levels of corticosterone and GR could be associated with the observed memory impairments in episodic-like memory. It is reasonable to speculate that this impairment could be reversed by blocking GR function as our preliminary data suggest. Many studies and theories were presented, however, the precise mechanism by which they contribute to AD onset remains to be fully elucidated.

- **How are the HPA axis dysregulation and early deficits in episodic-like memory related to Aβ?**

Two different experimental approaches have been used to show the link behavioural stress, dysregulation of axis and AD pathology : using analysis of AD mouse models which accumulate brain Aβ or infusion of Aβ peptides in rats (reviewed in (Rothman and Mattson, 2010)).

Several studies showed a direct relation between chronic stress and AD markers accumulation (APP metabolites, amyloid plaques and tau protein), as indicated below. In a triple-transgenic model of AD, the 3xTg mice, application of DEX accelerates the accumulation of intraneuronal $A\beta_{40}$ and $A\beta_{42}$ in vitro, implying that GCs can directly affect APP metabolites (Green, Billings et al., 2006). Also, chronic stress exposure and GC administration were shown to exacerbate APP metabolites accumulation, including Aβ, and increases Tau phosphorylation within the hippocampus (Cuadrado-Tejedor, Ricobaraza et al., 2012). Recently, it has been shown that chronic mild stress in the

3xTg-AD can lead to prolonged exacerbation of features of anxiety and elevated GC levels, leading to higher Aβ levels (Rothman, Herdener et al., 2012). A similar increase in extracellular and neuronal Aβ as well as an increase in phosphorylated tau were noted in the hippocampus in another transgenic model of AD following long-term immobilization stress (Jeong, Park et al., 2006). Comparatively, chronic stress isolation appeared to accelerate the underlying process of hippocampal and cortex amyloid plaque deposition in Tg2576 mice (Dong, Goico et al., 2004; Dong, Yuede et al., 2008). On the other hand, it had been hypothesized that in AD, an increase in Aβ plaques in the hippocampus of transgenic mice causes an increase in HPA axis activity, increasing corticosterone levels (Breyhan, Wirths et al., 2009; Nuntagij, Oddo et al., 2009). In rats, it was also been recently shown that Aβ injections causes HPA axis deregulation with corticosterone increase in corticosterone levels and in GR content (Brureau, Zussy et al., 2013). This implies that Aβ is perceived by the brain as a chronic stressor, suggesting that elevated GCs observed in AD could be a first a toxic consequence of altered APP processing (Zussy, Brureau et al., 2011).

The relation between chronic stress and cognitive decline in AD as also been investigated by others. AD transgenic mice exposed to immobilization stress for 8 months (from ages 3 to 11 months) resulted in severe learning and memory impairments and that increased extracellular amyloid plaque deposition, intraneuronal Aβ, and APP CTFs immunoreactivities, and neurodegeneration (Jeong, Park et al. 2006). In Tg2576 mice, induced stress increased Aβ levels and worsened spatial and fear memory compared to non-stressed animals (Carroll, Iba et al., 2011). In addition, also using this mouse model, it was observed that chronic stress exposure increased Aβ and phospho-tau in hippocampus and induced memory deficits in the spatial Morris-water-maze task (Cuadrado-Tejedor, Ricobaraza et al., 2012). Using rat models, *Catania and colleagues* revealed that like stress and GC, Aβ administration causes spatial memory deficits that are exacerbated by stress and GC (Catania, Sotiropoulos et al., 2007). Also in rat models, a marked impairment of learning and memory was observed when stress (induced by an intruder paradigm) was combined with Aβ, more so than that caused by Aβ alone (Srivareerat, Tran et al., 2009). All together the presented studies showed that cognitive decline and AD markers are accelerated by chronic stress situations

The present study showed that Tg2576 mice cannot cope as well as wt mice to elevated stress hormone levels, caused by the inability in turning the stress response axis off. Collectively, the previous evidence and our evidence strongly link adverse

stress inducing elevated corticosterone signalling, via GRs, with abnormal levels of Aβ, which could synergize to bring about hippocampal dysfunction and memory loss in AD. We propose a diagram linking these factors and explaining our results thus far (Figure 31).

Accordingly, AD human brains seem to be more susceptible to adverse stress possibly due to an impairment in hippocampus function and consequently in the HPA axis regulation in the early phase of AD. It had been shown that in elderly, chronic distress may enhance the incidence of mild cognitive impairment and contribute to the development of AD (Wilson, Evans et al., 2003; Wilson, Schneider et al., 2007). Nevertheless, the pathways linking changes in components of the HPA axis to changes in Aβ pathology still need to be clearly elucidated.

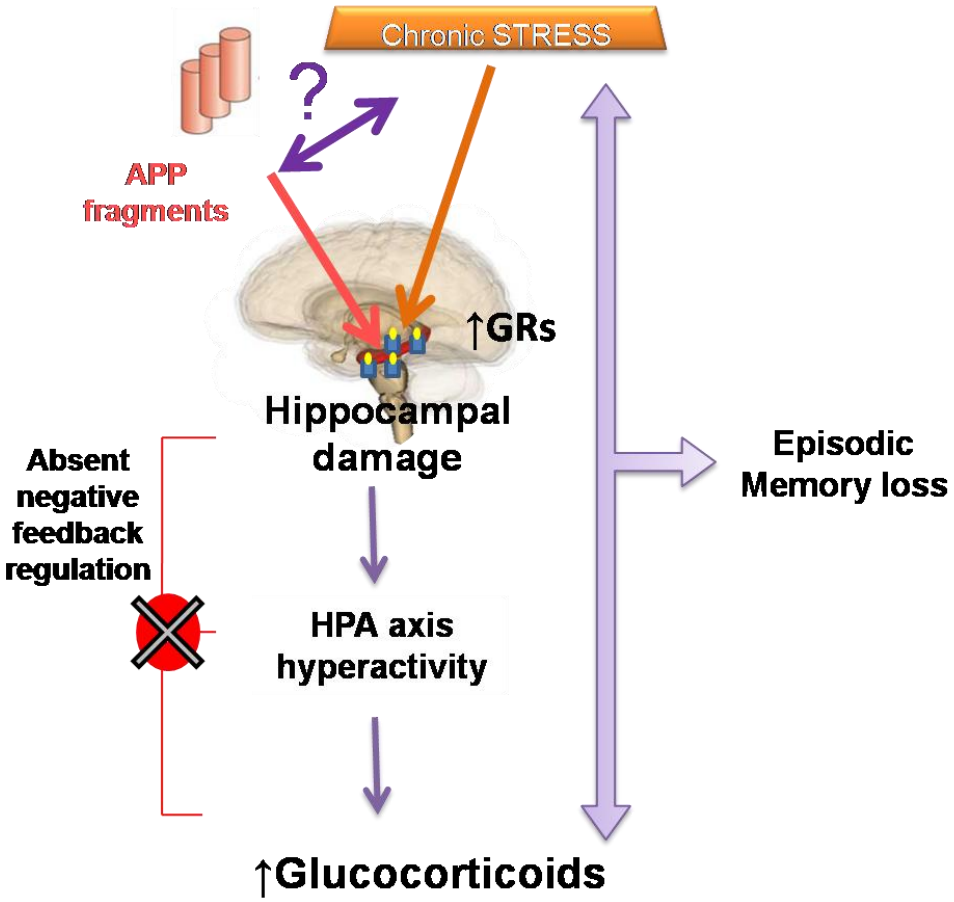

Figure 31: Schematic hypothesis of the link between the results obtained in the present study. At this 4 months of age, Tg2576 mice presents enhanced HPA axis activation characterized by increased glucocorticoids levels (corticosterone) and a fall in HPA axis negative feedback with enhanced GRs levels. Also, Tg2576 presents early deficits in episodic-like memory. Associated to this phenotype, Tg2576 mice present abnormal APP processing that in association with the chronic stress situation could contribute to the progression of AD pathology.

CONCLUSION and Perspectives

In summary, the results of this study indicate that at the early phase of AD pathology, when Aβ begins to accumulate, Tg2576 mice exhibit an over-activated central HPA axis, as measured by an abnormal increase in plasma corticosterone levels at the onset of the awake phase, an impairment in HPA axis negative feedback regulation and an increase in GR expression in the hippocampus. Also, Tg2576 mice present early cognitive alterations characterized by poor episodic-like memory, which might be rescued by blocking GR function. These results reinforce the increasing body of evidence demonstrating that GCs and hippocampal GRs might play important roles in initiating cognitive dysfunction in AD. The integrity of the hippocampus is crucial in controlling stress signalling and memory encoding, and stress clearly disrupts this ability. The present data impart a phenotype to the Tg2576 mice model suggestive of high vulnerability to stress at a very early age, and support our hypothesis that stress could be an important risk factor in AD neuropathology. However, the molecular mechanisms linking the appearance of AD like pathology to increases in the activity of the HPA axis in APP-transgenic mice remain unknown. Further research to clarifying these underlying mechanisms is needed, such as a differential diagnosis of the HPA axis function (eg. CRH, ACTH), understanding the GR regulation in these mice and evaluating GR necessity to induce the behavioural phenotype using GR knockout mice. It is also important to confirm the rescue in episodic-like memory with RU486 by increasing the number of animals, and confirming these data with a blocker of the GC synthesis (metyrapone). Thus, the present data, in conjunction with future studies, may be helpful for designing novel therapeutic interventions to halt progression of AD pathogenesis in humans, especially targeting the early phase of the pathology.

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