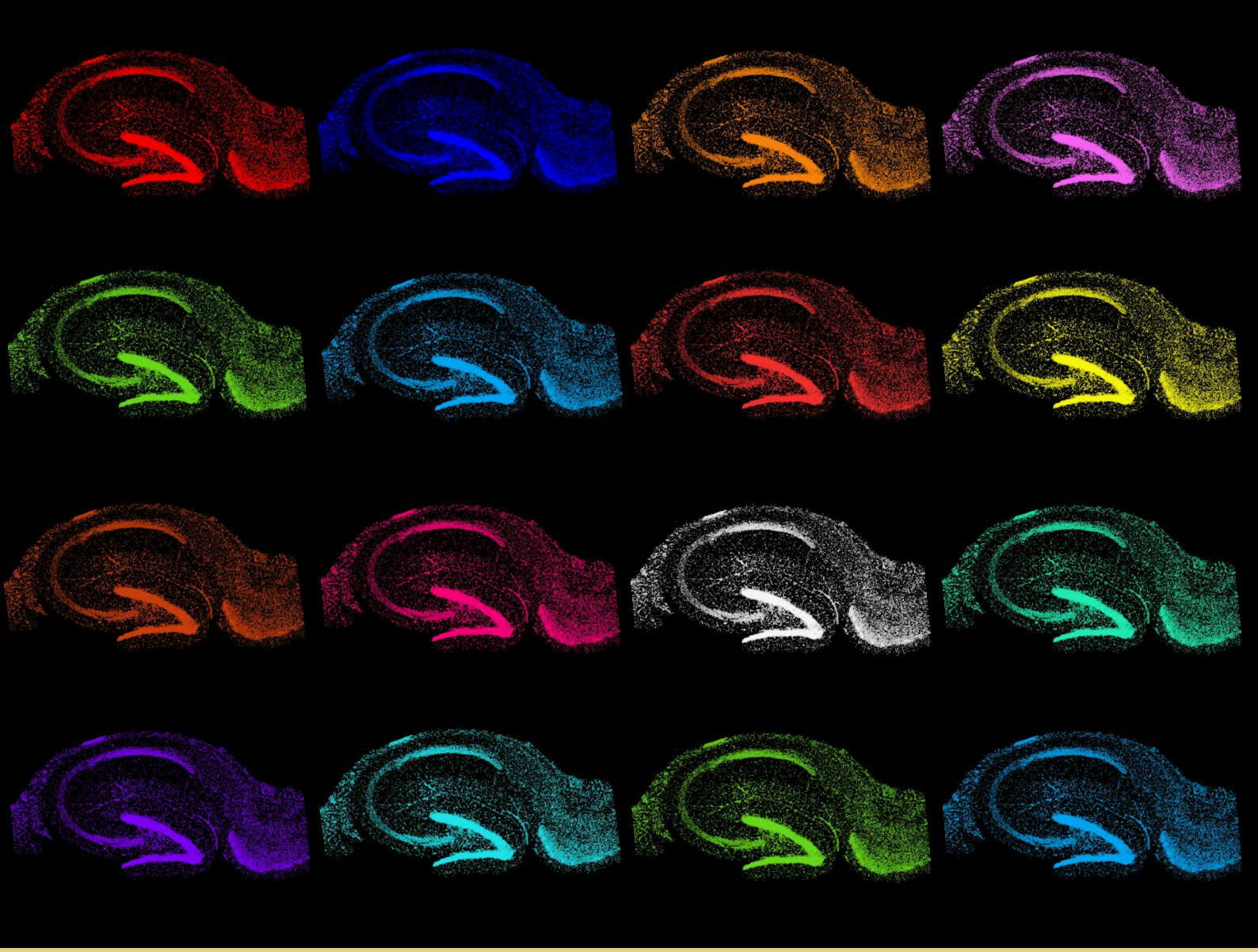

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Role of astrocytes in synaptic function and memory. Focus on adenosine A_{2A} receptors.

Dissertação de Mestrado em Bioquímica

Julho / 2017

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Universidade de Coimbra

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UNIVERSIDADE DE COIMBRA

Papel dos astrócitos na função sináptica e na memória. Foco nos recetores de adenosina A_{2A}.

Role of astrocytes in synaptic function and memory. Focus on adenosine A_{2A} receptors.

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de mestre em Bioquímica, realizada sob a orientação científica da Doutora Paula Maria Garcia Agostinho (Universidade de Coimbra) e do Professor Doutor Ângelo José Ribeiro Tomé (Universidade de Coimbra).

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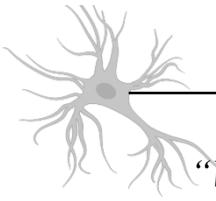

"What is the function of glial cells in neural centers? The answer is still not known, and the problem is even more serious because it may remain unsolved for many years to come until physiologists find direct methods to attack it"

Santiago Ramón-y Cajal (1909/1911)

Ramón-y-Cajal S. Histologie du Système Nerveux de l'Homme et des Vertébrés. Maloine; Paris, France: 1909, 1911. (reviewed and updated by the author, translated from Spanish by L. Azoulay) English translation: Ramón-y-Cajal, S. (1995): Histology of the Nervous system System of Man and Vertebrates, translated by N. Swanson & L. Swanson, OUP, New York.

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Abbreviations

Aβ	Amyloid-β peptide
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
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
BDNF	Brain-derived neurotrophic factor
CA	Cornu ammonis
Ca^{2+}	Free calcium ion
$[Ca^{2+}]i$	Intracellular calcium concentration
CaMKII	Calcium/calmodulin-dependent protein kinases
CBX	Carbenoxolone
CD73	Ecto-5'-nucleotidase
Cl^-	Free chloride ion
cm	Centimeter
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSF	Cerebrospinal fluid
Cx	Connexin
Cx43	Connexin43
DG	Dentate gyrus
DHK	Dihydrokainic acid
DMSO	Dimethyl sulfoxide
DP	Depotentiation
DV	Dorso-ventral
EAA1	Excitatory amino-acid transporter 1
EAA2	Excitatory amino-acid transporter 2
EC	Entorhinal cortex
ECM	Extracellular matrix
ECS	Extracellular space
EPSP	Excitatory post-synaptic potential
Fb-A_{2A}R KO	Forebrain selective A_{2A}R KO mice

fEPSP	Field excitatory post-synaptic potential
GABA	γ-amino butyric acid
GAT-1	GABA transporter 1
GAT-2	GABA transporter 2
GAT-3	GABA transporter 3
GFAP	Glial fibrillary acidic protein
GJ	Gap junction
GLAST	Glutamate aspartate transporter
Gln	Glutamine
GLT-1	Glutamate transporter 1
Glu	Glutamate
GluT	Glutamate transportes
GPCR	G-protein coupled receptor
GS	Glutamine synthetase
h	Hours
HCs	Hemichannels
HD	Huntington's disease
HFS	High-frequency stimulation
HS	Horse serum
Hz	Hertz
icv	Intracerebroventricular
IHC	Immunohistochemistry
IMF	Intermediate filaments
INFγ	Interferon-γ
IP	Intraperitoneal
ITI	Inter-trial interval
K^+	Free potassium ion
$[K^+]_o$	Extracellular K^+ concentration
KO	Knockout
L-AA	L-α-aminoadipic acid
LFS	Low-frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
M Ω	Megaohms
MEC	Medial entorhinal cortex
Mg^{2+}	Free magnesium ion
mGluR	Metabotropic glutamate receptor
min	Minutes
ML	Medial-lateral
mV	Milivolt
Na^+	Free sodium ion

NH_4^+	Ammonium
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NT	Neurotransmitter
NVU	Neurovascular unit
OFT	Open field test
PBS	Phosphate buffer saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PNS	Peripheral nervous system
RE	Recording electrode
ROS	Reactive oxygen species
RT	Room temperature
s	Seconds
SC	Schaffer collaterals
SCH58261	5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazole[4,3-e]-1,2,4-triazolo-[1,5c]pyrimidine
SE	Stimulation electrode
SEM	Standard error of the mean
SNARE	Soluble N-ethyl maleimide-sensitive factor attachment protein receptor
str.	*Stratum*
str. l-m	*Stratum lacunosum-moleculare*
str. ori.	*Stratum oriens*
str. pyr.	*Stratum pyramidale*
str. rad.	*Stratum radiatum*
TBOA	DL-threo-β-benzoyloxyaspartate
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
TGFβ	Transforming growth factor-β
TNFα	Tumor necrosis factor-α
VAMP	Vesicle associated membrane protein
VGLUT	Vesicular glutamate transporters
VNUT	Vesicular nucleotide transporter
VSERT	Vesicular D-serine transporter
WT	Wild type

Resumo

O estudo dos processos de plasticidade sináptica e de memória tem sido tradicionalmente centrada nos neurónios, porém, não deve ser ignorado nestes processos o possível papel dos astrócitos, as células maioritárias no cérebro. Existem evidências que suportam a existência de uma comunicação bidirecional entre astrócitos e neurónios ao nível sináptico, denominada de "sinapse tripartida", que reconhece os astrócitos como o terceiro elemento ativo na transmissão sináptica. Assim, os astrócitos, para além de captarem neurotransmissores, são capazes de libertar gliotransmissores para a fenda sináptica, modulando sincronicamente a transmissão sináptica. O presente estudo tem como objetivo avaliar o impacto dos astrócitos na plasticidade sináptica em circuitos do hipocampo, e consequentemente compreender a contribuição destas células no controlo de processos de formação de memórias, tanto em condições fisiológicas como patológicas associadas a défices cognitivos, como é o caso da doença de Alzheimer.

Diferentes ferramentas farmacológicas foram utilizadas para interferir com os astrócitos e, consequentemente, avaliar a sua contribuição em processos de plasticidade sináptica, nomeadamente na potenciação de longa duração (LTP, do inglês *long-term potentiation*) registada nos neurónios piramidais presentes na região CA1 do hipocampo na via proveniente dos colaterais de Schaffer (CS). A incubação aguda de fatias de hipocampo com duas gliotoxinas diferentes, L-α-aminoadipato (L-AA) ou trifluoroacetato (TFA), levou a uma redução significativa na amplitude da LTP (~38%). Estes resultados foram corroborados pelos efeitos observados quando se bloqueou funções específicas dos astrócitos, como é o caso da captação de glutamato da fenda sináptica, através do inibidor especifico dihidrokainato (DHK), e da libertação de gliotransmissores através de hemicanais (HCs), utilizando o bloqueador de HCs e junções gap carbenoxolone (CBX), pois os bloqueadores destas vias de transporte também diminuíram significativamente a LTP. No seu conjunto, os resultados mostraram que, independentemente da forma como manipulamos os astrócitos *in vitro*, obtemos uma redução consistente na LTP. Os recetores de adenosina do subtipo A_{2A} ($A_{2A}R$) são conhecidos por terem um papel fundamental no controlo da transmissão sináptica, através de mecanismos pré- ou pós-sinápticos. Foi também mostrado que após estímulos de elevada frequência, os astrócitos são capazes de induzir a libertação de ATP para a fenda sináptica, onde é metabolizado em adenosina, podendo ativar os $A_{2A}R$. Para investigarmos o papel dos $A_{2A}R$ astrocíticos na modulação da LTP no hipocampo, um

antagonista seletivo para estes recetores foi utilizado (SCH58261). Porém, como o número de condições é bastante limitado neste grupo de experiências, a conclusão que podemos retirar é que a plasticidade sináptica parece não ser alterada na presença de SCH58261 e L-AA, aparentando que os $A_{2A}R$ deixam de controlar a plasticidade sináptica quando os astrócitos estão disfuncionais.

Uma vez que os astrócitos são capazes de alterar a sua morfologia e as suas funções em resposta a diferentes lesões, é extremamente importante que sejam criados modelos *in vivo* para recolher dados acerca do envolvimento da astroglia em patologias cerebrais, como é o caso da doença de Alzheimer. Assim, fizemos a administração da gliotoxina L-AA em ambos os ventrículos laterais de murganhos adultos para investigar o impacto dos astrócitos na função sináptica e na memória. Avaliámos a memória dependente do hipocampo, através do teste de reconhecimento de objetos (NOR), que foi realizado 72 horas após a injeção de L-AA ou de solução salina. Os resultados obtidos não revelaram efeitos significativos do L-AA na memória dos animais; no entanto, o estudo da LTP mostrou uma diminuição significativa na amplitude da potenciação nos murganhos injetados com a gliotoxina relativamente aos controlos (~25%). Os resultados obtidos com a administração *in vivo* de L-AA na LTP foram consistentes com os observados quando se fez a incubação aguda da gliotoxina na fatia de hipocampo. Para além dos estudos funcionais de eletrofisiologia, fomos também investigar o impacto da gliotoxina (incubação aguda e injeção) em marcadores astrocíticos (proteína ácida fibrilar glial, GFAP e glutamina sintetase, GS), utilizando técnicas de imunohistoquímica. Nas secções de hipocampo de animais injetados com L-AA, observámos um decréscimo no número de células marcadas com GFAP, o que reforça as evidências de que a gliotoxina está a interferir com os astrócitos, o que provavelmente está a diminuir a LTP.

A capacidade das sinapses modificarem a transmissão sináptica, mantendo um estado dinâmico e saudável no cérebro é reconhecido como sendo um processo essencial na aprendizagem e formação de memórias, sendo por isso imperativo conhecer os mecanismos que medeiam estes processos. Este trabalho apresenta evidências firmes sobre a contribuição dos astrócitos na modulação da LTP no hipocampo.

Palavras-chave: Astroglia, recetores de adenosina A_{2A}, gliotoxina, hipocampo, plasticidade sináptica.

Abstract

Research on synaptic plasticity and memory has traditionally been neuron-centric. Nevertheless, it is crucial not to ignore the role of astrocytes in these processes since these glial cells are now known to play a more active part in complex neural processing in the healthy CNS than previously recognized. Increasing evidence 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 astroglial cells are the third active element of synapses. Thus, astrocytes release and uptake gliotransmitters within the synaptic cleft to fine-tune synaptic transmission. The present study aims to evaluate the impact of astrocytes on synaptic plasticity in hippocampal circuits to later understand the role of astrocytes to control memory processes in physiological and pathological conditions associated with cognitive deficits, such as in Alzheimer's disease (AD).

Different pharmacological tools were used to interfere with astrocytes to further evaluate the contribution of these star-shaped cells in synaptic plasticity events, namely long-term potentiation (LTP) in the Schaffer collateral (SC) - CA1 pyramidal neurons. Acute incubation with two distinct gliotoxins, L-α-aminoadipic acid (L-AA) and trifluoroacetic acid (TFA), led to a significant reduction (about 38%) in LTP amplitude. These results were supported by those showing that the blockade of exclusive astrocytic functions, such as glutamate uptake from the synaptic cleft (using a selective inhibitor of glutamate transporter-1 named dihydrokainic acid DHK) and hemichannels (HCs)-mediated gliotransmitter release (via inhibition of HCs and gap junction with carbenoxolone, CBX), also significantly decreased LTP. Taken together, these results showed that by manipulating astrocytic function in different ways *in vitro*, we were able to reproduce the same effect on mouse hippocampal LTP. Adenosine A_{2A} receptors (A_{2A}Rs) are known to have a key role in controlling synaptic transmission, either by pre- or postsynaptic mechanisms, and tetanic stimulation is known to trigger release of adenosine triphosphate (ATP) from astrocytes and ATP-derived adenosine activates A_{2A}Rs. Thus, we took advantage of a selective A_{2A}R antagonist (SCH58261) to probe the role of these receptors, which are also expressed in astroglial cells, in the modulation of LTP in hippocampal slices. However, due to the low number of experiments done in these

group of results, what we can say is that apparent astrocytic dysfunction abrogates the role of A_{2A}Rs on proper control of synaptic plasticity.

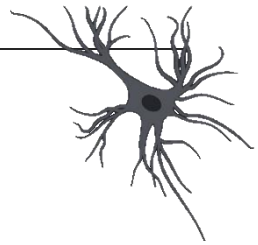
Since astrocytes alter their physiology drastically in response to injury or disease states, it is of utmost importance to create *in vivo* models to gather new information about the involvement of astroglia in brain pathologies, like Alzheimer's or Parkinson's (PD) disease, and pave the way to develop new therapeutic strategies. Bilateral intracerebroventricular (icv) injection of L-AA, a specific gliotoxin, into the two lateral ventricles of C57Bl/6 mice, we were able to work with an animal model in which astrocytic function were seriously compromised. From this point, memory performance and synaptic plasticity were accessed in these animals to evaluate astrocytic contribution. Mice were tested in novel object recognition (NOR) tasks 72 h after the injections (L-AA or vehicle); however, our results about memory performance in these two animal groups did not reach statistical significance. On the other hand, when LTP was measured in the SC-CA1 synapses of L-AA-injected mice a significant depression in LTP amplitude was observed (about 25%), when comparing with vehicle-injected mice, going in accordance with the previous results regarding acute incubation of the gliotoxin in hippocampal slices. Additionally, immunohistochemical analysis probing for astrocytic markers, such as the glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS), were performed in transverse hippocampal sections obtained from slices acutely exposed to the gliotoxin or slices from L-AA-injected mice. The results revealed a decrease in the number of GFAP-positive cells in hippocampal sections from L-AA-injected animals, reinforcing that indeed this gliotoxin was interfering with astrocytes.

The ability to rapidly alter the strength of synaptic connections between neurons is thought to be the molecular basis underlying learning and memory. Therefore, identifying the mechanisms that lead to changes in synaptic strength has fundamental implications for understanding proper brain function. The current work presents strong evidence for the involvement of astrocytes in modulating mouse hippocampal long-term potentiation.

Keywords: Astroglia, adenosine A_{2A} receptors, gliotoxin, hippocampus, synaptic plasticity.

Chapter ***1***

Introduction



1.1. The nervous system

1.1.1. Foundations

For hundreds of years, scientists have been studying the human brain including every single component inside that complex structure, and they all came from different scientific disciplines like medicine, biology, chemistry, physics, and psychology, among others. In the course of time, neuroscientists agreed that nervous tissue, like all other organs, is made up of these fundamental units called "neurons" (Gray, 1959; Purves et al., 2004; Mazzarello, 2006).

Nowadays, the nature of these neuronal cells is well known among scientific community. However, for neurobiologists in the nineteenth century it was tough to prove that with the microscopes and cell staining techniques that were available back then. Initially, some neuroscientists believed that each neuron was connected to its neighbors, forming a continuous neuronal cell network, or *reticulum* (Shepherd, 1991; Mazzarello, 2006; Yuste, 2015a). For that reason, this was called the "reticular theory" and supported by Camillo Golgi (1843-1926), an Italian neuroscientist. In contrast, Santiago Ramón y Cajal (1852-1934), a Spanish neuroscientist, claimed that the nervous system is comprised by individual cells, and therefore, a new concept was born – "neuron doctrine" (Ramón y Cajal, 1888; 1906; 1954; Guillery, 2005). Moreover, based on light microscopic examination of nervous tissue stained with silver salts, according to a method pioneered by C. Golgi, Cajal argued persuasively that neuronal cells are discrete entities, and that they communicate with one another by means of specialized contacts that Sherrington, an English scientist, called "synapses" (Gray, 1959; Yuste, 2015b). In addition, Cajal described axonal growth, a crucial process in the development of nervous system, and also characterized dendritic spines and glial cells (Figure 1), explaining their possible contribution in synaptic transmission (Garcia-Lopez et al., 2007; Garcia-Marin et al., 2007). Only after several years, Golgi's and Cajal's work was recognized by the award of the Nobel Prize for Physiology or Medicine in 1906 (Lopez-Munoz et al., 2006).

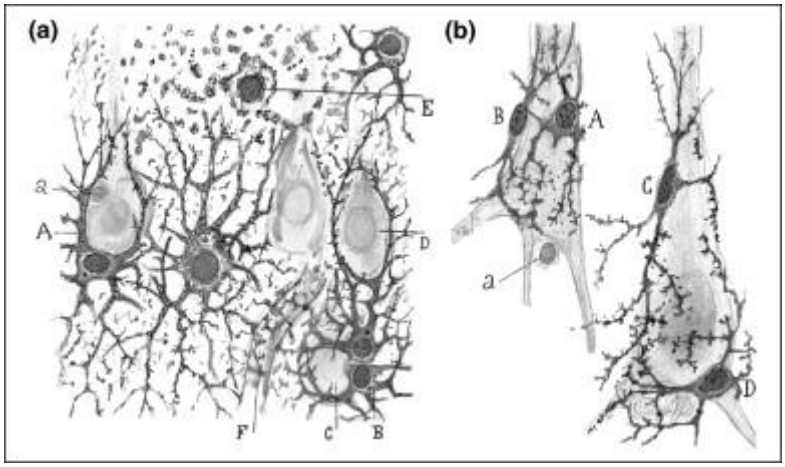


Figure 1 | Cajal's drawing of astroglial cells using the sublimated gold chloride method. **a |** Astrocytes (indicated by A) in the pyramidal layer of the human hippocampus (indicated by D), twin astrocytes (indicated by B) and a satellite cell called the 'third element' by Cajal (indicated by a). **b |** Different astrocytes (indicated by A, B, C and D) surrounding neuronal somas in the pyramidal layer of the human hippocampus (from García-Marín et al., 2007).

1.1.2. Overall organization of the nervous system

The nervous system is the control and communication system of an animal's body and has three main functions: sensory input, integration of information and motor output. In vertebrate species, the nervous system is comprised of two subdivisions, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is the most critical and sensitive system in the human body and is composed by the brain and spinal cord (Purves et al., 2004; Nieuwenhuys et al., 2007). In contrast, the PNS incorporates spinal nerves, which branch from the spinal cord, and cranial nerves that branch from the brain. The PNS includes the autonomic nervous system (divided into the sympathetic and parasympathetic nervous system), which controls vital functions such as breathing, digestion, heart rate, secretion of hormones, among many more (Kandel et al., 2013).

Several characteristics of the nervous system make its reactions to trauma and disease unique. Fundamentally, the brain is sheathed by the bony skull that grants protection from injury and plays also an important role in restricting its expansion. Between the skull and brain are the meninges, which consist of three layers of tissue that cover and protect the brain and spinal cord. From the outermost layer inward they are named the dura mater, arachnoid and pia mater. Moreover, the brain is bathed by the cerebrospinal fluid (CSF), produced by choroid plexus in the roof of the lateral, third, and

fourth ventricles, through a combination of processes, such as diffusion, pinocytosis and active transfer (Squire et al., 2008; Mai and Paxinos, 2011). The CSF provides brain protection, and is also capable of reducing the pressure at the base of the brain. Additionally, is able to handle the excretion of harmful metabolites and other substances away from the brain (Mai and Paxinos, 2011).

Proper neuronal function requires a highly regulated extracellular environment, wherein the concentrations of ions, such as sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}), which must be maintained within very narrow ranges. Since brain tissue is highly vascularized it is essential that the interface between the CNS and the peripheral circulatory system works as a dynamic regulator of ion balance, a facilitator of nutrient transport and a barrier to potentially harmful molecules (Hawkins and Davis, 2005; Barres, 2008). This homeostatic aspect of the cerebral microcirculation, referred to as the "blood-brain barrier" (BBB), performs all of these functions (Ehrlich, 1885; Lewandowsky, 1900). The BBB is a selective barrier formed by endothelial cells which are connected by tight cell-cell junctions (tight junctions) (Abbott et al., 2006; Banerjee and Bhat, 2007). Essentially, it has been proposed that the microvascular endothelium, astrocytes, pericytes, neurons, and extracellular matrix constitute a "neurovascular unit" (NVU), and that the components of the NVU maintain dynamic interactions with each other, playing an important role in cerebrovascular function (Neuwelt, 2004; Hawkins and Davis, 2005). Contact and communications between the cells of the NVU regulate CNS development and synaptic activity and also influence permeability properties of the BBB (Rubin and Staddon, 1999).

1.1.3. The cellular components of the central nervous system

All tissues and organs in the body consist of cells and the specialized functions of cells and how they interact determine the functions of organs. Clearly, neuronal cells are specialized for electrical signaling over long distances, and understanding this process represents one of the more dramatic success stories in modern neuroscience. In contrast, supporting cells, are not capable of communicating with other cells through action potentials. Nevertheless, they have essential functions in the developing and in adult brain. We must begin by learning how brain cells work individually and then see how they are assembled to work together.

1.1.3.1. Neuronal cells

The neuron is a highly specialized cell type, which can be electrically excitable and capable of process and transmit information through electrical and chemical signals. The human brain is estimated to contain at least 100 billion neurons (Drachman, 2005). However, these cells can be categorized according to their size, shape, neurochemical characteristics, location, and connectivity, which are important determinants of the particular functional role of each neuron in the brain (Bear et al., 2007; Squire et al., 2008). Neurons are interconnected with one another to form circuits, and many neural circuits connected to others, forming a neural system or network. The neural network is then able to process specific kinds of information and provide the foundation of sensation, perception and behavior (Purves et al., 2004). The synaptic connections, underlying this circuitry, are normally composed by dense tangle of dendrites, axon terminals or even varicosities (axon swellings).

Neurons transfer information by communicating with each other forming synapses, by a process named synaptogenesis. However, synaptic transmission is not always the same, and thus synaptic plasticity is the term used to describe the biological process by which specific patterns of synaptic activity result in changes in synaptic strength; which is thought to contribute to learning and memory (Alkon and Nelson, 1990; Zoghbi et al., 2000; Malinow and Malenka, 2002). Both pre- and postsynaptic mechanisms, along with other cell types can contribute to the processes of synaptic plasticity in the brain. The most intensively characterized examples of such synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD) (Figure 11) (Martin et al., 2000; Collingridge et al., 2004).

1.1.3.2. Glial cells

In addition to neurons, glial cells (also called neuroglia or simply glia) are great contributors to normal brain function, and for that reason are named supportive cells. These cells were first described by the famous German pathologist Rudolph Virchow (1821-1902), who named them “nerve glue”, for the reason that he considered glia as an inactive "connective tissue” holding neurons together in the CNS (Virchow, 1846; 1854; Wang and Bordey, 2008). In all parts of the CNS, glial cells outnumber neurons by about 9:1, and they are also major players in the reaction of the nervous system to trauma and

disease (Harris, 2013). According to their size, glial cells are divisible into microglia and macroglia.

Microglia are ubiquitously distributed in the CNS and comprise up to 20% of the total glial cell population in the brain. These cells are innate immune cells and form the first line of defense of the CNS (Gehrmann et al., 1995). They share many properties with macrophages, found in other tissues, and are primarily scavenger cells that remove cellular fragments from sites of injury. Additionally, microglia, like their macrophage counterparts, secrete signaling molecules (e.g. cytokines, chemokines and nitric oxide) that are able to modulate local inflammation and influence cell survival or death (Finsen et al., 1999; Dissing-Olesen et al., 2007).

Macroglia, on the other hand, can be categorized as astrocytes (also called astroglia) and as oligodendrocytes in the CNS. A major function of astrocytes is to maintain, in a variety of ways, an appropriate chemical environment for neuronal signaling (Figure 2). As for oligodendrocytes, which form one of the most highly specialized cellular structures in the body, the myelin sheath, contribute to speed up action potential propagation along the axons by allowing a saltatory conduction at myelin sheath gaps, named as nodes of Ranvier (Baumann and Pham-Dinh, 2001). The diversity of processes in which astrocytes and oligodendrocytes are involved show that these cells can respond in different ways to traumatic and neurodegenerative situations, in order to maintain the structural integrity of cerebral tissue. Although the role played by oligodendrocytes is less well-known than that ones played by astrocytes (Ramirez-Exposito and Martinez-Martos, 1998).

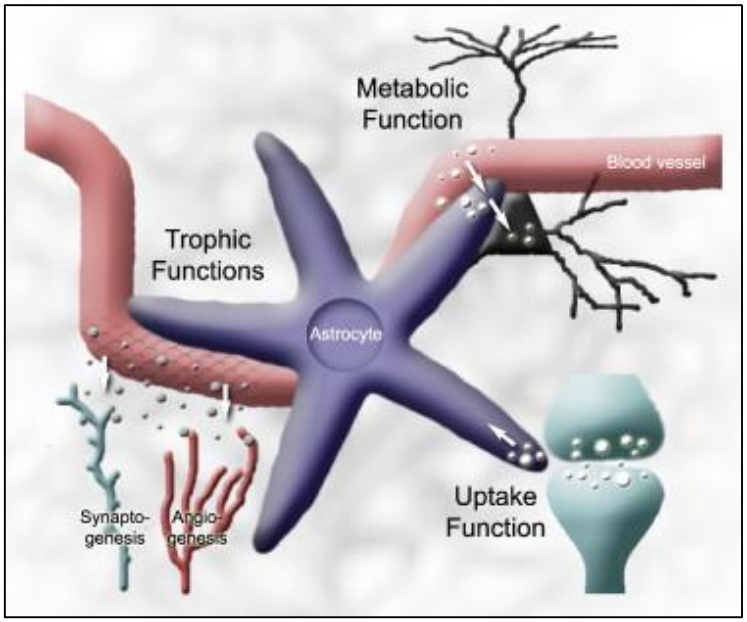


Figure 2 | Astrocytes have several homeostatic functions maintaining a viable nervous system environment for neurons. These functions include: 1 | providing metabolic support for neurons; 2 | taking up K+ and neurotransmitters; 3 | Synaptogenesis, angiogenesis, and BBB maintenance (from Wang and Bordey, 2008).

1.2. Astroglial cells in the CNS

1.2.1. Defining an astrocyte

As mention before, astrocytes are indispensable and specialized glial cells in the brain, and thus perform numerous essential functions in the healthy CNS. In the beginning of the twentieth century, astrocytes have been viewed as a homogeneous cell population that have a star-shaped morphology, extend numerous processes surrounding neighboring neurons and blood vessels, and contain intermediate filaments (IMF) or glial fibrils (Wang and Bordey, 2008). Currently, it is well known that this IMF consists mainly of one kind of intermediate filament protein, glial fibrillary acidic protein (GFAP) (Eng et al., 1971; Kimelberg, 2004).

Clearly, astrocytes may have specialized functions based on their microenvironment. That being said, based on their cellular morphologies and anatomical locations, astrocytes can be categorized in two main subtypes such as protoplasmic (type I) and fibrous (type II) (Figure 3) (Miller and Raff, 1984; Privat and Rataboul, 2007). Hence, protoplasmic astrocytes are found throughout all gray matter and exhibit a morphology of several stem branches that give rise to many branching processes in a uniform globoid distribution. This particular type of astrocytes is known to play many important and diverse roles, including i) development guiding (Hatten and Mason, 1990; Ullian et al., 2001), ii) regulation of the extracellular concentrations of ions, metabolites and neurotransmitters (Walz, 1989; Vernadakis, 1996), iii) support of neuronal and synaptic functions (Keyser and Pellmar, 1994; Araque et al., 1999; Sofroniew and Vinters, 2010); among others. Likewise, fibrous astrocytes are located mainly in white matter and tend to have long, thin, unbranched processes, whose endfeet envelop nodes of Ranvier (Ramón y Cajal, 1909; Wang and Bordey, 2008). Gray and white matter astrocytes differ not only in their spatial locations and morphologies, but also in their transcriptional regulation of gene expression. Thus, protoplasmic astrocytes have few IMF and, in contrast, fibrous astrocytes have many glial filaments composed mainly by GFAP (Peters et al., 1976).

Other studies have demonstrated that in healthy CNS, individual protoplasmic astrocytes have essentially non-overlapping domains and that one hippocampal astrocyte

can contact with around 100,000 synapses (Ogata and Kosaka, 2002; Nedergaard et al., 2003; Halassa et al., 2007). Additionally, this compartmentalization of labor by individual astrocytes allows a one-to-one astrocyte-to-synaptic unit communication without information mismatch (or noise) from a second astrocyte, receiving different inputs or being in a different activity state (Bushong et al., 2002; Wang and Bordey, 2008).

With the development of electrophysiology and with the arising of novel molecular and genetic tools, it is now clear that astrocytes represent a diverse population of cells with numerous functions, which are going to be enlightened during the dissertation.

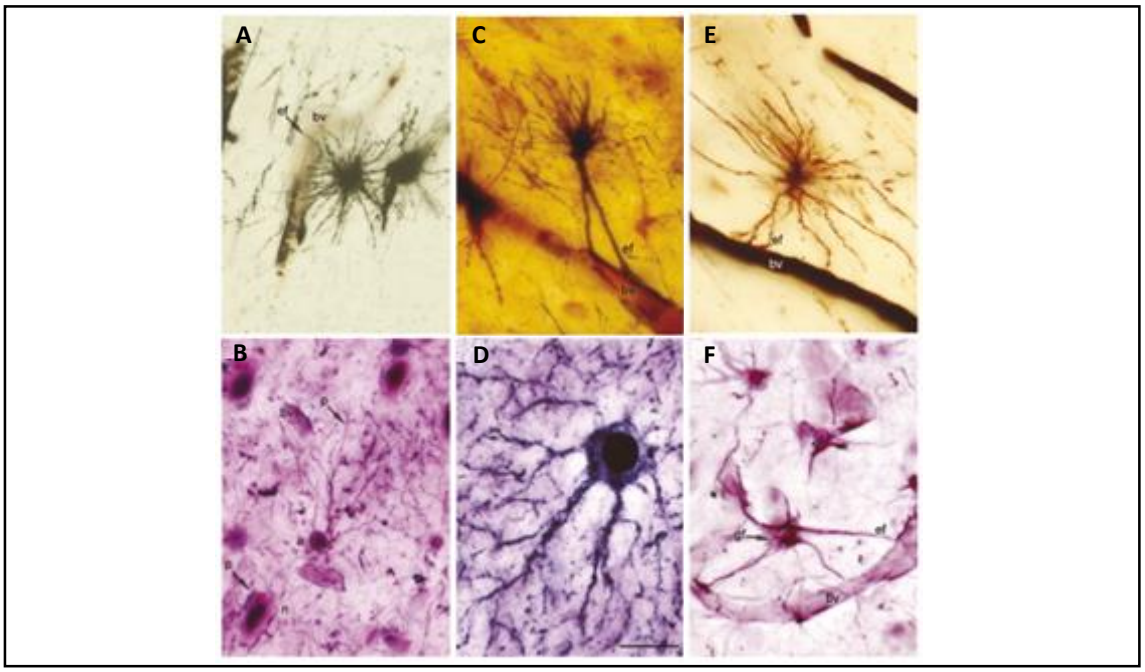


Figure 3 | Protoplasmic astrocytes (A,B,C,D) from Cajal's original slides impregnated by using: **A** | the Golgi-Cox method; **B** | gold chloride sublimated method; **C** | formol-uranium nitrate method; **D** | silver carbonated method. Fibrous astrocytes (E,F) impregnated by: **E** | the Golgi-Kenyon method; **F** | ammoniacal silver oxide method. Abbreviations are: a = astrocytes, bv = blood vessels, ef = endfeet, gf = gliofilaments, n = neuron, p = processes (adapted from García-Marín et al., 2007).

1.2.2. The role of astrocytes in the CNS

All structural, biochemical, and biophysical characteristics of astrocytes are tightly related to their functions in the CNS. Briefly, astrocytes have several critical functions, including: i) promotion of neuronal maturation, ii) modulation of synaptic activity via the release of gliotransmitters, iii) formation and remodeling of synapses, iv) promotion of

neuronal survival during development, v) angiogenesis regulation, vi) defense against oxidative stress and vii) maintenance of favorable microenvironment for neurons; among many more.

1.2.2.1. Synthesis of extracellular matrix, adhesion molecules and trophic factors

Astrocytes are the major source of extracellular matrix proteins (ECM) and adhesion molecules in the CNS, and thus can either promote or inhibit neurite outgrowth depending on the balance of ECM and adhesion molecules. Growth-promoting molecules include laminin (Liesi, 1985; Liesi and Silver, 1988; Chiu et al., 1991), N-cadherin (Neugebauer et al., 1988; Tomaselli et al., 1988), neural cell adhesion molecule (NCAM) (Neugebauer et al., 1988; Smith et al., 1990), and fibronectin (Price and Hynes, 1985; Liesi et al., 1986; Matthiessen et al., 1989;). Astrocytes also synthesize and secrete proteolytic enzymes, in particular the matrix metalloproteinases (MMPs) (Wells et al., 1996; Muir et al., 2002), which play a pivotal role in ECM degradation and remodeling (Shapiro, 1998; Yong et al., 1998).

In addition, astrocytes are able to release growth factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) (Rudge et al., 1992), and fibroblast growth factor (FGF) (Vaca and Wendt, 1992). These molecules control neuronal maturation and survival (Ojeda et al., 2000). *In vitro* studies showed that astrocytes control neuronal differentiation via activity-dependent neurotrophic factor release (Blondel et al., 2000). There are many more molecules secreted by astrocytes that regulate neuronal maturation and survival, under physiological conditions and following injury (reactive astrogliosis, figure 4a) (Mahesh et al., 2006).

1.2.2.2. Astrogliosis: astrocytic reactivity in the CNS

Astrocytes can actively participate to brain responses to toxic and traumatic insults, through a complex process called reactive astrogliosis or simply astrogliosis. This response involves morphological and functional changes including hypertrophy, upregulation of intermediate filaments, such as GFAP, and increased proliferation (Pekny and Nilsson, 2005; Sofroniew and Vinters, 2010; Parpura et al., 2012). Reactive astrocytes also release cytokines and many other factors that mediate inflammatory

responses and remodeling processes, thus playing both beneficial and detrimental roles in brain pathology (Colangelo et al., 2014). To put it simple, astrogliosis occurs prominently in response to all forms of CNS injury or disease (either acute or chronic). The functions of reactive astrocytes are not well understood, and both harmful and beneficial activities have been attributed to these cells. Under specific circumstances, reactive astrogliosis can lead to harmful effects. When the astrogliosis is pathological itself, instead of a normal response to a pathological problem, leads to the notion of "astrocytopathies" (Sofroniew, 2015). However, several studies point towards roles for reactive astrocytes in restricting inflammation and protecting neurons and oligodendrocytes, thereby helping to limit tissue degeneration and preserve function after CNS injury (Sofroniew, 2005, 2009, 2015).

According to this concept, reactive astrogliosis is not an all-or-none response, nor is it a uniform process. Instead, reactive astrogliosis is a finely gradated continuum with progressive changes in gene expression and cellular changes that are subtly regulated by complex inter- and intra-cellular signaling (Sofroniew and Vinters, 2010). In healthy tissue, the extensive network of finely branched processes of individual astrocytes occupy contiguous non-overlapping domains (Bushong et al., 2002). When it comes to mild-to-moderate reactive astrogliosis, there appears to exist preservation of the individual non-overlapping domains of reactive astrocytes, in spite of the hypertrophy of the cell body and processes (Wilhelmsson et al., 2006). Ultimately, at extreme level of activation in response to intense tissue damage and inflammation, reactive astrogliosis involves glial scar formation that incorporates newly proliferated cells and in which astrocyte processes overlap in manners not seen in healthy tissue (Sofroniew, 2009; Sofroniew and Vinters, 2010).

A wide range of intracellular signaling molecules have the ability of triggering reactive astrocytes to overcome injury (Figure 4b). The astrogliosis molecular mediators can be released by any cell type in CNS tissue, including neurons, microglia, oligodendrocyte lineage cells, endothelia, leukocytes and other astrocytes, in response to CNS insults ranging from subtle cellular perturbations to intense tissue damage and cell death. Recent data shows that different specific signaling mechanisms trigger different molecular, morphological and functional changes in reactive astrocytes, in a manner that reflects the gradated responses of reactive astrogliosis (Sofroniew, 2009).

To summarize, reactive astrocytes have vital roles in neural protection and repair. With this in mind, it is reasonable to say that astrocytes are active scouts in search of any

abnormality in the CNS. They are indeed critical to scar formation and function to reduce the spread and persistence of inflammatory cells, to maintain and repair the BBB, to offer protection from oxidative stress via glutathione production (Chen et al., 2001), to decrease tissue damage and lesion size and to decrease neuronal loss and demyelination. They are able to protect brain cells and structures through various pathways, such as uptake of excitotoxic glutamate, adenosine release, and even degradation of Aβ peptides (Bush et al., 1999; Sofroniew, 2009).

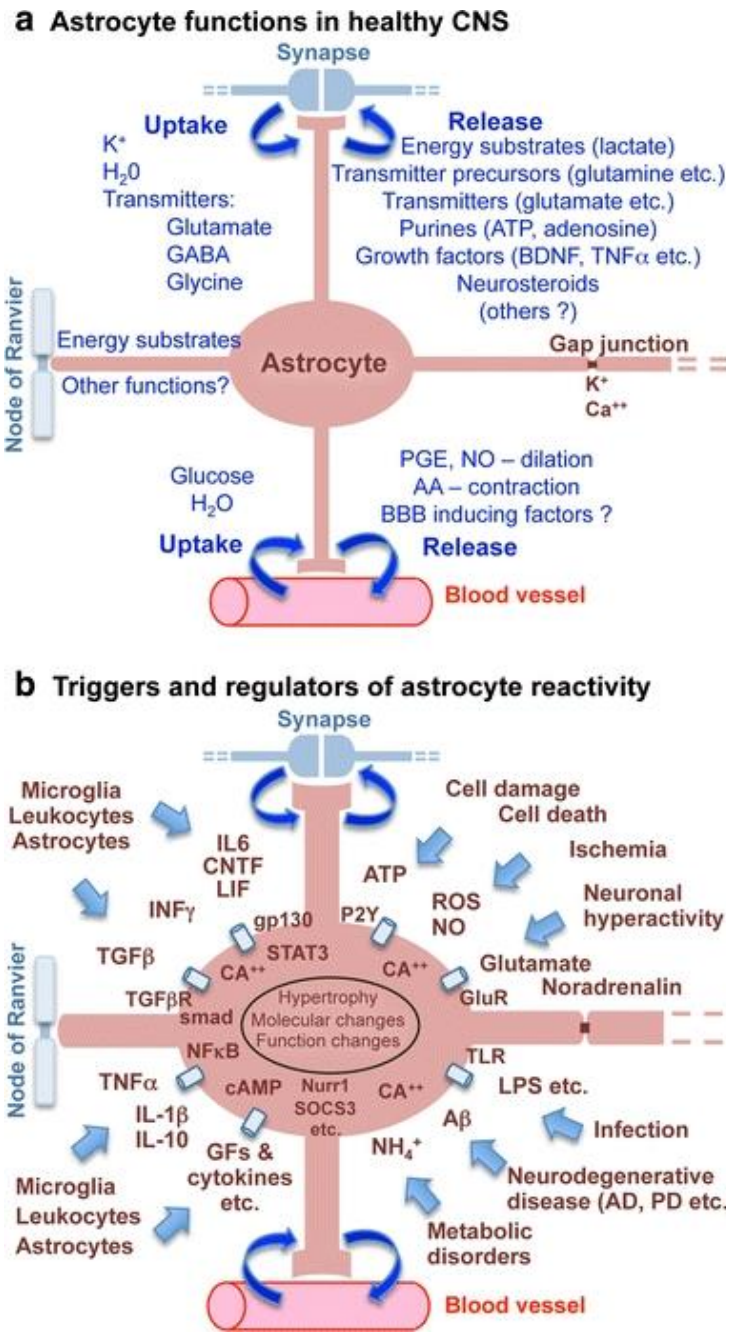


Figure 4 | Schematic representations that summarize: **a** | astrocyte functions in healthy CNS and **b** | triggers and molecular regulators of reactive astrogliosis. Some intercellular signaling molecules that can trigger this complex mechanism are growth factors and cytokines (IL6, LIF, CNTF, TNFα, INFγ, TGFβ, FGF2, among others), mediators of inflammation (e.g. Lipopolysaccharides, LPS), neurotransmitters (e.g. glutamate and noradrenalin), purines such as ATP and adenosine, and also reactive oxygen species (ROS), including nitric oxide (NO). Conditions of hypoxia and glucose deprivation, products associated with neurodegeneration such as Aβ peptide, molecules associated with systemic metabolic toxicity such as ammonium cation (NH_4^{+}), and regulators of cell proliferation (e.g. endothelin-1) can also induce reactive astrogliosis. Despite this information, molecular triggers that lead to proliferation of reactive astrocytes *in vivo* are not fully characterized, but include EGF, FGF, endothelin 1 and ATP (from Sofroniew and Vinters, 2010).

1.2.2.3. Angiogenesis: blood-brain barrier formation and maintenance

As mention before, the blood-brain barrier (BBB) is a selective barrier formed by the endothelial cells that line cerebral microvessels. This physical structure protects the brain from toxic substances in the blood, supplies the CNS with nutrients, and filters excess and toxic molecules from the brain to the bloodstream (Yamagata et al., 1997; Pardridge, 1999; Engelhardt, 2003; Zlokovic, 2008). Thus, it's possible to maintain a stable and optimal brain environment for a healthy neuronal function. However, for BBB to be formed, angiogenesis must occur in the first place.

Angiogenesis is the growth of blood vessels from the existing vasculature and is a normal and vital process in growth and development of the brain tissue. This process involves several steps, including basement membrane degradation, endothelial cell proliferation and recruitment, tube formation and maturation, including reconstitution of the basement membrane (Sofroniew and Vinters, 2010) (Figure 5). Jiang and colleagues (1995) showed that when astrocytes are cultured with endothelial cells occur formation of capillary-like structures. Astrocytes influence angiogenesis and formation of tight junctions between brain endothelial cells (BEC), during final stages of BEC differentiation (Yamagata et al., 1997; Zlokovic, 2005).

Because astrocytes are so important in the formation of that specialized system of brain microvascular endothelial cells, they need to make sure that everything is exactly how it is supposed to be. For instance, a dysfunction of the NVU contributes to several neurovascular pathologies, an event that usually is associated with AD (Takano et al., 2007; Barres, 2008). Defective clearance of amyloid-β peptide (Aβ) across the BBB, due to either aberrant angiogenesis (Carmeliet and Jain, 2000) or endothelial senescence associated with alterations in receptors involved in Aβ clearance or influx, could increase the concentration of soluble neurotoxic Aβ peptides in brain interstitial fluid and lead to deposition of Aβ fibrils and to the formation of vascular amyloid lesions (Shibata et al., 2000; Deane et al., 2003).

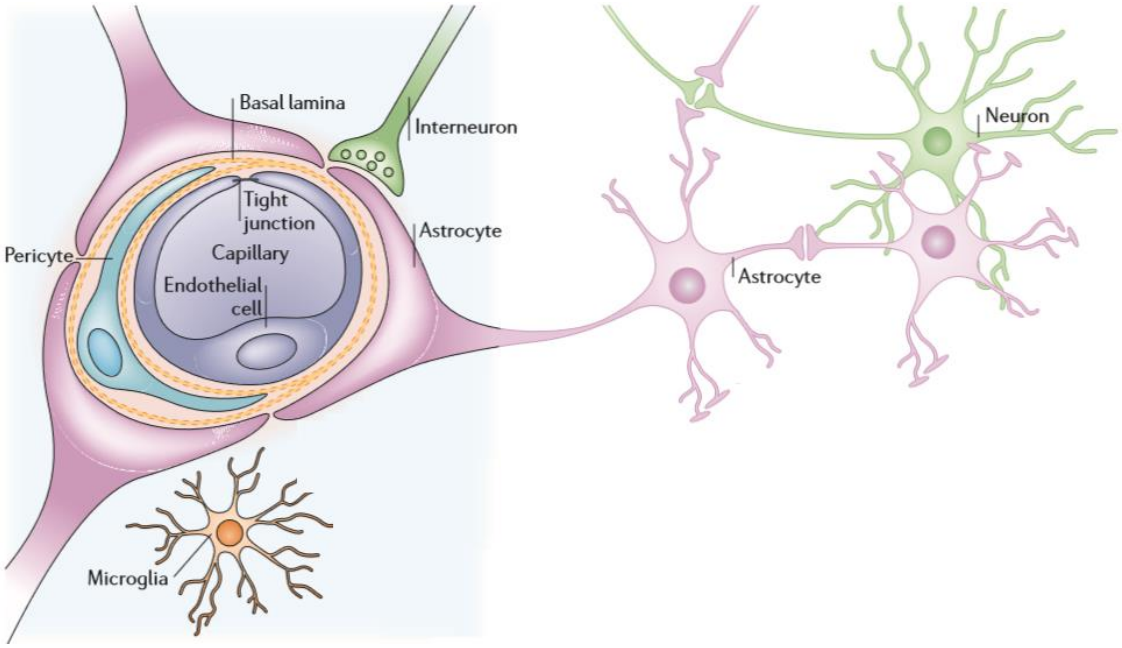


Figure 5 | Cellular constituents of the blood–brain barrier. The barrier is formed by capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular end feet. Astrocytes provide the cellular link to the neurons (adapted from Abbott et al, 2006).

1.2.2.4. Extracellular ion buffering: a focus on K^+ buffering

Neurons are bathed in an extracellular fluid that is rich in sodium (Na^+) ions and relatively poor in potassium (K^+) ions. The relative concentrations of these cations are reversed inside the cells, resulting in chemical gradients across the cell membrane that are crucial to many important processes, including fast electrical signaling involving Na^+ influx and K^+ efflux (Kofuji and Newman, 2004). Even modest neuronal K^+ effluxes may elicit considerable changes in extracellular K^+ concentration $[K^+]_o$ due to the limited volume of the CNS extracellular space (ECS) and the low baseline $[K^+]_o$ (Nicholson and Sykova, 1998). These $[K^+]_o$ changes can impact a wide variety of neuronal processes, such as maintenance of membrane potential, activation and inactivation of voltage-gated channels, synaptic transmission and electrogenic transport of neurotransmitters. When these mechanisms are disrupted, the extracellular $[K^+]_o$ can reach values as high as 60 mM (note that in the brain, $[K^+]_o$ is kept close to 3 mM) and CNS function is severely compromised (Somjen, 2001, 2002).

Around the twentieth century, a couple of scientists were the first to hypothesize that astrocytes are involved in this potassium clearance of the ECS and also postulated that astrocytes could use the manipulation of the $[K^+]_o$ as a means to control neuronal

excitability (Gerschenfeld et al., 1959; Hertz, 1965). Mechanisms of $[K^+]_o$ buffering can be broadly categorized, as either K^+ uptake or K^+ spatial buffering (Somjen, 2002; Kofuji and Newman, 2004). In the case of K^+ uptake, excess K^+ ions are temporarily sequestered into astrocytes by transporters or by K^+ channels. To preserve electroneutrality, K^+ influx into astrocytes is accompanied by either influx of anions, such as Cl^-, or by efflux of cations, such as Na^+. Eventually, K^+ ions accumulated in astrocytes are released back into the ECS, and the overall distribution of K^+ across the cellular compartments is restored. In contrast, for efficient K^+ spatial buffering the glial cells should form a syncytium (Figure 6) in which K^+ currents can cross relatively long distances, implicating that astrocytes should be highly and selectively permeable to K^+, which enters and exits through the glial membranes (Somjen, 2002; Kofuji and Newman, 2004). Several lines of evidence demonstrate that astrocytes do indeed form a functional syncytium that allows intercellular diffusion of ions and other signaling molecules (Nagy and Rash, 2000). Such extensive cellular coupling is due to the high density of gap junction or hemichannel proteins, such as connexins and pannexins, in astrocytes (Dermietzel, 1998; Rouach et al., 2002).

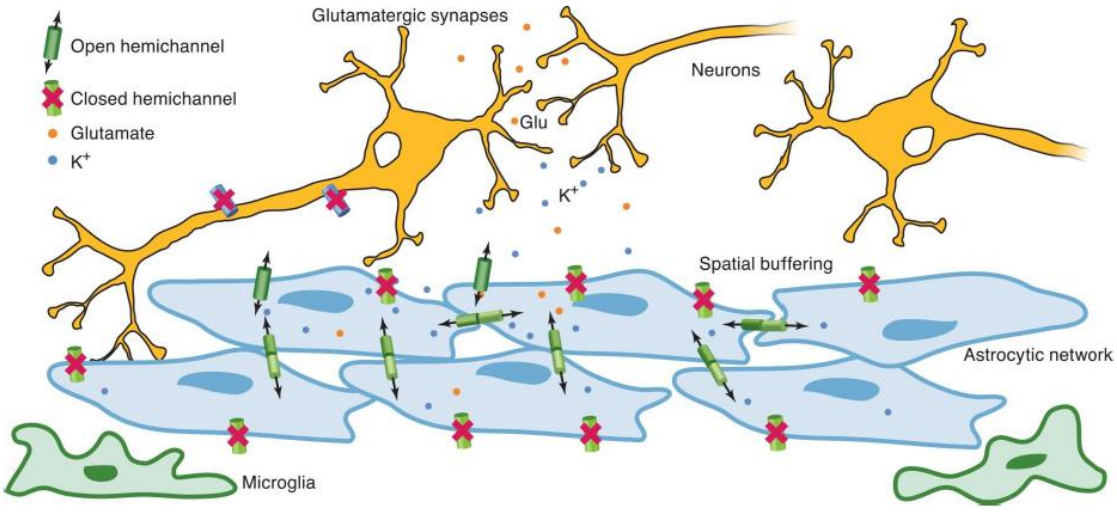


Figure 6 | Scheme showing functional roles of gap junction channels between astrocytes. Surrounding astroglia take up both K^+ (blue dots) and glutamate (orange dots) either through open hemichannels or specific K^+ channels or glutamate transporters, and distribute them through gap junctions throughout the astrocytic syncytium before releasing them at a distant site (from Byrne et al., 2014).

1.2.2.5. Metabolic support

As cited above, astrocytes play a central role in brain homeostasis, in particular via the numerous cooperative metabolic processes they establish with neurons, such as the supply of energy metabolites and neurotransmitter recycling functions. Taking this under consideration, in normal brain, astrocytes and neurons form a complex, symbiotic relationship for both maintenance of neuronal function as well as support of brain metabolism (Magistretti and Pellerin, 1996; Sokoloff, 1999; Escartin et al., 2006).

Most aerobic metabolism within the CNS is performed within neurons due to their high ATP requirements, particularly following neuronal activation, with astrocytes forming a smaller metabolic compartment in comparison. Astrocytes are important in buffering neurons from the bloodstream, and hence assisting in providing glucose to the ECS, as the primary initial fuel metabolite for the brain; however, they also store glycogen that is crucial during periods of hypoglycemia (Tsacopoulos and Magistretti, 1996; Gruetter, 2003). Glucose is the obligatory energy substrate of the adult brain. Nevertheless, under particular circumstances the brain has the capacity to use other blood-derived energy substrates, such as ketone bodies, during development and starvation (Nehlig, 2004; Belanger et al., 2011) or lactate during periods of intense physical activity (van Hall et al., 2009). Glucose enters cells trough specific glucose transporters (GLUTs) and is phosphorylated by hexokinase (HK) to produce glucose-6-phosphate. As in other organs, glucose 6-phosphate can be processed via different metabolic pathways: the main ones are glycolysis (leading to lactate production or mitochondrial metabolism), the pentose phosphate pathway and glycogenesis (in astrocytes only, Figure 7) (Belanger et al., 2011).

Metabolic supply is vital to neuron activity and survival. Therefore, impairments in astrocytic function are increasingly being recognized as an important contributor to neuronal dysfunction and, in particular, to neurodegenerative processes.

1.2.2.6. Neurotransmitter uptake

During neurotransmission, neurotransmitters and ions are released at high concentration to the synaptic cleft. The rapid removal of these substances is important so that they do not interfere with future synaptic activity or cause neurotoxicity. The presence of astrocyte processes around synapses position them well to regulate neurotransmitter uptake and inactivation. These arguments are consistent with the presence of transport systems for many neurotransmitters in astrocytes (Wang and Bordey, 2008; Belanger et al., 2011). For instance, glutamate reuptake is performed mostly by astrocytes (Figure 7), which convert glutamate into glutamine, via the glutamine synthetase pathway, and then release it into the ECS. Glutamine is taken up by neurons, which use it to generate glutamate and γ-aminobutyric acid (GABA), potent excitatory and inhibitory neurotransmitters, respectively (Bak et al., 2006).

Because glutamate is the major excitatory neurotransmitter in the brain (about 5-15 mmol/kg wet weigh), an overstimulation of glutamate receptors is highly toxic to neurons (a phenomenon referred to as excitotoxicity) (Persson and Rönnbäck, 2012). Glutamate uptake is primarily achieved by the astrocyte-specific sodium-dependent high-affinity glutamate transporters, such as glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST), which correspond to human EAAT2 and EAAT1, respectively (Chaudhry et al., 1995; Palacin et al., 1998; Anderson and Swanson, 2000; Bak et al., 2006) (Table 1). Astrocytes are known to take up and metabolize GABA as well (Jursky et al., 1994). GABA is cleared from the synaptic cleft by specific, high-affinity, sodium- and chloride-dependent transporters, which are located in surrounding glial cells and also in presynaptic terminals (Borden, 1996; Conti et al., 1999). Depending on the brain region, astrocytes express a high density of high affinity GABA transporters, such as GAT-1, GAT-2 and GAT-3 (Conti et al., 1999).

At elevated extracellular concentrations, glutamate and other neurotransmitters may turn into powerful neurotoxins that are capable of inducing degeneration of neurons (Gegelashvili et al., 2001; Bak et al., 2006; Belanger et al., 2011). This process, which is known as excitotoxicity (in the case of glutamate), contributes to many neurodegenerative such as Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) diseases, among other pathologies.

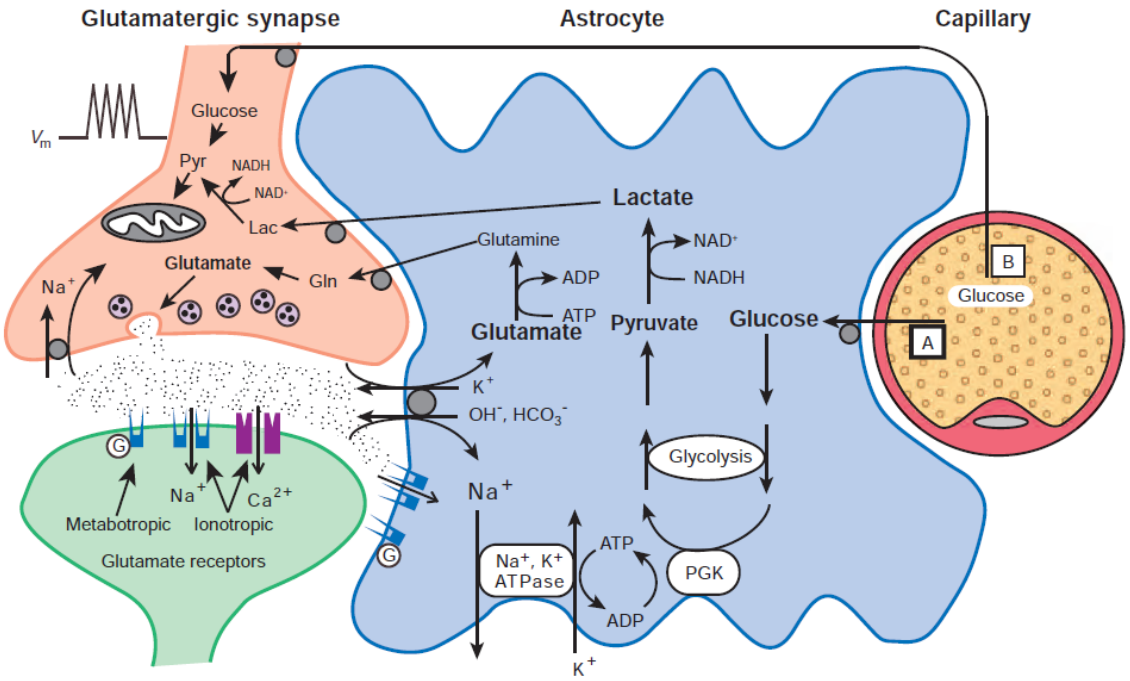


Figure 7 | Schematic representation of the mechanism for glutamate-induced glycolysis in astrocytes during physiological activation. At glutamatergic synapses, presynaptically released glutamate depolarizes postsynaptic neurons by acting at specific receptor subtypes. The action of glutamate is terminated by an efficient glutamate uptake system located primarily in astrocytes. Glutamate is co-transported with Na^+, resulting in an increase in the intra astrocytic concentration of Na^+, leading to an activation of the astrocytic Na^+, K^+ - ATPase. Activation of Na^+, K^+ - ATPase stimulates glycolysis (i.e., glucose utilization and lactate production). Within the astrocyte, one ATP fuels one "turn of the pump," and the other provides the energy needed to convert glutamate to glutamine by glutamine synthase (GS). Once released by astrocytes, lactate can be taken up by neurons and serve as an energy substrate (from Squire et al., 2008).

Table 1 | Nomenclature and expression pattern of glutamate transporter (GluT) subtypes. Information was collected from: (Milton et al., 1997; Anderson and Swanson, 2000; Sattler and Rothstein, 2006; Lin et al., 2012)

GLUTAMATE TRANSPORTERS			
GluT subtype	**Human homolog**	**Anatomical localization**	**Cellular localization**
GLAST	EAAT1	Cerebellum, cortex, spinal cord	Astrocytes, oligodendrocytes
GLT-1	EAAT2	Predominant GluT in the brain. Throughout brain and spinal cord.	Astrocytes. During development, in neurons and oligodendrocytes
GLT-1b	EAAT2b	Throughout brain and spinal cord	Astrocytes, neurons
EAAC1	EAAT3	Hippocampus, cerebellum, stiatum	Neurons
EAAT4	EAAT4	Cerebellum	Purkinje cells
EAAT5	EAAT5	Retina	Photoreceptors and bipolar cells

1.2.2.7. Neuromodulation: gliotransmission-mediated synaptic plasticity

Important progress has been made in the past two decades in understanding the role of astrocytes in the generation of neuron-astrocyte network outputs, resulting in behavior control. Astrocytes have emerged as important partners of neurons in information processing. Nowadays, there is evidence that astrocytes are, at least, involved across four different behavioral domains, such as cognition, emotion, motor and sensory processing (Oliveira et al., 2015). Additional studies also show that astrocytes can actively contribute to synaptic plasticity and activity by releasing neuroactive molecules, called gliotransmitters (Kimelberg et al., 1990; Martin, 1992; Haydon and Carmignoto, 2006). The concept that astrocytes release gliotransmitters to affect synaptic transmission has led to a paradigm shift in neuroscience research over the past decade. This concept suggests that astrocytes, together with pre- and postsynaptic neuronal elements, make up a functional synapse. These emerging functions imply that astrocytes are active participants in brain activity, rather than passive elements in maintaining the extracellular space.

In the tripartite conceptualization of the synapse, perisynaptic astrocytes are present along with the standard pre- and postsynaptic neurons (Araque et al., 1999; Halassa et al., 2009; Santello et al., 2012). The arborization and ramifications of astrocytes allow them to tightly enwrap the synaptic terminal in order to modulate synaptic processes (Araque et al., 1999; Derouiche et al., 2002; Ota et al., 2013).

Increasing studies suggest that astrocytes respond to neurotransmitter release by increasing their intracellular calcium levels ($[Ca^{2+}]i$), and that they control neuronal excitability through the release of gliotransmitters (Haydon and Carmignoto, 2006). More specifically, astrocytes sense synaptic activity through a broad variety of ion channels, transporters and receptors expressed on their surface, and depending on which synaptic inputs are activated and the glial receptors involved, a multitude of intracellular second messenger pathways are activated, most of them involving Ca^{2+} (Perea and Araque, 2005). In turn, this induces gliotransmission (the release of gliotransmitters), which can act either on neighboring glia or neurons.

Currently, the list of known gliotransmitters that mediate astrocyte to neuron signaling includes cytokines, taurine, D-serine, GABA, glutamate, ATP and adenosine, among others (Kimelberg et al., 1990; Haydon and Carmignoto, 2006; Turrigiano, 2006;

Ota et al., 2013). Several mechanisms have been implicated in the release of gliotransmitters (Figure 8). These mechanisms include: (i) release through functional hemichannels, constituted mainly by connexin 43 (Cx43) assembles (Torres et al., 2012; Chever et al., 2014; Orellana and Stehberg, 2014); (ii) through reverse operation of plasma membrane glutamate transporters (Malarkey and Parpura, 2008); (iii) through Ca^{2+}-dependent exocytosis (Parpura and Zorec, 2010; Khakh and McCarthy, 2015); iv) release through ionotropic purinergic receptors (Malarkey and Parpura, 2008; Delekate et al., 2014) and v) anion channel opening induced by cell swelling (Mongin and Orlov, 2001; Malarkey and Parpura, 2008).

Glutamate plays a key role in the regulation of synaptic activity and causes a response in astrocytes. Importantly, as mentioned before, astrocytes actively sequester up to 90% of glutamate that is released into the ECS between neurons (Chaudhry et al., 1995; Palacin et al., 1998; Bak et al., 2006). Glutamate causes a wide range of effects in astrocytes via metabotropic glutamate receptors (mGluR), and by ionotropic glutamate receptors of the sub-type N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Bains and Oliet, 2007). In addition to glutamate, ATP (discussed in section 1.4.2) is also able to induce or control persistent changes in synapse strength, through the insertion or removal of AMPA receptors (AMPARs) (Bains and Oliet, 2007). This includes effects on NMDA receptor-dependent long-term potentiation (LTP) and long-term depression (LTD), as well as homeostatic and activity-independent plasticity (Gordon et al., 2005).

These persistent changes in hippocampal synaptic strength are believed to be essential for cognitive processes, such as learning and memory (Martin et al., 2000; Moraga-Amaro et al., 2014; De Pittà et al., 2015).

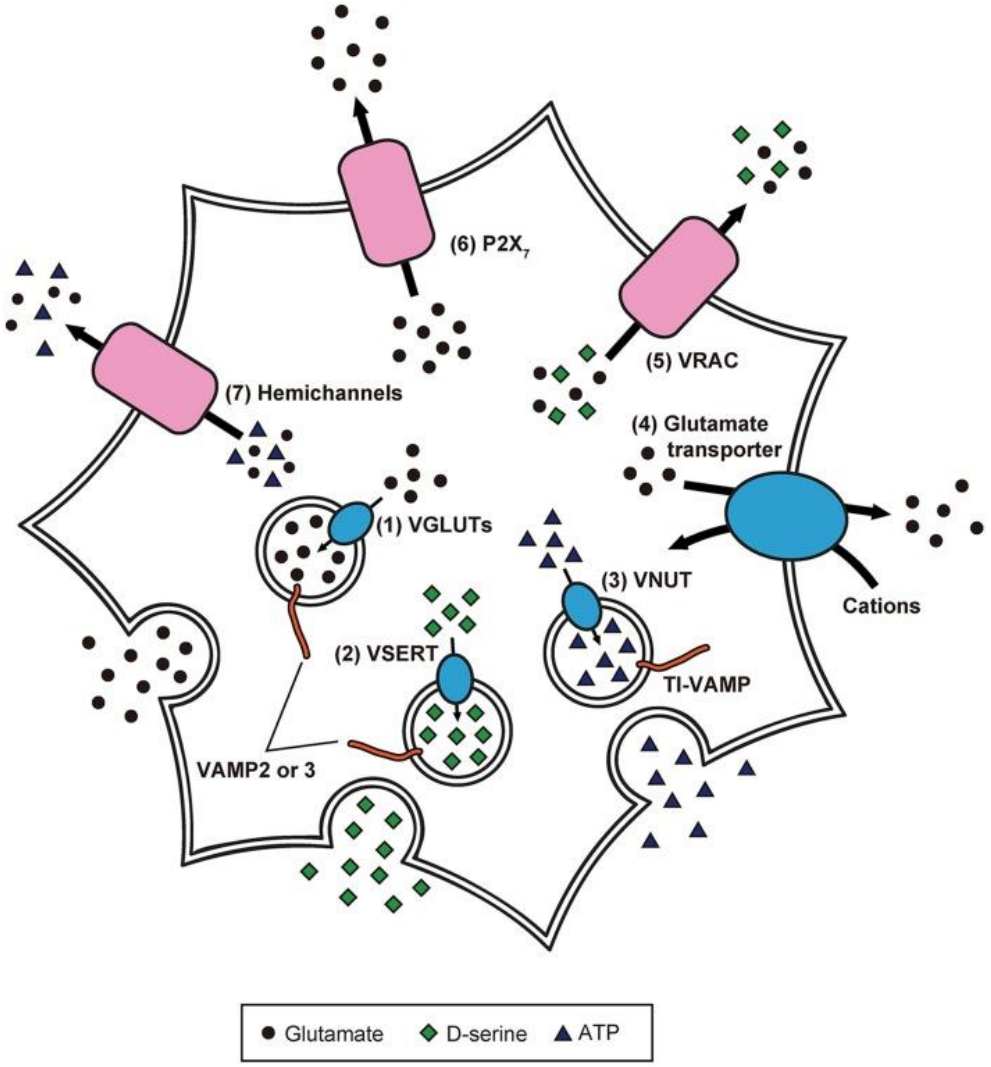


Figure 8 | Precise intracellular machinery involved in the release of glutamate, D-serine and ATP from astrocytes. Glutamate and D-serine are taken up into synaptic-like vesicles through: **(1)** VGLUT and **(2)** vesicular D-serine transporters (VSERT), respectively. These synaptic-like vesicles fuse to the plasma membrane, mediated by SNARE proteins including VAMP2 or VAMP3, in response to $[Ca^{2+}]i$ increase. In contrast, ATP is released through secretory lysosomes. Storage of ATP into secretory lysosomes is achieved by **(3)** vesicular nucleotide transporter (VNUT). Through the interaction of SNARE proteins including TI-VAMP, ATP-containing secretory lysosomes are Ca^{2+}-dependently exocytosed. Moreover, the existence of other release mechanisms has been discovered: **(4)** reverse operation of plasma membrane glutamate transporters, **(5)** cell swelling-induced anion transporter (VRAC) opening, **(6)** release via $P2X_7$ receptors, and **(7)** gap junction channels (hemichannels) on the cell surface of astrocytes (from Harada et al., 2016).

1.3. Hippocampal synaptic plasticity and cognition

1.3.1. Hippocampal anatomy and circuitry

The hippocampal formation is comprised of a group of cortical regions that includes the dentate gyrus (DG), hippocampal subicular complex, being subiculum (S), presubiculum (PrS) and parasubiculum (PaS), and finally entorhinal cortex (EC). Briefly, the hippocampus, which in Latin means seahorse (named for its shape), is a cortical structure in the medial portion of the temporal lobe responsible for the formation and storage of memory (Scoville and Milner, 1957; Kroes and Fernández, 2012), and consists of a heterogeneous population of neurons distinguished by their ontogeny, morphological characteristics and connectivity (Leuner and Gould, 2010).

The hippocampus can be viewed as a primitive form of three-layered cortical tissue. Accordingly, it has the following layers extending from the ventricular surface to the dentate gyrus: i) an external plexiform layer which contains axons of pyramidal cells that project outside the hippocampus as well as hippocampal afferent fibres from the EC; ii) *stratum oriens*, which contains basal dendrites and basket cells; iii) a pyramidal cell layer, which contains the pyramidal cells of the hippocampus; and iv) the *stratum radiatum* and *stratum lacunosum-moleculare*, which are two layers that contain the apical dendrites of the pyramidal cells and hippocampal afferents from the EC (Braak et al., 1996; Schultz and Engelhardt, 2014). The pyramidal cells of the hippocampus are arranged in a C-shaped fashion, which is interlocked with another C-shaped arrangement of the dentate gyrus (Figure 9).

The hippocampus is divided into a number of distinct sub-regions (CA1, CA2, CA3 and CA4). The pyramidal cells situated closest to the subiculum are referred to as the CA1 field, whereas the CA4 field is located within the hilus of the dentate gyrus (Agster et al., 2013; Schultz and Engelhardt, 2014). The CA2 and CA3 fields are located between the CA1 and CA4 fields. Collaterals of axons arising from CA3 pyramidal cells (called Schaffer collaterals, SC) project back to the CA1 field. The CA1 field is of particular interest because is one of the most studied regions for its association with impaired LTP and memory consolidation. Several studies have shown that when the CA1 region of the

dorsal hippocampus is temporarily inactivated in rodents during the various stages of NOR (novel object recognition test), memory of objects is impaired, producing deficits in both the encoding and retrieval of object recognition memory (Granger et al., 1996; Zola-Morgan, 1996; Remondes and Schuman, 2004).

Regarding the hippocampal connectivity, the intrinsic flow of information follows a sequencial and largely unidirectional and glutamatergic (excitatory) path that ultimately forms part of a closed circuit (Schultz and Engelhardt, 2014). Neurons in layers II and III of MEC give rise to projections to all constituents of the hippocampus. Layer II cells project to DG and CA3, whereas cells in layer III project to CA1 and the subiculum. The layer II projection to DG is the entry point of the trisynaptic pathway, which subsequently includes the mossy fibres projection from DG to CA3 and the Schaffer collateral projection from CA3 to CA1 (Figure 10). The two entorhinal inputs have become known as the direct (layer III to CA1) and the indirect (layer II via the trisynaptic pathway) pathways to CA1 (Agster et al., 2013; Witter et al., 2013).

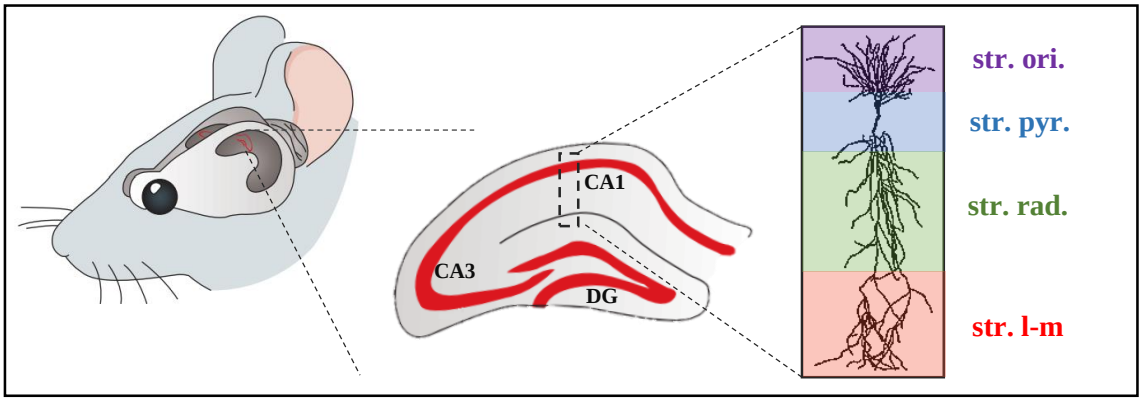


Figure 9 | Anatomical layers of a CA1 pyramidal neuron in a mouse brain. Abbreviations are: *stratum oriens* (str. ori.); *stratum pyramidale* (str. pyr.); *stratum radiatum* (str. rad.) and *stratum lacunosum-moleculare* (str. l-m).

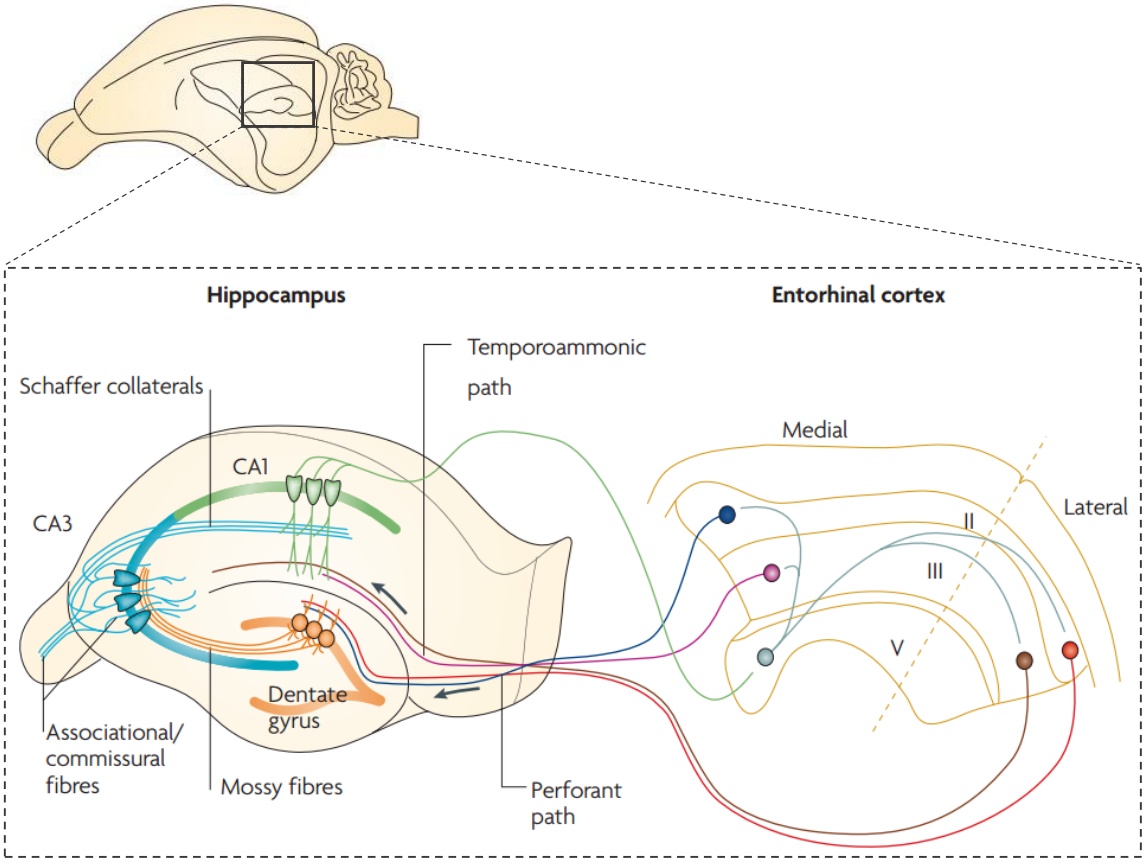


Figure 10 | Basic anatomy of the hippocampus formation. The wiring diagram of the hippocampus is traditionally presented as a trisynaptic loop. The major input is carried by axons of the perforant path, which convey polymodal sensory information from neurons in layer II of the entorhinal cortex to the dentate gyrus. Perforant path axons make excitatory synaptic contact with the dendrites of granule cells: axons from the lateral and medial entorhinal cortices innervate the outer and middle third of the dendritic tree, respectively. Granule cells project, through their axons (the mossy fibres), to the proximal apical dendrites of CA3 pyramidal cells which, in turn, project to ipsilateral CA1 pyramidal cells through Schaffer collaterals and to contralateral CA3 and CA1 pyramidal cells through commissural connections. In addition to the sequential trisynaptic circuit, there is also a dense associative network interconnecting CA3 cells on the same side. CA3 pyramidal cells are also innervated by a direct input from layer II cells of the entorhinal cortex (not shown). The distal apical dendrites of CA1 pyramidal neurons receive a direct input from layer III cells of the entorhinal cortex. There is also substantial modulatory input to hippocampal neurons. The three major subfields have an elegant laminar organization in which the cell bodies are tightly packed in an interlocking C-shaped arrangement, with afferent fibres terminating on selective regions of the dendritic tree (adapted from Neves et al., 2008).

1.3.2. Synaptic plasticity at CA1 pyramidal neurons

Synaptic plasticity is the biological process by which specific patterns of synaptic activity result in changes in synaptic strength. Long-term potentiation (LTP) and long-term depression (LTD) are the best described forms of synaptic plasticity in the CNS (Bear and Malenka, 1994; Malenka, 1994; Malinow and Malenka, 2002), and both pre- and postsynaptic mechanisms can contribute to the expression of synaptic plasticity. Likewise, the molecular mechanisms underlying both forms of dynamic changes in synaptic function (LTD and LTD) are distinct.

For LTP induction both pre- and postsynaptic neurons need to be active at the same time because the postsynaptic neuron must be depolarized when glutamate is released from the presynaptic neuron to fully relieve the Mg^{2+} block of N-methyl-D-aspartate receptors (NMDARs). As a consequence of coincident depolarization and glutamate binding, Ca^{2+} influx through NMDARs is maximal, which activates intracellular signaling cascades involving activation of Ca^{2+}/calmodulin-dependent protein kinases (CaMKII) that ultimately are responsible for the altered synaptic efficacy. NMDARs-dependent LTP is therefore an associative form of plasticity and fulfills the criteria for correlated activity as the origin of the synaptic strengthening between two neurons proposed by Donald Hebb more than 60 years ago (Hebb, 1949).

In contrast, LTD can be induced by repeated activation of the presynaptic neuron at low frequencies without postsynaptic activity. Because the driving force for Ca^{2+} entry is very large for a neuron at rest and the block of NMDARs by Mg^{2+} is incomplete even at resting potentials, significant Ca^{2+} enters the cell in response to synaptic stimulation during low-frequency synaptic stimulation (Jahr and Stevens, 1993; Lüscher and Malenka, 2012). Presumably, the repeated occurrence of this smaller NMDAR-dependent Ca^{2+} influx is the mechanism by which LTD is induced (Siegelbaum and Kandel, 1991; Malenka, 1994).

Therefore, if LTP involves the activation of CaMKII (and other kinases) and LTD represents the inverse of LTP, then a logical hypothesis is that LTD involves the preferential activation of protein phosphatases (Bear and Malenka, 1994) (Figure 11). A sufficiently robust rise in postsynaptic Ca^{2+}, associated with NMDAR activation, triggers a cascade of intracellular signaling events culminating in either insertion (during LTP) or removal (during LTD) of AMPARs at the postsynaptic terminal (Gordon et al., 2005).

Depotentiation (DP) is known as another form of neuronal plasticity and is also believed to be involved in the cellular mechanisms underlying information storage in the mammalian brain. Briefly, DP is the mechanism by which synapses that have recently undergone LTP can reverse their synaptic strengthening in response to low-frequency stimulation (LFS). Therefore, the scientific term metaplasticity, which refers to the plasticity of synaptic plasticity, can be defined as a higher order plasticity and reflects how the prior experience of a synapse may alter the subsequent ability of a synapse to modify its synaptic strength in response to a plasticity-inducing stimulus (Abraham and Bear, 1996; Abraham, 2008). Several authors hypothesized that DP prevents the saturation of synaptic potentiation by resetting synapses into a more efficient state to store new information and to protect against excitotoxicity or epilepsy (Abraham, 2008). However, detailed information about the cells and the mechanisms that underlie DP still remain unclear. For example, DP is reported to be NMDARs-dependent in some experimental conditions (Fujii et al., 1991) and metabotropic glutamate receptors-dependent in others (Fitzjohn et al., 1998; Kulla et al., 2008), which is similar to LTD case. Although NMDARs are thought to play a role in the induction of both forms of plasticity, there are some evidence that the NMDA receptor subunit NR2B is involved in LTD whereas NR2A is involved in DP (Zhu et al., 2005; Sanderson, 2012).

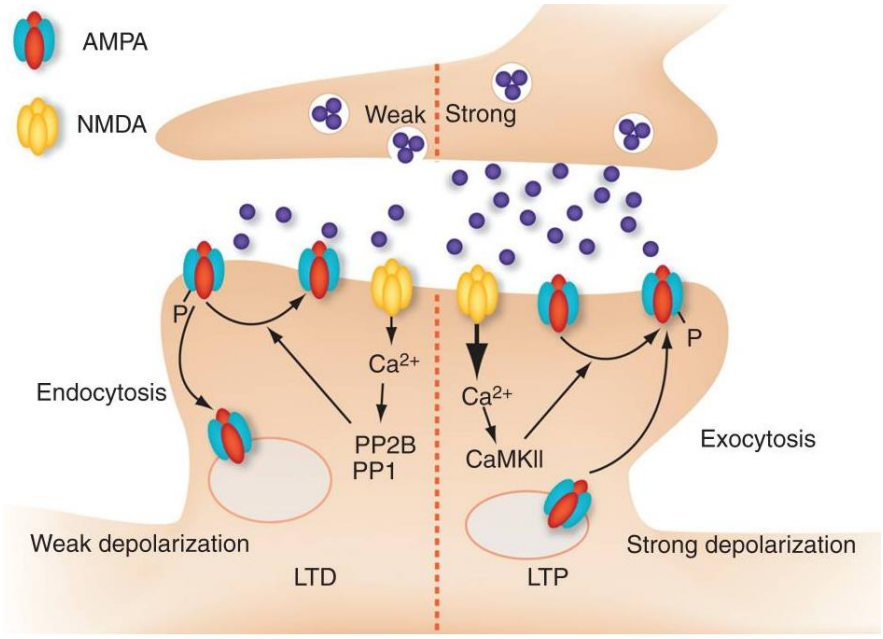


Figure 11 | Schematization of postsynaptic expression mechanisms of LTP and LTD. Left | Weak activity of the presynaptic neuron leads to modest depolarization and Ca^{2+} influx through NMDARs. This preferentially activates phosphatases that dephosphorylate AMPARs, thus promoting receptor endocytosis. Right | Strong activity paired with strong depolarization triggers LTP via CaMKII, receptor phosphorylation, and AMPARs exocytosis (adapted from Lüscher and Malenka, 2012).

1.4. Astrocytic purinergic signaling in synaptic plasticity

1.4.1. Purinergic receptors in the CNS

Extracellular purines (adenosine, ADP, and ATP) are important signaling molecules that mediate diverse biological effects via cell surface receptors termed purinergic receptors. Apart from being a source of cellular energy, the purine nucleotide ATP also functions as a potent extracellular messenger and neurotransmitter via activation of members of the P2 receptor family (Burnstock, 2007). This class of receptors comprise both metabotropic P2Y and ionotropic P2X receptor families (Ralevic and Burnstock, 1998; Le Feuvre et al., 2002).

On the other hand, adenosine, produced by ecto-enzymatic breakdown of ATP, act as a neuromodulator controlling neurotransmitter release, regulating synaptic transmission and commanding the action of some receptor systems (Cunha, 2001). Furthermore, this nucleoside plays an important part in the control of the innate and adaptive immune systems, and dysregulation of the adenosine system is involved in pathologies ranging from epilepsy to neurodegenerative disorders and psychiatric conditions (Cunha et al., 2008; Abbracchio et al., 2009; Gomes et al., 2011). Another class of purinergic receptors can be activated by adenosine and are named P1 receptors (Table 2). All P1 adenosine receptors are coupled to G proteins (metabotropic receptors), and they can be categorized in three subtypes (A_1, A_2, and A_3), in which A_1 seems to be the most abundant in the brain tissue (Cunha, 2005; Burnstock, 2007). Adenosine A_2 receptors are further subdivided into types A_{2A} and A_{2B}.

Besides the evidence that A_{2A} receptors are highly expressed in the basal ganglia, they are now recognized to display a widespread distribution in the brain (Lopes et al., 2004; Fredholm et al., 2005) and are mostly located in synapses (Rebola et al., 2005; Gomes et al., 2011). More specifically, A_{2A} receptors can be located in dendritic spines and post-synaptic densities (Rebola et al., 2005) of asymmetric contacts between cortico-thalamic glutamatergic projections (Cunha, 2005). A_{2A} receptors are also located in the presynaptic nerve terminals of limbic and neocortical regions of the brain, being also present in microglial cells and, most importantly, in astrocytes (Li et al., 2001; Cunha, 2005; Rebola et al., 2005; Matos et al., 2012a).

ATP and adenosine are involved in mechanisms of synaptic plasticity and memory formation (Wieraszko and Ehrlich, 1994; Cunha et al., 1996). However, modification of A_1 and A_{2A} receptor binding in aged striatum, hippocampus, and cortex of rats has been reported, suggesting alterations in the number of these receptors with the aging (Cunha et al., 1995). Moreover, evidence showed that ATP analogs can facilitate LTP through P2 receptor activation that triggers adenosine release, leading to activation of P1 (A_{2A}) receptors, which are claimed to be involved in modulating spatial recognition memory in mice (Wang et al., 2006). Moreover, in conditions were astrocytes become reactive, for instance, in response to abnormal Aβ peptide accumulation, evidence state that higher levels of A_{2A}Rs are expressed on their surface. However, this modification impairs the regular removal of glutamate from the extracellular space (Matos et al., 2008, 2012a, 2012b). If astrocytes fail to reuptake glutamate to the intracellular compartment, excitotoxicity will be continuously induced, leading to a complex cascade of events, and thus, being a risk factor for the early onset of AD. In addition, A_{2A} receptors are also involved in other brain processes, such as locomotion, feeding, sleep and arousal, mood and motivation (Burnstock, 2007).

The role of extracellular ATP and purinergic receptors in neurodegeneration is the focus of a rapidly expanding area of research. The ability of adenosine receptors to control excitatory transmission prompts the interest in considering this neuromodulatory system as a putative therapeutic target to manage brain disorders. This interest is further bolstered by recurrent observations showing that the extracellular levels of adenosine are modified upon brain damage. Thus, albeit the extracellular levels of adenosine increase with neuronal activity (Mitchell et al., 1993), they increase to considerable higher levels when brain damage occurs (Latini and Pedata, 2001; Gomes et al., 2011), for instance under neurodegeneration seen in several disorders like multiple sclerosis, PD, AD and HD (Cunha et al., 2008; Kaster et al., 2015).

Table 2 | Characteristics of metabotropic adenosine receptors (P1 receptors). Information was collected from: Cunha, 2005; Fredholm et al., 2005; Rebola et al., 2005; Cunha et al., 2008; Shen et al., 2008; Gomes et al., 2011.

ADENOSINE RECEPTORS				
Receptor subtype	**Localization in the CNS**	**Cellular and Subcellular localization**	**Mechanisms of action**	**Physiological properties**
A_1	Widespread. Highly expressed in cortex, cerebellum and hippocampus; intermediate levels in other areas	Pre-, post- and extra-synaptically in glutamatergic neurons and striatonigral GABAergic neurons; astrocytes, oligodendrocytes, microglia	Inhibits Ca^{2+} influx and decrease synaptic transmission (**inhibitory**); Inhibits the release of glutamate, GABA and other NTs; Leads to membrane depolarization; decreases astrocyte proliferation	Inhibits the direct pathway of the basal ganglia, resulting in a decrease on psychomotor activity; Seizure suppression; neuroprotection; spinal analgesia; can induce sleep and wakefulness; antidepressant
A_{2A}	Restricted. Highly expressed in striatum, nucleus accumbens, olfactory tubercle; low levels in other areas	High levels post-synaptically in striatopallidal GABAergic neurons; high levels in hippocampal active zones; fewer in astrocytes, oligodendrocytes, microglia	Stimulates Ca^{2+} influx and potentiate synaptic transmission (**facilitatory**); Stimulates the uptake and release of glutamate, GABA and other NTs from neurons; induce astrogliosis and astrocyte proliferation	Elicits the indirect pathway of the basal ganglia, resulting in a decrease on psychomotor; pro-inflammatory; inactivation improves learning and memory processes; induce vasodilation; promotes sleep.
A_{2B}	Widespread. Low levels in all areas	Residual levels in most brain cells	Stimulates Ca^{2+} influx	Pro-inflammatory activity
A_3	Widespread. Intermediate levels in cerebellum and hippocampus. Low levels in other areas	Residual levels in most brain cells	Inhibits Ca2+ influx and synaptic activity during hypoxia; uncouples A_1 and mGlu receptors	Anti-inflammatory activity; can elicit survival or apoptosis pathways.

1.4.2. Astrocytic involvement in neuromodulation: a focus in adenosine signaling

Transmitter-receptor interactions have been established as being fundamental to all levels of nervous system function. The existence of receptors on astrocytes was first unequivocally shown in primary astrocyte cultures (Van Calker and Hamprecht, 1980). Knowledge of the functions of these receptors is critical to understanding how astrocytes interact with the other brain cells. Currently, we know that astrocytes express a large

repertoire of receptors, including metabotropic (G-protein coupled receptors) and ionotropic receptors (Kimelberg, 1995). These cells express a variety of receptors for typical neurotransmitter molecules, like glutamate (glutamatergic receptors), acetylcholine (muscarinic and nicotinic receptors), ATP and adenosine (purinergic receptors), GABA (GABAergic receptors), norepinephrine (adrenergic receptors), among other neurotransmitters, and for retrograde messengers such as endocannabinoids (Porter and McCarthy, 1997; Haydon, 2001). Furthermore, astrocytes also express receptors for growth factors, chemokines, steroids, and receptors involved in innate immunity that participate in regulating astrocyte development and response to neurons and injury (Liu and Neufeld, 2007; Wang and Bordey, 2008).

In the CNS, ATP is released from the nerve terminals (mainly by exocytosis) and astrocytes (both by vesicular release and diffusion through Cx43 HCs (Luisa Cotrina et al., 1998; Burnstock, 2007; Araque et al., 2014; Chever et al., 2014). Release of ATP represents a powerful pathway of glia-neuron interaction implicated in the synaptic plasticity, metaplasticity and neurological disorders (Pascual, 2005; Hulme et al., 2014). ATP signaling regulates Ca^{2+}-dependent glutamate release via astrocytic P2Y receptors. The ATP released from astrocytes also interacts directly with pre- and postsynaptic neurons, serving to regulate their own glutamatergic transmission and to also enhance the concentration of AMPA receptors (Haydon and Carmignoto, 2006). Moreover, in astrocytes, the $P2Y_1R$ and $P2X_7R$ seem to play a major role in Ca^{2+} signaling and Ca^{2+} wave propagation (Fumagalli et al., 2003; Fischer et al., 2009).

Additionally, some of the ATP released by astrocytes is converted directly to adenosine, an important nucleoside in controlling synaptic function (Wieraszko and Ehrlich, 1994; Cunha et al., 1996; Zimmermann and Braun, 1996). In fact, there are a variety of ecto-nucleotidases responsible for extracellular hydrolysis of ATP to AMP; and the final hydrolysis of AMP to adenosine is mediated by an ecto-5′-nucleotidase (CD73), a key ecto-enzyme in adenosine production (Zimmermann and Braun, 1996; Cunha, 2005). ATP-derived adenosine acts on either A_1 or A_{2A} receptors to depress or enhance excitatory synaptic transmission, respectively (Lopes et al., 2002; Cunha, 2005; Costenla et al., 2010). Additionally, adenosine can also bind to astrocytic receptors (A_1 and A_{2A}) and induce the release of gliotransmitter through Ca^{2+}-dependent mechanisms (Nishizaki et al., 2002; Cristovao-Ferreira et al., 2011). Through astrocytes, adenosine, indirectly modulates the neuronal network.

Basal levels of adenosine, derived from astrocytic ATP, regulate the dynamic range for LTP generation (Pascual, 2005; Panatier et al., 2011; Araque et al., 2014). Moreover, it was shown that astrocytes regulate synaptic transmission and modulate plasticity through the control of extracellular adenosine (Pascual et al., 2005). These studies place astrocytes at center stage in the control of adenosine in the extracellular space, which in turn controls plasticity and memory processes. Because adenosine is highly expressed under pathological conditions, glial cells, in particular, astrocytes might become prime targets in pathological events that are associated with a deficit in synaptic plasticity, including stroke, epilepsy, peripheral neuropathies, PD, AD, HD and also schizophrenia.

1.5. Astrocytic blunting: impact on synaptic plasticity

Since our goal is to study how astrocytes impact on hippocampal synaptic plasticity (detailed in chapter 2), we selected different pharmacological tools, already described for many years, to target astrocytes. With this in mind, the following information refers to the different drugs used in this work and to their mechanism of action.

1.5.1. Gliotoxins: L-α-aminoadipic acid (L-AA) & trifluoroacetic acid (TFA)

L-α-aminoadipic acid (L-AA) was proposed to be a specific gliotoxin over four decades ago when Olney and colleagues (1971) noticed the toxic properties of L-AA during the neurotoxicity evaluation of a number of compounds. More specifically, they saw that L-AA induced changes in only one cell type of the retina, the non-neuronal Muller cell (Olney et al., 1971). Further *in vitro* studies about the properties of L-AA confirmed that it had specific cytotoxic effects towards astrocytes, but not neurons, and that it was taken up by astrocytes through sodium-dependent glutamate transporters like GLT-1 and GLAST before exerting its toxic effect on astroglial cells (Huck et al., 1984). Additionally, L-AA was tested *in vivo* and both Khurgel and Olney together with their colleagues presented anatomical evidence that intracerebral injections of L-AA resulted in localized and selective ablation of astrocytes (Olney et al., 1980; Khurgel et al., 1996). Furthermore, McBean reported that intracerebral application of the compound into the striatum of adult rats caused a significant reduction in the activity of the glial enzyme, glutamine synthetase (GS) (McBean, 1994). Other studies were conducted using this gliotoxin to target astrocytes without inducing toxicity to the surrounding neurons (Takada and Hattori, 1986; Brown and Kretzschmar, 1998; Banasr and Duman, 2008; Lima et al., 2014).

Trifluoroacetic acid (TFA) has been established as the toxic principle of the South African poison plant *Dichapetalum chymosum* and of other *Dichapetalum* plants as well (Peters, 1957). Numerous studies were performed with this compound, and now it is well known that fluorocitrate can be administered in several different ways to achieve specific inhibition of glial metabolism (Clarke et al., 1970; Paulsen et al., 1987; Clarke, 1991;

Hassel et al., 1994; Fonnum et al., 1997). TFA is exclusively taken up by astrocytes, where it is converted in fluorocitrate, which in turn is able to inhibit the Krebs cycle enzyme aconitase, the enzyme that catalyses the isomerization of citrate to isocitrate (Peters, 1957; Paulsen et al., 1987), and has been shown to depress astrocytic function. From this point forward, a massive metabolic dysfunction is generated in glial cells because the TCA cycle is compromised in the first step where citrate is converted into isocitrate (Hassel et al., 1995). Therefore, α-ketoglutarate, a TCA cycle intermediate, the precursor responsible for glutamate formation via glutamate dehydrogenase (GDH) inside astrocytic cells is reduced. Additionally, glutamate is responsible for glutamine formation inside astrocytes, via GS, which can subsequently be released into the ECS and taken up by neurons, and serve as precursor for neurotransmitter glutamate synthesis (Nissen et al., 2015). Finally, ATP formation is also decreased in astrocytes for the reason that TCA cycle is abolished.

1.5.2. Blockade of glial transporters

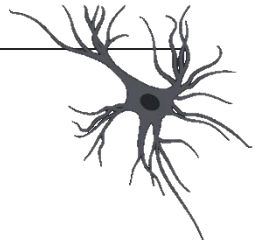
Glutamate transporters are expressed by all CNS cell types, but astrocytes are the cell type primarily responsible for glutamate uptake, mostly mediated by sodium-dependent systems (Chaudhry et al., 1995; Palacin et al., 1998; Anderson and Swanson, 2000; Bak et al., 2006). By blocking these mechanisms with selective inhibitors, we will be able to interfere with normal astrocytic function. The pharmacological tools available to block NA^+-dependent glutamate transporters are i) DL-threo-β-benzoyloxyaspartate (TBOA), an inhibitor of both GLAST and GLT-1 (Arriza et al., 1994; Tsukada et al., 2005), and ii) dihydrokainic acid (DHK), a selective GLT-1 blocker at μM concentration range (Oliet et al., 2001; Tsukada et al., 2005; Bernardinelli and Chatton, 2008).

Hemichannels (HC) and gap-junctions have been shown to play a crucial role in astrocyte-astrocyte communication and astrocyte-neuron signaling. The astrocytic HC are known to be involved in brain functions, such as synaptic plasticity and memory (Orellana and Stehberg, 2014). Astrocytic HCs are hexamers formed by two distinct protein families, namely connexins (Cx) and pannexins (Panx), which are differently regulated by Ca^{2+}, pH changes and ATP (Karpuk et al., 2011). Connexin43 (Cx43) are very abundant in astrocytes and constitute HCs that mediate the release of neuroactive molecules, such as K^+, ATP and glutamate (Dallérac et al., 2013; Giaume et al., 2013;

Chever et al., 2014a), which are known to modulate neuronal activity. Thus, astrocytic HCs blockade might be a way to have hints about the role of astrocytes in neuronal function, especially in synaptic plasticity. There are some pharmacological tools able to block HC and/or gap junction, such as the carbenoxolone (CBX), which we used in this study (Fischer et al., 2009; Dallérac et al., 2013), and others that are more specific for Cx43 HC, such as the blockade of Gap26 and Gap19 peptides (Abudara et al., 2014; Chever et al., 2014a).

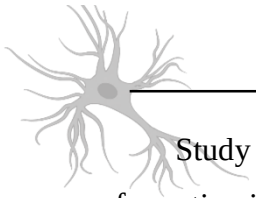
Chapter 2

Objectives



2.1. Aim of the work

Accumulating evidence support the existence of a bidirectional communication between neurons and astrocytes in the control of brain function; this gave rise to the concept of 'tripartite synapse' highlighting astrocytes as a third element of synapses. Astrocytes, the largest cell population in brain, have tentacle impact on information processing in neuronal circuits, thus these glial cells: i) enwrap most synapses, ii) provide metabolic support to synapses, iii) remove excitatory neurotransmitters, namely glutamate from synapses, iv) participate in ion homeostasis, v) shape synaptic volume, vi) regulate synaptic strength through the release of gliotransmitters, Particularly, astrocytes also control synaptic plasticity, the purported neurophysiological basis of memory processes. However, few experimental studies have attested the impact of astrocytes in synaptic function and morphology of neural circuitry, in part due to the lack of efficient tools. Thus, filling this gap of knowledge will set the stage for tackling astrocytic functions as targets to delay the onset of synaptic and memory deficits that are the phenotypic core of several brain disorders. Therefore, the major goal of this work is to:



Study the impact of astrocytes on the control of synaptic plasticity and memory formation in the mouse hippocampus under physiological conditions.

2.2. Work plan

2.2.1. Role of astrocytes in synaptic function (acute treatments)

In order to investigate the impact of astrocytes in hippocampal synaptic plasticity (mainly long-term potentiation, LTP), different pharmacological tools were used to selectively interfere with astroglial cells, namely:

- L-α-aminoadipic acid (L-AA)
- Trifluoroacetic acid (TFA)
- DL-threo-β-benzoyloxyaspartate (TBOA) / Dihydrokainic acid (DHK)
- Carbenoxolone (CBX)

By using two different gliotoxins (L-AA and TFA), already described in the previous chapter (section 1.5.1.), and by blocking specific function of astrocytes, such as glutamate uptake (TBOA, DHK) and hemichannels or gap junction-mediated gliotransmitter fluxes (CBX), we further show evidence about how astrocytes modulate synaptic plasticity on the Schaffer collaterals (SC) – CA1 pathway of adult mice.

2.2.2. Role of astrocytic A_{2A} receptors in hippocampal synaptic plasticity

Since adenosine receptors are expressed in glial cells, and given their importance in plasticity mechanisms, particularly A_{2A} receptors (A_{2A}Rs), we probed for the role of astrocytic A_{2A}Rs in hippocampal synaptic plasticity, mainly LTP at SC-CA1 synapses of adult mice. To successfully achieve this objective, pharmacological and genetic approaches were used:

- Selective A_{2A}Rs antagonist (SCH58261)
- Forebrain selective A_{2A}R KO mice (Fb-A_{2A}R KO)

After performing electrophysiological studies with the selective A_{2A}R antagonist in WT C57Bl/6 mice, fb-A_{2A}R KO mice were used to confirm the previous results.

2.2.3. Consequences of chronic L-AA injections in memory and synaptic function

After studying the acute effect of L-AA (*in vitro*) in mouse hippocampal LTP, an *in vivo* mice model was used to determine how astrocytes impact on memory formation and LTP at SC-CA1 pyramidal neurons.

Adult mice were intracerebroventricularly (icv) injected with the gliotoxin (L-AA and vehicle/control) and 72h later, recognition memory was evaluated. After the novel object recognition test, the same animals were used in electrophysiological studies.

2.2.4. Astrocytic modifications triggered by L-AA in hippocampal sections

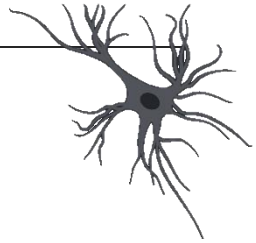
Neurochemical analysis was performed in transverse hippocampal slices from animals mentioned above to look for morphological and molecular alterations triggered by the gliotoxin. Immunohistochemistry in hippocampal sections from both acute and chronic treatments were used to probe for changes in astrocytic markers, namely:

- Glial fibrillary acidic protein (GFAP)
- Glutamine synthetase (GS)

With the present study, we expect to contribute for the validation of astrocytes as novel targets to treat or prevent the onset of brain disorders associated with cognitive deficits, mainly memory.

Chapter 3

Materials & Methods



3.1. Animals

Male C57 black 6 (C57Bl/6) and forebrain selective $A_{2A}R$ KO mice (fb-$A_{2A}R$ KO) mice with 8-12 weeks-old were obtained from Charles River laboratories (Barcelona, Spain). The animals were housed in standard cages with *ad libitum* access to food and water under controlled standard conditions (fixed 12 h dark/light cycle, controlled temperature (23 ± 2°C) and humidity (approximately 66%). The Cre-loxP strategy was used to generate fb-$A_{2A}R$ KO mice, which exhibit a deletion of $A_{2A}Rs$ in the neurons of striatum as well as cerebral cortex and hippocampus (Bastia et al., 2005; Shen et al., 2008). CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout (Fb-$A_{2A}R$ KO) mice and the generation and genotyping of fb-$A_{2A}R$ KO mice has been previously described (Bastia et al., 2005; Wei et al., 2011; Matos et al., 2015).

3.1.1. Ethical considerations

All efforts were made to reduce the number of animals used and to minimize their stress and discomfort. The studies were conducted in agreement with standard procedures to reduce animal suffering, in accordance with approved animal welfare guidelines and European legislation (ORBEA 128_2015/04122015) and the certification of Direção Geral de Alimentação e Veterinária (DGAV; 0421/000/000/2016 Ref 014420).

3.2. Materials

3.2.1. Chemical reagents, antibodies and their manufacturers / suppliers

The gliotoxins L-α-aminoadipic acid (L-AA) and trifluoroacetic acid (TFA), as well as the carbenoxolone disodium salt were acquired from Sigma-Aldrich (Missouri, USA). The two glutamate transporter blockers, dihydrokainic acid (DHK), DL-threo-β-benzoyloxyaspartate (TBOA) and the antagonist of adenosine A_{2A} receptors (SCH58261) were acquired from Tocris (Bristol, UK). Carbogen mixture (95% O_2/5% CO_2) was purchased from Linde (Lisbon, Portugal). Additionally, ultrapure low melting point agarose was purchased from Invitrogen- ThermoFisher Scientific (Massachusetts, USA). For immunohistochemistry studies, two primary antibodies were used: i) goat polyclonal anti-GFAP (-C-terminus) from Santa-Cruz Biotechnologies (California, USA); ii) rabbit polyclonal anti-glutamine synthetase from Molecular Probes-ThermoFisher Scientific (Massachusetts, USA). The goat and rabbit polyclonal secondary antibodies were purchased from MolecularProbes-ThermoFisher Scientific (Massachusetts, USA). Fluorescent mounting medium DAKO was purchased from Agilent Technologies (California, USA). The reagents needed to prepare the solutions of avertin, aCSF (artificial cerebrospinal fluid), PBS (phosphate buffered saline), sucrose, paraformaldehyde, anti-freezing, permeabilization and blocking solutions as well as *2-Bromo-2-chloro-1,1,1-trifluoroethane*, (halothane) were all from Sigma-Aldrich (Missouri, USA).

Table 3 | Solutions used in the methodologies.

FORMULATION				
aCSF **(mM)**	**PBS** **(mM)**	**Anti-freezing** **(mM)**	**Avertin** **(mM)**	**PFA** **(M)**
NaCl **124** KCl **3** $NaH_2PO_4.H_2O$ **1.25** $NaHCO_3$ **26** Glucose **10** $MgSO_4$ **1** $CaCl_2$ **2**	NaCl **137** KCl **2.7** $Na_2HPO_4.7H_2O$ **10** KH_2PO_4 **1.9**	$NaH_2PO_4.H_2O$ **12.3** $NaHPO_4.2H_2O$ **20.3** Ethylene glycol **30 %** Glycerol **30%**	2,2,2-Tribromoethanol **70.7** 2-methyl-2-buthanol **113.4** NaCl **138** Ethanol **12.5 %** (in PBS)	PFA **1.3** NaOH **5** (in PBS)

Table 4 | Drugs used throughout extracellular electrophysiological experiments.

DRUGS		
Compounds	**Dissolved in**	**Concentration**
L-AA	aCSF	100 µM
TFA	aCSF	100 µM
Carbenoxolone	aCSF	50 µM
DHK	Ultrapure water	15 µM (stock 15 mM)
TBOA	Ultrapure water	5 - 10 µM (stock 5 mM)
SCH58261	DMSO	50 nM (stock 5 mM)

Table 5 | Antibodies used in immunohistochemistry of 50 µm hippocampal sections.

PRIMARY ANTIBODIES			
Antibody	**Dilution**	**Origin**	**Laboratory**
Anti-GFAP	1:300	Goat	Santa-Cruz Biotechnologies
Anti-GS	1:1000	Rabbit	ThermoFisher Scientific

SECONDARY ANTIBODIES			
Antibody	**Dilution**	**Origin**	**Laboratory**
Anti-goat Alexa 488	1:1000	Donkey	Invitrogen
Anti-rabbit Alexa 594	1:1000	Donkey	Invitrogen

3.3. Methods

3.3.1. Stereotaxic surgeries

Stereotaxic surgery is a method used to manipulate the brain of living animals. This technique allows to accurately target deep structures within the rodent brain through the use of a stereotaxic atlas (Paxinos and Franklin, 2001), which provides the 3D coordinates of each area with respect to anatomical landmarks on the skull. Therefore, the animals, under anesthesia, are mounted on a specialized apparatus (Dual lab standard™ stereotaxic, Stoelting Co, Illinois, USA) which enables the precise placement of experimental tools at the defined coordinates.

In this work, the animals were divided into two groups that were bilaterally injected in the cerebral lateral ventricles (icv, intracerebroventricular): one group of animals was injected with L-AA (40 µg/µl), whereas, the other group (control) was administrated with vehicle (PBS), resulting in a delivery of the drug/saline into the CNS through the cerebrospinal fluid (CSF).

Mice were anesthetized with 15 µl/g of avertin (intraperitoneal injection, IP) and placed in a stereotaxic frame. Afterwards, an incision was made to expose the skull and to find bregma (intersection of the coronal and sagittal sutures), which represents our 0,0,0 point (Figure 12B). From this point forward, by using the digital coordinate display, we defined in the skull the X (antero-posterior) and Y (medial-lateral) desired coordinates (Figure 12A). After reaching the desired X and Y coordinates, a Hamilton syringe was used to pierce the brain tissue until the Z (dorso-ventral) coordinate value. After injecting the solutions (total of 4 µl, pH = 7.4, 0.5 µl/min) in the lateral ventricles, the syringe is retracted very slowly to avoid the reflux of the injected solution. Finally, we removed the animal from the stereotaxic frame and a suture was given to the animal's head. The mice were allowed to recover for 3 days. It is important to mention that correct icv administration was confirmed in preliminary experiments by injecting trypan blue into mice lateral ventricles.

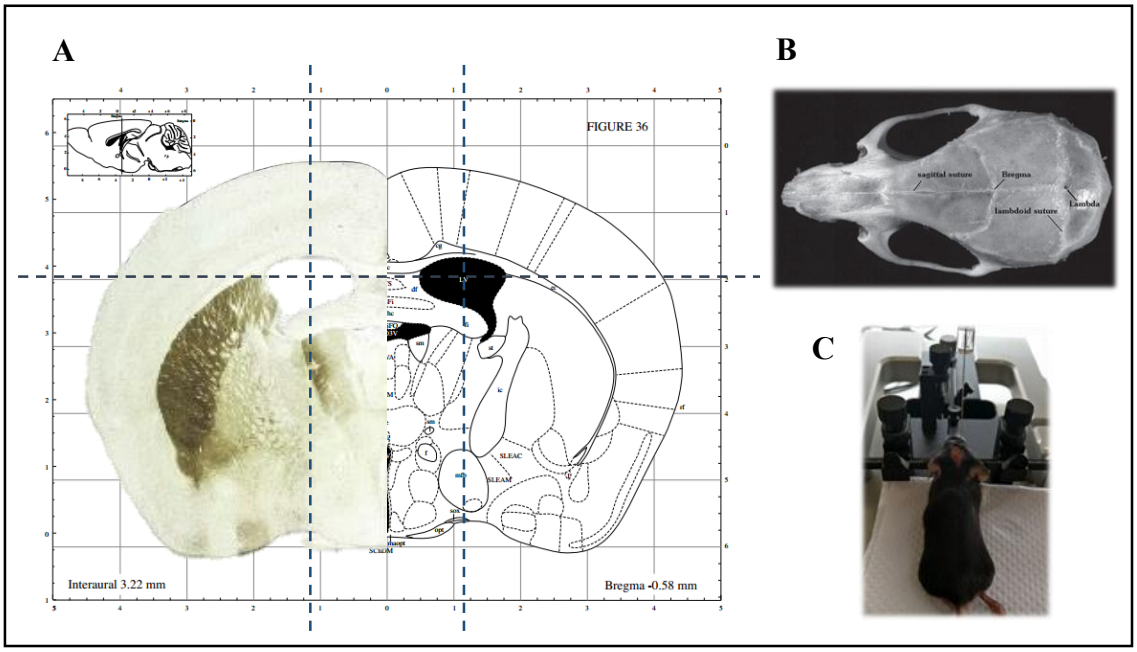


Figure 12 | Scheme of coronal sections showing the icv injection site. A) Coronal sections of adult mouse brain at coordinates: interaural line 3.22 mm, bregma - 0.58 mm. The dashed lines define the stereotaxic coordinates: AP: - 058; ML: ± 1.13; DV: - 2.00. B) The dorsal surface of the mouse skull showing the horizontal plane reference points, bregma and lambda. Bregma is the intersection of the coronal and sagittal sutures whereas Lambda is defined as the point of intersection of the projection of lines of best fit through the sagittal and lambdoid sutures. C) Representation of a mouse placed in the stereotaxic instrument at the end of the procedure (adapted from Paxinos and Franklin, 2001).

3.3.2. Behavioral experiments

Behavioral experiments have become important tools for the analysis of functional consequences of induced injuries (chemical or physical) or genetic mutations in experimental animals. A large variety of rodent behavioral tests are currently used to evaluate traits, such as sensory-motor function, social interactions, anxiety-like and depressive-like behavior, substance dependence and various forms of cognitive function (Gerlai and Clayton, 1999; Gerlai, 2001).

To investigate the impact of astrocytic ablation on memory impairment (hippocampus-dependent), we performed the following behavioral tests: 1) open field and 2) novel object recognition.

3.3.2.1 Open field

The open field test (OFT) is a common measure of exploratory behavior and general activity in both mice and rats, providing simultaneous information about locomotor activity and anxiety that allow to infer about the animal "health" state (Hall and Ballachey, 1932; Walsh and Cummins, 1976). Therefore, this behavioral test can be used as a control to check if the animals have severe complications due to icv administration (L-AA or PBS) mainly in motor activity or even in the animal's vision. Other parameters like distance moved, time spent in the center or in the periphery, rearing and fecal count can also be assessed in this test (Coelho et al., 2014).

The behavioral tests were carried out in a sound attenuated room with 15 lux illumination. The mice were placed in the center of the open field and their movements were monitored for 10 min and then the novel object recognition was performed, using also the same apparatus (Figure 13). The apparatus was cleaned before and after each behavior test or between different sessions using a 70% alcohol solution (Lopes et al., 2015).

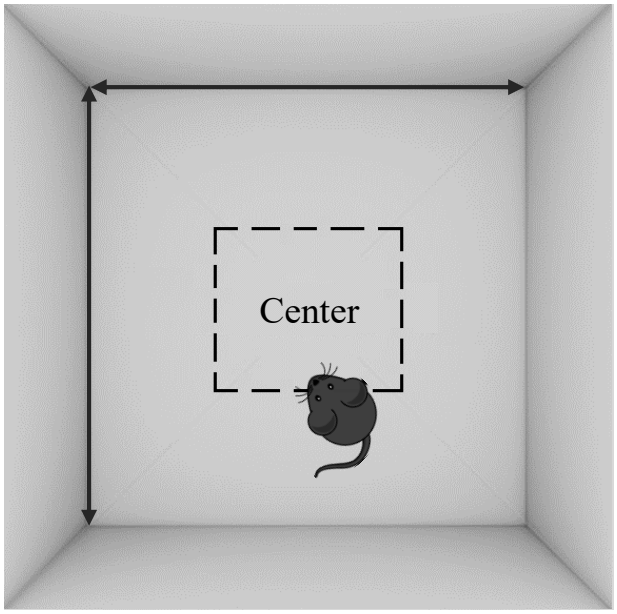


Figure 13 | The open field apparatus has a white floor of 40 cm × 40 cm. Each mouse was placed in the center of the open field and locomotor activity was measured. Rodents will typically spend a significantly greater amount of time exploring the periphery of the arena, usually in contact with the walls, than the unprotected center area (center).

3.3.2.2. Novel object recognition

The novel object recognition (NOR) test is used to evaluate cognition, particularly recognition memory (hippocampus-dependent), in rodent models of CNS disorders. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of learning and recognition memory (Ennaceur and Delacour, 1988; Wan et al., 1999; Broadbent et al., 2004). In this test, we measured the time spent exploring each object.

In brief, mice were first habituated to the open field arena and the NOR test consisted of two sessions: in the first session (familiarization session, 10 min) the mice were exposed to two equal objects and in a second session (test session, 5 min) mice were exposed to a familiar and a novel one (Figure 14). The inter-trial interval (ITI) between the two sessions was 90 min. Each session was recorded using the ANY-maze software v.4.99m and the time exploring each object was manually scored. Object exploration was defined as the orientation of the nose to the object, touching with forepaws or nose, sniffing and biting the objects, but climbing on the objects was not considered. The recognition index was calculated as time exploring the novel object / time exploring both objects) (Lopes et al., 2015).

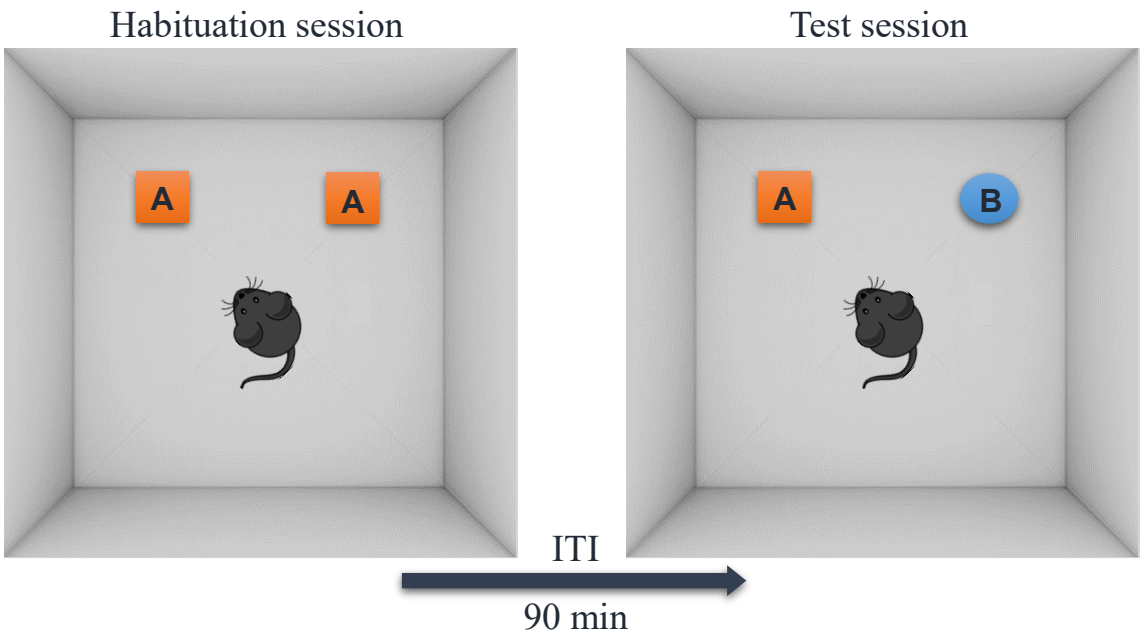


Figure 14 | NOR test schematization. This assay is conducted in an open field arena with two different objects. Both objects are consistent in height and volume, but are different in shape and appearance. After habituation, the animals are exposed to the familiar arena with two identical objects placed at an equal distance (familiarization session). After this, the mice are allowed to explore the open field in the presence of the familiar and a novel object (test session) to test long-term recognition memory.

3.3.3. Electrophysiological experiments

Dynamic changes in the efficacy of excitatory synaptic transmission underlie much of the plasticity in the CNS. The most well-studied forms of synaptic plasticity in the CNS are LTP and LTD and the molecular mechanisms underlying these variations have been extensively characterized and proposed to be cellular models of learning and memory (Alkon and Nelson, 1990; Zoghbi et al., 2000; Malinow and Malenka, 2002). In this study, electrophysiology experiments were performed to assess synaptic plasticity in Schaffer fibres-CA1 pyramid synapses (Figure 16A). More specifically, we performed extracellular recordings in hippocampal slices due to their ability to maintain stable recordings for several hours, which is very important if we want to study long-term changes in synaptic efficiency.

All experiments were performed on hippocampal slices from adult male C57Bl/6 mice. First, animals were anesthetized under halothane atmosphere and sacrificed by decapitation. Then, the brain was quickly removed and placed into a petri dish with ice-cold, oxygenated artificial cerebrospinal fluid (aCSF) and gassed with 95% O_2 / 5% CO_2 mixture (Costenla et al., 1999). Both cerebellum and olfactory bulb were removed and afterwards, a scalpel blade was used to cut through the intrahemispheric fissure to separate the two hemispheres. Both hippocampi were dissected and slices (400 µm thick) were cut perpendicularly to the long axis of the hippocampus with a Brinkmann McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, Guildford, UK) (Figure 15) (Cunha et al., 1994). Subsequently, transverse slices were allowed to recover functionally and energetically for at least 1 hour in a preincubation chamber (BSC-PC prechamber, Harvard Apparatus, Massachusetts, USA) with gassed aCSF at 32ºC. Each slice used for recording was then transferred to a 1 mL capacity submersion-type recording chamber (BSC-ZT Zbicz Top, Harvard Apparatus, Massachusetts, USA) and continuously superfused with aCSF (control) or other compounds (conditions tested) at a flow rate of 3 mL/ min at a temperature of 30.6ºC (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 the gliotoxins (L-AA and TFA) were pre-incubated for 2 hours and continuously superfused with the same solution during the recordings. On the other hand, TBOA, DHK, carbenoxolone and SCH58261 treatments consisted of a 20-30 min superfusion, which continued throughout the remaining time of the recording.

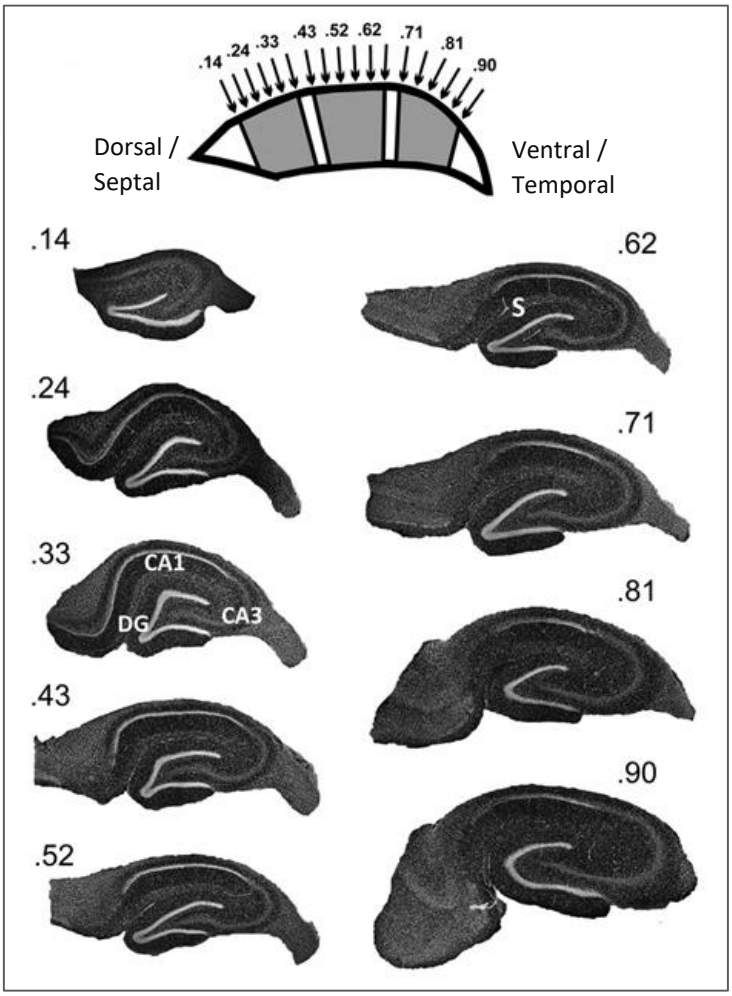


Figure 15 | Sampling scheme of a rodent hippocampus illustrated at the top. Hippocampal sections spaced across the septotemporal axis at the bottom. Slices with numbers 14 to 33 are classified as dorsal whereas slices with numbers 71 to 90 are ventral. In this study, only medial to dorsal-medial hippocampal slices were used (slices number 43 to 62). Letters stand for: DG - dentate gyrus; S - suprapyramidal blade of the dentate gyrus (adapted from Jason Snyder, Functional Neurogenesis).

Electrophysiological recordings of field excitatory post-synaptic potentials (fEPSPs) were obtained by electrical stimulation of Schaffer collaterals (SC) which generates excitatory postsynaptic potentials (EPSPs) in the postsynaptic CA1 pyramidal cells (Figure 16A). Stimulation was performed using either a Grass S44 or Grass S48 square pulse stimulator (Grass Technologies, Warwick, RI, USA), and after amplification (ISO-80, World Precision Instruments, Hertfordshire, UK) the recordings were digitized using an analog-to-digital converter (BNC-2110, National Instruments, Newbury, UK) (Lopes et al., 2015). The data acquisition and analysis software was performed using the WinLTP version 2.20.1 (WinLTP Ltd., Bristol, UK) (Anderson and Collingridge, 2001). To

quantify changes in the fEPSPs, the criteria used was the signal slope measured right after the presynaptic volley (dotted area in blue in figure 16B). Evoked fEPSPs were recorded using micropipettes obtained using a Flaming/Brown micropipette puller system, model P-87 (Sutter Instruments, USA) and filled with 4M NaCl with a resistance of 1-2 MΩ.

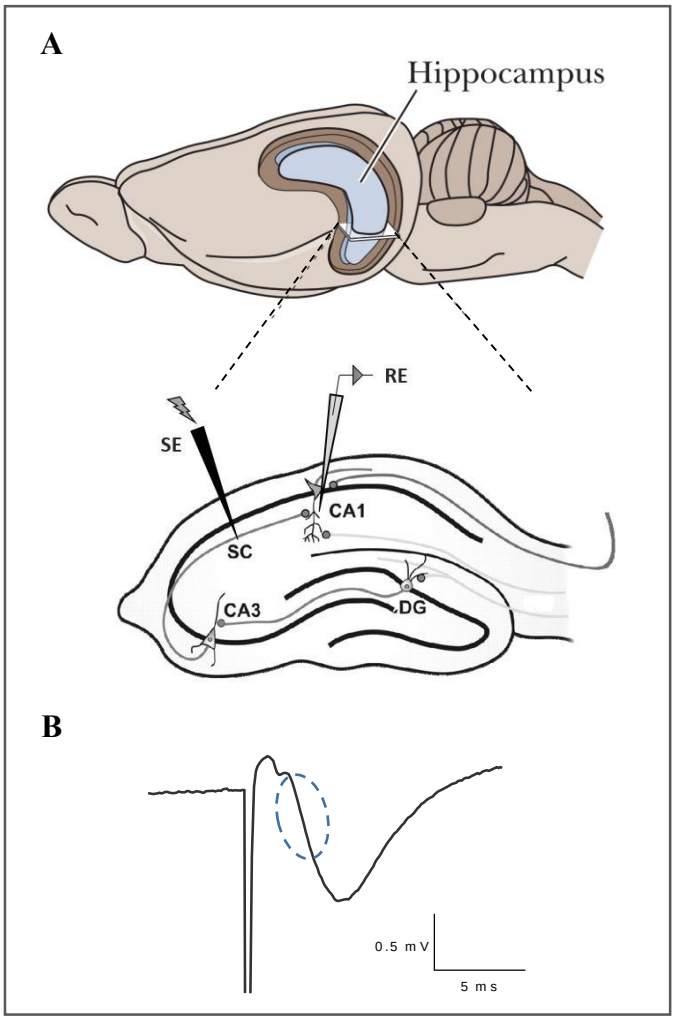


Figure 16 | Diagram of a section through the rodent hippocampus showing the recorded pathway. A) The stimulation electrode (SE) is placed in the Schaffer collaterals (SC) and fEPSPs are recorded in the dendrites of the CA1 pyramidal neurons where the recording electrode (RE) is located. B) Representative recording of a fEPSP obtained with the previous electrode positioning. The trace comprises the stimulus artefact, 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 (dotted area in blue).

Input/output curves (I/O curves; fEPSP slope *versus* stimulus intensity) were performed in all slices in order to determine the adequate level of electrical stimulation for the remainder of the experimental protocol as well as to evaluate changes in basal

synaptic transmission due to pharmacological manipulations. Stimulus intensity was increased in 0.2 mA increments from that which produced no detectable post-synaptic response to a stimulus that produced a maximal post-synaptic response, resulting in a sigmoidal-shaped curve. It's very important to mention that following treatment with DL-TBOA, DHK, CBX and SCH58261, a second I/O curve was performed to check for modifications in basal synaptic transmission (Figure 17B).

Prior to the induction of LTP, a steady baseline (always 40% of the maximum fEPSP slope with no apparent contamination) was recorded in an average of 3 sweeps of 20 s each, for at least 10 min. From this point forward, LTP was elicited by high-frequency stimulation (HFS) (one train of 100 1Hz pulses for 1 s) and the subsequent changes in synaptic transmission were recorded for 60 min (Figure 17A). In specific conditions, this protocol was followed by depotentiation induced by a low-frequency stimulation (LFS) (one train of 900 1 Hz pulses for 15 min) followed by a 60 min recording (Figure 17C). LTP was quantified as the percentage of change between the average slope of the ten potentials taken between 50 and 60 min after LTP induction in relation to the average slope of the fEPSP measured, during the 10 min that preceded LTP induction (earlier recorded baseline). Depotentiation was quantified in relation to the last 10 min of the previously induced potentiation.

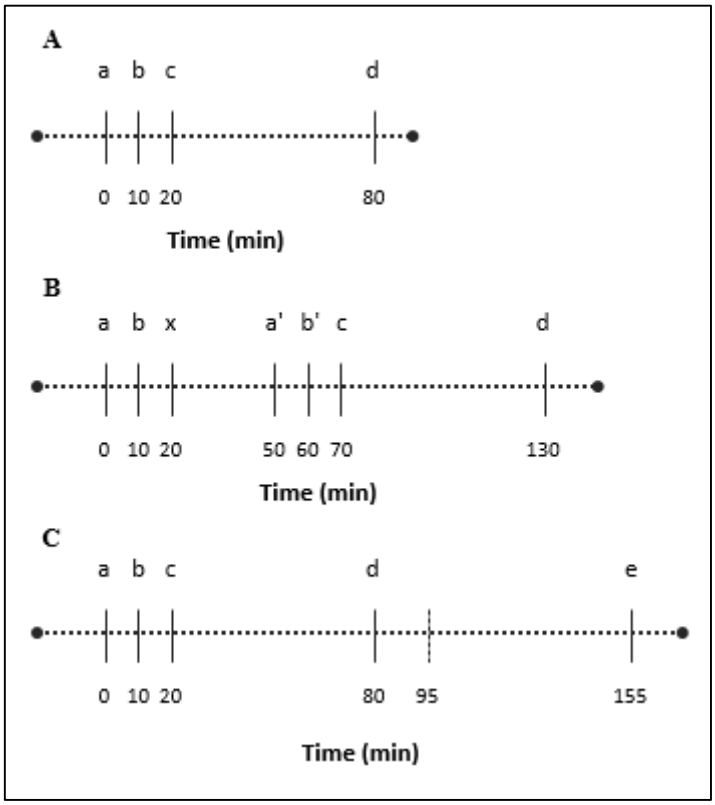


Figure 17 | Simple representation of the protocols used in extracellular electrophysiological recordings. **A |** LTP induction protocol used for aCSF (CTR), L-AA and TFA-incubated slices. **B |** LTP induction protocol applied to all slices exposed to TBOA, DHK, CBX and SCH58261. **C |** Depotentiation (DP) protocol was used in slices treated with aCSF and L-AA. Letters stand for: a) Input/ output curve; b) baseline; c) LTP induction (HFS, 1 s, 100 Hz); d) End of potentiation protocol and LFS induction in **C** (15 min, 1 Hz); e) End of DP protocol; x) Drug solution added to the system and superfused for 20-30 min; a') second input/ output curve; b') second baseline.

3.3.4. Sectioning hippocampal slices

After performing all the fEPSP recordings, the remaining slices (400 µm) were fixated by immersion in a 4% paraformaldehyde (PFA) solution overnight. After fixation, the slices were transferred into a 30% sucrose solution. Two days later, after dehydration, hippocampal slices were stored at -20°C in an anti-freezing solution until used in immunohistochemistry studies. The slices collected belong to: i) control group (incubated in aCSF at RT); ii) treatment with L-AA (incubated for 2 h at RT); and iii) treatment with TFA (incubated for 2 h at RT).

Afterwards, the hippocampal slices were washed in PBS to completely remove the anti-freezing solution. Next, the slices were immersed in a 3% ultrapure low melting point agarose solution (LMP) and then transferred into the cubes and arranged in the flattest way possible until the agarose is completely solidified (Figure 18) It is important to measure the temperature of the agarose before insert the slice in the cubes (not exceeding 50 °C). After removing the excess agarose, the slices were placed in a vibratome (Leica VT1200S, Leica Biosystems, Germany), where 50 µm thickness sections were cut and further transferred into a multiwell plate with PBS. These sections can be kept at -20 °C embedded in anti-freezing solution until the immunohistochemistry studies were performed.

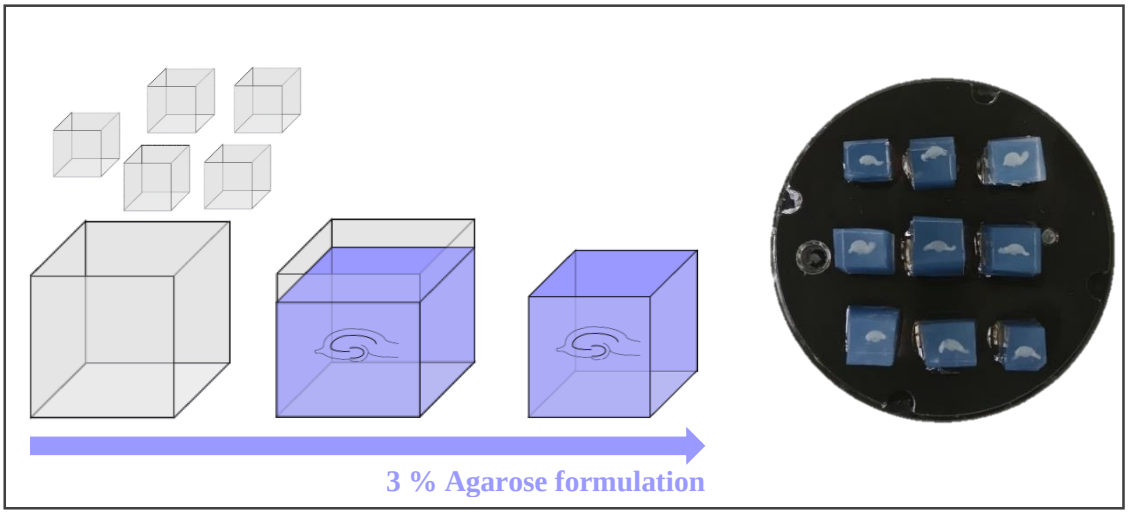


Figure 18 | Process of agarose LMP solidification using 1.5 x 1.5 x 1.5 cm molds A few minutes after adding the agarose and the hippocampal slices to the cubes, it becomes solid and can be placed in a vibratome to be cut. The parameters used were: speed: 0.22 mm/s; amplitude: 0.55 mm; thickness: 50 µm.

3.3.5. Neurochemical studies

After performing the behavioral and electrophysiological studies (mentioned above), *in vitro* studies, such as Western blot and immunohistochemistry were done. This information will be associated with all the previously acquired results. For the Western blotting analysis, the material used were total extracts from hippocampal dentate gyrus, CA1 and CA3 regions obtained by micro-dissection from 400-600 µm hippocampal slices. In contrast, for immunohistochemical studies, 50 µm hippocampal sections were used.

3.3.5.1. Immunohistochemistry

Immunohistochemistry (IHC) analysis is a method for demonstrating the presence and location of proteins in tissue sections. Though less sensitive quantitatively than immunoassays, such as Western blotting, it enables the observation of processes in the context of intact tissue. Immunohistochemical staining is accomplished with antibodies that recognize the proteins of interest. The antibodies used for protein detection can be polyclonal, a heterogeneous mix of antibodies that recognize several epitopes, or monoclonal, which show specificity for a single epitope of the protein. In our case, we performed this method to visualize astroglial proteins: glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS), amongst other astrocytic and synaptic markers (ongoing work).

Hippocampal sections were washed with PBS to remove the anti-freeze solution. Since we were interested in cytoplasmic proteins, a step of cell permeabilization was required. The sections were incubated in a permeabilization solution (0.1% Triton in PBS) for 15 min and then placed for 1 h in a blocking solution, containing 10% horse serum and 0.1% Triton, in PBS. This step is important to prevent the nonspecific binding of the antibodies, and the serum used was chosen according to animal origin (source) of the secondary antibody. Therefore, for the secondary antibodies which were made in donkey, we used horse serum (Table 5). After the blocking step, hippocampal slices were incubated with primary antibodies 4°C under gentle agitation. A 2-day incubation was performed due to the thickness of the hippocampal slices (50 µm). Then, the sections were washed with 0.1% Triton in PBS (2 times for 5 min) and incubated for 15 min with

10% horse serum and 0.1% Triton in PBS, before the incubation with the secondary antibodies for 2h at RT, under agitation. It should be mentioned that (for each IHC study), some slices were not incubated with the primary antibodies, only with the secondary antibodies, in order to have a control (negative control) of the experimental procedure. The sections were first washed with PBS and further stained with nuclear dye DAPI (1:5000) for 10 min at RT. Finally, another set of washes was performed and the slices were mounted in gelatin-coated slides using DAKO mounting medium until completely dried. Hippocampal sections were visualized in the epifluorescence microscope (Zeiss Imager Z2, Oberkochen, Germany), and the pictures were captured using the software Carl Zeiss AxioVision SE64 Rel. 4.8.2. The images taken were analyzed and quantified in ImageJ software (v.1.44p) using always the same area (Figure 19).

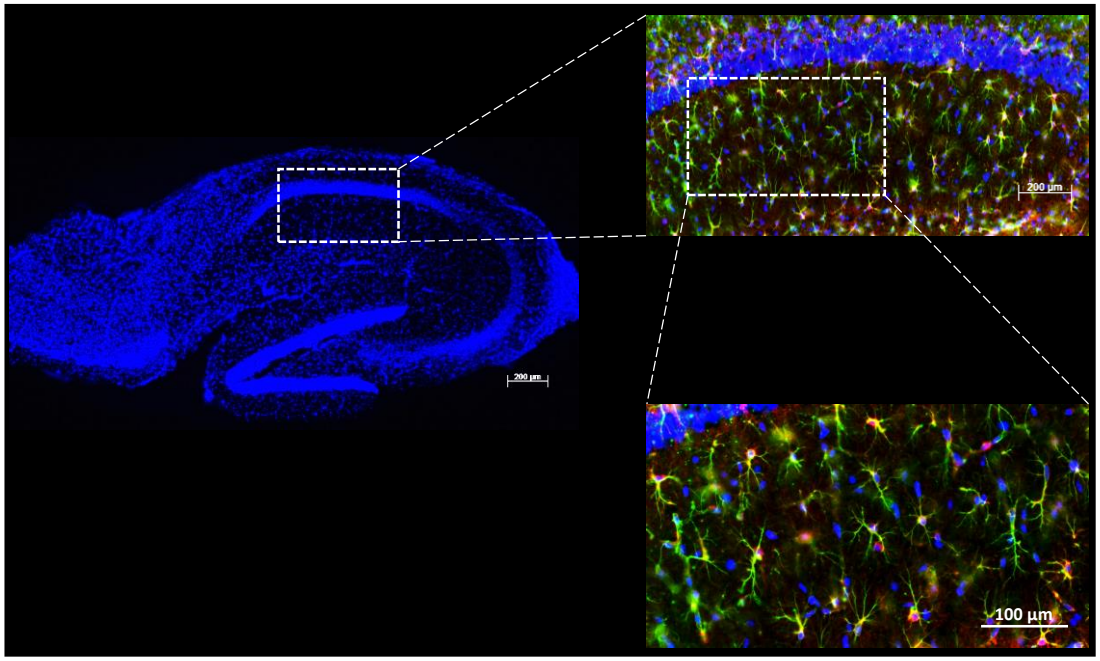


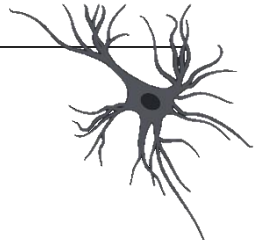
Figure 19 | Schematization showing how the IHC quantification was performed. Hippocampal sections were labeled with rabbit anti-Glutamine synthetase (GS), goat anti-GFAP antibodies and with the nuclei dye DAPI, and further visualized in the epifluorescence microscope. All images were obtained at ×20 magnification using a fluorescent microscope and the area quantified was within *stratum radiatum* (hippocampal CA1 region) using always the same shape.

3.4. Statistical analysis

Data are presented as mean ± SEM of *n* different animals (*n*, corresponding to independent mice) indicated in all figure captions. After assessing the normal distribution of the groups, parametric analysis was performed to all conditions. The significance of the differences between the means was calculated using an unpaired Student's *t*-test when comparing two experimental groups. Otherwise, when doing comparisons among more than two experimental groups, one-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test (comparing the mean values of each group) were performed. Values of $P < 0.05$ were considered to be statistically significant. All statistical analyses were performed with GraphPad Prism software (v. 6.05).

Chapter 4

Results



4.1. Role of astrocytes in synaptic function

Increasing evidence suggest that astrocytes release and uptake gliotransmitters within the synaptic cleft to fine-tune synaptic transmission (Halassa and Haydon, 2010; Rial et al., 2016). Therefore, it is of interest to investigate the impact of astrocytes on hippocampal synaptic plasticity before conducting any other type of experiments. To probe for the importance of astrocytes in hippocampal long-term potentiation (LTP) or depotentiation (DP) processes, we used different approaches to interfere specifically with astroglial cells and analyzed their impact by performing electrophysiological extracellular recordings in the Shaffer collaterals (SC) – CA1 pathway, as described before (section 3.3.3.). The first candidates chosen for this purpose were: i) L-α-aminoadipic acid (L-AA) and ii) trifluoroacetic acid (TFA), since these compounds can target astrocytes (Peters, 1957; Olney et al., 1980; Paulsen et al., 1987; Khurgel et al., 1996; Brown and Kretzschmar, 1998; Lima et al., 2014; Nissen et al., 2015). Additionally, two other pharmacological tools were used to blunt astrocytic function and to confirm if actually astrocytes play an important role in synaptic function. The first, dihydrokainic acid (DHK), a selective glutamate transporter-1 (GLT-1) inhibitor, and the hemichannels/gap junction blocker, carbenoxolone (CBX).

4.1.1. L-AA, a gliotoxin, decreased LTP in mouse hippocampus

We first studied if L-AA, a well-known selective gliotoxin, would have any impact on hippocampal LTP *in vitro*. For this purpose, and after optimizing the incubation period and the concentration of the gliotoxin, extracellular electrophysiological recordings were performed to assess synaptic plasticity in hippocampal Schaffer fibres-CA1 pyramid synapses. Here, we show that hippocampal slices, when incubated for 2 h in a solution of L-AA (100 μM, dissolved in aCSF), have diminished LTP magnitude when compared to the slices treated with aCSF, the control condition for this set of experiments, (CTR was the nomenclature used). More specifically, in CTR slices, the magnitude of LTP (induced by high-frequency stimulation, HFS) was of 80.95 ± 10.08 %, whereas in L-AA-treated slices it was of 43.40 ± 5.27 %, representing a reduction of 37.55 ± 11.66 % in

hippocampal LTP from adult C57Bl/6 mice (Figure 20). L-AA had no effect on basal synaptic transmission (data not shown). These observations imply that astrocytes are essential elements in shaping synaptic plasticity of mouse hippocampal SC-CA1.

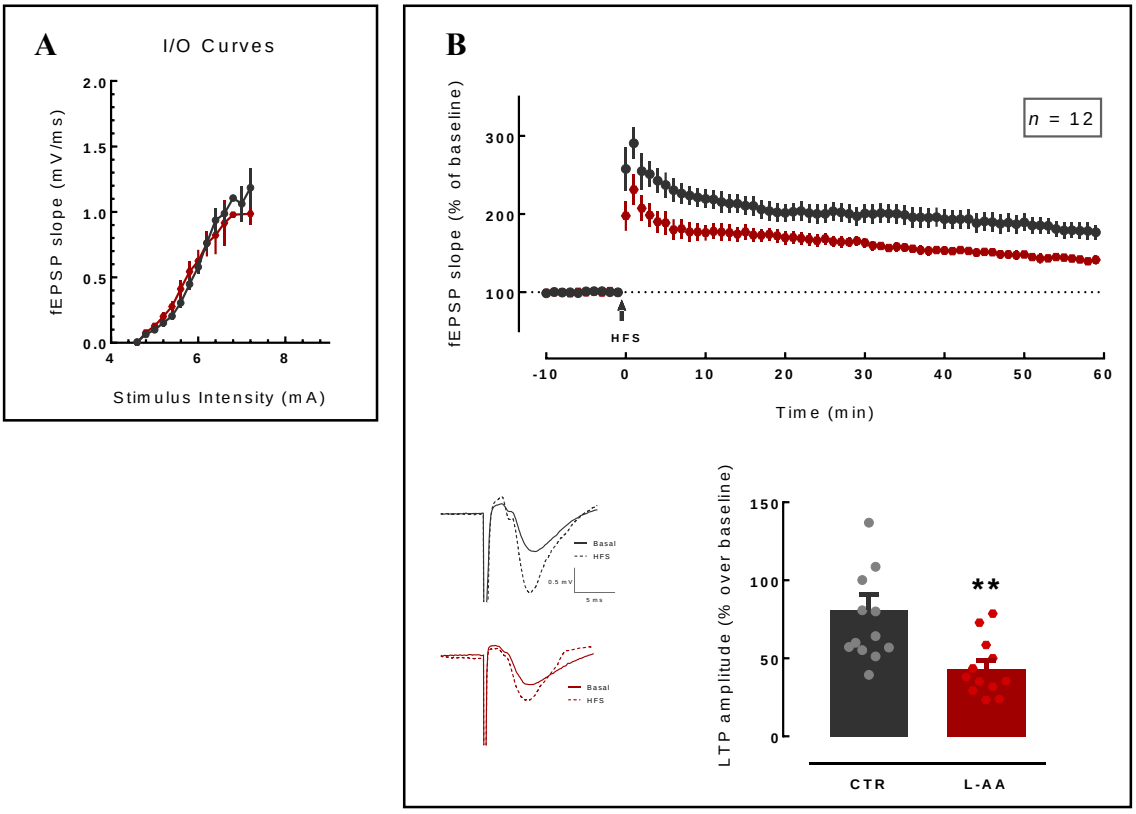


Figure 20 | Effect of L-AA, a specific gliotoxin, on field-potential LTP of mouse hippocampal slices. **A |** Input/output (I/O) curves in hippocampal slices incubated in artificial cerebrospinal fluid (aCSF, control, CTR, *n* = 12) and treated with L-AA (100 µM, *n* = 12) for 2h as described previously. I/O curves are not different between the two groups, indicating that changes in synaptic strength were unlikely to be responsible for eventual LTP differences among the slices incubated with the gliotoxin. **B |** The time course of changes in fEPSP slope is shown on the top of the panel. Arrow denotes timing of tetanic stimulation (HFS, 1 s, 100 Hz). fEPSP amplitude was recorded for 60 min following tetanization to measure LTP. On the lower left, representative recording of a typical fEPSP in mouse hippocampus by positioning electrodes in the SC-CA1 synapses. Each trace comprises the stimulus artefact, 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). LTP magnitude (shown on the bottom right), corresponding to the average fEPSP slope 50–60 min after LTP induction, was significantly decreased in all slices treated with L-AA (** $P < 0.01$ *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. L-AA-incubated slices showed a tendency for depotentiation

Next, we asked if slices where astrocytes were compromised (acute incubation of L-AA 100 µM, 2 h) would display a different behavior when subjected to low-frequency stimulation (LFS) following previous potentiation (HFS) for 60 min. Our data revealed that L-AA- treated slices indeed show a different pattern after LFS induction when compared to the CTR condition. Differences between the two treatments were accessed using a paired Students' *t*-test and the mean of differences was 13.19 ± 1.28 % (Figure 21B). However, these results are not significant when compared to the baseline recorded for the last 10 min of potentiation (analysis not shown). Additional experiments are required to confirm if hippocampal slices exposed to the gliotoxin are more sensitive to depotentiation protocols.

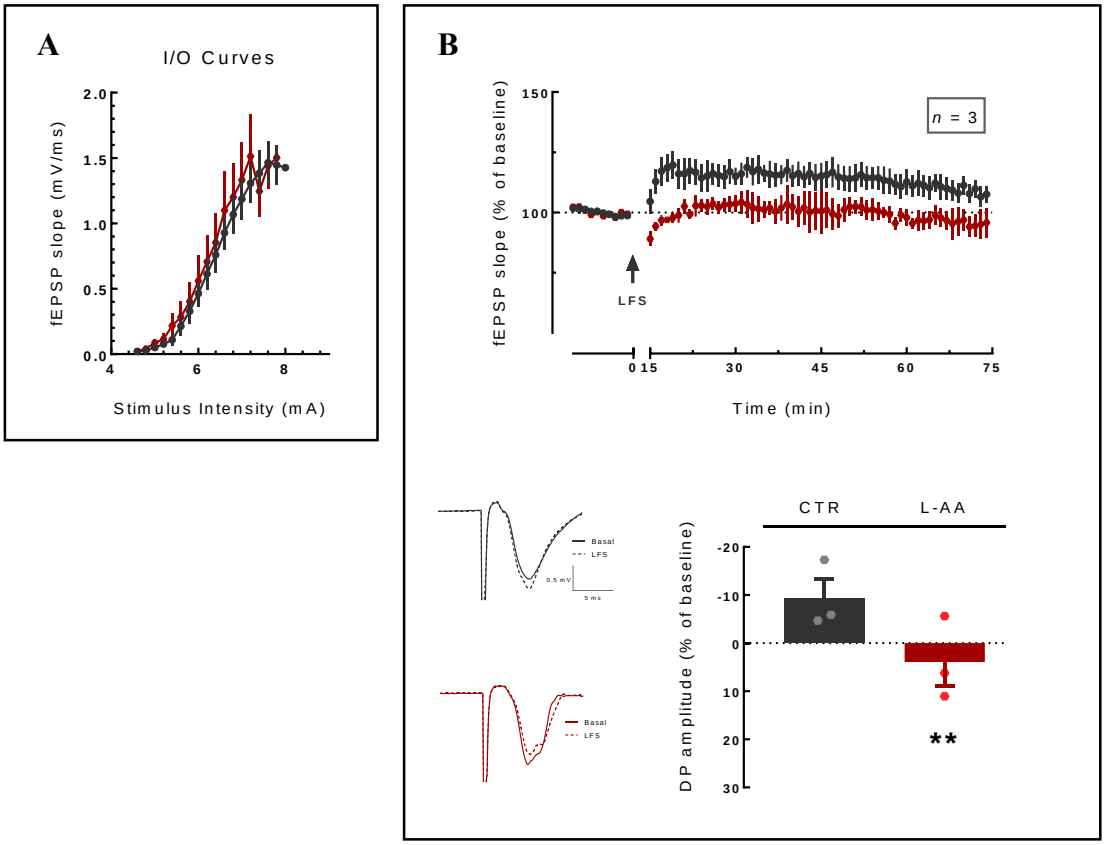


Figure 21 | Effect of L-AA on mouse hippocampal slice depotentiation. **A |** Stimulus-response curves in hippocampal slices incubated in artificial cerebrospinal fluid (aCSF, control, CTR, *n* = 3) and treated with L-AA (100 µM, *n* = 3) for 2h did not display significant differences, indicating that changes in synaptic strength were unlikely to be responsible for eventual depotentiation differences among the slices incubated with the gliotoxin. **B |** The time course of changes in fEPSP slope is shown on the top of the panel. Arrow denotes timing of depotentiation protocol (LFS, 15 min, 1 Hz). fEPSP amplitude was recorded for 60 min following stimulation. On the left, representative traces are shown for baseline (solid line) and depotentiation (dashed line) regarding both treatments. Magnitude of depotentiation estimated from the averaged fEPSP slope 50–60 min after LFS induction, was significantly decreased in slices treated with L-AA (** $P < 0.05$ *vs.* CTR, paired Students' *t*-test). All values are mean ± SEM of *n* independent experiments. The *n* values refer to the number of mice used per condition.

4.1.3. TFA, other gliotoxin, caused a similar decrease in LTP

Next, it was our goal to assess if L-AA was effectively targeting astrocytes. Thus, we took advantage of another well documented gliotoxin, named trifluoroacetic acid (TFA) and evaluated SC-CA1 LTP using the previously described protocol. In this group of experiments, hippocampal slices were incubated with L-AA (100 µM, 2 h), TFA (100 µM, 2 h) or the two gliotoxins together (L-AA + TFA 100 µM, 2 h each) or were kept in aCSF (control condition, CTR). As was previously shown, L-AA impacted on LTP (33.67 ± 2.81 %) triggering a significant decrease in magnitude when compared to CTR (73.41 ± 6.85 %). Strikingly, TFA treatment had the same effect as L-AA on LTP magnitude (33.40 ± 6.51 %), as well as the two gliotoxins combined (29.43 ± 5.09 %), meaning that astrocytic blunting mediated by different pathways led to the same effect on synaptic plasticity (Figure 22).

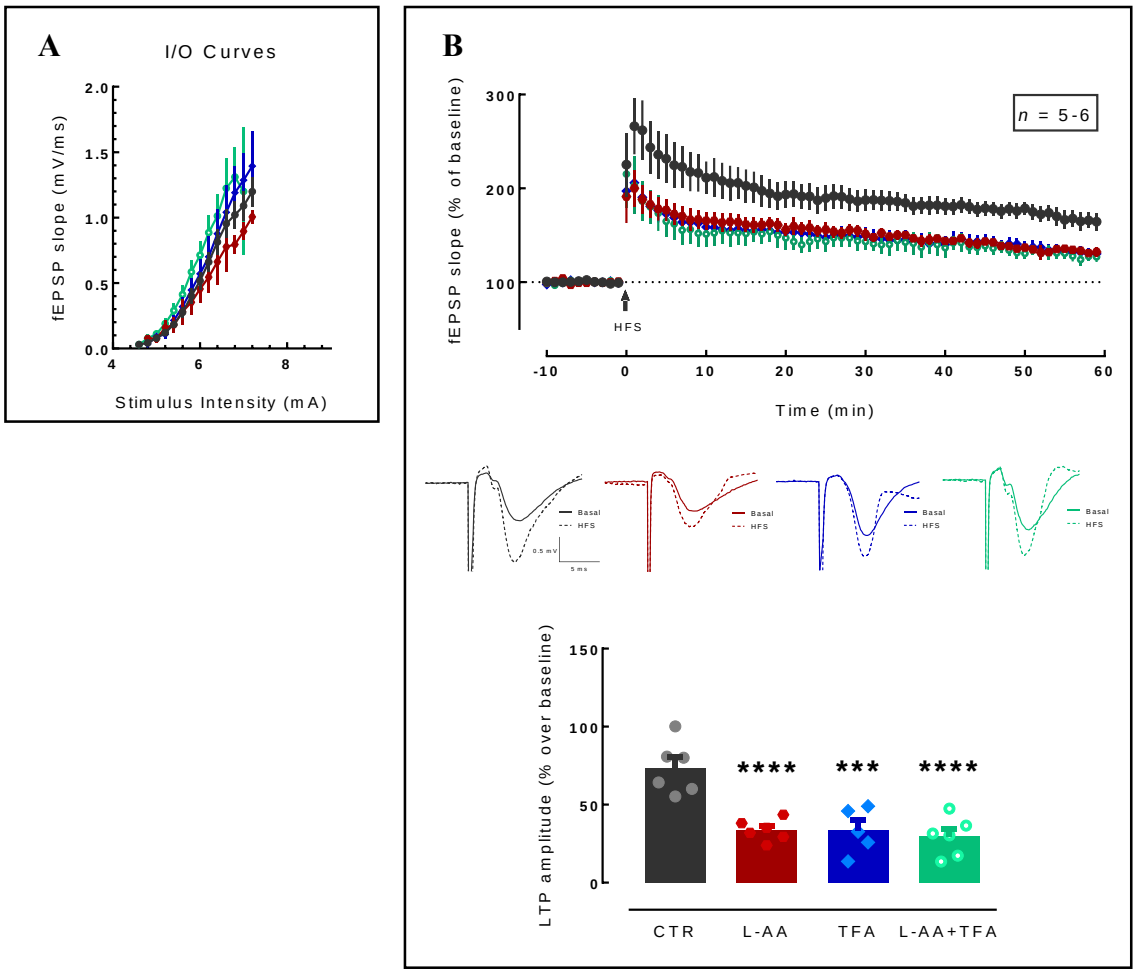


Figure 22 | Effect of TFA, a specific gliotoxin, on mouse hippocampal LTP. **A |** Stimulus-response curves in hippocampal slices incubated in aCSF (control, CTR, *n* = 6), L-AA (100 µM, 2h, *n* = 6), TFA (100 µM, 2h, *n* = 5) and TFA on top of L-AA (100 µM, 2h each, *n* = 6). I/O curves measuring

the magnitude of the fEPSP response across a range of stimulation currents was comparable between the all groups as seen in the figure. **B |** Time course of changes in fEPSP slope after stimulation at t=0 min with a HFS (1 s, 100 Hz) is shown on the top of the panel. Representative traces are shown for baseline (solid line) and LTP (dashed line) regarding each treatment. 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 (the depression after the stimuli artifact). fEPSP amplitude was recorded for 60 min following potentiation to measure LTP. Results are expressed as percent of an average fEPSP slope recorded during the last 10 min before HFS and 50-60 min post-HFS. LTP magnitude (shown on the bottom), was significantly decreased in all slices treated with the gliotoxins (*** $P < 0.001$, **** $P < 0.0001$ *vs.* CTR, one-way analysis of variance (ANOVA)). All values are mean ± SEM of *n* independent experiments. The *n* values refer to the number of mice used per condition.

4.1.4. The blockade of GLT-1 decreased LTP magnitude

Since glutamate is an active participant in memory and plasticity processes and GLT-1 and GLAST are the mainly astroglial glutamate transporters involved in maintaining its physiological extracellular concentrations, we probed whether this regulation was influencing LTP on SC-CA1 synapses. To test this hypothesis, we used a selective inhibitor of both transporters, named DL-threo-β-benzoyloxyaspartate (TBOA) (Arriza et al., 1994). Unfortunately, we were unable to work with TBOA since all slices exposed to this drug collapsed during basal synaptic transmission, even at different concentrations (5-10 μM). For that reason, we opted for DHK (15 μM), a selective GLT-1 blocker at μM concentration range, that interferes with glutamate uptake from astrocytes (Arriza et al., 1994; Oliet et al., 2001; Matos et al., 2008). After applying the LTP protocol to hippocampal slices, we again observed an impairment of LTP by L-AA (32.51 ± 6.64 %), when compared to CTR (57.74 ± 2.09 %). Interestingly, the blockade of GLT-1 caused a decrease (35.60 ± 6.52 %) in LTP identical to that triggered by L-AA *per se*. A similar reduction of LTP was detected upon a combined exposure to both L-AA and DHK (29.49 ± 6.08 %), thus excluding the possibility for an additive effect of both drugs on hippocampal synaptic plasticity (Figure 23).

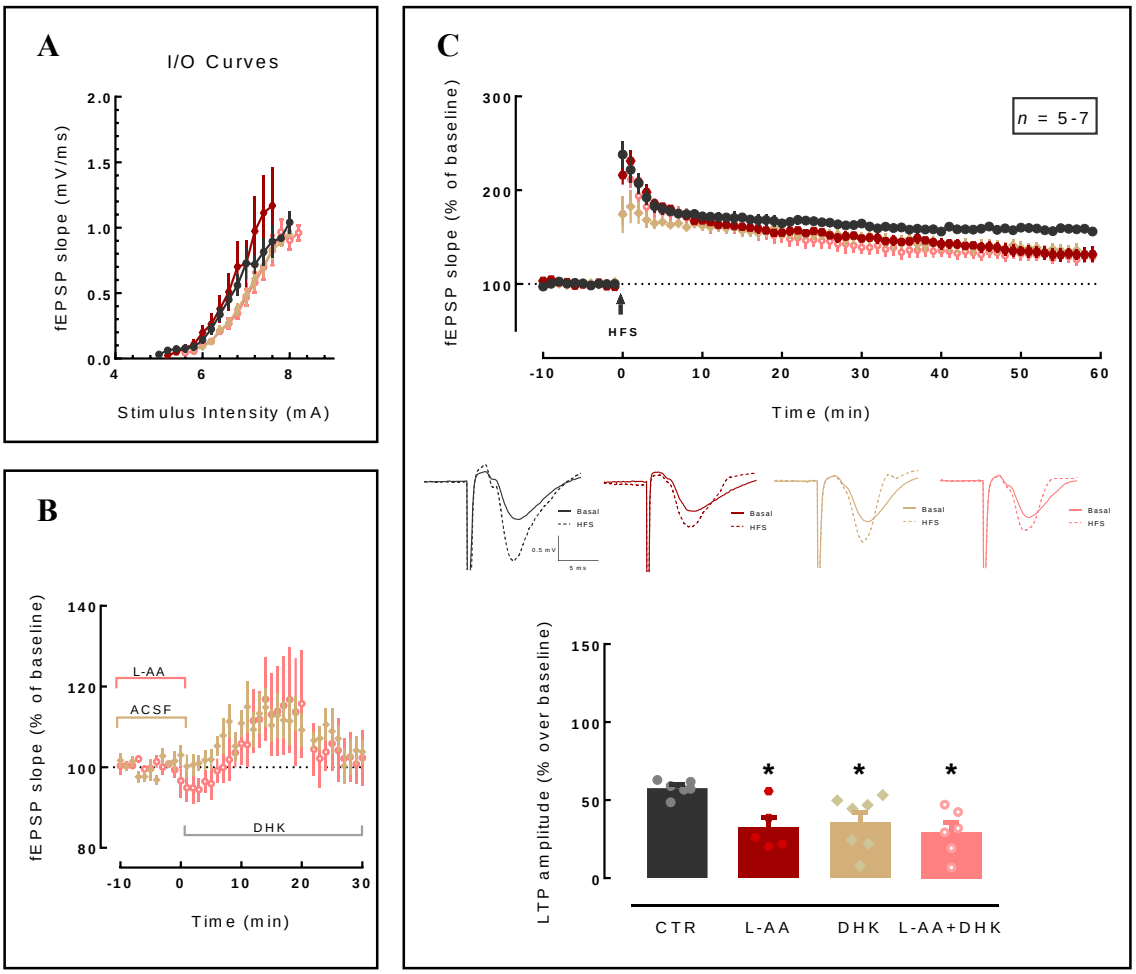


Figure 23 | DHK, a selective GLT-1 inhibitor, caused a significant decrease in LTP. **A |** Input/output curves obtained in hippocampal slices of adult mice treated with aCSF (control, CTR, *n* = 6), L-AA (100 µM, 2h, *n* = 5), DHK (15 µM, superfused for 30 min, *n* = 7) and with both L-AA and DHK (L-AA+DHK, 100 µM, 2h prior to recordings and further perfused for 30 min, *n* = 6) are nearly imposable. I/O curves are displayed as the relationship between fEPSP slope (ordinates) and stimulus intensity (in the abscissa). **B |** Effect of DHK (15 µM) on basal synaptic transmission. Hippocampal slices were exposed to DHK for 30 min prior to LTP induction (grey bar). The fEPSP slope is expressed as the percentage of the value immediately before the addition of DHK. DHK had a transitory effect on basal synaptic transmission, with no statistical significance. Following exposure to DHK (DHK and L-AA+DHK treatments), a new I/O curve was performed and no differences were detected between the two. **C |** The time course of changes in fEPSP slope is shown on the top of the panel. Arrow denotes timing of tetanic stimulation (HFS, 1 s, 100 Hz) and fEPSP amplitude was recorded for 60 min. Representative records of the CA1 evoked fEPSP are shown for each condition at baseline (solid line) and after tetanus (dashed line). Average normalized (against baseline) fEPSP rise slope 50-60 min after the LTP induction in slices from adult mice is shown on the bottom. All treatments are significantly different from control slices (* $P < 0.05$ *vs.* CTR, one-way ANOVA). The bar graph shows the mean ± SEM of independent experiments performed in *n* different mice.

4.1.5. The blockade of hemichannels and gap junctions abolished LTP

Astrocytes release gliotransmitters through different mechanisms and directly shape synaptic transmission. A major pathway for the release of neuroactive substances such as ATP or glutamate into the extracellular space is through hemichannels (HCs) (Fischer et al., 2009; Dallérac et al., 2013; Chever et al., 2014b). Taking this into account, we decided to use carbenoxolone (CBX, 50 μM), a blocker of hemichannels and gap junction, to determine whether LTP was being influenced. LTP changes in SC-CA1 synapses was measured and our data revealed a massive reduction of LTP amplitude in slices superfused with CBX (6.32 ± 8.10 %), when compared to CTR (81.14 ± 16.36 %) (Figure 24C). Additionally, basal synaptic transmission was altered after CBX exposure (Figure 24B), indicating that hemichannels and gap junctions are active under resting conditions and are able to modulate not only synaptic plasticity, but also basal synaptic transmission.

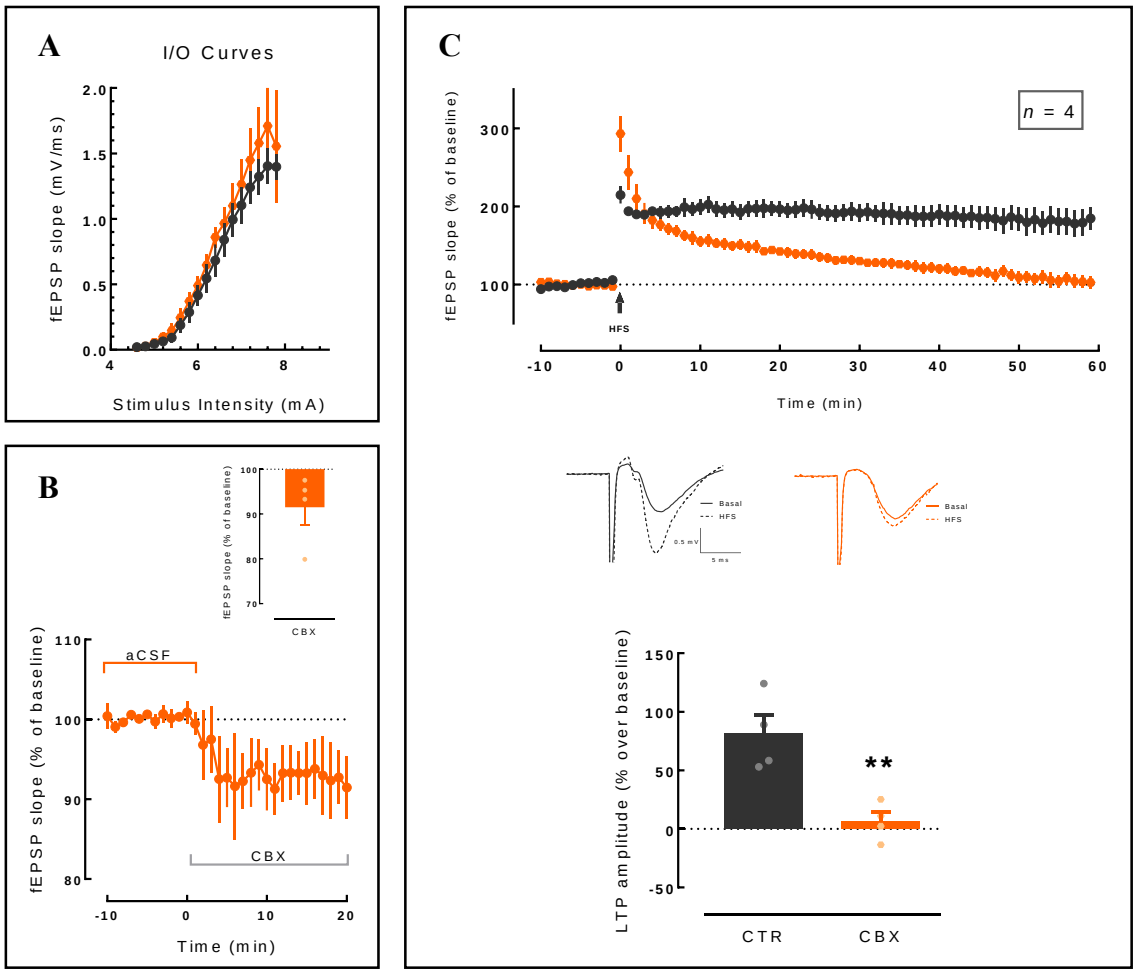


Figure 24 | Effect of CBX, a Cx43 HC blocker, on hippocampal LTP. **A |** Averaged input/output curves of the SC–CA1 fEPSP slope from hippocampal slices treated with aCSF (control, CTR, *n* = 4)

and CBX (50 μM, superfused for 20 min, $n = 4$). I/O curves, where the fEPSP slope was plotted versus the stimulus intensity, are nearly imposable throughout the 20 mA intensity increments. However, the slices treated with CBX seem to have a trend towards an increase in baseline synaptic activity (non-significant). **B |** Effect of CBX (50 μM) on basal synaptic transmission. Hippocampal slices were exposed to CBX during the time indicated by the grey bar to the end of the recordings. The fEPSP slope is expressed as the percentage of the value immediately before the addition of the drug. CBX reduced basal synaptic transmission as shown in the bar graph (average of the last 5 min), but with no statistical significance ($P = 0.07$, unpaired students' *t*-test vs baseline). Following exposure to CBX, a new I/O curve was performed and no differences were detected between the two (data not shown). **C |** The averaged time course of LTP in which fEPSP slopes were normalized in each experiment using the averaged slope value during the baseline (-10 to 0 min) is shown on the top. The arrow indicates tetanic stimulation (100 Hz, for 1 s) and was applied at time 0. The potentiation ratio was calculated using the slope value from 50 to 60 min. Sample traces of field EPSPs (average of 3 responses) are shown for both treatments at baseline (solid line) and after stimulation (dashed line). The potentiation is almost abolished in the slices treated with CBX, as shown in the bar graph (** $P < 0.01$ *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.

4.2. Role of astrocytic A_{2A} receptors in synaptic plasticity

Adenosine exerts a critical role in controlling hippocampal synaptic plasticity and astrocytes were shown to be equipped with all four types of adenosine receptors (Fredholm et al., 2001; Björklund et al., 2008; Boison et al., 2011), which controls the release of different molecules (ATP, glutamate, among others) with impact on neuronal activity. Moreover, the ATP released from astrocytes can also be completely catabolized into adenosine (Zimmermann and Braun, 1996; Haydon and Carmignoto, 2006; Abbracchio et al., 2009; Halassa et al., 2009; Lopes et al., 2011), and it is known that adenosine A_{2A} receptor (A_{2A}R) have a key role sustaining LTP (Cunha, 2005). Having demonstrated that astrocytes are important elements in synaptic plasticity, we decided to study if A_{2A}Rs expressed in glial cells would impact on LTP.

4.2.1. Astrocytic blunting abrogated the effect of adenosine A_{2A} receptor blockade on hippocampal LTP

To investigate if A_{2A}Rs are active participants in LTP when astrocytic function is compromised, we took advantage of a selective antagonist for these receptors (SCH58261). The supra-maximal concentration of 50 nM was used to block virtually all adenosine A_{2A}R (Lopes et al., 2004). LTP on SC-CA1 synapses was measured in hippocampal slices incubated with L-AA (100 μM, 2h), superfused with the selective A_{2A}Rs antagonist (SCH58261, 50 nM) or exposed to both drugs (L-AA + SCH58261). We observed a similar decrease on LTP magnitude in all mentioned conditions (Figure 25C). Briefly, L-AA once again displayed a decrease in LTP (43.09 ± 3.92 %) when compared to CTR (90.50 ± 18.97 %). In accordance with previous data from our group (Costenla et al., 2011), a reduction in LTP amplitude was observed in slices exposed to the selective A_{2A}Rs antagonist (44.83 ± 6.43 %). Finally, in slices treated with both drugs a similar depression in LTP was observed (45.41 ± 13.16 %), meaning that L-AA occluded the effect of A_{2A}Rs antagonist in hippocampal slices. Also, the basal synaptic transmission was altered after SCH58261 exposure (Figure 25B). However, statistical analysis did not yield any significant differences.

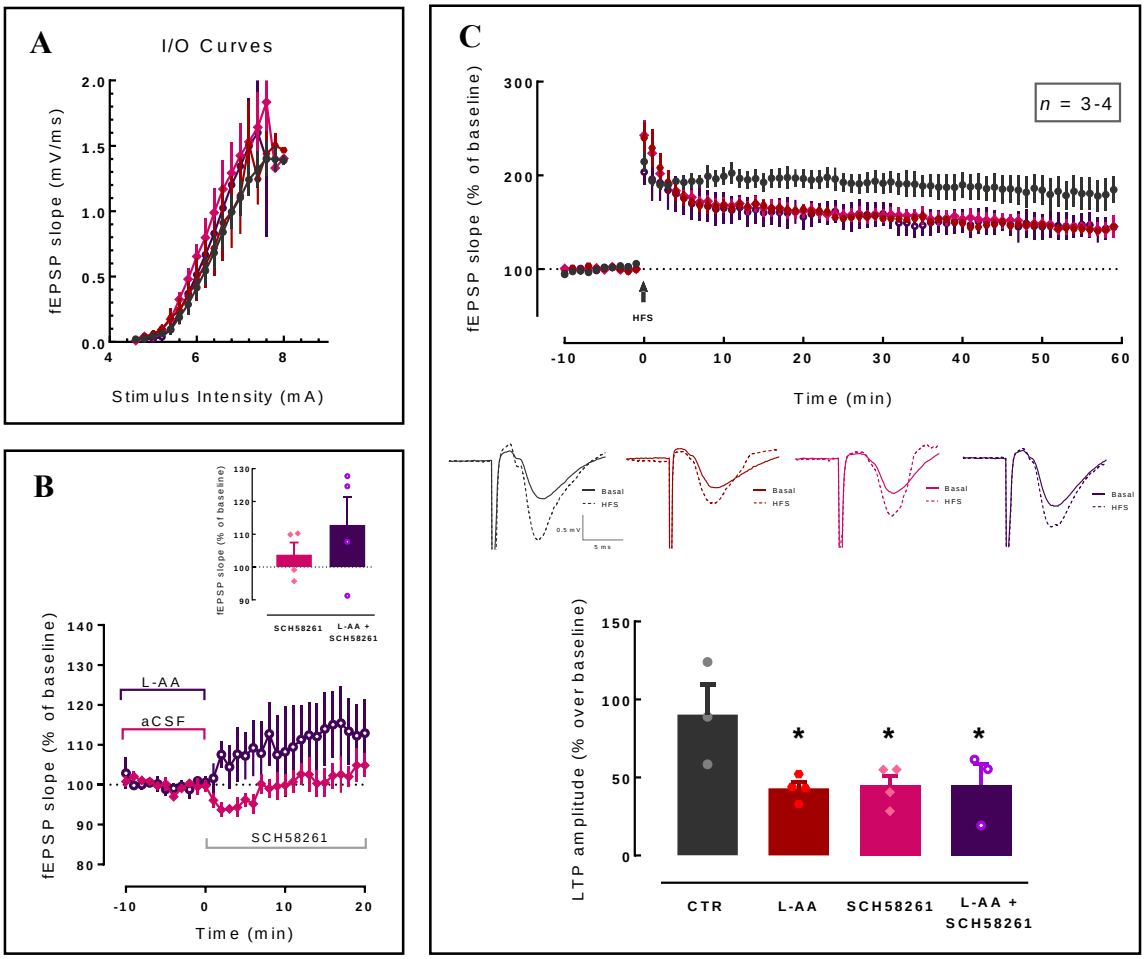


Figure 25 | Effect of astrocytic blunting on the impact of the selective $A_{2A}R$ antagonist SCH58261 on mouse hippocampal LTP. **A |** Input/output (I/O) curves obtained by plotting the slope of fEPSPs in the CA1 area of the hippocampus as a function of the stimulation intensity. I/O curves were similar in all hippocampal slices treated with aCSF (control, CTR, $n = 3$), L-AA (100 μM, 2h, $n = 4$), SCH58261 (50 nM, superfused for 20 min, $n = 4$) and with SCH58261 on top of L-AA (L-AA+SCH58261, 100 μM, 2h prior to recordings and further perfused for 20 min, $n = 3$). I/O curves determined in all groups were almost imposable, indicating that changes in synaptic strength were unlikely to be responsible for eventual LTP differences. **B |** Alterations in basal synaptic transmission following SCH58261 superfusion in the system are shown in the bar graph (average of the last 5 min), but this was not statistically significant. Hippocampal slices were exposed to SCH58261 during the time indicated by the grey bar to the end of the recordings. **C |** Averaged time course changes of fEPSP slope induced by HFS (1 s, 100 Hz) in hippocampal slices from adult mice. The ordinates represent normalized fEPSP slopes where 100% corresponds to the averaged slopes recorded for 10 min before the HFS and the abscissa represents the time of each recording. Recordings obtained in representative experiments are shown below and each one is the average of 3 consecutive responses obtained before (solid line) and 50–60 min after (dashed line) LTP induction. LTP magnitude (shown on the bottom), was significantly decreased in slices pharmacologically treated (* $P < 0.05$ *vs.* CTR, one-way analysis of variance (ANOVA)). All values are mean ± SEM of *n* independent experiments. The *n* values refer to the number of mice used per condition.

4.2.2. Genetic deletion of forebrain neuronal A_{2A} receptors did not affect the impairment of LTP triggered by gliotoxins

To confirm the previous set of results we decided to carry out a pilot experiment using mice genetically modified (forebrain $A_{2A}R$ conditional knockout, Fb-$A_{2A}R$ KO), which exhibit a deletion of $A_{2A}Rs$ in the neurons of striatum as well as cerebral cortex and hippocampus (Bastia et al., 2005; Shen et al., 2008). Hippocampal slices from fb-$A_{2A}R$ KO and their corresponding WT littermate mice were incubated in L-AA (100 µM, 2h) and TFA (100 µM, 2h). Our results showed no differences among the two genotypes (Figure 26C). L-AA and TFA incubation in slices from fb-$A_{2A}R$ KO resulted in a reduced LTP amplitude (34.82 ± 14.69 % and 28.08 ± 8.55 %, respectively), compared to the control (79.68 ± 6.48 %). Likewise, in WT littermate mice, L-AA and TFA triggered an identical depression of hippocampal LTP (34.42 ± 16.70 % and 45.82 ± 9.30 %, respectively) when compared to control slices (84.92 ± 18.32 %).

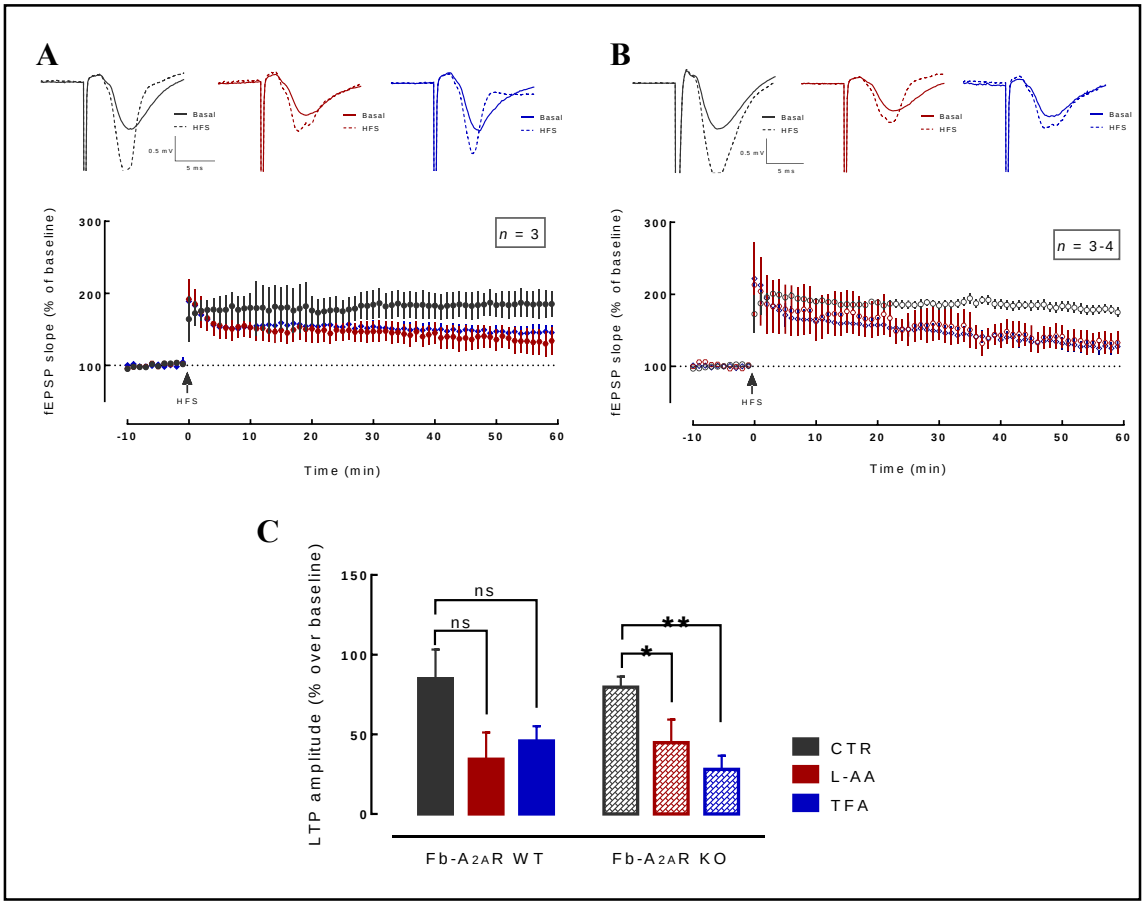


Figure 26 | Effect of two different gliotoxins on SC-CA1 hippocampal LTP from Fb-A_{2A} KO mice. **A-B |** Averaged time course changes of fEPSP slope induced by HFS (1 s, 100 Hz) in hippocampal

slices from Fb-A_{2A}R KO mice (right) and their corresponding WT littermates (left). Hippocampal slices from WT littermates were incubated in aCSF (control, CTR, *n* = 3), L-AA (100 μM, 2h, *n* = 3), TFA (100 μM, 2h, *n* = 3). Likewise, slices from Fb-A_{2A} KO mice were treated with aCSF (CTR, *n* = 4), L-AA (100 μM, 2h, *n* = 3), TFA (100 μM, 2h, *n* = 4). Representative traces are also shown on the top of the panel for all treatments at baseline (solid line) and after stimulation (dashed line) for both genotypes. Arrow denotes timing of tetanic stimulation (HFS, 1 s, 100 Hz) and fEPSP amplitude was always recorded during 60 min. **C |** LTP magnitude, corresponding to the average fEPSP slope 50–60 min after LTP induction. LTP amplitude was significantly decreased in slices treated with gliotoxins from Fb-A_{2A} KO animals (* $P < 0.05$, one-way ANOVA *vs.* CTR) whereas in WT littermates there are no significant differences between the treatments ($P > 0.05$ *vs.* CTR, one-way ANOVA). Additionally, there were no differences observed between the two animals. All values are mean ± SEM of *n* independent experiments. The *n* values refer to the number of mice used per condition.

4.3. Consequences of chronic L-AA injections (*in vivo*) in memory and synaptic function

Until now, by studying the changes in LTP elicited by the exposure to different gliotoxins (*in vitro)*, we have shown that astroglial cells actively participate in synaptic function. Because astrocytes are associated with deficits in synaptic plasticity, which in turn are associated with several brain disorders such as epilepsy, schizophrenia, PD and AD (Kimelberg et al., 1990; Martin, 1992; Haydon and Carmignoto, 2006), it is of utmost importance to create *in vivo* models to gather new information about the involvement of astrocytes in brain pathologies. For that purpose, we are creating an *in vivo* model in which C57Bl/6 mice are intracerebroventricularly (icv) injected with L-AA or vehicle, as mentioned before, to evaluate changes in hippocampal dependent-tasks and, in a near future, investigate the role of astrocytes on the early onset of AD or other forms of dementia. The nomenclature used in the following experiments is L-AA for mice injected with the gliotoxin and CTR for animals injected with PBS.

4.3.1. Recognition memory was not significantly affected by astrocytic blunting triggered by L-AA icv injected

Because astrocytes are thought to shape synaptic plasticity, the neurophysiological basis of learning and memory (Martin, 1992; Malinow and Malenka, 2002; Haydon and Carmignoto, 2006), and considering that the hippocampus is a crucial brain region for both spatial and recognition memory (Granger et al., 1996; Zola-Morgan, 1996), we tested whether L-AA icv injection in mice affects their performance in the novel object recognition (NOR) test. First, an OFT was performed in an empty open field arena to check if the animals have "healthy" complications due to icv administration. Briefly, both groups, PBS- (CTR) and L-AA-injected (L-AA), displayed a similar travelled distance (33.47 ± 3.45 m for L-AA compared to CTR, 36.03 ± 3.81 m) (Figure 27A). The time spent in the center zone (73.02 ± 4.73 s compared to CTR, 69.03 ± 7.23 s) and the time spent in the peripheral zone (527.00 ± 4.74 s compared to CTR, 530.90 ± 7.23 s) were also identical (Figure 27B-C). Finally, following quantification of NOR, our results

showed a slightly reduction regarding the recognition index (66.13 ± 2.53 % compared to CTR, 71.12 ± 3.12 %), without statistical significance ($P > 0.05$) (Figure 27D).

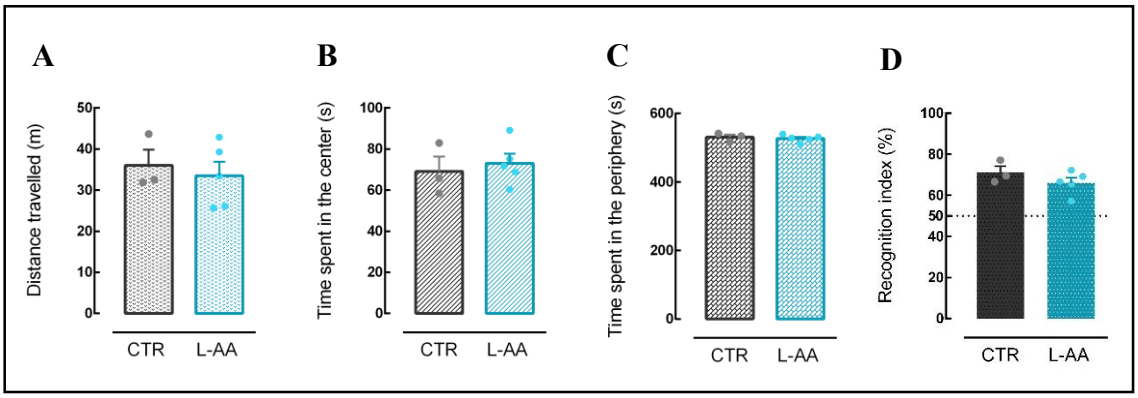


Figure 27 | OFT and NOR performed in mice icv injected with L-AA after 72 h. **A |** Locomotor activity of mice injected with vehicle (n = 3) and L-AA (n = 5). The results are expressed as distance travelled (m). **B-C |** Open field test for anxiety measures. The parameters used were the time spent in the central zone (in seconds) and the time spent in the peripheral zone (in seconds). Among these evaluations, there were no differences between the two conditions. **D |** Novel object preference (expressed as a recognition index) during novel object recognition testing. Both groups show comparable exploration of the novel object. The dashed line at 50% is indicative of chance performance. All values are mean ± SEM of n experiments. The n values refer to the number of mice used.

4.3.2. L-AA-injected mice displayed a significant reduction in hippocampal LTP

Following behavior analysis, we asked whether the same C57Bl/6 L-AA-injected mice would exhibit a decrease in LTP amplitude, since L-AA *in vitro* showed a consistent LTP reduction upon a 2h incubation. Therefore, 72 h after icv administration of L-AA or PBS, hippocampi from both groups of animals were dissected and the slices were probed for differences in LTP magnitude induced by HFS. According to our previous results, regarding L-AA acute exposure in hippocampal slices, L-AA- injected mice showed a significant reduction in hippocampal LTP (52.38 ± 6.16 % compared to CTR, 77.04 ± 8.58 %) (Figure 28).

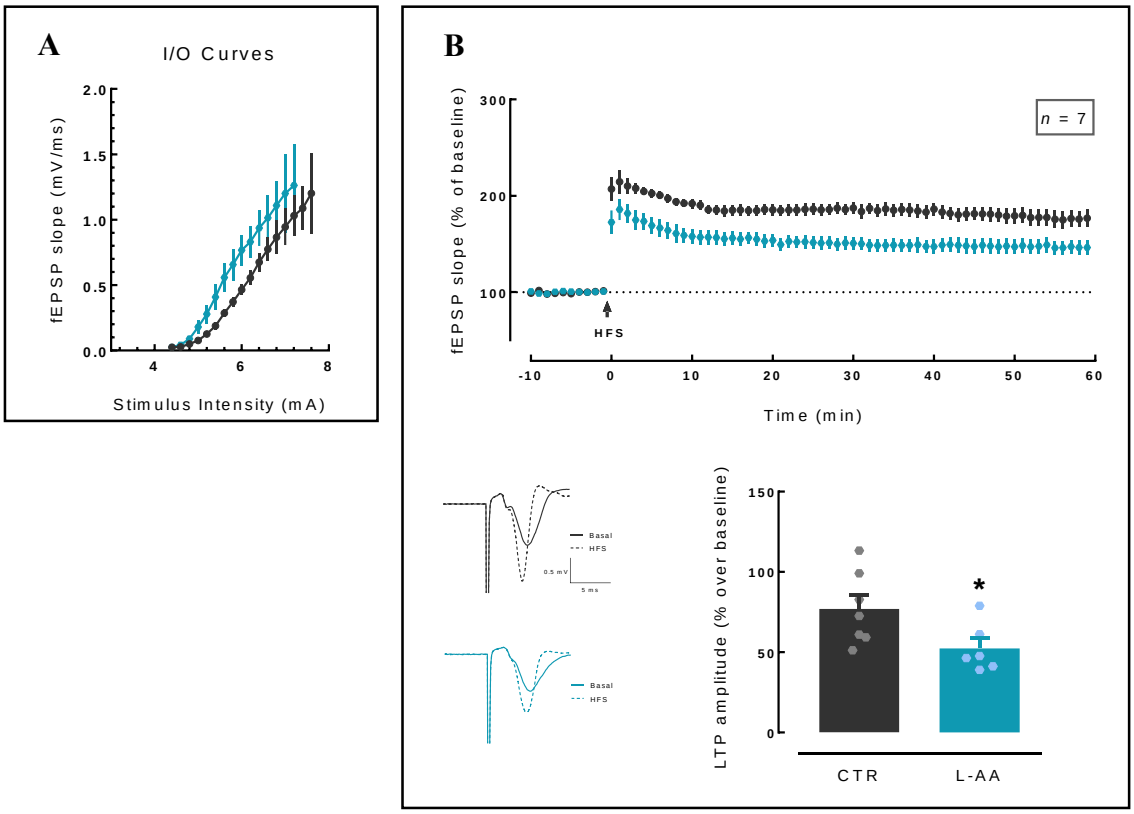


Figure 28 | CA1- LTP is significantly decreased in L-AA-injected mice. **A |** Input/output curves presenting fEPSP slope in response to increasing stimulus input in adult mice injected with PBS (CTR, *n* = 7) and L-AA (L-AA, *n* = 6). Average I/O slopes measured show no difference regarding the maximum fEPSP slope. However, slices from L-AA-injected animals display increased baseline synaptic activity (higher fEPSP slope compared to control group at all intensities). **B |** Averaged time course fEPSP slope compared to baseline is shown on top of the panel. Arrow denotes timing of tetanic stimulation (HFS, 1 s, 100 Hz) and fEPSP amplitude was recorded for 60 min following tetanization to measure LTP. On the left, traces show representative recordings of fEPSP before (baseline, solid line) and 50 min after the train (after LTP, dashed line). Each trace comprises the stimulus artefact, 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 (the depression after the stimuli artifact). LTP magnitude (shown on the bottom), corresponding to the average fEPSP slope 50–60 min after LTP induction, was significantly decreased in slices from L-AA- injected mice (* $P < 0.05$ *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.

4.3.3. Acute L-AA incubation did not alter LTP in mice previously injected with the gliotoxin

To further confirm that L-AA injected *in vivo* was mimicking the robust effects of acute exposure of this gliotoxin in hippocampal LTP *in vitro* (section 4.1.1.), we incubated slices from both treatments (L-AA- and PBS-injected) with L-AA (2 h, 100

μM dissolved in aCSF), as previously described. Our results revealed that L-AA acute incubation had no effect on C57Bl/6 mice injected with the gliotoxin (Figure 29). More specifically, compared to PBS- injected mice (CTR, 69.86 ± 14.81 %), the remaining treatments had a comparable reduction in hippocampal LTP (L-AA with 42.70 ± 3.63 %, CTR + L-AA with 34.19 ± 4.92 % and finally L-AA + L-AA with 36.18 ± 4.04 %). With these observations, we can conclude that this model of icv L-AA administration is reliable when studies in synaptic plasticity are performed.

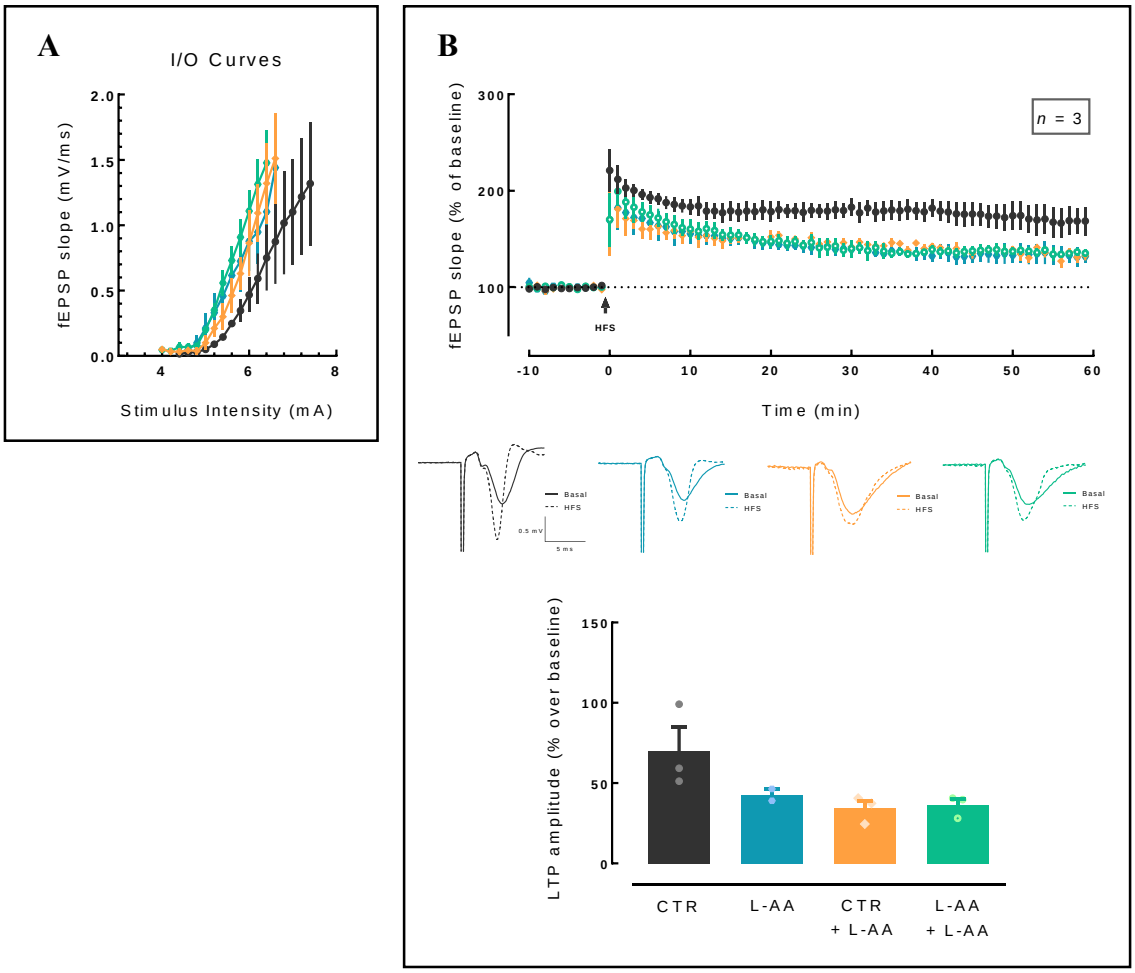


Figure 29 | Effect of L-AA acute incubation on hippocampal slices from injected mice. **A |** Stimulus-response curves in hippocampal slices from PBS- injected mice (control, CTR, *n* = 3), L-AA- injected mice (L-AA, *n* = 3) and further acute incubation in L-AA (CTR + L-AA, 100 μM, 2h, *n* = 3 and L-AA + L-AA, 100 μM, 2h, *n* = 3). I/O curves measuring the magnitude of the fEPSP response across a range of stimulation currents was comparable between the all groups as seen in the illustration. **B |** The time course of changes in fEPSP slope is shown on the top of the panel. Arrow denotes timing of tetanic stimulation (HFS, 1 s, 100 Hz). fEPSP amplitude was recorded for 60 min following tetanization to measure LTP. Representative recording of a typical fEPSP in mouse hippocampus by positioning electrodes in the SC-CA1 synapses is also shown. LTP magnitude (shown on the bottom), corresponding to the average fEPSP slope 50–60 min after LTP induction, was similar in slices with acute incubation to L-AA when comparing to mice injected with the gliotoxin ($P > 0.05$ *vs.* CTR, one-way ANOVA). All values are mean ± SEM of *n* independent experiments. The *n* values refer to the number of mice used per condition.

4.3.4. L-AA-injected mice exhibited a significant depontentiation upon acute incubation with the gliotoxin

Next, using the slices incubated with L-AA and following LTP, a LFS protocol was applied to observe if these animals would display a tendency for depotentiation, as seen before in section 4.1.2. Our data demonstrated a significant depotentiation for both groups exposed to the gliotoxin. Both PBS and L-AA- injected animals incubated with L-AA showed a depotentiation of 11.39 ± 2.29 % and 10.83 ± 3.07 %, respectively (Figure 30).

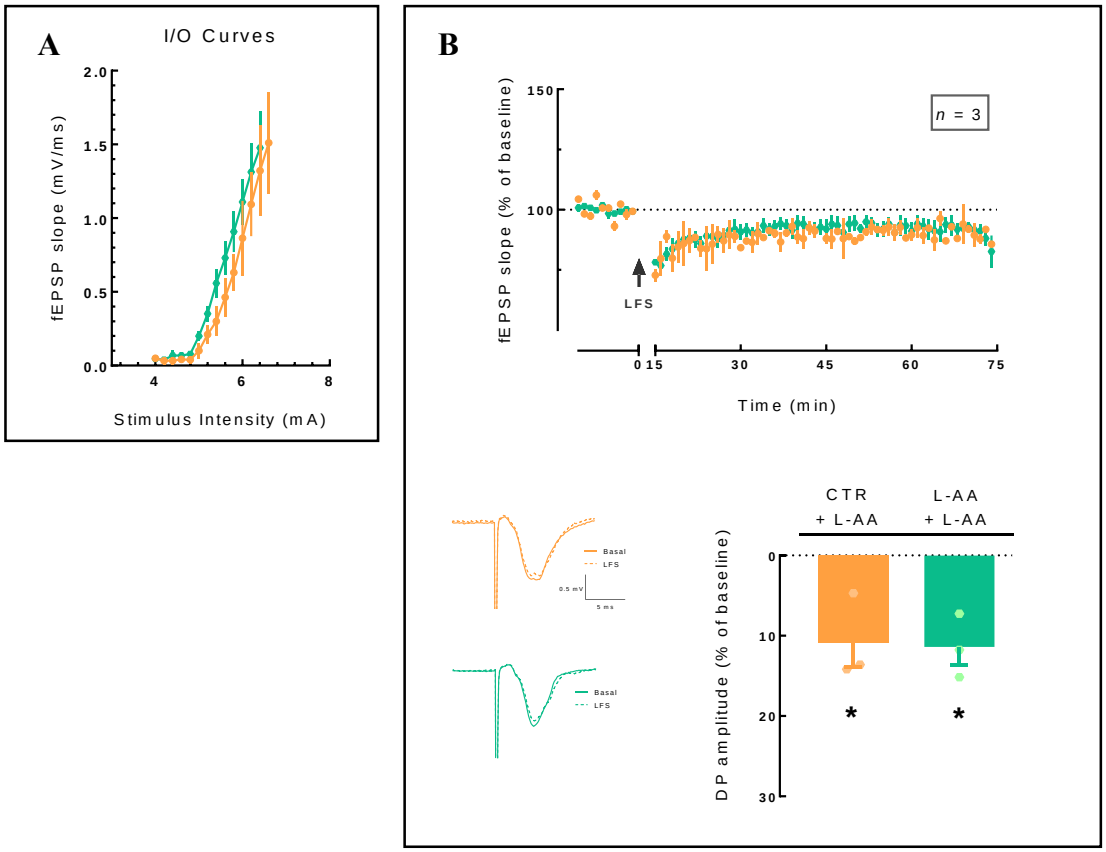


Figure 30 | LFS induction in L-AA incubated slices from injected mice led to a significant depotentiation. **A |** Stimulus-response curves of fEPSP slope (mV/ms) *vs.* stimulus intensity (mA) at the SC-CA1 synapses in hippocampal slices from mice injected with PBS and L-AA incubated with the gliotoxin (CTR + L-AA, 100 µM, 2h, *n* = 3 and L-AA + L-AA, 100 µM, 2h, *n* = 3). I/O curves were comparable between the two groups as seen in the panel. **B |** The time course of changes in fEPSP slope is shown on the top of the illustration. After potentiation for 60 min, a depotentiation (LFS, 15 min, 1 Hz) was induced as shown in the graph at time 0 indicated by the black arrow. fEPSP slope is plotted over time as percentage of the baseline before LFS and fEPSP amplitude was recorded for another 60 min following stimulation. Examples of fEPSP recordings before (solid lines) and 60 min after LFS induction (dashed lines) in the CA1 area of hippocampal slices are also shown. Magnitude of depotentiation estimated from the averaged fEPSP slope 50–60 min after LFS induction, was significantly decreased in both slices treated with L-AA (* $P < 0.05$ *vs.* baseline, one-way ANOVA). All values are mean ± SEM of *n* independent experiments. The *n* values refer to the number of mice used per condition.

4.4. Astrocytic modifications triggered by L-AA in hippocampal sections

Up to this point, we have shown that L-AA *in vitro* and *in vivo* is capable of inducing changes in hippocampal synaptic plasticity. However, one question remained unanswered: how are astrocytes being affected by L-AA? Literature states that this drug, once inside astroglial cells, is capable of inducing cell death by inhibiting glutamine synthetase (GS) (McBean, 1994). Taken all this into account, we performed immunohistochemical analysis using transverse hippocampal slices obtained from the slices previously analyzed by electrophysiology to look for changes in the number of astrocytes and the immunostaining intensity for GS.

4.4.1. Acute treatment of hippocampal slices with L-AA had no effect on the labelled astrocytic markers

Hippocampal slices (400 μm) incubated for 2 h with L-AA or aCSF and were sectioned into 50 μm sections to be used in immunohistochemical analysis, probing for astrocytic markers. Hippocampal sections were labeled with rabbit anti-glutamine synthetase (GS), goat anti-GFAP antibodies (Figure 31A) and with the nuclear dye DAPI (not shown). Following quantification, our results showed that a 2-hour incubation in L-AA was not sufficient to induce changes in the number of GFAP-positive astrocytes (23.67 ± 1.14 compared to CTR, 25.85 ± 0.24) or alter the immunoreactivity of GS (63.67 ± 1.89 AU compared to CTR, 54.79 ± 4.96 AU) (Figure 31B-C). CTR and L-AA nomenclature stands for hippocampal slices treated with aCSF or L-AA (100 μM, 2 h), respectively.

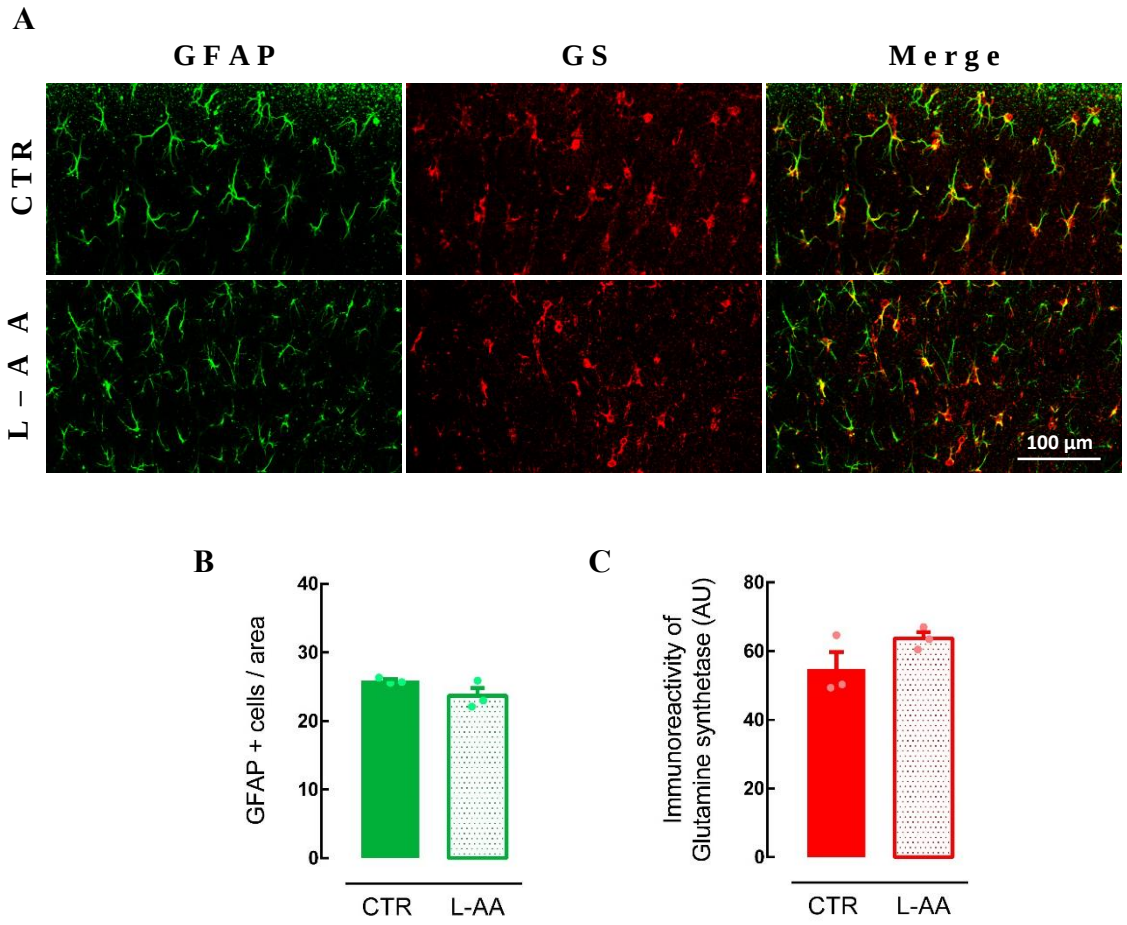


Figure 31 | Immunohistochemical labelling of astrocytes in hippocampal sections (50 µm thickness). **A |** Representative images of GFAP (green), glutamine synthetase (GS, red) and the overlay of both channels in hippocampal slices previously incubated with L-AA (100 µM, 2h, *n* = 3, with 3 slices per animal) and aCSF (CTR, *n* = 3, with 3 slices per animal). Scale bar of 100 µm for all panels. All images were obtained at ×20 magnification using a fluorescent microscope and the area quantified was within *stratum radiatum* (hippocampal CA1 region). **B |** Bar graph shows the number of GFAP-positive cells and **C |** immunoreactivity of GS for CTR and L-AA treatments. Number of positive cells and immunoreactivity of GS are expressed as the mean ± SEM of the *n* indicated. AU refers to arbitrary units.

4.4.2. L-AA-injected mice displayed a significant decrease in GFAP-positive cells

Considering the similarities in the alterations of LTP amplitude between acute L-AA treatment and the injection of the gliotoxin icv, we used hippocampal slices from L-AA- and PBS-injected animals previously used in electrophysiology and performed the same IHC protocol to look for changes in astrocytic markers in the *in vivo* model. By labelling hippocampal sections with rabbit anti-GS, goat anti-GFAP antibodies (Figure 32A) and

with the nuclear dye DAPI (not shown), we showed that 72 h after L-AA injections, a significant (P < 0.01, 24 % reduction) decrease in GFAP-positive cells was observed (19.42 ± 0.96) when compared with PBS-injected mice (25.56 ± 1.26) (Figure 32B). However, regarding the GS immunoreactivity the two groups presented similar values: The hippocampal slices from LAA injected mice displayed 56.16 ± 2.82 AU compared to CTR, 57.95 ± 2.37 AU) (Figure 32C). CTR and L-AA nomenclature stands for PBS and L-AA-injected mice, respectively.

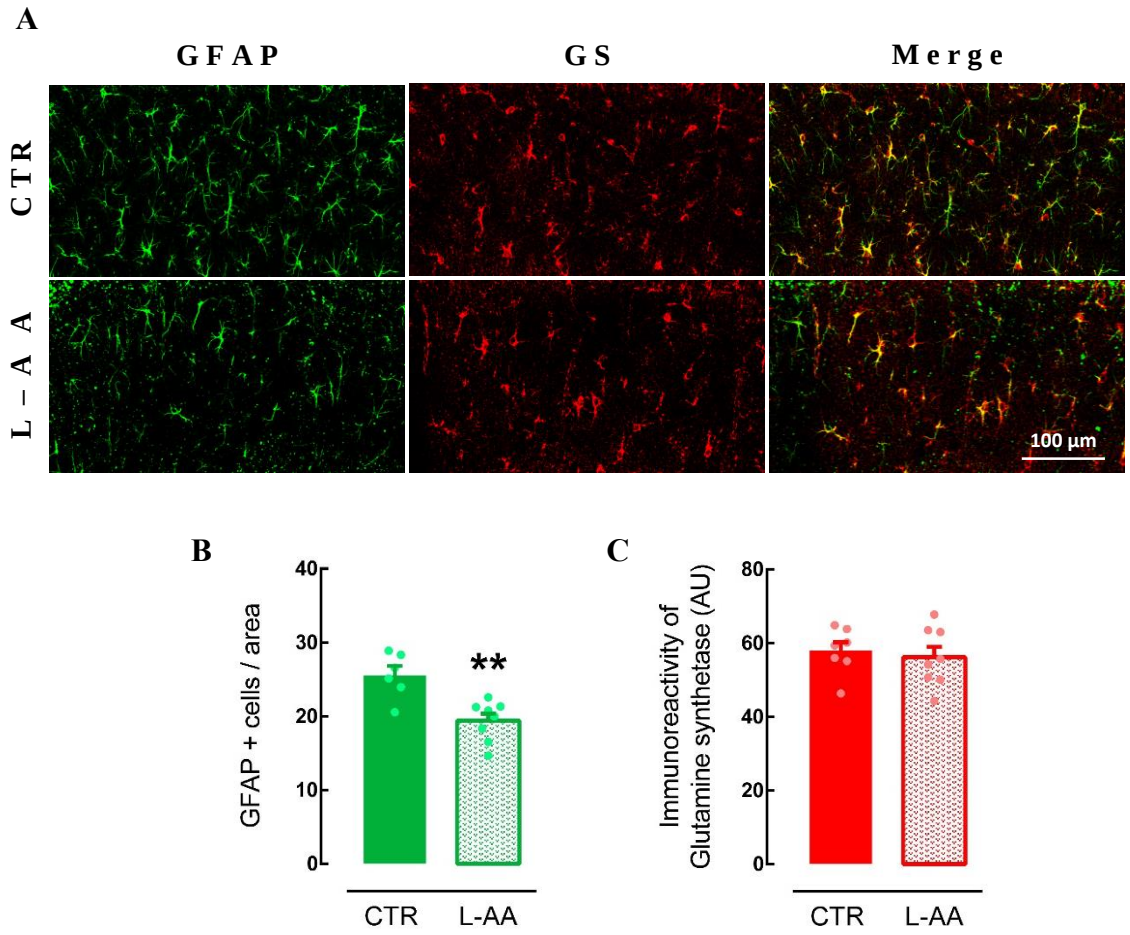
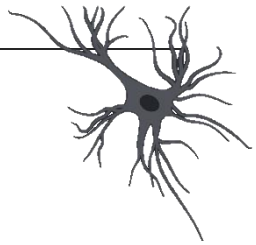


Figure 32 | Immunohistochemistry of hippocampal sections from L-AA-injected mice (50 µm thickness). **A |** 400 µm transverse hippocampal slices were kept from PBS (CTR, n = 6, with 3 slices per animal) and L-AA- (L-AA, n = 7, with 3 slices per animal) injected mice and used for labeling with GFAP (green) and glutamine synthetase (GS, red). Merged images of GFAP and GS are also shown. Scale bar of 100 µm for all panels. All photographs were obtained at ×20 magnification using a fluorescent microscope and the area quantified was within *stratum radiatum* (hippocampal CA1 region). **B |** Cell counting of GFAP revealed a significant decrease in GFAP-positive cells per area (* P < 0.01 *vs.* CTR, unpaired Students' *t*-test). **C |** Immunoreactivity of GS was also quantified, but no differences were found. Number of positive cells and immunoreactivity of GS are expressed as the mean ± SEM of the n indicated. AU refers to arbitrary units.

Chapter **5**

General discussion



5.1. Discussion

The potential involvement of glial cells in neuronal plasticity and in higher brain functions has long been suggested (Kimelberg et al., 1990; Martin, 1992; Araque et al., 1999; Oliet et al., 2001; Haydon and Carmignoto, 2006). The lack of experimental agents to selectively manipulate astrocytes has hindered the ability of researchers to explore the various possible roles of astrocytes, especially in *in vivo* preparations. The present study provides evidence for the involvement of astrocytes in mouse hippocampal synaptic plasticity and memory-related processes.

Distinct pharmacological tools were used to selectively interfere with astrocytes and to further evaluate how they can contribute to hippocampal long-term potentiation (LTP) from adult mice. First, we tested how incubation with L-AA, a specific gliotoxin to silence astrocytic function without providing harmful effects on surrounding neurons (Olney et al., 1980; McBean, 1994; Khurgel et al., 1996; Lima et al., 2014), would impact on LTP amplitude, and based on our first results (a reduction of 37 %, see figure 20), we can say that astrocytes were involved in this process. Although several studies also use this gliotoxin to specifically blunt astrocytic function, to this date, there are no reports of astrocytic involvement in SC-CA1 LTP using L-AA as a pharmacological tool. Instead, L-AA was used to probe the role of astrocytes in the modulation of NMDARs function in amygdala (in rats), and what Li and his team (2013) showed was that the incubation of amygdala slices with a solution containing L-AA (1 mM, 90 min) caused an inhibition of LTP, but no alterations in excitability or basal synaptic transmission (Li et al., 2013), which are in line with our results.

However, one simple approach is not sufficient to claim that astrocytes are active participants in synaptic plasticity. To strengthen our hypothesis, another gliotoxin, namely TFA, was used to blunt astrocytic function. Strikingly, acute incubation with TFA led to the same decrease on LTP (see figure 22), as well as the two gliotoxins combined. In other words, astrocytic blunting mediated by different pathways (inhibition of glutamine synthetase by L-AA and metabolic dysfunction caused by TFA) had the same impact on LTP. Additionally, the reason why L-AA and TFA combined displayed the same decrease on LTP (occlusive effect of TFA) is because we are only targeting

astrocytes, meaning that even though we were manipulating astrocytes in different ways, the final effect was the same. Several studies have claimed that TFA induced inhibition of glial metabolism by studying the specific release of D-serine, GABA and ATP (Zhang et al., 2003; Henneberger et al., 2010; Boddum et al., 2016). Also, Henneberger and colleagues (2010) showed that incubation of rat hippocampal slices with TFA (5 mM), for at least 50 min, led to a total LTP blockade, possibly due to the high concentration of the gliotoxin; nevertheless, these data are in accordance with our results (Henneberger et al., 2010). To this point, the only conclusion we can draw is that the blunting of astrocytes caused an impairment in mouse hippocampal LTP.

To sustain the above mentioned, we decided to interfere with specific functions of astrocytes in physiological conditions. It is well described that astrocytes play a prominent role in maintain glutamate homeostasis in the brain (concentration of ~2-4 μM in the extracellular fluid), hence protecting against high extracellular glutamate concentrations, which may result in neurodegeneration caused by the excitotoxic action of glutamate (Burnstock, 2007; Matos et al., 2008, 2012a, 2012b; Persson and Rönnbäck, 2012). For that reason, in the present study we used a selective inhibitor of glutamate transporter 1 (GLT-1), the predominant glutamate transporter (GluT) in astrocytes under physiological conditions (Anderson and Swanson, 2000; Sattler and Rothstein, 2006; Lin et al., 2012), to cause a malfunction of astrocytes and affect glutamate homeostasis. However, it is also known that microglia is able to express Na^+-dependent high affinity glutamate transporters during pathological situations, such as after traumatic brain injury, prion diseases, as well as infections with virus such as the human immunodeficiency virus (HIV) (López-Redondo et al., 2000; Van Landeghem et al., 2001; Chrétien et al., 2004; Persson et al., 2006, 2007; Persson and Rönnbäck, 2012). Our results concerning the inhibition of GLT-1 by DHK showed a similar reduction on LTP when compared to the effect of L-AA (see figure 23). Moreover, after exposing hippocampal slices to L-AA combined with DHK, we observed an occlusion of DHK, which indicates that in our experimental conditions the DHK was acting mainly in astrocytic GLT-1. Indeed, what is known about the presence of GLT-1 in microglia is that these transporters are only expressed under pathological situations, which is not the case of our conditions. Furthermore, our results are consistent with previous studies were mice lacking GLT-1 were used to perform extracellular recordings in pyramidal neurons of CA1 region and the results revealed that LTP was significantly reduced, due to either excessive NMDA

receptor activation or to AMPA receptor desensitization, caused by an increase in the concentration of glutamate in the synaptic cleft (Katagiri et al., 2001).

Astrocytes integrate neuronal inputs through their membrane channels, receptors and transporters and can transmit information by clearing (via GluTs) or releasing a number of neuroactive substances named gliotransmitters. Hemichannels (HCs)-mediated gliotransmitters release is believed to interfere with basal excitatory synaptic transmission and synaptic plasticity (Torres et al., 2012; Chever et al., 2014a; Orellana and Stehberg, 2014). In the present study, we evaluated the effect of carbenoxolone (CBX), a general blocker of HCs and gap-junction (GJ), which in astrocytes are mainly composed by connexin43 (Cx43), on hippocampal LTP. It is important to mention that this interest emerged due to data gathered by our group in cultured astrocytes, showing that CBX, significantly prevented HCs-mediated ATP release (unpublished data presented by Madeira and colleagues at XV SPN meeting 2017), which is known to control synaptic function (Haydon and Carmignoto, 2006). Notably, by inhibiting HCs and/or GJ with CBX, a slight decrease on basal synaptic transmission was observed, although with no statistical significance, and, most importantly, LTP was almost abolished (see figure 24B-C). Our results are in agreement with those showing that CBX exposure (100 μM, for 10 min) resulted in a progressive decrease of the basal synaptic transmission in adult rat hippocampal slices (Andersson et al., 2007). The gliotransmitter release from astrocytes can be sensed by pre- or postsynaptic glutamate receptors such as the metabotropic glutamate receptor (mGluR) or NMDARs, both of which are known to modify pre- and postsynaptic activities, leading to alterations in basal synaptic transmission or synaptic plasticity. This study from Anderson and colleagues (2007) also showed that CBX abolished the transient heterosynaptic depression, an intersynaptic communication in which active synapses decrease the efficacy of neighboring inactive synapses, which are associated to astrocytic calcium signals and the release of ATP/adenosine (Zhang et al., 2003; Pascual et al., 2005). Furthermore, more recent studies also showed that mice with conditional deletion of Cx43 in astrocytes ($Cx43^{-/-}$) had a decrease in basal synaptic transmission (Chever et al., 2014b); and notably in double knockout mice $Cx30^{-/-}Cx43^{-/-}$ (with conditional deletion of Cx43 in astrocytes and additional total deletion of Cx30), it was observed that LTP was nearly absent (Pannasch et al., 2011), which goes in accordance to our results as well (see figure 24C). The data gathered by us regarding the effect of CBX in mouse hippocampal LTP were from a pilot study, and we are aware that

the connexin-based channels in astrocytes exist either in intercellular GP or in unapposed HCs, being difficult to determine to what extent these different functions of connexin-based channels of astrocytes are important for synaptic plasticity events (Nagy and Rash, 2000; Dallérac et al., 2013). Thus, we plan on continuing this study to give clear evidence about the involvement of astrocytic HCs-mediated gliotransmitter release on synaptic function, by using a selective blocker of Cx43 HCs (gap19) with no effect on gap junctions (Abudara et al., 2014).

Adenosine is released into the extracellular space either by neurons or astrocytes, acting as a neuromodulator, thus controlling synaptic function (Lopes et al., 2011, 2002; Costenla et al., 2010). Therefore, we took advantage of an A_{2A} receptor ($A_{2A}R$) antagonist (SCH58261) to examine the impact of these receptors on regulating LTP together with the gliotoxin L-AA. What we saw by applying SCH58261 in hippocampal slices was that astrocytic blunting abrogates the effect of $A_{2A}R$ blockade, since we observed an occlusive effect of SCH58261 on L-AA-treated slices (see figure 25C), which seems to indicate that astrocytic ATP-derived adenosine is activating $A_{2A}R$ in pre-synaptic terminals (Cunha, 2005; Rebola et al., 2005). Furthermore, we carried out a pilot experiment using mice genetically modified (forebrain $A_{2A}R$ conditional knockout, fb-$A_{2A}R$ KO) to complement and strengthen our set of results obtained with SCH58261 in the presence of gliotoxin. Using this genetically modified mice, with silenced $A_{2A}R$ in neurons, we expected to have an idea about whether the astrocytic $A_{2A}Rs$ control hippocampal synaptic plasticity. Unfortunately, our results with hippocampal slices from fb-$A_{2A}R$ KO mice showed a peculiar effect because we had absolutely no differences in LTP magnitude range between KO mice and their littermates in the control conditions, preventing us from taking any conclusions. These results are opposed to data already obtained by our group, showing that in fb-$A_{2A}R$ KO animals there are a significant decrease in hippocampal LTP when comparing to the same condition in their littermate mice (Queirós et al, submitted manuscript). A control that should have been done was the confirmation of fb-$A_{2A}R$ KO genotype to know for sure if we were working with animals with conditional knockout for $A_{2A}Rs$. It is worth mentioning that studies concerning the impact of astrocytic $A_{2A}R$ in mouse hippocampal LTP were not reported to this date, although, it was shown by our group that $A_{2A}R$ are present in astrocytes, and that these receptors control glutamatergic activity through a mechanism that involves their functional interaction with Na^+/K^+ATPase (Matos et al., 2013). Our group have also shown that astrocytic $A_{2A}R$-

induced modification of GLT-1 function, which is associated with several striking neuronal adaptations at the glutamatergic circuitry, typified by an increase of synaptic glutamate release, an enhanced density of NR2B subunits of NMDA receptors, and an increased internalization of AMPA receptors (Matos et al., 2015). All these events can directly influence synaptic plasticity processes, thus reinforcing the idea that astrocytic A_{2A}Rs may modulate synaptic function. Moreover, in pathological conditions, such as in AD, it was shown that Aβ peptide accumulation is associated with increased levels of A_{2A}Rs in astroglial cells (Matos et al., 2012a; Orr et al., 2015), and that the genetic ablation of astrocytic A_{2A}R enhanced long-term memory formation (Orr et al., 2015). Taken together, given the high density of astrocytes in the brain, a deeper understanding of the roles operated by astrocytic A_{2A}Rs and their relationship within the tripartite synapse in plasticity phenomena might be crucial for the development of safe and effective therapies for brain disorders, such as AD.

Later, we moved on to an *in vivo* mice model in which we caused alterations in proper astrocytic functions, triggered by the injections of L-AA or vehicle in both lateral ventricles. Three days after the injections, mice were exposed to an open field to check if they had any complications, mainly in motor activity, and then the animals were habituated to two equal objects to further perform novel object recognition (NOR) test. Our data revealed a non-significant decrease in recognition index for L-AA-injected mice, when compared with vehicle-injected mice (control). Still in the same two animal groups, we performed extracellular recordings on SC-CA1 synapses to correlate the behavior results with plasticity events in the hippocampus, because this structure has been studied at almost every level of analysis, ranging from detailed behavioral studies to the functional and molecular mechanisms underlying synaptic transmission and plasticity (Gerlai and Clayton, 1999; Gerlai, 2001). Remarkably, L-AA-injected mice presented a significant reduction in hippocampal LTP (see figure 28), although we did not find a significant impairment in the NOR test. This lack of effect on recognition memory could be due to the fact that this test does not exclusively depend on the hippocampus, but also in part from cortex; indeed, there are studies showing that perirhinal cortex is crucial for novel-familiar object discriminations and medial prefrontal cortex (mPFC) for object-in-place associational and recency discriminations (Barker et al., 2007; Broadbent et al., 2010; Antunes and Biala, 2012). Additionally, since L-AA was injected in the lateral ventricles, meaning that the solutions injected spread into the CNS through the

cerebrospinal fluid (CSF), it is likely that the entire hippocampal structure was not affected in the same way. As an alternative, another more directed and also more complex hippocampal-dependent test, modified Y-maze, should be performed to check for differences in memory between the two groups of animals (Murray and Ridley, 1999) because if we want to evaluate differences in memory, we need find a task, which is not too easy, or else, all mice will be able to learn. Altogether, these limitations might explain the lack of effect on NOR test, despite the clear effect observed in hippocampal LTP. This gliotoxin was used in a variety of tests where, for instance, L-AA was injected in the PFC of rats and glial ablation was sufficient to induce depressive-like behaviors (Banasr and Duman, 2008). Likewise, other studies showed that injection of the gliotoxin in the dentate gyrus of mice inhibited the contextual fear memory expression after the fear conditioning, suggesting that the presence of astrocytes is critically required for the formation of long-term memory (Choi et al., 2016).

In this work, all experiments were performed to try to gather the most possible information. Therefore, we tried to study metaplasticity in hippocampal slices from mice, where they were incubated with L-AA for 2 h, and also from L-AA/vehicle-injected mice, with a 2 h incubation with the gliotoxin as well. The results showed no significant differences in the percentage of depotentiation, when comparing to the baseline recorded for the last 10 min of the previously induced potentiation; however, L-AA-incubated slices displayed a different profile of excitability when comparing to control slices (see figure 21B). Regarding the slices from L-AA or vehicle-injected animals and further incubation with L-AA, both conditions displayed a similar and significant depotentiation when compared with the baseline (see figure 30B). Unfortunately, a necessary control to this study is missing, namely the induction of LFS in slices from L-AA/vehicle-injected mice only exposed to aCSF to evaluate how injected animals respond to these stimulations *per se*. Astrocyte-mediated metaplasticity may be a particularly important concept in pathological conditions, as astrocytes alter their physiology drastically in response to injury or disease states (Sofroniew and Vinters, 2010; Sofroniew, 2015).

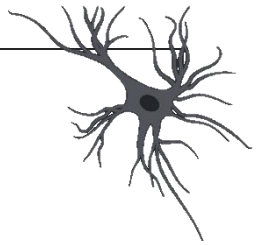
In this work, we also performed immunohistochemical assays to evaluate changes in astrocytic markers of hippocampal slices from L-AA/vehicle-injected mice and also from naïve animals where slices were incubated for 2h with the gliotoxin. Briefly, a two hour-incubation with the gliotoxin was not sufficient to induce changes neither in the number of astrocytes (quantified as the number of GFAP-positive cells) nor in glutamine

synthetase (GS) immunoreactivity (see figure 31). In contrast, in slices from L-AA-injected mice we detected a significant decrease in the number of GFAP-positive cells (-24 % compared to control), and there were no apparent alterations in GS immunoreactivity (see figure 32). Our results are in line with other studies showing that L-AA is able to trigger alterations in astrocytic markers, such as GFAP, however these studies are focused on other brain regions, such as prefrontal cortex and dentate gyrus and in different rodents, resulting in great variations in GFAP immunoreactivity, ranging from 20% to almost 100 % reduction (Banasr and Duman, 2008; Lima et al., 2014; Choi et al., 2016). On the other hand, Saffran and Crutcher (1987) reported that there was no evidence of astrocytic death at any of the concentrations used when they injected bilaterally L-AA into the dentate gyrus (Saffran and Crutcher, 1987). All these results showed different effects of L-AA *in vivo*, suggesting that astrocytes are more or less sensitive to the gliotoxin according to their location in the brain. This idea is plausible given that astrocytes are a heterogenous population of cells and have been shown to transport glutamate (L-AA is taken by astrocytes through glutamate transporters) in different amounts depending on the their position (Drejer et al., 1982; Balcar and Yi Li, 1992).

Astrocytes are multi-functional cells with pleiotropic effects on neuronal processes. Clearly, several experiments are required to end this work. Nevertheless, on the basis of the aforementioned results, we presented strong evidence for the role of astrocytes in synaptic plasticity and hope to continue to study their role in memory formation in both physiological and pathological conditions.

Chapter **6**

Conclusions



6.1. Highlights

- L-AA functions as a specific gliotoxin both *in vitro* and *in vivo*, inducing a similar depression in hippocampal long-term potentiation (LTP);
- L-AA administration in mice decreases the number of GFAP-positive cells in hippocampal sections;
- Astrocytes are indispensable for long-term potentiation (LTP) in the mouse hippocampus;
- Astrocytic blunting may impair memory processes;
- Adenosine A_{2A} receptors seem not to have apparent function when astrocytes are blunted;
- Astrocytes may mediate certain forms of metaplasticity;
- Astrocytes have great potential as therapeutic targets in diseases associated with memory deficits.

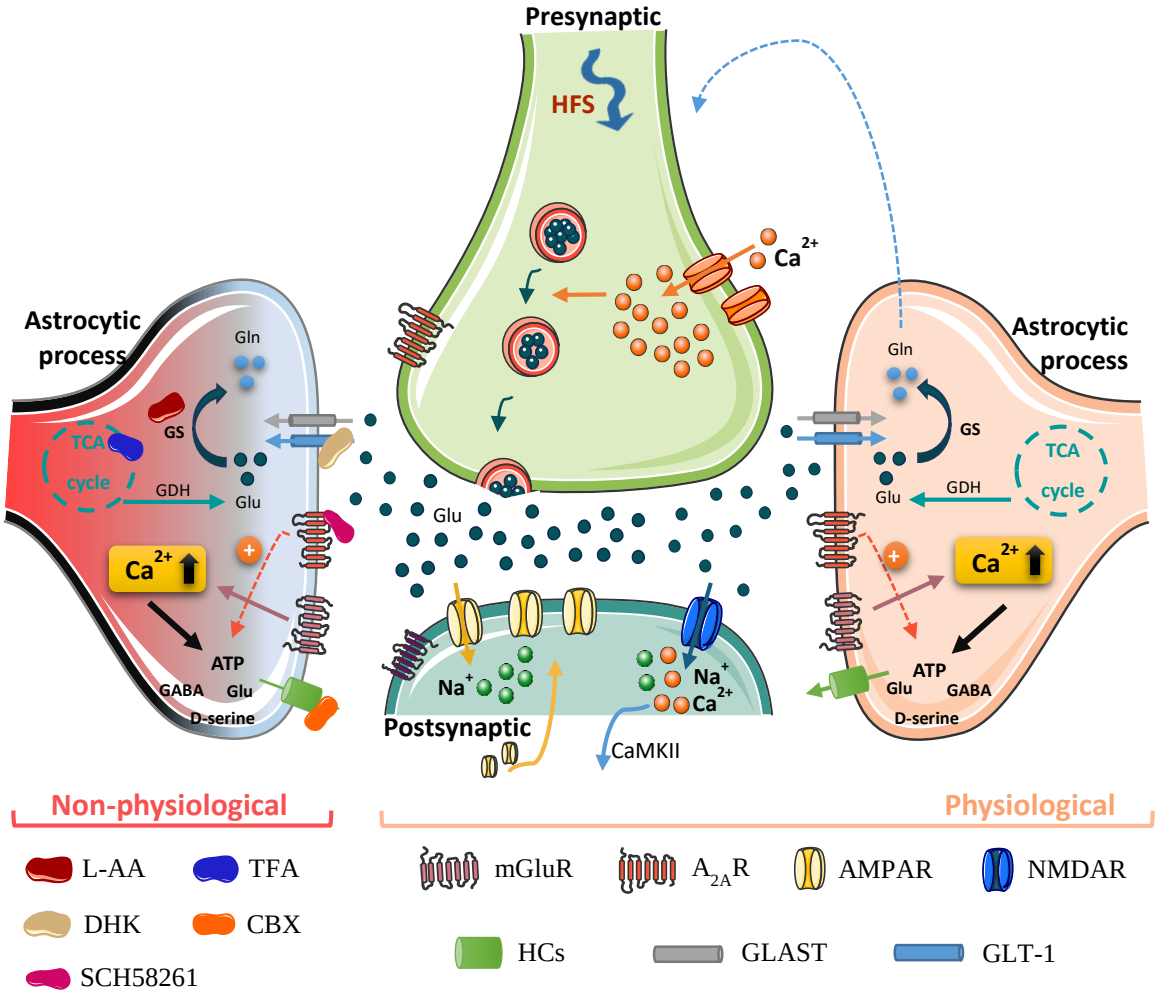
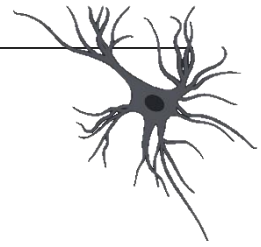


Figure 33 | Under physiological conditions, astrocytes sense and control synaptic transmission through the uptake and release of gliotransmitters. After tetanic stimulation, astrocytes control LTP (the mechanisms underlying LTP are described in section 1.3.2.). LTP is reduced when we blunt astrocytic function with: i) L-α-aminoadipic acid (L-AA), a specific gliotoxin, which is taken up by astrocytes through sodium-dependent glutamate transporters, and once inside astrocytic cells are capable of inhibit glutamine synthetase (GS); ii) trifluoroacetic acid (TFA), another specific gliotoxin, which is taken up by astrocytes and inhibits the Krebs cycle enzyme aconitase, leading to a depression in astrocytic function; iii) dihydrokainic acid (DHK), a selective GLT-1 blocker, prominent GluT in astrocytes responsible for glutamate (Glu) uptake and iv) carbenoxolone (CBX), blocker of HC and/or gap junction, compromising gliotransmission through HCs. Adenosine A_{2A} receptors, expressed both in neurons and astrocytes, were blocked using a selective antagonist (SCH58261).

Chapter 7

References



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