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ROLE OF ASTROCYTES IN SYNAPTIC PLASTICITY AND MEMORY IN ANIMAL MODELS OF ALZHEIMER'S DISEASE

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Dissertação de Mestrado na área científica de Neurociências orientada pela Doutora Paula Agostinho e Doutora Paula Canas apresentada à Faculdade de Medicina da Universidade de Coimbra.

Faculdade de Medicina
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Role of astrocytes in synaptic plasticity and memory in animal models of Alzheimer's disease

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Dissertação de mestrado apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de mestre em Investigação Biomédica, realizada sob orientação científica da Doutora Paula Maria Garcia Agostinho (Universidade de Coimbra) e da Doutora Paula Margarida Gomes Canas (Universidade de Coimbra).

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“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.”

William Lawrence Bragg

Index

List of figures

List of tables

Abbreviations

Aβ- Amyloid-β protein

AβPP- Amyloid-β precursor protein

aCSF- Artificial cerebrospinal fluid

AD- Alzheimer's disease

AICD- AβPP intracellular domain

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

AMPAR- α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

ANOVA- Analysis of variance

AP- Antero-posterior

ATP- Adenosine triphosphate

BBB- Blood-brain barrier

CA- *Cornu Ammon*is

Ca^{2+}- Calcium ion

$[Ca^{2+}]i$- Intracellular calcium concentration

CaMKII- Calcium/calmodulin-dependent protein kinases

CREB- cAMP response element-binding protein

CSF- Cerebrospinal fluid

cm- Centimeter

CNS- Central Nervous System

CTR- Control

Cx43- Connexin 43

DG- Dentate gyrus

DV- Dorso-Ventral

EC- Entorhinal cortex

EPSPs- Excitatory post-synaptic potentials

fEPSP- Field excitatory post-synaptic potential

fAD- Familial Alzheimer's Disease

GABA- γ-amino butyric acid

GFAP- Glial fibrillary acidic protein

Glu- Glutamate

GS- Glutamine synthetase

h- Hours

HFS- High frequency stimulation

Hz- Hertz

icv- Intracerebroventricular

IHC- Immunohistochemistry

IP- Intraperitoneal

ITI- inter-trial interval

K^{+}- Potassium ion

KCl- Potassium Chloride

KH_2PO_4- Potassium dihydrogen phosphate

L- Lateral

L-AA- L-α-aminoadipic acid

LFS- Low frequency stimulation

LTD- long-term depression

LTP- Long-term potentiation

Mg^{2+}- Magnesium ion

mGluR- Metabotropic glutamate receptor

min- Minutes

mL- Mililiters

ms- Miliseconds

mV- Milivolt

Na^+- Sodium ion

NaCl- Sodium Chloride

Na_2HPO_4- Sodium phosphate dibasic

NH^{4+}- Ammonium

NFTs- Neurofibrillary tangles

NMDA- N-Methyl-D-aspartate

NMDAR- N-Methyl-D-aspartate receptor

NVU- Neurovascular unit

OF- Open Field

PBS- Phosphate buffer saline

PFA- Paraformaldehyde

PNS- Peripheral Nervous System

PS1- Presenilin-1

PS2- Presenilin-2

RE- Recording electrode

RT- Room temperature

s- Seconds

sAD- Sporadic Alzheimer's Disease

sAβPPα- soluble AβPPα fragment

sAβPPβ- soluble AβPPβ fragment

SC- Schaffer collaterals

SE- Stimulation electrode

SEM- Standard error of the mean

T- Temperature

TCA- Tricarboxylic acid cycle

α-CTF- C-terminal fragment

β-CTF- AβPP β-carboxyl-terminal fragment

Resumo

Os astrócitos, a maior população celular do cérebro, têm um papel importante no processamento e metabolismo neuronal e no controlo da barreira hematoencefálica. Evidências apoiam a existência de uma comunicação bidirecional entre neurónios e astrócitos no controlo da função cerebral, originando o conceito de "sinapse tripartida", que postula que os astrócitos são o terceiro elemento ativo das sinapses, modulando a plasticidade sináptica. A plasticidade sináptica, nomeadamente a potenciação de longa duração (LTP, "long-term potentiation") e a depressão de longa duração (LTD, "long-term depression") representam os mecanismos neurofisiológicos associados à memória, na qual o hipocampo tem um papel crucial. A doença de Alzheimer (DA) caracteriza-se por uma acumulação de placas amilóides extracelulares, compostas maioritariamente por péptidos β-amilóide (Aβ) e por tranças neurofibrilares constituídas por proteína tau hiperfosforilada. Assim, Aβ é considerado causar DA, levando às perdas e disfunção sináptica subjacentes aos défices cognitivos associados a esta patologia neurodegenerativa. No entanto, o papel dos astrócitos, nomeadamente na atividade sináptica na DA continua pouco esclarecido. O presente estudo pretende definir o impacto dos astrócitos na plasticidade sináptica hipocampal, particularmente em LTP e LTD, bem como avaliar marcadores astrocíticos, em condições não-patológicas e de DA. Para mimetizar a DA, as fatias de hipocampo foram incubadas com o peptídeo $A\beta_{1-42}$ solúvel (exposição aguda, 50 nM, durante 40 min) ou administrado intracerebroventricularmente (icv, 0,5 mM) em murganhos C57Bl/6 jovens adultos. Além disso, também foi usado o modelo animal triplo transgénico da DA, os murganhos 3xTgAD.

Realizaram-se registos de eletrofisiologia nos neurónios piramidais presentes na região CA1 na via proveniente dos colaterais de Schaffer e mediu-se LTD primeiramente em condições de exposição aguda a $A\beta_{1-42}$. Os resultados obtidos demonstram que a $A\beta_{1-42}$ teve um impacto robusto na amplitude de LTD, levando a um desvio de LTD para LTP. Para silenciar a contribuição dos astrócitos na modulação da plasticidade sináptica, utilizámos a gliotoxina L-α-aminoadipato (L-AA), previamente validada na indução da patologia astrocítica. A incubação de fatias do hipocampo com L-AA (100 µM, 2 h) não teve efeito na amplitude LTD em condições não-patológicas (controlo). No entanto, a exposição a L-AA em condições patológicas de DA reverteu significativamente os efeitos de $A\beta_{1-42}$ na plasticidade sináptica, levando a uma recuperação da amplitude de LTD.

Os animais injetados (icv) foram sujeitos a testes comportamentais para avaliar o desempenho de tarefas dependentes do hipocampo e, os murganhos injetados (icv) com $A\beta_{1-42}$ apresentaram défices significativos de memória quando comparados com os injetados com veículo. À semelhança dos efeitos observados em condições de exposição aguda a $A\beta_{1-42}$, a LTD e também LTP foram significativamente comprometidas em comparação com os murganhos injetados com veículo. Adicionalmente fez-se estudos imunohistoquímicos para identificar as proteínas astrocíticas, proteína acídica fibrilar glial (GFAP) e S100β, em cortes transversais de fatias do hipocampo de murganhos injetados (icv). Os resultados revelaram um aumento na imunorreatividade da GFAP em animais $A\beta_{1-42}$. Em concordância com os estudos de exposição aguda a $A\beta_{1-42}$, a incubação com L-AA apenas teve efeito na amplitude de LTD sob condições que mimetizam a DA ($A\beta_{1-42}$ icv), revertendo significativamente os efeitos de $A\beta_{1-42}$ na plasticidade sináptica, recuperando o prejuízo na LTD. Contrariamente, em condições não-patológicas o L-AA diminuiu significativamente a LTP, sendo este efeito menos evidente em condições de DA ($A\beta_{1-42}$ icv). Ademais, a gliotoxina (L-AA) aumentou a imunorreatividade de GFAP observado em condições controlo (veículo icv) e não tendo efeito significativo na reatividade dos astrócitos em condições de DA. Estes dados reforçam a interferência desta gliotoxina na função astrocítica e a ideia de que os astrócitos são cruciais para a regulação da plasticidade sináptica, em especial na LTD em condições de DA.

A plasticidade sináptica foi avaliada em murganhos 3xTgAD (11 meses) onde se observou uma tendência para um aumento na amplitude LTP comparado com murganhos não transgénicos (nonTg). Além disso, as fatias de hipocampo de murganhos 3xTgAD exibiram uma intensa reatividade astrocítica avaliada pela imunorreatividade de GFAP nas regiões CA1 e CA3. A disfunção astrocítica induzida por L-AA pareceu reverter as alterações em LTP observadas em 3xTgAD. Em fatias de ambos os grupos de animais a incubação com L-AA causou um aumento da GFAP.

Este trabalho apresenta fortes evidências de que os astrócitos estão disfuncionais em condições de DA, comprometendo a plasticidade sináptica hipocampal e a memória, suportando o conceito de que os astrócitos podem ser um apropriado alvo para o desenvolvimento de novos tratamentos para a DA.

Palavras-chave: Astroglia, gliotoxina, hipocampo, plasticidade sináptica, péptido β-amilóide.

Abstract

Astrocytes, the largest cell population in the brain, have a key role in neuronal function and metabolism and in the control of blood brain barrier. Increasing evidences support the existence of a bidirectional communication between neurons and astrocytes in the control of brain function, giving rise to the concept of 'tripartite synapse', which postulates that astrocytes are the third active element of synapses with the capacity of fine-tune synaptic plasticity. The synaptic plasticity, such as the long-term potentiation (LTP) and long-term depression (LTD), constitute the neurophysiological mechanisms of memory, a process in which hippocampus has a key role. Alzheimer's disease (AD) is characterized by the accumulation of extracellular amyloid plaques, composed mainly by amyloid-β (Aβ) peptide, and by neurofibrillary tangles constituted by hyperphosphorylated tau protein. Aβ peptides are considered to be a causative agent of AD, which can cause synaptic loss and dysfunction that underlie the cognitive and memory deficits associated to this neurodegenerative disorder. However, the role of astrocytes, namely on synaptic function under AD conditions, is still not completely understood. The present study aims to define the impact of astrocytes on hippocampal synaptic plasticity, in particular on LTP and LTD, as well as to evaluate the astrocytic markers in physiological and AD-like conditions. To mimic AD-like conditions, hippocampal slices were incubated with soluble $A\beta_{1–42}$ (acute exposure, 50 nM, for 40 min) or administrated intracerebroventricularly (icv, 0.5 mM) in young adult C57Bl/6 mice. Moreover, a transgenic AD animal model, 3xTgAD mice, was used.

Electrophysiological recordings in the Schaffer collaterals-CA1 pyramidal synapses were performed and LTD was first measured under acute exposition to $A\beta_{1–42}$. The data obtained showed that $A\beta_{1–42}$ had a robust impact on LTD amplitude, leading to a shift of LTD toward LTP, compared with control mice. To silence astrocytic contribution in shaping synaptic plasticity we used the gliotoxin L-α-aminoadipate (L-AA), previously validated to induce astrocyte pathology. Incubation of hippocampal slices with L-AA (100 μM, 2 h) had no effect on LTD amplitude in non-pathological conditions (control). However, treatment with L-AA under AD-like conditions significantly reverted the effects of $A\beta_{1–42}$ on synaptic plasticity, rescuing LTD impairment.

The icv-injected mice were behaviorally characterized using hippocampal-dependent tasks, and it was observed a memory deficit in icv $A\beta_{1-42}$ mice when compared

with vehicle mice. Similarly, to that observed in hippocampal slices acutely exposed to $A\beta_{1–42}$, in icv $A\beta_{1-42}$ injected mice the hippocampal LTD and also LTP were significantly compromised, as compared with icv-vehicle mice. Furthermore, immunohistochemical analysis probing for astrocytic markers, such as the glial fibrillary acidic protein (GFAP) and S100β were performed in transverse hippocampal sections obtained from slices of icv-vehicle and $A\beta_{1–42}$ injected mice. The results showed an increase in GFAP immunoreactivity in slices from icv $A\beta_{1–42}$ injected mice. In agreement with our data obtained in conditions of acute $A\beta_{1–42}$ exposure, the incubation of hippocampal slices with the gliotoxin (L-AA) had no effect on LTD in control conditions. However, under AD-like conditions (icv injection), L-AA significantly reverted the effects of $A\beta_{1–42}$ on synaptic plasticity, rescuing LTD impairment. By contrast in non-pathological conditions, the LTP was significantly decreased by L-AA, being this effect less evident in icv $A\beta_{1–42}$ injected mice. Moreover, it was observed that this gliotoxin (acute exposure) increased hippocampal GFAP immunoreactivity in physiological conditions, but in AD-like conditions decrease the effect was the opposite. These data reinforce that indeed this gliotoxin was interfering with astrocytes function and these glial cells had a prominent role in shaping synaptic function, contributing to synaptic plasticity impairment, mainly LTD, in AD-like conditions.

We also evaluated the hippocampal synaptic plasticity in the 3xTgAD mice (with 11 months old) that showed a tendency to an increase in hippocampal LTP amplitude compared with littermates non-transgenic (nonTg) mice. Moreover, the slices from 3xTgAD exhibited an increased astrocytic reactivity, assessed by GFAP immunoreactivity in CA1 and CA3 regions of hippocampus. The astrocytes blunting triggered by L-AA tend to rescue the LTP alterations observed in 3xTgAD. The incubation of LAA of hippocampal slices from both 3xTgAD and nonTg mice seemed to cause an increase in GFAP immunoreactivity.

Overall, the current work demonstrates strong evidences that in AD-like conditions, the astrocytes are dysfunctional, impairing hippocampal synaptic plasticity and memory. Thus, these studies support that astrocytes can be viewed as a valid target for the development of novel treatments for AD.

Keywords: Astroglia, gliotoxin, hippocampus, synaptic plasticity, Amyloid-β peptide.

Chapter 1

Introduction

Introduction

1.1. Central Nervous System (CNS) and peripheral nervous system (PNS)

The nervous system coordinates its actions by the acquisition and processing of information and its response, giving rise to generating movements and other behaviors. These characteristics allow the nervous system to be simultaneously a sensory system and a motor system (Purves et al. 2004; Ghose et al. 2012). In addition to this distinction based on functional characteristics, the vertebrate nervous system can be divided into Central Nervous System (CNS) and Peripheral Nervous System (PNS), according with its anatomical characteristics (Ghose et al. 2012). The CNS comprises the brain (cerebral hemispheres, diencephalon, cerebellum, and brainstem) and the spinal cord. PNS consists mainly of sensory and motor neurons (figure 1) (Purves et al. 2004; Kandel et al. 2013).

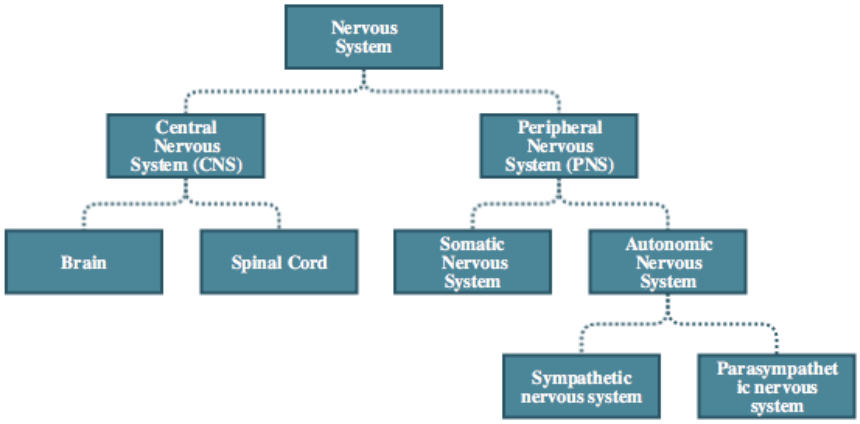

Figure 1. Simplified schematic representation of the Nervous System. The CNS consists of brain and spinal cord that connects brain and the PNS. PNS carries messages to and from CNS and its divided into somatic nervous system, which controls voluntary muscles and transmits sensory information to the CNS; and automatic nervous system that can control involuntary body functions. The last one comprises the sympathetic nervous system and parasympathetic nervous system, which arouses body to expend energy and appeases body to conserve and maintain energy, respectively (Adapted from Dietrich and Dragatsis 2016).

The brain requires special care because it is a very sensitive and important part of the human body. Thus, as a form of brain protection and maintenance are present the bony skull, meninges, cerebrospinal fluid (CSF) and blood-brain barrier (BBB). The bony skull is a crucial structure of human body that provides protection from injury and limits its expansion. Another important structure, the meninges, which are located between the skull and brain, consist of three layers of tissue that cover and protect the brain and spinal cord. Furthermore, the brain is bathed by CSF, a nutrient-rich fluid that provides brain protection. This fluid can handle the excretion of harmful substances away from the brain

and maintain the pressure at the base of the brain (Liddelow 2011; Brinker et al. 2014). CNS requires an extremely regulated extracellular environment which is achieved through the maintenance of ions concentration such as sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) at very narrow ranges (Hawkins and Davis 2005; Banerjee and Bhat 2007). Simultaneously, is of utmost importance the existence of an interface between the CNS and the peripheral circulatory system, which regulates dynamically the ionic balance, facilitates the transport of nutrients and at the same time acts as a barrier to potentially harmful molecules, forming a semipermeable membrane designated as BBB (Liddelow 2011; McConnell et al. 2017). The maintenance of cerebrovascular function implies a series of dynamic interactions between key elements that are of the utmost importance. These elements are the endothelial cells, astrocytes, pericytes, myocytes, neurons and the extracellular matrix, which together constitute a "neurovascular unit" (NVU) (figure 2) (Hawkins and Davis 2005; Eng and Gary 2009). This NVU influences the properties of BBB and has great relevance in CNS regulation and synaptic activity (Muoio et al. 2014).

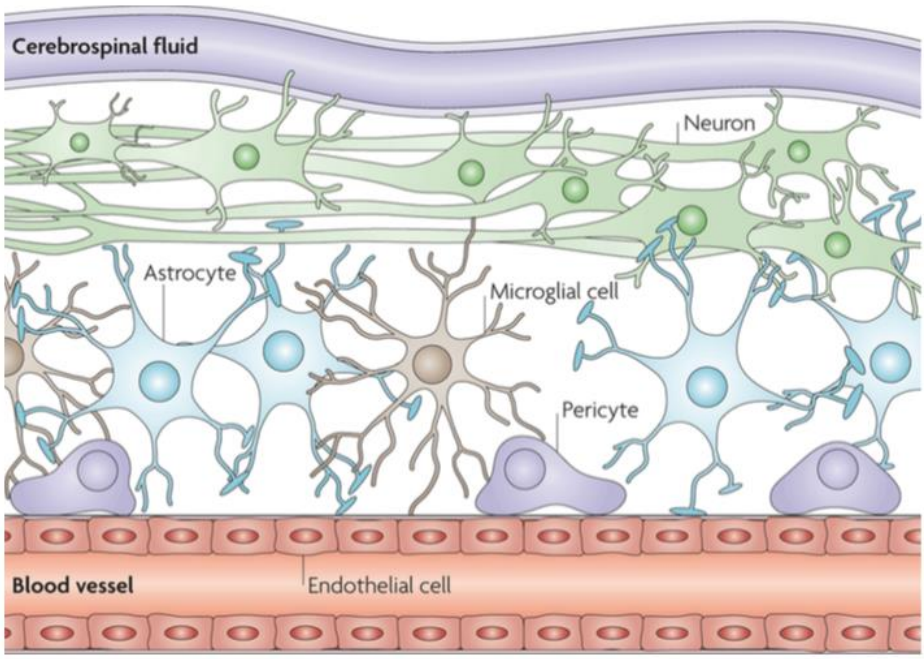

Figure 2. The blood-brain barrier. The blood-brain barrier is formed by brain microvascular endothelial cells, astrocytes and pericytes. It maintains the neural microenvironment by regulating the passage of molecules into and out of the brain, and protects the brain from any microorganisms and toxins that are circulating in the blood (Whitaker et al. 2008).

1.2. Cellular components of CNS

Considering the entire investigation of Cajal, Golgi and many other scientists who followed their research, it was realized that the nervous system could be broadly divided into two types of cells: nerve cells (neurons) that are specialized in electrical signaling through long distances, and neuroglia (glial cells) which are supporting cells for the organism and play important functions in developing and in adult brain (Purves et al. 2004; Kandel et al. 2012).

1.2.1. Neuronal cells

Neurons are essential elements in the CNS, since they are specialized cells in intercellular communication. This important function is related to their overall morphology, the specific organization of membrane components for electrical signaling and the functional and structural complexity of synapses (Squire et al. 2008). These neuronal cells have a complex arborization of dendrites that extend from the neuronal cell body promoting their specialization in neuronal communication through electrical signals. Dendrites are responsible for receiving and integrating information from other neurons, and the complexity of inputs received from a neuron depends on the complexity of its dendrites (McAllister 2007). Neurons have different functional roles in the brain, according to their size, shape, neurochemical characteristics, location and connectivity. Thus, neurons can be divided into different groups: inhibitory neurons, excitatory neurons, and neuromodulatory neurons. These neurons form circuits that are essential for the maintenance of neuronal function (Squire et al. 2008; Yuste 2015).

Between two adjacent neurons, an extracellular space is formed between the pre- and post-synaptic elements called synaptic cleft. These pre- and post-synaptic elements communicate through the secretion of molecules from the presynaptic terminal that bind to receptors present in the post-synaptic terminal (figure 3), being these synaptic contacts of extreme relevance for normal neuronal functioning. The information sent by synapses on the neuronal dendrites is integrated and "read out" at the origin of axon - specialized for signal conduction to the next site of synaptic interaction (Purves et al. 2004; Pereda 2014). To carry signals at long distances, there is an electrical event, called an action potential, which through a wave of electrical activity that propagates from its point of initiation at the dendrites to the terminus of the axon where synaptic contacts are made, allows this information to go through. The synapses can be distinguished in two different types: chemical and electrical, in which the information is transmitted through these

action potentials described before. The electrical synapses, the least represented in the nervous system, have the pre- and post-synaptic terminals connected through specialized channels, gap junctions, that allow a direct connection between the cytoplasm of the adjacent neurons. In addition, they allow the passage of molecules, such as ions, ATP or intracellular metabolites, between the two cells, representing a faster signal transmission (Pereda 2014). In the chemical synapses, synaptic vesicles with neurotransmitter molecules are secreted from the presynaptic terminals. These neurotransmitters alter the electrical properties of the target cell through binding to their receptors, located at the post-synaptic terminal (Kandel et al. 2012).

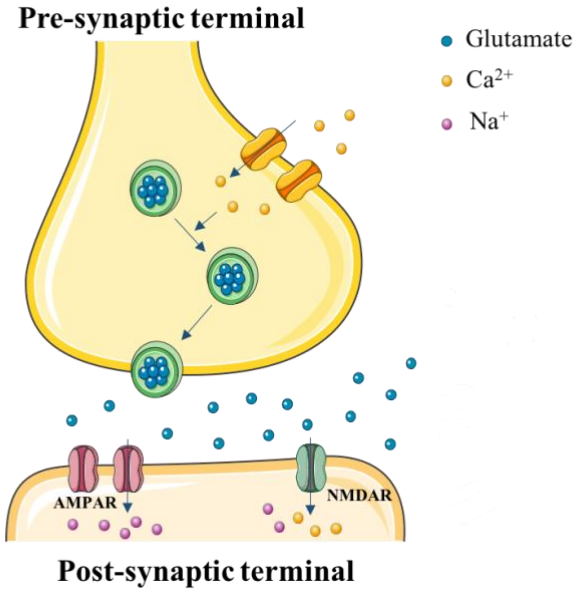

Figure 3. Illustrative representation of chemical synapse. The extracellular space between two adjacent neurons, called as synaptic cleft, are composed by pre- and post-synaptic elements. These elements communicate with each other through the secretion of molecules, and this communication is of extreme importance for normal neuronal functioning.

1.2.2. Glial cells

Glial cells are the large majority of the cells constituting the nervous system, (Jäkel and Dimou 2017), existing a ratio of 3 glial cells to 1 neuron in rodents (Purves et al. 2004) and in human this ratio becomes 9:1, favoring the glial cells (Harris 2013; Rose and Kirchhoff 2015). This means that increased brain complexity is accompanied by an increase in the ratio of glial cells to neurons. Accordingly, it is described that these cells participate actively in brain homeostasis (Barres 2008; von Bernhardi et al. 2016) and defense against pathological insults (Purpura et al. 2012). Currently, it is considered the existence of three types of glial cells in CNS – microglia, astrocytes, and

oligodendrocytes (figure 4) – that are classified into two main groups: microglia and macroglia (Freire et al. 2007; Squire et al. 2008).

Microglia are found in all regions of the brain, amounting about 5-20% of the total number of cells in the brain (Squire et al. 2008); and represents the innate immune system and the first line of defense of the CNS, being also the major players in its development. However, aberrant function of microglia, usually associated with the production of pro-inflammatory and neurotoxic factors are associated with several brain pathologies (Ladeby et al. 2007; Squire et al. 2008). Currently, it is accepted that this type of cells are originated from yolk sac - derived macrophages and share many features with the adult macrophages of peripheral immune system (Ginhoux and Prinz 2015). Microglia, as well as macrophages, eliminate cell debris from sites of injury, secrete signaling molecules (cytokines) to modulate inflammation and influence cell death or life (Gehrmann et al. 1995; Pivneva 2008). The macroglia cells also include oligodendrocytes and astrocytes (also named as astroglia) in the CNS. Oligodendrocytes provide a coating around some axons - called myelin - that is important for the efficiency of electrical signal transmission (Simons and Nave 2015). The other cells constituting the macroglia, the astrocytes, have a star-like morphology ("astro") and are responsible for maintaining a suitable chemical environment for neuronal signaling and survival (Stipursky et al. 2011).

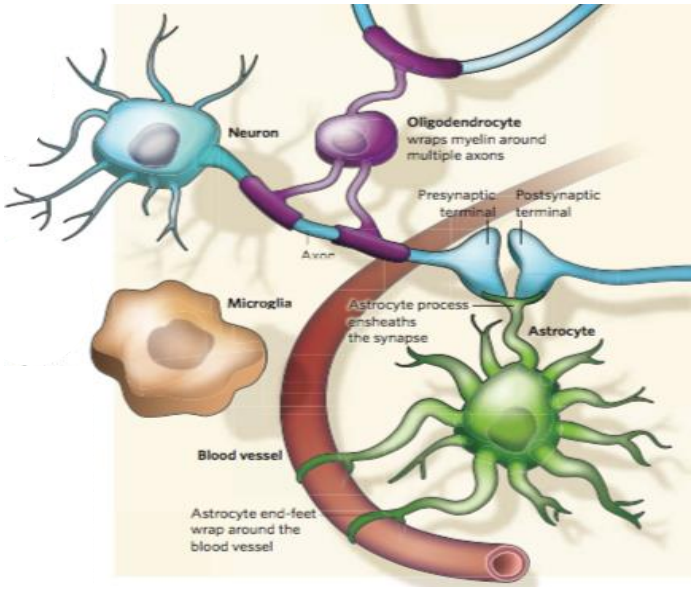

Figure 4. Glia-neuron interactions. Different types of glia interact with neurons and the surrounding blood vessels. Oligodendrocytes wrap myelin around axons to speed up neuronal transmission. Astrocytes extend processes that ensheath blood vessels and synapses. Microglia keep the brain under surveillance, providing protection against damage or infection, although the excessive activation of these cells can be harmful (Allen and Barres 2009).

1.2.3. Astrocytes: multitasking cell

Astrocytes are the largest cells in the brain, having a star-shaped morphology with numerous processes surrounding neighboring neurons and blood vessels, and constitute about 20-50% of the volume of most brain areas (Squire et al. 2008; Wang and Bordey 2008). These cells have complex morphologies and can be divided into three large groups based on their morphology, antigenic phenotype and location: i) protoplasmic; ii) fibrous and iii) radial, which are located mainly in the gray matter, white matter and in the periventricular space, respectively (Barres 2008; Squire et al. 2008; Sofroniew and Vinters 2010). Protoplasmic astrocytes have many branching processes that are responsible for the encircle of synapses and blood vessels, whereas fibrous (or fibrillar) astrocytes have long, thin, unbranched processes whose envelop nodes of Ranvier (Barres 2008; Wang and Bordey 2008). The radial glia control neuronal migration during development, and in the adult brain they are present as Müller cells of the retina or Bergmann glia of the cerebellum (Sofroniew and Vinters, 2010).

In the nervous system, astrocytes have critical functions which include: i) promotion of neuronal maturation and survival, ii) modulation of synaptic activity through the release of gliotransmitters and neuroactive molecules uptake (e. g. glutamate and GABA), ii) formation and remodeling of synapses, iv) promotion of neuronal survival during development, v) angiogenesis regulation, vi) defense against to toxic and traumatic insults of CNS, vii) metabolic support and viii) maintenance of favorable microenvironment for neurons (some astrocytic roles showed in figure 6) (García-Lopez et al. 2007; Wang and Bordey 2008; Purpura et al. 2012; Araque et el. 2014). Thus, these glial cells are known for their ability to synthetize and secrete several molecules, such as growth-promoting molecules (e. g. like N-cadherin), growth factors (e. g. brain-derived neurotrophic factor (BDNF)) or proteolytic enzymes (e. g. matrix metalloproteinases (MMPs)) that can control neuronal maturation and survival (reviewed in Wang and Bordey 2008). On the other hand, astrocytes can also remove neurotransmitters and ions that are released at high concentration to the synaptic cleft during the neurotransmission, such as glutamate (Belanger et al. 2011). These astroglial cells can also actively participate to brain responses to injury, through a process called astrogliosis (subject developed in section 1.3.3.3), which involves morphological and functional changes of these glial cells (Sofroniew 2015). Furthermore, increasing evidences show that these glial cells have the ability to maintain brain homeostasis, since astrocytes can clear extracellular space and it can provide the substrates necessary for neuronal function, such

as the supply of energy rich metabolites (Escartin et al. 2006). It is important to emphasize that astrocytes and neurons form a complex and crucial relationship that maintenance neuronal function and support brain metabolism (Bélanger et al. 2011). Astrocytes can also actively contribute to synaptic activity through the release of neuroactive factors, such as adenosine and ATP, which can also control blood flow, suggesting that these cells are not only passive elements in the maintenance of extracellular space but rather active participants in brain activity (reviewed in Wang and Bordey 2008; Suzuki et al. 2011).

Astrocytes contain glial fibrils, namely glial fibrillary acidic protein (GFAP), a type III intermediate filament protein (Jacque et al. 1978), which can be also expressed by other cells, such as ependymal cells during development (Roessmann et al. 1980); immature neurons (Liu et al. 2010); glomeruli and peritubular fibroblasts from rat kidneys (Buniatian et al. 1998) and stellate cells of pancreas and liver in rats (Apte et al. 1998); Leydig cells from testis in both hamsters and humans (Maunoury et al. 1991; Davidoff et al. 2002); human keratinocytes (von Koskull 1984), osteocytes and chondrocytes (Kasantikul and Shuanghoti 1989). However, this protein still is widely used as an astrocytic marker. Another astrocytic marker used is S100β that belongs to the S100 family of EF-band calcium binding protein that is produced and secreted to extracellular space by astrocytes. The secreted S100β exerts trophic or toxic effects depending on its concentration; however, high S100β levels in biological fluids (e. g. CSF) are considered a biomarker of pathological conditions, including neuroinflammatory process and neurodegenerative disorders, such as Alzheimer´s disease (AD) (Baudiers et al. 1986; Sen and Belli 2007; Michetti et al. 2012; Khakh and Sofroniew 2015).

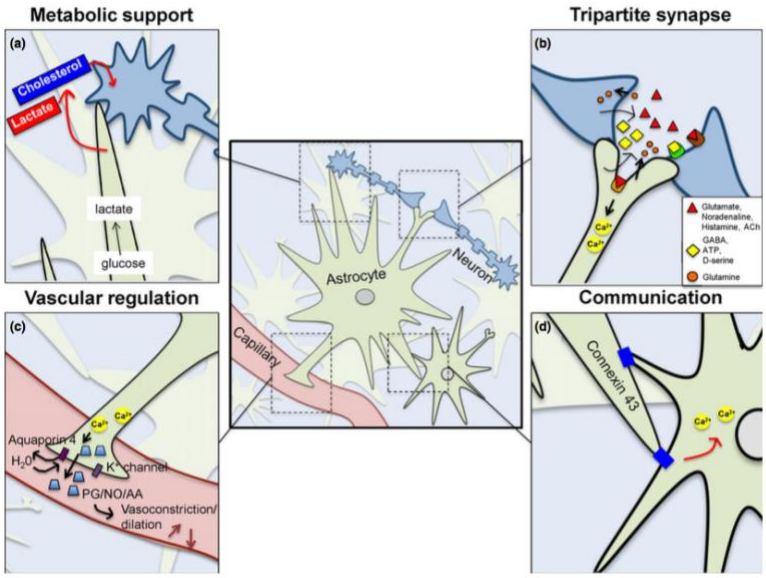

Figure 5. The many roles of the astrocyte: (A) Astrocytes provide energy substrates for neurons, primarily in the form of lactate which is converted from glucose as well as providing cholesterol to support neuronal function. **(B)** The intimate association of astrocytic processes with neuronal synapses enables the finely tuned regulation of synaptic transmission. Neurotransmitters are released from the presynaptic terminal such as glutamate, that is capable of binding to post-synaptic receptors or to receptors present on the surface of astrocytic processes. This binding can trigger a calcium response in the astrocyte that results in the release of gliotransmitters such as GABA, ATP or D-serine that will regulate synaptic activity. Glutamate can also be recycled back to the precursor glutamine, which is released into the extracellular space and taken up by neurons to be resynthesized into glutamate. **(C)** Astrocytes couple together synaptic activity with local blood flow to ensure neurons have a sufficient energy supply. **(D)** Gap junctions such as connexin 43 (Cx43) enable dialogue between neighbouring astrocytes, which is mediated by intracellular changes in calcium levels (Adapted from Garwood et al. 2017).

1.2.3.1. The third active element of synapses: astrocytes

The knowledge of astrocyte functions, especially the involvement in their function of synapses, suggested the existence of a bidirectional communication between astrocytes and neurons, with an impact on behavior expression (Achour and Pascual 2010; Oliveira et al. 2015). Astrocytes respond to a wide range of neurotransmitters and neuromodulators such as glutamate, GABA, noradrenaline, acetylcholine, and adenosine triphosphate (ATP) (Haydon and Carmignoto 2006), through the elevation of their internal Ca^{2+} concentration ($[Ca^{2+}]$). Thus, astrocytes control neuronal excitability through release of neuroactive factors (a process named gliotransmission), such as glutamate, GABA, ATP, taurine, D-serine and cytokines, which are responsible for the control of synaptic plasticity a process that regulate brain responses, such as memory (Robitaille et al. 2014; Oliveira et al. 2015; Petrelli et al. 2016; Verkhratsky et al. 2016; Sardinha et al. 2017).

The release of gliotransmitters can occur through several mechanisms that include: i) hemichannels constituted mainly by Cx43 assembles (Torres et al. 2012; Chever et al. 2014; Orellana et al. 2014); ii) reverse operation of plasma membrane glutamate

transporters (Malarkey and Papura 2008); iii) Ca^{2+}- dependent exocytosis (Parpura and Zorec 2010; Khakh and McCarthy 2015); iv) ionotropic purinergic receptors and v) anion channel opening induced by cell swelling (Malarkey and Papura 2008).

All these observations were crucial for the perception of the importance of astrocytes as a third level of information integration in neural networks, thus leading to the creation of the concept of "tripartite synapse" (Robitaille et al. 2014; Araque et al. 1999). Later on, another element, the microglia, was added to the concept of "tripartite synapse" giving rise to the "quad-partite synapse" (figure 6) (Schafer et al. 2013). Microglia are considered the cells responsible for innate immunity of the brain resembling the immune cells that migrate to the injury sites and elicit inflammatory responses through the release of cytokines and chemokines, becoming activated (Perry and Gordon 1988). In this new concept, microglia dynamically and regularly interact with neurons and astrocytes, having a role in controlling the brain homeostasis (Raivich 2005; Hanisch and Kettenmann 2007; Cherry et al. 2014).

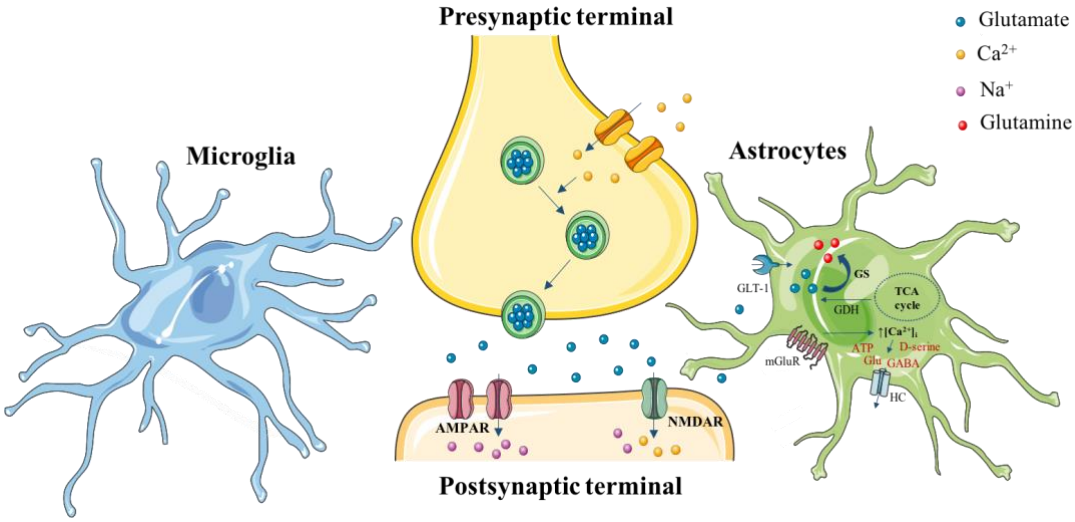

Figure 6. Representation of "Quad-partite" synapse. The processing of information in synapses is not only defined by neurons, but also by glia cells, namely by astrocytes, which enwrap synapses, and microglia, dynamically interacting with synapses in an activity-dependent manner. Thus, amongst other roles, astrocytes regulate the basal levels of glutamate, defining the basal excitability of neurons; also the extracellular levels of glutamate contribute to define the density of NMDARs and AMPARs in the plasma membrane of the post-synaptic compartment, which trigger and sustain alterations of synaptic plasticity, respectively. The excitability of neurons, their set-up of plasma membrane glutamate receptors, namely of NMDARs provided by glial cells are critical to allow the implementation of adequate synaptic plasticity traits, i.e., an appropriate encoding of both long-term potentiation (LTP) and depression (LTD) according to specific patterns of incoming information (adapted from Rial et al. 2016).

Glutamate is a neurotransmitter of great relevance to astrocytes, insofar as this amino acid is captured by the astrocytes (90% of glutamate that is released into the extracellular space between neurons), where it is metabolized into glutamine and further taken up by the neurons to synthetize *de novo* glutamate, forming the glutamate-glutamine cycle. This metabolic pathway involving a neuronal-astrocyte cross-talk is crucial to maintain the adequate supply of glutamate to CNS, and involves a sequence of events; briefly, glutamate is captured and metabolized by astrocytes into glutamine by glutamine synthetase, the glutamine released in the extracellular space, being taken up by neurons, mainly at presynaptic terminals, for *de-novo* synthesis of glutamate through the action of glutamine (Schousboe et al. 1995; Sonnewald et al. 1997; Palacín et al. 1998). Thus, this neurotransmitter allows the astrocytes to modulate neuronal excitability and synaptic functions (Bezzi et al. 2004; Singh and Abraham 2017).

Glutamate, which is released in high amounts by glutamatergic neurons, can affect astrocytes via metabotropic glutamate receptors (mGluR), ionotropic glutamate receptors of the sub-type N-methyl-D-aspartate (NMDARs), **α**-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPARs) receptors and kainate receptors (Pinheiro and Mulle 2006; Bains and Oliet 2007). Glutamate can triggers a wave of intracellular calcium changes in astrocytes (Cornell-Bell et al. 1990), leading to alterations at the synapse level that can modulate synaptic activity. Indeed, astrocytic calcium waves can influence neuronal responses and control synaptic strength by the release of gliotransmitters, such as glutamate, GABA and ATP (Haydon and Carmignoto 2006) through exocytosis, involving the fusion of SNARE-proteins of vesicles with the cellular membrane (Nedergaard 1994; Araque et al. 1999; Genoud et al. 2006).

All these facts suggest that astrocytes play an essential role in the regulation of synaptic force, thus contributing to brain communication processes, namely to cognitive processes like memory and learning and also to other behavioral domains, like emotion, motor, and sensory processing (Purpura et al. 2012; Oliveira et al. 2015; Sardinha et al. 2017).

1.2.3.2. Pharmacological tools to interfere with astrocytes function

Numerous efforts have been made to make it possible to understand astrocyte activity. However, the study of astrocyte function *in vivo* is a difficult task, since it is extremely difficult to separate the astrocytic component from the output of the neuron-astrocyte network. In order to overcome this difficulty, neuroscientists have tried to taking advantage of specific pharmacological and/or genetic tools that can manipulate astrocyte functions in animal models (Oliveira et al. 2015).

α-aminoadipic acid (AA) is the best-described toxin associated with astrocytes ablation or blunting. This toxin is a six-carbon amino acid, a homolog of glutamate that differs only by a single methylene group and it is an intermediate in the metabolism of lysine that exists naturally in the brain (Chang 1978; Chang 1982). Olney and colleagues (1971) proposed that AA was a selective toxin for astrocytes during the neurotoxicity evaluation of a number of compounds. More specifically, they noticed that AA induced changes in only one cell type of the retina - the Müller cell. **α**-aminoadipic acid has two isomers: L-**α**-aminoadipic acid (L-AA) and D-**α**-aminoadipic acid (D-AA) that are both gliotoxins, although L-AA is more toxic than D-AA (Bridges et al. 1992). Furthermore, it was shown that *in vivo* administration of L-AA had selective toxicity for astrocytes, but no effect on the survival neurons (Khurgel et al. 1996; Building et al. 1998). The gliotoxin is taken up by transport, which exists mostly in astrocytes, and once inside the astrocytes inhibits the glutamine synthetase (GS) – an enzyme crucial for the glutamate metabolism (figure 7) and, consequently interfere with glial metabolism (Huck et al. 1984; McBean 1994; Tsai et al. 1996). Taking these characteristics into account, L-AA was the gliotoxin chosen to blunting astrocytes in our study.

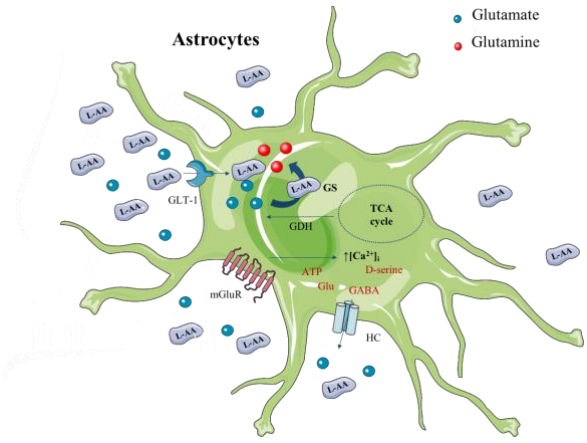

Figure 7. Pharmacological tool to astrocytes blunting. L-AA was a selective toxin proposed to blunting astrocytes, since it interferes with astrocytes metabolism, by causing a significant reduction in the activity of the glial enzyme, glutamine synthetase (GS) – an enzyme crucial for the glutamate metabolism.

1.3. The hippocampus: a brain structure crucial to memory

Many different mammalian forebrain systems own circuits with different architectures, proposing different functions even though can act together for the same purpose (Lynch et al. 1996). One of these brain systems – the limbic system – includes the hippocampus that represents the region involved in learning, memory and spatial navigation in rodents and in humans (Burgess et al. 2002; Anand and Dhikav 2012; Voss et al. 2017).

The hippocampus – named assigned due to its shape (*seahorse* in Latin) - is one of the most widely studied structure in the brain since 1957 when a patient lost the ability to form memories after surgical removal of the hippocampus (Scoviille and Milner 1957). After this discovery, numerous studies have been conducted in order to expand the knowledge about the neurobiological basis of memory (Scoville and Milner 1957; Knierim 2015).

1.3.1. Hippocampal anatomy and circuitry

The hippocampal formation of the mammalian brain is a cortical structure present in the medial portion of temporal lobe comprised by entorhinal cortex, dentate gyrus, "*Cornu Ammon*" (CA) areas and subiculum (O'Mara 2005; Lavenex et al. 2014).

In the hippocampus, two parallel circuits have been described: the trisynaptic and the temporoanatomic circuit. The trisynaptic circuit, which is composed by EC layer II projections to the DG, which relay activity to CA3 through the mossy fibers and then, CA3 neurons project to CA1 (Layer II – DG - CA3 - CA1) to finally reenter in the deep layers of EC. In this circuit CA3, neurons also receive a direct projection from EC layer II. The other circuit, the temporoanatomic circuit, in which CA1 neurons receive a direct projection from EC layer III (Layer III - CA1). These different circuits have been proposed to contribute for shaping learning and memory processes (Witter et al. 2013; López-Madrona et al. 2017).

The entorhinal cortex mediates the reception of the information and send it to be processed by the hippocampus, establishing bidirectional connections with the subicular complex. Through this complex system, information is sent from the hippocampus to the several cortical regions, which act plays a key role in memory and cognition (Takehara 2014; Witter et al. 2017). Distinct subregions can be distinguished in the hippocampus, also called CA by Rafael Lorente de Nó (1934), that comprises CA1, CA2, CA3 and CA4 regions (van Strien et al. 2009; Schultz and Engelhardt 2014). These hippocampal

subregions are constituted essentially by pyramidal cells (figure 8). From the ventricular surface to the DG, the hippocampus formation can be sectioned into an external plexiform layer – which contains axons of pyramidal cells and hippocampal afferent fibers; *stratum oriens* – which contains basal dendrites of the pyramidal cells and several types of interneurons; pyramidal cell layer – which contains the pyramidal cells of the hippocampus; and finally, the *stratum lucidum*, the *stratum radiatum* and *stratum lacunosum-moleculare* were located, which contain the apical dendrites and terminal branches of pyramidal neurons, respectively and hippocampal afferents from the EC. *Stratum lucidium* is only present in CA3 region, constituted by mossy fibers that travel and form synapses with proximal dendrites above the pyramidal neurons of CA3 (Schultz and Engelhardt 2014). What easily identifies the hippocampal region is its shape where the pyramidal cells are arranged in a C-shaped, which interconnect with the C-shaped arrangement of the DG (Schultz and Engelhardt 2014).

The pyramidal cells of the CA3 region have branched axons that project from the hippocampus to the other structures, but also organize into collateral fibers that form synapses with the dendrites of pyramidal cells in the CA1 region. These synapses are glutamatergic excitatory (use glutamate as a neurotransmitter) and allow the intrinsic flow of information thus forming a circuit (Schultz and Engelhardt 2014). The hippocampus organization on slices enables the selective stimulation of Schaffer collaterals and the recording of the evoked responses in the pyramidal cells of the CA1 region. Most of the long-term plasticity studies, such as long-term potentiation (LTP) and long-term depression (LTD), have focused on these synapses, since the electrical stimulation of Schaffer collaterals generates post-synaptic excitatory potentials in the cells of the CA1 region (Lynch et al. 1996; Broadbent et al. 2004; Anand and Dhikav 2012; Song et al. 2012; Schultz and Engelhardt 2014; Knierim 2015).

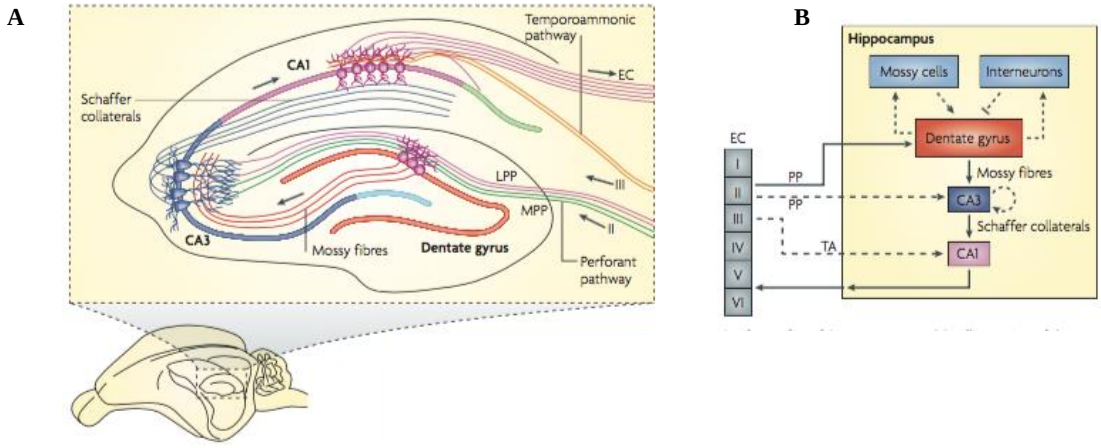

Figure 8. The neural circuitry in the rodent hippocampus. A. An illustration of the hippocampal circuitry. **B.** Diagram of the hippocampal neural network. The traditional excitatory trisynaptic pathway (entorhinal cortex (EC)–dentate gyrus–CA3–CA1–EC) is depicted by solid arrows. The axons of layer II neurons in the entorhinal cortex project to the dentate gyrus through the perforant pathway, including the lateral perforant pathway and medial perforant pathway. The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibers. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons through the PP. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway. The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells. (Adapted from Deng et al. 2010).

1.3.2. Hippocampal synaptic plasticity and memory

Nowadays, it is known that neurons do not perform their functions alone, they organize themselves in ensembles or neural circuits that process information and provide the formation of sensations, perception, and behavior (Kandel et al. 2012). During the development of neural circuits, the nervous system has the capacity to change and this characteristic is denominated as neuronal plasticity. However, this plasticity is also found in adult brains with the purpose of learning new skills, establishing new memories and responding to injuries throughout life. Neurons are in continuous activity, receiving and sending stimulus, and thus their function are always changing, and although the mechanisms responsible for these changes are not completely defined they are thought to be a consequence of changes in synaptic strength (Purves et al. 2004; Squire et al. 2008).

Synaptic activity in CNS presents different patterns that induce different changes in synaptic strength: a high frequency stimulation can induces a long-term potentiation (LTP), whereas a low frequency stimulation induce a long-term depression (LTD) (Kronberg et al. 2017). The most widely studied form of activity-dependent change in synaptic force is the "Hebbian" plasticity, proposed by Donald Hebb in 1949. These forms of plasticity have been mostly studied as the cellular bases of learning and memory processes (reviewed in Neves et al. 2012). After this proposal in 90's, experiments started

based on *ex vivo* studies, with functional hippocampal slices, focusing on synaptic connections between Schaffer collaterals (where stimulation takes place) and CA1 pyramidal cells (registration place), in which electrical stimulation generates excitatory post-synaptic potentials (Squire et al. 2008; Lüscher and Malenka 2012).

The cellular and molecular mechanisms underlying the processes of synaptic plasticity are not yet fully understood, but it is thought that their knowledge will help to conceive how the brain works when it comes to storing memories, learning, and organizing behaviors.

1.3.2.1. Long-term potentiation (LTP)

Several studies have been done with special attention to the LTP involved in the excitatory synapses in the hippocampus, not only because this is a brain structure in which the synaptic plasticity is necessary for learning and memory , but also because it allows *ex vivo* studies in hippocampal slices that preserve cell organization in circuits (Squire et al. 2008).

Timothy Bliss and Terje Lomo (1973) were pioneers in the study of the synaptic plasticity in the hippocampus, demonstrating that a few seconds of high-frequency electrical stimulation could improve synaptic transmission in rabbit hippocampus (reviewed in Nicoll 2017). Furthermore, according to Hebb (1949), to trigger LTP it is necessary that the presynaptic cell and the post-synaptic cell be simultaneously active, suggesting that the coordinated activity of a presynaptic terminal and a post-synaptic neuron leads to the reinforcement of the synaptic connection between them - "*cells that fire together wire together"* (Lüscher and Malenka 2012).

During resting conditions the NMDARs are blocked by Mg^{2+}, but following tetanic stimulation on the Schaffer collaterals of CA1 pyramidal cells occurs a strong temporal summation of excitatory post-synaptic potentials (EPSPs), triggering a large depolarization of the post-synaptic cell that is sufficient to relieve the Mg^{2+} blockade of the NMDARs. This phenomenon allows an increase in glutamate release by presynaptic terminals that leads to post-synaptic depolarization due to AMPARs activation. This depolarization leads to NMDARs activation and subsequent Ca^{2+} influx to the stimulated synapse. This increases in Ca^{2+} activates intracellular signaling cascades involving, such as the activation of Ca^{2+}/calmodulin-dependent protein kinases (CaMKII). The activation of CaMKII leads to the phosphorylation of AMPARs subunits, promoting thus the insertion of these receptors in posts-ynaptic terminals, that ultimately are responsible for

the altered synaptic plasticity (figure 9) (Collingridge et al. 2004; Santos et al. 2009; Lüscher and Malenka 2012). Taking this into account, it can be inferred that synaptic LTP induction, between the Schaffer collaterals and the CA1 pyramidal cells, depends not only on the post-synaptic depolarization, due to NMDARs activation and Ca^{2+} entry through these receptors, but also relies on AMPARs insertion at the post-synaptic terminal (Gordon et al. 2005).

Several studies have shown that learning and LTP induced by experimental protocols of high frequency stimulation share the same mechanisms (Gruart et al. 2006; Whitlock et al. 2006; Neves et al. 2008). For this reason, this mechanism is widely used to study changes in synaptic plasticity as the neurological basis of learning and memory (Martin et al. 2000).

1.3.2.2. Long-term depression (LTD)

LTD is a lasting activity-dependent decrease in the synaptic plasticity, which was first described in CA1 by Lynch and colleagues (1976). At the synapse between the Schaffer collateral fibers and the pyramidal cells of the hippocampal CA1 region, this phenomenon is triggered by a long-term and low frequency stimulation of the Schaffer collateral fibers (Muller et al. 1995; Martin et al. 2000).

In the hippocampus, LTD as well as LTP can be induced by activation of the pre-synaptic neuron. In this region, LTD may be dependent on the activation of NMDARs and subsequent the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ increase, in particular in post-synaptic terminals. However, although the LTD is associated with a low $[Ca^{2+}]_i$ increase in the post-synaptic terminals, the LTP is associated with high $[Ca^{2+}]_i$. The differences in $[Ca^{2+}]_i$ in post-synaptic terminals may be related with the different of enzymes of signaling pathways, thus at lowest $[Ca^{2+}]_i$ occurs mainly phosphatases activation, whereas highest $[Ca^{2+}]_i$ trigger kinases activation, such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (figure 9) (Jahr and Stevens 1993; Luscher and Malenka 2012).

Moreover, LTD can also occurs through a mechanism involving the metabotropic glutamate receptors that regulated on protein synthesis at the level of dendrites due to the activation of mitogen-activated protein (MAP) kinases and phosphatases of tyrosine residues, which ultimately contributes to decrease of AMPARs levels in the post-synaptic terminals (Gladding et al. 2009; Chater and Goda 2014).

Briefly, LTP involves the activation of CaMKII, as well as other kinases such as cAMP-dependent protein kinase (PKA) and Ca^{2+}-dependent kinases, whereas LTD

involves the preferential activation of protein phosphatases, which is consistent with the fact that these two synaptic plasticity phenomena are inverse mechanisms. Furthermore, the increase of Ca^{2+} levels in post-synaptic, associated with NMDARs activation leads to insertion (during LTP) or removal (during LTD) of AMPARs at the post-synaptic terminal (Muller et al. 1995; Gordon et al. 2005).

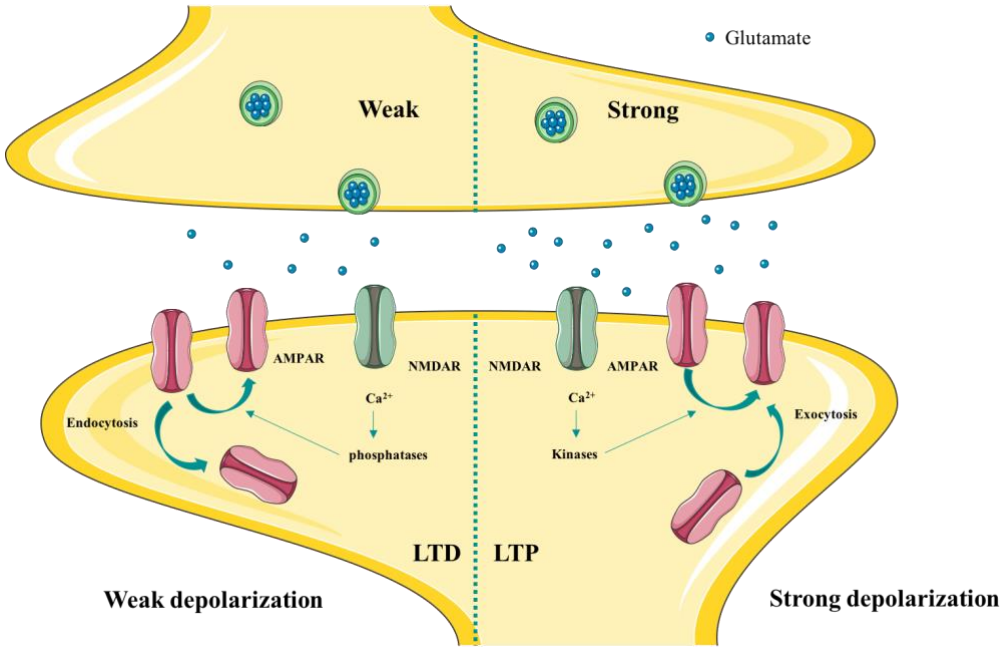

Figure 9. Post-synaptic expression mechanisms of LTP and LTD. A weak activity of the presynaptic neuron leads to modest depolarization and calcium influx through NMDA receptors. This preferentially activates phosphatases that dephosphorylate AMPARs, thus promoting receptor endocytosis. Strong activity paired with strong depolarization triggers LTP in part via CaMKII activation that trigger AMPAR phosphorylation, and their exocytosis, that is AMPARs insertion in cell membrane. (Adapted from Luscher and Malenka 2012).

1.4. Alzheimer's disease

Alois Alzheimer, in 1906, described clinical and pathological hallmarks of a serious disease in cerebral cortex, called "Alzheimer's disease" (reviewed in Cipriani et al. 2011). Alzheimer's disease (AD) is a more common and age-dependent neurodegenerative disease and the most common type of dementia in elderly people (60-80% of cases), affecting nearly 44 million people worldwide in 2016 and is expected to increase exponentially in the coming years (Agostinho et al. 2015; Scheltens et al. 2016; Laurent et al. 2018).

AD is a progressive and irreversible neurodegenerative disease, which is characterized by cognitive and non-cognitive dysfunctions, and usually it begins by memory impairment (Barage and Sonawane 2015; Silva et al. 2018). There are two forms of AD, sporadic AD (sAD) and familial AD (fAD) (Barão et al. 2016); being sAD the most common form and it has been suggested that the main cause of this type of AD is the inability to remove Aβ peptide from brain tissue (Masters et al. 2015). The sAD occurs as a consequence of aging and is associated to non-genetic and genetic risk factors, such as cardiovascular diseases, type-2 diabetes (T2D) and with the presence of gene allele ε4 that codified the apolipoprotein ε4 (Irie et al. 2008; Solomon et al. 2013). However, a small proportion (5%) of patients with this pathology have a rare form of this disorder that is entirely passed down through genetics, being inherited from a parent, being classified as familial AD (fAD). The fAD has an early age onset (mean age of ~ 45 years) and occurs due to the inheritance of autosomal dominant mutations in genes encoding amyloid-β precursor protein (AβPP), presenilin-1 (PS1), and presenilin-2 (PS2) which cause overproduction of Aβ peptide. Although the causes are different, sAD and fAD share the same neuropathological features (reviewed in Rocchi et al. 2003; Masters et al. 2015; Agostinho et al. 2015).

1.4.1. Neuropathological features of Alzheimer's disease

AD is characterized by the accumulation of insoluble forms of β-amyloid (Aβ) in plaques in the extracellular spaces, as well as in the walls of blood vessels, intracellular neurofibrillary tangles (NFTs) (figure 10) composed of hyperphosphorylated tau protein. Other features of this pathology include brain atrophy due to synaptic and neuronal loss in the hippocampus and cerebral cortex that likely contribute to cognitive deficits (Castellani et al. 2010; Masters et al. 2015). The presence of dystrophic neurites, activated

microglia and reactive astrocytes (astrogliosis) are also hallmarks of this pathology that contribute to clinical AD symptoms (Fonseca et al. 2015; Agostinho et al. 2015).

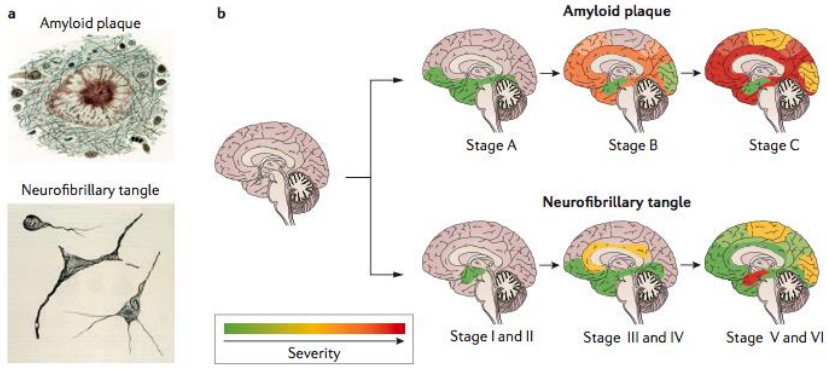

Figure 10. The pathological evolution of Alzheimer's disease. (a) Amyloid plaques and neurofibrillary tangles spread through the brain as the disease progresses. (b) In typical cases of AD, Aβ deposition precedes neurofibrillary and neuritic changes with an apparent origin in the frontal and temporal lobes, hippocampus and other structures of limbic system (top row**)** (Masters et al. 2015).

1.4.1.1. The Amyloid-β peptide

Originally, amyloid cascade hypothesis suggested that amyloid plaques are the causative agent of AD (Hardy and Higgins 1992). However, increasing evidences suggest that soluble Aβ correlates better with memory decline in patients with AD and in animal models of this pathology than amyloid plaques, contributed to an evolution in conceptual terms of this hypothesis, being currently widely accepted that the soluble Aβ oligomers are the toxic agents (Ñaslund et al. 2000; Lesné et al. 2006). The Aβ peptides with 40 or 42 amino acids, are able to self-aggregate originating Aβ dimers, oligomers and Aβ fibrils (Guedes et al. 2014). Accordingly, diverse studies using different experimental models of AD had shown that soluble Aβ oligomers are the causative agents of synaptic dysfunction and of other degenerative process that underlie the cognitive deficits even in early phases of AD (Hardy and Selkoe 2002; Haass and Selkoe 2007). Aβ peptides production and accumulation are thought to be related with a synaptotoxicity process that usually evolve to neuronal damage and it is described that these peptides also affect the glial cells (Agostinho and Cunha 2010; Zhang et al. 2012; Mucke and Selkoe 2012).

The Aβ peptide is metabolized by the proteolytic processing of amyloid-β precursor protein (AβPP), a type 1 transmembrane protein that is produced in brain cells (O'Brien and Wong 2011; Agostinho et al. 2015). The AβPP can be metabolized through two major pathways: the non-amyloidogenic pathway or the amyloidogenic pathway (Agostinho et al. 2015). The non-amyloidogenic pathway is mediated by an α-secretase that cleaves AβPP and generates: i) a large soluble α-secreted AβPP (sAβPPα) fragment and ii)

membrane-bound C-terminal fragment (α-CTF) that can be further cleaved by γ-secretase (a multimeric enzyme complex) to generate AβPP intracellular domain (AICD) and a p3 peptide (Thinakaran and Koo 2008; Agostinho et al. 2015). The other AβPP proteolytic processing - amyloidogenic pathway – involves a first cleavage mediated by β-secretases leads to the formation of soluble β-secreted AβPP (sAβPPβ) fragment and the remaining membrane-bound C-terminal fragment (β-CTF) can further be cleaved by a γ-secretase resulting in the formation of soluble Aβ peptide and in the production of AβPP intracellular domain (AICD) (figure 11) (Castellani et al. 2010; Agostinho et al. 2015; Scheltens et al. 2016). Both non-amyloidogenic pathway and amyloidogenic pathway are of extreme importance to ensure normal brain functioning. However, an imbalance in AβPP proteolytic cleavage, favoring amyloidogenic pathway, and AβPP mutations can result in increased Aβ production, mainly $A\beta_{42}$ (more toxic form) and $A\beta_{40}$ (most abundant form) (Lansbury 1997; Agostinho et al. 2015).

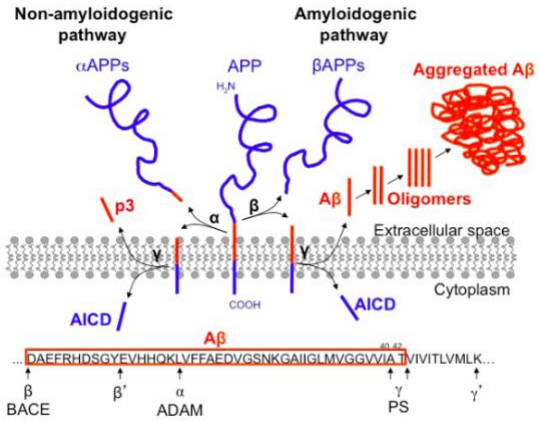

Figure 11. Amyloid-β Precursor Protein (AβPP) processing. α- or β-secretases may cleave AβPP through the non-amyloidogenic or amyloidogenic pathway originating αAβPPs or βAβPPs soluble fragments, respectively and membrane-retained fragments that are then cleaved by a γ-secretase originating the p3 fragment (non-amyloidogenic pathway) or the Aβ peptide (amyloidogenic pathway) and the AICD. Aβ monomers can form toxic soluble oligomers and higher molecular weight insoluble fibrils that aggregate and deposit in susceptible brain regions (Fonseca et al. 2015).

However, increasing evidences indicate that production and accumulation of Aβ *per se* are unable to account for all AD features (Agostinho et al. 2015). Therefore, the abnormal tau phosphorylation, and the subsequent formation of intracellular deposits of tau hyperphosphorylated that make up the NFT, is also considered to have a key role in AD pathogenesis (Laurent et al., 2018). Furthermore, there are evidences that Aβ peptides trigger signalling pathways that contribute to tau hyperphosphorylation, existing thus a relationship between these two proteins (Lopes et al. 2010; Martin et al, 2013).

1.4.1.2. Tau hyperphosphorylated

Tau is a family of neuronal proteins that belong to the family of microtubule-associated proteins (MAP) and that are produced by alternative splicing of mRNA from a single gene. There is increasing evidence that this protein has numerous important functions, namely in microtubules stability promoting through their interaction with heterodimers of **α**- and β-tubulin (Weingarten et al. 1975; Laurent et al. 2018).

The phosphorylation of tau is the most studied process, considering its importance in some diseases, namely AD. The levels of phosphorylated tau within the cells depends on the activity of kinases and phosphatases; when this balance is interrupted and abnormally phosphorylation of tau occurs, which can contribute to the development of "thauopathy" (Hutton et al. 1998; Poorkaj et al. 1998; Yancopoulou and Spillantini 2003; Laurent et al. 2018). This irregular process leads to a significant reduction of the binding tau microtubules causing microtubule destabilization, which can trigger neuronal death. In addition, it has been shown that soluble Aβ fragments can control cleavage and phosphorylation of tau, which is essential for NFT formation (O'Brien and Wong 2011). Accordingly, reducing levels of Aβ peptide can prevent tau pathology from developing and abrogates spatial memory problems (Oddo et al. 2006).

1.4.1.3. Astrogliosis in Alzheimer's disease

Astrocytes respond to all forms of CNS toxic and traumatic injury or disease with a variety of morphological and functional changes, called "astrogliosis". These changes include hypertrophy of the cell soma and astrocytic processes become thickening, as well as increased gene expression of a number of astrocytic structural proteins, such as GFAP. Usually the reactive astrocytes proliferate to the region of the tissue lesion, forming of astrocyte scars (Sofroniew and Vinters 2010; Parpura et al. 2012; Sofroniew 2015; Assefa et al. 2018). These scars, formed by astrocytes processes strongly overlapped, surround the dying cells in the lesion tissue, limiting thus the spreading of the damage; however, these scars formation can also hinder the regeneration of injured regions sites by prevent the incoming of new cells, mainly neuronal or glial precursor cells (Sofroniew 2009; Sofroniew and Vinters 2010).

In the literature, it is described that different signaling mechanisms trigger different molecular, morphological and functional changes in reactive astrocytes, such as growth factors and cytokines, mediators of innate immunity, neurotransmitters, purines and oxygen species (ROS) (Sofroniew 2009). Accordingly, reactive astrocytes can lead to

harmful or beneficial effects, under specific circumstances. An example of their harmful activity is when astrogliosis is pathological *per se*, that is the astrocytes instead mediate a "normal" response to a pathological insult in order to resolve it act as a promotor of damage and lead to astrocytopathies (Sofroniew 2015). On the other hand, reactive astrocytes can be beneficial in the preservation of CNS function, through various mechanisms, such as uptake of excitotoxic glutamate and adenosine release (Sofroniew 2009; Sofroniew 2015).

The astrocytes have been implicated in the onset and progression of neurodegenerative diseases, either as consequence of loss of their normal homeostatic functions or due to a gain of toxic functions (figure 12) (Barreto et al. 2011; Garwood et al. 2017). Indeed, astrocytes can internalize Aβ, and although this can be a way of hamper the accumulation of the peptide, it can also promote the astrocytes activation (Barreto et al. 2011; Delekate et al. 2014). Accordingly, the reactive astrocytes are found in *postmortem* AD human brain and in animal models of AD nearly Aβ deposits (Simpson et al. 2010; Verkhratsky et al. 2010; Delekate et al. 2014). Thus, intracellular aggregates of Aβ interfere with normal astrocytic functions, and the aberrant astrocytes dysfunction that can be harmful by triggering neuronal injury (Agostinho and Cunha 2010; Barreto et al. 2011). The amyloid plaques observed in AD are surrounded by reactive astrocytes and also by activated microglia (Osborn et al. 2016; Assefa et al. 2018), which are usually associated with a neuroinflammatory process (another AD feature). The inflammatory response in AD includes an increase in number, size and motility of astrocytes and also of microglia, a process named as gliosis (Glass et al. 2010; Sofroniew and Vinters 2010). These glial cells release of pro- and anti-inflammatory mediators as well as neurotoxic factors, such as nitric oxide due the overexpression of inducible nitric oxide synthase (iNOS), which that can trigger neuronal damage, being the synapses particularly affected (Agostinho and Cunha 2010; Purpura et al. 2012; Chung et al. 2015). Therefore, the amyloid plaques are usually surrounded by reactive astrocytes and microglia and also by dystrophic neurites, giving rise to senile plaques (or neuritic plaques) (Wyss-coray et al. 2003; Osborn et al. 2016). Moreover, during the process of neuronal damage and glial activation occurs the production of inflammatory factors (e.g chemokines and cytokines) that promote the recruitment of peripheral immune cells, such as lymphocytes, which sustain and exacerbate the neuroinflammatory process. Therefore, the neuroinflammation process that encompasses brains cells and the entry of immune peripheral cells due to alterations in BBB permeability, tending to be a chronic and harmful process. The pro-

inflammatory cytokines, released by astrocytes or microglia, can have impact on synaptic function, having often a repressive effect on LTP that might contribute to cognitive dysfunction at an early stage of AD (Agostinho and Cunha 2010; Purpura et al. 2012; Chung et al. 2015).

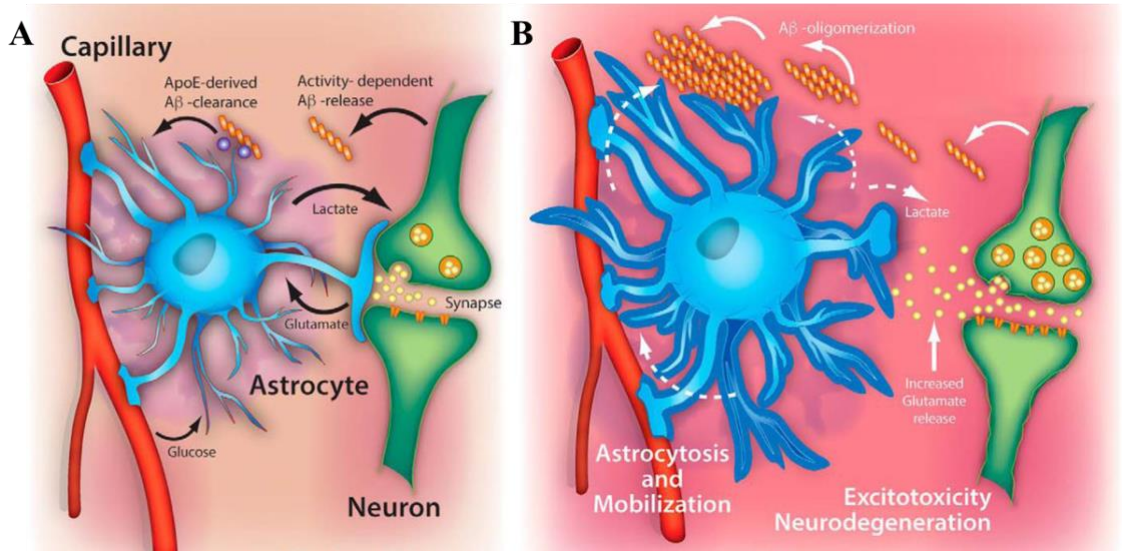

Figure 12. Astrocytes are active players in AD. A. Astrocytes can be crucial in the preservation of CNS function, through various mechanisms, such as uptake of excitotoxic glutamate and glutamate release. Thus, astrocytes can reduce extracellular levels of Aβ through their internalization, preventing Aβ oligomerization and aggregation. **B.** The astrocytes have been implicated in the onset and progression of neurodegenerative diseases, such as AD, either as consequence of loss of their normal homeostatic functions or due to a gain of toxic functions. AD may interfer with Aβ clearance mechanisms while simultaneously promoting the further release Aβ, thereby permitting Aβ oligomerization and subsequent plaque formation. Without proper functioning astrocytes, it has an increase in glutamate release and generate excitotoxicity damage, leading to neurodegeneration and cognitive dysfunction. (Adapted from Vanderheyden et al., 2018).

Considering these pieces of evidence, astrocytes are active players in AD. Nevertheless, new perspectives of astroglia roles in this brain disease can be crucial to define if astrocytes manipulation may provide a novel principle for treatment of AD at early stages (Colangelo et al. 2014; Verkhratsky et al. 2010).

Chapter 2

Aims of the study

Aims of the study

The general goal of this study is to provide new insights about the role of astrocytes in the two cardinal features of AD: synaptic dysfunction and memory deficits.

Having that in mind, this study was designed to evaluate:

i) The impact of AD-like conditions and astrocytes blunting (triggered by a gliotoxin, L-AA) on hippocampal synaptic plasticity, with particular focus on long-term depression (LTD).

ii) The involvement of astrocytes in early phases of AD, using a mouse model of intracerebroventricular (icv) injection of $A\beta_{1-42}$, particular on hippocampal synaptic efficacy, through long term potentiation (LTP) and LTD phenomena and, simultaneously, correlate the putative alterations in synaptic function with memory deficits of icv $A\beta_{1-42}$ injected mice.

iii) Changes in astrocytes reactivity under AD-like conditions, defining whether the blunting of astrocytes by the gliotoxin (acute exposure) have impact on astrocytic markers; to further correlate alterations in astrocytes morphology with the synaptic dysfunction.

iv) The impact of astrocytes blunting on hippocampal synaptic plasticity, and its correlation with alteration in astrocytic markers, in another mouse model of the AD, the triple transgenic mice of AD (3xTgAD mice).

In the first task, hippocampal slices of young adult mice (C57/Bl6) were acutely exposed to $A\beta_{1-42}$ or to L-α-aminoadipic acid (L-AA), mimicking AD-like conditions (Canas et al. 2009; Matos et al. 2012) or astrocytic blunting (Lima et al. 2014), respectively. Synaptic plasticity, namely LTP and LTD, was evaluated using electrophysiological recordings, measuring field excitatory post-synaptic potentials in the Schaffer collaterals-CA1 pyramidal neurons.

To address the tasks ii and iii, we used an animal model of the AD, consisting in the icv $A\beta_{1-42}$ injection, which was very similar to a model previous validated by our group (Canas et al. 2009), except that the one used in the present study exhibited astrogliosis (see results section). In this model we assessed hippocampal dependent memory changes, validating thus our AD model, and further we evaluated the synaptic plasticity, namely LTD and LTP, and identified alterations in astrocytic proteins, markers of astrocytes reactivity. In addition, we also evaluated the impact of astrocytic blunting

(triggered by acute L-AA exposure) in pathological conditions of AD in synaptic plasticity (LTD and LTP) and in astrocytic markers; since we have hypothesized that the astrocytes behave differently under pathological conditions.

In the last task, we used the 3xTgAD and littermate (as control) mice to define the impact of astrocytic blunting on synaptic plasticity (LTP). Thus, we used hippocampal slices obtained from 3×TgAD and control mice, superfused or not with gliotoxin L-AA (acute exposure), measuring field excitatory post-synaptic potentials to gauge synaptic plasticity alterations in Schaffer fibers-CA1 pyramidal synapses. Alterations in astrocytic markers, mainly in GFAP, in hippocampi of this AD mice model exposed or not to L-AA was also evaluated.

With this study we expect to give novel insight to validate astrocytes as key players in shaping synaptic function and to strength the idea that these glial cells are a valuable therapeutic target in neurodegenerative disorders associated with memory deficits, such as AD.

Chapter 3

Reagents, Solutions and Methods

3.1. Animals

In most of the studies we used mice young adult male C57 black 6 (C57Bl/6) mice with 8 to 10 weeks old, obtained from Charles River Laboratories (Barcelona, Spain), which are animals widely used as an experimental model to assess physio-pathological human conditions (Mekada et al. 2009). Additionally, we also use the triple transgenic model of Alzheimer's disease (AD) (3xTgAD; Oddo et al. 2003) and its background strain, wild-type non-transgenic (nonTg) mice (C57BL6/129sv), obtained from our colony (Lopes et al. 2009; Silva et al. 2018). The animals were maintained under a controlled environment with 23 ± 2˚C temperature 12-hour dark/light cycle, and approximately 66 % humidity, and the animals had *ad libitum* access to food and water. The studies were conducted in agreement with the approved animal welfare guidelines and European legislation for the use of experimental animals (ORBEA 128_2015/04122015) and the certification of Direção Geral de Alimentação e Veterinária (DGAV; 0421/000/000/2016 Ref 014420). Furthermore, all efforts were made to reduce the number of animals used and to minimize their stress and discomfort. All experiments were conducted between 9 a.m. and 7 p.m. to avoid the influence of circadian rhythms on their performance in behavioral tests.

3.1.1. Animal models of Alzheimer's disease

Although, no mice model recapitulates all of the features of AD spectrum, the use of these animal models allow study to detail cellular and molecular aspects of this pathology, which is not readily possible for ethical issues with human patients and the data obtained from peripheral samples of the patients did not also reproduce all the neuropathological AD features (LaFeria and Gree 2012).

3.1.1.1. $A\beta_{1-42}$ injection model

To model sporadic AD, we used young adult mice (9-11 weeks) that were intracerebroventricular (icv) injected with the synthethic amyloid-β peptide fragment 1-42 ($A\beta_{1-42}$, 0.5 mM, Bachem). In fact, our group and others had previously shown that a single intracerebroventricular (icv) injection of $A\beta_{1-42}$ can induce AD-like behavioral alterations, in particular long-term deficits in learning and memory (Nakamura et al. 2001; Medeiros et al. 2007; Cunha et al. 2008; Canas et al. 2009). $A\beta_{1-42}$ peptide was

dissolved in water and stored until used (Canas et al. 2009). This peptide was administered intracerebroventricularly in the lateral ventricle (see figure 13B) in a total of 4 µl of $A\beta_{1-42}$ solution, containing 2 nmol of peptide and vehicle animals were intracerebroventricularly infused with a similar volume of filtered water (Dall'Igna et al. 2007; Canas et al. 2009). Animals were anesthetized with 13-15 µl/g (according to their weight) of avertin (2.2.2-tribromoethanol 70.7 mM, 2-methyl-2-butanol 113.4 mM, NaCl 138 mM, pure ethanol 12.5% in PBS, Sigma) (intraperitoneal injection, IP) and placed in a stereotaxic frame (figure 13C). Posteriorly, the cranium was perforated in order to insert a stainless steel needle on the following coordinates from bregma: antero-posterior (AP): - 0.58 mm; lateral (L): ± 1.13 mm; dorso-ventral (DV): - 2.00 mm (Paxinos and Franklin 2001), as previously described by our group (Canas et al. 2009), and the $A\beta_{1-42}$ solution or vehicle (water) injected at a rate of 1 µl/min for approximately 4 min. These coordinates allow a proximity to the hippocampus, a region particularly linked to learning and memory (Broadbent et al. 2004). The behavioral performance of these animals was evaluated 17 days after Aβ administration (Dall'Igna et al. 2007).

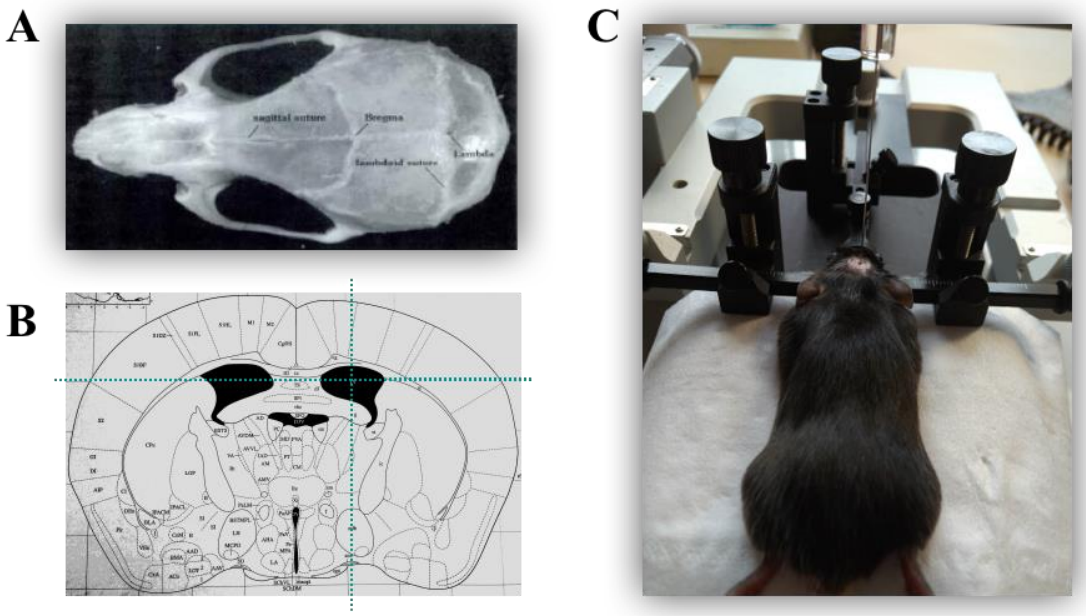

Figure 13. Representation of icv injections. **A.** The dorsal surface of the mouse skull showing the horizontal plane reference points, bregma and lambda. Bregma is the point intersection between coronal and sagittal sutures whereas lambda is the intersection of the projection of lines of best fit through the sagittal and lambdoid sutures. **B.** Coronal sections of young adult brain at coordinates: interaural line 3.22 mm, bregma - 0.58 mm. The dashed lines define the stereotaxic coordinates used (AP: - 0.58 mm; L; ± 1.13 mm; DV: - 2.00 mm). **C.** Illustration of a mouse placed in the stereotaxic instrument at the time of icv injection (Paxinos and Franklin 2001).

3.1.1.2. Triple transgenic mice model of AD (3×TgAD)

In the literature, there are numerous models that mimic AD. However, the most widely used that covers almost the entire neuropathological spectrum of AD is the triple transgenic model, 3×TgAD. The triple transgenic mice harbor three mutant transgenes: $PS1_{M146v}$, APP_{Swe}, and tau_{301L}. This animal model develops an age-dependent and progress neuropathology that includes the plaque and tangle pathology. Furthermore, this model is consistent with the amyloid cascade hypothesis since Aβ deposition has been shown to occur first, proceeding from the overexpression of AβPP and tau pathology (Oddo et al. 2003).

Therefore, the triple transgenic mice model was used to evaluate the synaptic plasticity in Schaffer fibers-CA1 pyramidal synapses, namely LTP and afterwards immunohistochemistry studies.

3.2. Reagents and solutions

3.2.1. Electrophysiology experiments

Table 1. Drugs used in extracellular electrophysiology experiments.

Compound	Dissolved in	Concentration	Laboratory
(L-α-Aminoadipic acid) L-AA	aCSF	100 µM	Sigma - Aldrich (USA)
Aβ_{1-42}	aCSF	50 nM	Bachem (Switzerland)

Table 2 Composition of solutions used in electrophysiology experiments.

Reagent	Composition	Concentration (mM)	Laboratory
Artificial cerebrospinal fluid (aCSF)	NaCl	124	Sigma - Aldrich (Missouri, USA)
	KCl	3	
	Sodium bicarbonate ($NaHCO_3$)	26	
	Sodium phosphate monobasic (NaH_2PO_4)	1.25	
	(2R,3S,4R,5R)-2,3,4,5,6-pentahydroxyhexanal (D-Glucose)	10	
	Magnesium sulfate ($MgSO_4$)	1	
	Calcium chloride ($CaCl_2$)	2	

3.2.2. Immunohistochemistry

Table 3. Composition of solutions used in immunohistochemistry.

Reagents/Solutions	Components	Concentration (M) or (mM)	Laboratory
Paraformaldehyde (PFA) 4%	PFA	1.3 M	Sigma - Aldrich
	Sodium hydroxide (NaOH)	5 M	Sigma
	(in PBS)		
Sucrose 30%	(2R,3R,4S,5S,6R)-2-[(2S,3S,4S,5S)-3,4-dihydroxy-2,5-bis(hydroxymethyl)oxolan-2-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol) (Sucrose)	30%	Sigma - Aldrich
	(in PBS)		
Anti-freezing solution	Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$)	12.3 mM	Sigma - Aldrich
	Sodium hydrogen phosphate dihydrate ($NaHPO_4.2H_2O$)	20.3 mM	
	Ethylene glycol ($HOCH_2CH_2OH$)	30%	
	$HOCH_2CH(OH)CH_2OH$ (Glycerol)	30%	
Ultrapure low melting point agarose		3%	Invitrogen
Fluorescent mounting medium DAKO			Agilent Technologies

Table 4. Antibodies used in immunohistochemistry of 50 µm hippocampal sections.

Primary antibodies			
Antibody	**Dilution**	**Origin**	**Laboratory**
Anti-GFAP	1:1000	Rabbit	Millipore
Anti-S100β	1:100	Mouse	Abcam
Secundary antibodies			
Antibody	**Dilution**	**Origin**	**Laboratory**
Anti-rabbit Alexa 488	1:1000	Donkey	Invitrogen
Anti-mouse Alexa 594	1:1000	Donkey	Invitrogen
Anti-rabbit Alexa 594	1:1000	Donkey	Invitrogen

3.3. Methods

3.3.1. Behavioral studies

To study the involvement of different brain areas and circuits that might be involved in mice response to different environments or stimulus, a wide range of behavioral tests has been developed. The most appropriate behavioral test have been selected for each experiment, according to what is intended to be evaluated, such as motor function, anxiety-like behavior, spatial and recognition memory or cognitive function (Gerlai and Clayton 1999; Gerlai 2001).

Before performing the behavioral studies, the mice were handled for 5 min every day for 16 days (from injection to behavioral analysis) and there was a period of habituation to the room for 1 hour before the tests. All behavior experiments were performed in a sound-attenuated room with controlled dimmed lighting conditions, predominantly set with a red source of light (10 lx). Between each test, the equipment was carefully cleaned with ethanol 10% to remove the smell traces left by the mice.

To investigate the impact of icv $A\beta_{1-42}$ injection on memory hippocampus-dependent impairment, novel object recognition and modified Y-maze tests were performed. Behavior was video-monitored, and the videos were later analyzed with the ANY Maze video tracking system (Stoelting) or manually scored.

3.3.1.1. Open Field

The open field test was originally designed by Hall (Hall 1934) to measure behavioral mice responses and evaluate the exploratory and general activity of mice, providing simultaneous information about locomotor activity and anxiety that allow inferring about the animals' "health" state (Hall and Ballachey 1932; Walsh and Cummins 1976).

In this experiment, locomotor activity was evaluated 17 days after surgery using an open field arena with a gridded floor (30 × 30 × 50 cm), as illustrated in figure 14 (Broadhurst 1957; Canas et al. 2009). Each animal was positioned in the periphery of the arena, facing the wall and the exploratory behavior of the animals was evaluated distance travelled and time spent in each zone over a 10 min period (Kaster et al. 2015). In parallel, occupation plots allow to measure anxiety as the time spent in the center of the apparatus (Carmo et al. 2014; Gonçalves et al. 2015; Lopes et al. 2015). The open field test also worked as habituation for the subsequent behavior tests (Novel Object Recognition), so two consecutive days of open field test were performed before the beginning other behavior tests. Therefore, this behavioral test was used as a control test to check if the animals had complications due to the icv injection ($A\beta_{1-42}$ or water), mainly in motor activity.

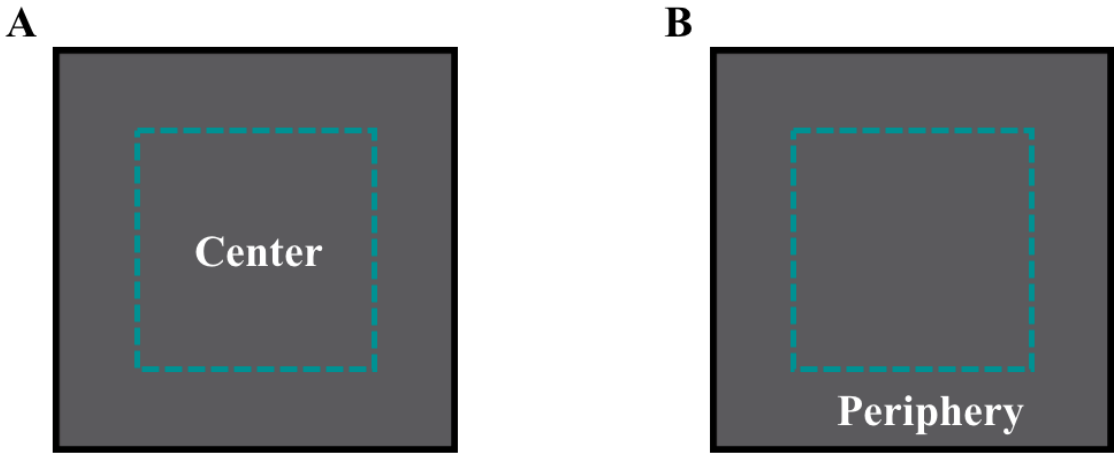

Figure 14. Open Field Habituation. The Open Field apparatus has 30 × 30 cm, with 50 cm high black walls and a white floor. Each mouse was placed in the periphery of the open field and locomotor activity was measured. Rodents typically spend a significantly greater amount of time exploring the periphery of the arena than the unprotected center arena.

3.3.1.2. Novel Object Recognition (NOR)

Mice have a tendency to interact more with a novel object than with a familiar object. Therefore, this tendency has been used to study cognition performance of rodents, considering that the choice to explore the novel object reflects the use of learning and recognition memory (Ennaceur and Meliani 1998; Wan et al. 1999; Broadbent et al. 2004). Hence, mice performed the NOR test to probe recognition memory dependent of hippocampus and cortical regions (Assini et al. 2009).

As we can see in figure 15, the NOR test consisted of two sessions: the first with two identical objects (training session, 10 min) and the second with two dissimilar objects – a familiar and a novel one - (test session, 5 min) with an inter-trial interval (ITI) of 90 min. The locations of the objects were counterbalanced in each section to avoid any basal preference for any side of the maze that might have existed. It is expected that when placing a new object and an object that mice are already familiarized within the same apparatus, the rodent directs its interest towards the novel object, exploring the familiar object for a shorter time period. This reflects the use of recognition memory, a hippocampal-dependent memory performance since it is dependent on NMDARs of this brain region (Rampon et al. 2000). Object exploration was defined as the orientation of mouse nose to the object, touching with forepaws or nose, sniffing and biting the objects. The recognition index was calculated as a ratio of the time spent exploring novel object over total exploration time of both objects (Canas et al. 2009; Lopes et al. 2015).

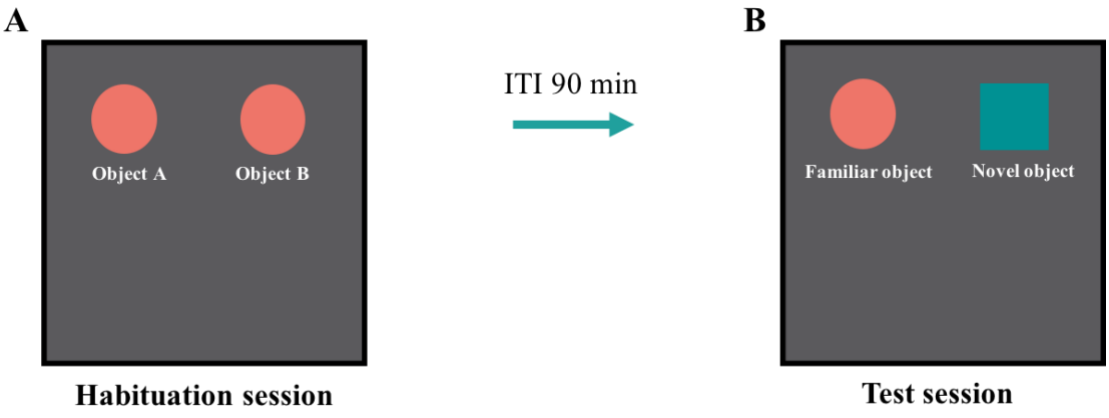

Figure 15. Novel Object Recognition. This assay is conducted in an open field arena with two different objects. The objects are different in shape and appearance. **A.** In the habituation session, the animals are exposed to the familiar arena with two identical objects placed at an equal distance. **B.** After an ITI of 90 min, the mice are allowed to explore the same arena but, in this case, in the presence of a familiar and a novel object to evaluate intermediate-term recognition memory.

3.3.1.3. Y-Maze

The Y-shaped maze has been used to perform the modified Y- test that can evaluate memory for spatial location, in which hippocampus is involved; and it is based on mice innate tendency to explore novelty, which permits dissociating memory from learning (Dellu et al. 1992; Dellu et al. 1997).

The test took place 24h after the NOR test and it consists of two trials: the acquisition and the retention phase with an ITI of 90 min (figure 16). In the first phase, each animal was placed at the beginning of one arm and was free to explore two of the three arms for 5 min, because one of the arms was closed with a door. The arm where the mouse was put in was called "start" arm, while the other arm was called "other". After the acquisition phase was finished, the animal was put back in its holding cage and remained there for 90 min. After this inter-trial interval, the door was removed to start the second phase of the experiment. The mouse was put back in the same "start" arm as before, but this time having access to all arms of the maze: "start", "other" and "novel" and the animal was allowed to explore the maze for 5 min. In both phases, the number of entries and the time spent in each arm and the total distance travelled were registered. What is expected is that, in the second trial, mice that randomly explore the maze would retrieve 33 % of entries and time in the "novel" arm, while mice with good memory performance would explore the "novel" arm for longer time periods and more times.

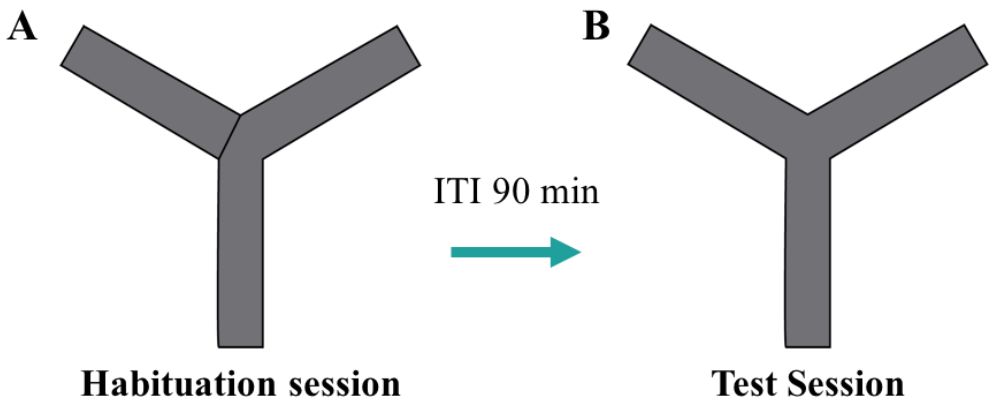

Figure 16. Modified Y-maze. This assay is conducted in a three-arm apparatus and each arm of the maze was 35 cm long, 10 cm wide and 25 cm high. The test consists of two trials: **A.** the acquisition phase (each animal was free to explore two of three arms) and **B.** retention phase (each animal was access to all arms of the maze). Modified Y-maze test was done to evaluate the memory performance of mice.

3.3.2. Electrophysiological Recordings

Synaptic plasticity consists of dynamic changes in the efficacy of excitatory synaptic transmission and it is manifested as long-term potentiation (LTP) and long-term depression (LTD), two major molecular mechanisms that are thought to be involved in learning and memory processes (Kochlamazashvili et al. 2011). Considering that we wanted to study long-term changes in synaptic efficiency, we performed extracellular recordings in hippocampal slices due to their ability to maintain stable recordings for several hours. The experiments were performed in Schaffer fibers-CA1 pyramidal synapses to assess synaptic plasticity.

In these experiments, we used the animals described in section 2.1. After anesthetize profoundly the animals in a halothane (2-Bromo-2-chloro-1,1,1-trifluoroethane, Sigma) atmosphere, they were sacrificed by decapitation. Thereafter, the brain was removed and placed into a petri dish with ice-cold, oxygenated artificial cerebrospinal fluid (aCSF) and gassed with a carbogen mixture (95% O_2/5% CO_2, Linde) (Costenla et al. 1999). This step should be performed as soon as possible so that the brain maintains its physiological properties. Considering that for all experiments hippocampal slices were used, it was necessary to dissect the hippocampus. Both hippocampi were dissected and 400 µm thick slices were cut perpendicularly to the long axis of the hippocampus (transverse slices) with a Brinkmann McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, Guildford, UK) (Cunha et al. 1994). In animals injected with $A\beta_{1-42}$ or vehicles, only the injected hemisphere was used for electrophysiological recordings. In naïve C57BL/6 and in 3×TgAD animals, both hemispheres were used. Posteriorly, slices were allowed to recover functionally and energetically for at least 1 h (for LTP) or 1 h and 30 min (for LTD) in a preincubation chamber (BSC-PC prechamber, Harvard Apparatus, Massachusetts, USA) with gassed aCSF at 30.6 ºC or 32.0 ºC for LTP or LTD, respectively. After this pre-incubation time, individual slices were transferred to a submersion recording chamber with 1 mL capacity (BSC-ZT Zbicz Top, Harvard Apparatus, Massachusetts, USA) and continuously superfused with aCSF (control) or L-AA at a flow rate of 3 mL/min with gassed aCSF kept at 30.6 ºC (LTP) or 32.0 ºC (LTD) (TC-202A Bipolar Temperature Controller, Harvard Apparatus, Massachusetts, USA). Control slices were only exposed to aCSF before and during the recordings, whereas slices treated with L-AA were pre-incubated for 2 h and continuously superfused with the same solution during the recordings. On the other hand, $A\beta_{1-42}$ treatment consisted of a

40 min incubation, followed by aCSF superfusion throughout the remaining time of the recording.

A bipolar concentric electrode was placed onto Schaffer collateral-commissural pathway and stimulated every 20 s with regular pulses of 0.1 ms. The orthodromically-evoked field excitatory post-synaptic potentials (fEPSP) were recorded through an extracellular microelectrode pipette (filled with aCSF, 1-2 MΩ (MegaOhms resistance), obtained using a Flaming/Brown micropipette puller system, model P-87 (Sutter Instruments, USA) (Costenla et al. 1999; Kaster et al. 2015; Gonçalves et al. 2015) (figure 17A). Recordings were obtained with an ISO-80 amplifier (World Precision Instruments, Hertfordshire, UK), stimulated with either Grass S44 or Grass S48 and digitized using an analog-to-digital converter (ADC) (BNC-2110, National Instruments, Newbury, UK). Both the data acquisition and its subsequent analysis were performed using the software WinLTP version 2.20.1 (WinLTP Ltd., Bristol, UK) (Costenla et al. 1999; Anderson and Collingridge 2001; Lopes et al. 2015; Gonçalves et al. 2015).

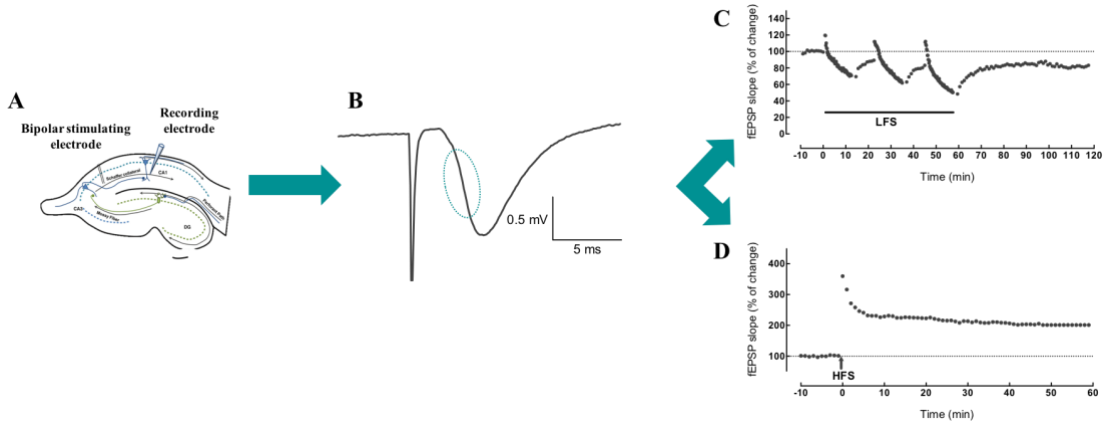

Figure 17. Schematic representation of electrophysiology recordings in hippocampal slices. A. The stimulation electrode (SE) is placed in the Schaffer collaterals (SC) and fEPSPs are recorded in the dendrites of the CA1 pyramidal neurons where the recording electrode (RE) is located. **B.** Representative recording of a fEPSP obtained with the previous electrode positioning. The trace comprises the stimulus artifact, followed by the presynaptic volley and the fEPSP. The intensity of the stimulus was adjusted to evoke a fEPSP without population spike contamination and responses were quantified as the initial slope of the averaged fEPSPs. **C. D.** Representative time course was recorded over the entire experiment. This time course comprises fEPSP slope recorded during: baseline, LFS or HFS and slope recorded during 60 min.

Firstly, input-output curve (I/O) (a sigmoidal-shaped curve: fEPSP slope versus stimulus intensity) was obtained to select the intensity of the stimulus to evoke a fEPSP (~40% or 60% of maximal amplitude for LTP or LTD, respectively, with no apparent contamination) (figure 17B); to ensure that modifications of stimulus amplitude were not due to changes in baseline synaptic efficiency and to evaluate changes in basal synaptic

transmission due pharmacological manipulations. After obtaining a stable baseline for at least 10 min, LTP was induced by a high-frequency stimulation (HFS) pattern (one train of 100 Hz pulses for 1 s), as we see in figure 17D (Lopes et al., 2015) and LTD was induced by a low-frequency stimulation (LFS) consisting of 1500 pulses at 2 Hz (0.2 ms pulse-width) and was applied for 10 min and repeated three times with a 10-min interval between completion of one LFS-train and the start of the successive one (figure 17C). LTP and LTD were quantified as the percentage changed between the average slope of the ten potentials taken between 50 and 60 min after the induction protocol related to the average slope of the fEPSP measured during 10 min that preceded the induction protocol (Van der Jeugd et al. 2011; Lopes et al. 2015; Laurent et al. 2016; Silva et al. 2018).

The effects of L-AA and of $A\beta_{1-42}$ on LTP or LTD (figure 18A and C, respectively) were evaluated by comparing the amplitude of this stimulus in the absence of drugs (control) with the amplitude in the presence of these drugs (test). L-AA condition was obtained by slices incubation during 2 h out of the setup and L-AA solution remained in the system until the end of the experiment, while the $A\beta_{1-42}$ (acute application on slices) were added 40 min before, before starting the recordings that are performed in aCSF, as it shown in figure 18B.

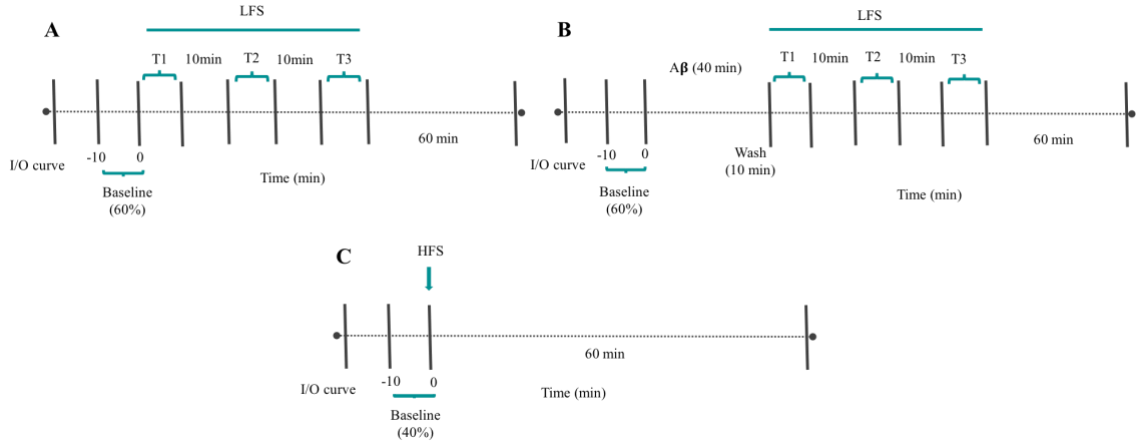

Figure 18. Experimental design used in extracellular electrophysiological recordings. A. LTD induction protocol used for aCSF (CTR) and L-AA incubated slices. **B.** LTD induction protocol applied to slices exposed to $A\beta_{1-42}$. **C.** LTP induction protocol used for aCSF (CTR) and L-AA incubated slices. LTD induction (LFS, 1500 pulses at 2 Hz (0.2 ms pulse-width) applied for 10 min and repeated three times with a 10-min interval between completion of one LFS-train and the start of the successive one) and LTP induction (HFS, 1 s, 100 Hz).

3.3.3. Sectioning hippocampal slices

After performing the fEPSP recordings, the remaining slices (400 µm) were collected and these slices belong to the following conditions: vehicle group (incubated in aCSF or L-AA (2 h)), $A\beta_{1-42}$ group (incubated in aCSF or L-AA (2 h)), 3×TgAD (incubated in aCSF or L-AA (2 h) and nonTg (incubated in aCSF or L-AA (2 h). The slices were fixated by immersion in a 4% paraformaldehyde (PFA) solution during two days. After fixation, the slices were transferred into a 30% sucrose solution. Two days later after dehydration, hippocampal slices were stored in an anti-freezing solution until used in immunohistochemistry studies.

To perform the sectioning of hippocampal slices (400 µm), we first washed them with phosphate buffered saline (PBS; NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.9 mM, Sigma) to remove the anti-freezing solution completely and then the slices were placed carefully inside the molds (cubes) containing 3% ultrapure low melting point agarose solution (LMP) (T ± 40 ˚C), (figure 19A). As it is possible to observe in figure 19B, after slices submersion in the agarose, the cubes were placed in ice until solidified the agarose completely. At this point, the slices are ready to be placed in the vibratome, (Leica VT1200S, Leica Biosystems, Germany), where 50 µm thickness sections were cut and further transferred into a multiwell plate with PBS (figure 19C). These sections can be stored embedded in anti-freezing solution until the immunohistochemistry studies were performed.

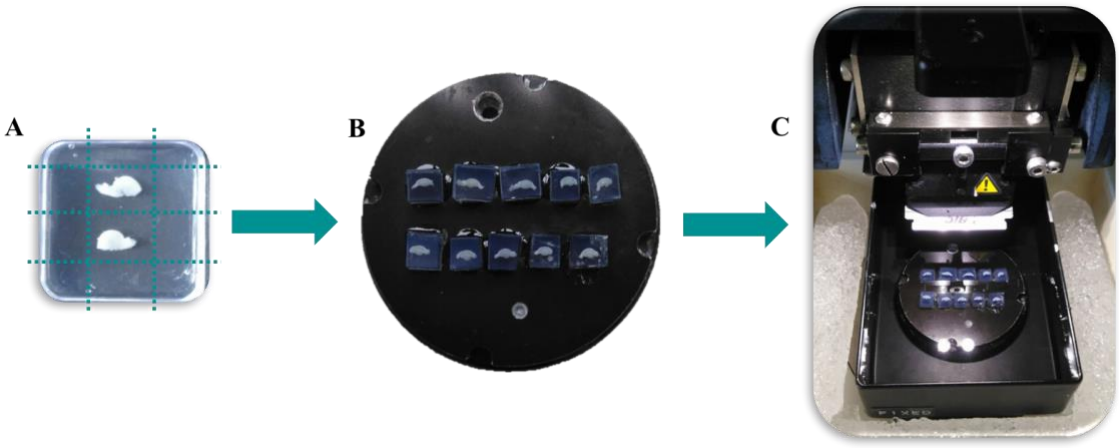

Figure 19. Sectioning hippocampal slices. A. The hippocampal slices were placed into molds, which were further filled with agarose. **B.** Few minutes after adding the agarose, it becomes solid and can be cut into cubes and placed in a vibratome. **C.** In the vibratome, cubes can be sectioned with 50 µm thickness (using the folowing parameters: 0.22 mm/s speed and 0.55 mm amplitude).

3.3.3.1. Immunohistochemistry

Immunohistochemistry (IHC) is a microscopy-based technique for detecting the location of cellular components (proteins or other macromolecules) in tissue sections using antibodies. Since antibodies are highly specific, the antibody will bind only to the antigen of interest in the tissue section. In this case, the antibody-antigen interaction is visualized through a fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy (Kabiraj et al. 2015).

The hippocampal slices (50 µm), prepared as described in 3.3.3. were washed to remove the anti-freezing solution, and then incubated with 0.1% Triton in PBS (permeabilization solution, Sigma - Aldrich) during 15 min, since we are interested in cytoplasmic proteins. To prevent nonspecific antibody binding, the sections were incubated with a blocking solution (10% horse serum + 0.1% Triton in PBS, Sigma - Aldrich), during 1 h under gentle agitation at RT. Afterwards, the slices were incubated with primary antibody (anti-GFAP 1:1000 and anti-S100β 1:100) diluted in a blocking solution for 2 days at 4 ˚C (because of the thickness of the hippocampal slices – 50 µm). The sections were further washed twice with 0.1% Triton in PBS for 5 min under gentle agitation and then, the sections were blocked with 10 % Serum (horse) + 0.1 % Triton in PBS for 15 min under agitation. Afterwards, the slices were incubated with the secondary antibody (anti-rabbit Alexa 488 1:1000, anti-mouse Alexa 594 1:1000 and anti-rabbit Alexa 594 1:1000) for 2 h at RT (some slices were only incubated with secondary antibody to have a negative control of the experimental procedure). After that, the slices were washed 3 times with PBS during 10 min and then, 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI 1:5000) was used for staining the nucleus during 10-15 min at RT under agitation. Finally, the slices were washed 3 times with PBS during 5 min. The next step was the assemble of slices into gelatin-coated slides using DAKO mounting medium and its visualization in the epifluorescence microscope (Zeiss Imager Z2, OberKochen, Germany). The images were captured using a monochromatic camera and processed by the AxioVision SE64 Rel 4.8.2. software (Carl Zeiss). The ImageJ software (v.2.0.0) was used to analyze and quantify the obtained images (figure 20).

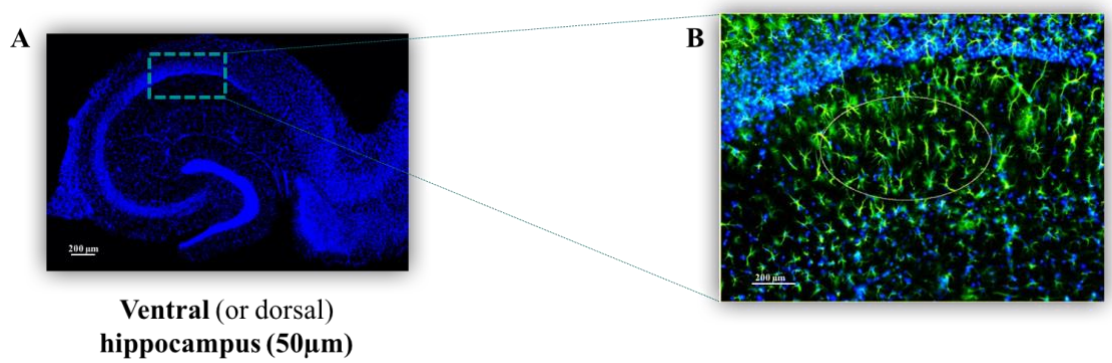

Figure 20. Illustration of the IHC quantification. Sections were labeled with rabbit anti-GFAP and mouse anti-S100β antibodies and with the nuclei dye DAPI, and further visualized in the epifluorescence microscope. **A.** All images were obtained at × 5 ampliation to identify the morphology of hippocampal slices and then, **B.** images were obtained at × 20 ampliation using a fluorescent microscope. The area quantified and used to identify and account for $GFAP^+$ cells was within stratum radiatum (hippocampal CA1 region) always using the same shape. Scale bar of 200 µm for all images.

3.4. Statistical analysis

Data are expressed as mean ± SEM of the indicated number of independent experiments (n) in different animals. Firstly, the normal distribution of the groups was assessed and then, a parametric analysis was performed to all conditions. Statistical analysis to compare the mean value of a single group with a hypothetical value was performed a one-sample *t*-test. Student's *t*-test was used in order to test the significance of the difference between two independent experimental groups. Otherwise, to compare more than two groups, either one or two-way analysis of variance (ANOVA) for independent means was used, followed by a Sidak's multiple comparisons test. Statistical differences were considered statistically significant at $P < 0.05$ and all tests were performed using the GraphPad Prism Software® (StatSoft Inc., La Jolla, CA, USA).

Chapter 4

Results

4.1. Impact of astrocytes blunting on synaptic plasticity in physiological and AD-like conditions

Astrocytes release and uptake gliotransmitters within the synaptic cleft to fine-tune synaptic transmission (Halassa and Haydon 2010; Rial et al. 2016). However, the impact of astrocytes on hippocampal synaptic plasticity in physiological and pathological AD conditions remains to be unravel. In this part of the study we investigate first how AD conditions, mimicked by the exposure to $A\beta_{1\text{-}42}$, affect hippocampal synaptic plasticity, in particular long-term depression (LTD), to later uncover about the role of astrocytes in this plasticity phenomena in physio-pathological conditions. To assess the involvement of astrocytes in synaptic function we used a gliotoxin L-**α**-aminoadipic acid (L-AA), which was previously validated by our group (Pereira 2017, unpublished data) and by others (Khurgel et al. 1996; Brown and Kretzschmar 1998; Lima et al. 2014), as causing astrocyte blunting in different brain regions.

4.1.1. Acute exposure to $A\beta_{1\text{-}42}$ induced a shift in LTD toward LTP

The first step of this work was to evaluate if $A\beta_{1\text{-}42}$ acute exposure induces changes on synaptic plasticity, namely in hippocampal LTD (induced by low-frequency stimulation - LFS). For this purpose, electrophysiological recordings were performed in Schaffer fibers-CA1 pyramidal synapses in hippocampal slices of young adult C57/Bl6 mice that were exposed or not (CTR) to soluble oligomeric $A\beta_{1\text{–}42}$ (50 nM) for 40 min in gassed aCSF, before the recordings. As can be seen in figure 21D, in hippocampal slices incubated only with aCSF (CTR), the amplitude of LTD was of -45.78 ± 10.75 % of baseline, whereas in $A\beta_{1\text{-}42}$ incubated slices it was of 19.61 ± 13.02 % of baseline, representing a significant ($P < 0.05$) reduction in hippocampal LTD from C57Bl/6 mice (figure 21 C and D). It should be referred that were not found differences between I/O curves of the two groups, indicating that changes in synaptic strength were unlikely to be responsible for the observed LTD differences in the slices incubated with $A\beta_{1\text{-}42}$. These data showed that $A\beta_{1\text{–}42}$ exposure, although did not affect significantly the basal synaptic transmission (figure 21B), caused a shift in LTD-to-LTP; that is the application of a LFS in slices exposed to $A\beta_{1\text{–}42}$ triggered a LTP instead of a LTD phenomenon, as observed in control conditions, which suggest that $A\beta_{1\text{-}42}$ acute exposure strongly affected hippocampal synaptic plasticity, impairing LTD maintenance.

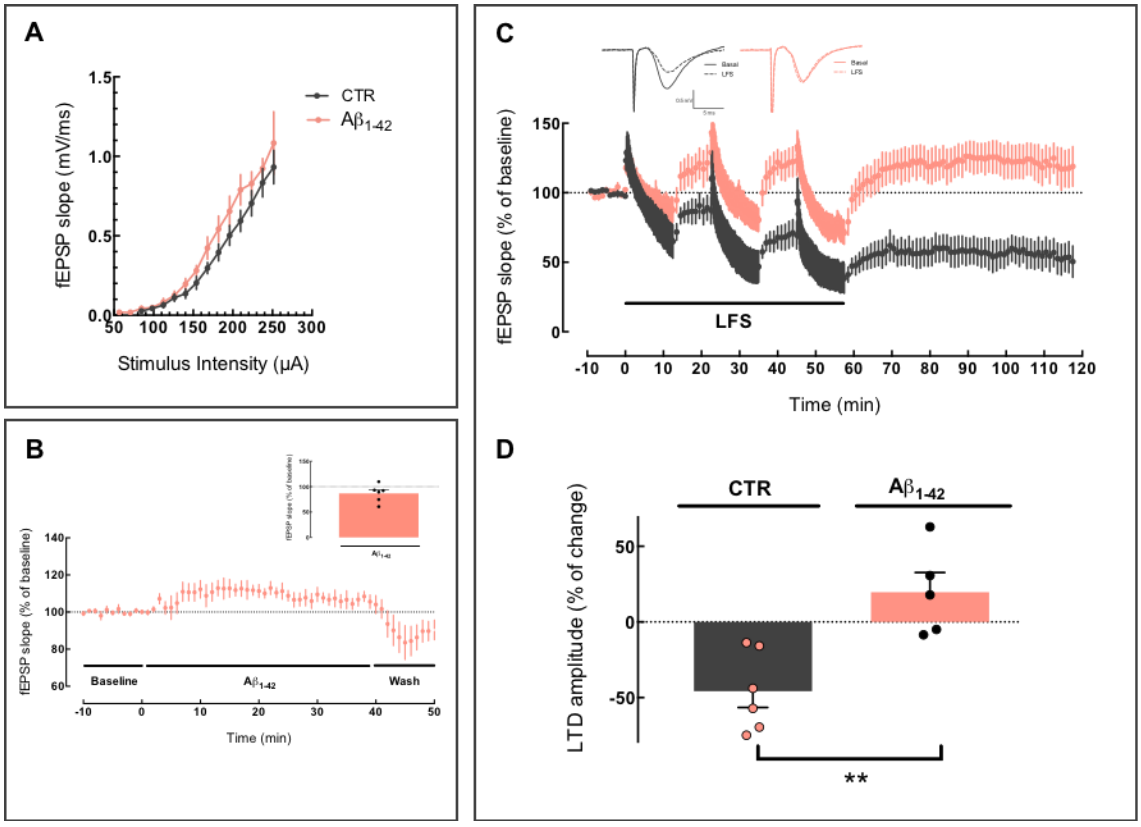

Figure 21. Acute exposure to Aβ $_{1\text{-}42}$ induced a LTD-to-LTP shift. A. Input/output (I/O) curves in hippocampal slices incubated in artificial cerebrospinal fluid (aCSF, control, CTR, n = 6) and with $A\beta_{1\text{-}42}$ (50 nM, 40 min, n = 5). I/O curves were not different between the two groups, indicating that changes in synaptic strength were unlikely to be responsible for the LTD differences in the slices incubated with $A\beta_{1\text{-}42}$. **B.** Effect of $A\beta_{1\text{-}42}$ (50 nM) on basal synaptic transmission. Hippocampal slices were exposed to $A\beta_{1\text{-}42}$ for 40 min prior to LTD induction. The fEPSP slope is expressed as the percentage of the mean of ten points before the addition of $A\beta_{1\text{-}42}$. $A\beta_{1\text{-}42}$ had transitory effect on basal synaptic transmission, with no statistical significance. **C.** Time course of changes in fEPSP slope were shown in graphic C, and there it is denoted the timing of low frequency stimulation (LFS, 3 trains of 1500 pulses at 2 Hz each separated with a 10-min interval). fEPSP amplitude was recorded for 60 min following tetanization to measure LTD. On top of the graphic, representative recording of a typical fEPSP in mouse hippocampus by positioning electrodes in the Schaffer-collaterals-CA1 synapses. Each trace comprises the stimulus artifact, followed by the presynaptic volley and the fEPSP. The intensity of the stimulus was adjusted to evoke a fEPSP without appreciable population spike contamination and responses were quantified as the initial slope of the averaged fEPSPs (the depression after the stimuli artifact). **D.** LTD amplitude corresponding to the average fEPSP slope 50 – 60 min after LTD induction, was significantly decreased in all slices treated with $A\beta_{1\text{-}42}$ (**P < 0.01 vs. CTR, unpaired Students' t-test). All values are mean ± SEM of n independent experiments. The n values refer to the number of mice used per condition.

4.1.2. The gliotoxin L-AA rescued LTD impairment trigged by $A\beta_{1-42}$ acute exposure

In the second part of this study, we evaluated if the blunting of hippocampal astrocytes (with L-AA) affects synaptic plasticity in both physiological and pathological conditions. To achieve this goal, electrophysiological recordings in Schaffer fibers-CA1 pyramidal synapses were performed in slices incubated only with L-AA (100 μM, 2h) or with slices previously incubated with $A\beta_{1-42}$ (50 nM, 40 min) and further incubated with L-AA (100 μM, 2h), to assess the impact of astrocytic dysfunction in physiological and AD-like conditions, respectively. The incubation of hippocampal slices with L-AA had no effect on basal synaptic transmission, as well as no apparent impact on LTD amplitude in physiological conditions. However, the L-AA treatment under AD-like conditions reverted significantly the effects of $A\beta_{1–42}$ on synaptic plasticity, rescuing the induction of LTD when compared with controls (figure 22C). It is important to mentioned that were not found differences between I/O curves, indicating that changes in synaptic strength were unlikely to be responsible for the LTD differences in the slices incubated with $A\beta_{1-42}$ or L-AA. In this set of experiments, we observed that in hippocampal slices incubated only with aCSF (CTR), the amplitude of LTD was of -36.48 ± 8.85 % and slices incubated with L-AA was of -23.36 ± 4.23 % of baseline. On the other hand, in $A\beta_{1-42}$ incubated slices, the amplitude of LTD was of 11.17 ± 7.76 % and $A\beta_{1-42}$ with L-AA was of - 25.43 ± 9.86 % of baseline, representing a significant difference between CTR and $A\beta_{1-42}$ ($P < 0.01$) and between $A\beta_{1-42}$ and $A\beta_{1-42}$ with L-AA ($P < 0.05$) in hippocampal LTD from young adult C57Bl/6 mice. With these observations, it is possible to concluded that astrocytes had an important role in shaping hippocampal synaptic plasticity in AD-like conditions.

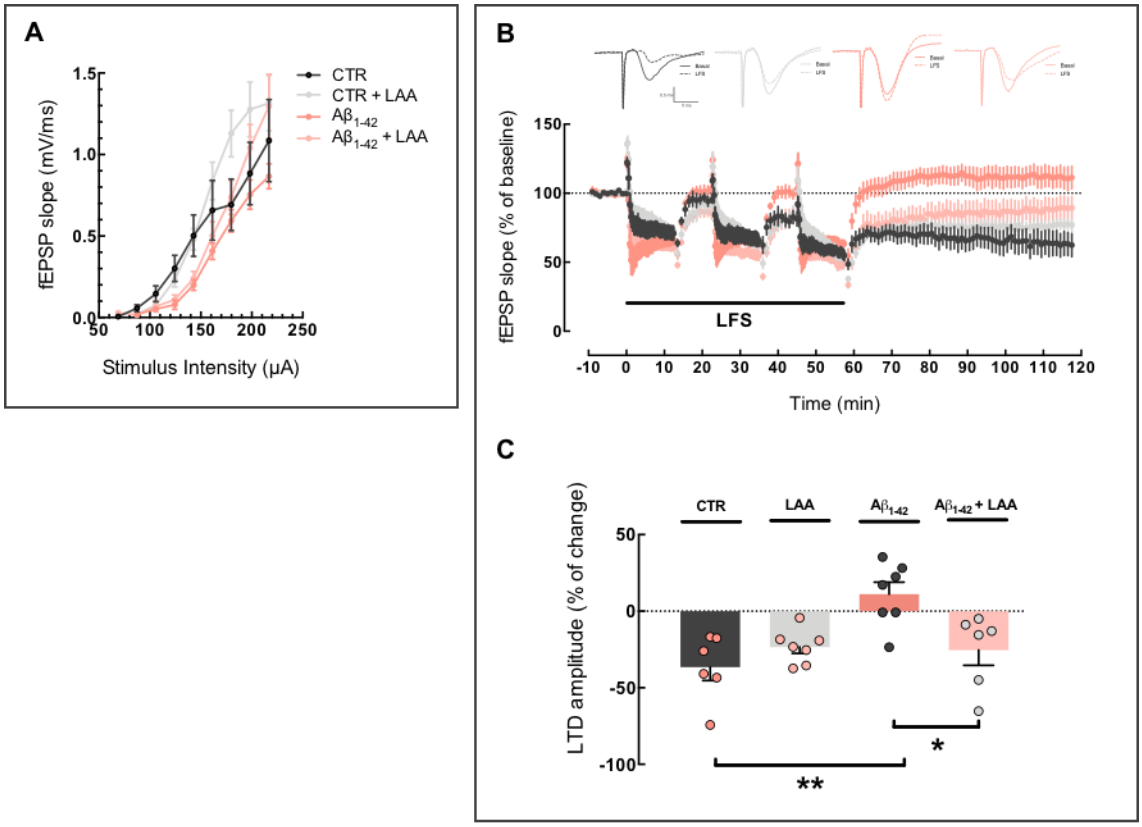

Figure 22. L-AA rescued LTD impairment trigged by $A\beta_{1\text{-}42}$ acute exposure. A. Input/output (I/O) curves in hippocampal slices treated with aCSF (control, CTR, n = 6), L-AA (100 µm, 2 h, n=7), $A\beta_{1\text{-}42}$ (50 nM, 40 min, n = 7) and with both $A\beta_{1\text{-}42}$ (50 nM, 40 min) and L-AA (100 µm, 2 h) n=6. I/O curves are not different between the four groups, indicating that changes in synaptic strength were unlikely to be responsible for eventual LTD differences among the slices incubated with $A\beta_{1\text{-}42}$ or L-AA. **B.** Time course of changes in fEPSP slope were shown in graphic B, and there it is denoted the timing of low frequency stimulation (LFS, 3 trains of 1500 pulses at 2 Hz each separated with a 10-min interval). fEPSP amplitude was recorded for 60 min following tetanization to measure LTD. On top of the graphic, representative recording of a typical fEPSP in the mouse hippocampus by positioning electrodes in the Schaffer-collaterals - CA1 synapses. Each trace comprises the stimulus artifact, followed by the presynaptic volley and the fEPSP. The intensity of the stimulus was adjusted to evoke a fEPSP without appreciable population spike contamination and responses were quantified as the initial slope of the averaged fEPSPs (the depression after the stimuli artifact). **C.** Incubation of hippocampal slices with L-AA (100 µM, 2h) had no effect (n=6) on LTD amplitude in physiological conditions (n=7), corresponding to the average fEPSP slope 50 – 60 min after LTD induction. However, treatment with L-AA under AD-like conditions (*n=6*) reverted significantly the effects of $A\beta_{1\text{–}42}$ on synaptic plasticity, rescuing the LTD impairment when compared with controls (*n=7*), (**P* < 0.05, ***P* < 0.01, one-way ANOVA). All values are mean ± SEM of n independent experiments. The n values refer to the number of mice used per condition.

4.2. Impact of astrocytic blunting on synaptic dysfunction triggered by AD-like conditions

The above described results, although relevant to assess the impact of astrocytes in non-pathological and AD conditions, allows to pave the way to evolve to more complex *in vivo* AD conditions. Therefore, in this part of the study we used an animal model of AD previously validated by our group (Canas et al. 2009), in which C57Bl/6 mice were intracerebroventricularly (icv) injected with $A\beta_{1-42}$ (0.5 nM). This mice model (after 20 days of $A\beta_{1-42}$ overload) were behaviorally evaluated in hippocampal-dependent tasks, namely both recognition and spatial memory through the novel object recognition (NOR) and a modified Y-maze test; and further evaluated regarding alterations in the synaptic function and reactivity of astroglial.

4.2.1. $A\beta_{1-42}$ icv-injected mice displayed memory deficits

Although previous studies of our group and of others have described that the intracerebral $A\beta_{1-42}$ administration causes memory deficits in rodents (Nakamura et al. 2001; Medeiros et al. 2007; Cunha et al. 2008; Canas et al. 2009) it was our purpose to get a mice model of sporadic AD with a robust alteration astrocyte reactivity. So, taking advantage of the experience with the mice model of AD, consisting in the icv $A\beta_{1-42}$ injection, we determined the time after administration of peptide that would occur gliosis by performing immunohistochemical brain analysis of mice injected with $A\beta_{1-42}$, and respective icv-vehicle injected mice. The data obtained revealed that after 20 days of $A\beta_{1-42}$ injection occurs a robust alteration in astrocytes reactivity (figure 27).

To achieve our aim of defining whether the astrocytes contribute in some way to synaptic dysfunction associated with early phase of AD alteration, we performed a set of behavioral tests to assess memory alterations in the model of AD (icv injected with $A\beta_{1-42}$ for 20 days) that we used, which allowed also the validation of our AD mice model. For that, icv-injected mice were subjected to three different behavioral tests: open field (OF), novel object recognition (NOR) and modified Y-maze. OF was performed to check the general animals' "state of health". After three habituations to the OF arena, NOR and Y-maze test were performed in order to evaluate mice performance in hippocampal-dependent tasks, namely both recognition and spatial memory. In OF it was possible to observe that vehicle and $A\beta_{1-42}$ mice displayed a similar travelled distance in the habituation phase (39.73 ± 2.05 m, 26.89 ± 2.07 m and 28.78 ± 2.51 m for vehicles and

34.87 ± 2.07 m, 26.09 ± 1.20 m and 28.30 ± 2.13 m for $A\beta_{1-42}$ mice) and this travelled distance decreased from the first to the third habituation (figure 23A). Furthermore, the time spent in the center (97.35 ± 13.34 s for vehicle and 96.22 ± 8.86 s for $A\beta_{1-42}$ mice) and the time spent in the peripheral area (502.60 ± 13.34 s for vehicle and 503.8 ± 8.86 s for $A\beta_{1-42}$ mice) were also identical in the two groups of animals (figure 23B) as well as the latency of the first entry in the center during the first habituation (1.89 ± 0.59 s to vehicles and 0.94 ± 0.30 s for $A\beta_{1-42}$ injected mice) (figure 23C). As we see in figure 23D, in the NOR test it was possible to observe that the two animal groups displayed a similar preference for both objects available to mice during the training session (28.25 ± 2.01 s for object A and 29.18 ± 2.78 s for object B ($P > 0.1$, n=9-12). In the NOR test session, $A\beta_{1-42}$ injected mice displayed a decrease in recognition index for the novel object (B) compared with vehicle mice (69.77 ± 3.16 % for the vehicle and 60.37 ± 3.12 % for the $A\beta_{1-42}$ injected mice, ($P < 0.05$, n= 9-12) (figure 23E). As it was possible to observe in figure 23F and G, in the modified Y-maze test, $A\beta_{1-42}$ injected mice travelled a lower distance in novel arm (39.50 ± 1.23 % for vehicle and 35.47 ± 1.02 % for $A\beta_{1-42}$ injected mice) and also the number of entries in novel arm was lower (38.88 ± 1.59 % for vehicle and 34.87 ± 0.84 % for $A\beta_{1-42}$ injected mice) as compared with control mice, existing a significant difference between these two groups ($P < 0.05$, n= 9-12). Overall, our behavioral results showed that icv $A\beta_{1-42}$ administration caused changes in hippocampal-dependent tasks, leading to memory deficits compared with icv vehicle injected mice.

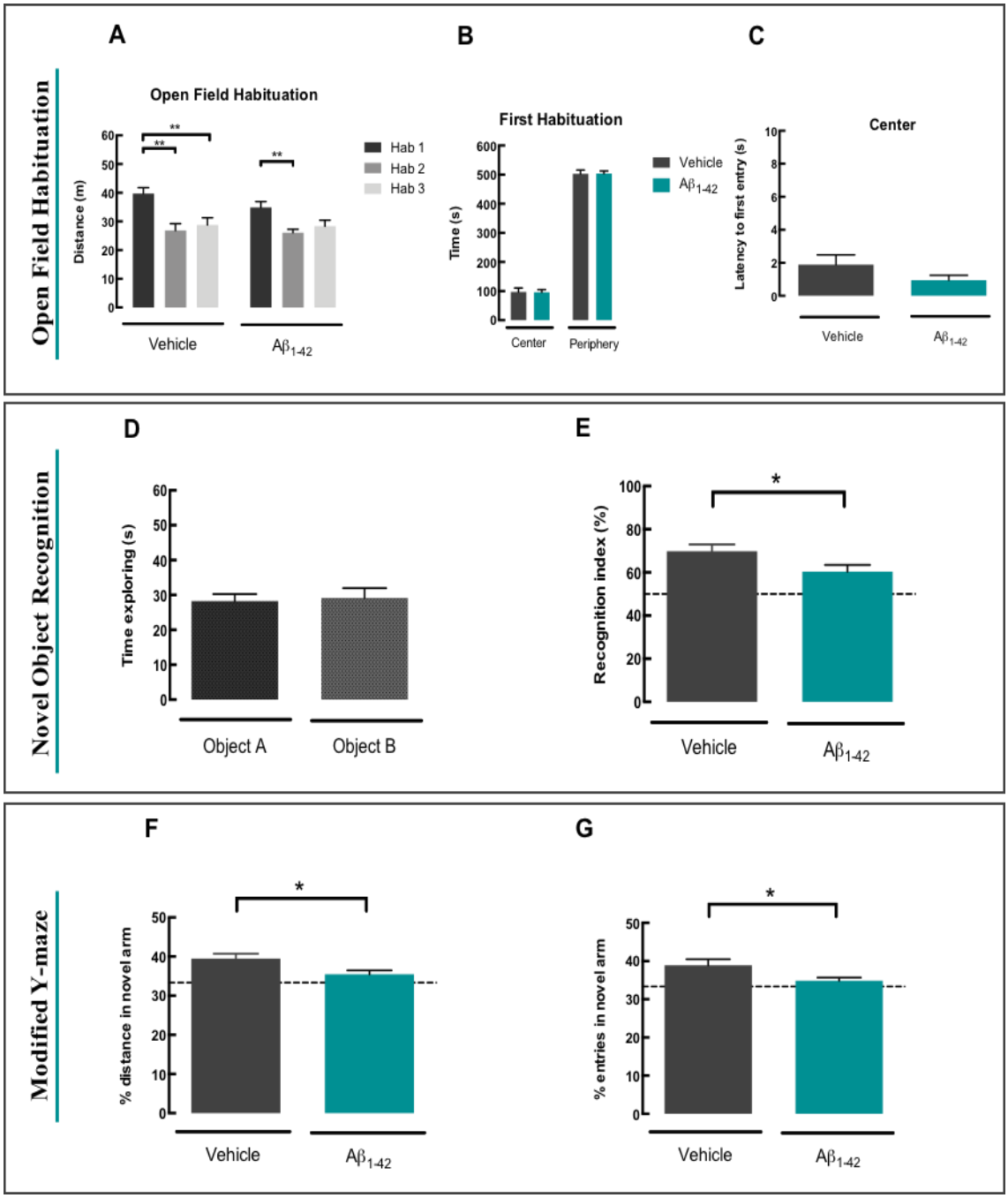

Figure 23. $A\beta_{1\text{-}42}$ icv-injected mice displayed memory deficits. OF was performed in mice icv injected with pure water (vehicle) or $A\beta_{1\text{-}42}$ after 17 days. **A.** Locomotor activity of mice injected with vehicle (n=10) and $A\beta_{1\text{-}42}$ (n=12). The results are expressed as distance travelled (m). $^{**}P < 0.01$, two-way ANOVA. **B. and C.** Open field test for anxiety measures. The parameters used were the time spent in the center (in seconds), the time spent in the peripheral zone (in seconds) and latency to first entry (in seconds). Among these evaluations, there were no differences between the two groups of animals. **D.** Learning and memory performance were evaluated by the novel object recognition test that was performed in icv-$A\beta_{1\text{-}42}$ and icv-vehicle injected mice. It was possible to evaluate the time exploring each object (in seconds) and novel object preference (expressed as a recognition index) during novel object recognition testing. Both groups showed comparable exploration of both objects in sample test ($P > 0.1$, n=9-12). **E.** In the test phase, $A\beta_{1\text{-}42}$ injected mice (n=12) displayed a decrease in exploration of the novel object compared with vehicles (n=9). **F. and G.** Modified Y-maze test performed in vehicle and $A\beta_{1\text{-}42}$ injected mice. $A\beta_{1\text{-}42}$ injected mice (n=12) displayed a decrease in distance travelled in novel arm (% distance) and in entries in novel arm (% entries) compared with vehicle injected mice (n=9) ($^{*}P < 0.05$, unpaired Students' t-test), which can confirm an impairment in learning and memory of $A\beta_{1\text{-}42}$ mice compared with vehicle-injected mice. All values are mean ± SEM of n experiments. The n values refer to the number of mice used.

4.2.2. Intracerebral Aβ$_{1-42}$ overload caused robust alterations in synaptic plasticity

After behavioral characterization of the icv-injected model of AD using hippocampal-dependent tasks, it was important to confirm that Aβ$_{1-42}$ administration had an impact on synaptic plasticity, since these parameters are correlated (Schacher and Hu 2014; Cunha and Agostinho 2010). For this purpose, electrophysiological recordings in Schaffer fibers-CA1 pyramidal synapses were performed under AD-like conditions, that is in hippocampal slices obtained from the mice icv injected with oligomeric Aβ$_{1–42}$ (0.5 nM) or with vehicle injected mice. As can be seen in figure 24 B and C, the icv administration of Aβ$_{1–42}$ significant (P <0.0001) affected the amplitude of LTD, leading to a LTD-to-LTP shift in the mouse hippocampus. Furthermore, we did not found differences between I/O curves, indicating that changes in synaptic strength were unlikely to be responsible for the LTD differences in the slices of both groups of animals. In this set of experiments, we observed that in hippocampal slices of vehicle mice the amplitude of LTD was of -44.00 ± 6.35 % of baseline, whereas in Aβ$_{1-42}$ icv-injected mice it was of 1.83 ± 4.12 % of baseline. Hence, the memory deficits perceived in Aβ$_{1–42}$ icv-injected mice are likely associated with an impaired LTD.

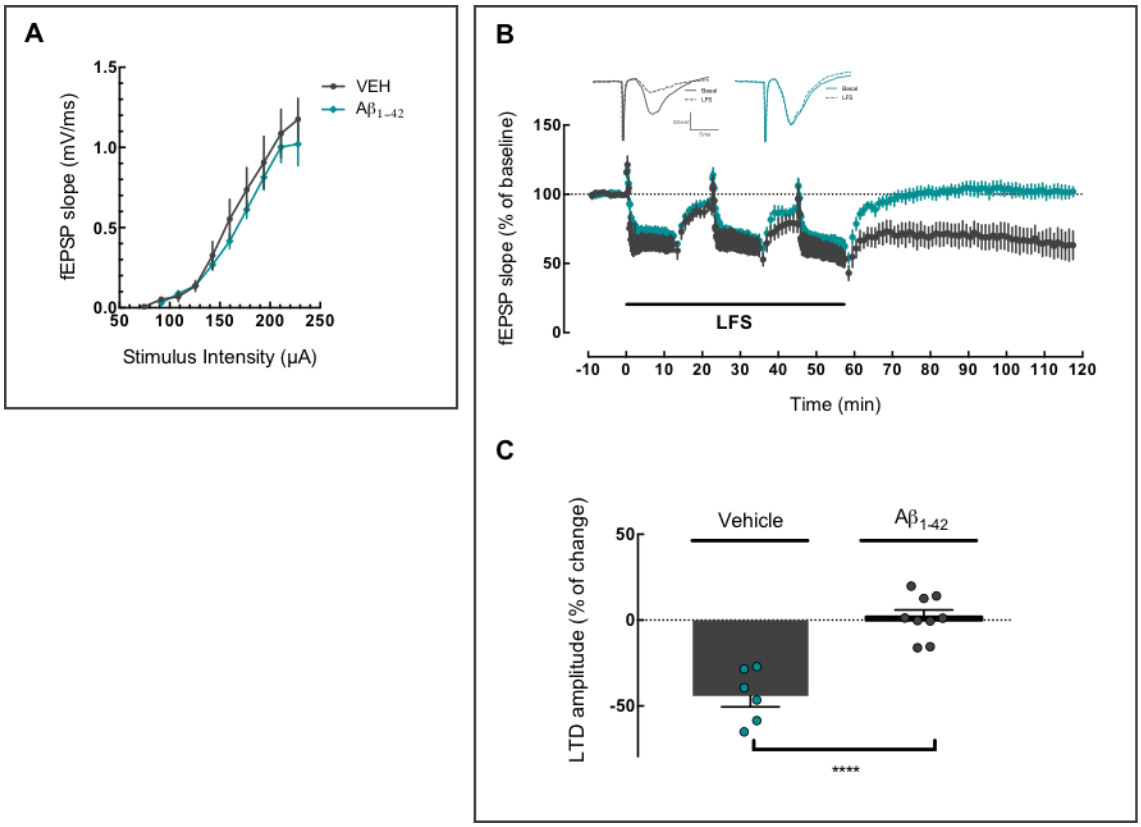

Figure 24. Intracerebroventricular Aβ$_{1-42}$ administration triggered a LTD-to-LTP shift. A. Input/output (I/O) curves in hippocampal slices of young adult mice injected with pure water (Vehicle, CTR, n=6) and Aβ$_{1-42}$ (Aβ$_{1-42,}$2.25 mg/ml, n=9). I/O curves are not different between the two groups, indicating that changes in synaptic strength were unlikely to be responsible for eventual LTD differences among the slices incubated with Aβ$_{1-42}$. **B.** Time course of changes in fEPSP slope were shown in graphic

B, and there it is denoted the timing of low frequency stimulation (LFS, 3 trains of 1500 pulses at 2 Hz each separated with a 10-min interval). fEPSP amplitude was recorded for 60 min following tetanization to measure LTD. On top of the graphic, representative recording of a typical fEPSP in the mouse hippocampus by positioning electrodes in the Schaffer-collaterals-CA1 synapses. Each trace comprises the stimulus artifact, followed by the presynaptic volley and the fEPSP. The intensity of the stimulus was adjusted to evoke a fEPSP without appreciable population spike contamination and responses were quantified as the initial slope of the averaged fEPSPs (the depression after the stimuli artifact). **C.** LTD amplitude corresponding to the average fEPSP slope 50 – 60 min after LTD induction, was significantly decreased in all slices treated with $A\beta_{1-42}$ (**** $P < 0.0001$, unpaired Students' *t*-test). All values are mean ± SEM of n independent experiments. The n values refer to the number of mice used per condition.

4.2.3. The gliotoxin L-AA rescued the alterations in synaptic plasticity induced by icv $A\beta_{1-42}$ administration

Following the evaluation of the impact of $A\beta_{1-42}$ administration on synaptic plasticity, we sought to establish the impact of astrocyte dysfunction (using L-AA) in synaptic function both in physiological or "non-pathological" and AD-like conditions. Thus, electrophysiological recordings in Schaffer fibers-CA1 pyramidal synapses were performed in hippocampal slices obtained from the icv vehicle- and icv $A\beta_{1-42}$-injected mice that were further incubated with L-AA (100 μM for 2h). Incubation of hippocampal slices with L-AA had no effect on basal synaptic transmission as well as no apparent impact on LTD amplitude in mice icv injected with vehicle. However, with the gliotoxin, L-AA, under AD-like conditions (slices from icv $A\beta_{1-42}$ injected mice) ($P < 0.05$) reverted significantly the effects of peptide on synaptic plasticity, rescuing the impairment of LTD when compared with the vehicle injected mice (figure 25 B and C). Additionally, we did not found differences between I/O curves, indicating that changes in synaptic strength were unlikely to be responsible for the LTD differences in the slices of the different groups of animals. Therefore, we are able to observe that in hippocampal slices of icv vehicle mice the amplitude of LTD was -44.19 ± 6.35 % and in slices of the same mice incubated with L-AA was -21.22 ± 4.93 % of baseline, whereas in hippocampal slices of $A\beta_{1-42}$ injected mice it was of 1.83 ± 4.12 % and in slices of the same mice incubated with L-AA it was of -24.88 ± 5.68 % of baseline. Indeed, there were significant ($P < 0.001$) differences between the vehicle and $A\beta_{1-42}$ and between $A\beta_{1-42}$ and $A\beta_{1-42}$ with L-AA of in hippocampal LTD from young adult C57Bl/6 mice. With these observations, it was possible to conclude that astrocytes play an important role in shaping synaptic plasticity of the mouse hippocampal Schaffer collaterals CA1 in this mice AD model.

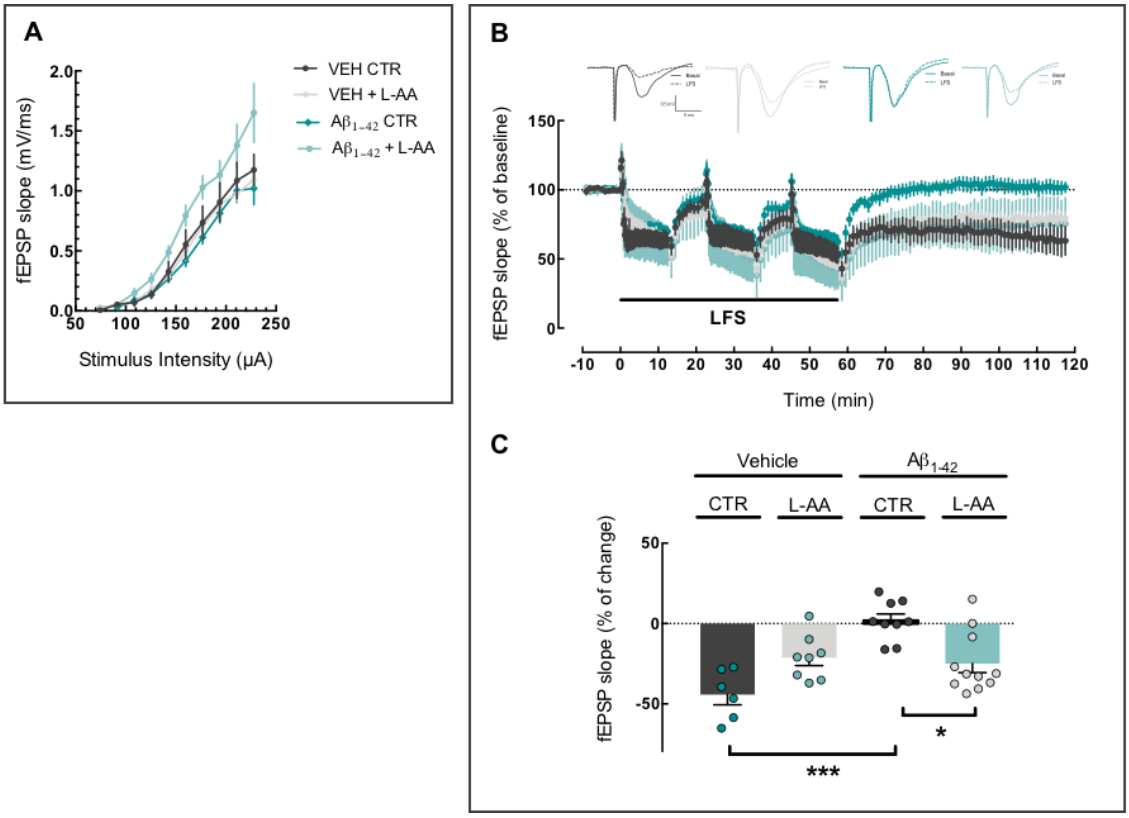

Figure 25. Astrocytic blunting (with L-AA) rescued LTD impairment after Aβ1-42 administration. A. Input/output (I/O) curves in young adult mice injected with pure water (Vehicle, CTR, n=6) and Aβ1-42 (Aβ1-42, 0.5 nM, n=9) and with both slices incubated with L-AA (100 µm, 2 h, n=8-11). I/O curves are not different between the two groups, indicating that changes in synaptic strength were unlikely to be responsible for eventual LTD differences among the slices incubated with Aβ1-42 or L-AA. **B**. Time course of changes in fEPSP slope were shown in graphic B, and there it is denoted the timing of low frequency stimulation (LFS, 3 trains of 1500 pulses at 2 Hz each separated with a 10-min interval). fEPSP amplitude was recorded for 60 min following tetanization to measure LTD. On top of the graphic, representative recording of a typical fEPSP in mouse hippocampus by positioning electrodes in the Schaffer-collaterals - CA1 synapses. Each trace comprises the stimulus artifact, followed by the presynaptic volley and the fEPSP. The intensity of the stimulus was adjusted to evoke a fEPSP without appreciable population spike contamination and responses were quantified as the initial slope of the averaged fEPSPs (the depression after the stimuli artifact). **C.** Incubation of vehicle hippocampal slices with L-AA (100 µM, 2h) had no effect (n=8) on LTD amplitude in physiological conditions (n=6), corresponding to the average fEPSP slope 50 – 60 min after LTD induction. However, treatment with L-AA under AD-like conditions (n=11) reverted significantly the effects of Aβ1–42 on synaptic plasticity, rescuing the impairment of LTD (n=9), (**P* < 0.05, ****P* < 0.001, two-way ANOVA). All values are mean ± SEM of n independent experiments. The n values refer to the number of mice used per condition.

4.2.4. Aβ1-42 administration decreased LTP amplitude

Synaptic plasticity manifested as LTD and LTP, two major molecular mechanisms that are thought to be involved in learning and memory processes (Kochlamazashvili et al. 2011). Therefore, we investigated the impact of astrocyte blunting (using L-AA) on plasticity LTP phenomena both in physiological and AD-like conditions (mice icv injected with Aβ1-42). Thus, electrophysiological recordings in Schaffer fibers-CA1 pyramidal synapses, were performed in hippocampal slices from the icv vehicle and icv Aβ1-42 injected mice that were further incubated with L-AA (100 µM, 2h). As can be seen

in figure 26 B and C, the gliotoxin L-AA had no effect on basal synaptic transmission, but reduced significant ($P < 0.01$) the LTP amplitude in physiological conditions. Furthermore, hippocampal slices of icv $A\beta_{1-42}$ injected mice showed a significant ($P < 0.05$) reduction in hippocampal LTP compared with the icv vehicle injected mice. Moreover, no differences between I/O curves were found, indicating that changes in synaptic strength were not responsible for the LTP differences in the slices of these animals. In this set of experiments, it was possible to observe that in hippocampal slices of the icv vehicle mice, the amplitude of LTP was of 79.00 ± 7.28 % and in the slices of the same mice incubated with L-AA it was of 37.95 ± 8.15 % of baseline, whereas in hippocampal slices of $A\beta_{1-42}$ injected mice it was 48.04 ± 6.96 % and in the slices of the same mice incubated with L-AA it was of 22.69 ± 4.24 % of baseline. These observations imply that astrocytes in physiological conditions are important elements in shaping synaptic plasticity. In AD conditions, the astrocytes blunting had no additional effect on the $A\beta_{1-42}$-induced LTP decrease.

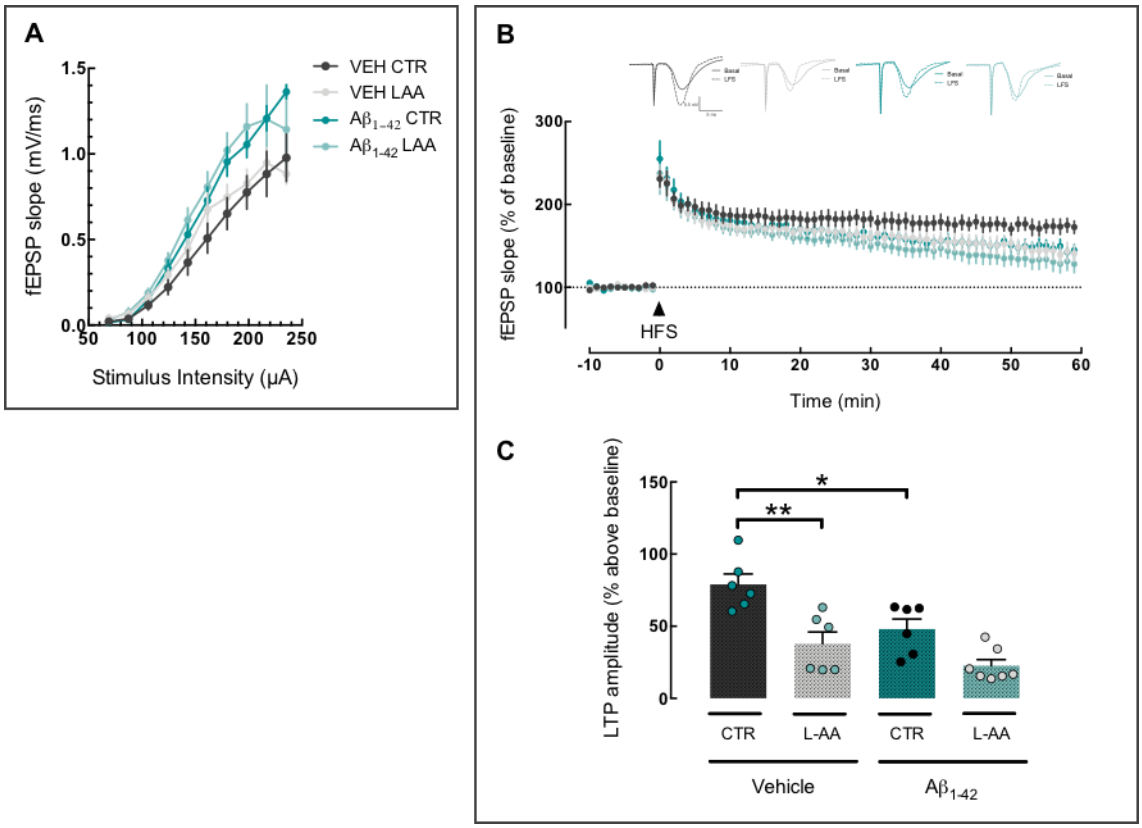

Figure 26. $A\beta_{1-42}$ administration decreased LTP. A. Input/output (I/O) curves in young adult mice injected with water (Vehicle, CTR, n=6) and $A\beta_{1-42}$ ($A\beta_{1-42}$, 0.5 nM mg/ml, n=6) and with both slices incubated with L-AA (100 µm, 2 h, n=6-7). I/O curves are not different between the four groups, indicating that changes in synaptic strength were unlikely to be responsible for eventual LTP differences among the slices incubated with $A\beta_{1-42}$ or L-AA. **B.** Time course of changes in fEPSP slope were shown in graphic B, and there it is denoted the timing of high frequency stimulation (HFS, 1 s, 100 Hz). fEPSP amplitude was recorded for 60 min following tetanization to measure LTP. On top of the graphic, representative recording of a typical fEPSP in mouse hippocampus by positioning electrodes in the Schaffer-collaterals - CA1 synapses. Each trace comprises the stimulus artifact, followed by the presynaptic volley and the fEPSP.

The intensity of the stimulus was adjusted to evoke a fEPSP without appreciable population spike contamination and responses were quantified as the initial slope of the averaged fEPSPs (the depression after the stimuli artifact). **C.** $A\beta_{1-42}$ administration (n=6) induced a significant LTP decrease compared with the vehicles (n=6). Incubation of vehicle hippocampal slices with L-AA (100 μM, 2h) had a significant effect (n=6) on LTP amplitude in physiological conditions (n=6), corresponding to the average fEPSP slope 50 – 60 min after LTD induction. However, treatment with L-AA under AD-like conditions (n=11) had no effect on synaptic plasticity. (*$P < 0.05$, **$P < 0.01$, two-way ANOVA). All values are mean ± SEM of n independent experiments. The n values refer to the number of mice used per condition.

4.2.5. Acute treatment of hippocampal slices with L-AA increased labelled astrocytic markers

The data obtained in the above reported studies showed that the AD mice model, consisting in icv $A\beta_{1-42}$ injection, displayed memory deficits that were accompanied by a significant alteration in hippocampal synaptic plasticity, in both LTD and LTP. Moreover, it was possible to infer that astrocytes were important for shaping synaptic plasticity both in physiological and pathological conditions. Several studies reported that $A\beta_{1-42}$ accumulation lead to astrocytes activation and subsequent release of pro- and anti-inflammatory mediators, namely cytokines, which can trigger synaptic dysfunction (reviewed in Agostinho et al. 2010; Purpura et al. 2012). Thus, we considered that it was important to realize whether astrocytic markers are affected by $A\beta_{1-42}$ overload (in icv $A\beta_{1-42}$ injected mice) and, by the addition of L-AA. In this study, we quantified the immunoreactivity of two astrocytic proteins, GFAP and S100β, and counted the number of astrocytes labeled with GFAP (GFAP positive cells). Therefore, hippocampal slices of icv vehicle and icv $A\beta_{1-42}$ injected mice used in electrophysiological recordings (400 μm) were incubated with L-AA or aCSF (control) for 2 h and, afterward, were sectioned into 50 μm sections to be immunolabelled for GFAP and S100β and with nuclear dye DAPI. The data obtained showed a significant increase ($P < 0.05$) in GFAP immunoreactivity in $A\beta_{1-42}$ icv injected mice (118.90 ± 7.03 %) as compared with vehicle injected mice (100.00 ± 4.05 %). However, the immunoreactivity of S100β was slightly lower in icv $A\beta_{1-42}$ mice (89.28 ± 3.28 %) than in icv-vehicle mice (100.00 ± 6.35 %). Furthermore, no significant ($P > 0.05$) alteration were found in the number of GFAP positive cells in these two group of animals (100.00 ± 2.04 % for the vehicles and 101.60 ± 1.75 % for $A\beta_{1-42}$ mice) (see figure 27 A, B C and D).

Regarding the effect of gliotoxin in non-pathological and AD-conditions, we found that hippocampal section of icv vehicle mice (non-pathological) exposed to L-AA exhibited a significant increase ($P < 0.01$) in GFAP (120.00 ± 4.01 %) immunoreactivity compared with respectively vehicle injected mice, but no significant effect were detected

in S100β immunoreactivity (107.20 ± 3.94 %) nor in GFAP positive cells (100.60 ± 2.52 %). However, the hippocampal slices of the $A\beta_{1-42}$-injected mice incubated with L-AA did not present any alterations in GFAP (95.88 ± 4.10 %) nor in S100β (95.30 ± 3.53 %) immunoreactivity as well as in GFAP positive cells (95.58 ± 1.41 %) compared with their control condition ($A\beta_{1-42}$-injected mice without L-AA) (figure 27 E, F and G).

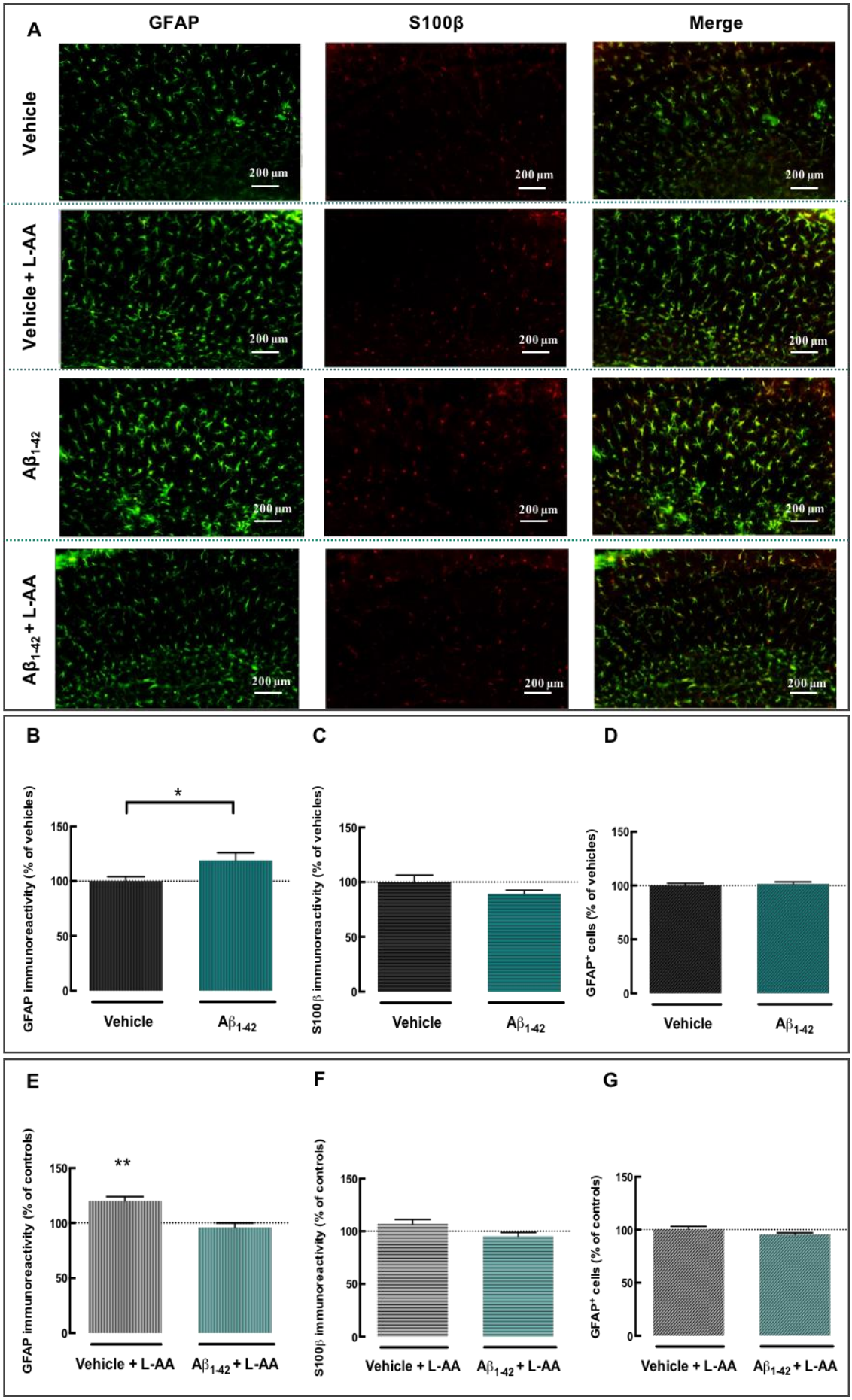
A
GFAP
S100β
Merge
Vehicle
Vehicle + L-AA
Aβ1-42
Aβ1-42 + L-AA
200 µm
B
C
D
GFAP immunoreactivity (% of vehicles)
S100β immunoreactivity (% of vehicles)
GFAP+ cells (% of vehicles)
*
150
100
50
0
Vehicle
Aβ1-42
E
F
G
GFAP immunoreactivity (% of controls)
S100β immunoreactivity (% of controls)
GFAP+ cells (% of controls)
**
Vehicle + L-AA
Aβ1-42 + L-AA

Figure 27. Acute treatment of hippocampal slices with L-AA increased astrocytic markers A. Immunolabelling of astrocytes in hippocampal sections (50 μm thickness). Representative images of GFAP (green), S100β (red) and merged channels obtained from hippocampal slices of icv-vehicles and icv $A\beta_{1-42}$ administrated mice that were further incubated with aCSF (CTR, with 2 slices per animal) or with L-AA (100 μM, 2h, with 2 slices per animal). Scale bar of 200 μm for all panels. All images were obtained at ×20 amplification using a fluorescent microscope and the area quantified was within *stratum radiatum* (hippocampal CA1 region). **B.** Bar graph shows GFAP immunoreactivity f (**P* < 0.05, unpaired Students' t-test) and **C.** S100β immunoreactivity in hippocampal sections of icv vehicles (n = 8) and icv $A\beta_{1-42}$ (n = 5) injected mice (results were normalized as % of vehicles). **D.** Number of $GFAP^+$ positive cells, normalized in function of the results obtained in icv vehicle mice. **E**. Bar graph of GFAP immunoreactivity (***P* < 0.01, One sample test (hypothetical value = 100%) and **F.** S100β immunoreactivity in hippocampal sections of icv vehicle (n = 8) and icv $A\beta_{1-42}$ (n = 8) injected mice that were both incubated with L-AA (results were normalized in % of their respective controls). **G.** Number of $GFAP^+$ positive cells, normalized their relatively to respective controls. GFAP and S100β immunoreactivities and number of GFAP positive cells are expressed as the mean ± SEM of the n indicated.

4.3. Impact of astrocytes blunting on synaptic plasticity and astrocytic markers in a transgenic mice model of AD

The triple transgenic mice model (3×TgAD) has three mutations associated with familial AD and develop both amyloid plaques and NFT (Oddo et al. 2003), and has been widely used. In this study we used this animal model of fAD to gather more information about the involvement of astrocytes in AD-like conditions. The nonTg and 3xTgAD mice used in this study had 11 months old; at this age, it is described that the 3xTgAD mice presented Aβ deposits in brain, cognitive impairment, alterations in synaptic plasticity and gliosis (https://www.alzforum.org/research-models/3xtg). To assess the impact of astrocytes blunting on synaptic function in this AD mice model it was used in hippocampal slices, obtained from 3×TgAD and age-matched nonTg mice, superfused or not with gliotoxin L-AA for 2 h, and fEPSP was measured to gauge synaptic plasticity in Schaffer fibers-CA1 pyramidal synapses, namely LTP. Moreover, it was also identified alterations in astrocytic markers, mainly in GFAP, by immunohistochemistry analysis of hippocampal sections (50 μm).

4.3.1. The triple transgenic AD mice displayed alterations in synaptic plasticity

Electrophysiological recordings in Schaffer fibers-CA1 pyramidal synapses performed in hippocampal slices of nonTg and 3×TgAD mice incubated with L-AA (100 μM, 2h) revealed that L-AA had no effect on basal synaptic transmission but tend to increase LTP amplitude in nonTg mice. Contrary to icv-$A\beta_{1-42}$ injected mice, hippocampal slices of 3×TgAD mice showed a slight increase in hippocampal LTP compared with the age-matched nonTg mice (figure 28). The amplitude of LTP in nonTg was of 21.80 ± 8.43 % and in slices of the same mice incubated with L-AA was of 42.43 ± 12.83 % of baseline. In hippocampal slices of 3×TgAD mice the LTP amplitude was

90.72 ± 14.84 % and slices of the same mice incubated with L-AA it was of 37.07 ± 16.92 % of baseline (figure 28B and C). These results contrast with the commonly held view that 3xTgAD mouse model correlates with a reduction in LTP efficacy; the increased LTP amplitude observed in the 3xTgAD used in our studies could be due to the age of the animals. Indeed, it would be useful use more animals (larger n) to perform the study and evaluate synaptic function in animals with different moths of age.

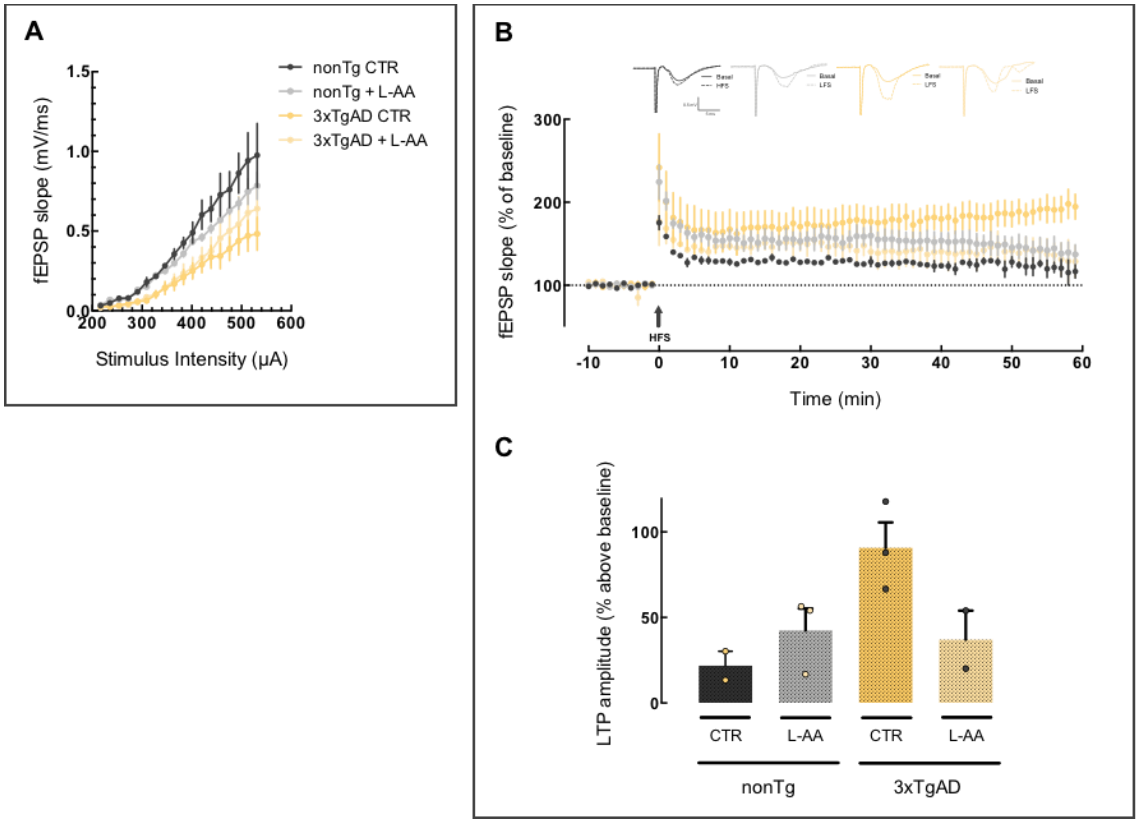

Figure 28. Triple transgenic AD mice displayed alterations on synaptic plasticity. A. Input/output (I/O) curves in non-transgenic mice (nonTg, n=2) and triple transgenic mice (3xTg-AD, n=3) incubated or not with L-AA (100 µm, 2 h, n=2-3). I/O curves were not different between the four groups, indicating that changes in synaptic strength were unlikely to be responsible for the eventual LTP differences among the different experimental groups. **B.** Time course of changes in fEPSP slope were shown in graphic B, and there it is denoted the timing of high frequency stimulation (HFS, 1 s, 100 Hz). fEPSP amplitude was recorded for 60 min following tetanization to measure LTP. On top of the graphic, representative recording of a typical fEPSP in mouse hippocampus by positioning electrodes in the Schaffer-collaterals-CA1 synapses. Each trace comprises the stimulus artifact, followed by the presynaptic volley and the fEPSP. The intensity of the stimulus was adjusted to evoke a fEPSP without appreciable population spike contamination and responses were quantified as the initial slope of the averaged fEPSPs (the depression after the stimuli artifact). **C.** 3xTg-AD (n=3) displayed an increase in LTP amplitude, compared with nonTg (n=2). In nonTg hippocampal slices the L-AA incubation (100 µM, 2h) had no significantly effect (n=3) on LTP amplitude n=2), corresponding to the average fEPSP slope 50 – 60 min after LTD induction. However, treatment with L-AA of hippocampal slices of 3xTgAD (n=2) caused a decrease in LTP amplitude. All values are mean ± SEM of n independent experiments. The n values refer to the number of mice used per condition.

4.3.2. Acute treatment of hippocampal slices with L-AA increased astrocytic markers immunoreactivity

Considering previous results, we thought it would be interesting to grasp whether astrocytes are affected in these 3xTgAD that were or not further challenged with the gliotoxin, L-AA; since this model had changes in hippocampal synaptic plasticity. For this purpose, immunohistochemical studies were performed using hippocampal slices of triple transgenic mice and their littermates (nonTg) to quantify GFAP immunoreactivity. Both hippocampal slices of nonTg and 3×TgAD mice were incubated with L-AA (100 µM during 2 h) and, after that, slices were sectioned into 50 µm sections to be used in immunohistochemistry. To identify and quantify astrocytes, hippocampal sections were labeled with anti-GFAP antibody and with DAPI, a nuclear dye. The GFAP immunoreactivity was slightly higher in 3×TgAD (118.00 ± 9.08 % for CA1 and 107.10 ± 7.56 % for CA3) as compared with nonTg mice (100.00 ± 2.03 % for CA1 and 100.00 ± 0.94 % for CA3) in CA1 and CA3 hippocampal regions (figure 29A and B). When the slices were incubated with L-AA, there were also no differences in GFAP immunoreactivity between nonTg (107.50 ± 2.03 %) and 3xTgAD (91.12 ± 1.72 %) conditions in CA1 region, even though we were able to detect a tendency to increase in nonTg (129.60 ± 9.64 %) and 3×TgAD (144.50 ± 17.24 %) mice in CA3 region (see figure 29 A and C).

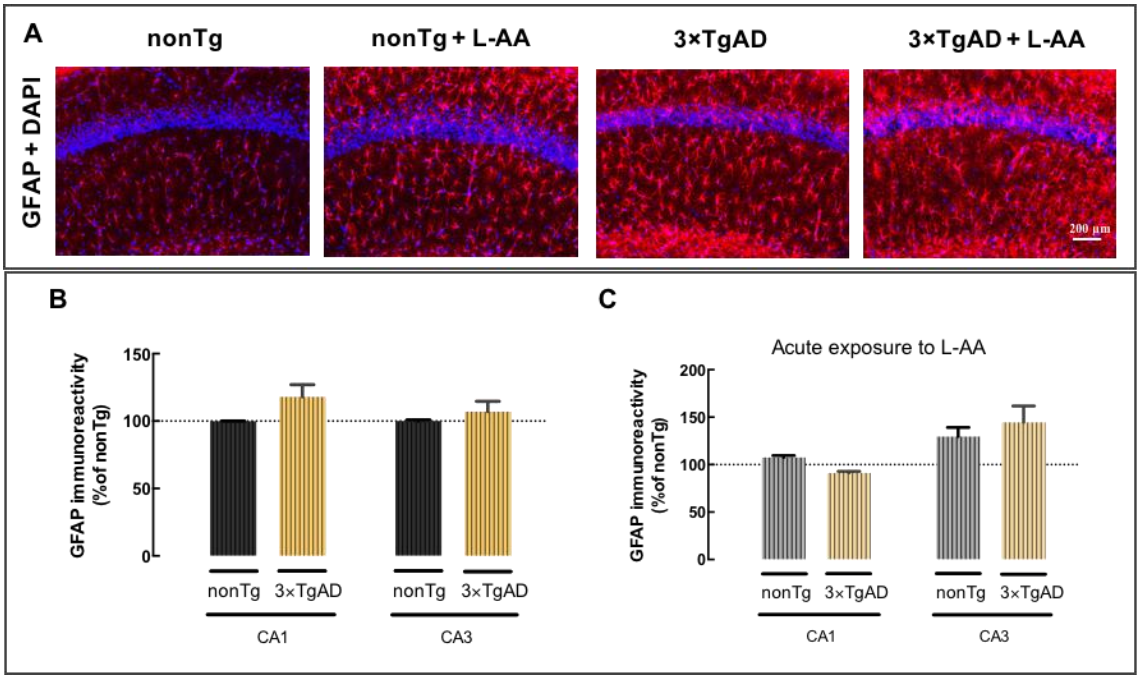

Figure 29. Acute treatment of hippocampal slices with L-AA increased astrocytic reactivity in nonTg and 3xTgAD. Immunohistochemical labelling of astrocytes in hippocampal sections (50 µm thickness). **A.** Representative images of immunolabeling of GFAP (red) and DAPI staining (blue) in hippocampal slices of nonTg and 3×TgAD mice previously incubated with aCSF (CTR, n = 2, with 2 slices per animal) or

incubated with L-AA (100 μM, 2h, n = 3, with 2 slices per animal). Scale bar of 200 μm for all panels. All images were obtained at ×20 amplitude using a fluorescent microscope and the area quantified was within hippocampal CA1 and CA3 region. **B.** Bar graph shows immunoreactivity of GFAP in hippocampal sections (CA1 and CA3) of nonTg and 3×TgAD mice (results were normalized as % of nonTg). **C**. Bar graph of immunoreactivity of GFAP in CA1 and CA3 of nonTg and 3×TgAD mice both incubated with L-AA (results were normalized in % of their respective controls). Immunoreactivity of GFAP are expressed as the mean ± SEM of the n indicated.

Chapter 5

Discussion

Discussion

In the present study, we mimicked AD-like conditions, through the exposition to oligomers of synthetic $A\beta_{1-42}$ peptides for hours (acute exposure) in hippocampal slices of mice or for days (chronic exposure by icv injection in living mice), with the aim of engendering the Aβ overload that occur in sporadic AD, in which the Aβ overproduction is not associated with genetic mutations (Hardy and Selkoe, 2002). The $A\beta_{1-42}$ peptides have been widely used by our group and others groups to model AD conditions and to study the associated neurodegenerative processes (reviewed in Pereira et al. 2005; Resende et al. 2008; Agostinho and Cunha 2010; Hernández-Zimbrón and Rivas-Arancibia 2014). In particular, our group has a large experience in a mice model of AD, consisting in the icv injection of $A\beta_{1-42}$, in which after at least 15 days occurs memory deficits, dysfunction of mitochondria in synapses, astrocytic glutamate clearance impairment and loss of synaptic markers, mainly of glutamatergic terminals, as well as overactivation of cyclin-dependent kinase 5 that is involved in tau hyperphosphorylation (Canas et al. 2009; Lopes et al. 2010; Matos et al. 2012; Amorim et al. 2017). In this study, we injected the synthetic peptide $A\beta_{1-42}$ in the one of the lateral ventricles, which are in close proximity with hippocampi, a region responsible for memory processing that is affect in early AD phases (Paxinos and Franklin 2001; Dall'Igna et al. 2007; Cunha et al. 2008; Canas et al. 2009). Accordingly in the present study we showed that seventeen days after the icv injection, $A\beta_{1-42}$ mice had spatial and recognition memory deficits (hippocampal-dependent tasks), evaluated by NOR and Y-maze tests (figure 23), which are the first evident behavioral modifications found in several AD models (Silva et al. 2018), which were neither associated with alterations in locomotor activity nor anxiety, as assessed the distances travelled in open field and the time spent in the center and peripheral area, respectively.

The data obtained with the present study showed that both the acute exposition to $A\beta_{1-42,}$ directly applied to hippocampal slice (figure 21) as well as the icv injection of $A\beta_{1-42}$ in mice had robust impact on synaptic plasticity, mainly in LTP and LTD (see figure 24 and 26, respectively), causing a reduction in these synaptic phenomena. It should be refereed that a study from our group had also shown that the acute application of $A\beta_{1-42}$ to hippocampal mice slices also lead to a reduction in LTP (Pereira et al. 2018, manuscript in preparation). Thus, these data strongly support that the overload of $A\beta_{1-42}$ is sufficient

to trigger long-lasting changes in the efficacy of synaptic potentiation (LTP) and depression (LTD), two events believed to represent the neurophysiological basis for memory (Tsien et al. 1996; Martin et al. 2000; Whitlock et al. 2006; Luscher and Malenka 2012), which is compromised even in early stages of AD (Tamagnini et al. 2012; Koch et al. 2012; Prieto et al. 2017; Silva et al. 2018). LTP has been extensively studied in AD animal models. However, LTD, the usual counterpart of LTP, has been neglected over time even though some recent studies show the crucial role of this mechanism to hippocampus-dependent learning (Van der Jeugd et al. 2011; Tamagnini et al. 2012; Silva et al. 2018). The results obtained in our study are in agreement with data previously published by other groups showing that Aβ peptide decrease the LTP amplitude leading to synaptic plasticity deterioration and, consequently, memory and learning impairment (Chen et al. 2000; Martin et al. 2000; Hughes and Herron 2018). In addition, other studies described the effects of soluble Aβ peptides or AβPP fragments on LTD, although in most of these cases this phenomenon was facilitated (Kim et al. 2001; Cheng et al. 2008; Shankar et al. 2008; Li et al. 2009; O'Riodan et al. 2018). However, this apparent contradiction may be explained by the difference in protocols used to induce LTD. In a model of AD-like tauopathy (Van der Jeugd et al. 2011; Laurent et al. 2016), using the same protocol we used, it was observed a decrease in hippocampal LTD amplitude, that is in accordance with the results obtained our study. Furthermore, a decrease in LTD amplitude caused by sAβPPα was also observed in hippocampal slices of adult rats, where it was used a stimulation protocol (LFS) similar to the one used in our study (Ishida et al. 1997). Since LTP involves insertion of more AMPARs into synapses, whereas LTD may involve their removal or endocytosis from synapses, we can speculate that AD-like conditions are affecting the turning over of AMPARs. The control of AMPARs number at synapses requires a balance between biosynthesis (receptors production), membrane trafficking (exocytosis and endocytosis of receptors), and degradation of receptors; and there are some studies that already reported that Aβ peptides impair AMPARs trafficking with a harmful effect on memory (Minano-Molina et al. 2011; Guntupalli et al. 2016). Moreover, both LTP and LTD can be dependent on NMDARs thus, these changes in synaptic plasticity may be related to modifications in intracellular signaling pathways or in the trafficking of these receptors (Kumar 2011; Van der Jeugd et al. 2011). The direct application or the accumulation of Aβ peptides or oligomers can be synaptotoxic, turning its "friendly" to "foe" action onto synaptic function, and may inhibit LTP and triggers LTD-like changes. The Aβ toxicity can also decreases synaptic NMDARs, since it affects

the density and stability of the dendritic spines, targeting many receptors present on the surface of dendritic spines, which could impair LTP (Kamenetz et al. 2003; Luscher and Malenka 2012; Peineau et al. 2018).

Other major goal of this study was to evaluate the impact of astrocytes on synaptic plasticity in physio-pathological conditions, thus we used a pharmacological tool to blunt astrocytes function and thus infer the role of these glial cells in conditions of AD caused by Aβ over-exposition. We used a selective astrocyte toxin L-α-aminoadipate (L-AA), previously validated by our group (Pereira 2017) as a trigger of astrocyte dysfunction. This gliotoxin that is taken by astrocytes, through glutamate transporters, inhibits glutamine synthetase, causing glutamate dyshomeostasis, and a decrease in astrocytes viability in prefrontal cortex and amygdala of rodents (Khurgel et al. 1996; Lima et al. 2014). Nevertheless, few (one) studies had addressed the impact of L-AA on hippocampal related function, in particular memory (Choi et al. 2016) and currently none had looked into hippocampal synaptic dysfunction associated with astrocytes blunting. Our data showed that L-AA (100 μM, 2h) had no effect on hippocampal basal synaptic transmission (data not shown) as well as no apparent impact on LTD amplitude in physiological conditions (figure 22 and 25). However, treatment with L-AA under AD-like conditions (both acute application and icv injection of $A\beta_{1–42}$) significantly reverted the effects of $A\beta_{1–42}$ on synaptic plasticity, rescuing the impairment of LTD when compared with controls or vehicles (figure 22 and 25, respectively). In addition to LTD phenomenon in the $A\beta_{1-42}$ icv-injected animals, the changes in synaptic LTP changes were also been evaluated in this model (figure 26), since both these plasticity phenomena are involved in memory that is impaired in AD. The application of L-AA only had an impact on physiological conditions decreasing the amplitude of LTP, which had already been demonstrated by the group (Pereira et al. 2018). These data led us to speculate that astrocytes can have a particular role in shaping the synaptic function in non-pathological conditions, whereas in conditions of AD these cells seem to be already dysfunctional by the $A\beta_{1-42}$ exposure, since their blunting by L-AA did not exacerbated the effects triggered by this peptide. It is known that astrocytes are essential elements in synaptic plasticity modulation under physiological conditions, namely by their active participation in tripartite synapses. Thus, several studies have already been done to deepen the knowledge about the astrocytic function, using tools that blunt the astrocytes function, namely using L-AA (Huck et al. 1984; Mcbean 1994; Tsai et al. 1996; Oliveira et al. 2015). However, data previously collected in our group (Pereira 2017, unpublished data) reported the

involvement of astrocytes in LTP SC-CA1 synapses using L-AA as a pharmacological tool. Currently, data published with this gliotoxin demonstrate the role of astrocytes in the modulation of NMDARs function in the amygdala, leading to an inhibition of LTP, without changes in excitability or basal synaptic transmission, which support our results (Li et al. 2013). The present study allowed us to infer that L-AA influences hippocampal synaptic plasticity under physiological conditions, through a LTP event (see figure 26). Since L-AA is a homologue of glutamate, it interferes with astrocytic glutamate transport and metabolism and, consequently with astrocytes function. As a consequence the levels of glutamate in the synaptic cleft can be altered, occurring likely an increase that impair synaptic plasticity and hence memory deficits (Huck et al. 1984; Mcbean 1994; Tsai et al. 1996; Oliveira et al. 2015). Thus, these evidences demonstrated in an animal model of AD may highlight the astrocytes as a potential therapeutic target for AD. In fact, during the progression of neurodegenerative disorders, such as AD, there is an increase in neuroinflammation associated with glial cells, namely astrocytes (Sofroniew and Vinters 2010; Sofroniew 2015; Garwood et al. 2017). Thus, these cells are responsible for acting on different AD-associated mechanisms to control synaptic plasticity and memory dysfunction (Singh and Abraham 2017).

The model of $A\beta_{1-42}$ icv injection had already been validated by our group; however, fifteen days after the injection neither neuronal loss nor microgliosis or astrogliosis was observed (Canas et al. 2009). Considering that our goal was to study astrogliosis, a preliminary study was developed to evaluate this hallmark of AD. Thus, we increased the time between the injection of $A\beta_{1-42}$ and the sacrifice and immunohistochemistry analysis of brain mice from 15 to 20 days. Curiously, after 20 days of $A\beta_{1-42}$ injection, astrogliosis was already observed through the increase in GFAP immunoreactivity, which delineated the time-point chosen for this study. In the present study, following the extracellular recordings on SC-CA1 synapses, immunohistochemical assays were performed to evaluate changes in astrocytic markers of hippocampal slices from $A\beta_{1-42}$-injected mice and vehicles, as well as in slices from both groups of animals incubated with L-AA for 2 h. It was observed that icv $A\beta_{1-42}$-injection induced a significant increase in GFAP immunoreactivity, without affecting the number of GFAP positive cells, nor S100β immunoreactivity in the CA1 region of the hippocampus (figure 27). These results are in agreement with other studies, where it is shown that Aβ induces alterations in astrocytic markers, namely in GFAP (Perez et al. 2010; Assefa et al. 2018). With this information we demonstrated that in icv $A\beta_{1-42}$ injected mice there was astrogliosis, as described in

the literature in other pathological models of AD, thus reinforcing the presence of astrogliosis as a hallmark of this neurodegenerative disease (Webster et al. 2006; Colangelo et al. 2014; Assefa et al. 2018). Surprisingly, the acute L-AA exposure *per se* (in icv vehicle mice) significantly increased GFAP immunoreactivity but had no effect on astrocytic proteins, neither GFAP nor S100β, in hippocampi (CA1 region) of icv $A\beta_{1-42}$ mice. Since in this AD mice model the GFAP immunoreactivity "basal" (without L-AA) was already up-regulated, this lack of L-AA effect, might suggest that in pathological AD conditions the astrocytes are dysfunctional, which might explain the changes observed in synaptic LTP and LTD phenomena observed in icv $A\beta_{1-42}$-injected mice. We can also speculate that the posterior L-AA incubation of hippocampi from icv $A\beta_{1-42}$-injected mice, which seem to rescue the effect of $A\beta_{1-42}$ on synaptic plasticity, caused a silencing of astrocytes that is beneficial for the synaptic alterations caused by AD-like conditions.

In the present study we used also another AD animal model, the 3xTgAD, which mimics the two most hallmarks of this pathology: amyloid plaques and neurofibrillary tau tangles and also presented astrogliosis and changes in synaptic plasticity at 6-7 months of age (Oddo et al. 2003; Pietropaolo et al. 2008). Therefore, this mice model of familial AD seemed us to be a good model to investigate the impact of astrocytes in AD. Unexpectedly, and despite the number of experiments is small to draw conclusions, we observed a tendency for an increase in hippocampal LTP amplitude in 3xTgAD animals, compared with nonTg (see figure 28). Noteworthy, these animals had 11 months of old and the low amplitudes of LTP observed in nonTg animals might be due to the age of animals that cause a shift in synaptic plasticity favoring, in this case, a decrease in synaptic transmission with reduced ability to induce LTP, as reported by others researcher groups (Kumar 2011; Ris and Gordaux 2018; Temido-Ferreira et al. 2018). It has been demonstrated that aging comprehends functional and structural alterations in the hippocampus that drive cognitive decline and moreover, aging is also characterized by an abnormal Ca^{2+} signaling, leads an age-associated increase in $[Ca^{2+}]i$ levels. Others evidences have been described that strengthens this hypothesis, like the fact that aged CA1 pyramidal neurons showed an increase in the duration of NMDARs-mediated responses, which can lead an altered Ca^{2+} metabolism. This increase can cause CREB dephosphorylation associated with an increase in calcineurin (phosphatase, PP2B) activity and subsequently suggesting a differential phosphatases and kinases activation that may have an directly impact on LTP shift (Temido-Ferreira et al. 2018). Furthermore,

it was reported that the shift from normal aging to AD could be related with dysregulation of homeostatic network in brain structures, particularly in hippocampus, mainly caused by increased adenosine A_{2A} receptors levels (Temido-Ferreira et al. 2018). On the other hand, the increased LTP amplitude observed 3xTgAD mice could be a consequence of an increase in tau pathology observed around this age particularly in hippocampal CA1 pyramidal neurons (Oddo et al. 2003; Pietropaolo et al. 2008). This observation can be interesting, since in our $A\beta_{1-42}$ icv-injection model we did not explore the tauopathology and, accordingly we observed an impairment in LTP and not an increase like in 3xTgAD mice. Alternatively, this increase may be a consequence of an increase in glutamatergic excitatory function and a decrease in the GABAergic inhibitory function or the combination of both (Ishida et al. 1997; Jolas et al. 2002).

Regarding the impact of astrocytic blunting, triggered by acute L-AA treatment of hippocampal slices it seemed that this gliotoxin have a slight effect in CA1 region, reducing GFAP immunoreactivity (see figure 29), but unfortunately it is not possible to draw conclusions from these preliminary results. However, we observed a strong increase in GFAP immunoreactivity in 3xTgAD mice as compared with nonTg mice, indicating the presence of astrogliosis. Therefore, our findings are in agreement with the data previously reported in this AD mice model (Guedes et al. 2014) and in other transgenic mice model of AD, Tg2576 (Boscia et al. 2017).

Overall, the data obtained show that AD-like conditions (acute $A\beta_{1-42}$ application and $A\beta_{1-42}$ icv injection), affected hippocampal synaptic plasticity, both LTD and LTP mechanisms are significantly affected, and the hippocampal-dependent memory. The astrocyte selective toxin (L-AA) *per se* decrease the synaptic plasticity, but in pathological conditions of AD this gliotoxin rescued the impact of impact of $A\beta_{1-42}$ on LTD (a shift of LTD to LTP) and also in 3xTgAD mice seems to restrain the increased LTP observed in this AD model. Therefore, we considered that the astrocytes are a potential therapeutic target for AD and other neurodegenerative disorders (Colangelo et al. 2014), which is a possibility that has been supported for more and more scientific evidences in the last few years.

Chapter 6

Conclusion

Conclusions

In Resume:

- $A\beta_{1-42}$ exposure both acutely (acute exposure) or chronically (icv-injection in mice) induced a decrease in LTD and LTP amplitude.
- $A\beta_{1-42}$ administration into lateral ventricle causes an impairment in hippocampal-dependent tasks, namely spatial and recognition memory.
- Astrocytes play an important role in shaping hippocampal synaptic plasticity, namely LTP in physiological conditions.
- In AD-like conditions, of both acute or chronic $A\beta_{1-42}$ exposure, the astrocytes blunting recover synaptic dysfunction in both AD mice models (icv $A\beta_{1-42}$ injected and 3xTgAD mice).
- A reactive astrogliosis, assessed by increased GFAP immunoreactivity, are present in the hippocampus of both AD mice models.
- The gliotoxin (L-AA) only increased GFAP immunoreactivity in non-pathological conditions, having no significant effect on AD-like conditions.

Taken together the data gathered in this study (above briefly listed), we can speculate that the astrocytes are functional affected by AD conditions contributing to the changes in synaptic plasticity and memory observed this pathology. However, we consider that further studies are necessary, such as the application of gliotoxin *in vivo* (e.g. intracerebral administration - chronic effect) in AD mice model, and we should also use other tools to identify astrocytes dysfunction or reactivity, which is not a simple all-or-none phenomenon but rather a finely gradated continuum process regulated temporally by specific signaling events (Sofroniew and Vinters 2010). Indeed, the establishment of astrocytes role in brain disorders has been hampered by a limited knowledge of how astrocytes function, and the limited number of markers to identify and discriminate potentially heterogeneous astrocyte subtypes, as well as by the lack of markers for assess astrocytes pathology.

Chapter 7

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