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GLUCOCORTICOID RECEPTORS MODULATE DENDRITIC SPINE PLASTICITY AND INFLAMMATION IN AN ANIMAL MODEL OF ALZHEIMER'S DISEASE

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Coordinator: Prof. Gianluigi Zanusso

Tutor: Prof. Mario Rosario Buffelli

Doctoral Student: Dott. Matteo Pedrazzoli

Glucocorticoid Receptors Modulate Dendritic Spine Plasticity and Inflammation in an Animal Model of Alzheimer's Disease

Matteo Pedrazzoli

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ABSTRACT

Chronic exposure to high dose of glucocorticoids (GC) is a key risk factor for the development of Alzheimer's Disease (AD), as recently described by clinical and genetic studies. Furthermore, hyper-activation of glucocorticoids receptors (GR) induces, in brain, alterations comparable to those produced by AD. In a transgenic mice model for AD, GC induces the increasing production of Aβ40, Aβ42 and Tau total, the most important and typical hallmarks of this dementia. Two of the key roles of GC in brain are the regulation of dendritic spine turnover and the inflammation state, two phenomena strongly altered in AD. The aim of my project was to investigate the correlation between glucocorticoids and Alzheimer's Disease. In particular, I focused my attention on how dendritic spine plasticity and microglia activation in CA1 region of hippocampus of 3xTg-AD mice are modified by modulation of glucocorticoid receptor with agonist and antagonist. Using an innovative combined Golgi Cox and immunofluorescence technique, we found that 5 days of treatment with 8mg/kg of dexamethasone, an agonist of GR, was able to vigorously reduce dendritic spine density in CA1 region of 3xTg-AD mice, both at 6 and 10 months of age and induced proliferation and activation of microglia. The activation of microglia could contribute to spine damage. On the contrary, the treatment with 20mg/kg of mifepristone, an antagonist of GR, strongly enhanced dendritic spine density in CA1 region, at both ages, results confirmed also by electron microscopy analyses. Moreover, the antagonist was able to improve the 3xTg-AD mice performance in Y-maze task at 10 months of ages and the proliferation of microglia, but it was not able to reduce the activation of microglia. I speculated that these apparently ambiguous results could be explained by the well-known biphasic behavior of GC in brain, as already observed for spine plasticity and memory. Additionally, *in vitro* experiments, using immunofluorescence and immunoblotting techniques, revealed that dexamethasone, clearly, induced activation of microglia in *vitro*, a result never described before. On the contrary, mifepristone promoted both activation and inhibition of microglia inflammatory state, suggesting the existence of a biphasic behavior of GC also on inflammation regulation. In conclusion, my data demonstrates that stress induced by dexamethasone exacerbate AD and promote a more rapid progression of the pathology through a premature reduction of dendritic spine density and enhancement of inflammation. Consequently, the use of antagonist, like mifepristone, could represent a promising therapeutic strategy to delay the onset and slow down the progression of AD. Taking in account the biphasic behavior of GC, the right dose and time of treatment need to be found, in order to obtain the best improvement: the increasing of spine turnover together with the reduction of inflammation and improvement of behavioral performances.

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INTRODUCTION

1. Alzheimer's disease

Alzheimer's disease (AD) is the most diffuse form of neurodegenerative disease. AD principal characteristic is its capability to produce a slow and relentless neurodegeneration of the central nervous system (CNS) associate with impairment of memory, thinking and behavior. 50 million people are affected by dementia and AD accounts for 60-80% of all cases. AD affects predominantly elder people over 65 years of age, although there is a growing of cases with the onset at younger ages (*World Health Organization, 2018*).

Auguste Deter, a 51 years old woman with an $\varepsilon 3/\varepsilon 3$ Apolipoprotein E (APOE) genotype and presenilin 1 mutation, was the first cased diagnosed of AD (Graeber et al. 1998). The case was of extreme importance, indeed, the early disease onset and the absence of the risk gene $\varepsilon 4$ suggested that Auguste Deter had a familiar form of AD. Dr. Alois Alzheimer studied this case and , later, described the typical hallmarks of this dementia defining them "plaque" and "tangle". He named the pathology as Presenile Dementia due to the early onset of dementia in Auguste.

Today, even if the knowledge of the AD pathophysiology is incomplete, it is now well documented that inheritance of specific genes plays an important role in making susceptible to the onset and/or modifying the disease progression. The discovery of "risk genes" explains the existence of a familial (rare) and a non-familial (common) forms, also known as "sporadic", even if risk genes exist also for the sporadic form and promote an early onset of the disease.
Both the familial and sporadic form of the AD start with memory loss of more recent events while the incapability of maintaining the memory of remote events appears later; finally, patients lose their sense of self. The gradual decline of memory slowly increases in severity until the symptoms become disabling and begin to involve other areas of cognition such as language, abstract reasoning and executive functions, including decision making. Changes in mood and affect as well

as non-typically presenting traits like delusions and hallucinations accompany memory decline contributing to dramatically invalidate life at work or in social situations. Neurological symptoms, typically occurring later, comprise seizure, hypertonia, incontinence, mutism. Death is commonly caused by general inanition, malnutrition and pneumonia (Bird, 2008). In addition, in order to anticipate the clinical diagnosis of AD before the declared stage of dementia, a novel clinical construct, the "mild cognitive impairment" (MCI), was proposed as a new diagnostic entity that accompanies the transition between normal aging and AD dementia. Patients with MCI have already some cognitive disturbs, however, they do not interfere with their activities of daily life as it occurs in dementia.

Nowadays, there is no cure for AD and the drugs available are involved only in marginally improving symptoms. Therefore, discovering innovative and effective therapies becomes urgent.

1.1. Genetic of Alzheimer's disease

Only 5% of all AD cases can be attributable to early-onset familial AD (Tanzi, 1999). These familial forms of the disease - rare, but with very penetrant mutations in amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*) genes - are often transmitted as an autosomal dominant trait with an onset that is typically below 65 years of age. Mutations in these genes might result in alteration of amyloid-β production (both Aβ40 and Aβ42) - one of the hallmarks of AD - leading to apoptosis of neurons and dementia (Sorbi et al., 2001). Figure 1 shows a timeline of AD onset according to the age.

APP is encoded by a gene on chromosome 21. It is a type I transmembrane protein and exists in several isoforms. APP is anterogradely transported along the axon to nerve terminal (Buxbaum et al., 1998).

APP is cut by three enzymatic complexes: α-secretase, β-secretase (β-site APP cleaving enzyme I) and ɣ-secretase. The different actions of these complexes produce different molecules, some of them are linked to AD – in particular Aβ 40-42 species. BACE1 cleavage of APP is a pre-requisite for Aβ formation (Cole & Vassar, 2007). In Figure 1 and Figure 2 it is reported a schematic representation of APP processing.

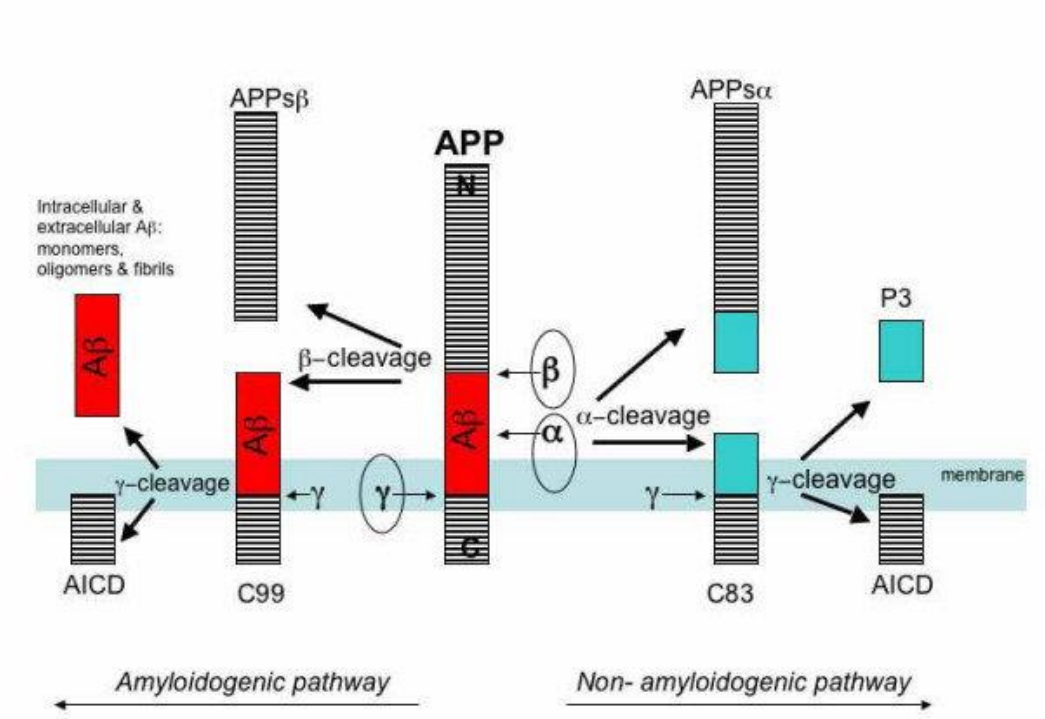

Figure 1. ***APP metabolism by the secretase enzymes****. APP can be cleaved by two different subsequences of enzymatic reactions. For Amyloidogenic pathway APP is sequentially cleaved by BACE1, the β-secretase, and by the enzymatic complex of γ-secretase, composed of presenilin, nicastrin, Aph1 and Pen2, to generate Aβ. In detail BACE1 cleavages APP and forms two protein: APPsβ and C99, a membrane bound fragment. C99 is the substrate for γ-secretase, and C99 cleavage generates the AICD and the fragment of Aβ. For non-Amyloidogenic pathway, α-secretase, which has been identified as TACE, ADAM9 and ADAM10, cleaves APP to generate the secreted ectodomain, APPsα and membrane bound fragment, C83. C83 is subsequently cleaved by the γ-secretase complex to yield the 3 KDa fragment, P3 and the AICD (Cole & Vassar, 2007).*

APP can undergo two different pathways: amyloidogenic and non-amyloidogenic. For the first, APP is cleaved by β-secretase within the extracellular domain, forming the Aβ N-terminal domain and two fragments: the secreted ectodomain, APPsβ, and a transmembrane protein C99 (Vassar et al., 1999). BACE 1, or β-site APP cleaving enzyme I, is a transmembrane aspartyl protease, (also called Asp-2 and memapsin-

2). The principal BACE cleavage site in native APP is between Glu +11 and Val +12 of the Aβ peptide. The second proteolytic event in APP processing involves intramembranous cleavage of C99 by γ -secretase, that liberates AICD, or p3 (3 kDa), and Aβ (4 kDa) peptides, respectively, into the extracellular compartment. The principal components of γ -secretase are presenilin-1 or -2 (PS1 or PS2), nicastrin, APH-1, and PEN-2 (Edbauer et al., 2003; Iwatsubo, 2004). PS1 or PS2 are the catalytic subunit of the γ -secretase. A pair of conserved aspartate residues, within the transmembrane domains 6 and 7 of PS1 and PS2, is fundamental for γ -secretase right activity. APH-1 and PEN2 are thought to stabilize the γ –secretase complex and nicastrin to mediate the recruitment of C99 to the catalytic site of the γ -secretase. The major sites of γ -secretase cleavage correspond to positions 40 and 42 of Aβ. Greater than 90% of secreted Aβ ends in residue 40, as a consequence, Aβ-42 represents less than 10% of total Aβ. In addition, γ –secretase cleavage at a distal site generates a cytoplasmic polypeptide, termed APP intracellular domain (AICD). Familial AD-linked mutations in APP, near the γ-secretase cleavage site, could favorite the Aβ-42 sites. So, mutation in APP and γ-secretase can promote the formation of pathological Aβ protein. An historical supporting evidence is represented by the triplication of chromosome 21, that contains APP gene, typical of Down Syndrome, triples the production of APP and consequently the probability of formation of Aβ. Indeed, Down syndrome patients develop the AD pathology earlier in comparison to those without Down syndrome (Prasher et al., 2004).

A lots of APP mutations were well identified. One of the most important is the APP Swedish mutation or APP KM670/671NL that is the only known mutation immediately adjacent to the β-secretase site in APP: it was firstly identified in two large Swedish families by Mullan (Mullan et al., 1992). Swedish mutation is a double mutation, resulting in a substitution of two amino acids, lysine (K) and methionine (M) to asparagine (N) and leucine (L). This mutation is present also in the transgenic mouse model for AD named 3xTg-AD (Oddo et al., 2003) and used in our experiments. Other AD mice models that express this mutation are Tg2576 mouse (Hsiao et al., 1996), one of the first AD mice, and J20 mouse (Mucke et al., 2000).

Preseniline 1 (PS1) and 2 (PS2) are important component of γ-secretase code by *PSEN1* and *PSEN2* on chromosome 14 (14q24.2) and chromosome 1 (1q42.13), respectively. The vast majority of mutations that promote AD are one of 90 PSEN1 gene mutation (Bertram & Tanzi, 2008). Mutation in PSEN1 lead to AD with early onset ages - during 40s – promoting a stronger activation of γ-secretase. It is thought that the presenilins are involved in the cleavage of the Notch receptor too, a pathway critical for cell fate decisions (Selkoe & Kopan, 2003).

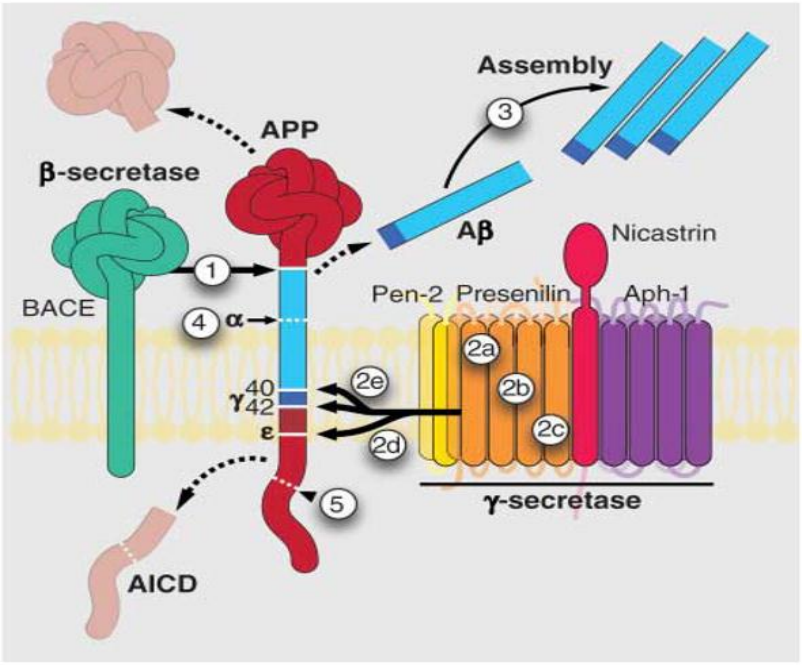

Figure 2 Schematic illustration of Aβ formations with the enzymatic complex involved. BACE1 and γ –secretase complex are responsible for the cut of APP (amyloid precursor protein). For the mechanism see paragraph 1.1 (Roberson & Mucke, 2006).

Even if it is not linked to familial form, Tau protein is a central protein involved in AD. Tau protein is coded by *MAPT* (microtubule-associated protein tau) gene on chromosome 17 (17q21.31). The whole family of Tau proteins are the products of MAPT alternative splicing (Figure 3). In human brain tissue, 6 isoforms of this protein exist, and they differ for the number of binding domains (3 or 4).

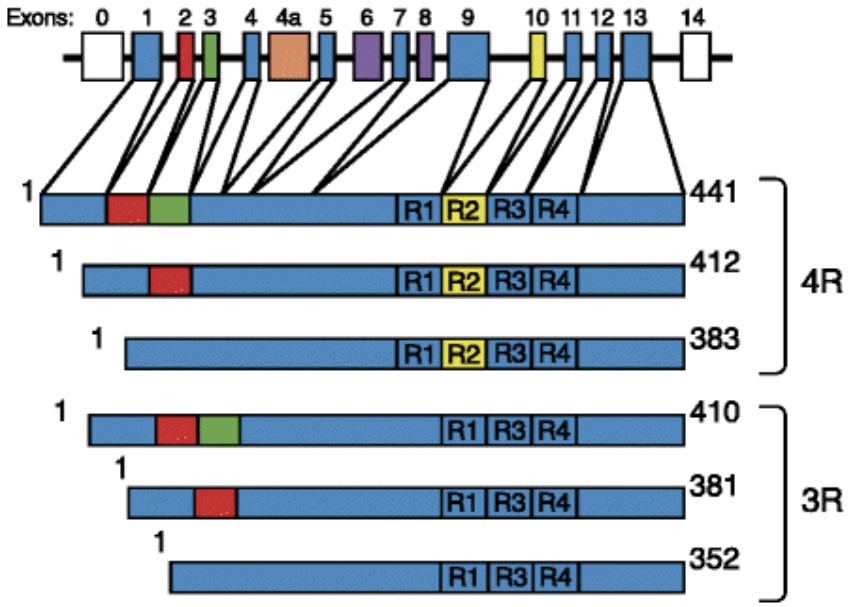

Figure 3. ***MAPT gene with six Tau isoforms express in human brain****. MAPT has 16 exons (E) and alternative splicing of mRNA of E2(red), E3 (green) and E10 (yellow) code for all these isoforms. "R" indicates the binding domains, 3 for three isoforms and 4 for the others three isoforms (Michel Goedert & Spillantini, 2017).*

Tau proteins are the major component of neurofibrillary tangle, a intracellular protein aggregate typical of AD (Goedert, Spillantini, Cairns, & Crowther, 1992). Hyper-phosphorylation of Tau is the common characteristic of these aggregates and are typical not only of AD, but also of a lot of diseases, generically named Tauopathies.

1.2. Sporadic forms of Alzheimer's disease

Sporadic forms of AD, generally, appear later during life and so they are named Later Onset AD (LOAD).

The most known and important risk factor for development of LOAD is linked to mutation in *APOE* gene.

The *APOE* gene is located on chromosome 19 and codes for Apolipoprotein E (ApoE) - which is predominantly expressed by astrocytes and strongly up-regulated by microglia in Aβ pathology (Krasemann et al., 2017). It is the major cholesterol carrier in the brain, which is involved in neuronal maintenance and repair. ApoE is

expressed on the cell surface where it binds to several receptors which are involved in lipid transport, glucose metabolism, neuronal signaling, and mitochondrial function. Interestingly, ApoE is able to bind to Aβ peptide, playing a role in its clearance (Bu, 2009).

Two polymorphic sites, located at codon 112 and 158, have been described in the human *APOE* gene and three main variations of the *APOE* gene have been identified, referred as "ε2," "ε 3," and "ε 4" alleles. *E3* is the most common allele and presents a Cys at codon 112 and Arg at codon 158. Two other *APOE* alleles have been described: the ε 2 allele in which Arg 158 is substituted by Cys; ε 4 allele in which Cys in 112 is substitute by Arg (Rihn et al. 2009; Green et al. 2009). Studies show that ε 2 allele, that could be involved in neuronal maintenance and repair, could be protective against AD (Mahley & Huang, 2006), while the ε4 allele is associated with increased risk of AD in both homo- and heterozygous phenotype (Bu, 2009). In the ε4 allele, the altered orientation of Arg61 in the C-terminal domain, promotes different interaction between C- and N-terminal domains, driving conformational changes of ApoE protein, which may finally lead to neuronal cell death. Unlike the mutations for familial AD, no one of the genes involved is sufficient and/or necessary for AD development, but act as a risk factor decreasing the onset age in a dose-dependent manner (Brady, Siegel, Albers, & Price, 2012).

If ApoE is the most important genetic risk for LOAD, other genes were identified after the advent of genome-wide screening technology, as reported in Table 1.

Table 1 Risk gene for sporadic AD. (Brady et al., 2012)

Gene	Protein	Location	% Risk change‡	Proposed molecular effects/pathogenic relevance*
APOE	apolipoprotein E	19q13	~400%	aggregation & clearance of Aβ; cholesterol metabolism
BIN1†	bridging integrator 1	2q14	~15%	production & clearance of Aβ
CD33†	CD33 molecule (siglec 3)	19q13.3	~10%	innate immune system response
CLU†	clusterin	8p21.1	~10%	aggregation & clearance of Aβ; inflammation
CR1†	complement component (3b/4b) receptor 1	1q32	~15%	clearance of Aβ; inflammation
PICALM†	phosphatidylinositol binding clathrin assembly protein	11q14	~15%	production & clearance of Aβ; synaptic transmission

Only genes/loci showing genome-wide significant ($P \leq 5 \times 10^{-8}$) risk effects and independent replication are included. For an up-to-date overview of these and other potential susceptibility genes see the AlzGene database at http://www.alzgene.org.
†Indicates genes/loci originally identified by GWAS.
‡Approximate change in disease risk (increase or decrease) per copy of minor allele as compared to non-carriers of minor allele.
*Selection of proposed effects; note that the functional evidence for these loci is often scarce (see text for more details).

1.3. The neuropathological markers of Alzheimer's disease

The hallmarks of AD are generally the so-called lesions that could be positive or negative. Typical "positive" lesions are represented by amyloid plaques (Aβ plaques) and neurofibrillary tangles (NFTs), neuropil threads, and dystrophic neurites that contain hyperphosphorylated tau (Crews & Masliah, 2010), followed by astrogliosis and microglia activation (Itagaki, McGeer, Akiyama, Zhu, & Selkoe, 1989). Characteristic "negative" lesions are loss of neurons, dendrites and synaptic structure.

Aβ plaques are the most common marker of AD. Aβ is a polypeptide composed by 39-43 amino-acids (Tamagno et al., 2018). As described in paragraph 1.1, Aβ plaques are formed from APP cleavage by BACE1 and γ-secretase. Mutations that promote the cleavage of BACE1 on APP or the γ-secretase activity is, indeed, well identified as the cause of familial AD. The possibility that the Aβ represents the main pathogenetic factor and the primary responsible of brain damaged and AD progression, have given rise to the so called "amyloid hypothesis" (Daniela Puzzo, Privitera, & Palmeri, 2012). However, pharmacological treatments promoting the removal of Aβ plaques, are not able to restore the cognitive deficit probably because Aβ has a physiological role too, rather than only a pathological one (Pearson & Peers, 2006; Daniela Puzzo et al., 2011).

Recent studies demonstrated that not only Aβ plaques but also the smaller Aβ assemblies commonly known as Aβ oligomers or protofibrils, which are formed before β-amyloid fibrils, are involved in the pathology and promote the main toxic effect on brain (Stephen W. Scheff, Price, Schmitt, & Mufson, 2006). Furthermore, evidence suggested that also the monomers of Aβ are involved in pathogenesis of AD: they are able to increase the activity of BACE1, reducing the capability of lysosome to degrade this enzyme (Tamagno et al., 2018).

The staining of Aβ plaques presents a fundamental pathogenic relevance because it allows to distinguish between Thioflavin-S negative diffuse amyloid plaques, that are found, primary, in the brain of elderly people without dementia, and Thioflavin-S positive dense-core plaques, typical of AD patients, that are linked to synaptic

loss, neuron degeneration and activation of both astrocytes and microglial cells (Itagaki et al., 1989).

It is common to find neuronal degeneration and microglia activation near Aβ plaques.

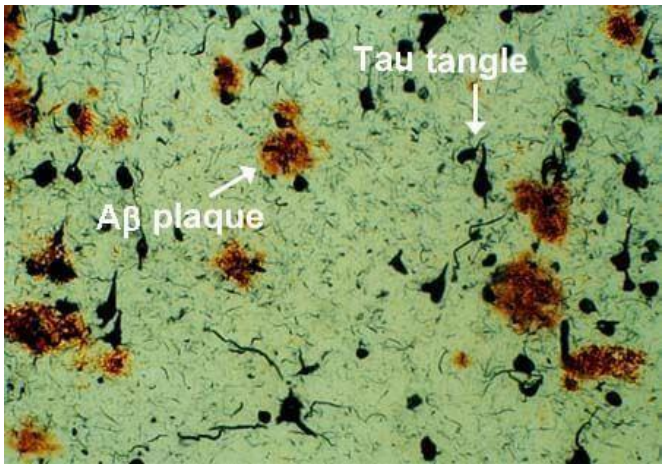

Figure 4. Histological preparation of brain slices in which it is clearly visible the presence of brown Aβ plaques and dark neurofibrillary tangle. The first are extracellular protein agglomerates that can disrupt the synaptic activity and burst inflammation; the second are intracellular protein aggregates that break the cytoskeleton stability and induce neuron degeneration (photo credit Dr. Dale Bredesen).

Neurofibrillary tangles are the second most important hallmark of AD. Its principal component is Tau protein. Tau is a microtubule-binding protein indispensable for intracellular transport in particular in axons. This protein is normally soluble, but after hyper-phosphorylation that occurs in AD, it loses its capability to bind to the cytoskeleton and begins to associate with paired helical filaments forming the NFT. The consequence axonal transport impairment compromises synaptic stability and at the end can promote neuronal death. After death, neurofibrillary tangles remain in extracellular space and can enhance inflammation.

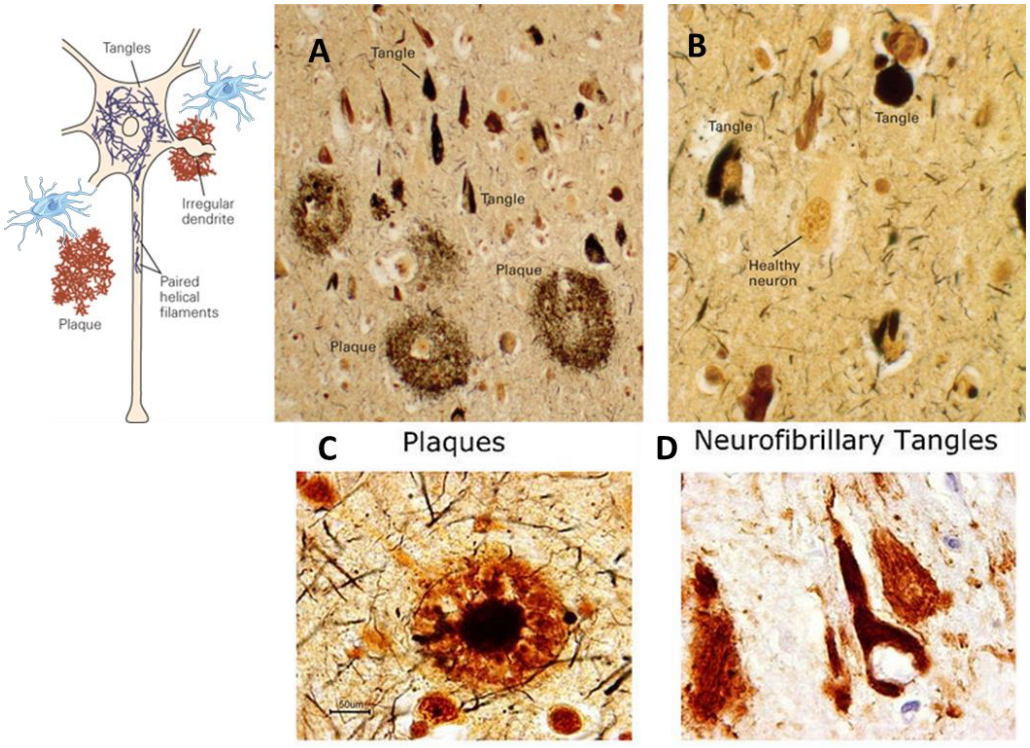

Figure 5. Images of brain slice of patient affected by severe AD. Aβ plaques and neurofibrillary tangles are clearly visible. In the diagram Aβ plaques surround a dendrite, inducing a dysfunction of information transmission. The plaques induce activation of microglial cells (in light blue) that can contribute to neuronal damage promoted by AD. At the same time, neurofibrillary tangles, composed by bundle of paired-helical of hyper-phosphorylate Tau protein are present in both cell body and axon disrupting the normal intracellular transport of the cell (modified from Principle of Neural Science, 2013, page 1337 that reproduced the images with permission from James Goldman). C) Magnification of Aβ plaques D) Magnification of neurofibrillary tangle.

1.4. Hormesis: the case of biphasic behavior of Aβ.

The term hormesis is used to identify a dose-response relationship that shows opposing effects at low and high doses. This biphasic behavior is well described for a lot of chemical molecules like Cd2+ and Cu2+ and phenol; however, also physical phenomena, like radiation, show this response. Starting from the late 19th century, a lot of components following this dose-response relationship were discovered, but due to the absence of a unique terminology, a lot of terms were used to described this phenomenon, like “Arndt-Schulz law”, U-shaped or inverted U-shaped,

biphasic, bidirectional, opposite effects, dual effects, and paradoxical effects (Kendig, Le, and Belcher 2010).

The term hormesis, instead, was used for the first time by Southam and Ehrlich in 1943 to describe how low doses of antimicrobial drug, extracted from the Red Cedar tree, were able to promote the growth of fungal species (Southam and Erlich 1943; Calabrese 2014).

Kendig, Le and Belcher, in 2010, proposed this unique definition for hormesis:

"Hormesis is a dose-response relationship for a single endpoint that is characterized by reversal of response between low and high doses of chemicals, biological molecules, physical stressors, or any other initiators of a response" (Kendig, Le, and Belcher 2010).

Recently, it was discovered that also Aβ presented hormetic effects (Daniela Puzzo et al., 2012). Aβ at low doses promotes important physiological effects, while the typical brain damage is produced essentially by plaques of the amyloid protein. One of the first evidence on the physiological role of Aβ was published by Plant in 2003. His team demonstrated that the use of inhibitors of β- or γ-secretases, in order to avoid the production of Aβ, caused cell death in primary neuron cultures. This death could be prevented through injection of low doses of Aβ that revert the physiological concentration of the peptide. Interestingly, this effect seems to be cell specific since the same experiments tested on non-neuronal culture had no effect on cell surviving (Pearson & Peers, 2006).

Moreover, in vivo and in vitro experiments showed that endogenous Aβ physiologically regulates synaptic plasticity and memory (Morley et al., 2010; Puzzo et al., 2011), and that administration of low concentrations of the peptide improve synaptic plasticity and memory (Gulisano et al., 2018; Puzzo et al., 2008)

Moreover, Aβ effects on brain seem to present an hormetic effects. Low concentration promotes positive effects on neurons survival, high and chronic concentrations, on the contrary, produces the typical damaged induced by AD, as

clearly demonstrated for synaptic plasticity and memory by Puzzo et al. using electrophysiological recordings and behavioral tests (Daniela Puzzo et al., 2012).

1.5.Alzheimer's disease and neuron dysfunction

Alzheimer's disease affects principally neuronal cells; it promotes the degeneration of neuronal connections, disrupts the spine turnover, reduces the dendrite arborization and finally causes the death of neuron. All these damages could be the results of a multi-factor mechanism that involves biochemical abnormalities, like the accumulation of Aβ and Tau protein, the dysfunction of neuronal plasticity and the enhancing of inflammations. In detail, the mechanism could be explained by pathological activation of NMDA receptors during excitotoxicity (mediated by Aβ) or disruption of dendritic transport (mediated by Tau) that both induce spine loss. Analogously, disruption of protein synthesis at the spine level can promote changes in spine densities and morphology (Herms & Dorostkar, 2016). Finally, rising of inflammation can induce the production of interleukin 1β, which antagonizes the action of BDNF, disrupting the spine turnover (McCullers, Sullivan, Scheff, & Herman, 2002).

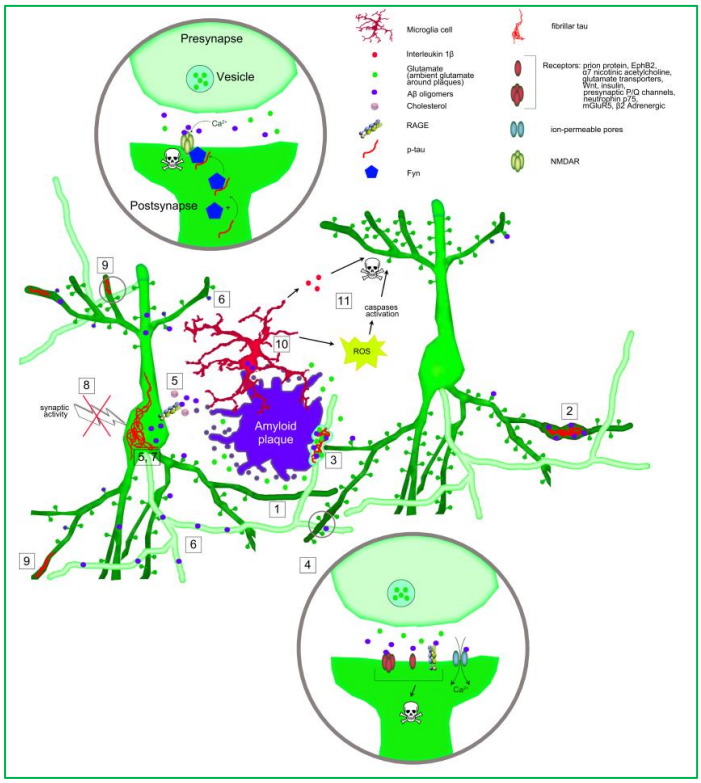

Figure 6. Reconstruction of spine damages produced by different intracellular and extracellular mechanisms: amyloid plaques with Aβ oligomers, microglia activation and intracellular fibrillar Tau can induced degeneration of dendritic spines through different mechanisms not completed elucidated (Dorostkar, Zou, Blazquez-Llorca, & Herms, 2015).

Since the cognitive impairment appears before the degeneration of neurons or even in the absence of neuronal degeneration, it has been proposed that synapses and dendritic spines are the first structures to be affected by AD, maybe through exposure to toxic Aβ oligomers (Stephen W. Scheff et al., 2006). Confirming this hypothesis, we know that postmortem studies using quantitative electron microscopy in mild cognitive impairment and mild AD patients showed that synapse loss is an early structural correlate in the process of AD also in human (S W Scheff et al., 2007; Stephen W. Scheff et al., 2006).

The damages on neuronal structures occur in many brain regions, primarily in the hippocampus, both in CA1 and CA3 regions and in dental gyrus. In CA1 the degeneration is stronger probably because of the greater accumulation of neurofibrillary tangles (Ferrer & Gullotta, 1990). Furthermore, the damage occurs

also in the entorhinal cortex, in particular layer two (Gómez-Isla et al., 1996), and in the prefrontal cortex (DeKosky & Scheff, 1990; Ferrer & Gullotta, 1990; Hamos, DeGennaro, & Drachman, 1989; S W Scheff & Price, 1998).

Despite the neuron degeneration is a common symptom of AD, in animal model of Alzheimer's Disease it is not a common primary mark. In 3xTg-AD mice, the model we have used, this phenomenon occurs only in the late phase of AD (Bittner et al., 2010). On the contrary, in APP/PS1, degeneration is common at 10 months of age in particular in CA1 region (Wirths & Bayer, 2010): in Figure 7, it is visible the extensive neuron loss (>50%) in the hippocampus; this degeneration is correlated with the accumulation of intraneuronal Aβ and Thioflavin-S positive intracellular material.

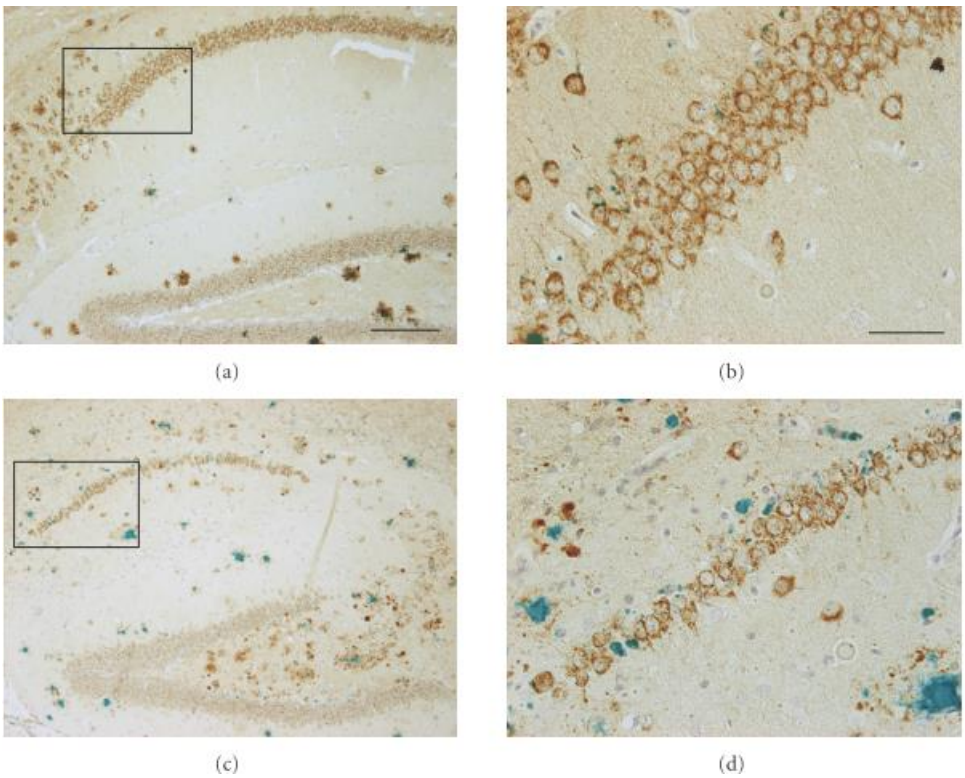

Figure 7. Slice of APP/PS1 mouse at 2 (a&b) and 10 months of age (c&d) marked for APP (in brown) and Aβ (in green). Magnification shows a strongly degeneration of neurons and a reduction of APP compared to Aβ at 10 months of age (Wirths & Bayer, 2010).

Together with neuron degeneration, AD progression is associated with the enhancement of inflammation in particular near the amyloid deposition where the principal immunomodulatory cells of the brain become activated: the microglia.

2. Microglia

Microglia are the resident and phagocytic immune cells of the brain and spinal cord. These cells represent about 15% of the total population of cells within the brain and exhibit distinct morphologies and functions across different anatomical regions (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011).

Even if the history of microglia analyses begun in the second half of 1800, the modern study of the microglia started in the 1960s, thanks to Georg Kreutzberg through the facial nerve lesion model, which allowed the possibility to investigate the activity of microglia in tissue with intact blood brain barrier (BBB), as well as to distinguish the behavior of resident microglia and periphery monocytes that invade the brain tissue (Blinzinger & Kreutzberg, 1968). His studies helped to define the concept that microglial cells are key players in both degeneration and regeneration of the brain. In Figure 8, you can appreciate the history of the discoveries related to microglial cells and their functioning.

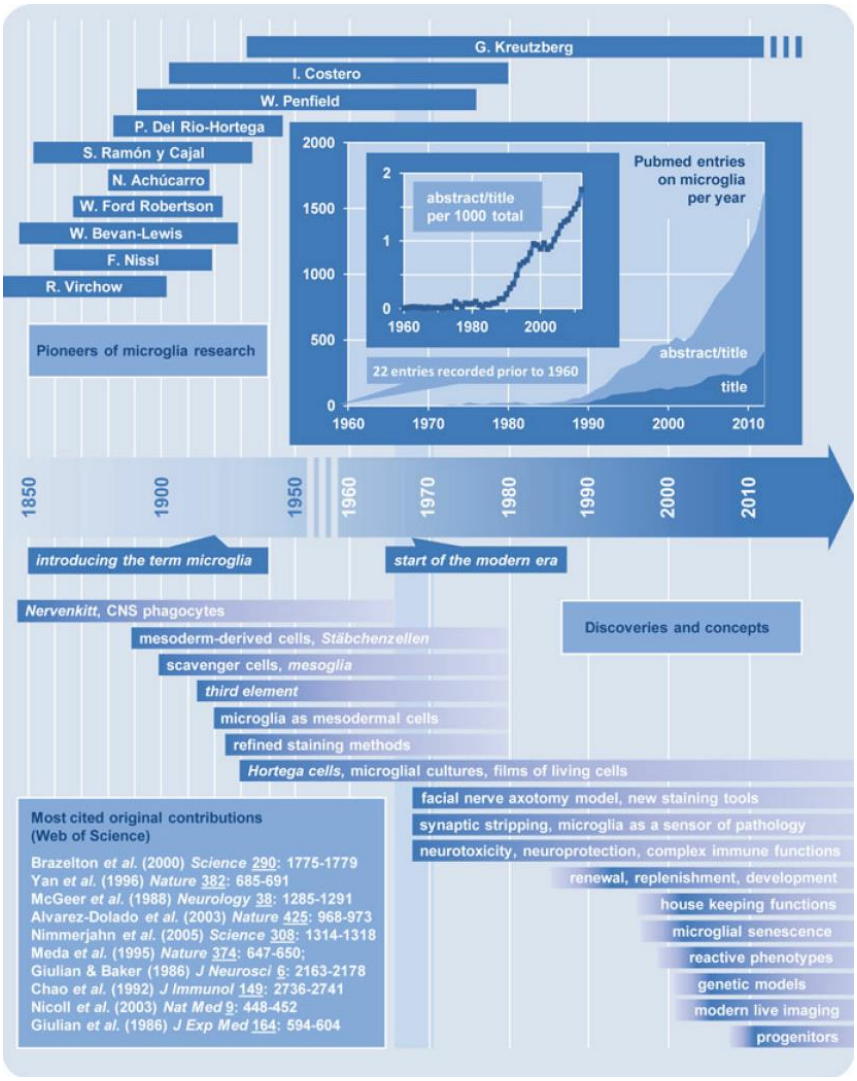

Figure 8. Historical overview of research and discovery related to microglia. The graphs (inset) illustrate the growth in research on microglia, according to the number of publications per year, carrying the term 'microglia' in the abstract and/or title (based on PubMed entries); the box lists the 10 most cited original contributions (by the topic microglia, based on the Web of Science). Published in Microglial in Health and Disease, Chapter 2, page 9 (Rezaie & Hanisch, 2014).

In the brain parenchyma, microglial cells acquires a ramified phenotype. This phenotype is different from the typical macrophagic state and it has been associated with microglial "resting" state. Toxic stimuli like infection, trauma and ischemia or neurodegenerative pathologies like AD, generate profound changes in the

morphology of microglial cells, producing a state that is named as "microglial activation" or M state.

Morphologically, in the activated state, microglial cell reduces its ramifications, while increases their thickness, it increases the dimension of body and, at least in *in vitro* model, assumes an amoeboid form, more similar to the macrophage one. The process of activation is completed by the induction of surface molecules, release of cytokines, chemokines and neurotrophic factors and the acquisition of a phagocytic activity (Ransohoff & Brown, 2012).

Two different states of activation can be distinguished: a pro-inflammatory one, M1, and an anti-inflammatory one, named M2 – schematically represented in Figure 9. Although these conformations are considered the fundamental polar states of microglia, they represent a simplification of the several functions and differently evolve during an inflammatory process (Manuel B Graeber, 2010).

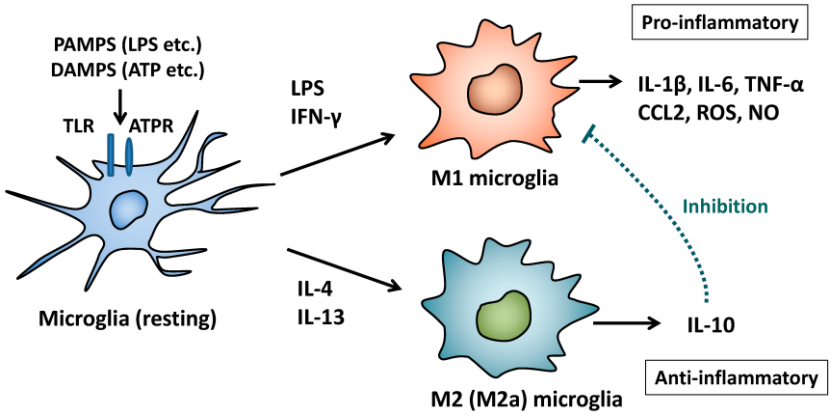

Figure 9. Graphic representation of Microglial states: resting, and phagocytic microglia in M1 or pro-inflammatory state, and M2 or anti-inflammatory state.

When classically activated, microglia acquired the M1 phenotype, characterized by the release of pro-inflammatory and pro-killing molecules - some of them are depicted in Figure 9 and Table 2 - such as interleukin-1β (IL-1β), IL-6, IL-12, IL-17, IL-18, IL-23, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), nitric oxide (NO) and chemokines like CCL2 (Subramaniam & Federoff, 2017). M1 microglia can, also, express specific markers like inducible NO synthase (iNOS),

cyclooxygenase-2 (COX2), major histocompatibility complex class II (MHC-II), CD86 (cluster of differentiation marker 86), reactive oxygen species (ROS) and prostaglandin E2 (Chhor et al., 2013). All these molecules are induced as the first line of defense in order to eliminate pathogens or the cause of injuries.

To prevent further damage after an injury, microglia can also promote tissue repairment and regeneration. In this sense, microglia may convert themselves to a repair/restoration-oriented state for tissue regeneration, called M2.

Table 2. List of cytokines and chemokines produced by microglial cells after the stimulus reported in the "condition" column. The table is published in "Microglial in Health and Disease" book (Hanamsagar, Cardona, Kielian, & Cardona, 2014). *Tabled data are obtained from a lot of contributors* (Aloisi, Penna, Cerase, Menéndez Iglesias, & Adorini, 1997; Cunha et al., 1997; Hanisch, 2002; S. C. Lee, Liu, Dickson, Brosnan, & Berman, 1993; Lokensgard et al., 2001; Lue, Walker, & Rogers, n.d.; Mandrekar et al., 2009; Schwab, Schluesener, Seid, & Meyermann, 2001).

Cytokine	Condition
IL-1α	In vitro, viral nucleocapsid, LPS
IL-1β	In vitro, viral nucleocapsid, LPS, Aβ
IL-3	In vitro, IFNγ
IL-6	In vitro, viral nucleocapsid, LPS, Aβ, cytomegalovirus
IL-8	In vitro, IFNγ, HIV
IL-10	In vitro, IFNγ/LPS,
IL-12	In vitro, IFNγ/LPS, IL-12
IL-15	In vitro, IFNγ
IL-16	In vivo, fetal tissues
IL-23	In vitro, IFNγ/LPS
M-CSF	In vitro, β-amyloid
TGFβ	In vitro, IL-1
TNFα	In vitro, viral nucleocapsid, LPS, HIV

Cytokine	Condition
CCL1	Ex vivo, *S. aureus*
CCL2	In vitro, viral Tat protein
CCL3	In vitro, LPS,
CCL4	In vitro, viral Tat protein
CCL5	In vitro, LPS
CCL7	In vitro, TLR2 signal
CCL8	In vitro, TLR2 signal
CCL9	In vitro, TLR2 signal
CCL11	In vitro, neuropeptide
CXCL1	In vitro, TLR2 signal
CXCL2	In vitro, ATP
CXCL4	In vitro, TLR2 signal
CXCL5	In vitro, TLR2 signal
CXCL8	IFNγ/sCD40L, viral Tat protein, *S. aureus*
CXCL9	In vitro, TLR2 signal, IFNγ
CXCL10	In vitro, LPS, TLR2 signal, IFNγ, viral Tat protein, *S. aureus*
CXCL16	Ex vivo, glioma

Microglia is abnormally activated by the Aβ deposition. In AD, the amyloid burden is the results of the combination between Aβ production and elimination through microglia activity. (Bradley et al. 2018). Interestingly, the pathogenesis of sporadic

form of AD seems to be related primarily to an impairment of plaque elimination rather than their excessive production (Mawuenyega et al., 2010). This deficit is probably due to a reduce efficiency of microglia. Indeed, microglia, representing the principal phagocytic component of the CNS, is the principal responsible of the clearance of Aβ, through two different mechanism: phagocytoses or local degradation by the release of Aβ degrading enzymes (Heneka, 2017). Specifically, the expression of pattern recognition receptors (PRRs) on the cell membrane allows microglia to bind both PAMPs and DAMPs, such as Aβ. Microglia TLR2 and TLR4, are upregulated in AD and can induce the pro-inflammatory effects of Aβ (Arancio et al., 2004).

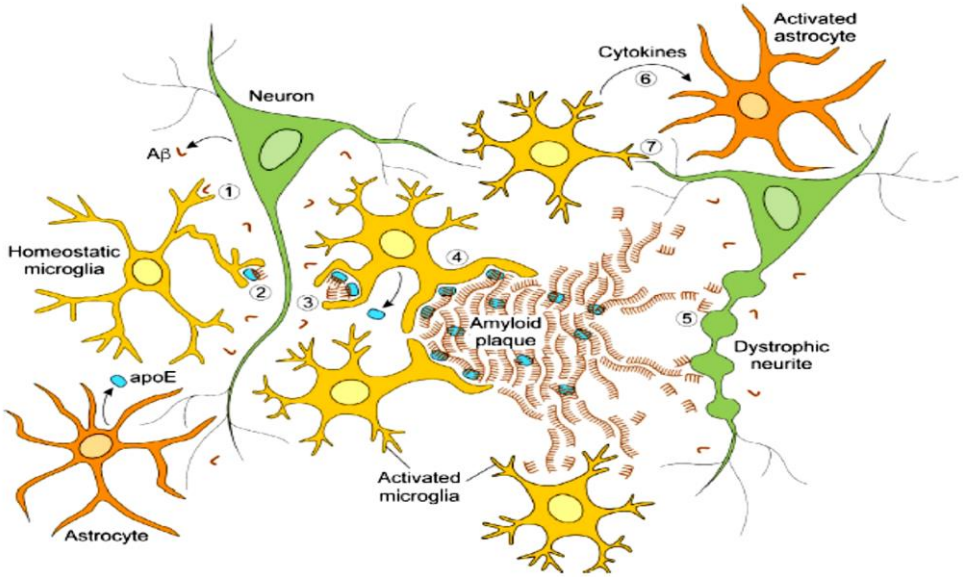

Figure 10. ***Representation of microglial cellular activities related to β-amyloid pathology****. Left: protective microglial activities that try to slow down the progression of AD: microglia can clear Aβ peptides via macropinocytosis of soluble Aβ* ***(1)*** (Mandrekar et al., 2009)***,*** *via uptake of lipoprotein-associated Aβ* ***(2)****, or via the phagocytosis of fibrillar Aβ deposits* ***(3)****. Microglia also surround larger deposits of Aβ in plaques* ***(4)*** *and try to contain them to reduce the damage to neurons. Right, the disease states when microglial are not more able to contain the damage promoted by AD because of their defective or insufficient activity. Aβ fibrils on the outskirts of the plaque act as substrate for the formation of amyloid plaques becoming a reservoir of toxic Aβ species that induce axon and dendrite dystrophy* ***(5)****. Moreover, microglia can release factors that promote*

*the activation of astrocytes (**6**) and participate in amyloid-dependent synapse loss (**7**) (Hansen, Hanson, & Sheng, 2018).*

In addition to microglia activation, in AD patients, an increase of microglia proliferation has been observed in the brain (Gomez-Nicola, Fransen, Suzzi, & Perry, 2013) as well as in several murine AD models (Kamphuis, Orre, Kooijman, Dahmen, & Hol, 2012).

Based on these observations, the reduction or modulation of inflammatory state has been widely investigated to slow down the beginning and progression of AD. Corticosteroids, the classical anti-inflammatory drugs - part of glucocorticoid hormones - were proposed as therapeutic strategy for AD (Alisky, 2008), but it was demonstrated that glucocorticoids chronic administration promoted extensive damage in the same brain region affected by AD and sometimes can induces the so called Steroid Dementia Syndrome, characterized by a lot of cognitive deficits, in particular related to memory formation. Taking in account the actual knowledge of corticosteroids mechanism of action, the apparently ambiguity between the different effects promoted by acute-low and chronic-high dose of the hormones can be explained by the well-known biphasic behavior of glucocorticoid hormones: that is their ability to induce opposite effects depending on the dose and time of administration in the brain. The glucocorticoid role in physiological and pathological conditions will be deepened in the next chapter.

3. Glucocorticoid Hormones

Glucocorticoids (GC) are a subclass of steroid hormones produced in the zona fasciculata of the adrenal cortex. Together with mineralocorticoids, they are the principal component of corticosteroid class of steroid hormones. The principal glucocorticoid hormone is the cortisone in human and the corticosteroid in rodents.

These hormones are involved in a lot of metabolic processes, such as metabolism, immunity, cognition, circadian learning and allostatic response. A summary of this activity is presented in the Figure 11, reported from (Kadmiel & Cidlowski, 2013).

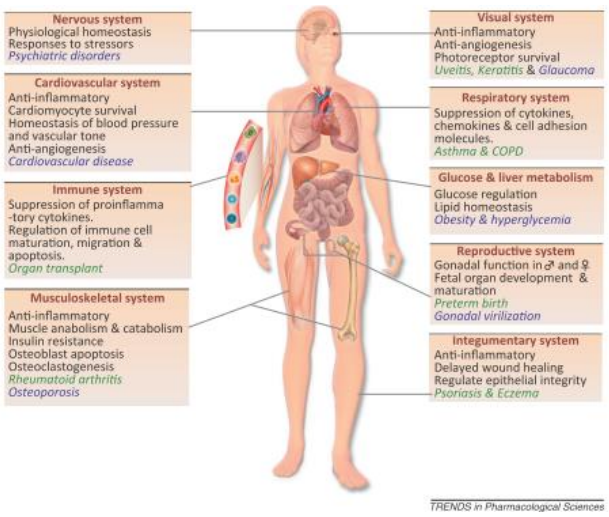

Figure 11. The role of glucocorticoids in health and disease. GC regulated a lot of systems. For our experiments the most important are the capability to alter dendritic spine plasticity, behavior and inflammation (Kadmiel & Cidlowski, 2013).

The production of GC is finely controlled by the hypothalamic–pituitary–adrenal (HPA) axis, a complex neuroendocrine system that involves the paraventricular nucleus of hypothalamus (PVN), the pituitary gland and the adrenal cortex. GC act on brain, through mineralocorticoid (MR) and glucocorticoid receptors (GR) (Wang & Harris, 2015), regulating physiological and behavioral responses under baseline conditions and after stress.

Cortisol and corticosterone are synthesized from cholesterol in adrenal cortex. The production is stimulated by adrenocorticotropic hormone (ACTH) – see paragraph 3.1 – that bind ACTH receptor, a G protein–coupled receptor of the melanocortin receptor family. Its activation induces increasing cholesterol availability through both rapid and slow mechanism (Beuschlein, Fassnacht, Klink, Allolio, & Reincke, 2001) and promotes the production of CORT. In Figure 12, it is shown the molecular structure and the principal chemical groups of cortisol and corticosterone.

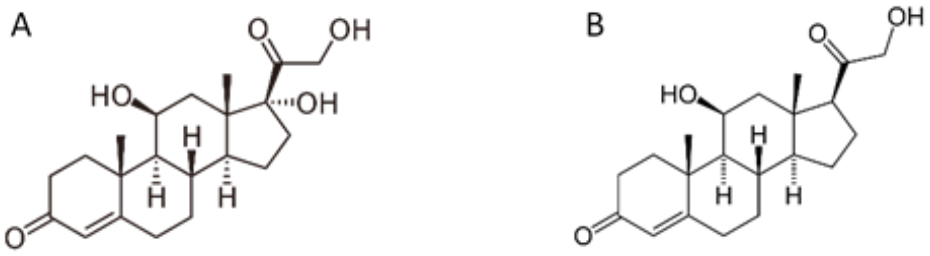

Figure 12. Chemical structure of Cortisol (A) and Corticosteroid (B), the principal human and rodent glucocorticoid, respectively.

The availability of CORT is negatively regulated by the 11β-Hydroxysteroid dehydrogenase (11β-HSD) that converts the biologically active cortisol to the inactive cortisone. The meaning of this regulation is attributed to the affinity of MR for CORT: MR, indeed, mediate also the effect of aldosterone whose affinity for the receptor is similar to CORT itself, but its availability is extremely lower and, as a consequence, 11β-HSD is necessary for the binding of aldosterone to MR.

3.1. HPA Axis

The production of GC is finely controlled by HPA axis, a complex neuroendocrine system that involves the paraventricular nucleus of hypothalamus (PVN), the pituitary gland and the adrenal cortex. As visible in Figure 13, during stress, parvocellular neurons of PVN induces the release of Corticotropin Release Factor (CTF or CRF) in the median eminence and reach pituitary gland through hypothalamus-hypophyseal System. There, CTF induces corticotroph cells to release adrenocorticotropic hormone (ACTH) in blood, that, in turn, reaches adrenal cortex and stimulates the production of cortisol (in human) or corticosterone (in rodent) (CORT). This system is finely regulated both in positive and in negative ways.

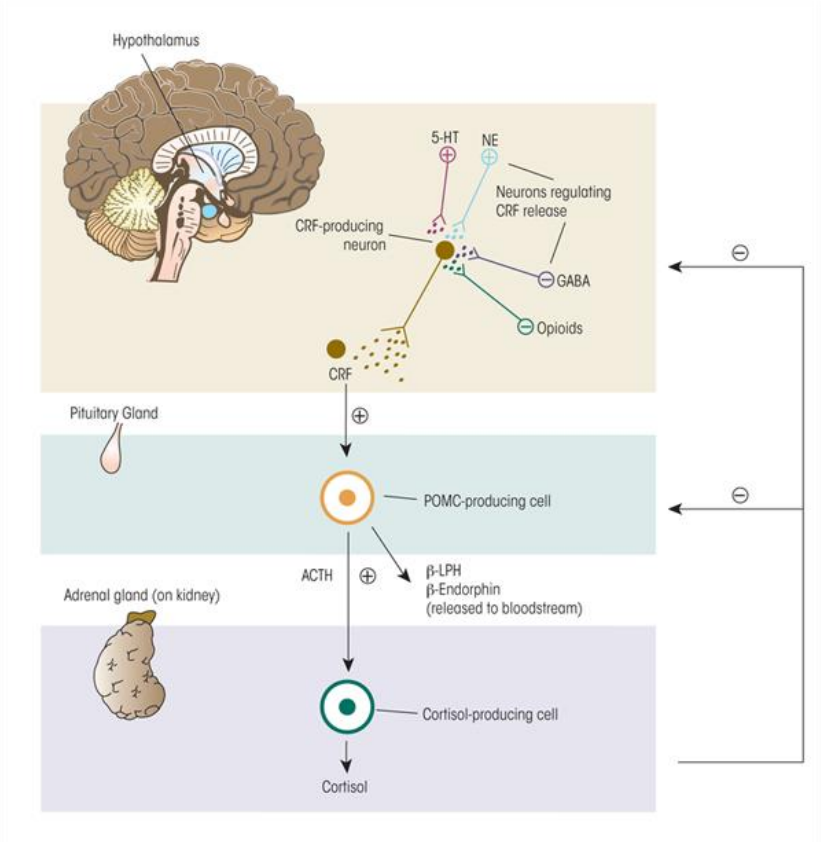

Figure 13. The major components of the stress response mediated by the hypothalamic–pituitary–adrenal (HPA) axis. Stress through amygdala activation, promotes the production of CFR. CRF is transported to anterior pituitary gland. There, CRF promotes production of proopiomelanocortin (POMC) that is the basis for a number of stress-related hormones, including adrenocorticotropic hormone (ACTH), β-lipotropin (β-LPH), and β-endorphin. ACTH, in particular, induces cells of the adrenal glands to produce and release the stress hormone cortisol, in human and corticosteroid in mouse. When cortisol levels reach a too high level, CRF and ACTH release would be reduced thanks to negative feedback promoted by GC themselves. NOTE: = ⊕ excites; ⊖ = inhibits. (Stephens & Wand, 2012).

At rest, the HPA axis shows a circadian activity: during active period, it increases the secretion of CORT that reaches a pick before the beginning of inactive period. For human the pick is reached in the evening, for rodents, whose active period is the night, in the morning. This circadian rhythm is regulated by suprachiasmatic

nucleus that project directly to PVN promoting the activation of the Axis during the active period (Chung, Son, & Kim, 2011; Lightman & Conway-Campbell, 2010).

In details, all the molecules produced by the nucleus of the Axis seem to exert a negative feedback. The most well-known mechanism is the GC one (see Figure 14). GC are able to activate hippocampus that in turn exerts a negative feedback on PVN resulting in a reduction of production of CTF and finally of GC themselves. At the same time, they exert a direct negative feedback to PVN and pituitary too. On the contrary, amygdala is able to increase the activity of PVN and so the production of GC, in particular after stressor stimuli (Brureau et al., 2013; Jankord & Herman, 2008; Stratakis & Chrousos, 1995).

In normal conditions, HPA activation results in a maximal rise in circulating GC after 15–30 min, and returns to baseline levels one hour after the termination of a stressor thanks to intervention of negative feedback (Shirazi, Friedman, Kaufer, & Sakhai, 2015) - (Wang & Harris, 2015). Therefore, alterations of these feedbacks can induce a major dysfunction of the Axis with the consequently uncontrolled production of GC. These alterations occur in diseases but also during aging: indeed, levels of circulating glucocorticoids increase with age in human (Kudielka, Buske-Kirschbaum, Hellhammer, & Kirschbaum, 2004) and rat (Brett, Chong, Coyle, & Levine, 1983). In the following paragraph it is illustrated the principal alteration of Axis in pathologies.

3.2. HPA Axis during disease

HPA axis disfunction is the bedrock of a lot of disease, in particular depression and chronic stress. Recently, this alteration was demonstrated in schizophrenia (Bennett Ao, 2008) and bipolar syndrome (Szczepankiewicz et al., 2011), but a dysregulation of glucocorticoids production was already found in 1975 for autism (Nir et al., 1995; Yamazaki, Saito, Okada, Fujieda, & Yamashita, 1975).

Furthermore, in the 90's it has been discovered that HPA Axis activity is early altered in AD and lead to cognitive impairment and psychiatric abnormality (Swanwick et al., 1998). This observation is coherent with the subsequent finding

that cortisol plasma concentration is higher in patients affected by AD, in comparison to healthy subject (Armanini et al., 2003). Moreover, Wilson in 2005 discovered that cortisol level in blood is a key risk factor to develop AD in elderly patients (Wilson et al., 2005), and more recently, an Australian research demonstrated that the risk is present in every people independently from their ages (Pietrzak et al., 2017).

The alterations of the axis can be promoted by the failure of the negative feedbacks or the enhancement of the positive ones. In the graph below, it is reported a schematic representation of the Axis activity and its regulation in normal and in pathological conditions. In chronic stress or depression, but also in AD, the chronic exposure to GC induces the degeneration of hippocampus, in particular in CA1 and CA3 regions (Sapolsky, Uno, Rebert, & Finch, 1990; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000; Stein-Behrens, Mattson, Chang, Yeh, & Sapolsky, 1994; Uno, Tarara, Else, Suleman, & Sapolsky, 1989), promoting a significant reduction of neurogenesis activity in Dental Gyrus too (Lemaire, Koehl, Le Moal, & Abrous, 2000; Mirescu, Peters, Noiman, & Gould, 2006). All these factors reduce the complex activity of Hippocampus and, consequently, its capability to inhibit the PVN release of CTF. At the same time Amygdala can become strongly reactive promoting a vigorous activation of the Axis. In this way, an irreversible loop is generated, and the production of glucocorticoid continues incessantly, altering the course and progression of the diseases.

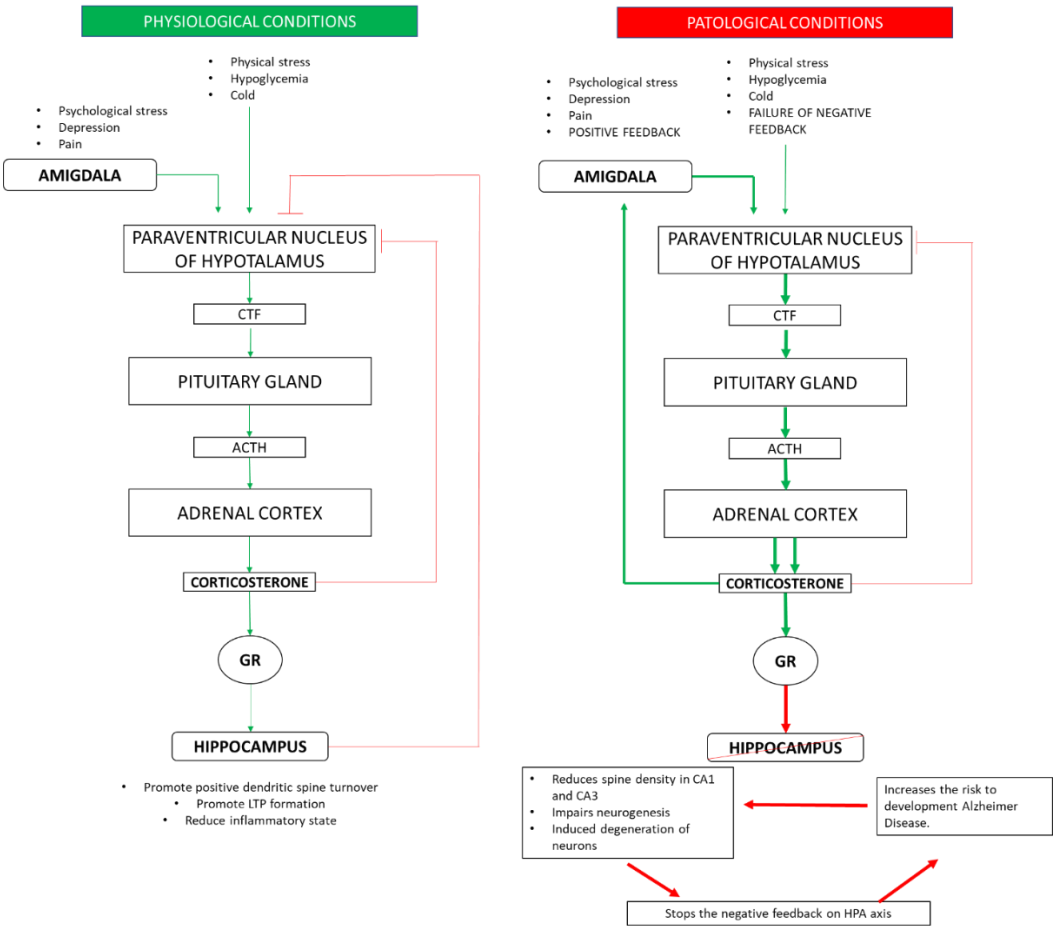

Figure 14. HPA axis regulation in physiological (left) and pathological conditions (right). In normal activity, psychological or physical stressor stimuli can activate the Axis that induces the production of GC, cortisol in Human and corticosteroid in rodents increasing the level of hormones in blood that mediate the physiological response to stress. In order to avoid excessive release of GC, negative feedback promoted by the cortisol/corticosterone themselves can inhibit directly both PVN and Adrenal Cortex; furthermore, GC, binding GR, induce the activation of hippocampus that, in turn, exert a strong inhibition activity on the Axis. All these negative feedbacks restore the initial condition. In pathological condition the negative feedbacks fail. In particular, chronic exposure to GC promotes the opposite effects on hippocampus, inducing the degeneration of CA1 and CA3 region, impairing the dental gyrus neurogenesis. Consequently, hippocampus is not able anymore to stop the Axis activity. Moreover, this chronic exposure promotes positive effects on amygdala that exert a positive feedback on the Axis. The final results of these alterations are the generation of an unstoppable loop that induces the continuous production of GC. The alterations of the Axis are typical not only of chronic

stress and depression but also of AD that can contribute to the formation and maintenance of this loop promoting the degeneration of hippocampus.

3.3. Glucocorticoid Circadian Rhythms

Glucocorticoid activity oscillates in synchrony with circadian rhythms. In rodents, corticosterone binding affinity to MR (Kd of 0.1–0.3 nM) is very high compared to that of GR (Kd of 2–5 nM) (Reul, De Kloet, & Kloet, 1985). One consequence is the relatively high occupation of MR with endogenous CORT during the whole circadian rhythm (Wang & Harris, 2015). On the contrary, activation of GR only occurs in the presence of high levels of GC in the blood. This condition could be present during circadian peak or after acute stressor stimulus (Liston et al., 2013). This circadian rhythm is the consequence of ACTH circadian one that precedes the GC rhythm of some minutes as reported in Figure 15.

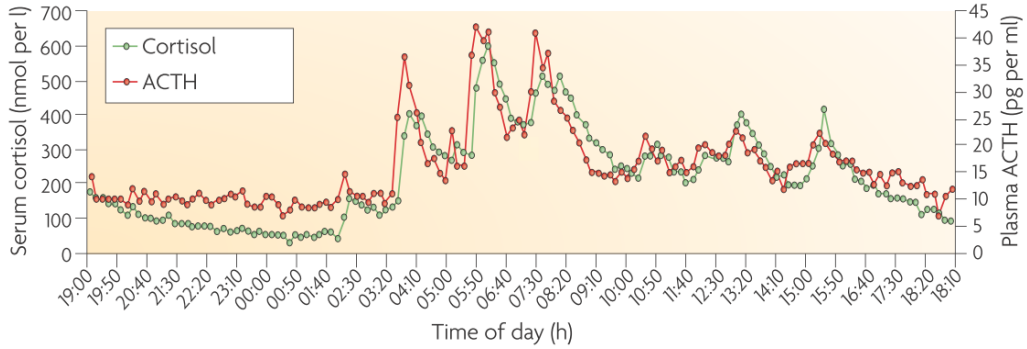

Figure 15. Changing in cortisol and ACTH concentration in blood during the Circadian Rhythm. Note that as expected the oscillation of ACTH precedes the cortisol one. Reproduced by Lightman &Conway-Campbell, 2010.

Moreover, there is an oscillatory pattern of glucocorticoid secretion with an hourly ultradian rhythm (Lightman & Conway-Campbell, 2010).

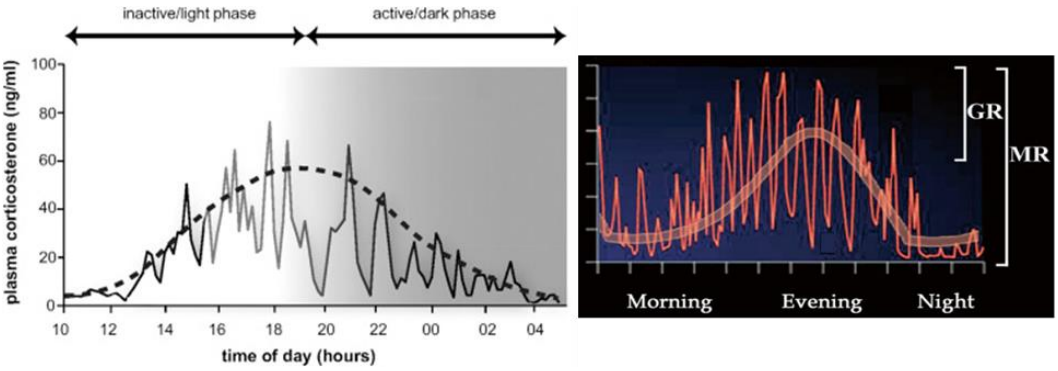

Figure 16. Circadian and Ultradian rhythm of GC with indication of activation of MR and GR. MR show high affinity for GC and so they are constantly activated, while GR are activated only during the circadian pick and in some ultradian peak (den Boon & Sarabdjitsingh, 2017; Wang & Harris, 2015).

However, the chronic presence of high GC may be a symptom of a dysfunction of the HPA axis, a condition typical of diseases such as chronic stress (Finsterwald & Alberini, 2014), depression (Dienes, Hazel, & Hammen, 2013), but also of Alzheimer's disease (AD), as previously demonstrated (Csernansky et al., 2006; Swaab et al., 1994; Umegaki et al., 2000; Zvěřová et al., 2013).

3.4. Glucocorticoid Receptors

Glucocorticoids exert their effects through at least two type of cytoplasmic receptors: mineralocorticoid receptor (MR), or type 1 receptor, and glucocorticoid receptors (GR), or type 2 receptor (Reul et al., 1985).

3.4.1. Genetic

Both receptors belong to steroid receptor of the nuclear hormone receptor family (Wang & Harris, 2015 Ch. 2, pag 37-38). MR are encoded in human by *NR3C2* gene that is located on chromosome 4q31 (Fan et al., 1989) and on chromosome 15 in mice (Martinerie et al., 2013), while human GR are encoded by *NR3C1* gene, which is located on chromosome 5q31 (Francke & Foellmer, 1989).

Human *NR3C2* gene - that codes for MR - is composed of 10 exons and 8 introns. The first two exons can undergo alternative splicing but both transcripts give rise to the 984 amino-acids mineralocorticoid receptors.

Human NR3C1 gene has 9 exons (Nicolaides, Galata, Kino, Chrousos, & Charmandari, 2010) and 11 introns; alternative splicing of exon 9 produce to different isoforms: the GRα, the predominant one with 777 amino-acids, and GRβ with shorter C-terminus transactivation region and 742 amino-acids. GRβ functions as inhibitor of GRα and resides in nucleus (Kadmiel & Cidlowski, 2013).

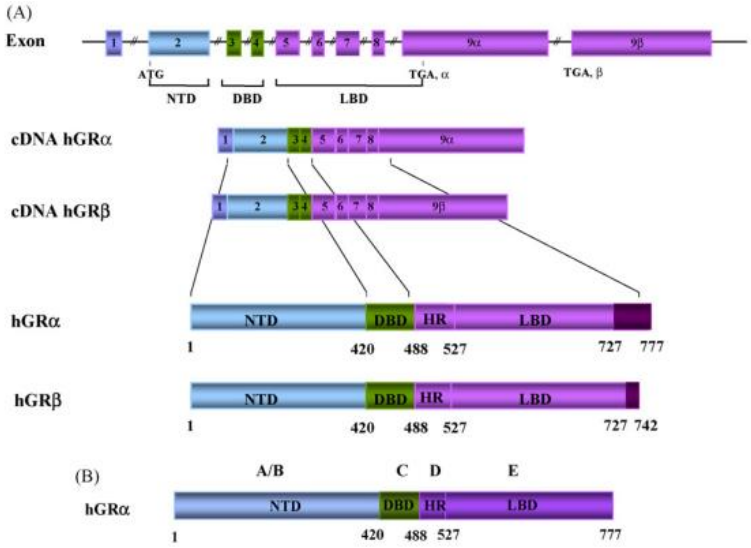

Figure 17. Schematic representation of the glucocorticoid receptor (hGR) gene and its domains. Through alternative splicing of the primary transcript it produces two mRNA and protein isoforms named hGRα and hGRβ (Nicolaides et al., 2010).

3.4.2. Type 2 Glucocorticoid Receptor (GR)

GR receptor is composed by 4 domains:

- A/B - N-terminal regulatory domain (NTD)
- C -DNA-binding domain (DBD)
- D- Hidden region
- E- Ligand-binding domain (LBD)

- C-terminal domain

Between amino acids 77 and 262 of the hGRα, N terminal region contains the activation function (AF)-1 a major ligand-independent transactivation domain, that promotes the interaction between the receptor and molecules that allow transcription (Nicolaides et al., 2010; Wang & Harris, 2015).

The DBD domain is the most conserved domain in steroid receptors and it contains two zinc fingers motif through which receptor binds the DNA in a specific promoter regions of target genes named Glucocorticoids - Response – Element (GRE), composed of this sequence: GGTACAnnnTGTTCT (Nicolaides et al., 2010; Wang & Harris, 2015).

The hinge region or region D is a variable sequence between DBD and ligand-binding domains. It is responsible for dimerization of DBD through its amino terminus. The hinge region confers structural flexibility in the receptor dimmers.

The ligand-binding domain (LBD) of the hGRα corresponds to amino acids 481–777, binds to glucocorticoids and it is essential for the binding of ligand to the receptor. The LBD is composed also by a second transactivation domain, called AF-2, which is ligand-dependent, as well as sequences necessary for the dimerization of receptor and their translocation from cytoplasm to nucleus. Moreover, it regulates the binding with the heat shock proteins and with coactivators (Nicolaides et al., 2010).

Some receptors contain an additional highly variable carboxyl-terminal region of unknown function. Of these functional domains, the NTD is the most variable and is the major target for ligand-dependent phosphorylation at multiple serine residues (Wang & Harris, 2015).

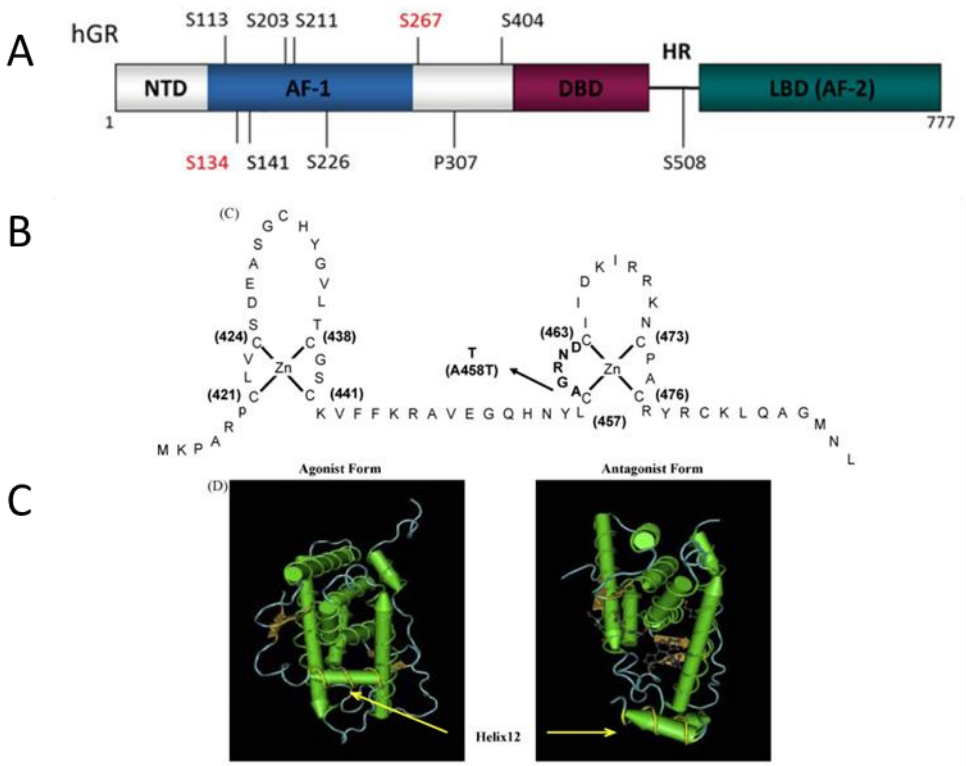

Figure 18. Schematic representation of the structure of the human glucocorticoid receptor (hGR) gene with the functional domains and best characterized phosphorylation sites of human GR. Sites in red are BDNF-dependent sites. (B) Enlargement of part of the DNA-binding domain (DBD) showing the amino acid sequence (single letter codes) of the two zinc fingers and the dimerization loop (in bold). The A to T mutation presented at position 458 could produce a defective dimerization of receptor. (C) Crystal structure of the ligand-binding domain (LBD) of the human glucocorticoid receptor-α (hGRα). Stereotactic conformation of the agonist (left) and antagonist (right) form of the LBD of hGR. The yellow arrows indicate the position of Helix 12, which is critical for the formation of AF-2 surface that allows interaction with activators. (NTD amino terminal domain, AF-1 activation function-1, DBD DNA-binding domain, HR hinge region, LBD ligand-binding domain. AF-2activation function-2, S serine, and P proline) (Nicolaides et al., 2010; Wang & Harris, 2015; Yankner, 1996).

Glucocorticoid receptors are crucial for normal development and are present not only in the brain but also in many different cell types and tissues, such as liver, lung and adrenal medulla; meanwhile, MR have a more limited distribution. The distribution of GR throughout the brain is widespread in neurons and glial cells,

particularly high in the limbic system, hippocampus (CA1, CA2,CA3 regions), septum and amygdala, in the parvocellular neurons of PVN and in the supraoptic nucleus (De Kloet et al., 2000).

3.5. Glucocorticoid mechanism of action

The binding of ligand with the LBD of hGRα produces a conformational change by compacting the receptor structure in order to increase its stability (Nicolaides et al., 2010). The binding of ligand allows the induction of the transactivation domain AF-2. The LBD is composed by 12 α-helices (H) and 4 small β-strands (Bledsoe et al., 2002). After this binding, H11 and H12 alter their position in order to allow the binding of coactivators to AF-2. On the contrary, the binding of an antagonist to the receptor, like mifepristone, promotes a different movement of H12 preventing the binding of coactivators to AF-2 (Kauppi et al., 2003).

In the absence of ligand, GR remains principally in the cytoplasm and form a hetero-oligomeric complex, composed of chaperone heat shock proteins (HSPs) 90, 70 and 50, immunophilins, and other proteins (Pratt, 1993). HSP90 is the principal protein responsible of the regulation of ligand binding: it exposes the ligand-binding site and hide the two nuclear localization sequences (NLS), NL1 and NL2 (Nicolaides et al., 2010).

After the binding of GC to GR, the receptor complex is dissolved, HSPs leave the receptor and GR translocate into the nucleus through a mechanism involving NLS.

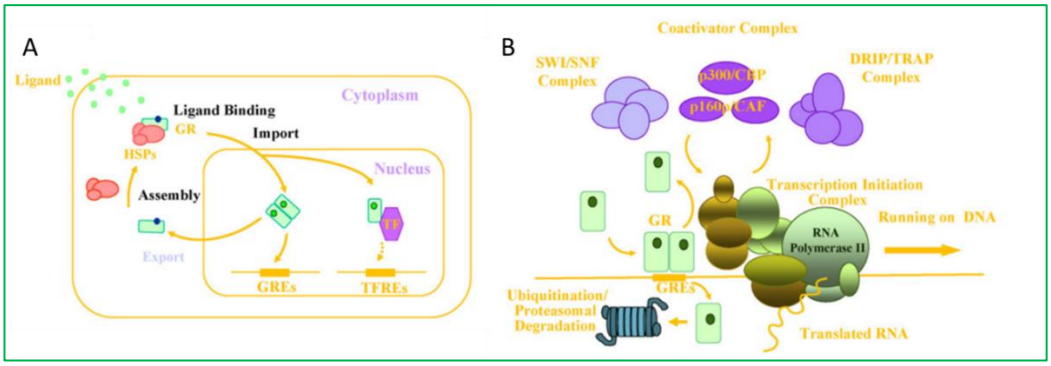

Figure 19. A) Translocation of receptor from cytoplasm to nucleus where it homodimerizes and bind to GRE. B) Interaction of AF-1 and AF-2 of hGRα with coactivators that promote

the gene transcription. AF: activation function; DRIP/TRAP: vitamin D receptor-interacting protein/thyroid hormone receptor-associated protein; GR: glucocorticoid receptor; GREs: glucocorticoid-response elements; HSP: heat shock protein; SWI/SNF: switching/sucrose non-fermenting; TF: transcription factor; TFRE: transcription factor-response element (Nicolaides et al., 2010).

Within the nucleus, the receptor binds cis-DNA element like GREs in the promoter regions of target genes, regulating their expression both positively and negatively depending on the promoter and the cofactors involved (Schaaf & Cidlowski, 2002). Alternatively, the ligand-activated hGR can modulate gene expression without involvement of GREs, binding with other transcription factors, such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB), p53 and signal transducers and activators of transcription (STATs) (Scheinman, Gualberto, Jewell, Cidlowski, & Baldwin, 1995) (Figure 19). In particular, trans-repression of the proinflammatory transcription factors AP-1 and NF-κB is induce by steroids without the binding to DNA (Nicolaides et al., 2010). Finally, GR can bind both DNA elements and transcription factors (J. Zhou & Cidlowski, 2005) (Figure 20).

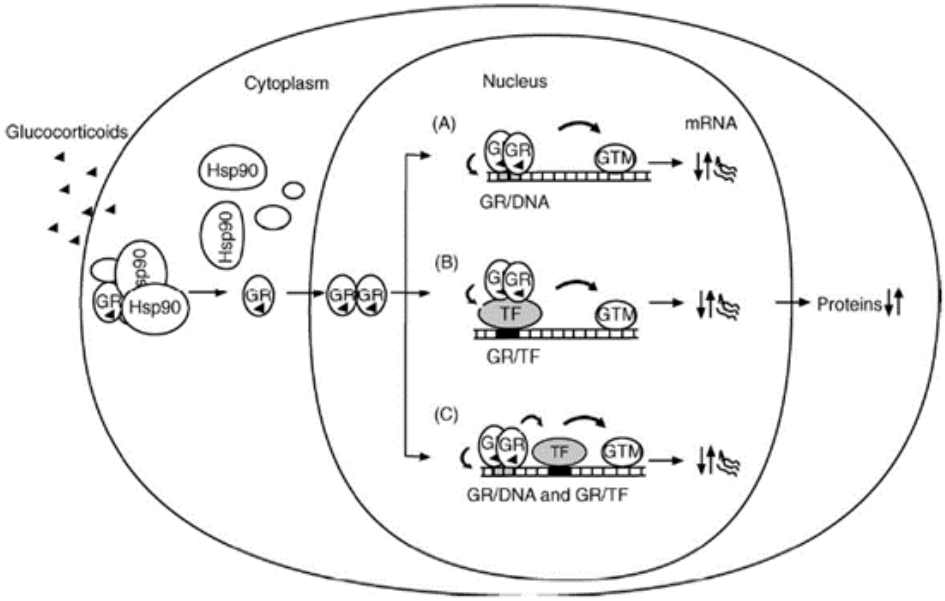

Figure 20. Schematic representation of the three different modalities of regulation of transcription by GR activation. First: dimer of GR binds GRE; Second: dimer or monomer of GR bind transcription factors (TF) that promote DNA transcription; Third: dimer of GR bind GRE and TF (E. Ronald De Kloet et al., 2000).

Emerging evidence suggests that glucocorticoids can exert more rapid actions (within minutes) in cytosol through non-genomic signal mechanism, that does not require nuclear mediated transcription or translation (Kadmiel & Cidlowski, 2013).

3.6. Glucocorticoid biphasic behavior

Like the Aβ peptides (Daniela Puzzo et al., 2012) (see *paragraph 1.4*), the behavior of GC, at least on brain, is hormetic, since it doesn't follow the classical linearity of dose/response, but, rather, the inverted-U shape dose-response relationship (Roozendaal, 2000). Differently from other hormetic compounds, the biphasic behavior of glucocorticoids seems to be more complex. Specifically, their hormetic effects is in function of the dose, but, above all, of the time of exposure to a determined dose. That means that high long-lasting dose and low-long lasting dose of GC produce comparable effects; on the contrary acute and medium dose produce the opposite.

This phenomenon it has been described for memory formation (for memory performance see *Figure 22*) and consolidation, for dendritic spine turnover (see *Figure 26*) and hypothesized for inflammation regulation.

3.6.1. Glucocorticoid and memory

The role of glucocorticoids in the regulation of memory formation and storage was first found in adrenalectomized rats, which present a great impairment in the production of corticosterone: these rats, indeed, were affected by spatial and contextual fear memory deficits, suggesting an involvement of GC in the mechanism. The central role of GC was, subsequently, investigated also in humans: the reduction of cortisol production results in a deficit of long-term declarative memory (Finsterwald & Alberini, 2014).

Since glucocorticoid acts through two kind of receptors (MR and GR), some hypotheses had been proposed to explain how their activity can be related and whether they code for different effects. One of the most important evidence is that activation of MR seems to regulate the initial phase of memory encoding, including the response to novelty, whereas GR are important in memory consolidation (E. Ron de Kloet, Oitzl, & Joëls, 1999; Finsterwald & Alberini, 2014; ter Horst et al., 2012). In support of these observations, the administration of spironolactone, an antagonist of MR impairs contextual memory both after 3h and 24h after treatment; while the use of mifepristone, the antagonist of GR, impaires memory only after 24h and has no effects before (M. Zhou et al., 2010) (See Figure 1). Blockade of GR may be able to disrupt the consolidated memory: indeed, following the learning of a new memory task, healthy subjects treated with synthetic glucocorticoids, present a significant reduction of the blood flow in temporal lobe, detected using positron emission tomography (PET), during memory recalled (D. de Quervain et al., 2003).

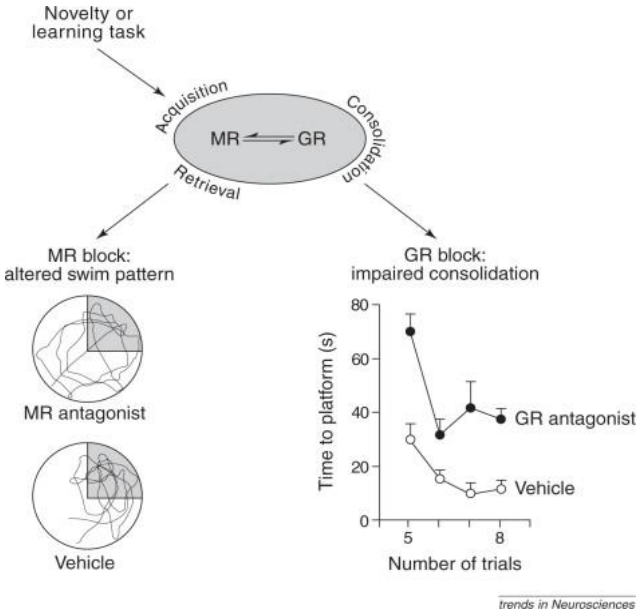

Figure 21. Schematic representation of the role of MR and GR on memory consolidation. MR is responsible of behavioral reactivity to novel stressor stimuli: inhibition of MR 30 or 45 minutes before the retrieval (day 2), in Water Morris Maze, reduces the time last near the platform in comparison to control. On the contrary, inhibition of GR immediately after acquisition (day 1) impaired performance 24h later (E. Ron de Kloet et al., 1999).

Regardless, the molecular mechanism promoted by the two receptors are unlikely independent: indeed, the ratio of activation of GR and MR is critical for mediation of positive or negative effects on cognitive performance (S. J. Lupien, Maheu, Tu, Fiocco, & Schramek, 2007): in particular, de Kloet in 1999 (E. Ron de Kloet et al., 1999) found that memory facilitation and improvement seem to be the results of the concomitant strongly activation of MR and intermediate activation of GR. So, a possible explanation of biphasic behavior of GC could be explain by the ratio of activity MR/GR as symbolized by the graph below.

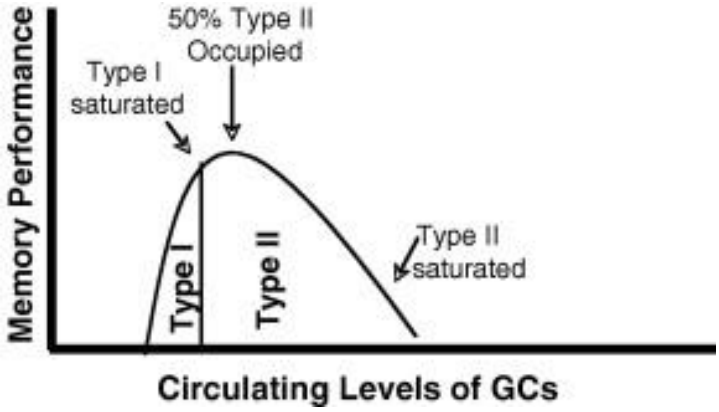

Figure 22. The MR (Type I)/GR (Type II) glucocorticoid ratio hypothesis of the association between "circulating levels of glucocorticoids", and "memory performance" (de Kloet et al., 1999). The figure shows occupancy of GC receptors as a function of circulating levels of GC and their capability to modulate memory. Maximization of memory is achieved when Type I glucocorticoid receptors (MR) are saturated and there is low occupancy of Type II glucocorticoid receptors (GR), while when both MR and GR are not occupied (left side of the inverted-U shape function) or are saturated (right side of the inverted-U shape function), there is an impairment in memory performance (S. J. Lupien et al., 2007).

Interestingly, hyper-activation of GR or GR deletion, seems to dramatically impair memory formation independently by activity of MR (E. Ron de Kloet et al., 1999); this observation can lead to other hypothesis that MR can play a little facilitation or obstruction activity but the principal promoters of biphasic behavior are GR (see also ***Discussion*** *page 87).*

3.6.2. Glucocorticoid and spine turnover

The effects promoted on memory are strictly linked to the capability of GC to regulate spine turnover acting on potentiation and depression of synapses and on physical remodeling of spines and dendrites and of their number and arborization, respectively.

Dendritic spines are a typical specialization region of neurons that originate, like a little protrusion, from dendrites, but also from axon hillock and soma. Spine represents the post synaptic element of an excitatory synapse and are presents in

more than 90% of this type of synapse. Spines are present in various neuron population of all vertebrates and some invertebrates (Nimchinsky, Sabatini, & Svoboda, 2002). Human brain contains more the 10^{13} spines (Nimchinsky et al., 2002).

Spines show characteristic structure indispensable for their biochemical and electrical function and can be distinguished in three main categories (or class) on the basis of their shape (Stephen W. Scheff et al., 2006): the mushroom-like spines which have a large head and a narrow neck, the thin spines which have a smaller head and a narrow neck and stubby spines which have no constriction between the head and the attachment to the shaft; finally the filopodium is consider the precursor of the mature spines and has a hair like morphology. A schematic representation of spine classes and the structure of post synaptic domain is reported in Figure 23.

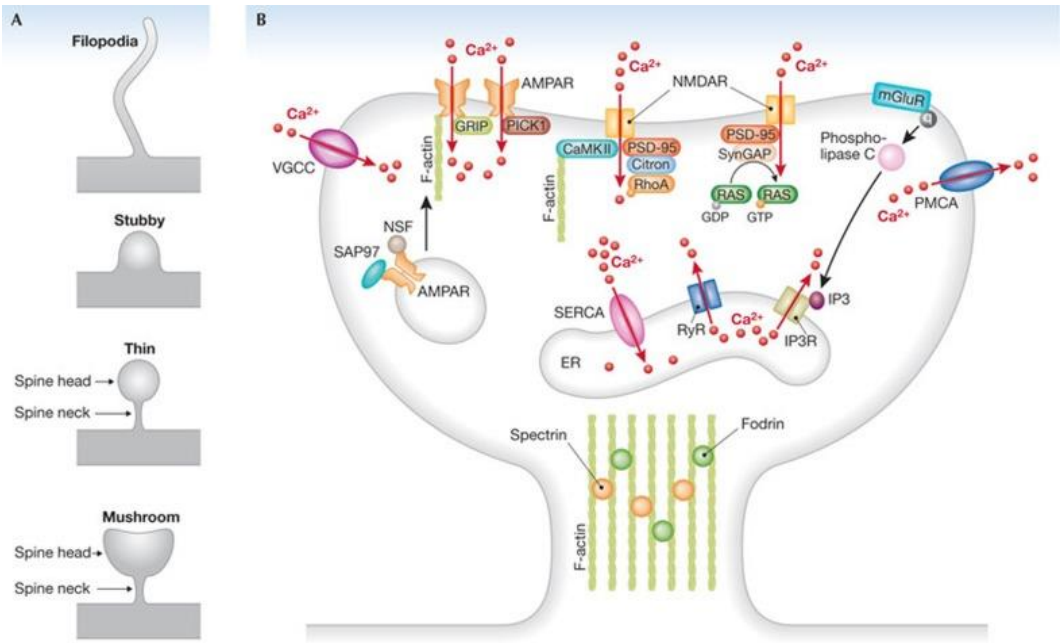

*Figure 23. Structural and molecular organization of spines. (**A**) It is reported the structure of the typical 4 different classification of dendritic spines. (**B**) Receptors and molecules related to calcium (Ca2+) signaling in spines. Red arrows indicate flux of calcium ions. AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CaMKII, Ca2+/calmodulin-dependent kinase II; ER, endoplasmic reticulum; GAP, GTPase-activating protein; GRIP, glutamate-receptor-interacting protein; IP3(R), inositol*

trisphosphate (receptor); mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; NSF, N-ethylmaleimide sensitive factor; PICK1, protein interacting with C kinase; PMCA, plasma membrane Ca2+-ATPase; PSD, postsynaptic density; RyR, ryanodine receptor; SAP97, synapse-associated protein 97; SERCA, sarco/endoplasmic reticulum Ca2+-ATPase; VGCC, voltage-gated calcium channel (Rochefort & Konnerth, 2012).

Whether the morphological differences of spines are link to specific activity is not clear. Electron microscopy showed that all classes of spines can contain the postsynaptic density (PSD) that are a protein dense specialization near the membrane of postsynaptic element (i.e. the spine) and so act as normal synapses (Rochefort & Konnerth, 2012). The PSD consists of the receptors, channels, and signaling systems involved in synaptic transmission and the coupling of synaptic activity to postsynaptic biochemistry (Nimchinsky et al., 2002).

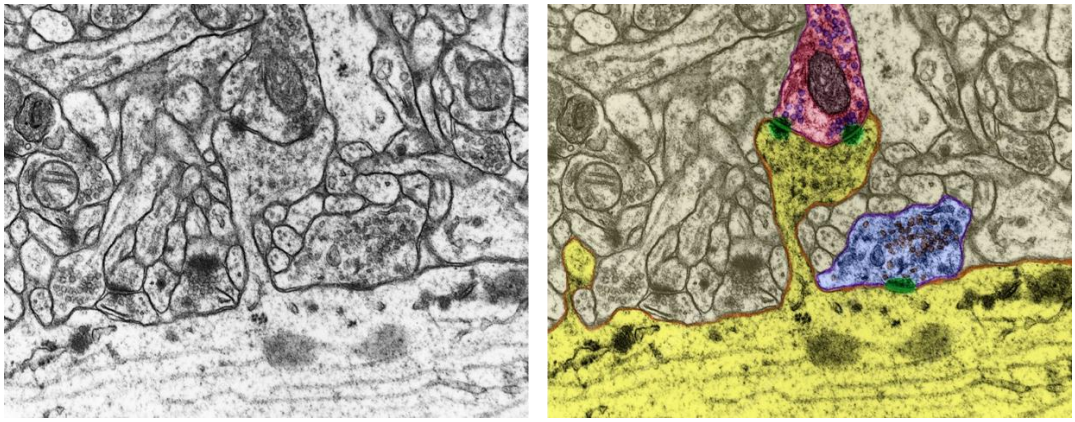

Figure 24. Imaging of neuron and synapses acquired using transmission electron micrograph (TEM). In yellow it is marked the dendrite and the mushroom-like spine that makes an excitatory synapse with the presynaptic neuron terminal (in purple). In blue it is marked the presynaptic terminal of an inhibitor synapse. The postsynaptic density (PSD) is colored in green. Interestingly, the spine presents two PSD that are typical of perforated synapses, a particular conformation of spine that can be assumed during the process of memory formation (i.e. LTP) (from Dennis Kunkel Microscopy / Science Photo Library).

Recently, some authors have suggested that both stubby and thin and filopodium are pre-mature spines while only mushroom-like ones can stabilized forming synapses, as schematically suggested in the graphical representation below (Miermans, Kusters, Hoogenraad, & Storm, 2017).

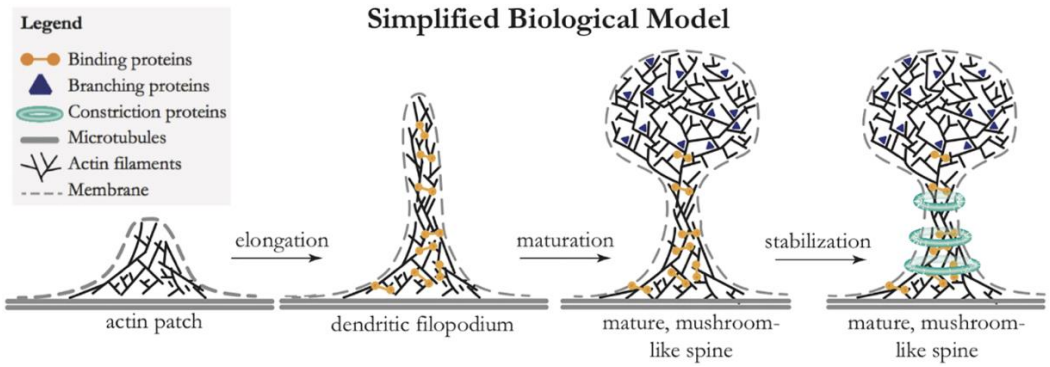

Figure 25. Spine morphological differences could represent the process of maturation and stabilization of the spine itself. In this picture, published by Miermans of Ducth University of Technology of Eindhoven, we can appreciate the modification of cytoskeleton of the spine from the first phase of elongation, promoting by actin patch, to the final stabilization of mushroom-like spines. This model considers the first spine as the previously called stubby spines and the second as the previously called thin spine. Moreover, it proposed that only mushroom-like spines present the typical characteristic of post synaptic elements, like organelles and vesicles. (Miermans et al., 2017).

Independently from their classifications, dendritic spines are a dynamic specialization of neurons that are subjected to continuously changes in number and shape. These changes are the result of the actin cytoskeleton modifications (Sekino, Kojima, & Shirao, 2007)

It is well known that glucocorticoids play an important role in regulation of dendritic spine plasticity. Conor Liston in 2011, demonstrated that glucocorticoids show a biphasic behavior on spine turnover. In particular acute high dose of corticosteroids in rats promote the formation of new spines and elimination of older

ones; on the contrary, chronic administration increases the elimination rate but not the formation of new spines, inducing newborn spine degeneration too (Liston & Gan, 2011) (See Figure 26). These effects are especially known in neurons of CA1 and CA3 regions of hippocampus but also in prefrontal cortex (Swanson et al., 2013).

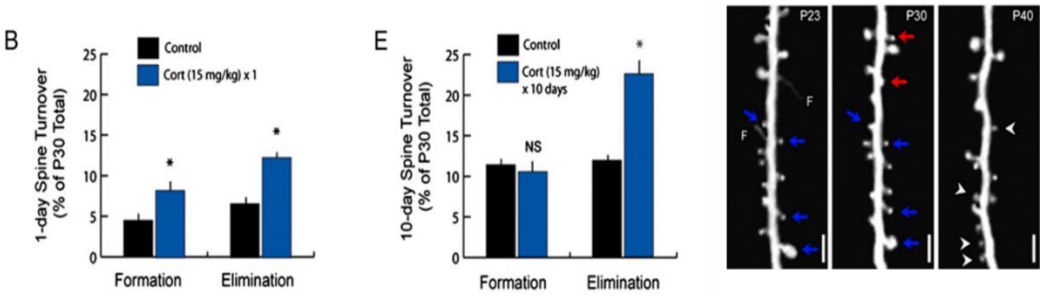

Figure 26. A single corticosterone injection significantly increased formation and elimination of spines over 24 h (B). Chronic glucocorticoid exposure increased elimination rates but had no significant effect on formation (E) (Liston & Gan, 2011).

High concentration of GC is present during the peak of circadian rhythm or after an acute stressor stimulus. Circadian glucocorticoid peaks seem to promote postsynaptic dendritic spine formation, not only in hippocampus, but also in the mouse cortex, whereas troughs are required for stabilizing newly formed spines that are important for long-term memory retention (Liston et al., 2013). Chronic exposure to GC, induced by drug administration or through physiological stress, can alter the rhythm preventing the consolidation of memory as described in the previous paragraph.

Chronic stress, saturating the GR, produces also dendrite atrophy reducing the neuron arborization. This phenomenon is described in particular in CA3 region of hippocampus by Ana Maria Magarinos already in the 90's (Magariños, McEwen, Flügge, & Fuchs, 1996) – see *Figure 28* - and more recently also in CA1 region, in particular in pyramidal and granular cells (Sousa et al., 2000). But hippocampus is not the only region affected: the reduction of the dendritic arborization occurs also on prefrontal cortex (McEwen, Nasca, & Gray, 2016). Interestingly, the regions

damaged by chronic stress are the same affected by AD as reported in the *paragraph 3.7*.

On the contrary, in amygdala, chronic stress promotes arborization and it is important to underline that hippocampus and amygdala are the principal regulator of the HPA axis (see paragraph 3.1). Therefore, chronic stress induces an hyperactivation of this Axis through simultaneously reducing the activity of hippocampus (the inhibitor the Axis) and increasing the activity of amygdala (the activator of the Axis). The final result of this process is the creation of unstoppable loop that continuously promote the production of GC, as described in *paragraph 3.2* and in *Figure 14* and *Figure 27* below. For a more in-depth analysis, consult the review and works of McEwen or see the picture below (McEwen, 2007; McEwen et al., 2016; McEwen & Milliken, 1999).

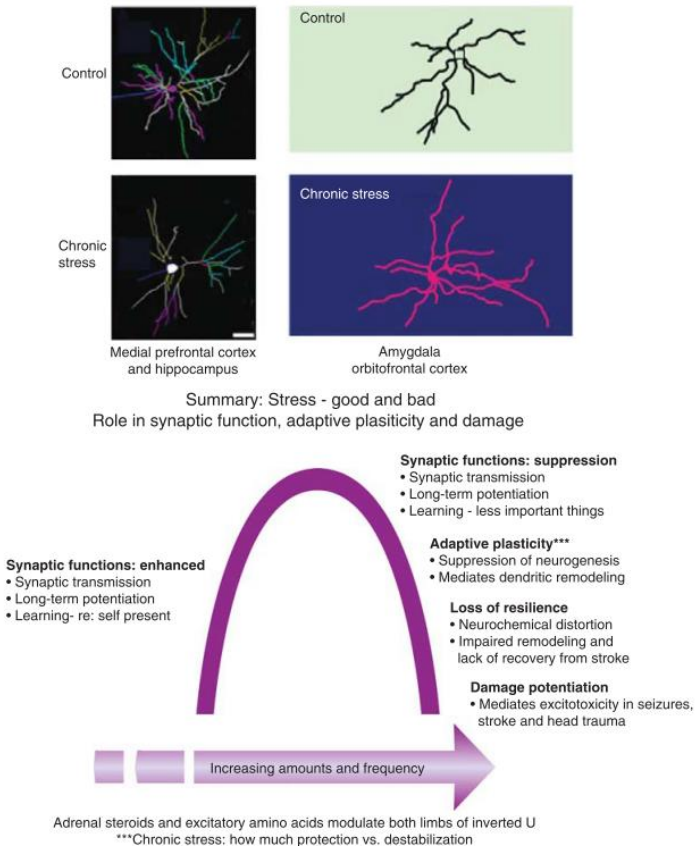

Figure 27. Representation of biphasic effects of GC on brain, and the different response of hippocampus-prefrontal cortex and amygdala to stress. Effects of acute and chronic stress

operate in space and time in an inverted U-shaped. Intermediate concentrations of GC enhance synaptic functions; on the contrary, chronic exposure to high concentrations of GC suppresses synaptic functions and neurogenesis and promotes neurochemical disfunctions. The chronic stress (or exposure to high GC), showed also a powerful effect on arborization: in medial prefrontal cortex and hippocampus it induces a strong reduction of arborization, while on amygdala it produces the opposite effect. These alterations may explain the hyper-activation of HPA axis: hippocampal negative feedback on Axis is reduced, while amygdala positive one is enhanced (McEwen et al., 2016).

Saturation of GR after treatment with high doses of glucocorticoids are also able to reduce LTP and enhance LTD. Indeed, enhanced activation of GR reduces the ability of hippocampal neurons to produce LTP and increases the threshold for synaptic strengthening, suggesting that activation of GR may play a role in reducing the accessibility of novel information to the same neural network. (Finsterwald & Alberini, 2014); Furthermore, long lasting activation of GR, like in chronic stress, strongly impairs the ability of neurons to induce LTP.

3.6.3. Glucocorticoid and inflammation

Activation of inflammation in brain can promote neuron damage through activation of microglia, the principal immunity cell of Central Nervous System (CNS) (Carrillo-De Sauvage et al., 2013). The action of microglia is regulated by glucocorticoids too: microglia, indeed, present both GR and MR.

GC are typical anti-inflammatory drugs and previous works found that administration of agonist of GC can reduce activation of microglia *in vitro* (Colton & Chernyshev, 1996; Drew & Chavis, 2000; Tanaka et al., 1997). Furthermore, both GR activation - using dexamethasone - and MR activation - with aldosterone - can reduce the proliferation of microglia *in vitro* (Ganter, Northoff, Männel, & Gebicke-Härter, 1992).

The results *in vitro* are, apparently, in contrast with the *in vivo* observations. Indeed, chronic exposure to GC can produce on brain an enhancement of inflammation, while opposite effects have been observed in the periphery, as commonly expected (Sorrells & Sapolsky, 2007). Munk observed the different effect of GC on brain already in 1984 and he suggested that the activation or the suppression of inflammation strictly depend on plasma concentration of GC (Munck, Guyre, & Holbrook, 1984). Preliminary hypothesis distinguished from low dose, that promoted pro inflammatory effects, and high dose that promoted anti-inflammatory action. This hypothesis is, now, incompatible with the observations that prolonged exposition to GC enhances the inflammation through activation of microglia (Minami et al., 1991; Uz et al., 1999). Nair in 2006 suggested that the activation of glucocorticoid receptors on microglia always promotes an anti-inflammatory effect on the cell, independently from the dose and time of exposure to glucocorticoids. Consequently, the increased activation of microglia observed *in vivo* after chronic exposure to glucocorticoids is exclusively due to an indirect mechanism involving neuron (Figure 53). In details, chronic exposure to GC, through hyper-activation of GR, damages neurons inducing an excessive release of glutamate. The consequently strong activation of NMDA receptors promotes the production of pro-inflammatory molecules that are able to compensate and overcome the anti-inflammatory effect directly exerted by GC on microglia, activating these cells (Nair & Bonneau, 2006).

Interestingly, intracisternal administration of mifepristone, a GR antagonist, effectively reduced immune-activated proinflammatory responses, specifically from hippocampal microglia and prevented Escherichia coli induced memory impairments in aged rats (Colton & Chernyshev, 1996; Drew & Chavis, 2000; Tanaka et al., 1997) underlining a critical role of GR activity on promoting inflammation in brain.

3.7. Glucocorticoid and Alzheimer's Disease connections

In recent years, attention has been focused on the role of glucocorticoids in the onset and progression of several neuropathologies, including Alzheimer's Disease. Indeed, if an acute dose or a physiological concentration of GC show positive effects on spine turnover (Liston et al., 2013), high and chronic dose of GC, through a prolonged activation of GR, induces profound alterations in brain that are comparable to those produced by AD, as showed in Figure 28 and in Figure 29.

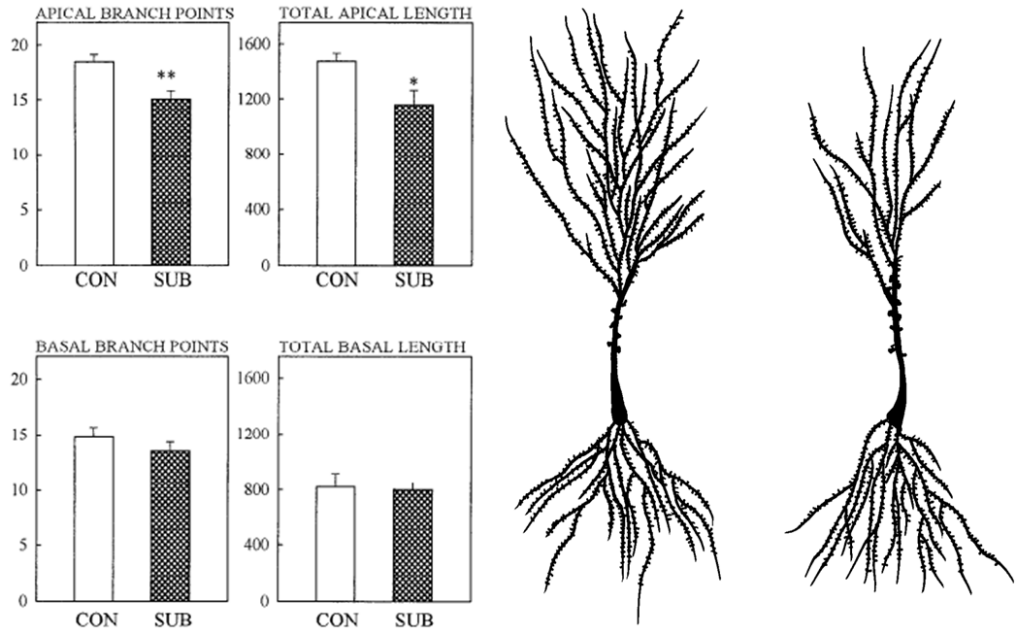

Figure 28. 28 day of psychosocial stress, in male tree shrews promote degeneration of apical dendrites in CA3 pyramidal neurons of hippocampus. Graphs presented on the left show that the degeneration is significant for the apical dendrites, but not for the basal ones. On the right, the changes occurred in pyramidal neurons of stress treated animals are represented (Magariños et al., 1996).

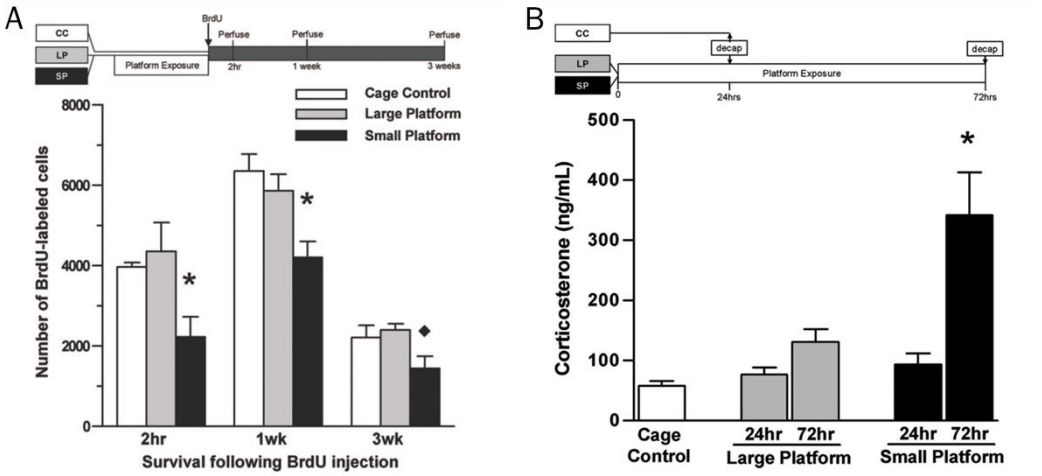

Figure 29. Mirescu in 2006 demonstrated that sleep deprivation induced impairment of neurogenesis through the enhancement of corticosterone level in blood. A) Rats were subjected to sleep deprivation using the "small-platform" (SP) method, while "large-platform" (LP) and no treatment (CC) were used as control. After 72 hours of treatment, animals received an intraperitoneal injection of BrdU (200mg/kg) and were sacrificed after 2hr,1 weeks or 3 weeks. The number of BrdU-labeled cells were reported in the graph: LP significantly reduced this number in all three time points. B) The concentration of corticosterone level was checked after 24hr and 72hr from the beginning of treatment. After 72hr, the concentration of the hormone was significantly higher in rats treated with SP demonstrating a correlation between neurogenesis impairment and corticosterone levels (Mirescu et al., 2006).

In details, chronic exposure to glucocorticoids is linked to degeneration of dendritic spines in hippocampus and prefrontal cortex (Liston & Gan, 2011; Magariños et al., 1996) as previously mentioned (compare Paragraph 3.6.2); it induces an impairment of hippocampal neurogenesis (Lemaire et al., 2000; Mirescu et al., 2006) and of behavior (Hammar, 2009; Wilson et al., 2003) related to declarative memory, executive functions and attention.

Therefore, the overlap of these symptoms with those typical of Alzheimer's disease suggested the hypothesis of the existence of a link between GR activity and dementia itself. Two important discoveries have made it possible to reinforce this hypothesis.

Firstly, it is known that a rare haplotype of *hsd11b1* gene, that codifies for a cortisone reductase, is associated with a 6-fold increased risk for sporadic AD (D. J. F. de Quervain et al., 2004). This enzyme is a NADPH-dependent enzyme that reduce cortisone in cortisol, the active form of the hormone promoting the stress-activity.

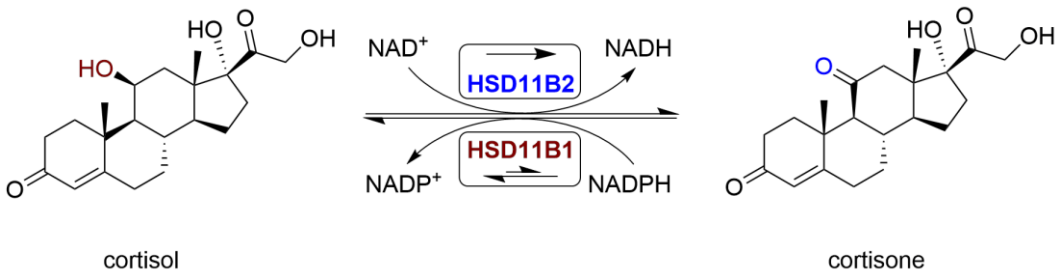

Figure 30. HSD11B1 catalyze the reduction of inactive cortisone to the active hormone cortisol. On the contrary, HSD11B2 promote the opposite reaction (en.wipedia.org).

De Quervain demonstrated that this rare haplotype is significantly more diffused in AD patients of Swiss and south Europe (named "Mediterranean": Italy and Greece) origin, rather than control one (2.9% in AD compared to 0.5% in control group). Furthermore, only for Mediterranean group, MC2R gene, that codified for ACTH receptor, is also associated with AD (*Figure 31*).

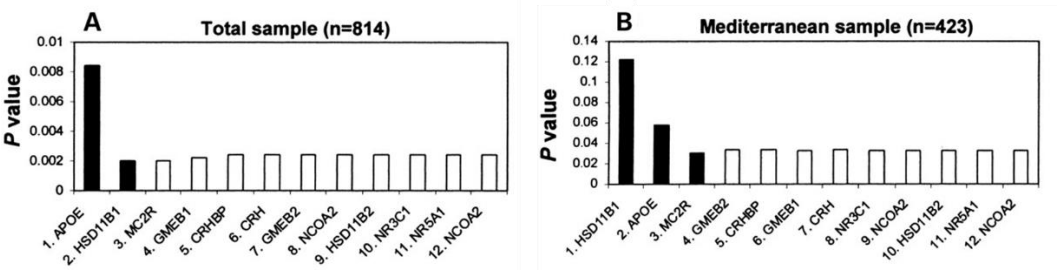

Figure 31. Significance level P as a function of Single Nucleotide Polymorphism in glucocorticoid-related genes and in APOE. Dark bars indicate the genes that represent a key risk for the development of AD. HSD11B1 (A), but also MC2R for Mediterranean sample (B), are susceptibility genes for AD (D. J. F. de Quervain et al., 2004).

Secondly, some previous works, like the one published by Green in 2006, demonstrated that the administration of dexamethasone, the agonist of GR, increased the production of Aβ 1-40 and 1-42 by 60% and Tau total in 3xTg-AD mice (K. N. Green, 2006). As a matter of fact, Green discovered the existence of one correlation between GR hyper-activation and the production of two of the typical hallmarks of AD. Stress induced by dexamethasone is, so, able to worsen the pathology.

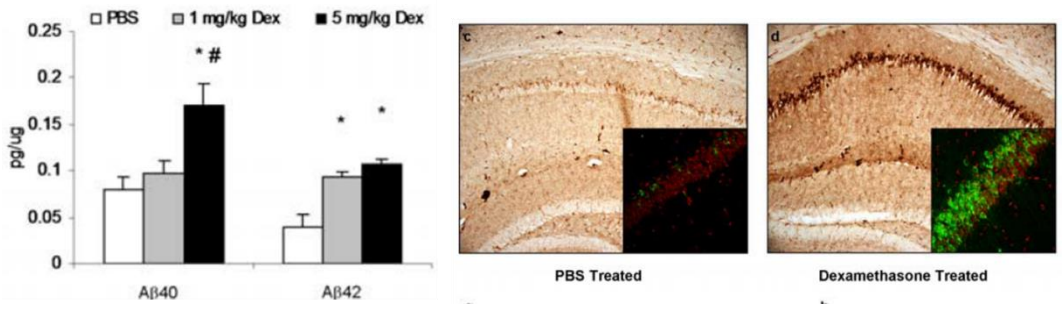

Figure 32. 3xTg-AD mice treated with PBS or with 1 or 5mg/kg of dose of dexamethasone, the agonist of GR. In the histogram on the left, it is reported the significantly increased concentration of Aβ40 and 42 after 5mg/kg agonist treatment. On the right, the Aβ staining of hippocampal slices of mice treated with PBS or dexamethasone is shown. The expression of Aβ is clearly increased in treated mice (K. N. Green, 2006) .

4. AIMS

Taking in account all the literature data described in the introduction, the **main aim** of my project was to investigate the correlations between AD and glucocorticoids. In particular, I focused my attention on the effects of GR modulation on spine plasticity and inflammation in an Alzheimer's Disease animal model, the 3xTg-AD mice.

Therefore, I studied the hippocampal structure, in particular on CA1 region, the area negatively affected both by AD (Padurariu, Ciobica, Mavroudis, Fotiou, & Baloyannis, 2012; West, Kawas, Martin, & Troncoso, 2000) and by high GC levels (McEwen, 2007; McEwen et al., 2016; Sousa et al., 2000).

The **first aim** of my project was to verify whether the modulation of GR activity could interfere, positively or negatively, with dendritic spine plasticity in an AD mouse model, the 3xTg-AD (3xTg) mouse (Oddo et al., 2003), using different techniques like Golgi Cox, Electron Microcopy and Behavioral tests.

Probably, GR effects on dendritic spines are not only the result of intra-neuron mechanisms, but also of glial cells. It is widely known that glucocorticoids have a potent anti-inflammatory activity and long-lasting high levels of GC are able to activate the principal immune cells of brain: the microglia (Nair & Bonneau, 2006). Even if the mechanism is not yet completed elucidated, microglia indeed shows pruning activity on spines during development and a similar action has been supposed also in adult brain. Therefore, the **second aim** of this work was to verify whether chronic activation of GR was able to promote the proliferation and activation of microglial cells. In order to do that we implemented a combined technique that allowed us to mark and reconstruct together neurons, using Golgi Cox staining, and microglia, using immunofluorescence.

Finally, to understand the possibility of existence of a biphasic behavior of GC also on inflammation, we investigated the activity of GC agonist and antagonist on microglial cells, *in vitro*, in order to confirm, or not, the existence of a direct pro-inflammatory activity of GR on microglia. We used immunofluorescence technique to verify the changes in fluorescent signal of IBA1, marker of microglia, and CD68, marker of M1 pro-inflammatory microglia state.

METHODS

1. *Ex Vivo* Studies

1.1. 3xTg Mice

We used 3xTg-AD mice expressing three mutant human transgenes—PS1 (M146V), βAPP (Swedish) and tau (P301L)24 that were purchased from The Jackson Laboratory (Sacramento, CA). Although the 3xTg-AD mice were originally derived from a 129/C57BL6 background, genetic analysis showed that our 3xTg-AD mouse colony matched ~80% of the allelic profiles of C57BL/6 mice after ten generations of random mating. All experiments were performed in accordance with the EU guidelines (2010/63/UE) and Italian law (decree 26/14) and were approved by the local authority veterinary service and by our university ethical committee. All efforts were made to minimize animal suffering and to reduce the number of animals used. Animal use was approved by the Italian Ministry of Health, in agreement with the EU Recommendation 2007/526/CE.

1.2. Experimental design

6 and 10 months old 3xTg-AD male mice were treated with the GR agonist, dexamethasone (D4902 Sigma-Aldrich), or the GR antagonist, mifepristone (M8046- Sigma-Aldrich), or only vehicle through intraperitoneal injections (i.p., four animals per group). Injections were performed for 5 consecutive days at 11 o'clock to not interfere with glucocorticoid circadian rhythm (Chung et al., 2011). Dexamethasone and mifepristone stock solutions were prepared using DMSO, respectively at 20mg/ml and 5mg/ml. The day of the injection the stock solution was diluted in 10% Tween-20 plus distilled water to obtain a dexamethasone concentration of 8mg/Kg and a mifepristone concentration of 20mg/kg. Three days after the last injection, animals were anesthetized using Tribromoethanol (TBE) drug, and perfused transcardially with 0.1 M phosphate buffer solution (PBS) followed by formaldehyde 10% V/V, buffered 4% W/V (Titolchimica-Italy). Finally, brains were extracted and left in fixative overnight. At that point, we

proceeded with Golgi Cox or combined Golgi Cox and Immunofluorescence technique.

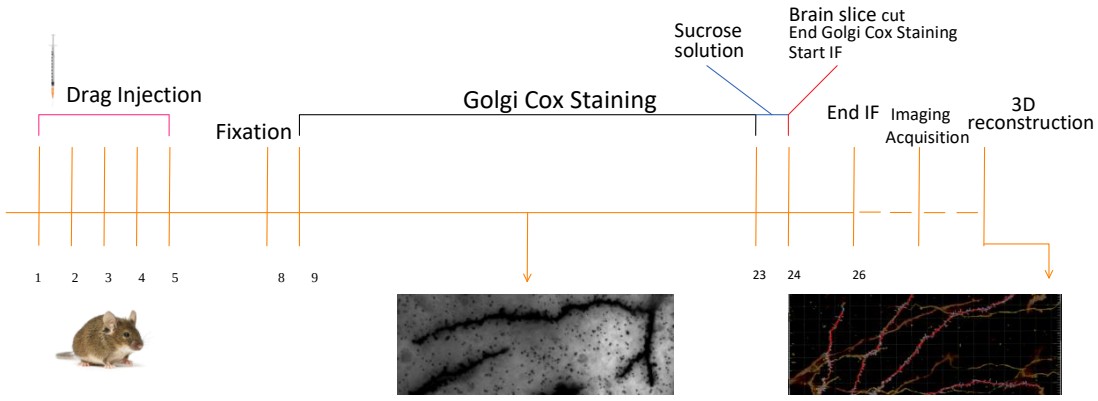

Figure 33. Schematic experimental procedures. The drug or vehicle are intraperitoneal injected starting at day 1 and until day 5. At day 8, animals are fixed using formaldehyde and brains extracted and left overnight in post fixation. At day 9, brains are left for 2 weeks in Golgi Cox solution; at day 23, brains are put in 30% sucrose solution for 24h and at day 24 they are cut in 100 or 60 µm thickness slices using vibratome. The 100 µm slices are put on slides for completing the Golgi Cox protocol; the 60 µm slices, are put in 24 well plates and treated for immunofluorescence using the combined Golgi Cox-IF protocol until day 26. Finally, images are collected using Neurolucida software, for Golgi staining neurons, and with Confocal microscopy for slices treated with combined techniques. The pics report neurons stained with Golgi Cox and the reconstruction of dendrites using Imaris Software (BitPlane).

1.3. Golgi Cox Staining

Brains were transferred in 200 ml of Golgi Cox solution at dark for 2 weeks.

The Golgi Cox solution contains 1% Mercury Chloride, 1% Potassium Dichromate and 1% Potassium Chromate in distilled water (Das, Reuhl, & Zhou, 2013b) (Zaqout & Kaindl, 2016).

[*The Golgi Cox is a technique conceived by Camillo in Golgi in 1873 as a "dark reaction" for the capability to mark neurons in black* (Golgi, 1873), *as visible in the original figure* .

The original protocol requires two different solutions: the first of potassium dichromate and the second of silver nitrate or gold chloride.

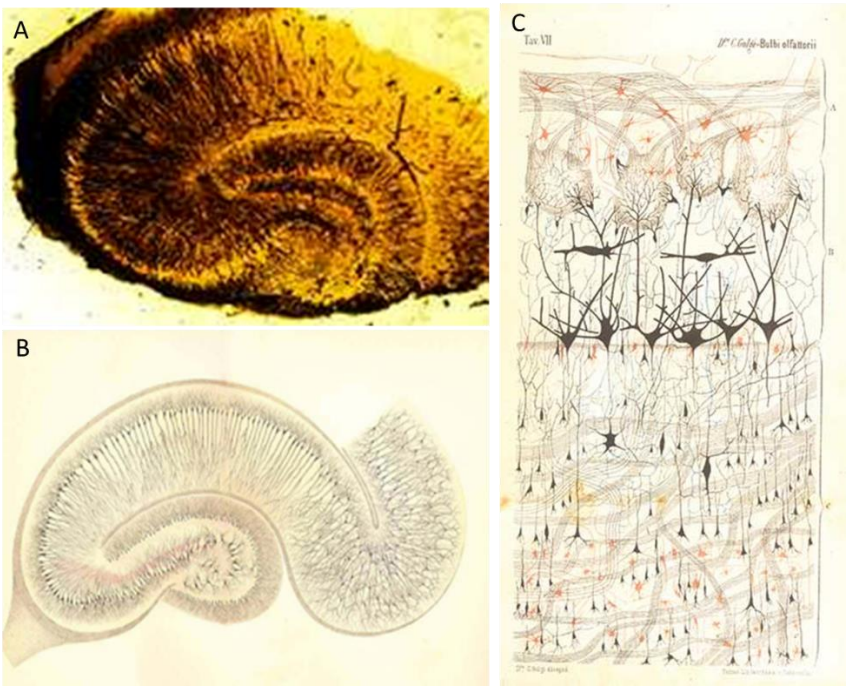

Figure 34. Original Golgi staining and graphical reconstruction performed by Camillo Golgi in the 19th century on hippocampus (A and B) and olfactory bulb (C). A) Hippocampal region slices of mouse brain after staining with dark reaction: body of neurons and neurites are marked in black; the brown background is produced by chromate deposition. B) manual drawing of Golgi stained hippocampus performed by Camillo Golgi: it is clearly visible the body of neurons and their filaments. C) manual drawing of Golgi stained olfactory bulb that shows different type of neurons and their connections. [From an original preparation from Golgi's laboratory, conserved in the former Institute of Pathology of the University of Pavia, now Golgi Museum]

Nowadays, the Golgi Cox technique, an evolution of the original one, is one of the most used protocol to stain neurons, dendrites and spines. This protocol is more rapid and requires only one solution for staining that is composed of potassium chromate, potassium dichromate and mercury chloride in distilled water. Generally, the concentration of these solution varies between 1% and 5% (Bayram-Weston, Olsen, Harrison, Dunnett, & Brooks, 2016; Das, Reuhl, & Zhou, 2013a; Rosoklija et al., 2003; Zaqout & Kaindl, 2016). *In our experiments 1% is enough to obtain a clear staining of neurons and their dendrites and spines. The power of Golgi staining is its capability to mark, randomly, a limited number of neurons in*

almost every brain regions, underlining clearly all neuron structures (Bentivoglio, 1998).]

Golgi Cox protocol.

For 1 liter of stock solution, three separate solutions called A, B and C were prepared and mixed. Solution A contains 1mg of Mercury Chloride (Thermo Fisher Scientific – 10219800) in 200ml of H2Omilliq, solution B contains 1mg of Potassium Dichromate (Thermo Fisher Scientific – 10791062) in 200ml of H2Omilliq and solution C contains 1mg of Potassium Chromate (Thermo Fisher Scientific – 10388430) in 600ml of H2Omilliq. Firstly, solution A was heated up to 80 degrees under hood to complete the dissolution and then it was allowed to cool down. Then solution B was poured into solution A and mixed. Finally, the solution C was poured into solution AB and mixed. At that point, stock solution was filtered and then conserved for 48 hours in dark at room temperature (RT) before use.

After the staining, brains were left in 30% Sucrose solution (in PBS) for 24h at 4° degree to reduce the tissue fragility for the next cut (Gibb & Kolb, 1998). Then, 100 µm thick slices were collected using vibratome (Leica VT1200, Leica Biosystems, Germany) and put on slides. At that point slices were passed in Kodak Developer (GBX Carestream Dental) for 5 minutes and then washed in distilled water for 5 minutes. Then, they were treated using Kodak Fixer (GBX Carestream Dental) for 15 minutes and again washed in distilled water for 5 minutes. Finally, slices were dehydrated using increasing concentrations of Ethanol (50%-60%-75%-85%). To avoid slices fragmentation, the dehydration did not reach 100%. At the end slices were mounted on slides using Eukitt (Sigma Eldrich, USA).

[***Guided Procedures****:*

1. Preparation of the Golgi Solution in Water milliq with:

or 1% HgCl2

or 1% K2Cr2O7

or 1% K2CrO4

To prepare a liter of solution, perform the following steps:

1. Add 10g HgCl2 in 200ml H2Oqq (A)

2. Add 10 gr K2Cr2O7 in 200 ml H2Oqq (B)

3. Heat A up to 80 ° C then cool down

4. Slowly pour B into A

5. Add 10 gr K2CrO4 in 600 ml H2Oqq (C)

6. Pour C into A-B

7. Filter to avoid deposits

2. Leave the solution 24 hours in the dark.

3. Immerse the whole brain for 24 hours in a solution volume between 50 and 100 times the volume of the preparation.

4. After 24 hours, change the solution and keep coloring for two weeks (recommended but we demonstrated this is not an indispensable step).

5. After two weeks, remove the brain and immerse it in a 30% sucrose solution (in milliq water) for 24 hours.

6. After 48 hours dissect the vibratome brain into 6% sucrose (milliq water). Make slices of 100 -150 um and place them on slides (use gelatinized slides 4 times).

7. Once mounted, immerse the slides in the Developer (GBX Carestream Dental) for 5 minutes (attention to toxicity)

8. Immerse in water milliq for 5 minutes

9. Immerse in the Fixer (GBX Carestream Dental) for 15 minutes

10. Immerse in water milliq for 5 minutes

11. Dehydrate with alcohol 50%, 75%, 90% for two minutes each (check the state of the slices, it is advisable not to exceed 90% because the slices are likely to break, especially if thin).

12. Add Eukitt mast (Sigma 03989-500ML) and cover with a slide.

13. Wait for it to dry and observe under a microscope.

Note:

• The solution, once prepared, can be used for about 1 month without any problems. It must however be protected from light and well closed in a ventilated cabinet or under a hood at room temperature.

• The change of the solution after 24 hours, as indicated in all the protocols, does not seem to be necessary, if sufficient quantities of solution are used.

• For a single mouse brain 200 ml of solution are efficient for staining the whole brains, except for some brain area like olfactory bulb in which impregnation seem to be absent.

• We tried to keep the brain in 30% sucrose for different times. The most suitable, for cutting, seems to be 24 hours.

• After cutting it may be difficult to adhere the slices to the slide. Two precautions are important: keep the slices wet with the solution, even when they are on the slide (put a drop of 6% sucrose, with a tip, directly on the slice) and crush them, so that they adhere better, using moist Whatman paper in order to avoid the drying of the slices.

• To improve the quality of the images that can be collected, it should be used developer and a fixer. Frequently, KODAK developer and fixer are used, but in Italy it is difficult to find. However, it is possible to obtain good results through the ones used in photography for dental radiographs too.

• Dehydration should be done in steps to understand if the slices remain intact in the various treatments. At the beginning we stopped at 75% because, beyond this

level, the slices were broken; after using the sucrose solution for cutting, we observed that it was possible to continue up to 90%. Although most of the protocols expect to reach 100%, we do not manage to reach this level and at the same time keep the slices intact.

• The Permount should be used as a mullion, but we have also had good results with EUKITT. Containing it Xylene, it is recommended to proceed up to 90% dehydration.]

1.4. Brightfield Microscopy and Dendrite Analysis

Images were collected using Olympus BX63 microscope (Olympus Corporation, Japan) and acquired by Neurolucida 64-Bit software (MBF Bioscience, USA). CA1 region of hippocampus was primarily identified at 10x then, acquisition of dendritic spines occurred at 100x. We collected images of 117 x 88 µm and analyzed three slices per mice between -1.955 mm to - 2.355 mm Bregma. Every stack was acquired using a Z stack unit of 35 µm.

We collected dendrites of layer 5 and 6 both of pyramidal neurons and interneurons of CA1 regions.

The anatomical structure of Hippocampus with CA layer is illustrated in Figure 35.

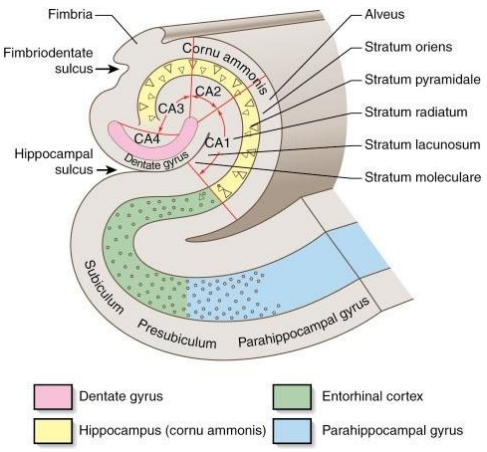

Figure 35 Schematic representation of hippocampal anatomical structure. Hippocampus is commonly described as the union of two structure: the "Cornu Ammonis" that contain the CA regions and the "Dentate Gyrus" that contain the granular cells and the one of the few adult neurogenetic areas of brain. Cornu Ammonis is divided in three or four CA regions: CA1, CA2, CA3, CA4 - non-always presents in literature. Each of them is constituted by 6 layers named "strata". In particular CA1 region presents: a Stratum oriens, that contains inhibitor basket cells and the basal dendrites of pyramidal cells that receive input from contralateral projection; a Stratum pyramidale that contains the cell body of pyramidal neurons and interneurons; finally the Stratum radiatum, lacunosum and moleculare that contains distal dendrites of pyramidal neurons, that makes synapses with Schaffer collateral fibers from CA3 and with the fibers of performant path form entorhinal cortex. We focused our attention on these last layers because they degenerate rapidly in AD and after chronic exposure to glucocorticoids.

After acquisition, images were deconvolved through AutoQuant software, converted in 8bit images through ImageJ software and, then, black signal was inverted to allow the analyses with Imaris - Bitplane Software.

Dendrites and spines of neurons in the CA1 region were reconstructed using Autopath system of Imaris. This system allows us to manually trace the dendrite in which we were interested, and then automatically rebuilt the dendrite. Finally, Imaris automatically found spines and reconstructed them. Every single spine detected by the Software was manually checked to avoid false positive signals. At that point, Imaris gave us information about the dendritic length and the number of spines. To reduce the bias related to different dendrite lengths, we calculated the medium spine density for each animal dividing the total numbers of spines with the total length of every dendrite. The medium total length built for mouse was about 500µm, for a total of about 6000µm.

1.5. Electron Microscopy

For the ultrastructural analysis, 4 Vehicle 3xTg and 4 Mifepristone 3xTg 6-7 months-old mice were perfused with 2% formalin and 2.5% glutaraldehyde in phosphate buffer pH 7.4. The brain was then excised, fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer pH 7.4 for 3 h at 4°C, washed in PBS

and sectioned in slices of 40 µm. The slices were then post-fixed with 1% OsO4 for 2 h at 4° and dehydrated with acetone. CA1 region was cut out and embedded in Epon resin. Ultrathin sections were observed with a Philips Morgagni transmission electron microscope (FEI Company Italia Srl), operating at 80 kV and equipped with a Megaview III camera for digital image acquisition and analysis. For morphometrical evaluation, ten images of longitudinally sectioned dendrites were taken at a fixed magnification (14000x) per animal.

The index of dendritic membrane irregularity (expressed as the ratio between the real length of the membrane profile and the corresponding linear length) and the density of spines with or without pre-synaptic terminal (the ratio between spine number and linear length) were assessed by using ImageJ software. The synaptic contact was identified by the presence of both the pre-synaptic element containing the typical vesicles and the post-synaptic electrondensity.

The results were pooled according to the experimental groups and the means ± standard deviation (SD) values were calculated.

1.6. Behavioral Test

3xTg mice (n=8 per group), together with C57BL/6 mice (N=8) for wild-type controls, were tested with Open field arena and Y-Maze test. The 3xTg were treated like exposed in paragraph 55, and tested after 2 days from the last treatment. The effects of vehicle, dexamethasone and mifepristone on locomotor activity and spatial learning and memory capabilities were investigated using the open field arena (OF, Open Field Cages, Ugo Basile, Varese, Italy) and the Y-Maze test (Y-Maze System for mice, Ugo Basile, Varese, Italy), respectively. Each test was performed at 6 and 10 months of age. Prior to treatment, 3xTG-AD mice were randomly assigned to the 3 groups: vehicle, dexamethasone, and mifepristone. For the OF test, mice were placed in center of the arena (44x 44x 30 cm) and video-monitored for 20 min. Video were analyzed (AnyMaze) for distance moved, immobility, entries and time spent in the center part of the arena.

For the Y-Maze test, each mouse was placed in the center of a symmetrical Y-maze with the three arms arranged at 120° to each other. Mice were allowed to freely explore the maze for 8 minutes and the total number of transitions and the sequence

of arms entered were recorded. Alternation was defined as successive entries into the three arms in overlapping triple sets. To reduce odor cues, the maze was cleaned with 10% ethanol solution after each session. Experimenters were blinded with respect to the mice treatment.

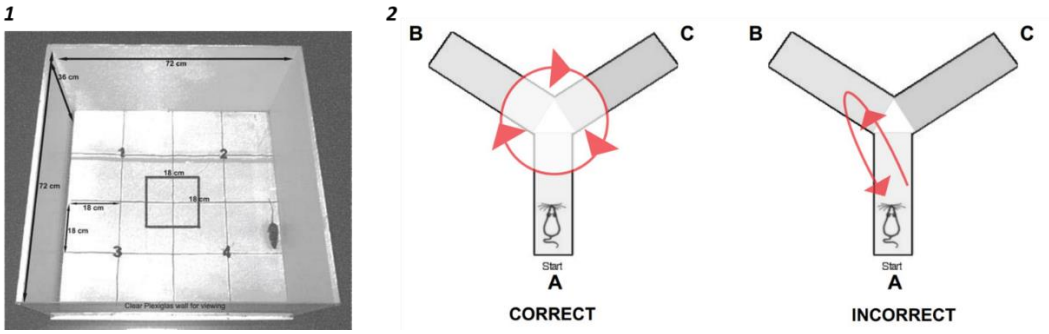

Figure 36 . 1) Open field task is used to measure the locomotor activity and the level of anxiety of animals. Generally, it takes place in a cube box of 1 meter long, 1 meter wide and 1 meter high (Carter, Shieh, Farra, & Harris, 2010). 2) Y-maze is a behavioral test used to verify the functionality of spatial and work memory. The labyrinthine structure is extremely simple: there are only 3 arms arranged in Y and called A, B, C. The mice are left free to explore the maze for a few minutes and the total number of movements between one arm and the other and the sequence are recorded. The measure that is analyzed is the alternation that is defined by the number of successive entries in three different arms, the "correct performance in comparison to all the entrances". The greater this measure, the greater the cognitive performance.

1.7. Combined Golgi Cox and Immunofluorescence Technique

The original protocols for this combined technique was initially published by Spiga in 2011. Spiga used his protocols to mark neurons using both Golgi staining and Immunofluorescence for Tyrosine Hydroxylase (TH) to detect presumably DAergic neuronal elements (Spiga et al., 2011). An example of how the protocols works is reported in Figure 37.

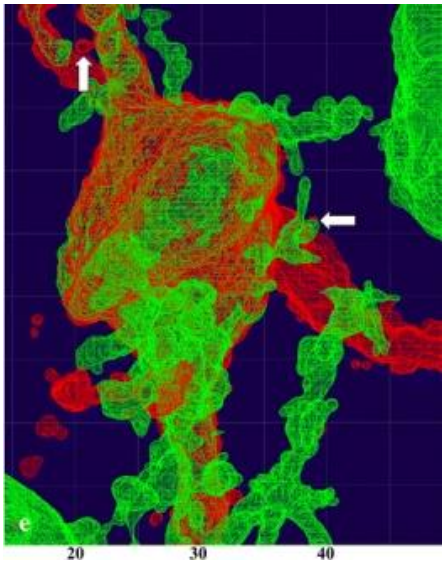

Figure 37. 3D reconstructions of neurons marked with combined Golgi Cox and Immunofluorescence published by Spiga in 2011 (Spiga et al., 2011). *In the figure, the neuron marked with Golgi Cox is shown in red, whose brightfield signal was rebuilt using reflectance confocal microscopy* (Batta, Kessler, White, Zhu, & Fox, 2015)*; instead, in green, it is shown the TH positive neurons. The white arrows indicate some dendritic spines-like structures visible only by impregnation.*

We modified the protocol to stain together different cells in particular neurons and microglia but we were able to mark astrocytes too. In detail the protocol we follow is described below.

6 and 10 months old 3xTg-AD male mice were treated with Dexamethasone or only the vehicle by using the same method expose in paragraph 55 (four animals per group). After the perfusion and the overnight post-fixation, each mouse brain was transferred in Golgi Cox solution for 2 weeks. Then, brains were cut, and slices were immersed in PBS into a 24 well plate and treated with Kodak Developer and Fixer like previously described. At that point we stopped the Golgi protocol and proceeded with the immunofluorescence staining (modified by Spiga et al., 2011). We treated slices for 30 minutes with the blocking solution, composed of 3% Bovine Serum and 0.3% triton in PBS. After we stained slices with primary Rabbit IBA1 antibody (WAKO - 1:500), diluted in the blocking solution, to mark microglia

activation and proliferation. The staining lasts for 36 hours at 4°C, at dark, then we washed slices using PBS solution 3 times for 10 minutes each. Subsequently, we proceeded with secondary antibody staining: we used Donkey Anti-Rabbit Alexa Fluor 488 (Invitrogen – Thermofisher) diluted in the blocking solution, for 2 hours at dark at room temperature. After 3 washes with PBS we stained slices with DAPI (1:2000 in PBS) for 5 minutes then we performed the last wash in PBS. Finally, slices were mounted on Xtra slides using Para Phenylenediamine (PPD).

1.8. Confocal Microscopy and Image Analysis

Images were acquired using Leika-Sp5 Confocal Microscopy. 20x magnification was used to detect microglial cells for the density analyses; glycerol 63X objective was used to acquire images for morphological analyses of microglia. Brightfield signal of Golgi-stained neurons was reconstructed through confocal reflection channel. We collected stack of 50 μm for a total volume of 0.0144 mm^3. Dendrites and spines of CA1 neurons were reconstructed by using the "Filaments" Autopath system of Imaris like previously described. Microglial cells were 3D rebuilt through the "Cell" function system of Imaris, taking advantage of the threshold that allowed us to reconstruct the whole visible cells. For each mouse, the microglia density was calculated dividing the total number of cells, counted using Imagej, with the volume of slice. Area and volume were calculated directly by "cell analysis" function of Imaris.

2. ***In Vitro* experiments**

2.1. Microglial Cell Culture

The *in vitro* part was performed thanks to the collaboration with Dr Silvia Coco and PhD student Morris Losurdo of University of Milano-Bicocca.

Primary cultures of microglial cells were isolated from mixed cultures of cortical and hippocampal astrocytes, established from brains of postnatal 1-2 days old C57BL/6 mice.

Briefly, after the removal of meninges, cortices and hippocampi were isolated and subjected to mechanical digestion. The obtained cell suspension was filtered with

70µm nytex membrane, centrifuged 10 min at 800x g at RT and finally resuspended in complete glial medium [Eagle's minimal essential medium (MEM, Gibco®)], 20% fetal bovine serum (Sigma-Aldrich), 33 mM Glucose (Sigma-aldrich), 1% Na-Pyruvate (Lonza), 2mM L-ultra glutamine, 100µg/ml streptomycin and 100U/ml penicillin (all from Euroclone)].

After about 14 days microglial cells were harvested by shaking the flasks at 230rpm for 45min at RT and seeded at a concentration of about 200.000 cells on 24mm plate well, previously pre-coated with 0.05 mg/ml poly-Ornithine (Sigma Aldrich®). To minimize activation, microglia cells were grown in a medium consisting from 5/6 glial medium without serum and 1/6 astrocyte-conditioned 0,22 um filtered medium.

Microglial cells were plated for 24h before being exposed to GR agonist and antagonist drugs [dexamethasone (1µM), diluted in DMSO and PBS, and mifepristone (1µM), diluted in DMSO] for 4 hours. Afterwards, medium was removed, and cells were fixed using formaldehyde 10% V/V, buffered 4% W/V (Titolchimica-Italy) or lysed for the analysis of specific markers by Western Blot (WB).

Cultures treated only with vehicle were used as control.

2.2. Immunofluorescence analyses

After fixation, microglial cells were treated for immunofluorescence for staining nucleus, IBA1 and CD68, marker of M1 pro-inflammatory microglial state. In detailed, the protocol was similar to that exposed in paragraph 1.7, but with different timing.

We treated slices for 15 minutes with the blocking solution, composed of 3% Bovine Serum and 0.3% triton in PBS. Afterwards, we stained slices with primary Rabbit IBA1 antibody (WAKO - 1:500) and Rat CD68 antibody (Biorad – 1:200), diluted in the blocking solution, to mark microglia state of activation. The staining lasts overnight at 4°C, at dark, then we washed slices using PBS solution 3 times for 10 minutes each. Subsequently, we proceeded with secondary antibody staining: we used Donkey Anti-Rabbit Alexa Fluor 488 (Invitrogen – Thermofisher) – for bind IBA1 primary antibody – and Donkey Anti-Rat Alexa Fluor 594 – for staining

CD68 primary antibody – both diluted in the blocking solution, for 1 hours at dark at room temperature. After 3 washes with PBS we stained slices with DAPI (1:2000 in PBS) for 5 minutes then we performed the last wash in PBS. Finally, slices were mounted on Xtra slides using Para Phenylenediamine (PPD) and acquired using confocal microscopy as described in paragraph 1.7.

2.3. Western Blot Analyses

Microglia cells were lysed for the analysis of specific markers by WB. Cell phenotype was investigated by the following antibodies: rat monoclonal anti-CD68 (1:400, Biorad, Hercules, CA, USA), rat monoclonal anti- CD206 (1:400, Biorad, Hercules, CA, USA). Total protein amount was evaluated by means of bicinchoninic acid assay. 15 µg of each sample were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare). All the data were normalized to β-actin (1:1000, Sigma). The immunoblotting and the analyses, like the harvesting of primary microglia culture, were both performed by PhD Student Morris Losurdo of the School of Medicine and Surgery of University of Milano-Bicocca.

CD68 and CD206 microglia markers were investigated in non-reducing conditions, as specified by manufacturer's instructions. At this aim, protein samples were prepared in loading buffer, without 2-mercaptoethanol.

Blocking and antibody probing occurred in TBS-T 0.1% buffer containing 5% non-fat milk. Secondary anti-rat antibodies (1:5000, *Genetex, Irvine, CA, USA*) were used for detection of rat anti-mouse CD68 and CD206 antibodies.

3. Statistical Analyses

For statistical analyses, one-way Anova were used in presence of more than 2 condition, followed by Tukey's post-hoc test. In the other cases t test was performed. Normalization test was not performed. In every experiment, an alpha of 0.05 is used as the cutoff for significance.

RESULTS

1. GR modulation significantly altered dendritic spine density of neurons in the CA1 region of hippocampus

Previous studies have shown that the exposure to a chronic glucocorticoid treatment alters dendritic arbors and spine density in several cortical regions in fixed brain tissue, through activation of GR (Liston & Gan, 2011) (Brady et al., 2012). These degenerations are typically present in Alzheimer's Disease too. To verify whether the activity of glucocorticoid receptors interferes positively and/or negatively with spine plasticity in an animal model of AD, we administered 8mg/kg of dexamethasone (i.p.) or 20mg/kg of mifepristone or only vehicle to 3xTg mice, as previously described. As presented in Table 3, we were able to reconstruct about 1500µm of dendrites for group at 6 months of ages and about 2500µm for 10 months of ages animal; we counted a total of 7586 spines for the first age and 13068 spines for the second one.

Table 3. Summary of the data analyzed. The dendritic spine density is calculated for every mouse, dividing the total numbers of spines with the total length of every dendrite. The mean spine density, shown in the table, is the average of densities of every animal in each group (vehicle or dexamethasone and mifepristone).

TABLE 6 MONTH	Mice n°	Dendrites n°	Spines n°	Length Dendrites µm	Mean Density/ 10µm	SD
Vehicle	5	46	2748	1596.19	**17.37**	1.46
Dexamethasone	4	75	1982	1490.14	**13.34**	0.70
Mifepristone	4	43	2856	1426.62	**20.06**	0.40

TABLE 10 MONTH	Mice n°	Dendrites n°	Spines n°	Length Dendrites µm	Mean Density/ 10µm	SD
Vehicle	4	106	4920	3030.68	**16.23**	0.12
Dexamethasone	4	84	3707	2616.71	**14.16**	0.16
Mifepristone	4	80	4441	2441.39	**18.20**	0.67

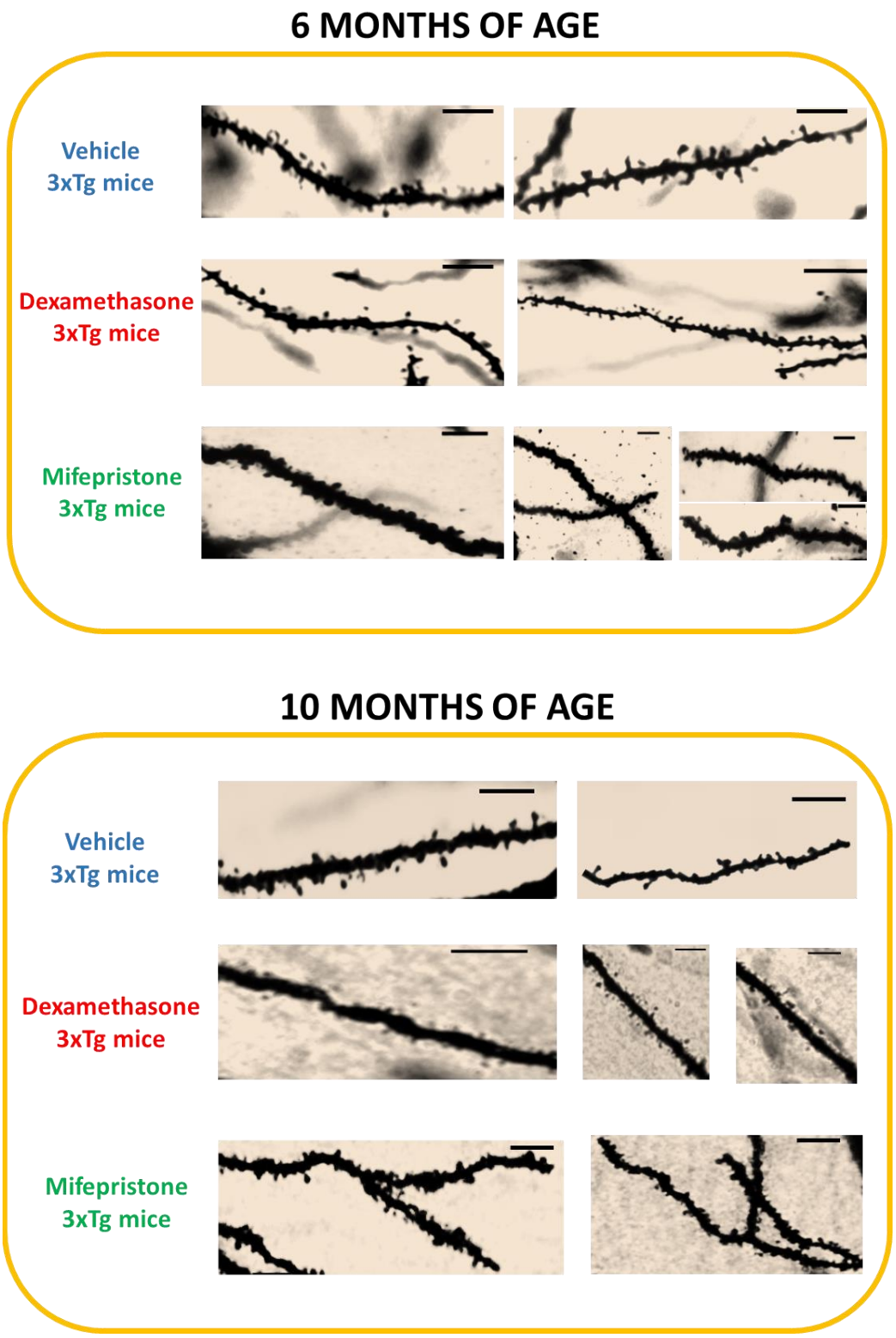

Figure 38. Qualitative comparison of hippocampal dendrites between control and dexamethasone or mifepristone treated 3xTg mice at 6 and at 10 months of age after Golgi Staining. Difference in numbers of dendritic spines between untreated and treated mice is clearly visible. ***Scale bars = 5µm***

We performed experiments both at 6 and 10 months of ages because they could be considered, respectively, the beginning and the medium late phase of AD in our 3xTg-AD male mice.

After collecting images using Neurolucida software, we reconstructed the Golgi signal of dendrites using Imaris Software, as described in the Chapter of ***Method***.

The Imaris analyses revealed the significant effects of stress and the potential role of anti-stress therapy in AD. The data are reproduced in the histograms below (Figure 39).

In details, our results showed that agonist of GR, dexamethasone, was able to strongly reduce the dendritic spine density: in particular, 5 days of treatment was enough to decrease the density in CA1 region of the hippocampus of about 23% at 6 months of age (dendritic spines/10 μm = 17.37 ± 1.79 and 13.34 ± 0.90 for Vehcile and Dexamethasone, respectively; $F(2,10)=28.62$, $p<0.01$) and of about 12.7% at 10 months of age (dendritic spines/10 μm = 16.23 ± 0.14 and 14.16 ± 0.18 for Vehcile and Dexamethasone, respectively; $F(2,9)=74.91$, $p<0.001$;).

Dendritic spine loss is a common alteration in AD (S W Scheff et al., 2007), however, it has never been described before in 3xTg mice younger than 10 months of age (Bittner et al., 2010). In addition, in dexamethasone treated mice we qualitatively observed the presence of several dendrites in atrophy, a condition typical of AD, but not previously seen in this model (Oddo et al., 2003) (Wirths & Bayer, 2010).

Indeed, the only early alteration related to neuronal plasticity, in 3xTg mice is the LTP dysfunction in hippocampus (Bertoni-Freddari et al., 2008).

On the contrary, administration of GR antagonist produced the opposite effects. Mifepristone induced an increase of dendritic spine density of about 15.5% (Vehicle: 17.37 ± 1.6; Mifepristone: 20.06 ± 0.516; $p<0.05$) and 12.1% (Vehicle: 17.37 ± 1.6; Mifepristone: 18.20 ± 0.78; $p<0.001$), at 6 and 10 months of age, respectively. These data were in contrast with those previously published related to spine turnover in mifepristone-treated rodents: blockade of GR showed the same negative effects seen with dexamethasone (Liston & Gan, 2011). However, our

results could explain the behavioral improvements found in other AD mice model treated with mifepristone (Lanté et al., 2015): the positive increment of hippocampal dendritic spines, that we found, could be linked to the amelioration of performance during "What–When–Where" object recognition protocol.

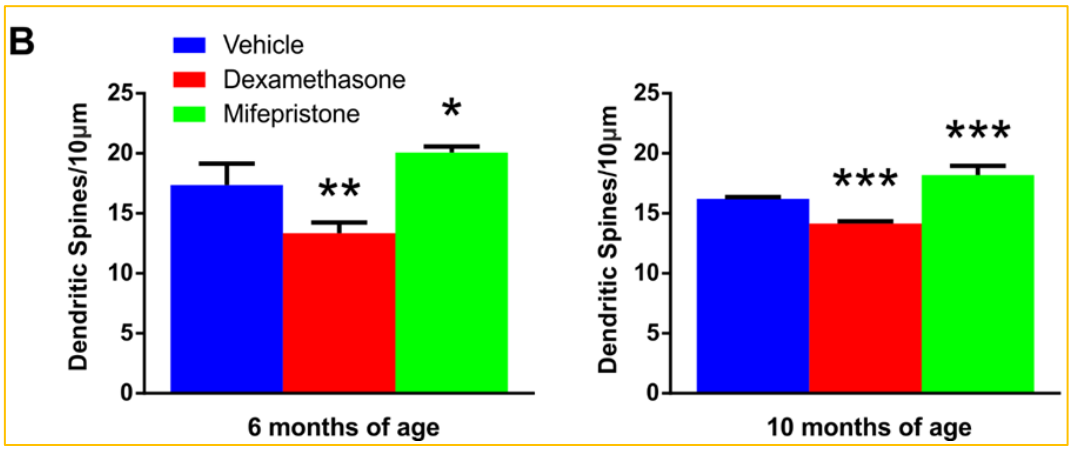

*Figure 39. Quantitative comparison of hippocampal dendrites between vehicle and dexamethasone or mifepristone treated 3xTg mice, at 6 months (F(2,12)=28,58; P=0.0000732) and at 10 months of age (F(2,11)=74,86; P= 0.00000246), after Golgi Staining. Dexamethasone reduced the CA1 dendritic spine density both at 6 and 10 months of age. On the contrary mifepristone produced the opposite result. The effects of GR agonist are prominent at 6 rather than 10 months of age (*p<0.05; **p<0.01; ***p<0.001).*

The effects of GR hyper activation were stronger at the beginning of the pathology (6 months of age) rather than in the middle late phase (10 months of age), but the tendency was the same at both ages. This data is coherent with Swanwick hypothesis (Swanwick et al., 1998) that stress would be more influent in the initial phase of the pathology rather than in the late stage (see ***Discussion*** *chapter*).

As described in the introduction of my thesis, we confirmed that there is no significant difference related to dendritic spine density in 3xTg of control between 6 and 10 months of age. This data enforces the hypothesis that dexamethasone strongly accelerates the progression of AD promoting a premature degeneration of spines in hippocampus.

1.2. Electron microscopy analysis confirmed the Golgi Cox results.

We know that Golgi Cox signal is rather noisy, and overestimation of spines can be a common consequence. In order to avoid this possibility, we performed experiments for electron microscopy. Consequently, we treated again 3xTg mice using only vehicle or mifepristone following the protocol described in paragraph 1.5.

The density of dendritic spines without synaptic contact significantly increased in mifepristone 3xTg in comparison to vehicle 3xTg mice (Pvalue < 0.05). Conversely, the density of dendritic spines with synaptic contact was similar in the two experimental groups (Figure 40). This observation is of extreme importance reviling that there was probably no overestimation of number of spines in Golgi Cox data analyses.

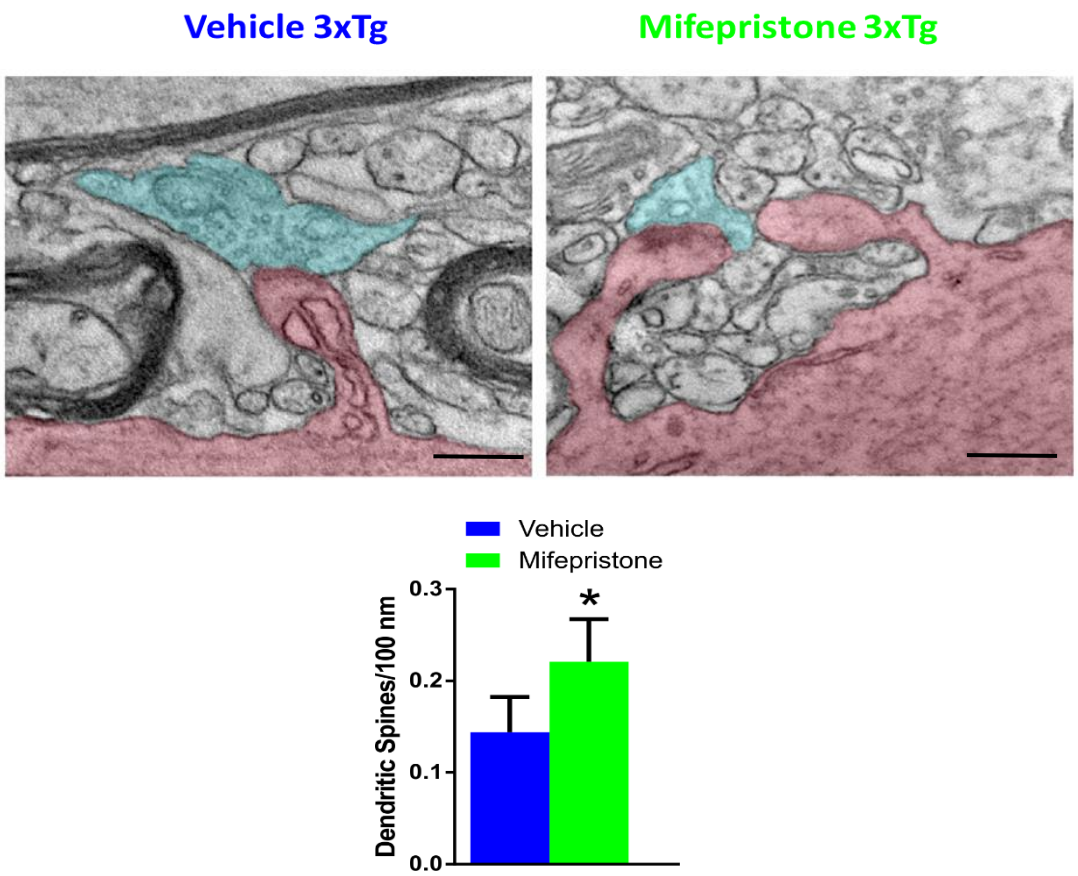

Figure 40. Spine density. High magnification details of dendritic spines from Vehicle 3xTg (d) and Mifepristone 3xTg (e) mice. The pre-synaptic terminal was digitally colored in azure and the post-synaptic element (dendritic spines) in pink. Note in e) the occurrence of

a dendritic spine with a synaptic contact and a dendritic spine without pre-synaptic terminal (arrowhead). f) Histogram showing the means ± (SD) of the spine densities; the asterisk indicates statistical significance. ***Scale bar = 200nm.*** *(*p<0.05; **p<0.01; ***p<0.001).*

The ultrastructural observation highlighted in mifepristone 3xTg an evident increase in the irregularity of the dendritic membrane (Figure 41), as confirmed by the statistical analysis. The irregularity index was, in fact, significantly higher in the mifepristone treated samples. We speculated that this irregularity could be linked, at least in part, to the process of spine formation.

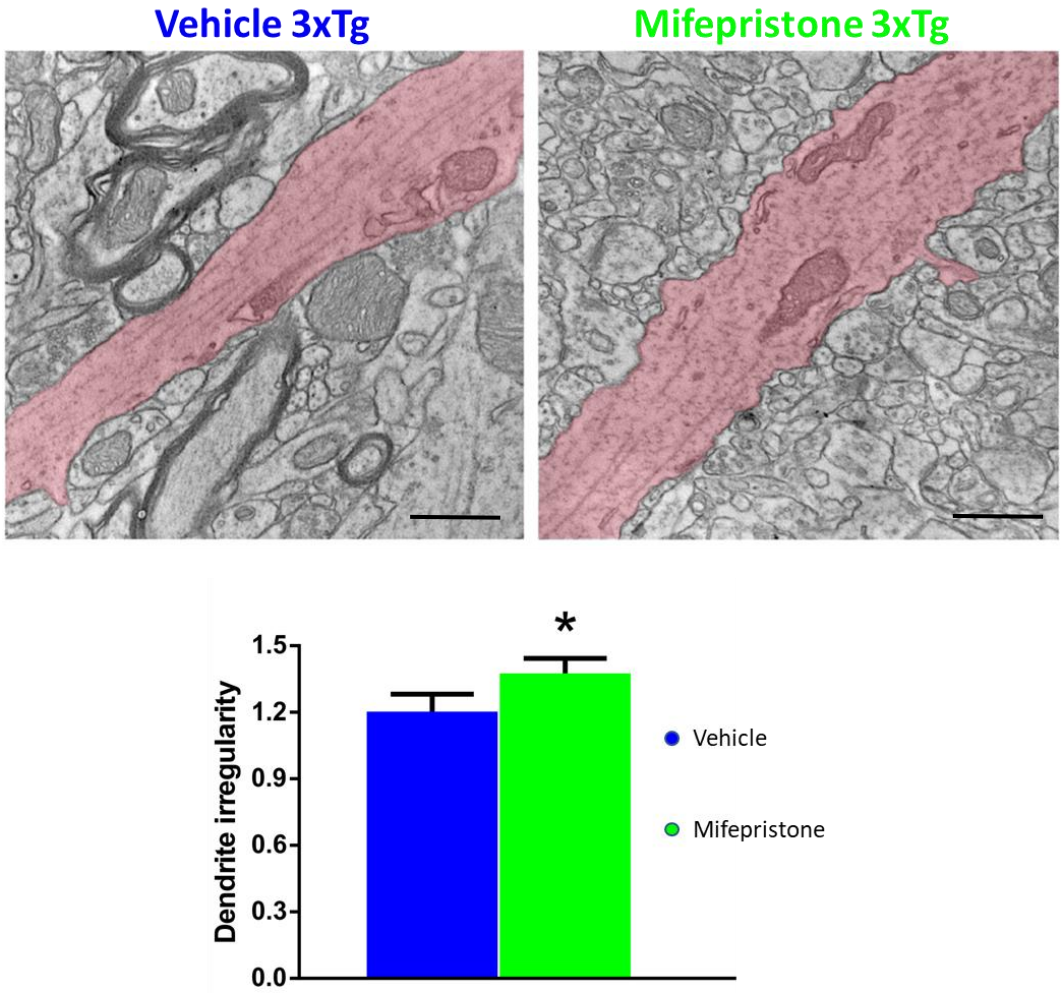

Figure 41. Dendrite irregularity. Transmission electron micrographs of dendrites from Vehicle 3xTg (a) and Mifepristone 3xTg (b) mice. The dendrites were digitally coloured in pink in order to highlight the membrane profile. c) Histogram showing the means ± (SE)

of the index of dendritic membrane irregularity; the asterisk indicates statistical significance. ***Scale bar = 500nm.*** *(*p<0.05; **p<0.01; ***p<0.001).*

Furthermore, we were able to find few perforated synapses (*Figure 42*) in treated mice but no one in control mice. Consequently we suggested that mifepristone can revert, at least in part, the deficit of perforated synapses whose formation represents the only early impairment of 3xTg-AD mice related to spines (Bertoni-Freddari et al., 2008).

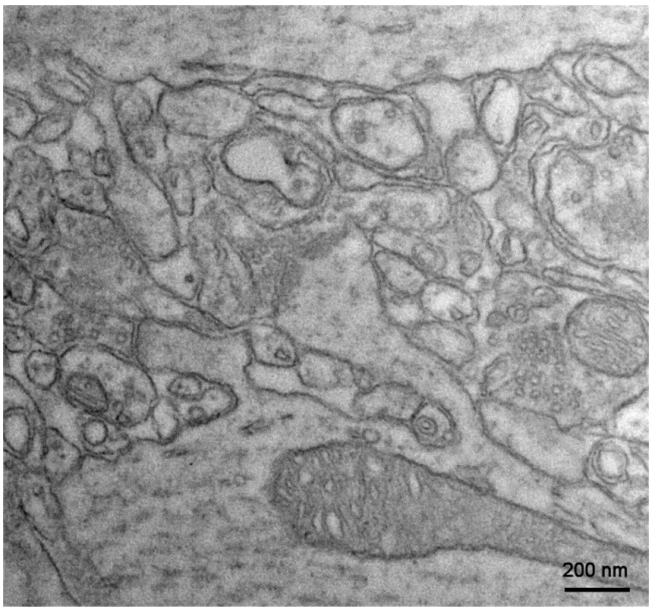

Figure 42. Perforated Synapse. The presence of perforated synapses in 3xTg mice is uncommon. Indeed, it represents the only early deficit related to dendritic spines in 3xTg-AD mice. In our experiments we were able to find few perforated synapses in mifepristone treated mice but not in control ones.

2. GR modulation alters Open Field Arena and Y-Maze Test performance in 3xTg Mice

Since the alterations in spine is often associate with changes in cognitive performance, we decided to test 3xTg mice through two behavioral tasks: Open

Field and Y-Maze. The mice were divided in 4 groups: one wild type groups, and three 3xTg groups treated with vehicle, dexamethasone or mifepristone.

In Open Field, as expected, distance travelled decreased over time in all groups (main effect of time: $F(1,28)=22.46$, $p<0.001$). In addition, the omnibus ANOVA revealed a significant interaction between time and treatment (time x treatment: $F(3,28)=4.89$, $p=0.011$), however more detailed analyses showed that distance travelled was significantly reduced in 3xTG mice compared to WT mice although 3xTG mice motor functions were not affected by any of the drug treatments. Similar pattern were observed at both time points (main effect of genotype, 6 months: $F(1,31)=94.22$, $p<0.001$; 10 months: $F(1,31)=56.44$, $p<0.001$).

Although spatial learning memory performance, tested by Y-Maze task, was also affected by time, (main effect of time $F(1,2)=11.24$, $p=0.002$), the omnibus ANOVA also revealed a main effect of treatment ($F(3,27)=16.85$, $p<0.001$), in fact when animals were tested at 6 months of age, all 3xTG mice performed significantly worse than WT mice, instead, when tested again at 10 months of age, the performance of 3xTG mice treated with mifepristone resulted significantly higher compared to vehicle treated mice and comparable to the level of performance exhibited at 6 months of age.

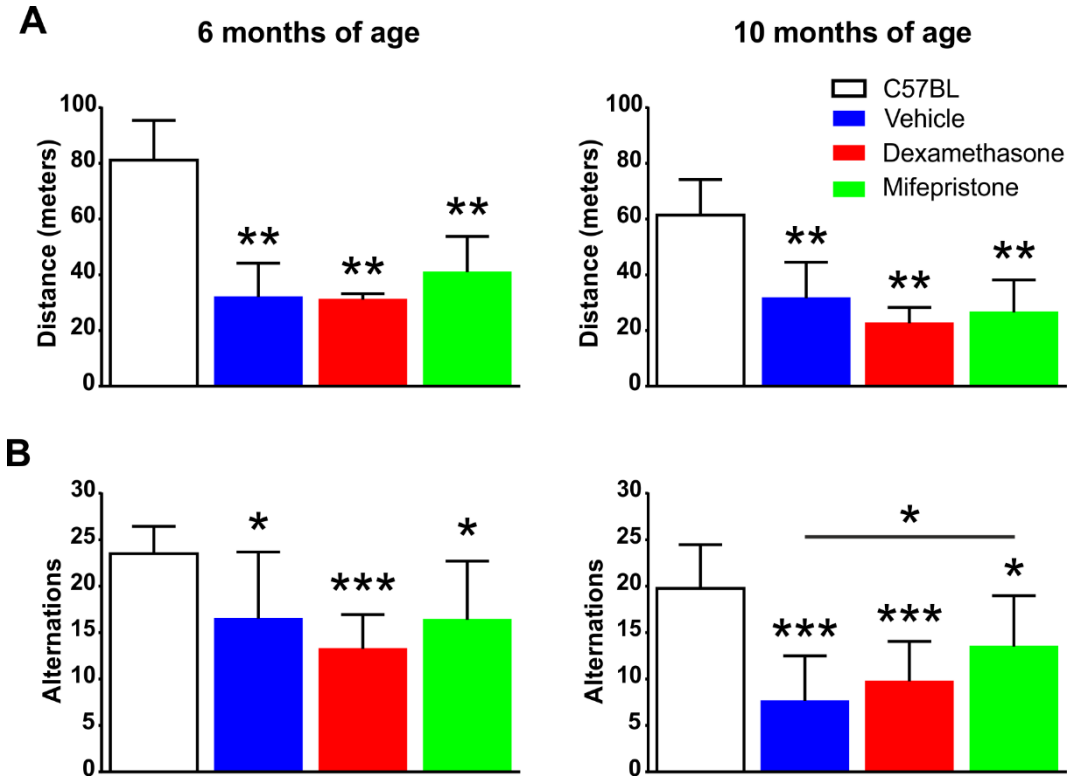

*Figure 43. Open Field and Y Maze performance. Four groups of mice were tested both at 6 and 10 months of age: wild type mice, presented in white, vehicle-treated 3xTg mice, in blue, dexamethasone-treated 3xTg mice, in red, and mifepristone-treated 3xTg mice, in green. For Open Field it is reported the average distance, in meters, covered by every mice groups. For Y-maze task, it is reported the number of alternations reached by every groups. (*p<0.05; **p<0.01; ***p<0.001).*

3. Dexamethasone induced proliferation and activation of microglia in CA1 region of hippocampus

Inflammation plays an important role in AD and, in the past, there were attempts to reduce inflammation in AD patients hopefully to delay the progression of pathology. Use of glucocorticoids was one of the treatments investigated as mentioned in the chapter of ***Microglia***. In particular, agonists of GR, like dexamethasone, are used in therapy against inflammatory pathologies like rheumatoid arthritis and bronchospasm (The American Society of Health-System Pharmacists, 2018). However, like for spine turnover, long-lasting high levels of GC are able to activate the principal immune cells of brain: the microglia (Nair & Bonneau, 2006). We suggested that the reason could be linked to biphasic behavior

of glucocorticoids in the brain (Lupien et al., 2005) as disserted in ***Discussion chapter***: pro-inflammatory state in the brain tissue of wild type mice can be induced both in the presence of high and chronic concentrations of GC and in absence of GC, like after an adrenalectomy surgery (Biondi & Zannino, 1997). As a consequence, according to the hormetic effect, medium concentration of GC could produce the opposite effect (Liston & Gan, 2011; Swanson et al., 2013). Taking in account these observations, we hypothesized that stress induced by dexamethasone treatment could produce an activation of microglial cells, that could contribute to dendritic spine degeneration, in 3xTg mice.

Therefore, we treated 3xTg male mice with dexamethasone (8mg/kg; i.p.) or only the vehicle, as previously described using the combined Golgi Cox and Immunofluorescence technique.

Firstly, we check that the new protocol for combined technique works correctly. Below you can see some of the reconstructions we obtained: these data clearly demonstrated the validity of the protocols.

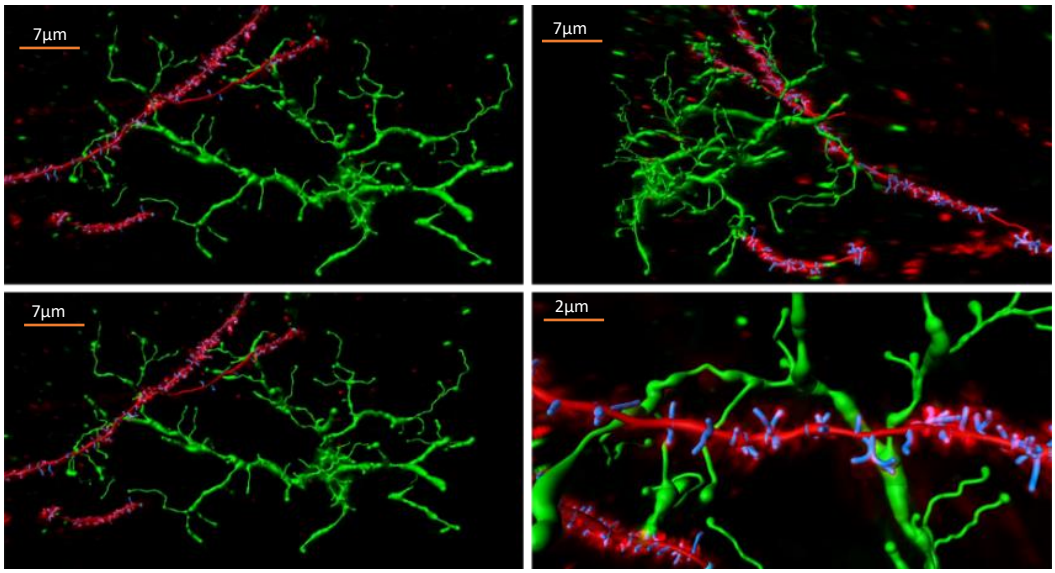

Figure 44. Reconstruction of dendrites and microglia using the combined technique of Golgi Cox and Immunofluorescence. We adapted protocol from the one published by Spiga in 2011. Neuron (red) is marked using Golgi Cox Staining, while microglia (green) using anti IBA1 antibody. Microglial cell was built using the cell autopatch system of Imaris. Dendrites and spines brightfield signal were detected by reflection channel of confocal microscope and then 3D rebuilt by filaments autopatch system of Imaris.

As visible in *Figure 45* qualitative comparison between slice of CA1 region of hippocampus of 3xTg mice treated with vehicle and with dexamethasone suggested that proliferation of microglia occurred after dexamethasone treatment. Even just by a qualitative observation it is possible to conclude that IBA1 signal, in green, is clearly enhanced in 3xTg mice stressed with dexamethasone compared to non-stressed 3xTg mice. This increment can be explained by an increase number of microglial cells and/or by a higher expression of IBA1: both of them suggested that microglia have proliferated and have assumed an activate conformation.

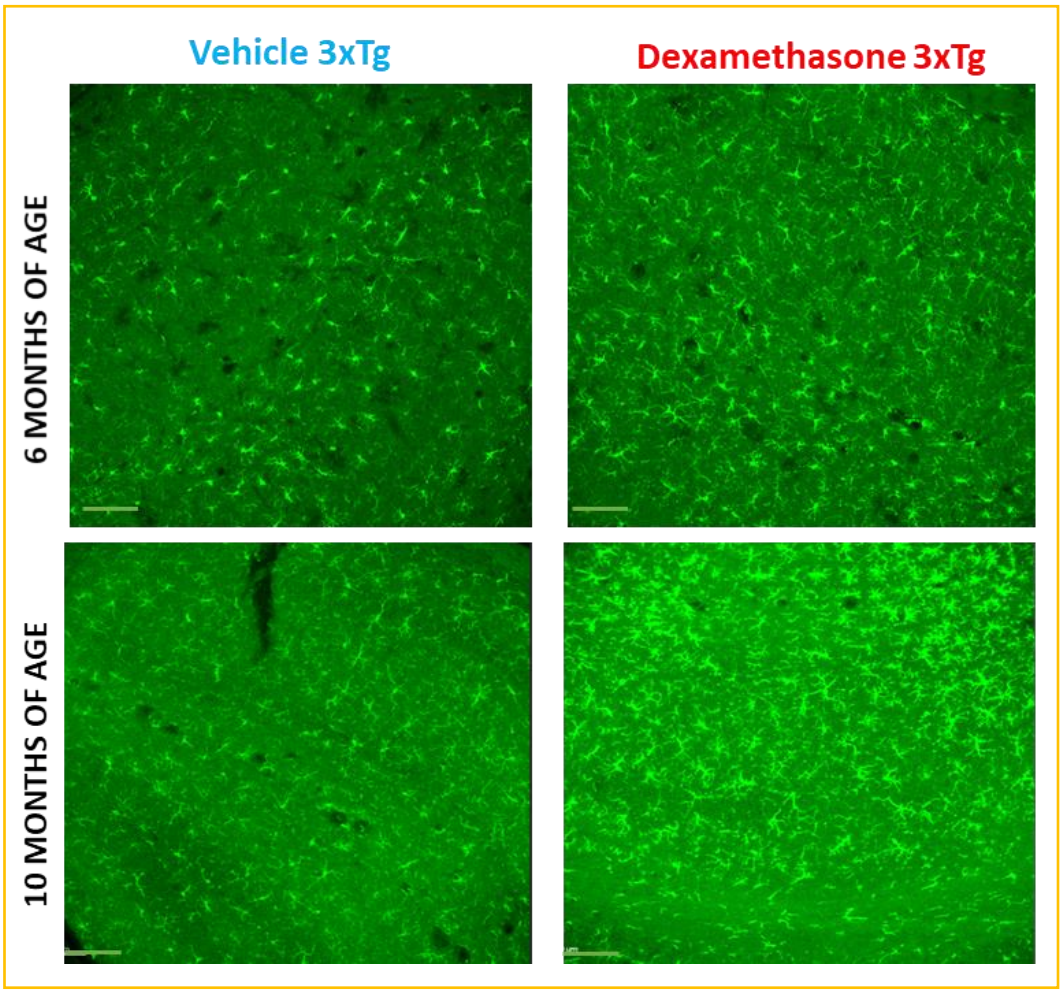

Figure 45. Qualitative comparisons between 3xTg mice treated with dexamethasone or only vehicle. The images were obtained from CA1 region of hippocampus using 20x objective at confocal microscopy. Microglia was marked, in green, using rabbit IBA1 antibody as described in paragraph 1.7. ***Scale bar = 100µm.***

As expected, quantitative analyses confirmed the previous suppositions. Dexamethasone induced proliferation of microglia in the CA1 region of Hippocampus both at 6 and 10 months of age: the density was increased of 57.17% (P=0.0051) and 31% (P=0.0095), respectively, in comparison with mice treated only with vehicle (t Test; P<0.01). The effects of GR agonist and antagonist on microglia proliferation are more prominent at 6 rather than 10 months of age (P=0.023), such as those produced on dendritic spines. Both our experiments (on dendritic spine and microglia activation) supporting the hypothesis that stress is an early key risk factor to develop AD and the idea that degeneration of spines and microglial activation are strictly linked and correlated. The activation is promoted also in the late phase of AD even if with low strength.

Another interesting result is related to the basal level of microglia in 3xTg mice: indeed, the density of the inflammatory cells is 37% higher at 10 months than 6 months of age (P=0.047). This finding is coherent with the observation that at 10 months of age Aβ plaque appears and this phenomenon could induce activation of the cells. In Figure 46, it is reported the quantitative analyses related to microglial density changes and all the comparisons between groups and ages.

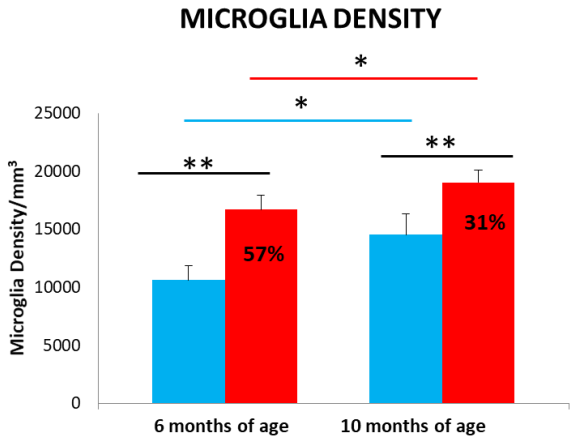

Figure 46. Quantitative analyses of microglia proliferation. As visible dexamethasone was able to increase the microglia density both at 6 and 10 months of age. The effects is

*prominent at 6 rather than at 10, like for spine density (see Figure 39). (*p<0.05; **p<0.01; ***p<0.001).*

As a matter of fact, proliferation of microglia is a known indicator of their activation. Therefore, we increased magnification on microscope to observe the morphology of microglia and how it has changed between vehicle and dexamethasone-treated 3xTg mice. As you can see in Figure 47 and on supplementary videos, in treated mice IBA signal is stronger than in control ones, moreover, microglia seemed to be hypertrophic and less ramified. All these observations suggested that microglia were in activation state. In order to confirm this hypothesis, we quantified microglia volume and area using Imaris software as reported in *paragraph 1.8* of ***Method*** part.

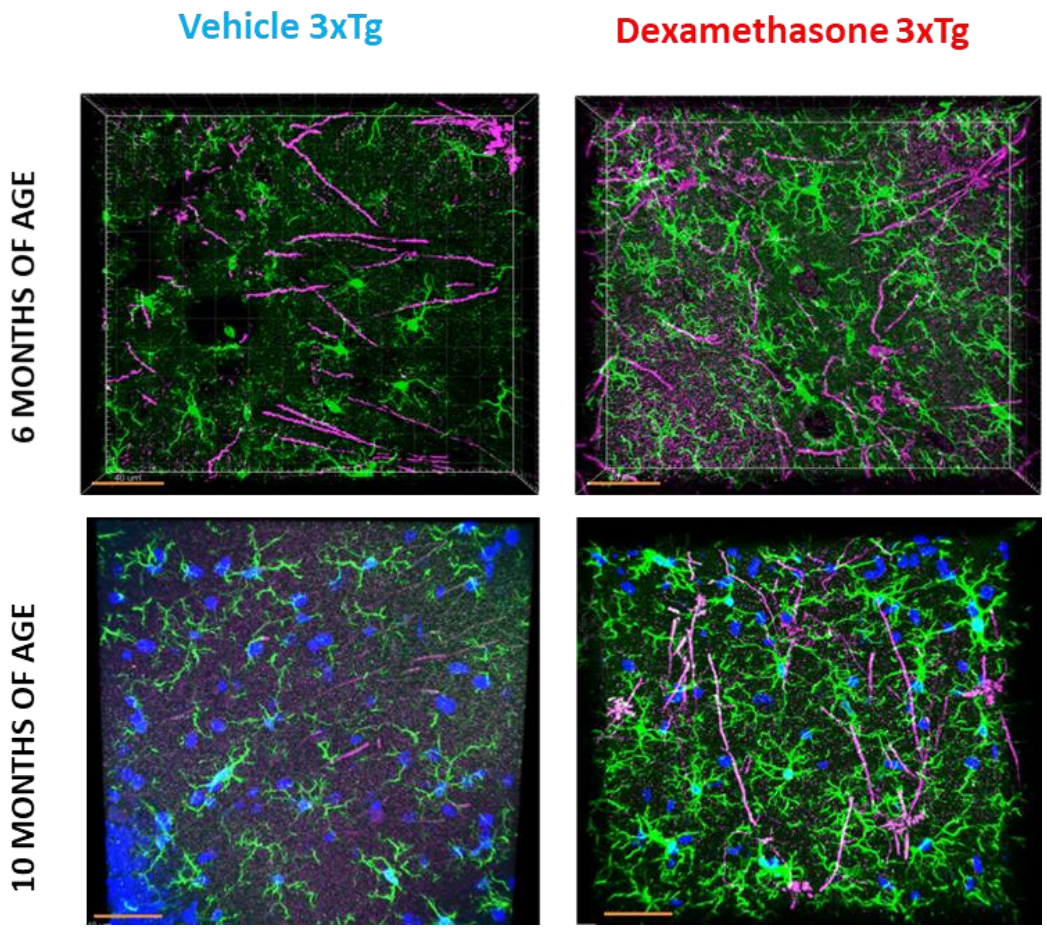

Figure 47. Magnification of CA1 region of hippocampus of mice treated with vehicle or dexamethasone. Microglia is marked in green (with anti-rabbit secondary antibody 488) using rabbit IBA1 antibody. Neurons in purple is built through reflective confocal

microscopy. As clearly visible, in dexamethasone treated animals, IBA1 signal is stronger, microglial cells are less ramified and seem to be hypertrophic. All these observations suggested that microglia were activated by hyper-activation of GR. DAPI is marked in blue (only in the lower part of the figure), IBA1 in green, neurons in pink. ***Scale bar = 40µm.***

Indeed, following dexamethasone treatment, both area and volume were significantly increased compared to vehicle alone both at 6 and 10 months of age: the area was increased of 47.4% ($P<0.05$) and 59.3% ($P<0.01$), respectively; the volume was increased of 83.0% ($P<0.05$) and 61.5% ($P<0.05$), respectively (Figure 48).

In conclusion, stress induced by dexamethasone promoted a strongly activation of microglia that was forced to proliferate and abandon the resting state phenotype. Consequently, these data suggested that the degeneration of spines observed in dexamethasone treated 3xTg mice (*Figure 39*) can be at least in part promoted by the activation of microglia.

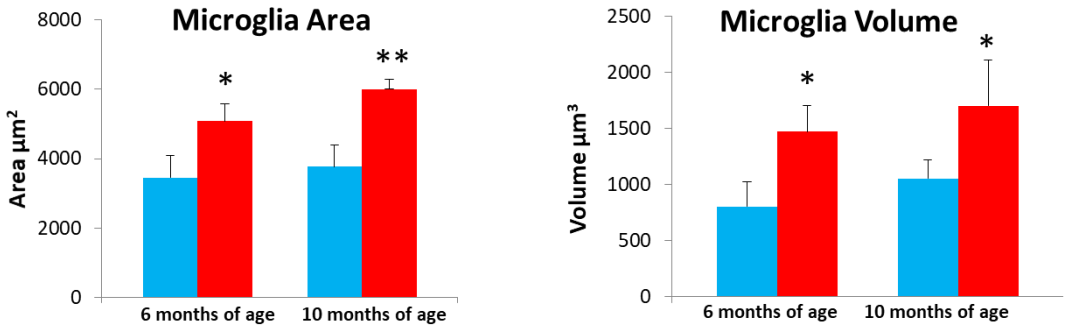

*Figure 48. Quantitative analyses of area and volume of microglial cells rebuilt using cell path of Imaris software. (*p<0.05; **p<0.01; ***p<0.001).*

3.1. Preliminary observations of mifepristone effects on Microglia *in vivo*

Finally, we repeated the same experiments using mifepristone instead of dexamethasone at 6 months of age. We treated three animals per group. We found that mifepristone produced a little reduction of microglia density (15.86%) in 3xTg mice rather than control ($P=0.046$).

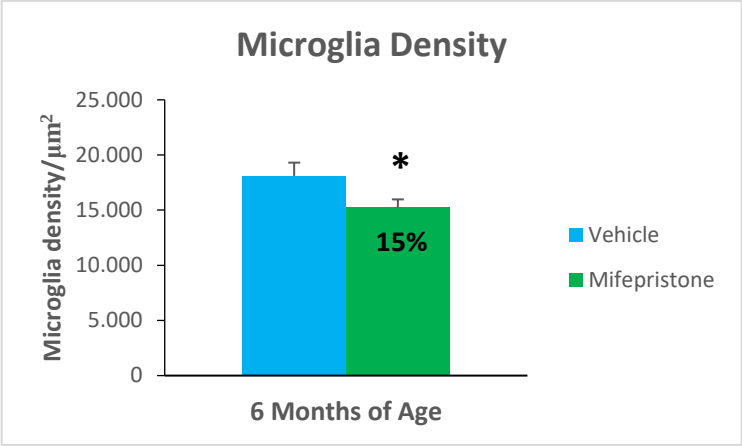

*Figure 49. Quantitative analyses of microglia proliferation in vehicle (blue) and mifepristone-treated 3xTg mice (in green). As visible mifepristone slightly reduces the microglia density at 6 months of age. (*p<0.05; **p<0.01; ***p<0.001).*

However, no difference between treated and control mice were found related to area (P=0.28) and volume (P=0.55) suggesting that the blockade of GR could not completely de-activate microglia.

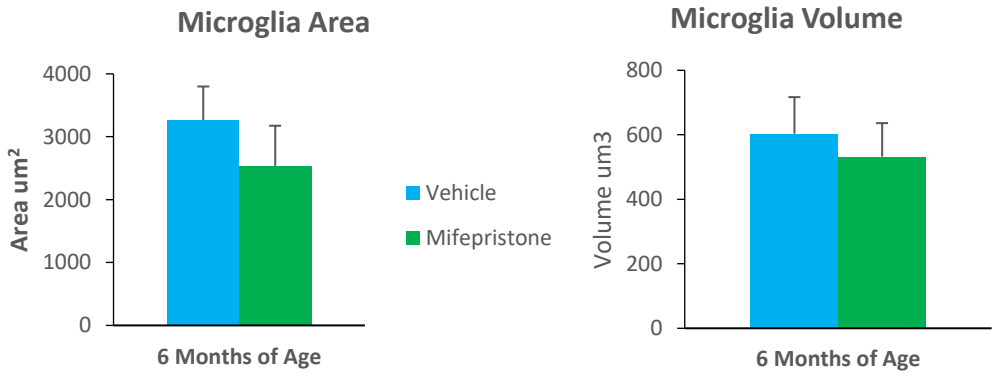

*Figure 50. Quantitative analyses of area and volume of microglial cells rebuilt using cell path of Imaris software in vehicle and mifepristone-treated 3xTg mice. Surprisingly, anti-stress treatment had no significant effects on reducing activity state of microglia as suggest by the lack of changes in area and volume after treatment. (*p<0.05; **p<0.01; ***p<0.001).*

4. High dose of GR modulators induced morphological changes in microglia primary cultures

How dexamethasone, a typical anti-inflammatory drug, could activate microglia in brain, was previously investigated in 2006 by Nair (Nair & Bonneau, 2006). He hypothesized that the dexamethasone-dependent activation of the microglia was not due to a direct mechanism but to an abnormal activation of NMDA receptors that promoted the release of pro-inflammatory molecules – see also ***Discussion*** and *paragraph 3.6.3*.

In fact, previous papers demonstrated that the microglial activation *in vitro* was stopped by dexamethasone administration (Colton & Chernyshev, 1996; Drew & Chavis, 2000; Woods, Poulsen, & Gall, 1999). Moreover, microglia from microglia GR KO mice also had increased activation, supporting the anti-inflammatory role of glucocorticoids in regulating microglial activation status (Wang & Harris, 2015, *p. 243*). To verify these previous observations, we treated microglial primary culture of non-transgenic mice using 1 µM dexamethasone or 1 µM mifepristone or only vehicle for 4 hours, as described in methods.

Immunofluorescence, unexpectedly, showed that dexamethasone treatment increased the signal of CD68 that is a marker of M1 phagocytic-pro inflammatory state of microglia. Immunoblotting confirmed the IF: CD68 was increased of about 209% (P = 0,03), but dexamethasone had no significant effects on CD206 expression (P=0.31), a marker of M2 phagocytic-anti-inflammatory state. Mifepristone treatment, instead, seemed to be able to activate cells both in M1 and M2. In every testing sample, Immunoblotting showed an increase of CD68 and CD206, but with a great variability that prevented us to obtain a significance both for CDD68 (P=0.26) and CD206 (P=0.06).

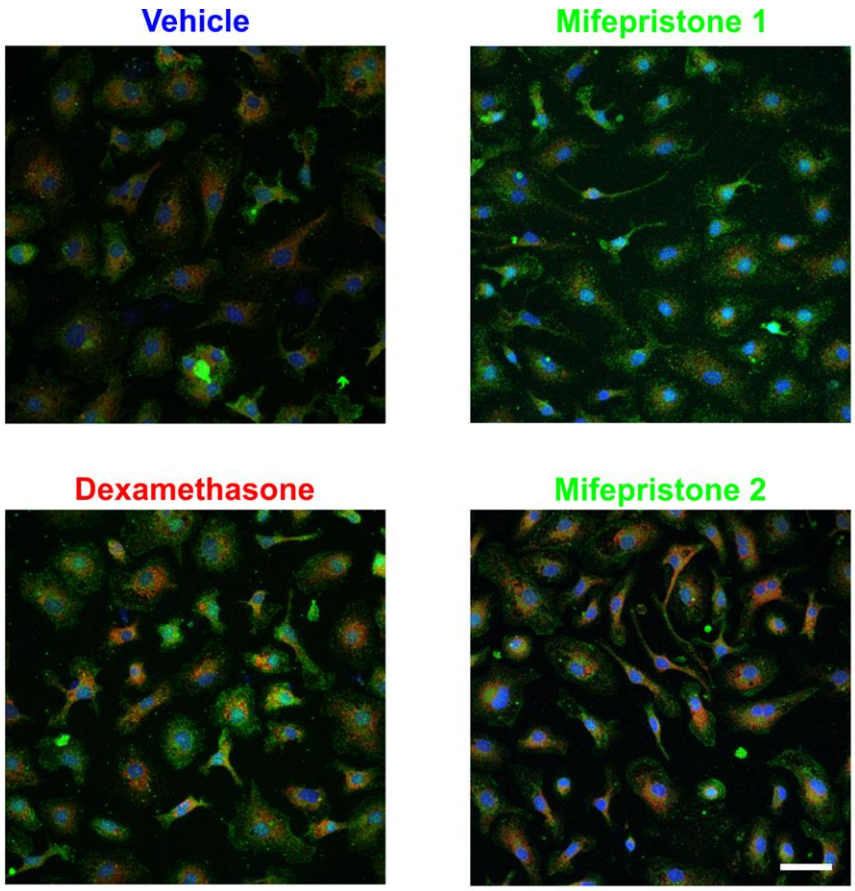

Figure 51. Primary microglia culture treated with vehicle or 1µM of dexamethasone or 1µM of mifepristone. Immunofluorescence result: Dexamethasone apparently increased CD68 (M1 marker) red-signal while mifepristone promoted ambiguous results: some cells present a low or absent CD68 signal, other ones a stronger CD68 signal. DAPI is marked in blue, IBA1 in green and CD68 in red. ***Scale bar = 40µm.***

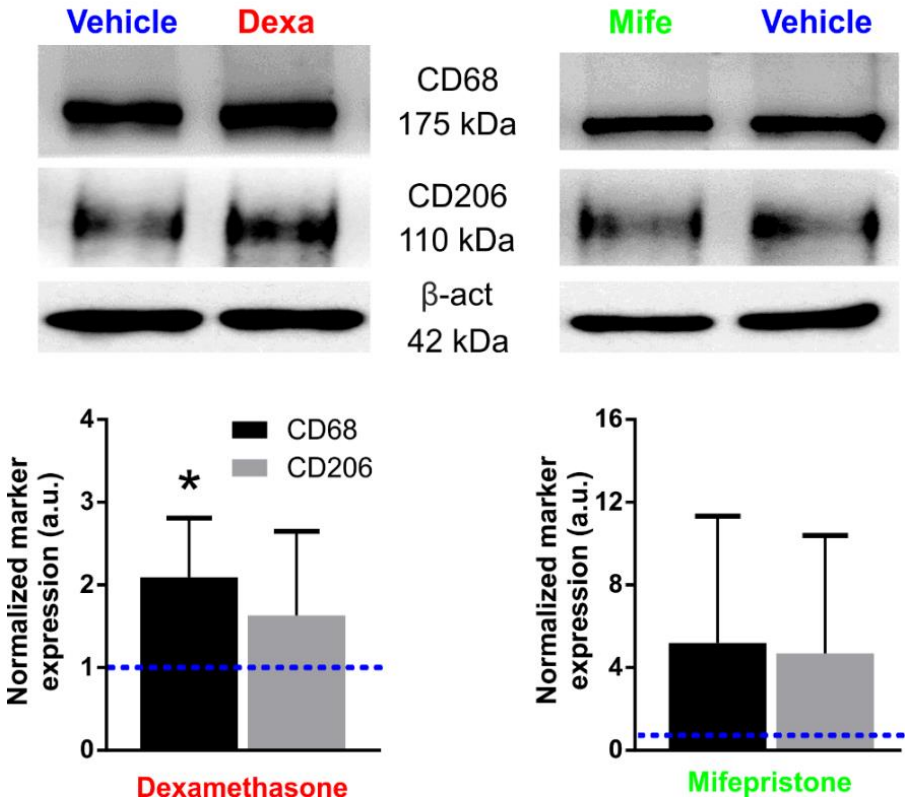

*Figure 52. Primary microglia culture treated with vehicle or 1µM of dexamethasone or 1µM of mifepristone. Immunoblotting confirmed data observed with immunofluorescence: dexamethasone was able to significantly increase CD68 expression but not the expression of CD206, M2 marker. On the contrary, treatment with mifepristone seemed to be able to increase both CD68 and CD206 expressions but with great variability. (*p<0.05; **p<0.01; ***p<0.001).*

As a matter of fact, our data are different from those previously published and show that dexamethasone is able to activate microglia *in vitro* through a direct mechanism too. On the contrary, the mifepristone effects on microglia are not clear also *in vitro* and require further investigation to better understand the molecular pathway involved after GR inhibition.

DISCUSSION

In summary our results show that:

1) the chronic administration of dexamethasone promoted a strong degeneration of dendritic spines of CA1 region of hippocampus of 3xTg-AD mice and increased the proliferation and activation of microglia both at 6 and 10 months of age.

2) mifepristone treatment, instead, increased the density of dendritic spine in CA1 region in both ages and the performance of 3xTg in Y-maze task at 10 months of age.

3) the treatment of primary microglia culture revealed that dexamethasone increased the expression of pro-inflammatory microglia (M1 state), while mifepristone apparently was able to increase the expression of both pro and anti-inflammatory microglia.

In detailed, our data show that stress level is a key factor in development and progression of Alzheimer's Disease. The stress induced by chronic administration of 8mg/kg of dexamethasone promoted a strong degeneration of dendritic spines of CA1 region of hippocampus, resulting in a drop of the spine density both at 6 at 10 months of age (*Figure 39*), that represent respectively the beginning and the medium late phase of AD, in 3xTg-AD male mice. On the contrary, a prolonged exposure to 20mg/kg of mifepristone was able to increment the dendritic spine density (*Figure 39*). As disserted below in this chapter, we don't know exactly the level of suppression of receptor activity produced by this treatment, but we know that the induced effects need to be explained by the hormetic role of GC. Indeed, even if the mifepristone was able to improve spine plasticity both at the beginning and in the medium late phase of AD, as revealed also by electron microscopy results (*Figure 40*), its efficacy on behavior tasks was observed only at later AD states (*Figure 43*). In the same way, the supposed anti-inflammatory role of antagonist of GR had been demonstrated only in part by our treatment: at 6 months of age, indeed, the drug reduced the density of microglia (*Figure 49*) but not their activation (*Figure 51*), suggesting that the dose used is, probably, not the best one to obtain a

strong anti-inflammatory effect together with the improvement of spine turnover. Additionally, *in vitro* experiments revealed that 1µM of mifepristone apparently was able to increase and decrease contemporaneously the expression of CD68 (M1 pro-inflammatory microglial state) in different cells but of the same culture (*Figure 51*), as immunofluorescence has revealed. Immunoblotting supported this ambiguity, showing that the antagonist of GR increased the expression of both CD68 and CD206 (marker of M2 anti-inflammatory microglial state) (*Figure 52*). On the contrary, the effects of agonist of GR (dexamethasone), that simulates the chronic stress condition, strongly enhanced the expression of CD68 but not the one of CD206, underlining that stress is able to induce a pro-inflammatory effect, also *in vitro*, directly on microglia, and opening to the possibility of the presence of a hormetic (biphasic) effect of glucocorticoid also on inflammation. Considering the single results three aspects are important to discuss: the role of stress in worsening the AD; the prominence of an early stress effects on AD; the biphasic behavior of GC and the possibility that GR antagonists could represent a promising therapeutic target to slow down the beginning and progression of AD. Finally, it is relevant to underline that the combined Golgi Cox and immunofluorescence technique, which we implemented in our lab, worked perfectly (*Figure 44; Figure 47*) and we were able to mark together neuron, with Golgi Cox, and microglia, with immunofluorescence, opening up important perspectives for future research.

Dexamethasone-induced stress worsen and exacerbates the AD

Dendritic spine loss is a typical early alteration of Alzheimer's Disease (Stephen W. Scheff et al., 2006), but it is well known from literature that this phenomenon occurs only later in the 3xTg-AD mice, and it becomes evident after Aβ plaque appearance (Bittner et al., 2010) during the last stage of AD.

Our results showed that stress induced by dexamethasone was able to promote a premature decrease of dendritic spine density in CA1 region of hippocampus of 3xTg mice, even months before the appearance of the plaques. Consequently, our

and Green's data (K. N. Green, 2006), have demonstrated that stress is able to worsen and speed up the AD progression, promoting an early Aβ-40/42 deposition and spine degeneration in 3xTg-AD mice.

Furthermore, stress strongly enhanced inflammation state in CA1 region of hippocampus, promoting the proliferation and activation of microglia. This phenomenon is controversial: dexamethasone is a typical anti-inflammation drug, but, in brain, it is able to increase inflammation after chronic exposure. The mechanism underlying this effect was investigated by Nair in 2006 (Nair & Bonneau, 2006) and it is schematically illustrated in the Figure 53. He suggested that activation of microglia is not directly promoted by GR activation on microglia themselves, but through a neuron dependent mechanism, involving NMDA receptor hyper-activation.

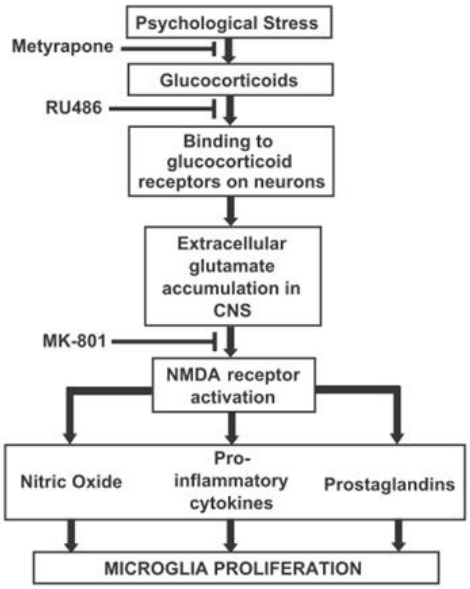

Figure 53. Schematic representation of the mechanism that promote the activation of microglia in the brain after chronic exposure to glucocorticoids. The blockade of the single steps prevents the activation of microglia. Interestingly, the use of RU486 (the pharmacological name for mifepristone) maintains the rest state of microglia.

Coherently with Nair hypothesis, some *in vitro* researches had already demonstrated that cortisone and GR agonists were able to exert both anti-

inflammatory and anti-oxidant effects on microglial cells (Colton & Chernyshev, 1996; Drew & Chavis, 2000; Tanaka et al., 1997). Moreover, a recent publication demonstrated that dexamethasone treatment, on human microglia culture, reduced the volume and increased the ramification of cells, suggesting a possible morphological change from M1 towards the resting state (van Olst, Bielefeld, Fitzsimons, de Vries, & Schouten, 2018). In summary, all these experiments, apparently proved that activation of GR promoted a reduction of microglia activation. Therefore, the apparent ambiguity between *in vivo* and *in vitro* effects, could be resolved by Nair observation.

Surprisingly, when we performed *in vitro* experiments to confirm the anti-inflammatory role of GR on microglia, we obtained exactly the opposite effect. Indeed, dexamethasone was able to activate inflammation even *in vitro*, acting directly on microglial cells.

This further ambiguity could be explained by the well-known biphasic behavior of glucocorticoids in brain, a phenomenon called hormesis and explained in *paragraph 1.4* and *3.6*. A lot of physiological molecules are likely to show a hormetic effect (Calabrese, 2014; Kendig, Le, & Belcher, 2010; Daniela Puzzo et al., 2012), producing opposite effects depending on high-chronic or acute-low exposure. Related to glucocorticoids, we observed that administration of high and chronic doses of GR agonist, disrupts the spine turnover promoting their premature degeneration. On the contrary, we know from literature that acute or low doses promote spine turnover in brain (Liston & Gan, 2011). We speculated that this biphasic behavior is not present only on spine turnover and memory formation but also on inflammation state. The existence of this direct pro-inflammatory effects on microglia demonstrated, indeed, that Nair hypothesis is not the only explanation for the activation of microglia *in vivo* after chronic exposure to GC. Consequently, our results suggested the existence of a biphasic behavior of GC also on inflammation. In this way, the activation of microglia *in vitro*, induced by dexamethasone, could be related to different dosage and time of treatment that we used, compared to those reported in previous works. In summary, the microglia activation observed *in vivo* after GC treatment, could underline both a direct mechanism on microglia cells, as

we demonstrated, and an indirect effect mediated by neuron damage, like proposed by Nair.

Stress-induced damage is stronger at the beginning of AD

It is interesting to observe that the negative effects produced by dexamethasone-induced stress is prominent at the beginning of AD rather than at the medium-late phases of AD, as we demonstrated both for dendritic spine degeneration and microglia proliferation. These data are coherent with the observation that healthy and elderly individuals with high cortisol levels were significantly more likely to develop Alzheimer's disease. Moreover, the cortisone level arise, in human, could accelerate the cognitive impairment in preclinical AD patients (Pietrzak et al., 2017). On the contrary the cortisol levels on senile AD patients were similar to the levels of unaffected controls (Swaab et al., 1994), indicating the importance of stress especially at the beginning of AD. Therefore, the stronger effects observed at 6 months of age compared to 10 months of age in 3xTg mice could be correlated to the prominent role of stress in preclinical stages of AD in human (Pietrzak et al., 2017; Swanwick et al., 1998; Wilson et al., 2005).

Blockade of stress through mifepristone produces positive but ambiguous results

If the stress induced by hyper-activation of glucocorticoid receptors was a negative factor that contribute to the worsening of AD, blockade of GR showed encouraging results. It is, indeed, known that mifepristone can reduce amyloid plaques in 3xTg-AD mice and in other AD mice models (Baglietto-Vargas, Medeiros, Martinez-Coria, Laferla, & Green, 2013; Pineau et al., 2016). We found that mifepristone treatment also promoted spine formations in CA1 region of 3xTg mice, both at 6 and 10 months of age, although with no significant difference in the two ages. This dendritic spine formation was, also, investigated using electron microscopy that, at 6 months of age, confirmed the results obtained with Golgi Cox, related to dendritic spine density. This is not an expected result because optical microscopy allowed us

to count thousands of spines, while with electron microscopy we were able to detect much lower number, but with a difference strong enough to obtain a significance. Moreover, the great irregularity of dendrites, in 3xTg mice treated with mifepristone, could be related to the process of maturation of spines. Lastly, we would like to highlight the presence of some perforated synapses in mifepristone treated 3xTg compared to vehicle treated 3xTg mice, in which we had not found any of them (*Figure 42*). Consequently, we suggest that mifepristone can partially revert the dendritic spine deficit and we will investigate this hypothesis in future experiments.

The positive improvement promoted by mifepristone was, also, observed with Y maze task. Indeed, at 10 months of age, mifepristone treatment improved the performance of 3xTg mice. Likely, the positive increment of dendritic spine density in CA1 region could be correlated to the cognitive improvement. The no significant difference found at 6 months of age could be related to the strong variability in performance between animals of the same group.

Related to microglia, the effects of mifepristone were, instead, ambiguous. Indeed, in the *in vivo* experiments, mifepristone slightly reduced microglia density at 6 months of age but had no effect on morphology. Indeed, microglia morphology suggested an activation in both vehicle and mifepristone treated animals. Surprisingly, *in vitro*, after treatment, microglia showed an increase of both CD68 and CD206 in every experiment but with a great variability. As previously described, CD68 is a marker of M1 pro-inflammatory microglia and CD206 a marker of M2 anti-inflammatory microglia. Thus, it is unusual that a drug treatment promotes an opposite polarization in different cells of the same culture. A physiological mechanism that could explain these contrast observations need to be found in the future. Now, we speculate that the biphasic behavior of GR activity could explain our finding.

Into biphasic behavioral mechanism hypotheses

Our data are coherent with both the two possible explanations of biphasic behavior of GC in brain. As explained in paragraph 3.6.1, one of the hypotheses is that the hormetic effects promoted by the hormones are only due to GR activation (McEwen & Magarinos, 2001; Sorrells & Sapolsky, 2007). So, in that case, the strong reduction of GR activity, induced by administration of high dosage of antagonist or, physiologically, by absence or very low level of plasma glucocorticoids, could be dangerous like their hyperactivation induced by administration of agonist or physiologically by chronic stress. Instead, a medium activation of GR should produce the best results on spine plasticity, memory formation and on inflammation reduction, as physiologically promoted by acute stressor stimulus. This possibility is supported by the fact that MR are always activated by GC, also at very low level, and by aldosterone, while GR, in physiological condition, are activated only for short times in the presence of acute stressor stimulus or at the peak of circadian rhythm. In pathological condition the MR state of activation is unaltered, but GR can be hyper-activated for an abnormal long time period. Moreover, the depletion or hyper-activation of GR through agonist, can impair memory consolidation independently by MR activity.

The second possibility, proposed by de Kloet in 1999 and confirmed by the works of Lupien, suggested that the combination of activity of MR and GR is the right explanation of the hermetic effect of GC. The best performance can be achieved with a high proportion of active MR and a low proportion of active GR (E. Ron de Kloet et al., 1999; Sonia J. Lupien et al., 2005; S. J. Lupien et al., 2007).

Interestingly, we suggested that the ambiguity results obtained with mifepristone can be explained by both hypotheses. In details, 20mg/kg of mifepristone treatment for 5 days was effective in improving spine plasticity, but not to reduce inflammation, *in vivo*. Moreover, *in vitro* experiments, a dose of 1µM of drug was not able to significantly reduce microglia activation. For these reasons, if the first hypothesis is correct, it is possible that our dosage was excessively high, and we inhibited the GR too much. On the contrary, if the second hypothesis is correct, we can speculate that our dosage was not enough elevate to blockade the receptors.

Indeed, mifepristone treatment does not interfere with MR activity. As a consequence, the outcome will depend on the ratio between the activity of MR and GR and the best compromise would be reached through a strong inhibition of GR combined with a high activation of MR.

Future perspective.

Therefore, in accordance with hormetic effect, we suggested that a strong reduction of GR activity, rather than their total inhibition, could be the best solution to slow down the beginning and progression of AD. We will verify this hypothesis using different dose and time of exposure to assure what is the best treatment to obtain simultaneously an improvement of spine turnover, of memory formation and of behavior performance and the reduction of inflammatory state. This represents the bedrock of the future progression of my project. Moreover, the implementation of the combined Golgi Cox and Immunofluorescence technique will allow us to investigate deeply the changes in the contacts between microglia and neurons, in order to verify whether the microglial cells are able to disrupt the dendritic spine of the neurons also in adults.

CONCLUSIONS

Our data support the existence of a strong correlation between Alzheimer's Disease (AD) and glucocorticoids. Stress is a key risk factor for the beginning of AD and for the rapidity of the disease progression, as demonstrated by our dexamethasone treatment of the transgenic mouse model of AD, the 3xTg-AD mouse. A similar pattern was found also in human, thanks to biological and psychiatric researchers that, already in the 80's, begun to investigate the connection between high levels of GC (like in depression) and the risk of AD development (Berrios, 1985; Hammar, 2009; Pietrzak et al., 2017; Wilson et al., 2005). Our results showed that the treatment with antagonist of GR is able to stimulate synaptic plasticity, implement cognitive abilities and behavioral performance and decrease the general state of inflammation in the brain. Consequently, the reduction of stress, promoted by suppression of GR activity, could represents a promising therapeutic strategy to postpone the onset of AD and slow down its progression. However, the biphasic behavior of GC requires an in-depth study to understand the ideal dosage and times. The recent development of some specific GR antagonists, with lower side effects, encourages the research (Canet, Chevallier, Zussy, Desrumaux, & Givalois, 2018). Thus, the literature data and our results strongly support the need to continue these studies in order to collect further data for a possible transition to the clinical research.

SUPPLEMENTARY PROJECTS

During my PhD course, I was involved in other projects and experimental tasks. In particular, I collaborated in 2 projects: 1) related to AD, we were interested to understand the capability of extracellular vesicles, released by human mesenchymal stem cell, to reduce the inflammation in 3xTg mice and to promote the spine plasticity; 2) related to Autism, we investigated the changing of dendritic spine density in a transgenic animal model for autism. The results were published in the 2018 (Bertero et al., 2018).

HUMAN MESENCHIMAL EXTRACELLULAR VESCICLES REDUCE INFLAMMATION AND INCRESE SPINE DENSITY IN 3xTg MICE.

The topic of this research is part of the PhD thesis of Morris Losurdo, of the laboratory of Dr. Silvia Coco of the School of Medicine and Surgery of University of Milano-Bicocca. Together with Morris, our lab performed the *in vivo* treatment and the e*x vivo* analysis in an animal model of AD. The aim of this project was to verify whether extracellular vesicles, released by human mesenchymal stem cell, pre-stimulated with pro-inflammatory molecules, could reduce the state of inflammation. *In vitro* he tested primary microglia culture of non-transgenic mice, using immunoblotting and immunofluorescence technique. E*x vivo*, we investigated whether these vesicles could reduce the state of activation and proliferation of microglia and could increase the dendritic spine density in hippocampus, entorhinal and prefrontal cortex of 3xTg-AD mice. The Ms. of these results is in preparation.

INTRODUCTION

Extracellular Vesicles

Extracellular Vesicles are membranous structures that are released by the cells both in physiological and pathological conditions and play an important role in the mechanisms of cellular communication. Among the different types of EVs, the most common are the exosomes (50-150nm) and the microvesicles (MVs, 50-1000nm) (Raposo & Stoorvogel, 2013). These vesicles differ in size, content (proteins, lipids, nucleic acids) and in biogenesis mechanism. The exosomes originate from the endocellular vesicle system that begins with the formation of a multivesicular endosome (MVE), which has intraluminal vesicles (ILV). Following the fusion of MVE with the plasma membrane, the vesicles are released into the extracellular environment (Figure 54).

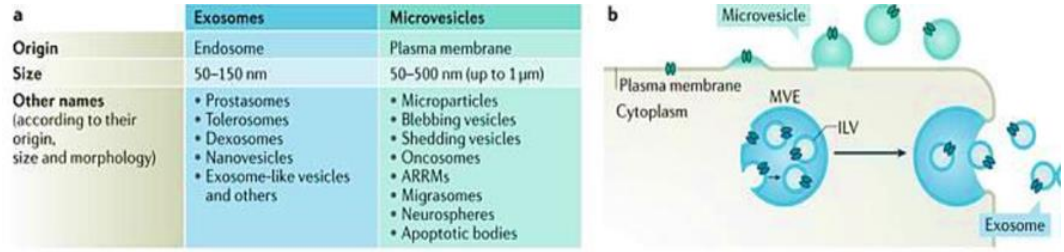

Figure 54. Classification of extracellular vesicles (EVs). a) In recent years the EVs have been classified according to their origin, size and morphology. To date, there are two major categories in which all types of EVs can be included: the exosomes and the microvesicles (MVs). b) Schematic representation of the process of biogenesis and release of exosomes and microvescicles. Exosomes originate from the system of endocellular vesicles, MVs originate by direct budding of the plasma membrane. MVE: multivesicular endosomes; ILV: intraluminal vesicle (Van Niel, D'Angelo, & Raposo, 2018).

In contrast, MVs originate by direct budding of the plasma membrane. To date there are no isolation protocols that allow to separate a type of vesicle from the other, due to both the size and the lack of specific markers for each of them. The role of EVs in cellular communication is also important in the CNS, indeed, the connection

between neurons and microglia also makes use of secreted vesicles (Paolicelli, Bergamini, & Rajendran, 2018). Their role in different pathological conditions has been demonstrated; in particular, an altered intra-cellular communication mediated by EVs seems to be involved in neurodegenerative diseases such as AD (Trotta et al., 2018).

Mesenchimal Stem Cells

Mesenchymal stem cells (MSCs), is one of the principal types of human adult stem cell. EVs released by MSCs promote an important immunomodulator activity (François, Romieu-Mourez, Li, & Galipeau, 2012). Indeed, MSCs have proven to be able to remove Aβ protein aggregates and reduce neurofibrillary tangles. Furthermore, they are able to recruit microglial cells and regulate their activity in an anti-inflammatory sense (Turgeman, 2015). This anti-inflammatory capacity has been largely attributed, not only to soluble factors, but specially to the release of EVs, which contain molecules (especially single-helical nucleic acids such as miRNAs), which can turn off specific inflammatory pathways in the microglial cells, directing them to a protective phenotype (M2).

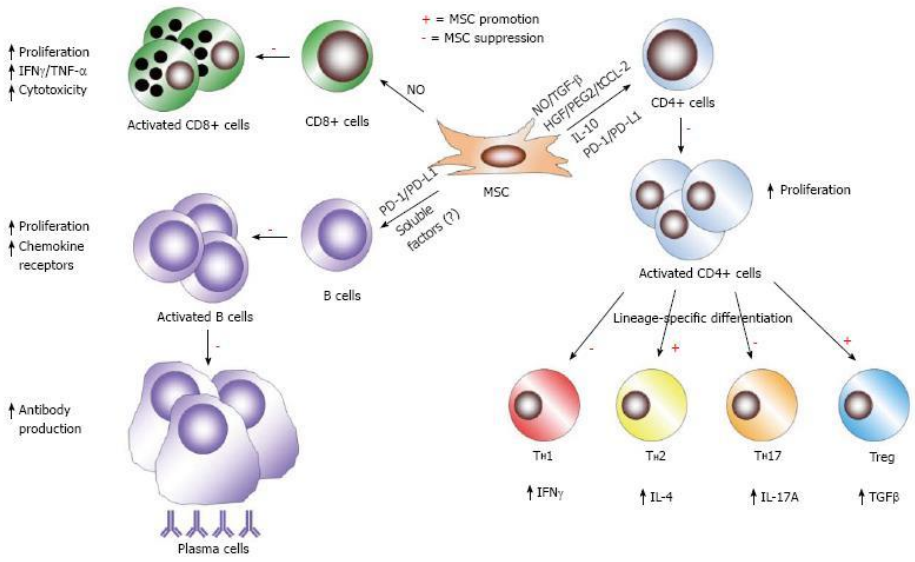

Figure 55. ***Mesenchymal stem cell immunosuppression of adaptive immune cells****. Mesenchymal stem cells (MSCs) promote the inactivation of B cells activity: it reduces proliferation, chemokine receptor expression, and differentiation, in order to avoid release of antibody by these cells. The mediation of this effects is not clear, but it is promoted by*

soluble factors and by PD-1/PD-L1 ligation. MSC have been shown to induce NO in response to inflammatory cytokine detection to suppress CD8+ T cell proliferation, cytokine production, and cytotoxicity. CD4+ T cells can differentiate into numerous effector populations. MSCs produce soluble factors (NO, TGF-B, HGF, PGE2, truncated CCL-2, and IL-10) and membrane-bound molecules (PD-1 ligation) to achieve suppression of CD4+ T cell proliferation and the polarization of CD4+ T cells towards TH1 and TH17 cells. MSCs favor the development of TH2 and anti-inflammatory Treg populations (Glenn & Whartenby, 2014).

MSCs are also capable to prevent accumulation of Aβ plaques, by inducing the rapid clearance of amyloid aggregates in an acutely induced AD model obtained by injecting Aβ into the dentate gyrus (DG) of the hippocampus of C57BL/6 mice (J. K. Lee, Jin, & Bae, 2009). Moreover, it seems that ADSCs promote anti-oxidative effects after their transplantation into hippocampal region of 8 months APP/PS1 AD mice (Yan et al., 2014). This is an important observation, in fact, it is well-known that the positive increment of Aβ plaques accumulation induce an increasing production of ROS, as a consequence of impairment in mitochondrial function, (Sheng et al., 2009). Moreover, MSC treatment improves hippocampal neurogenesis in the sub granular zone of Dental Gyrus. These studies suggest that a therapeutic approach able to reduce inflammation as well as the oxidative stress may have relevant neuroprotective effect in AD.

In our study, we used vesicles derived from human MSCs (hMSCs) that were administered to animal models through intranasal injections. The anti-inflammatory effect of vesicles derived from hMSCs was enhanced by the preconditioning of hMSCs through the administration of pro-inflammatory cytokines.

For this and all the previous analyzes, we used one of the most used research models to study the development and the course of AD disease. This is the triple-transgenic mouse for AD (3xTg-AD) by La Ferla (Oddo et al., 2003).

METHODS

To investigate microglial cell density in different brain areas, EVs, derived from preconditioned hMSCs, were injected intranasally in 3xTg female rats of 7 months with 100µl of solution containing EVs or only vehicle for controls.

Intranasal injections are a method of treatment that allows the administration of the vesicles directly on the internal surface of the airways, exploiting their amplitude and maintaining a concentration of the product sufficiently low to reduce undesirable effects, but able to guarantee adequate and relatively rapid absorption.

The vesicles were resuspended in sterile PBS solution at a concentration of 300µg /mL: in that way, 100µl of solution contains about $15x10^9$ vesicles. Injections were performed through a micropipette, using 5µL of PBS solution (for controls) or 5µL of EVs in PBS (for the treated models) at a time, distributed between the two nostrils. There were 5 injections, on the first day, with an interval of 5 minutes between one and the other. At a distance of 18h, additional injections were performed for a total of 50µL injected. 21 days from the last treatment mice were perfused with 30ml of phosphate buffered saline solution (PBS) and, immediately after, with 100ml of formaldehyde 10% V/V, buffered 4% W/V (PFA) (Titolchimica-Italy) to allow the fixation of the brain. Thanks to special surgical instruments, the brain was extracted and placed in a Falcon containing PFA.

After that, we performed our combined Golgi Cox and Immunofluorescence technique as described in the ***Method*** chapter of my thesis (*paragraph 1.7*).

Brains were last in Golgi Cox solution for two weeks then they were left in 30% sucrose solution for 24 hours and finally cut with vibratome. The coronal sections, 60µm slices, thus obtained, are divided into wells of a 24Well plate filled with PBS. Subsequently, we treated the slices with Kodak and after PBS wash, we started immunofluorescence as follow: 1 hour of blocking solution containing 0.3% Triton and 3% BSA; 36 hours of incubation with the primary antibody for rabbit-IBA1 (WAKO 1:500) and Rat-CD68 (Biorad 1:200) or Rabbit-IBA1 and Rat-CD206

(Biorad 1:100); 2 hours with the secondary antibody Anti-Rabbit 488 and Anti-Rat 594; finally DAPI staining for 5 minutes.

The images were reconstructed using Imaris software in the same way described in in *Method paragraph 1.8.*

A further experiment was performed for detect the position of vesicles using the label PKH26.

RESULTS AND DISCUSSION

The EVs, derived from human MSC and labeled with PKH26, were detected in microglial cells labeled with IBA1 in the CA1 area of the hippocampus, entorhinal cortex and prefrontal cortex. No internalization has been seen in astrocytes, on the contrary, although to a lesser extent, it has been observed in pyramidal neurons of the CA1 area.

We found that the treatment with vesicles significantly reduced the microglial density after 21 day of treatment in all of three regions, as reported in the figure below.

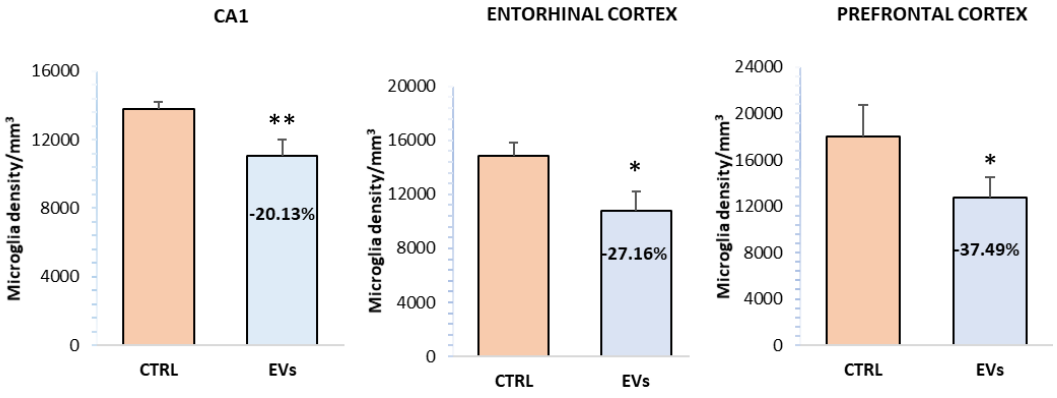

Figure 56. Representation of microglia density reduction following intranasal EV injections in areas: hippocampal CA1, entorhinal cortex and prefrontal cortex.

In details, compared to controls, the EVs treatment reduced the density of microglia of about 20.13% in the CA1 area of the hippocampus (*CTRL: 13,807.79mm^{-3}± 368.26mm^{-3}; EVs: 11,027.06mm^{-3} ± 952.99mm^{-3}; P value < 0.01*), of about 27.16% in the entorhinal cortex (*CTRL: 14830.59mm^{-3} ± 994.44; EVs: 10803.32 ± 1444.36; P value < 0.05*), and of about 37.49% in the prefrontal cortex (*CTRL: 18,026.64 mm^{-3} ± 2,738.72mm^{-3}; EVs: 12,769.01mm^{-3} ± 1,711.93mm^{-3}; P value < 0.05*). Furthermore, the number of microglial cells expressing CD68, marker of pro-inflammatory M1 state, was significantly lower compared to control ones, even if there was no significant difference related to the expression of CD206, marker of anti-inflammatory M2 state.

Interestingly, the reduction of microglia density is associate with the increase of dendritic spine density in all the three regions, as pictured in the figure below.

Table 4. Summary of dendrites lengths measured, and the number of spines counted for CTRL and EVs 3xTg mice groups per every region considered: CA1 region of hippocampus, Entorhinal Cortex and Prefrontal Cortex.

Spine analyses	**CA1**		**ENTORHINAL CORTEX**		**PREFRONTAL CORTEX**	
Group	CTRL	EVs	CTRL	EVs	CTRL	EVs
Mice (n°)	4	4	4	4	4	3
Dendritic Length µm	**1882.37**	**1698.94**	**1716.38**	**1029.77**	**669.17**	**695.52**
Dendritic Spines n°	**2735**	**3099**	**2181**	**1562**	**794**	**956**
Mean density/ 10µm	**14.44**	**18.30**	**12.58**	**15.20**	**11.89**	**13.80**

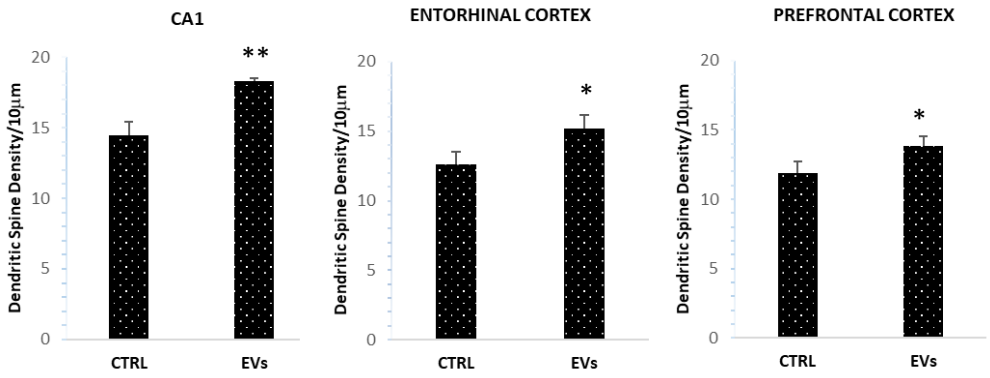

Figure 57. ***MSC-derived EVs increase dendritic spine density in 3xTg mice.*** *Histograms show the quantification of dendritic spine density (spines/10µm). Animals treated with EVs display a significative higher number of dendritic spines compared to the non-treated group in all the three regions.*

CONCLUSIONS

The treatment with the extracellular vesicles (EVs) of human mesenchymal stem cell was able to reduce the overall inflammatory state in key regions of the brain affected by AD, like CA1 regions of hippocampus and entorhinal and prefrontal cortex. In details, 2 day of treatment using 15 billion EVs promoted a strong reduction not only of microglia proliferation but also of their state of activation, resulting in a significant reduction of the expression of CD68, the typical marker for M1 pro-inflammatory state of microglia. The reduction of inflammation is an important goal for AD, but in future, this experiment could be extending also to other neuropathology diseases in which inflammation plays a fundamental role.

These discovers reinforces the hypothesis that the inflammation and spine plasticity are strictly correlated. Indeed, in my principal projects of glucocorticoids, we observed that the reduction of spine density in CA1 region is associated with the reduction of microglia density. Furthermore, this project demonstrated that the relation between microglia activity and spine loss, is not region specific: indeed, this phenomenon is present also in entorhinal and prefrontal cortex, crucial regions in the development and progression of the disease.

In future, together with Dr Silvia Coco lab., we will verify whether the effects are expressed also in older mice with a more advanced state of AD and whether the changes of treatment dose and time can produce more long-lasting improvements.

DENDRITIC SPINE DENSITY ALTERATION IN AN ANIMAL MODEL OF AUTISM

INTRODUCTION

Autism is a neurological disorder belong to the group of Pervasive Development Disorder (PDDs) together with Asperger's Disease, Rett's Disorder, atypical autism and childhood disintegrative disorders (DSM IV, American Psychiatric Society 1994) (Anderson, 2012).

The term "autism" was used for the first time by Leo Kanner in 1943, as the condition affected children in social relating (Anderson, 2012). Now, both International and American diagnostic criteria described autism as characterized by 3 conditions:

- Problem with communication.
- Impairment of social interaction.
- Presence of repetitive, rigid and stereotypic behaviors.

Despite these criteria, it is difficult to find the exact symptomatology of the autism: indeed, they differ a lot between individuals. Consequently, it is general used the expression "autism spectrum disorders" (ASDs) to indicate the heterogeneity of the pathological forms of the autism (Chen, Peñagarikano, Belgard, Swarup, & Geschwind, 2015).

A lots of autism patients present an EEG abnormality with diffuse and focal spike activity and paroxysmal wave patterns (Rapin, 2002). Furthermore, more than half of autism-affected patients suffers of intellectual inability and an important minority of language disfunctions (Anderson, 2012).

Brain Abnormalities and Genetic Background.

In the last years, one of the most important goal of autism-related research, was understanding the genetic basis of autism spectrum disorders (ASD) (Bertero et al., 2018).

Post-death studies on the brains of individuals with autism have detected abnormalities in the size of the brain and abnormalities that affect the areas of the trunk, the cerebellum, the limbic system and the cortex. The region with the most consistent abnormalities is the region of the anterior cingulate cortex (*Figure 58*) , a key component of the limbic system that contributes to affective and cognitive behaviors and to motor activities (Simms, Kemper, Timbie, Bauman, & Blatt, 2009), through neural circuits modulated by the neurotransmitter dopamine (Portmann et al., 2014). The projections of the dopaminergic neurons of the ventral mesencephalon reach the cortex, as well as the striatum. The latter contains medium spiny neurons (MSN), sensitive to dopamine, and forms the entry point of the circuit of the basal ganglia, which play an important role in motor control, motivation and attention. From the genetic point of view, these abnormalities are related to a variation in the number of copies on the human chromosome 16p11.2. In particular, children with 16p11.2 del carriers present these brain structural abnormalities (Bertero et al., 2018; Zufferey et al., 2012). Indeed, a deletion shows in the subject motor deficits, language delay, autism spectrum disorders (ASD), attention deficit disorder (ADHD), convulsions and hearing disorders; a duplication, on the other hand, is associated with both ASD and schizophrenia (Horev et al., 2011; Portmann et al., 2014). The deletion in the p11.2 locus of chromosome 16 associated with ASD causes a loss of 550kb of DNA and an aploinsufficiency of the 26 genes present in the chromosome. However, heterogeneous symptoms may occur in different individuals that present this mutation (Horev et al., 2011).

Chromosomal copy number variations (CNVs) have been associated with 5-10% of patients with ASD (de la Torre-Ubieta, Won, Stein, & Geschwind, 2016). Microdeletion of human chromosome 16p11.2 (16p11.2 del) is one of the most CNV in ASD, representing the 0.5–1% of all cases (Kumar et al., 2007). It is reported that ASD is diagnosed in about 18% of 16p11.2 deletion carriers and that this CNV is followed by a strong reduction in IQ (Zufferey et al., 2012) .

AIM

In the paper published in 2018, we showed that 16p11.2 del impairs prefrontal functional connectivity, producing a global connectivity reduction and impairing long range coupling in the parieto-temporal associative regions of the default mode network (Bertero et al., 2018). Since the clinical samples are limited in number, we investigate whether these findings can be reported also in a mouse model of 16p11.2 del (Horev et al., 2011). Out findings suggest that deletion in 16p11.2 may lead to impaired cognition and ASD-like symptoms via dysregulation of long-range prefrontal functional synchronization.

In detailed, my work was concentrated on understand whether specific brain area of transgenic mice presented different dendritic spine density compared to wild type ones.

In particular, we focused our attention on anterior cingulate cortex and on primary sensory cortex. Indeed, fMRI revealed that the first area presents abnormality in the 16p11.2 del mouse model, while the second one seems to be not altered in that model compared to wild type one.

METHODS

Brains of transgenic (HT) and wild type (WT) were fixed and collected by Gozzi group at Italian Institute of Technology of Rovereto (They collected 8 WT and 8 HT mice). In our lab, firstly we washed them in PBS; then we put the brains in Golgi Cox solution using the same protocol described in *paragraph 1.7* of the ***Introduction***: staining brains for 2 weeks, cut of the brains in 100µm slices, treatment of slices with Kodak, dehydration of slices using Ethanol and finally mounting the slices on slides using Eukitt.

We identified the two brain regions using Mouse Brain Atlas coordination: about 1.94mm Bregma for Anterior Cingulate Cortex, and about -0.82mm Bregma for Primary Sensory Cortex.

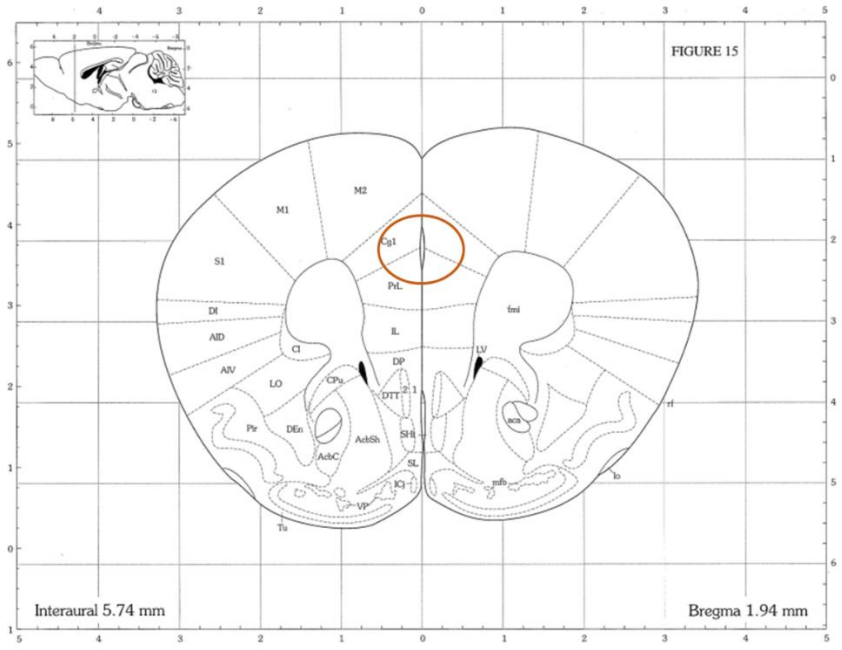

Figure 58. Anterior Cingulate Cortex coordinate form Mouse Brain Atlas. The circle identified the area of the prefrontal cortex we were interested to analyze (Franklin & Paxinos, George, 2008).

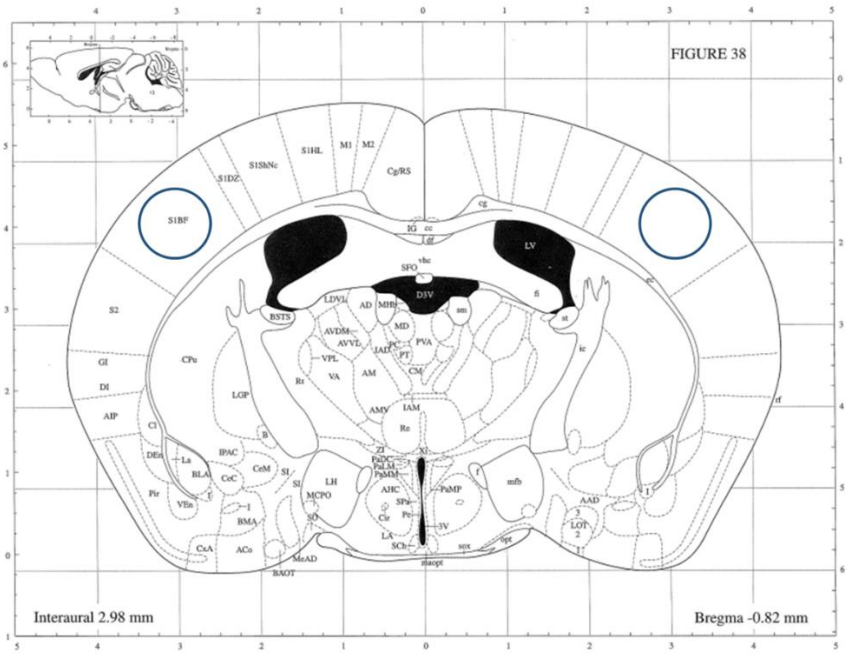

Figure 59. Primary Somatosensory Cortex coordinate form Mouse Brain Atlas. The circle identified the bilateral areas of the cortex we were interested to analyze (Franklin & Paxinos, George, 2008).

Finally, we acquired images using Neurolucida software (see *paragraph 61*), images were rebuilt using Imaris Software, and we calculated dendritic spine density of mice dividing the total numbers of spines counted for a single mouse for the total length of the dendrites of the mouse. For statistical analyses, we used T test and we accepted a significance lower of 5%.

RESULTS AND DISCUSSION

The analyses revealed that there is a connection between abnormality of the brain areas presented in 16p11.2 del mice and spine plasticity. Indeed, in Anterior Cingulate Cortex the dendritic spine density of 16p11.2 del mice (HT) was significantly reduced by 16.54% compared to wild type (WT) (t-test, Pvalue = 0.00012).

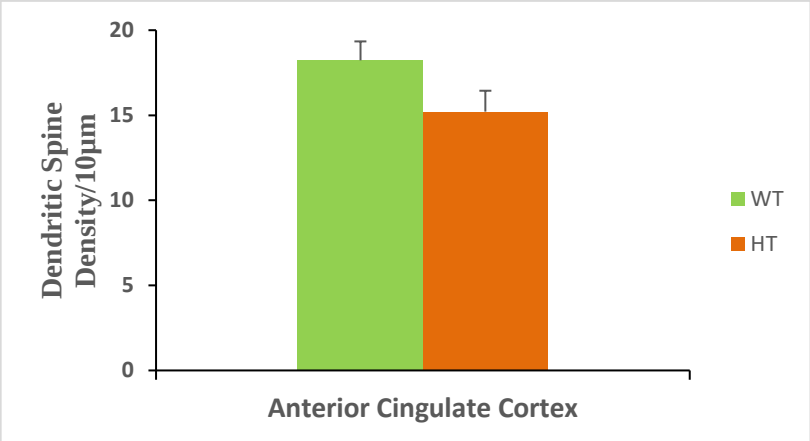

Figure 60. Dendritic spine density of Anterior Cingulate Cortex (Bregma 1.34mm) of WT mice (in green) and HT mice (in orange).

On the contrary, in the Primary Somatosensory Cortex the density of HT mice is comparable to those of WT ones: difference is lower than 0,5% (t-test, Pvalue = 0.68).

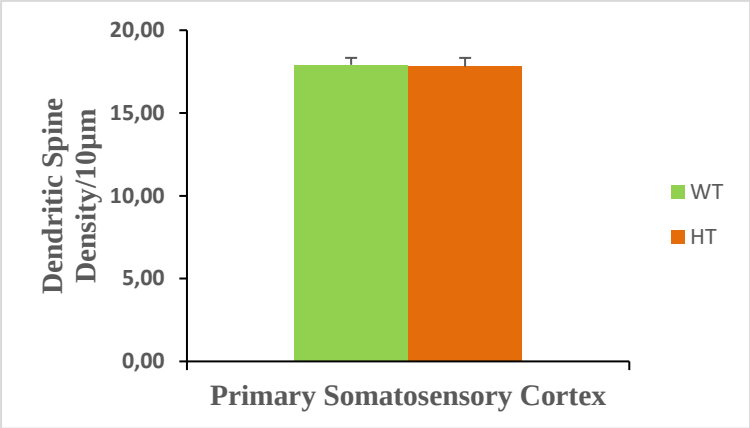

Figure 61. Dendritic spine density of Primary Somatosensory Cortex (Bregma -0.82mm) of WT mice (in green) and HT mice (in orange).

CONCLUSIONS

The results confirm the pathological characteristics of the 16p11.2 del mice. This model presents patterns similar to those of human autistic patients. The principal area affected in human and mice with 16p11.2 del is the anterior cingulate cortex. MRI revealed the abnormality of this area and our data showed that this alteration is associated with the strongly decrease of dendritic spine density. This data can be the indicator of a reduction of neuron connectivity in the area.

On the contrary, in the primary somatosensory area, where MRI revealed no difference, we didn't observe changing in dendritic spine density.

Consequently, we speculate that also in human, the abnormality observed in specific brain area can be linked to degeneration of neurons connection and activity like for other neurological pathology like Alzheimer's Disease.

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