The impact of astroglial dysfunction on excitatory synaptic transmission in neuropathological conditions: the epileptic hippocampus

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I N F O R M E D E A P R O B A C I O N

T E S I S D E D O C T O R A D O

Se informa a la Facultad de Ciencias que la Tesis de Doctorado presentada por:

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A mi madre, por todo.

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ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AD	After-discharge
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor
AP	Action potential
ATP	Adenosine triphosphate
A_{2A}R	Adenosine A_{2A} receptor
BLA	Basolateral amydala nucleus
CA1-CA3	*Cornu ammonis* area 1-3
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
EEG	Electroencephalography
ER	Endoplamatic reticulum
ESPC	Excitatory postsynaptic current
eEPSC	Evoked excitatory postsynaptic current
FT	Fast Ca^{2+} transients
GABA	γ-Aminobutyric acid
GPCRs	G-protein coupled receptors
GFAP	Glia fibrilar acidic protein
IP3	Inositol 1,4,5-triphosphate
IP3R	Inositol 1,4,5-triphosphate receptor

LTD	Long-term depression
LTP	Long-term potentiation
mEPSC	Miniature excitatory postsynaptic current (TTX-resistant)
meEPSC	Minimal evoked excitatory postsynaptic current
mGluR	Metabotropic glutamate receptors
mRNA	Messenger ribonucleic acid
MI	Multiplicity index
NMDAR	N-methyl-D-aspartate glutamate receptor
PAR1	Protease-activated receptors
PPF	Pair pulse facilitation index
P2YR	P2Y-subtype of purinergic receptor
P2XR	P2X-subtype of purinergic ionotropic receptor
RK	Rapid kindling protocol
sEPSC	Spontaneous excitatory postsynaptic current
SE	*Status epilepticus*
SO	*Stratum oriens*
SR	*Stratum radiatum*
SR101	Sulforhodamine 101
ST	Fast Ca^{2+} transients
TLE	Temporal lobe epilepsy
TNFα	Tumor necrosis factor-alpha
TRP	Transient receptor potential

DRUGS

DHPG	(*S*)-3,5-Dihydroxyphenylglycine
GDPβS	Guanosine 5′-[β-thio]diphosphate trilithium salt
LY367	(LY367385) (1)-2-methyl-4-carboxyphenylglycine
MPEP	2-methyl-6-phenylethynyl-pyridine
MRS2179	2'-Deoxy-N6-methyladenosine 3', 5'- bisphosphate tetrasodium salt
PPADS	Pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid tetrasodium salt
PTX	Picrotoxin
TTX	Tetradotoxin

CONTENTS

ABSTRACT

Astroglial cells are essential components of brain machinery. Indeed, astrocytes modulate synaptic transmission, neuronal excitability and plasticity in healthy brain, through the Ca^{2+}-dependent release of neuroactive substances, process referred to as gliotransmision. Despite that altered astroglial physiology has been observed in several neuropathological conditions including epilepsy, whether the astroglial Ca^{2+}-dependent modulation of synaptic transmission is also altered in such pathologies remains poorly understood. By using a chronic model of epilepsy, the kindling, we investigated how astroglial physiology is affected by the epileptogenesis induction, and what is the functional impact of altered astroglial physiology on neuronal transmission. Because spontaneous astroglial Ca^{2+}-mediated glutamate gliotransmission is believed to modulate the hippocampal excitatory synaptic efficacy, spontaneous astroglial Ca^{2+} elevations as well as CA3-CA1 synapses electrophysiological properties were recorded from control and epileptic rats. Astrocytes from epileptic slices display slow spontaneous Ca^{2+} transients and higher frequency of glutamate gliotransmission -evaluated the astrocyte-dependent slow inward currents (SICs) recorded form CA1 neurons- than control slices. CA1 SC-evoked, spontaneous and miniature excitatory postsynaptic currents (eEPSC, sEPSC and mEPSC respectively) from epileptic slices showed an increased synaptic efficacy compare to control slices. The increased mEPSC frequency with no changes in mEPSC amplitude, the lowered pair-pulse facilitation index (PPF) and the increased number of successful responses evoked by minimal stimulation (meEPSC) obtained in epileptic slices suggest that the elevated excitatory synaptic efficacy was mainly mediated by an increase in the probability of neurotransmitter release (Pr). Remarkably, when astroglial Ca^{2+} signal were blocked by the intracellular dialysis of BAPTA, there was a strong decrease on the synaptic efficacy of CA3-CA1 synapses from epileptic slices down to control values. P2Y purinergic receptors and group I

glutamatergic metabotropic receptors (mGluR) antagonists also produced a decrease of the Pr. Specifically, the data suggests that P2Y1R are involved in the astroglial Ca^{2+}-signal required for gliotransmission; and mGluR5 presynaptic receptors directly modulate neurotransmitter release. These findings showed that astroglial Ca^{2+}-signaling is increased in the epileptic tissue strongly impacting synaptic function, which likely contribute to the pathophysiology of epilepsy.

INTRODUCTION

The classic vision of astrocytes as the supporting cells of the central nervous system has become obsolete. Nowadays, astrocytes are considered as active modulators of synaptic transmission that display Ca^{2+}-based excitability leading to the release of neuroactive substances, a process referred to as gliotransmission. Despite the evidence suggests that astrocytes play a key role in the modulation of synaptic physiology, deeper knowledge is required to better understand the impact of astrocyte signaling in neuronal function and behavior in healthy and pathological conditions. In this study, we investigate whether and how astroglial physiology and gliotransmission are affected in the epileptic brain, as well as the impact on the excitatory synaptic transmission of the hippocampal formation.

I Astroglial physiology

Astrocytes represent the most abundant type of glial cells in the central nervous system (CNS). The widely recognized neuroanatomist Santiago Ramón y Cajal was the first who described the classic astroglial anatomy by applying Golgi's technique, a state-of-the-art technology in the latest nineteenth century (Fig. 1), and also the first who suggested the physiological relevance of these cells for neuronal working (Ramon y Cajal 1913). The great technological advance of the last decades confirms Cajal's early observations. Astrocytes present a characteristic star-shape morphology with small somas and numerous branches and processes allowing them to be in close anatomical relation with synapses, blood vessels and other astrocytes (Bushong et al., 2002; Theodosis et al., 2008). Many functions have

been attributed to astrocytes, from neurogenesis to the regulation of brain homeostasis. Classically, astrocytes are thought to tune synaptic activity by controlling extracellular [K+] and the clearance and recycling of neurotransmitters as glutamate from the synaptic cleft (Kimelberg and Nedergaard, 2010). However, growing evidence suggests that astrocytes modulate directly synaptic transmission (Jourdain et al., 2007; Perea and Araque, 2007), plasticity (Henneberger et al., 2010; Min and Nevian, 2012a) and neuronal excitability (Fellin et al., 2004) by the active release of neuroactive substances. While astrocytes are electrically unexcitable cells, they display both spontaneous and neurotransmitter-evoked elevations of intracellular [Ca^{2+}] (Cornell-Bell et al., 1990). These Ca^{2+} signals were first observed in cultures where the mechanical stimulation of one astrocyte produced an elevation of cytoplasmatic [Ca^{2+}] that propagates to neighboring astrocytes resulting in a

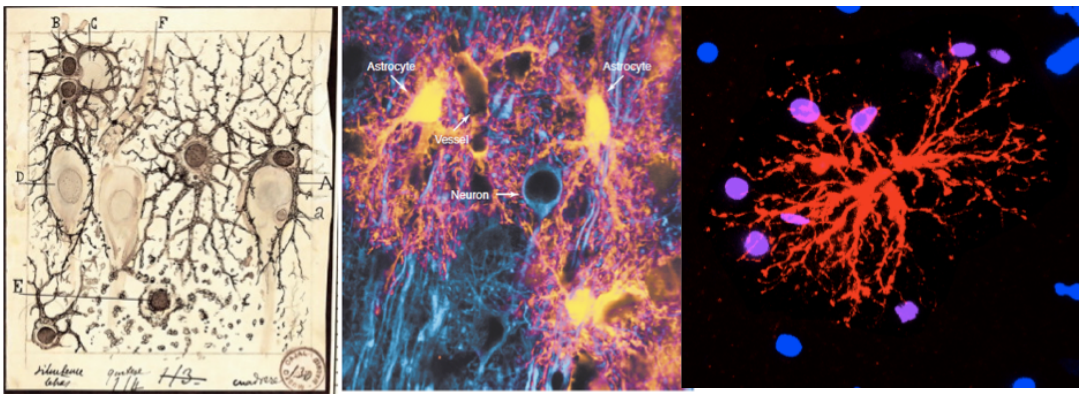

Figure 1. The complex morphology of astrocytes. (Left) The neuroanatomist Santiago Ramón y Cajal was the first who described the astroglial anatomy and the tight relation of astrocytes with blood vessels and neurons by taking advantage of Golgi potassium dichromate/silver staining method (Golgi, 1895). (Middle) Lastest microscopy techiques futher reveal the anatomical relation of astrocytes with neurons and vessels. Neurons are labeled with an antibody to microtubule-associated protein 2 (MAP-2; blue), whereas astrocytes are expressing enhanced green-fluorescent protein (eGFP; yellow) (Nedergaad et al., 2003). *(*Right) Confocal Z-stack reconstruction of an hippocampal astrocyte loaded by intracellular dyalisis of Alexa-594 (red) surrounded by other Hoecht-labeled cellular nuclei (blue) obtained in our lab.

Ca^{2+} wave (Cornell-Bell et al., 1990). Later, both evoked and spontaneous intracellular Ca^{2+} elevations were also confirmed *in situ* - in slices (Aguado et al., 2002; Nett et al., 2002) and *in vivo* (Navarrete et al., 2012))- in multiple brain areas including cortex and hippocampus (Hirase et al., 2004). Because one consequence of intracellular Ca^{2+} elevations or Ca^{2+} transients is the release of neuroactive substances that may modulate neuronal function

(Araque et al., 2000; Fellin et al., 2004), astrocytes are nowadays considered as active partners of synapses regulating information transfer between neurons. The discovery of the impact of astroglial function on synaptic properties led to coin the term *tripartite synapse* (Araque et al., 1999) which refers to astrocytes as the third active element of synapses in addition to the pre and postsynaptic neuron, where intracellular Ca^{2+} elevations represent the substrate of astroglial excitability (Agulhon et al., 2008; Haydon, 2001).

II Tripartite synapses

Tripartite synapses have been described in most encephalic structures including cerebral cortex, cerebellum, and hippocampus. In this type of synapses, astrocytes and neurons establish a complex and specialized bidireccional communication. Specifically, astrocytes detect synaptic activity through receptors adapted to the type of synapses they interact with. In parallel, astrocytes release substances widely known as gliotransmitters that modify neuronal properties through the activation of specific receptors present in the pre and postsynaptic membrane. Gliotransmitters represent a great variety of substances including neurotransmitters (i.e., glutamate, GABA), neuromodulators (i.e., endocannabinoids) and neurotrophines (i.e., BDNF) usually attributed to neuronal release. There are still some controversies about the effective neuron-astrocyte communication. Even though astrocytes are in close proximity with neurons, the distance to the synaptic cleft and the neurotransmitter recapture mechanisms are claimed to represent a limitation to effective signaling. However, both astrocytes and neurons express metabotropic receptors that have been described as high-affinity slowly desensitizing receptors (Araque et al., 2002; Panatier et al., 2011), indicating that astrocytes express a specialized machinery to effectively perceive neuronal signaling just as neurons do the other way around (Fig. 2).

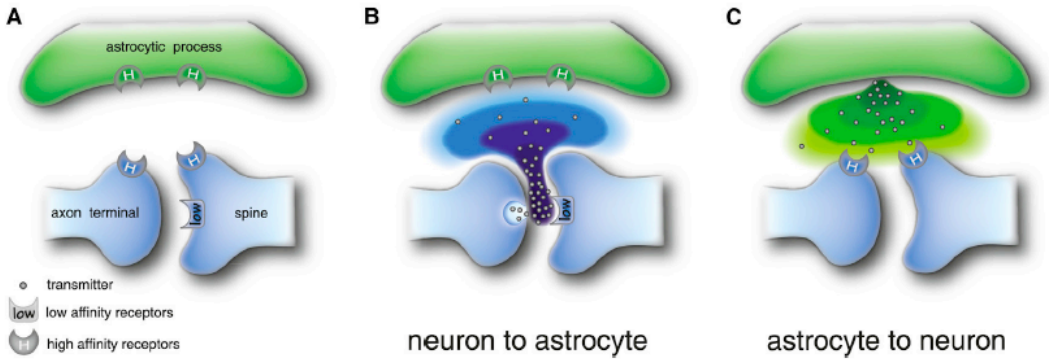

Figure 2. Tripartite synapses. A) Schematic illustration of tripartite synapses, showing the pre (axon terminal), postsynaptic element (spine) and the astrocytic process. B) Neurotransmitter concentration drops away from the synaptic cleft, but the high affinity receptors (H) present in astroglial membrane allow astrocyte to detect low levels of neurotransmitters. C) The activation of astroglial H produces Ca^{2+} signals that, in turn, lead to the release of neuroactive substances or gliotransmitters, that activate neuronal H receptors situated in extrasynaptic loci. (Araque et al., 2014)

III Astroglial Ca^{2+} signaling

The activation of specific G-protein coupled receptors (GPCRs) located at the astroglial membrane, mainly through IP3 production and Ca^{2+} release from intracellular stores (Verkhratsky et al., 1998); or the activation of ion channels also present in the astrocyte membrane produces characteristic Ca^{2+} transients that can cause the release of neuroactive substances depending on the ligand and the subtype of receptor activated (Shigetomi et al., 2008). GPCRs are activated by several extracellular signals as neurotransmitters, neuromodulators or substances released from other astrocytes among others. Diverse astroglial Ca^{2+} elevations according to Ca^{2+} source (intra or extracellular), their spatiotempotal properties and mechanisms involved in their generation have been described. Regarding the diversity of Ca^{2+} signaling and the functions of astrocytes, a current issue is to determine whether different Ca^{2+} signals carry out specific functions. Thus, it is highly required to explore the mechanisms involved in Ca^{2+} signal generation, the diversity of Ca^{2+} signals and their consequences on brain physiology.

a. Ca^{2+} sources in astrocytes

Diverse Ca^{2+} signals have been related to diverse Ca^{2+} sources. There are three major Ca^{2+} sources in astrocytes: 1) The endoplasmatic reticulum (ER) mostly mediated by inositol-1,4,5-phosphate receptors (IP3R, subtype 2 mainly) and ryanodine-cafeine sensitive stores; 2) The extracellular space mediated by store-operated receptors as TRP channels; and 3) Mitochondria, which play a major role in Ca^{2+} buffering (Verkhratsky et al., 2012).

Hippocampal somatic global Ca^{2+} are mainly mediated by Ca^{2+} release from intracellular IP3R2-dependent stores, since are largely reduced in IP3R2 KO mice (Srinivasan et al., 2015). However, in those KO mice there are IP3R2-independent Ca^{2+} elevations in astrocytic branches mainly mediated by Ca^{2+} from extracellular space. For example, the transient receptor potential A1 (TRPA1), a non-selective cationic channel, has been shown to mediate the spotty Ca^{2+} signal in microdomains and to set the resting [Ca^{2+}] in hippocampal astrocytes *(Shigetomi et al., 2012)*. In *Stratum lucidum* (SL) astrocytes from wild-type mice, nominally Ca^{2+}-free buffer decreased both waves and microdomain Ca^{2+} elevations within astroglial processes, whereas global and somatic signals remain unchanged (Srinivasan et al., 2015). Interestingly, these extracellular Ca^{2+}-dependent signals are mediated by non-TRPA1 receptors, suggesting a regional specificity of Ca^{2+} signals. Taken together this evidence shows that different Ca^{2+} sources are in charge of diverse Ca^{2+} signals within the same astrocyte. Moreover, the diversity of receptors mediating Ca^{2+} elevations seems to be characteristic from each brain area, which increase the complexity of astroglial signaling as discussed below. However**,** whether synaptic alterations related or not to neuropathologies, may affect the patterns and kinetics of Ca^{2+} elevations or vice versa remains poorly understood.

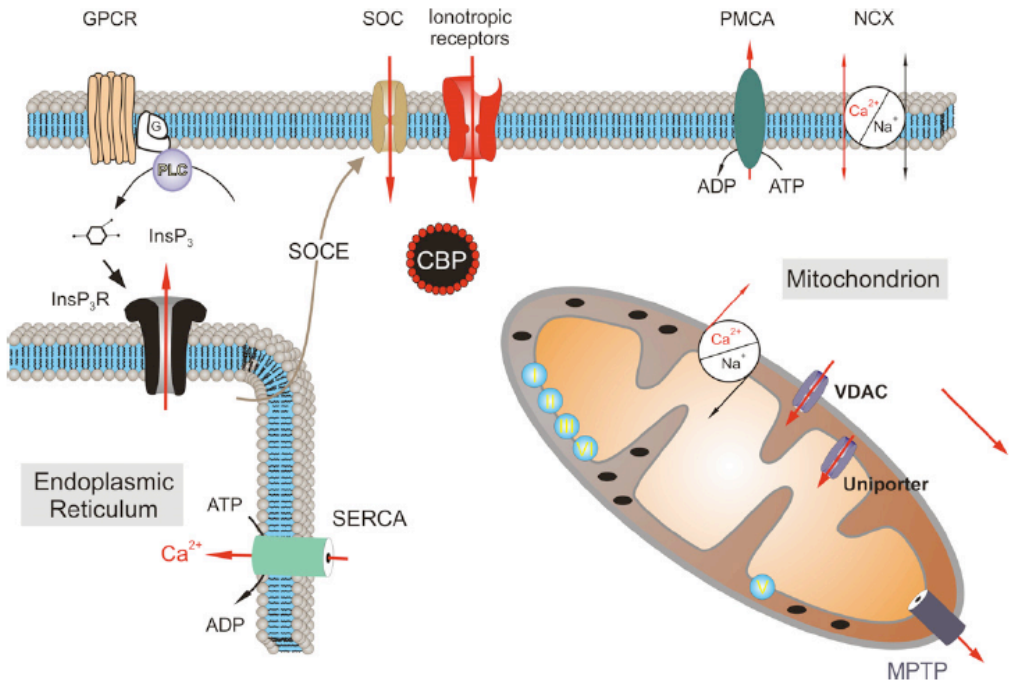

Figure 3. Principles of astroglial Ca^{2+} signaling. Schematic drawimg showing the main Ca^{2+} stores in astrocytes. The activation of GPCRs leads to PLC activation and the production of IP3 that activates InsP3R present in the endoplasmatic reticulum membrane triggering the Ca^{2+} release to the cytoplasm. The activation of store opened channels (SOC) and other ionotropic receptors present in the astroglial membrane also contribute to intracellular Ca^{2+} elevations. Whereas mitochondrions are closely related to astroglial Ca^{2+} buffering they also contribute to cytoplasmatic Ca^{2+} elevations through transient opening of the mitochondrial permeability transition pore (MPTP) and the functioning of the Ca^{2+}/Na+ exchanger also present in the astroglial membrane (Verkhratsky et al., 2012).

b. Diversity of Ca^{2+} signals in astrocytes

Diverse types of intracellular Ca^{2+} elevation have been observed in astrocytes according to their spatial-temporal properties, of which the main types described are: spontaneous Ca^{2+} elevations confined to strict microdomains of astrocytic branches, partially action potential (AP) independent; local waves that spread to several microdomains and encompasses to the entire branches and occasionally the soma; and spontaneous somatic Ca^{2+} elevations (Di Castro et al., 2011; Srinivasan et al., 2015). These Ca^{2+} signals have been differentially observed in several areas triggered by specific physiological events. In the hippocampus, CA1 and DG astrocytes display both global and local Ca^{2+} events restricted to branches. The Ca^{2+} activity restricted to microdomains is produced in response to single vesicle release of

neighboring synapses and it is thought to be relevant for the basal synaptic function (Di Castro et al., 2011; Panatier et al., 2011). Otherwise in *SL* and CA3 astrocytes, Ca^{2+} signals triggered by one vesicle-release are undetectable. Since CA3 and *SL* astrocytes only respond to intense neuronal firing, they are believed to control neuronal network synchronization (Haustein et al., 2014; Srinivasan et al., 2015). Therefore, astrocytes seem to be physiologically specialized to each microcircuit exerting distinct functions even within the same structure.

Interestingly, it has been shown recently that astrocytes *in vivo* also respond to high-level circuit activity between separate brain areas by changing the Ca^{2+} signaling. Bergman glia and cortical astrocytes display local and somatic spontaneous Ca^{2+} transients, but respond with global Ca^{2+} signal during locomotion, startle reflect and electrical stimulation of Locus Coeruleos (LC) that mediates sensory inputs to cortex (Bekar et al., 2008; Kerr and Nimmerjahn, 2012; Srinivasan et al., 2015). The change in the astroglial Ca^{2+} signaling mode from disorganized spontaneous events to global Ca^{2+} elevations -mediated by α-adrenergic receptors- suggests that astrocytes from cortex and cerebellum respond according to the brain state locked to sensorial inputs. These findings suggest that astroglial Ca^{2+} signaling is not only relevant to microcircuit function but also important for the integration of coordinated activity between separate areas.

To increase the computational ability of astrocytes, the activation of determined GPCRs produces specific intracellular Ca^{2+} transient dynamics. Indeed, the multiple and varied patterns and kinetics of Ca^{2+} elevations (i.e. changes in amplitude, duration or frequency of Ca^{2+} elevations) depends on the synaptic system involved (Perea and Araque, 2005), the brain area and the frequency and intensity of synaptic activity (Haustein et al., 2014). For example, the synaptically evoked release of glutamate from Shaffer collaterals (SCs) and acetylcholine (ACh) from alveus hippocampal synapses produces different astroglial Ca^{2+} signals when their release is stimulated separately or simultaneously (Perea and Araque, 2005), suggesting that astrocytes may decode and integrate different synaptic activities producing appropriate responses according to network requirements.

Altogether, this evidence suggests that astrocytes are able to decode neuronal activity since specific Ca^{2+} signals occur in response to specific inputs and physiological events, and they are mediated by different mechanisms in each brain area. However, how astrocytes encode different signals to generate specific physiological responses (i.e. the specific release or not of different neuroactive substances) remains widely unknown. Thus, it is highly required to investigate the specific consequences of Ca^{2+} signals for astroglial physiology and pathophysiology to gain a better understanding of the role of astrocytes in brain function.

IV Inter-astrocyte communication

Astrocytes also communicate with other astrocytes by both extracellular and intracellular pathways. Actually, they form extensive GAP junction-mediated astroglial networks (mostly constituted by Cx43 and Cx30), which allow the intracellular communication by the passing of small molecules (i.e. IP_3), ions (i.e. Ca^{2+}) and neuroactive substances (i.e. ATP) that also might mediate the propagation of Ca^{2+} waves. Although the astroglial coupling function was initially thought to provide metabolic support, ion and neurotransmitter homeostasis, it is now showed to modulate the activity of neuronal circuits too. Indeed, the propagation of the Ca^{2+} signal through the astroglial network has been hypothesized to underlie hippocampal heterosynaptic depression required for learning and memory (Serrano et al., 2006). In addition, constitutive Cx43 and Cx30 KO have severe behavioral consequences (Pannasch et al., 2011), suggesting that astroglial networks may play a role key role in brain physiology by modulating neuron-glial communication and then contributing to local and distal neuronal activity (Pannasch et al., 2011).

Otherwise, ATP signaling represents the main extracellular pathway for astrocyte-astrocyte communication through the activation of purinergic receptors P2X and P2Y (Cotrina et al., 2000), which also affects to astroglial-neuron signaling. Specific P2Y1Rs activation produces a Ca^{2+} signal in astrocytes that triggers the release of astroglial glutamate (Domercq et al.,

2006), which increases the synaptic strength of dental granular cells through the activation of presynaptic NMDARs (Jourdain et al., 2007). Remarkably, ATP is also released by connexin-forming hemichannels (Orellana et al., 2012; Stout et al., 2002), thus providing an additional role for connexins for extracellular communication beyond their role in GAP junction formation. According to this idea, it has been shown recently that Cx30 hemichannels regulate the morphology of astroglial processes controlling their insertion into the synaptic cleft, which result to be important to effective astroglial signaling (i.e., gliotransmission) in setting the basal synaptic strength (Pannasch et al., 2014). Therefore, connexin-forming hemichannels or GAP junction and ATP signaling result essential for astrocyte-astrocyte communication, which in turn modulates neuronal and synaptic physiology.

Altogether, these findings indicate that astrocytes are not only local but also global modulators of synaptic physiology throughout the brain. Nevertheless**,** it is not fully understood how astrocyte-astrocyte communication changes during several physiological events or pathological processes, and how this could affect to astroglial excitability and synaptic transmission.

V Astrocyte-neuron signaling: the role of gliotransmission

Astrocytes modulate synaptic transmission through the release of gliotransmitters. Gliotransmitters are released into extracellular space by using several different mechanisms:

1) Through ion channels induced by cell swelling (Pasantes Morales and Schousboe, 1988), conexines or panexines hemichannels (Cotrina et al., 1998; Iglesias et al., 2009) and ionotropic purinergic receptors (P2X, (Duan et al., 2003)).

2) Through the reversal uptake of astroglial transporters (Szatkowski et al., 1990)

3) Through Ca^{2+}-dependent release via exocytosis (Bezzi et al., 2004; Parpura et al., 1994), on which we will focus in our study.

Once released, gliotransmitters act onto receptors situated in other astrocytes or in the pre and postsynaptic membrane, thus affecting to different aspect of neuronal function and synaptic transmission. Multiple gliotransmitters have been reported to affect synaptic physiology in addition to glutamate such as ATP, D-serine and TNF (Araque et al., 2014). ATP, which provides the main energetic source for intracellular processes, also acts as intercellular signaling molecule. ATP mediates intercellular signaling directly by acting onto purinergic receptors (P2Y or P2X receptors) mainly expressed in astroglial membrane, therefore it is believed to represent the main pathway mediating inter-astrocyte communication (Cotrina et al., 2000). Otherwise, once released ATP is rapidly converted into adenosine by the action of extracellular enzymes. Adenosine itself also modulates excitatory synaptic transmission acting on A1 or A2A presinaptic receptors, depressing or enhancing the glutamate release respectively (Panatier et al., 2011; Pascual et al., 2005). Another important gliotransmitter for synaptic modulation is the D-serine, which is tonically released by astrocytes and required for NMDAR-mediated synaptic plasticity (Henneberger et al., 2010). Despite the high variety of gliotransmitters, in this work we focus on glutamate gliotransmission, known to occur spontaneously in the hippocampus setting the level of excitatory synaptic transmission.

a. Glutamate gliotransmission

Pioneer works described that the experimentally evoked somatic Ca^{2+} signals produce the exocytotic release of glutamate from hippocampal astrocytes, which affects to both pre and postsynaptic elements of glutamatergic synapses. At postsynaptic level, evoked astroglial glutamate produces the appearance of slow inward currents (SICs) in pyramidal neurons, which are mediated by the activation of extrasynaptic NR2B-containing NMDA receptor (Angulo et al., 2004; Fellin et al., 2004). These early works showed that SICs might synchronize neuronal activity. Interestingly, not every signal that produces a Ca^{2+} elevation

entails the appearance of SICs (Shigetomi et al., 2008), suggesting the specific astroglial glutamate release in response to appropriate signals.

At presynaptic level, evoked glutamate gliotransmission by Ca^{2+}-uncaged methods or by acute application of the specific GPCRs agonist, regulates the excitatory synaptic transmission by increasing its efficacy, modulating both short and long-term synaptic plasticity in several areas including hippocampus and cortex (Jourdain et al., 2007; Min and Nevian, 2012b; Perea and Araque, 2007). Remarkably, it has been shown that glutamate gliotransmission also occurs spontaneously in hippocampal slices in response to spontaneous Ca^{2+} elevations. This spontaneous glutamate release contributes to setting the basal synaptic efficacy at excitatory synapses as well as the threshold for synaptic plasticity (Bonansco et al., 2011), suggesting the important role of astroglial glutamate in the synaptic physiology. However, whether glutamate-mediated astroglial modulation of synapses could be affected or play some role in diverse pathological processes, either beneficial or deleterious, is still controversial.

VI Astrocytes in neuropathologies

Since astrocytes may profoundly affect to brain physiology and synaptic transmission, it is not surprising that astroglial dysfunction have been related with several neuropathologies including epilepsy (Tian et al., 2005), neurodegenerative diseases (Kuchibhotla et al., 2009) and other neuroinflammatory proccesses. This is because astrocytes respond to aforementioned pathologies and to many other brain injuries through a process known as reactive astrogliosis, which represents indeed a hallmark of brain damage. Reactive astrogliosis encompasses several morphological alterations characterized by hypertrophic soma and processes, spatial overlapping and different functional changes including altered protein expression (Sofroniew and Vinters, 2010). These alterations represent a continuous process of progressive changes that vary with severity of the insult. Although reactive

astrocytes are characterized by well-described morphological changes, physiological consequences are less understood. Still, there is evidence from clinical and experimental studies that reactive astrocytes have the potential to exert detrimental effects. For example contributing to inflammation (Brambilla et al., 2005), releasing potentially excitotoxic glutamate (Ding et al., 2007) or compromising the blood brain barrier (BBB, Argaw et al., 2009), which may contribute to the development of several neuropathologies. There is some evidence suggesting that astroglial Ca^{2+} signaling and gliotransmission are altered in reactive astroglia. Several components involved in astrocyte signaling are deregulated in gliotic conditions including glutamate and purinergic metabotropic receptors (Aronica et al., 2000; Bennett et al., 2012; Franke et al., 2012). In addition, increased Ca^{2+} wave frequency and synchrony have been observed in cortical astrocytes *in vivo* in a mouse model of Alzheimer disease (Kuchibhotla et al., 2009) as well as increased Ca^{2+} signaling during evoked epileptic seizures (Tian et al., 2005) However, the consequences or the possible contribution of deregulated astroglial Ca^{2+} signaling to pathological proccesses is still poorly understood.

a. Astrocytes in epilepsy

An increasing body of evidence has documented the deregulation of astroglial function particularly in epilepsy. Epilepsy is a complex and multifactorial pathological brain condition characterized by hyperexcitable and hypersynchronic state of the neural networks that leads to unpredictable but recurrent epileptic seizures within a local region or that spreads throughout the brain (Goldberg and Coulter, 2013). Altered excitation-inhibition balance is believed to play an important role in the epileptic syndrome (McCormick and Contreras, 2001). As in most of brain injuries, an overall chronic change of the epileptic brain is a prominent astrogliosis (Binder and Steinhäuser, 2006). Initial findings about the role of astrocytes to the pathophysiology of epilepsy focused on the ability of astrocytes to buffer extracellular K^+-through Kir channel expression-, water homeostasis –through water channel aquaporines- and the recycling of glutamate and GABA from extracellular space –through aminoacid transporters-. Altered expression and activity of astroglial Kir4.1 channels

(Bordey and Sontheimer, 1998), aquaporine 4 (Lee et al., 2004) and the excitatory aminoacid transporter (EEAT) have been observed in epileptic tissues suggesting a disruption of the astroglial role in brain homeostasis.

b. Astroglial Ca^{2+} signaling and epilepsy

Because astrocytes modulate both excitatory and inhibitory synaptic transmission through gliotransmission, a deregulation of astrocyte-neuron signaling may alter the excitation-inhibition balance thus also contributing to the expression and establishment of the epileptic syndrome (Wetherington et al., 2008). According to that, altered expression of proteins involved in astroglial Ca^{2+} signaling are deregulated in the hippocampal formation of temporal lobe epilepsy (TLE) models (Aronica et al., 2000). Recent works have shown pro-epileptic features of astroglial signaling. During evoked epileptic-like discharges (also known as ictal discharges) in slices, astrocytes display an increased frequency of Ca^{2+} transients mediated by mGluR5 and P2Y receptor activation (Gómez-Gonzalo et al., 2010; Tian et al., 2005). This evoked Ca^{2+} transients facilitate the propagation of ictal discharges probably by improving excitatory synaptic transmission (Gómez-Gonzalo et al., 2010). Following this idea, antiepileptic drugs reduce both ictal discharges and astroglial Ca^{2+} transients, suggesting the involvement of astrocyte signaling in seizure propagation (Tian et al., 2005). However, what is the precise astroglial mechanism involved in seizure propagation is not completely understood.

Although most of works have focused on the role of astroglial signaling on ictal discharges propagation as mentioned above, the consequences of the epileptogenic process on astroglial function is poorly understood. Epileptogenesis is the process whereby a healthy brain transforms into an epileptic one (Goldberg and Coulter, 2013). This process entails altered functionality of neural networks towards the generation of abnormal electrical activity that promotes chronic seizures. An enhancement of excitatory transmission is believed to underlie the pathophysiology of epilepsy, which is support by the high extracellular glutamate levels (Cavus et al., 2005, 2008) and increased expression of glutamate receptors found in epileptic tissue (Aronica et al., 2000). Since astrocytes can release glutamate, they have

been proposed as non-synaptic source of glutamate in epileptic tissue. In agreement with this, an increase in glutamate gliotransmission after the status epilepticus (SE) has been shown to contribute to neuronal death related to SE (Ding et al., 2007). However, whether astroglial signaling could be chronically affected during epileptogenesis is poorly understood. Moreover, since astrocytes modulate the efficacy of excitatory synaptic transmission through the Ca^{2+}-dependent release of glutamate (Bonansco et al., 2011; Perea and Araque, 2007), deregulation of astroglial-neuron communication could be produced as a part of the alterations caused by the epileptogenic process. Thus, by using the kindling model, which reproduces the progression of epilepsy, we asked whether and how astroglial Ca^{2+} excitability and its role in the modulation of excitatory synaptic transmission are affected during epileptogenesis. In particular, we tested whether the astrocyte-neuron signaling pathway may be altered in the epileptic brain. We propose that increased astrocyte-neuron signaling could contribute to enhancing the excitatory tone of the epileptic brain and ultimately predispose neurons to seizures.

Summary points

- Astrocytes modulate the synaptic efficacy of the excitatory synaptic transmission by the Ca^{2+} dependent release of glutamate namely glutamate gliotransmission.
- Morphological, functional and molecular alterations of astrocytes have been related to several neuropathological conditions, especially to epilepsy.
- Different proteins involved in astrocyte-neuron signaling are upregulated in the epileptic tissue, from both human and animal models, including purinergic and glutamatergic receptors.
- During evoked epileptic-like discharges in slices, astrocytes display an increased frequency of Ca^{2+} transients, which are strongly reduced by antiepileptic drugs, suggesting an involvement of astrocytes in the propagation of epileptic discharges.

Since we showed that spontaneous glutamate gliotransmission sets the basal synaptic efficacy of excitatory synaptic tranmission of hipocampal synapses (Bonansco et al., 2011), we asked whether spontaneous glutamate gliotransmission is altered in the epileptic hippocampus and if so how this affects the basal excitatory synaptic transmission.

HYPOTHESIS

Our general hypothesis is that **astroglial Ca^{2+} excitability and glutamate gliotransmission are altered in the epileptic hippocampus affecting the basal excitatory synaptic efficacy thus contributing to the high excitation level characteristic of epileptic tissue.**

GOALS

Our general goal is to determine the functional alteration of astroglial excitability and glutamate-mediated gliotransmission and the consequences for excitatory synaptic efficacy in the hippocampus of epileptic-like rats.

SPECIFIC AIMS

1.Examine the pattern of astroglial spontaneous Ca^{2+} elevations and the glutamate-mediated gliotransmission in control and epileptic hippocampus.

2. Determine the effects of altered glutamate-mediated gliotransmission on the glutamatergic synaptic efficacy of CA3-CA1 synapses from epileptic hippocampus and control hippocampus

2.1 Evaluate quantal parameters of spontaneous and evocated glutamatergic neurotransmission of CA3-CA1 synapses.

2.2 Assess the contribution of astroglial Ca^{2+} signaling in the modulation of excitatory synaptic transmission.

3. Determine the precise cellular pathway of astrocyte-neuron communication in the epileptic hippocampus.

METHODS

The procedures of animal care; surgery and recording were in accordance with the guidelines laid down by the Institutional Animal Care and Ethics Committee at the Faculty of Sciences, *Universidad de Valparaíso* (DIUV Law 20380, Chile) and NIH (USA).

I. Kindling model of epilepsy

a) Justification of the animal model

We used Sprague-Dawley rats of 35-45 postnatal days that were subjected to the epilepsy induction. Specifically, we used the kindling protocol. This protocol has been widely used as a chronic model to induce epileptogenesis in several structures, including cortex and hippocampus, which mimics the progression of seizures and epileptiform activity spreading throughout the brain (Goddard, 1983) and resemble some of the plastic and progressive changes observed in temporal lobe epilepsy (TLE). Kindling protocol consists in repetitive electrical stimulation of subcortical areas (epileptogenic focus) that elicits gradual and progressive epileptic-like discharges and convulsive behavior, culminating in generalized seizures. One advantage of kindling is that allow the control of the epileptogenic focus, where the stimulation electrode is implanted. In order to avoid any effect resulting from the direct local lesion due to electrode implantation in the hippocampus, we decided to induce fully kindled state by chronic stimulation in the basolateral amygdala (Rebola et al., 2003) Specifically, we employ a new variant of rapid kindling protocol (RK) developed in our laboratory, recently published in Morales and colleagues (2014) briefly described bellow.

b) **Stereotaxic surgery**

Briefly explained, male Sprague-Dawley rats (35 postnatal days) were anesthetized and subjected to stereotactic surgery procedures for electrode implantation. The surgical procedures for electrode implantation were as previously described (Corcoran et al., 2011; Greenwood et al., 1991) with minor modifications. The stimulation electrode was implanted in the right basolateral amygdale complex (BLA). Two pairs of electrodes for cortical recording and anchorage were bilaterally implanted in the primary motor cortex (Br. -2.12 mm, ML. ± 2.0 mm, DV. 1.5 mm) and in the visual cortex (Br. -6.12 mm, ML. ± 2.5 mm, DV. 1.5) according to the stereotaxic atlas of Paxinos and Watson, 1998. Following surgery, rats had at least one week of recovery period before the start of the kindling procedure.

c) Kindling protocol

RK protocol consists of ten daily trains of biphasic rectangular current pulses at subthreshold after-discharges (ADs) intensity, for three days. Rats subjected to RK protocol display progressive epileptic activity throughout the brain, assessed by EEGs recordings of ADs, accompanied by severe convulsive behavior evaluated by the well-characterized Racine scale (Racine 1972). Rats were considered as fully kindled when at least three consecutive secondary generalized epileptic seizures (i.e.: Racine stages 4 or 5) were reached (10.0±1.6 number of seizures reached per animal) and long term repetitive ADs occurred (Fig. XX). Under these conditions, cortical and mesolimbic structures of fully kindled rats are considered epileptic tissue, including hippocampal formation (Morales et al., 2014). Although intracellular recordings of CA1 pyramidal neurons from kindled slices showed higher excitability than control slices (Morales et al., 2014), neither spontaneous ictal nor interictal discharges were observed in acute hippocampal slices.

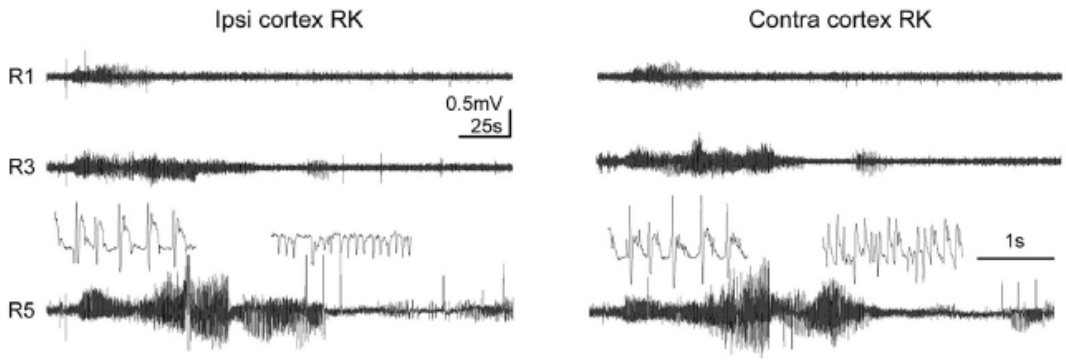

Figure 3. EEG recordings from cortical electrodes during RK protocol. Afterdischarges (AD) simultaneously recorded on ipsi and contralateral cortex during different Racine stage (R1, R3 and R5) showing the spreading of epileptiform activity throughout the brain. Published in (Morales et al., 2014).

The control group consisted of implanted rats that remained non-stimulated (i.e.: sham) or non-implanted rats. All the experiments were performed at the most one week after fully kindled states were reached (i.e.: 45-60 postnatal days). Rats were maintained *ad-libitum* before, during and after all procedures.

II. Electrophysiological recordings of hippocampal slices

a) Hippocampal slice preparation

Acute hippocampal slices were obtained from control and kindled rats as previously described (Bonansco et al., 2002; Fuenzalida et al., 2009). Briefly put, the brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) at 4ºC, and gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). Coronal brain slices (300-350 µm) were cut with a Vibroslice microtome (VSL, WPI, USA) and incubated for 1 h at room temperature (21–24°C) in ACSF containing (in mM): 124.0 NaCl, 2.7 KCl, 1.25 KH2PO4, 2.0 Mg2SO4, 26.0 NaHCO3, 2.5 CaCl2 and 10.0 glucose. In some cases, we prepare the hipocampal slices with chilled sucrose-based cutting solution, which improve slices quality in older animals (in mM): 215.0 sucrose, 2.5 KCl, 20.0 glucose, 26.0 NaHCO3, 1.6 NaH2PO4, 1 CaCl2, 4 MgCl and 4 MgSO4 saturated with 95% O2/5% CO2. Slices were then transferred to an

immersion-recording chamber (2 ml), fixed to an upright microscope stage (FN100 IR; Nikon Inc.; Japan) equipped with infrared and differential interference contrast imaging devices and with a 40X-water immersion objective. Slices were perfused with carbogen-bubbled ACSF (2ml/min) and maintained at room temperature (21-24ºC). All recordings were made in presence of the $GABA_AR$ antagonist picrotoxin (PTX, 10 μM).

b) **Recording, stimulation and analysis in neurons**

Whole-cell voltage clamp recordings using a EPC-7 amplifier (Heka Instruments, Germany) were made from CA1 pyramidal cells voltage clamped at -60 mV, unless otherwise stated, using patch-type pipette electrodes (~3–5 MΩ) containing (in mM): 100.0 Cs-gluconate, 10.0 TEA, 10.0 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, 1.0 $MgCl_2$, 10.0 ethyenebis-(oxonitrilo) tetracetate and 4.0 sodium salt (Na-ATP), pH 7.2 (adjusted with CsOH). When indicated, GDPβS, a non-hydrolysable GTP used to block the intracellular pathway of metabotropic receptors, was added to intracellular solution. Excitatory postsynaptic currents (EPSCs) were elicited at 3 s. intervals, digitally filtered at 3.0 kHz, acquired at 4.0 kHz using an A/D converter (ITC-16; Instrutech, Germany) and stored with Pulse FIT software (Heka instruments, Germany). Experiments started after a 5–10 min stabilization period following the establishment of whole-cell configuration. Cells that exhibited a significant change in access resistance (>20%) were excluded from analysis. Stimulation of Schaffer collaterals was performed using a concentric electrode (platinum／iridium, 125 μm OD diameter; FHC Inc., USA) placed at the *stratum radiatum*. The fibers were activated by bipolar cathodic stimulation (50–100 μs, 0.3–0.5 Hz, 20–100 μA; Master 8, AMPI, Israel) through an isolation unit (Isoflex, AMPI, Israel). We used a paired-pulse protocol to estimate putative presynaptic changes by calculating the paired-pulse facilitation (PPF) index. PPF index was defined as the ratio between the second and the first EPSC amplitude. As described in (Cabezas and Buño, 2006) minimal stimulation of Schaffer collaterals (meEPSC) was elicited using a theta electrode that was placed in the stratum radiatum close to apical dendrites, near the recording pipette (~100 μm). In order to activate single or few SC fibers single square pulses (~20-200 μA; 60μs) were delivered. Synaptic

efficacy was calculated as the mean peak amplitude of all responses, successes and failures, whereas synaptic potency only considered the mean peak amplitude of the successes. The Pr was calculated as the ratio between the number of successes and the total number of stimuli, expressed as percentage of successes or failures as indicated in the text. In most cases, minimal and conventional stimulation was performed simultaneously in the same experiment using the two electrodes (concentric and theta capillary) positioned as mentioned before, in order to stimulate different subsets of fibers. Off-line recording analysis was performed with pClamp software of Molecular Devices (USA).

Spontaneous excitatory postsynaptic currents (sEPSC) and miniature excitatory postsynaptic currents (mEPSC) were recorded from CA1 pyramidal cells without any synaptic stimulation. For mEPSC, tetradotoxin (TTX, 0.5 μM) was added to the ACSF to prevent action potential-dependent neurotransmitter release. Both sEPSC and mEPSC were analyzed off-line using analysis software (Minianalysis, Synaptosoft, USA), which allowed visual detection of events. Only events with amplitudes higher than 5.0 pA, an exponential decay and a monotonic rising phase were considered mEPSCs. In order to compare the synaptic connectivity in kindled and control rats, the multiplicity index (MI), whose values indicate changes in the number of synaptic contacts between CA3-CA1 neurons, was calculated (Fuenzalida et al., 2009; Hsia et al., 1998). Briefly, the amplitude and frequency mean values of sEPSC and mEPSC alone were obtained, recorded respectively before and after adding tetrodotoxin (TTX; 0.5 μM). Multiplicity was calculated as the mean amplitude of action potential-driven events divided by mean quantal size (i.e.: mean amplitude of mEPSC recorded in TTX). MI variations indicate changes in the number of simultaneously releasing active sites onto the recorded neuron.

In order to evaluate the glutamate release from astrocytes, we recorded the slow inward NMDAR-dependent currents (SICs; APV-sensitive). To prevent the Mg^{2+}-blockade of NMDAR without changing the Ca^{2+}/Mg^{2+} ratio, all SICs were recorded at depolarized holding potential (≈+35mV; during brief periods), obtaining slow outward currents, and in presence of

TTX (0.5 μM). Only events with amplitude greater than +50 pA, a rise time slower than 20 ms (tenfold of mEPSC rise time) and APV-sensitive were classified as a SIC (Fig. 5).

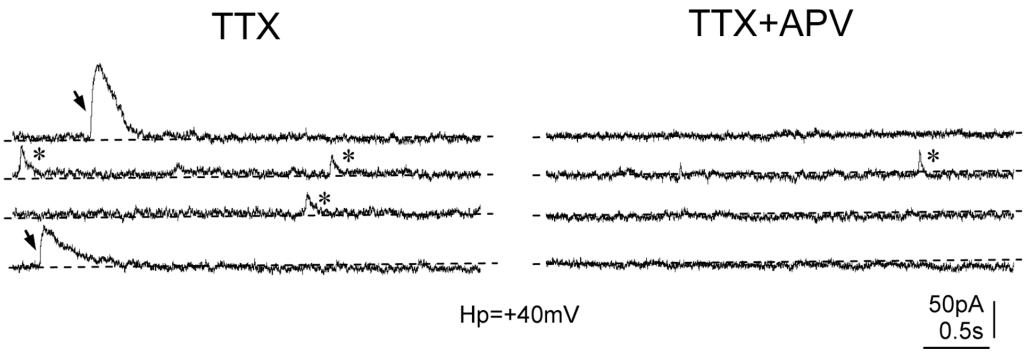

Figure 4. NMDA-mediated SIC in kindled hippocampal slices. Representative traces of spontaneous mEPSC (asterisks) and slow currents recorded as outward currents (arrow) at holding potential of +40mV in presence of TTX (0.5mM), before and after adding APV (50mM). Similar effects were observed in five cells.

c) Intracellular recording and dye loading in astrocytes

We also performed intracellular recordings from astrocytes, which were first identified by their shape and size: round or oval somas of 5-15 μm (Perea and Araque, 2005). Whole-cell recordings were performed with patch micropipettes (5-7 MΩ) filled with control solution that contained (in mM): 97.5 K^+ gluconate, 32.5 KCl, 10.0 HEPES, 1.0 MgCl2 $6H_2O$, 5.0 EGTA and 3.0 Na^+ 2ATP, adjusted with KOH (295-300 mOsm). Astrocytes were maintained at a holding potential of -70 mV and were identified by their current responses induced by holding potential changes and input resistance. Current-voltage curves were performed by applying 10 mV depolarizing steps from -170 to 10 mV with 50 ms of duration. Astrocytes with passive current responses were identified as GluT (Matthias et al., 2003). For the BAPTA experiments, only GluT astrocytes -which are interconnected by gap junctions- were selected, while GluR astrocytes were rejected (Jabs et al., 2008). For BAPTA experiments, 40 mM of the Ca^{2+} chelator were added in the pipette where K^+ gluconate and KCl was equimolarly substituted. In order to assess the BAPTA diffusion area over the astroglial syncitium (Fig. 5), we checked the diffusion of fluorescent dye Alexa Fluor 594 (0.2 - 0.15

mM) or Alexa 488 (0.1-0.2 mM) included in the recording pipette, and continuously monitored it with the fluorescence microscope. Then, the BAPTA-loaded area was estimated as 50μm radius from the patched astrocyte. Those astrocytes patched below 20 minutes were rejected. In some slices, Alexa Fluor 594 images were also obtained in post-fixed slices with a confocal microscope Eclipse 80i Nikon (Three excitation laser: Violet diode 408nm, Ar 488nm and He-Ne 543nm; emission filters: 450/35, 515/30 and 605/75). Z-stack reconstructions were performed each 1 μm with Niss-Element software (Nikon, Japan). In some experiments DAPI was used for nuclear staining.

To check intracellular dye spreading, Alexa 488 (0.1 mM) was included in the intracellular solution. Astrocytes were patched at least for 20-30 minutes, when then dye gets the maximum spreading.

III. Ca^{2+} imaging in astrocytes

To evaluate astroglial excitability, spontaneous intracellular Ca^{2+} variations were measured by monitoring the changes in fluorescence intensity of FLUO4-AM, a commonly used Ca^{2+} sensitive dye, applied by bulk loading. Slices were incubated with FLUO4-AM. To prepare FLUO4-AM, 4μl of DMSO and 3μl of pluronic acid (20% in DMSO) were added to FLOU4-AM vial; 1-2 μl of the dye with was dropped over the hippocampus, obtaining a final concentration of 5-10 μM,) for 45-60 minutes at room temperature. Then, slices were transported to normal ACSF and maintained at least for 30 minutes before the recording of the Ca^{2+} transients. Under these conditions, most of the cells loaded are astrocytes (see Fig. 6 and also in (Perea and Araque, 2005). Slices were previously incubated with the astroglial morphological marker sulforhodamine-101 (SR101; 0.5-1 μM) for 20-30 minutes in low Ca^{2+} (high Mg^{2+}) ACSF at 32-34ºC to check that the cells with fluorescence elevations were indeed astrocytes. A CCD camera Andor DR328G (Andor Technologies plc, Ireland) attached to a Nikon microscope (Nikon, Japan) was used to monitor astrocytes. Cells were illuminated with a xenon lamp at 490 nm; 200-400 ms exposure duration and images were

sampled every second for 5 min per 36700 μm2 area, regulated by a shutter (Lambda SC Smart shutter, Sutter Instrument Company). We used Niss-Elements AR 3.2 (Nikon, Japan) as control and off-line analysis software, and pClamp software of Molecular Devices (USA) for quantitative analysis of Ca^{2+} elevations. Ca^{2+} elevations were quantified by defining elliptical regions of interest (ROIs) manually adjusted to the shape and size of the areas with variations in fluorescence intensity of FLUO4-AM, using colocalization with SR101 as well as morphology recognition through IR microscopy. Ca^{2+} variations were estimated as changes in the fluorescence signal over the basal fluorescence (ΔF/F0) after background subtraction. A Ca^{2+} elevation was considered as an event where the ΔF/F0 intensity exceeds the fluorescence of the baseline in two standard deviations, at least for two consecutive frames. The parameters used to characterize spontaneous Ca^{2+} transients were duration (seconds), expressed as full-length duration due to the high complexity of the transients (i.e.: multiple fluorescence peaks by transient that does not permit to calculate the half-width duration used in other works); frequency (min-1 or Hz as indicated) and the number of cells per area displaying Ca^{2+} transients. Those events where the ΔF/F0 intensity dropped to half of the maximum fluorescence in relation to the baseline were considered independent events.

IV. Inmunolabeling procedures

To assess astroglial reactivity, the immunoreactivity to glial fibrilary acidic protein (GFAP) was evaluated. For immunolabeling analysis, hippocampal slices from control and fully kindled rats were processed in parallel. Briefly explained, slices were fixed in 4% v/v p-formaldehyde for 12 hrs, then the slices were incubated with anti-GFAP (1:200, DAKO) diluted in tris-phosphate buffer and bovine serum albumin 1% p/v, overnight (Cortés-Campos et al., 2011). The samples were then incubated with Cy2-conjugated rabbit anti-IgG (Jackson Immuno Research, Pennsylvania, USA) for 2 hrs at 22ºC. Hoechst was used for nuclear staining. Images were obtained by using a confocal spectral microscope Zeiss 780 (ZEISS, Germany). GFAP and Hoechst expression levels were quantified by using the Imaris software (Bitplane AG, Swizerland) and ImageJ (NIH, USA). Results were shown as

the area with immunoreactivity (area of individual particles with inmunorreactivity expressed in μm^2 or pixels) and as the average intensity of immunoreactivity (expressed in arbitrary units).

For confocal imaging, an LSM780 confocal microscope (Axio Observer.Z1, Carl Zeiss) with ZEN 2011 software v8.0, SP 2 (Carl Zeiss, Germany) was used to acquire multi-channel fluorescence images. EC Plan-Neofluar 20 x/0.50 M27 objectives were used with the following channels (laser and excitation wavelength, fluorophores, beam splitters, emission spectral filter): laser DPSS 561-10, 561 nm, DyLight549, MBS 488/561/633, 558 – 623 nm; laser Diode 405-30, 405 nm, Hoechst, MBS -405, 412-499 nm. Channels were acquired sequentially with an eight-bit dynamic range, and the pinhole size was set at 1.05 Airy Units for the 561 nm laser and 2.01 Airy Units for the 405 nm laser, giving the channels optical slice thicknesses of 13.5 µm and 17.9 µm, respectively. Sampling resolution was 1.66 µm x 1.66 µm x 1 µm voxels. The images were processed in the same software, ZEN 2011, where the fluorescence intensities were changed lineally (Min and Max values shifted) and some of the images were 3D processed in the 3D module of ZEN 2011 to visualize the data using Transparent-rendering mode. Research team leading by Dr. Francisco Nualart performed inmunolabeling experiments in the laboratory of Cellular Biology Department, Centro de Microscopia Avanzada (CMA BIOBIO) University of Concepcion (Chile).

V. Reagents

Cell-permeable fluorescent calcium indicator FLUO4-AM and fluorescent dyes SR101, Alexa 594 and Lucifer Yellow were purchased from Molecular Probes (USA). Chemicals were purchased from Sigma-Aldrich (USA) and Tocris (UK). The antagonists of group I mGluRs (+)-2-methyl-4-carboxyphenylglycine (LY367385; LY367) and 2-methyl-6-phenylethynyl-pyridine (MPEP) were dissolved in NaOH (1M). TTX (0.5-1.0 µM), picrotoxin (PTX; 10 µM), the specific antagonist of P2Y1Rs, 2'-Deoxy-*N*6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS2179; 10 µM) and the P2Rs generic antagonist, Pyridoxalphosphate-

6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS; 25-50 μM) were added directly to the perfusion system. GDPβS was directly dissolved in intracellular recording solution.

VI. **Statistical analysis**

Data are expressed as mean ± SEM, unless otherwise noted. All data were analyzed to determine if they fitted to normal distribution (Shapiro-Wilk test, Kolmogorov-Smirnov test). According to that, a parametric (Student's two-tailed t test) or a non-parametric test (Mann-Whitney test) was performed as indicated. ANOVA with Bonferroni correction *post-hoc* analysis or ANOVA repeated measures with Tukey post-hoc analisys were performed for multiple groups' comparisons. Differences were considered statistically significant at $p<0.05$; $p<0.01$ and $p<0.001$ as indicated.

RESULTS

I. Reactive astrogliosis in the hippocampus of kindled rat

In this work we used a variant of the rapid kindling protocol (RK) that was developed in our laboratory. As aforementioned, the electrically stimulated area was the amygdale complex (BLA), avoiding the direct manipulation of the hippocampus, our study area. Since astrogliosis is a hallmark of epileptic tissue, we assessed whether these epilepsy-related morphological and functional changes are present in hippocampal slices obtained from rats in RK-induced kindled state. First, we evaluated immunoreactivity to GFAP, whose increased expression is a widely reported indicator of astrogliosis (Pekny and Nilsson, 2005). Astrocytes from hippocampal formation of kindled slices showed hypertrophic morphology with thicker and tortuous processes compared to control slices (Fig. 6A). Specifically, astrocytes located in both *stratum oriens* (SO) and *radiatum* (SR) of CA1 region from kindled slices exhibited higher values of GFAP staining relative area (Kindled: 7.07±1.7% vs. Control: 1.8±0.24% values for SR; p=0.035) and staining intensity than the control group (Kindled: 22.58±2.85 vs. Control: 8.89±1.9, values for SR expressed in AU; n=8 areas, 4 slices from 2 rats for each condition processed in parallel both conditions; p=0.005; Fig. 5B). After using the nucleus marker Hoechst, we found no differences in any of the measured parameters between kindled and control slices (13.41±4 and 13.56±3, respectively, Hoechst staining intensity from SR expressed in AU; p=0.489; Fig. 6C), suggesting that the total number of cells in kindled rats did not change significantly. Confirming the above, morphological analysis of SR101-positive cells showed higher mean cellular size without changes in the number of astrocytes per area (Fig. 6D-F). These results indicate that the amygdale kindling protocol induces strong reactive astrogliosis in all hippocampal formation, characterized by hypertrophic morphology, without noticeable

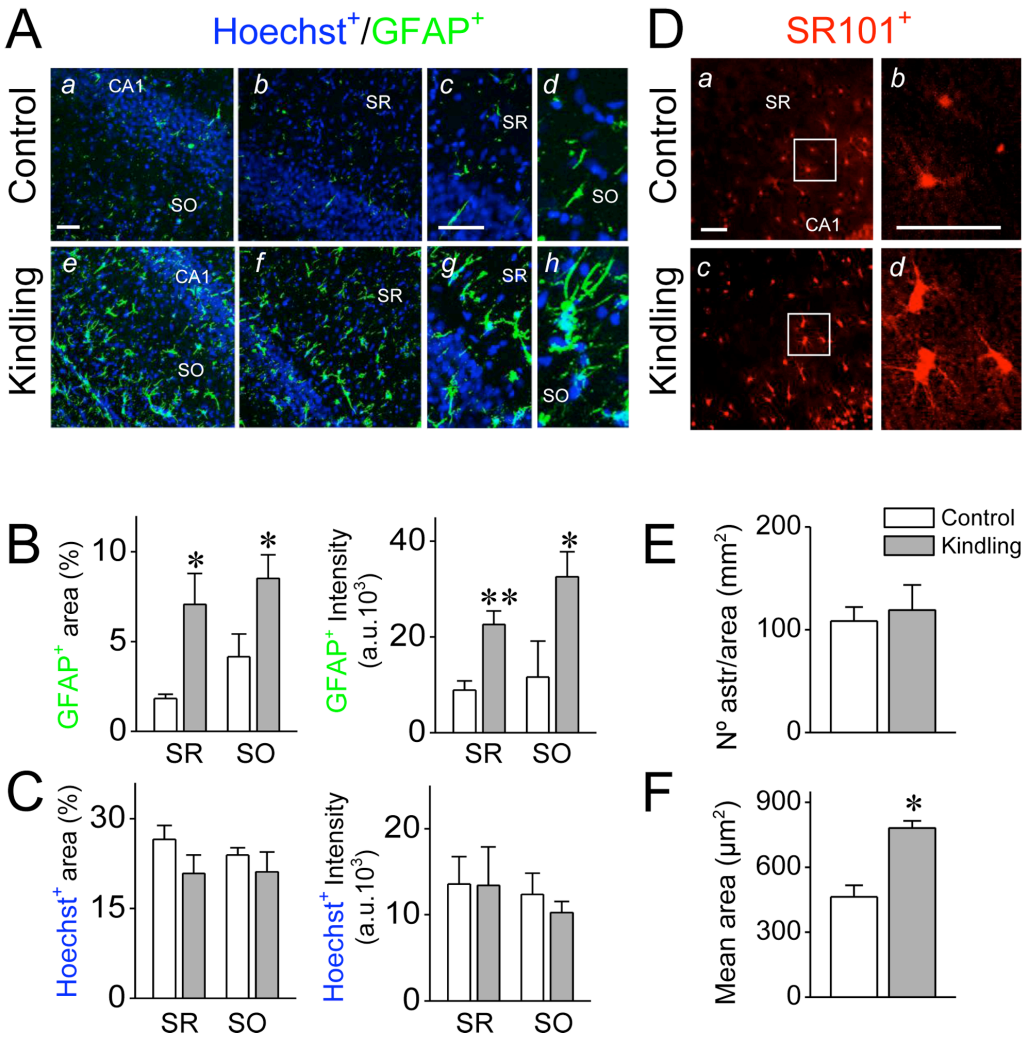

Figure 5. Kindled hippocampal slices showed pronounced astrogliosis and astroglial hypertrophy. (A) Immunostaining for GFAP (green) and Hoechst (blue) throughout the hippocampus sections from control and kindled rats (SO: Stratum oriens, SR: Stratum radiatum), and their respective high-magnification images (Aa-d and Ae-h). Sections of fully kindled hippocampus exhibit higher pattern of GFAP$^+$ staining and hypertrophic morphology typical for reactive astrocytes. (B-C) Expression levels of GFAP$^+$ and Hoechst$^+$ immunostaining detected in SR and SO in control and kindled groups, quantified as average values for relative area (%) and immunofluorescence intensity (arbitrary units, AUx103). (D) SR1011 cells from SR and their respective high magnification images of kindled and control groups. Quantitative analysis of SR1011 cells showed as (E) the number of astrocytes per area and as (F) the mean staining area. Notice that the mean area shows higher values in kindled than control slices, without changes in the number of astrocytes, revealing marked astroglial hypertrophy. Calibration bars: 100 lm for all images. Statistics: Mann-Whitney-test or T-test (*P <0.05; ** P <0.01). Published in Álvarez-Ferradas et al., 2015.

neuronal loss or astroglial hyperplasia, as previously described (Osawa et al., 2001). In addition, these findings confirm the effectiveness of our RK protocol by corroborating that the amygdala stimulation is sufficient to generate epilepsy-related morphological and functional changes in all hippocampal formation. These changes can be directly attributed to the pathophysiological plastic process behind epilepsy development and establishment, because there was no surgery or direct electric stimulation over the hippocampus.

II. Intracellular coupling of astrocytes in epileptic and control hippocampus

Tipically, astrocytes abundantly express Cx43 and Cx36, which mediated the formation of extensive astroglial networks (Pannasch and Rouach, 2013). Since increased expression of conexines have been reported in multiple pathological conditions and particularly epilepsy (Fonseca et al., 2002), we ask whether in addition to hypertrophy and reactive astrogliosis the inter-astrocyte coupling may be also increased in epileptic slices. To assess the fluorescent dye spreading through astroglial syncitium, we patch-clamped astrocytes from the hippocampus of control and kindling rats with an intracellular solution containing the fluorescent dye Alexa488 (100μM, see methods), able to cross through GAP junctions. In all the experiments, we performed the intracellular dialysis for at least 20-30 minutes, were the dye seems to get the maximum of its spreading. It is noteworthy that we previously used Alexa594 to check dye loading, but in contrast to other works we did not observed clear dye spreading to other astrocyte somas both in control and kindled slices. Alexa488 spreading reach over 40±5 astrocytes in kindled slices and 27.5±3.3 in control (n=4 and n=6 respectively, p=0.11, Fig7). Still, these data are not conclusive since astroglial networks present high variability; so more experiments are needed to further confirm whether astroglial synctium is actually increased or not in the epileptic hippocampus.

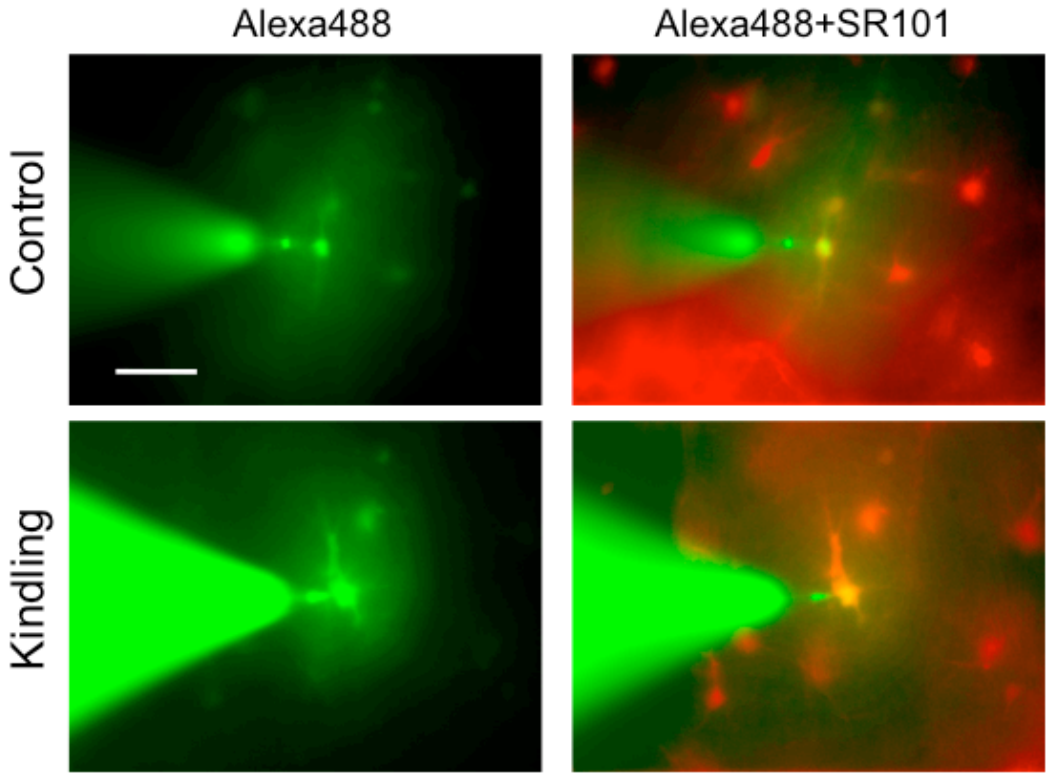

Figure 7. Intercellular dye coupling in control and kindled hipocampal slices. Representative images of the fluorescent dye Alexa488 (left, 100μM) applied by intracellular dyalisis in one SR101+ astrocyte, and (left) merged images showing colocalization between Alexa488 loaded astrocytes and SR101.

III. Abnormal pattern of spontaneous astroglial Ca^{2+} elevations in epileptic hippocampus

Astroglial Ca^{2+} transients, who constitute the basis of astroglial excitability, produce the release of glutamate form astrocytes. This spontaneous astrocyte glutamate release has been proposed as a non-synaptic source of glutamate excess in focal epileptiform activity (Fellin et al., 2006). However, whether spontaneous Ca^{2+} dynamics in astrocytes and Ca^{2+} dependent glutamate release from astrocytes is altered in the epileptic brain remains unknown. To test this possibility we evaluated the astroglial Ca^{2+}-excitability by comparing

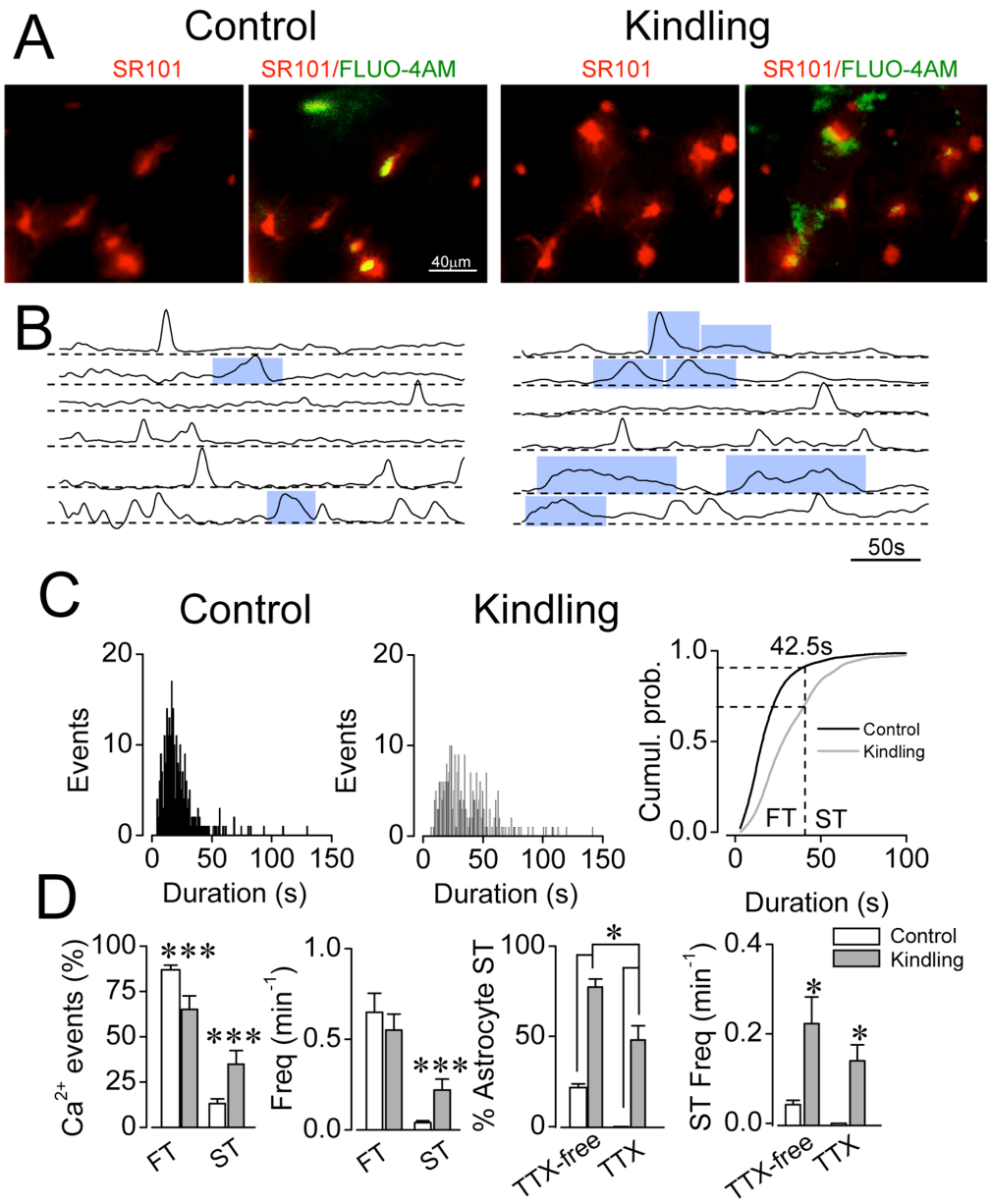

Figure 8. Kindled hippocampal slices display spontaneous astroglial Ca21-mediated hyperexcitability. (A) Representative fluorescence microscopy images of SR1011 astrocytes (red) and maximum intensity projection obtained from 5 min video of fluorescence transients for FLUO4-AM-loaded astrocytes of CA1 area (stratum radiatum) showed colocalization (yellow) both in the control (left) and the kindled (right) hippocampal slices. B) Representative Ca^{2+}-fluorescence traces of spontaneous $[Ca^{2+}]i$ elevations from six astrocytes. Blue boxes identify the slow Ca^{2+} elevations namely slow transients (ST; i.e.,≥42.5 s); and fast transients (FT; i.e., <42.5 s without box), detected in the control (left) and the kindled (right) slices. C) Distribution histograms of the spontaneous Ca^{2+} signal durations obtained from all astrocytes analyzed for control (n=74; left) and kindling (n=75; middle). Cumulative distribution plot of the spontaneous Ca^{2+} events duration obtained from all astrocytes analyzed for control and kindling (right). Dashed line indicates 42.5 s-duration cut-off criterion to classify FT and ST populations from both conditions. D) ST and FT percentage for each astrocyte analyzed and ST and FT mean frequency for both groups (left). Percentage of astrocytes that exhibited ST and ST frequencies values, recorded before and after adding tetrodotoxin (TTX; 0.5-

lμM) in control and kindled groups (right). Notice that most astrocytes in the kindled hippocampus still display a great number of ST in presence of TTX, whereas in control group they were completely abolished. Statistics: for distribution analysis of Ca^{2+} elevations K-S test, for others Mann-Whitney-test or T-test (*P <0.05; ** P <0.01; *** P <0.001). Published in Álvarez-Ferradas et al., 2015.

the spontaneous intracellular [Ca^{2+}] ([Ca^{2+}]i) elevations (or Ca^{2+} transients) from control and kindled hippocampal slices pre-incubated with the astrocyte-specific marker SR101 and Fluo4-AM (Fig. 8A). Kindled slices exhibited spontaneous Ca^{2+} events of higher duration than control, whereas the number and frequency of events by astrocyte were similar in both groups (Fig. 8B, 3.8±0.3; 0.7±0.05 min^{-1}; n=74 in kindling and 3.6±0.3; 0.7±0.06 min^{-1}; n=75 in control, respectively). Individual Ca^{2+} transient duration showed a non-normal distribution in both groups as shown in the histograms (Fig.8C). Regarding individual duration, Ca^{2+} transients were classified into slow and fast transients (ST and FT) based on the 75-percentile of cumulative distribution -42,5s as cut-off criterion- (dashed line; Fig.8C). These FT (<42.5s) and ST (≥42.5s) occurred randomly in the same astrocyte. Remarkably, 34.8±7.7% of the astrocyte [Ca^{2+}]i elevations from kindled slices were STs whereas in the control group they accounted for only 13.1±2.8% of total transients (p=0.0001; Fig. 8D). Moreover, in kindled hippocampus, 77.3±4.4% of astrocytes exhibited STs, while only 21.3±2.0% exhibited them in control group (Fig. 8D). Also, STs were more frequent in kindled than in control group (Kindled: 0.22±0.06min^{-1} vs. Control: 0.04±0.01min^{-1}; p=0.0001), while FT frequency was indistinguishable between groups (Fig. 8D). We next assessed whether Ca^{2+} transients were dependent on endogenous spontaneous synaptic activity. Bath application of tetrodotoxin (TTX) to block action potential-dependent neurotransmission did not affect FT frequency either in control or in kindled slices (Kindled; TTX-free: 0.56±0.09 and TTX: 0.41±0.03min^{-1}, p=0.48; vs. Control; TTX-free: 0.65±0.1 and TTX: 0.55±0.35min^{-1}, p=0.42). TTX completely abolished STs in control slices, whereas 47.8% of all astrocytes in the kindled group continued displaying STs activity at similar frequency (TTX-free: 0.22±0.06 min^{-1} and TTX: 0.14± 0.04 min^{-1}; p=0.185; Fig. 8D), representing about 62% of the total amount of astrocytes that exhibited them in TTX-free conditions. Together these results indicate that astrocytes from kindled hippocampus exhibit abnormal patterns of spontaneous Ca^{2+} STs, which are partially synaptic-activity

independent. Since astroglial excitability is based on $[Ca^{2+}]i$ variations, the increased temporal course of Ca^{2+} events directly represents a form of hyperexcitability in these cells (Halassa et al., 2007; Zorec et al., 2012)

IV. **Increased glutamate gliotransmission in epileptic hippocampus**

Spontaneous astroglial Ca^{2+} elevations in CA1 area are known to induce astroglial glutamate release, which can be detected as spontaneous SICs in adjacent pyramidal neurons (Angulo et al., 2004; Fellin et al., 2004; Perea and Araque, 2005; Shigetomi et al., 2008). Therefore, we next tested whether Ca^{2+} hyperexcitability exhibited by astrocytes of the kindled group affects the glutamate-mediated gliotransmission. To achieve this, the spontaneous SICs were recorded in presence of TTX (0.5 mM). Both control and kindled groups showed a similar proportion of CA1 pyramidal neurons displaying SICs (70% and 73%, respectively). However, CA1 neurons of kindled group exhibited a higher SICs frequency than those of control (Kindled: $0.18\pm0.06 min^{-1}$; n=6 cells vs. Control: $0.07\pm0.06 min^{-1}$; n=8 cells, $p<0.05$, Fig 9A), without changes in SICs amplitude (Kindled: 0.082±0.026 nA vs. Control: 0.078±0.015 nA; p=0.32, Fig 9B). No significant differences were observed in both rise and decay times of SICs between groups (Figure 9B). Altogether, these results show that the glutamate-mediated gliotransmission is increased in kindled hippocampus, presumably as a consequence of the astroglial hyperexcitability.

V. **Spontaneous glutamatergic neurotransmission is increased in epileptic hippocampus**

Given that changes in glutamate-mediated gliotransmission can modulate neurotransmission (Bonansco et al., 2011), we asked whether upregulation of the astrocyte-neuron signaling is

sufficient to modify the synaptic properties of glutamatergic transmission in kindled hippocampus. First, we tested whether synaptic glutamatergic transmission could be altered in the kindled hippocampus by recording sEPSC and mEPSC excitatory postsynaptic currents (Fig. 9C). Notably, the sEPSC frequency was significantly higher in the kindled than in the control groups (Kindled: 3.4±0.3 Hz; n=6 cells vs. Control: 2.5±0.1 Hz; n=5, $p<0.03$; Fig 9C), without significant changes in the sEPSC amplitude (Kindled: 45.6±3.9 pA vs. Control: 36.8±3.4 pA, p=0.06; Fig 9C). Similarly, the frequency (Kindled: 1.3±0.1 Hz vs. Control: 0.4±0.1 Hz, p=0.00002), but not the amplitude of the mEPSC (Kindled: 26.2±2.1 pA vs. Control: 23.7±1.7 pA, p=0.18; Fig. 9D) was also higher in the kindled group compared to the controls. This increase in the sEPSC and mEPSC frequency could reflect an increase in the number of synaptic contacts, changes in the Pr or both. To test this possibility, the multiplicity index (MI; see Methods) was analyzed, as an indirect measure of the total number of synaptic contacts (Hsia et al., 1998). As shown Fig.9E, MI values were similar between groups (Kindled: 1.7±0.1 vs. Control 2.4±0.6, n=7, p=0.34), indicating that in hippocampal-kindled slices the upregulation of glutamatergic neurotransmission could be due to an increase of the Pr.

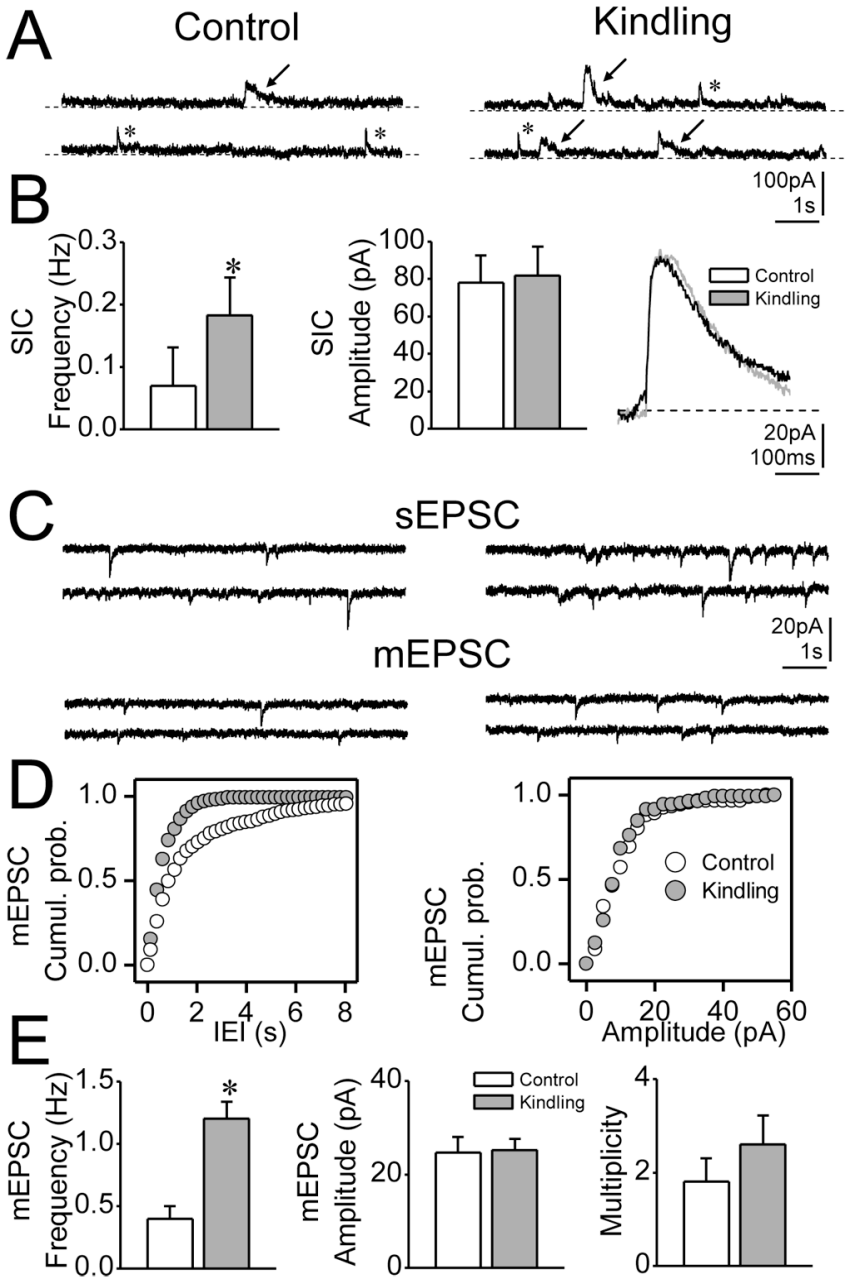

Figure 8. Spontaneous glutamate-mediated gliotransmission is increased in epileptic hippocampus. (A) Representative patch-clamp recordings of spontaneous excitatory postsynaptic activity (sEPSC; asterisk) and slow inward currents (SIC; arrow) recorded in CA1 pyramidal neuron at depolarized holding potential (i.e., +40 mV) in control and kindled hippocampal slices. (B) Summary graphs of SICs frequency and amplitude, showing higher SICs frequency in kindled hippocampus compared with control conditions. Superimposed average traces show that both amplitude and kinetic properties of SICs were undistinguishable between groups (right). (C) Spontaneous postsynaptic activity from control (left) and kindled group (right) recorded at -60 mV before and after adding TTX (0.5 μM), obtaining sEPSC and miniature (mEPSC), respectively. (D) Cumulative probability graphs of both mEPSC inter-event interval and amplitude for control and kindled groups. (E) mEPSC frequency (left) and amplitude (middle) means and MI (right) for both conditions. Statistics: T-test (*P <0.01). Published in Álvarez-Ferradas et al., 2015.

VI. Probability of neurotransmitter release is increased in epileptic hippocampus

To more directly test whether the increase in neurotransmitter release in kindled rats is due to a presynaptic change, we recorded the EPSC evoked by conventional stimulation of Schaffer collaterals, using a paired pulse protocol and minimal stimulation. Paired-pulse facilitation index (PPF; see methods) was 27.63% lower in kindled compared to control rats (Kindled: 1.1±0.05; n=14 vs. Control: 1.52±0.1; n=6, p=0.0014; Fig 10A). As changes in the PPF inversely correlate with Pr (Dobrunz and Stevens, 1997), the decrease of the PPF in kindled rats can reflect an increase in Pr. Correspondingly, the coefficient of variation of EPSC amplitude was higher in the kindled rats compared to control (0.37±0.03 vs. 0.25±0.02, Fig.10A). Moreover, minimal stimulation responses (meEPSC) showed a decrease in the failure rate (Control: 61.8±2.7%, n=8 vs. Kindled: 37.6±5.9%, n=17, p=0.0002; Fig. 10B) and an increase in the synaptic efficacy (Control: 14.8±1.8pA vs. Kindled 22.9±1.2pA, p=0.033; Fig. 10B) without differences in the synaptic potency (Kindled 23.7±1.2 pA vs. Control 26.4±2.8 pA, p=0.11; Fig 10B). These results further show that the abnormal rise in synaptic efficacy in glutamatergic synapses of hippocampal-kindled slices is due to the increase in basal Pr in the synaptic terminals.

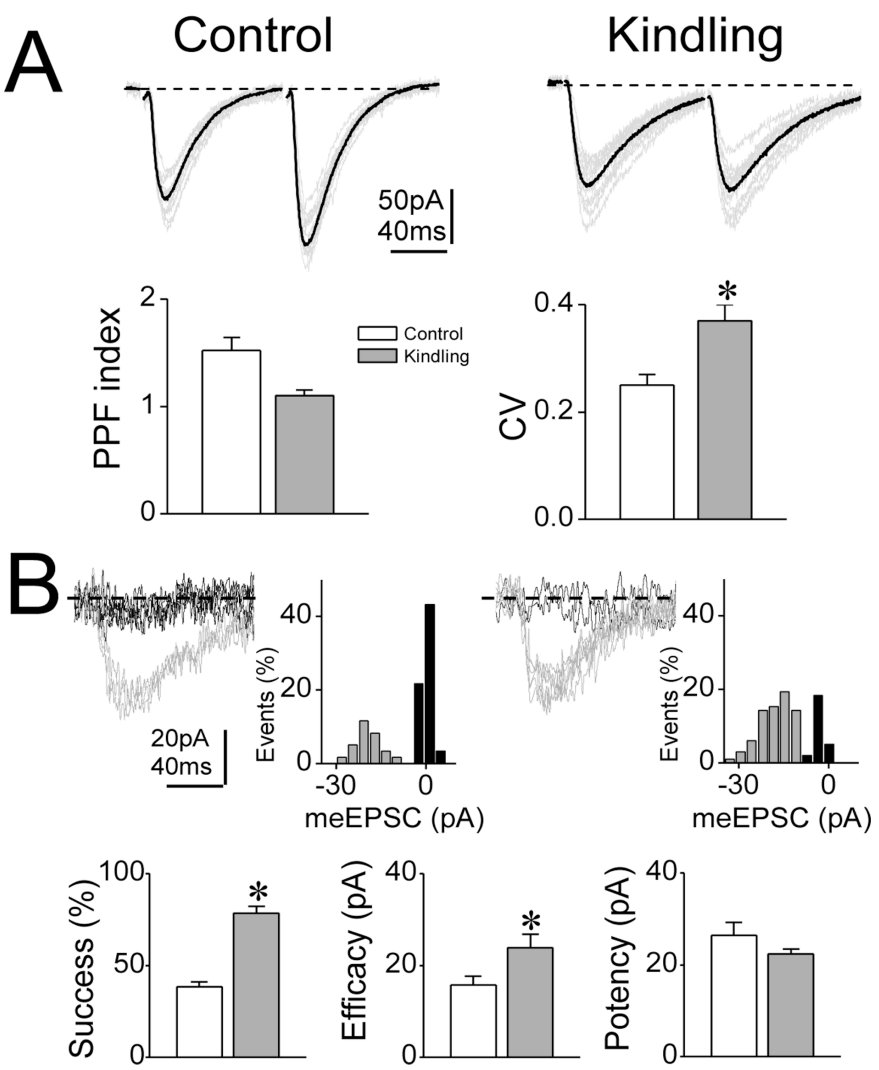

Figure 10. Increased synaptic efficacy in CA3-CA1 synapses of kindled hippocampus. (A-top) Superimposed consecutive EPSCs traces (10 sweeps; grey traces) and average (black trace) evoked by pair pulse protocol (PPP) in control and kindled hippocampal slices. Summary graphs of paired PPF and coefficient of variation of EPSC amplitude for control and kindled group (bottom). (B-top) Representative traces of minimal evoked EPSC (meEPSC) for control and kindled condition and their respective distribution histograms, showing failures and successful responses. Mean values of probability of neurotransmitter release (Pr; percentage of successful responses; left), synaptic efficacy (success and failure amplitude average; middle) and synaptic potency (success amplitude average; right) for control and kindled rats. Statistics: T-test (*P <0.01). Published in Álvarez-Ferradas et al., 2015.

VII. **Astroglial Ca^{2+} transient inhibition relieves upregulated Pr in epileptic hippocampus**

Having established that glutamate-mediated gliotransmission and neurotransmission are increased in kindled hippocampus, we next asked whether the astroglial hyperexcitability is implicated in the rise of basal Pr at glutamatergic synapses. If this were the case, selective inhibition of astroglial Ca^{2+} signaling should reduce glutamate gliotransmission, therefore diminishing the Pr. To test this possibility, the spontaneous Ca^{2+} signal was selectively inhibited by loading GluT astrocytes (see methods, Fig. 11B-C); with the Ca^{2+}-chelator BAPTA (40mM), applied by intracellular dialysis at least for 20 min. The region within a diameter of 100μm from the patched astrocyte to nearby astroglial processes with clear DAPI nuclear staining was established as a BAPTA-loaded area; and the field outside the perimeter (see Methods; BAPTA and non-BAPTA fields, respectively; Fig. 11A) as BAPTA non-loaded area. Intracellular Ca^{2+}-transients were quantified before and after 20-30 min of BAPTA dialysis. Within the BAPTA-loaded area, the number of astrocytes with spontaneous Ca^{2+} transients diminished by 78.6%. For the same kindled slice, the number of spontaneous astroglial Ca^{2+} signals per area was also diminished when compared to the non-BAPTA field (BAPTA: 9.25±4.5 and non-BAPTA: 27.5±8.8 number of transients per area; n=3 slices from 3 rats; Fig. 11D). We next evaluated the synaptic efficacy of CA1 pyramidal neurons within the BAPTA versus non-BAPTA field from the same kindled slice. We found that the PPF index were significantly higher in neurons within the BAPTA field compared to those located at the non-BAPTA field (BAPTA: 1.781±0.20, n=5 vs. non-BAPTA: 1.1±0.05 n=8 neurons of 6 slices from 5 kindled rats, p=0.023; Fig. 11E). Moreover, the success rate of meEPSC recorded in CA1 pyramidal neurons from the BAPTA field was significantly lower than those recorded from the non-BAPTA field (BAPTA: 28.5%±7.31 n=5 vs. non-BAPTA 63.6%±5.87, n=6, p=0.014; Fig. 11F) whereas the synaptic potency remained unchanged (Fig. 11F).

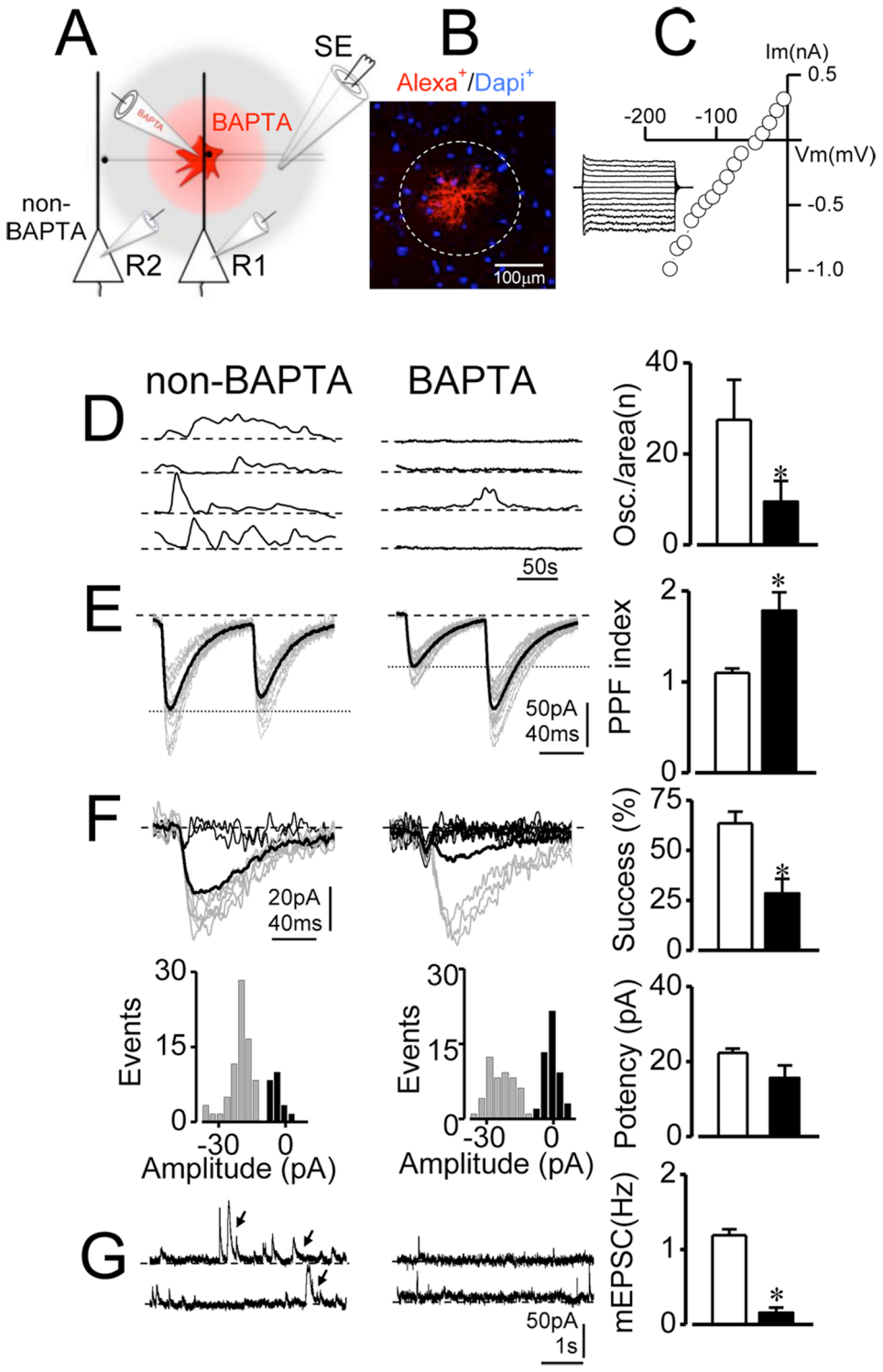

Figure 11. Blockade of astroglial Ca^{2+} signal abolishes the rising of synaptic efficacy in epileptic hippocampus. (A) Experimental arrangement of the intracellular dialysis of BAPTA (40 mM) into single patch-clamped astrocyte, which spreads through the syncytium (BAPTA loaded field; red), surrounded by a non-BAPTA astrocyte field (black). In these experiments, electrophysiological

recordings and synaptic stimulation (by stimulation electrode; SE) were performed in CA1 pyramidal neurons located in BAPTA (R1; < 75 lm from the patched astrocyte) and non-BAPTA fields (R2; >150 lm from the patched astrocyte) in the same kindled hippocampal slice. (B) Maximal intensity projection of Alexa594-labelled astrocyte syncytium after patching a GluT astrocyte, showing several somas colocalized with $Dapi^{+}$. (C) Representative recording of patch-clamped GluT astrocyte. (D) Representative fluorescence traces of spontaneous Ca^{2+} transients recorded in astrocytes located in non-BAPTA (left) and BAPTA-loaded fields (middle) after 30 minutes of BAPTA-dialysis, and mean number of transients by area (right). Notice that astroglial Ca^{2+} signals close to BAPTA-dialyzed astrocytes were virtually abolished with respect to those located in non-BAPTA fields. (E) Representative PPP-evoked EPSC traces, recorded in CA1 pyramidal neurons located in non-BAPTA (left) and BAPTA-loaded fields (middle), and their respective PPF index values (right). (F) Representative meEPSC traces, recorded in CA1 pyramidal neurons located in previously mentioned fields (left and middle), with their respective amplitude distribution histograms; and summary graph of success rate and potency (right); and (G) mEPSC and slow inward currents (SICs, arrows) traces (left and middle) and EPSC frequency mean values (right). Notice that in BAPTA-loaded area no SICs were observed. Statistics: T-test (*P <0.05). Published in Álvarez-Ferradas et al., 2015.

Consistent with these results, the mEPSC frequency was also lower in neurons within the BAPTA field compared to those neurons in the non-BAPTA field (BAPTA: 0.15±0.07Hz n=4 vs. non-BAPTA: 1.19±0.08Hz, n=6, p=0.0001; Fig. 11G). In addition, in the recordings performed at depolarized potential (+40mV), no SICs were observed (n=4; Fig. 11G), suggesting that BAPTA-Ca^{2+} chelating in astrocytes abolished both pre (i.e., mEPSC frequency) and postsynaptic effects (i.e., SIC) of glutamate released from astrocytes. Altogether, our results show that the selective inhibition of astroglial Ca^{2+}-transients in hippocampal-kindled slices decreases the basal Pr and therefore the synaptic efficacy of both evoked and spontaneous glutamatergic neurotransmission.

VIII. mGluR5 receptor antagonist decrease the Pr in epileptic hippocampus

We next investigated the astrocyte-neuron signaling pathways involved in the upregulation of synaptic efficacy and astroglial hyperexcitability in hippocampal kindled slices. Since in physiological conditions spontaneous glutamate released from astrocytes modulates the synaptic efficacy of CA1 synapses via activation of presynaptic group I mGluRs (Bonansco et al., 2011), we tested if these receptors are also implicated in the upregulation of excitatory neurotransmission in the epileptic hippocampus. To achieve this, MPEP (100μM) and

LY367385 (50μM), two specific antagonists of mGluR5 and mGluR1 respectively, were perfused while PPF-EPSC and astroglial spontaneous Ca^{2+}-activity was recorded. After 20-30 min of MPEP plus LY367385 co-application, the PPF index showed a relative increase of 193±12 % with respect to basal level (n=5, p=0.02), which indicates a decrease of Pr, reaching values similar to those in the control rats. Moreover, this increase over the PPF index was replicated by MPEP alone and partially by LY367385 (MPEP: 148.1±21%; n=5; p=0.02 and LY367385: 111.5±3.7%; n=4; p=0.03; Fig. 12F), indicating that astrocyte-mediated regulation of the Pr requires mGluR5 activation more than mGluR1 activation. Otherwise, astroglial Ca^{2+}-signaling was completely insensitive to MPEP and LY367385, applied either jointly or separately (Fig. 12A and 12E, respectively). In addition, these antagonists do not affect either ST or FT incidence (Fig. 12B), strongly suggesting that spontaneous astroglial Ca^{2+} transient in kindled slices is independent from group I mGluRs activation. Taken together, these findings strongly suggest that mGluR5 is the main receptor implicated in Pr modulation and their activation may occur downstream of the astroglial Ca^{2+} signal.

IX. P2Y1R antagonist decrease the Pr and the spontaneous Ca^{2+} transients in epileptic hippocampus

Increasing evidence suggests that astroglial ATP-mediated signaling through P2YRs is involved in astroglial $[Ca^{2+}]i$ elevations (Fields and Burnstock, 2006), directly related to glutamate release (Domercq et al., 2006), and consequently with the control of neuronal transmission (Jourdain et al., 2007; Di Castro et al., 2011). To determine whether purinergic receptors could be involved in the astroglial hyperexcitability and in the upregulation of the

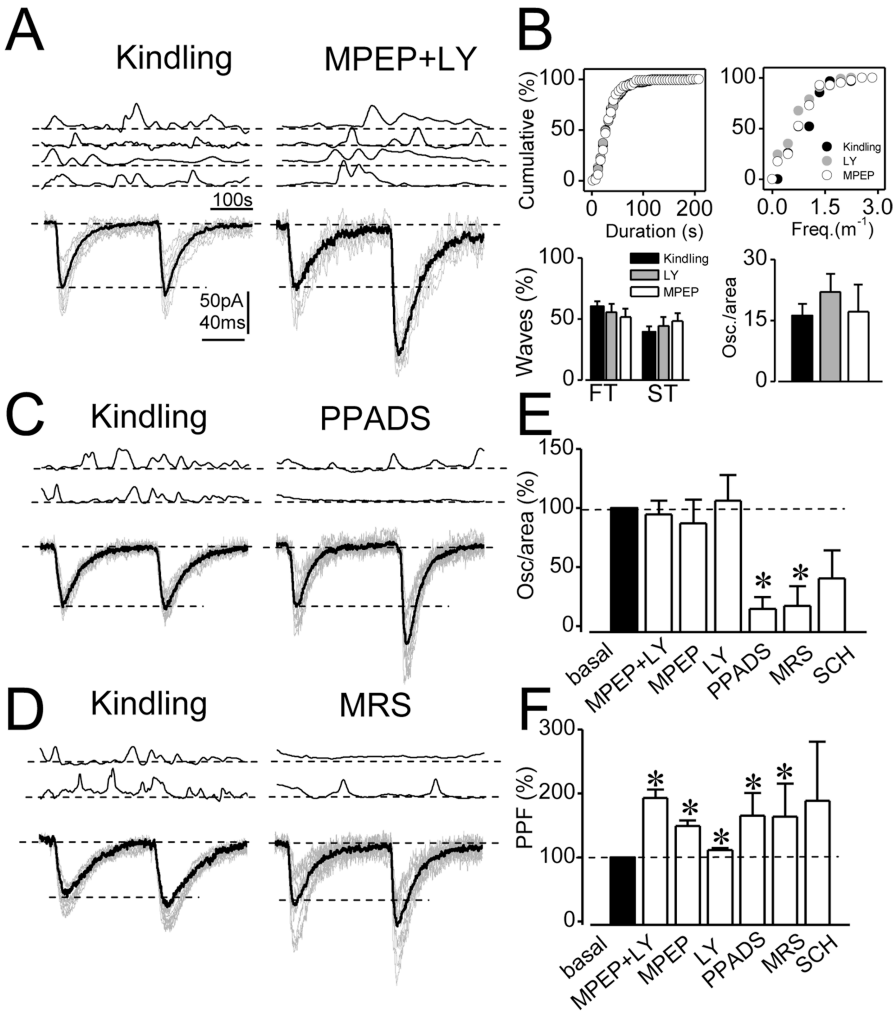

Figure 12. Purinergic and glutamatergic metabotropic receptors involved in astrocyte-neuron signaling in kindled hippocampus. (A) Representative simultaneous recordings of spontaneous Ca^{2+} signals from four astrocytes (top) and PPP-evoked EPSC in a neighboring CA1 pyramidal cell (PPP; bottom) before (left) and 20 minutes after (right) the addition of group 1 mGluR antagonist, MPEP (100 μM) plus LY367 (50 μM) in kindled hippocampal slices. (B) Cumulative graphs of duration (top-left) and mean frequency (top-right) for Ca^{2+} transients; FT and ST percentage per astrocyte (bottom left) and number of Ca^{2+} events per area (bottom right) in kindled slices, before and after group I mGluR antagonists perfusion. Notice that MPEP or LY did not modify any of the Ca^{2+} event parameters measured. (C-D) Astroglial $[Ca^{2+}]i$ elevations (top) and PPP-evoked EPSC (bottom), before (Kindling) and 20 minutes after the addition of the nonselective P2R antagonist, PPADS; and the selective P2Y1R antagonist, MRS2179. E) Number of Ca^{2+} events by area and F) PPF index summary graphs. Mean values are expressed as the percentage of change with respect to basal conditions after adding MPEP plus LY367; MPEP alone (50 μM); LY367385 alone (100 μM); PPADS (50 μM); MRS2179 (10 μM) and SCH58261 (A2AR antagonist; 100 nM). Statistics; Paired T-test for single comparisons; Mann Whitney test for Ca^{2+} experiments with MRS2179; ANOVA with Bonferroni post hoc analysis for multiple comparisons (**P <0.01; * P <0.05). Published in Álvarez-Ferradas et al., 2015.

Pr in synapses of kindled slices, we recorded simultaneously the PPF index and astroglial Ca^{2+} transients in presence of PPADS (50μM), a non-selective purinergic P2 (P2R) antagonist, as well as in presence of MRS2179 (10μM), a specific P2Y1R antagonist (Fig. 12C-D). Both drugs induced an increase in the PPF index (PPADS: 163.7±5 and MRS2179: 165.3±4%; n=5 neurons each; Fig.12F) and a strong reduction in the number of Ca^{2+} transients (Fig. 12E). Moreover, MRS2179 produces a decrease in mEPSC frequency of 52±8.5% in all the cells we recorded (n=8, paired T-test p=0.0017) without changes in mEPSC amplitude, suggesting that P2Y1R activation is required for spontaneous astroglial signaling.

X. A2AR does not participate in astrocyte-neuron signaling in epileptic hippocampus

Because ATP is rapidly converted into adenosine, which is known to facilitate transmitter release via presynaptic adenosine A_{2A} receptor ($A_{2A}R$) in the hippocampus (Cunha et al., 2001), it is also possible that the increase of Pr in kindled slices requires $A_{2A}R$ activation. To test this possibility, we used the specific $A_{2A}R$ antagonist (SCH58261, 100nM). SCH58261 showed highly variable effects on the PPF index in different CA1 cells (188.5±92 %; n=5 neurons, p=0.39; Fig. 12F), with an increase in the PPF index observed just in 2 out 5 neurons, whereas no effects were observed in the other 3. This variability was also observed in astroglial Ca^{2+} transients (40.3±23 %; n=3 areas, p=0.11; Fig. 12E), which suggests that the $A_{2A}R$ activation cannot be considered as a main astrocyte-neuron signaling pathway in the epileptic hippocampus.

XI. Occlusion effects of mGluR5 and P2Y1R antagonist on synaptic transmission

The previous results strongly suggest that the upregulation of synaptic efficacy in epileptic hippocampus requires the activation of presynaptic mGluR5 receptors that directly modulate the Pr, while P2Y1R activation regulates spontaneous astroglial $[Ca^{2+}]i$ elevations and subsequent glutamate exocytosis required for synaptic modulation. If this were the case, the effect of P2Y1R blockade on synaptic efficacy should be occluded in presence of MPEP.

Consistent with this hypothesis, the decrease in the success rate and in the synaptic efficacy induced by MPEP was unchanged after adding MRS2179 (MPEP: 41.9±0.05 and MPEP + MRS2179: 43.2±0.06 %, n=5, p=0.94; Fig. 13A-B), suggesting that mGluR5 blockade completely occluded the effects of P2Y1 inhibition on synaptic efficacy. In the same experiments, MPEP-insensitive astroglial Ca^{2+} signals were strongly reduced after adding MRS2179, confirming that the presynaptic mGluR5 activation occurs down-stream, after the P2Y1R-mediated astroglial $[Ca^{2+}]i$ elevation (Fig. 13A-B). These results strongly suggest that in kindled slices, astroglial P2Y1Rs are responsible for spontaneous Ca^{2+} hyperexcitability that likely produces the raised spontaneous glutamate gliotransmission, upregulating the basal Pr in excitatory synapses via presynaptic mGluR5 activation.

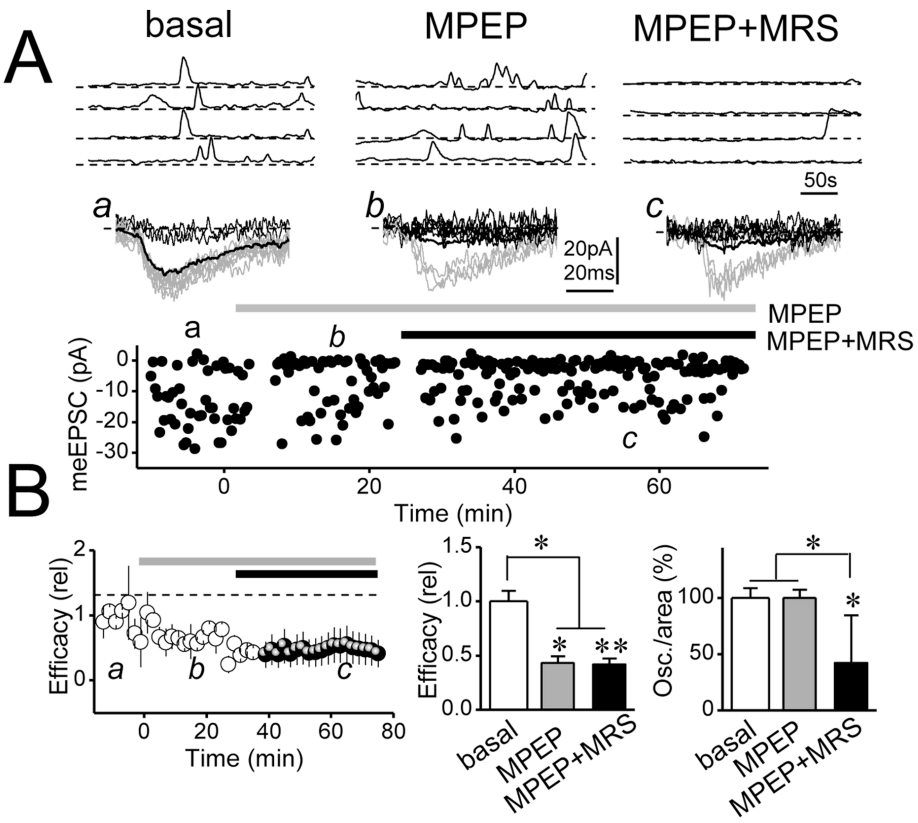

Figure 13. Astroglial P2Y1Rs and presynaptic mGluR5 blockade reverses abnormal astrocyte-neuron signaling in kindled hippocampus. (A) Representative occlusion experiment showing simultaneous recordings of spontaneous astroglial Ca^{2+} signals (top) and meEPSC (middle) before (basal, a), in presence of MPEP alone (b) and in MPEP plus MRS2179 (c) performed in a kindled slice. Time course graph of the occlusion experiment (bottom) shows the changes in the meEPSC success rate for each condition. Interestingly, MPEP itself did not affect astroglial Ca^{2+} signaling, which was fully abolished after MRS2179 addition, whereas the effect of MRS2179 on synaptic efficacy was occluded by MPEP effect. (B) Synaptic efficacy expressed as relative change plotted against time during occlusion experiments (left), mean values of synaptic efficacy (middle) and number of Ca^{2+} events per area (right) in conditions a, b and c. Statistics; Paired T-test for single comparisons; Mann Whitney test for Ca^{2+} experiments with MRS2179; ANOVA with Bonferroni post hoc analysis for multiple comparisons (**P <0.01; * P <0.05).

XII. mGluR5 activation restore the frequency of mEPSC decreased by P2Y1R inhibition

First of all, in the aforementioned experiments we focused in to determine the specific astrocyte-neuron signaling pathway in kindled-epileptic hippocampus. We established that mGluR5 and P2Y1R are the two main receptors implicated this via. Therefore, we decided to assess whether these receptors may contribute to astrocyte-neuron signaling in control conditions. Occlusion experiment strongly suggests that mGluR5 present in presynaptic membrane is required to increased the Pr. If this is true, the experimental activation of astrocytes or mGluR5 pathway should increase the Pr of excitatory synaptic transmission while endogenous astroglial signaling is blocked or reduced. To test this possibility, we evaluate the endogenous excitatory transmission before and after the addition of MRS2179 and the subsequent application of the group I mGluR agonist DHPG (50μM) both in kindling and control rats. In kindled rats, the mEPSC frequency was profoundly reduced to the 37.7±8.7% 20-30 min after adding P2Y1R antagonist with no changes in mEPSC amplitude (from 0.9±0.18 to 0.31±0.06 Hz, n=7, $p<0.01$, Fig. 14A-B) whereas no effect was observed in control rats (from 0.27±0.04 to 0.16±0.02, $p>0.1$). Remarkably, when DHPG was applied to the bath there is a recover of mEPSC frequency to the 69.9±14.5% of the initial frequency in epileptic slices (n=6, $p<0.05$, Fig. 14A-B) without changes in their amplitude. To ensure that any effect is mediated by postsynaptic mGluR, in all the experiments, GDPβS, a non-hydrolizable GTP used to block G-protein transduction, was added to the intracellular solution. Since in control slices there is no effect of DHPG application (from 0.16±0.02 in MRS, to 0.23±0.07 after the addition of DHGP, n=7, $p=0.44$, Fig. 14A-B), these data suggest that the activation of mGluR5 is not only needed but also sufficient to increase the Pr in pathological conditions. Thus, these findings further confirm that presynaptic group I mGluRs is the main pathway by which enhanced astroglial signaling contributes to upregulate the excitatory synaptic efficacy of kindled hippocampus. Moreover, since neither group I mGluR nor P2Y1R are involved in astrocyte-neuron signaling in control conditions, these results

indicate that this pathway is exclusively activated during pathologic conditions, more specifically as a consequence of the epileptogenic process.

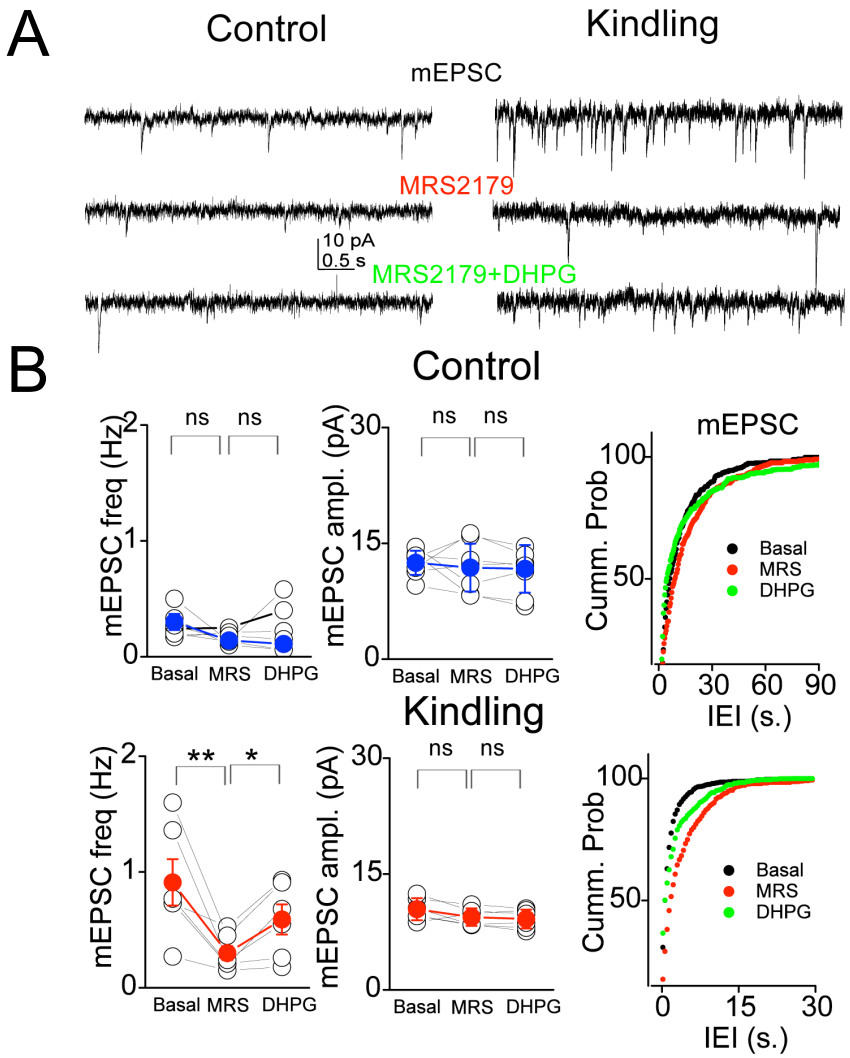

Figure 14. Presynaptic mGluR5 recover the synaptic efficacy decreased by MRS2179. A) Representative recordings of mEPSC from control and kindled slices obtained in TTX-presence (up), MRS2179 (middle) and MRS2179+DHPG (bottom). B) Summary graphs of experiments from control (up) and kindled (bottom) slices. mEPSC frequency (left) and amplitude values (right) during the basal condition and after the addition of MRS2179 and MRS2179+DHPG. Notice that the mEPSC amplitude remains unchanged both in control and kindled slices whereas MRS and DHPG profoundly affect the mEPSC frequency in kindled slices. (Right) Cummulative graphs of mEPSC inter event intervals (IEI) expressed in percentage from all the recorded cells form control (up) and kindled slices (bottom). Paired t-test **p<0.001, *p<0.05.

DISCUSSION

The main goal of this work was to explore whether astroglial function could be affected in a rat model of epileptogenensis, the kindling; as well as the consequences on synaptic physiology. Here, we showed that astrocytes from epileptic slices display an abnormal spontaneous Ca^{2+}-dependent excitability that directly affects the synaptic physiology in a pathological brain condition. An increased astroglial Ca^{2+}-signaling likely via increased glutamate gliotransmission, upregulates the synaptic efficacy of the CA3-CA1 circuit through a mechanism that requires the activation of astroglial P2Y1R and presynaptic mGluR5 (Fig. 15).

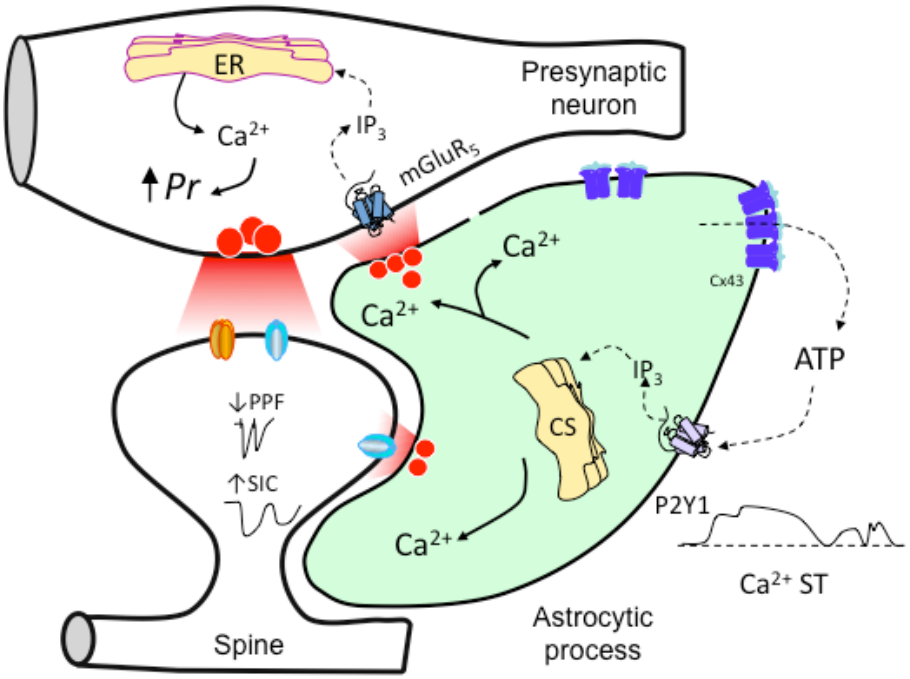

Figure 15. Pathological astrocyte-neuron signaling in the epileptic hippocampus. Astrocytes from epileptic hippocampus display Ca^{2+} dependent hyperexcitability, observed as an increase in ST incidence, likely mediated by an elevated P2Y1R-dependent purinergic signaling, where ATP could be released by neurons, astrocytes of microglia. Probably as a consequence of the astroglial hyperexcitability glutamate-mediated gliotransmission is increased, measured as an elevated SICs frequency –mediated by the activation of postsynaptic NMDAR-, which increases presynaptic Pr – through the activation of presynaptic group I mGluR-. We propose that this mechanism could contribute to the enhancement of the excitatory tone characteristic of the epileptic brain that ultimately predisposes neurons to seizures.

CHAPTER 1: MORPHOLOGICAL ALTERATIONS OF ASTROCYTES FROM KINDLED HIPPOCAMPUS

1.1 Reactive and hypertrophic astrocytes in hippocampus of kindled rats

We carried out our study in an epileptic brain that resembles many of the changes observed in neurons and astrocytes during epileptogenesis. The hippocampus of kindled rats presents a widespread reactive astrogliosis and cellular hypertrophy. This is the first evidence of astroglial alteration (Fig 6), an alteration highly conserved in most of epileptic tissues including those from TLE patients (Wetherington et al., 2008). Thus this finding showed that amygdala RK protocol induces the main morphological and functional alteration in astrocytes observed in the hippocampus of several epilepsy models and humans. In addition, neuronal hyperexcitability, characterized by an increase of discharge frequency and a decrease in AP accommodation, were obtained from CA1 intracellular recordings (Morales et al., 2014), further validating the RK protocol.

Reactive astrogliosis occurs as a consequence of the inflammatory response caused by epileptic-like seizures. Several substances as glutamate, cytokines and proinflammatory molecules released by microglia, neurons or other astrocytes during the seizures are known to induce astrogliosis (Sofroniew and Vinters, 2010; Vezzani et al., 2011). Therefore, reactive astrogliosis can be directly attributed to the epileptogenesis process, since any surgical intervention or electric stimulation was done over the hippocampus. Under our conditions, the astroglial hypertrophy was observed in absence of both hyperplasia and neuronal loss since the number of cells or nuclei was equal in kindled than control slices (Fig. 6). This result proves that neuronal death is not necessary to induce reactive astrogliosis in agreement with previous work (Khurgel et al., 1995). In conclusion, these findings suggest that amygdale RK is a reliable protocol to induce epileptogenesis-related alterations in the hippocampal formation. While reactive astrogliosis is one of the most common pathological lesions in epilepsy and other brain insults, it continues to be poorly

understood. Since reactive astrogliosis entails not only morphological but also protein expression alterations, it is highly likely that other functional alterations may occur in hippocampal astrocytes. However, we have to point out that animals subjected to RK protocol do not display spontaneous seizures (i.e., only as a result of exogenous electrical stimulation). Therefore, functional alterations observed here may be linked to a critical step of the epileptogenic process but not necessary to the complete establishment of epileptic syndrome (i.e., the appearance of spontaneous epileptic seizures). An important limitation that our model shared with all chronic models of epilepsy is that the study area –the hippocampus- is not the epileptic focus *per se*, which should be the most affected area in the epileptic brain. According to this idea, the alterations produces within the epileptic focus should be far severe to what we observe here.

1.2 Astroglial coupling in kindled-epileptic hippocampus

In addition to reactive astrogliosis, dye-coupling experiments suggest that astroglial network is not significatively increased in kindled hippocampus (Fig. 7). Even though preliminary, kindled slices showed slight tendency to present an increased anatomical coupling of astrocytes. Previous reports in human epileptic tissue and animal models of epilepsy showed an increased Cx43 expression and increased dye coupling in astrocytes (Fonseca et al., 2002; Takahashi et al., 2010). Since animals lacking astroglial coupling (Cx30-/-Cx43-/- KO mice) present a decreased glutamate clearance and K+ buffering (Pannasch et al., 2011), the increased astroglial network may result an adaptative response to neuronal hyperactivity to limit the progression of seizures. However, there are some inconsistencies regarding this issue since astroglial networks also present proepileptic aspects. For example, increased astroglial coupling also facilitate intracellular Ca^{2+} waves leading to the release of neuroactive substances synchronizing the activity of large neuronal populations (Angulo et al., 2004; Fellin et al., 2004). An intriguing hypothesis is that the increasing in astroglial coupling may occur during epileptic-like discharges to facilitate seizure ending (i.e., promoting K^+ buffering), but finally resulting detrimental for circuit performance by promoting inter-astrocytic Ca^{2+} signaling and thus facilitating epileptogenesis.

CHAPTER 2: FUNCTIONAL ALTERATIONS OF ASTROCYTES FROM KINDLED-EPILEPTIC HIPPOCAMPUS

2.1 Increased astroglial Ca^{2+} signaling in the epileptic brain

In addition to morphological alterations (Fig. 6), astrocytes from the kindled hippocampus showed an altered pattern of spontaneous Ca^{2+} signals. Astrocytes showed a Ca^{2+}-mediated hyperexcitability, observed as a higher incidence of STs partially TTX-insensitive. In control slices STs were also observed but in about a 3-fold smaller proportion, being completely abolished with TTX (Fig. 8B-D), indicating that STs in control conditions may be mediated by neuronal APs. APs-modulation of Ca^{2+} signaling seems to be specific from each brain area. While other groups showed that expanded Ca^{2+} elevations in the processes of DG astrocytes are partially inhibited by TTX (Di Castro et al., 2011), in other brain areas as CA3 are largely unaffected by TTX (Haustein et al., 2014). Since astroglial Ca^{2+} dynamics is altered because epileptogenesis induction, the mechanism generating Ca^{2+} signaling may be linked to specific physiological conditions. Thus, this data suggests that astroglial function can be affected during other neuropathological conditions.

The TTX-insensitivity of a ST subpopulation from epileptic hippocampus indicates that a signaling pathway neuronal hyperactivity-independent (i.e.: increased sEPSC frequency; Fig. 9C) must mediated the astroglial hyperexcitability, as it is also suggested by P2R blockade experiments (Fig. 12). Remarkably, similar TTX-insensitivity of astroglial $[Ca^{2+}]i$ elevations have been described in tissue resected from epileptic patients (Navarrete et al., 2013). Despite synapses from epileptic hippocampus present an increase mEPSC activity, it seems unlikely that they mediate TTX-insensitive STs, since it has been showed that mEPSCs induce local Ca^{2+} events almost exclusively confined to astroglial processes and not to somatic signals as observed here (Di Castro et al., 2011). However, further investigation is required to study whether the local Ca^{2+} signals confined to processes are also affected in the epileptic hippocampus. Altogether these data suggest that during the epileptogenic process there must be an alteration in protein expression that implicate a change in astroglial Ca^{2+} signaling pathway resulting in a type of hyperexcitability. Despite astroglial

reactivity is present in epileptic slices, we cannot conclude whether the hyperexcitability is directly associated to reactive astrogliosis-related changes. Independently, we can conclude that this astroglial hyperexcitability could be considered as a novel pathological property of astrocytes in the kindled-epileptic hippocampus.

2.2 Glutamate gliotransmission in kindled hippocampus

Astroglial Ca^{2+} signaling produces the release of neuroactive substances as glutamate. In our conditions, we detect an increased frequency of SICs in CA1 neurons from epileptic hippocampus (Fig. 9A,B). Despite it is not well understood the precise mechanism of glutamate release by astrocytes, we can establish a correlational link between STs-mediated astroglial hyperexcitability and increased glutamate gliotransmission. It is noteworthy that not every Ca^{2+} signal leads to the release of gliotransmitter. For example, the activation of astroglial PAR1 trigger by the apparence of SICs, which is not observed by the activation of other receptors like P2YR (Shigetomi et al., 2008). As an interesting observation, Ca^{2+} signals produced by PAR1 activation display longer durations than the Ca^{2+} signals produced by others receptors. Following this idea, pharmacological manipulation to increase the duration of cytoplasmatic Ca^{2+} signals in astrocytes as the blockade of mitochondrial Ca^{2+} buffering by Ru360, produces an increased glutamate exocytose in cultures (Reyes and Parpura, 2008). In addition, numerous reports have provide evidence to suggest that only very large evoked astroglial Ca^{2+} elevations are required to produce enough glutamate gliotransmission to stimulate neuronal receptors (Navarrete et al., 2012). Thus, according to this piece of evidence, the increase duration of Ca^{2+} signals may be a determining factor in increasing glutamate release from astrocytes. Otherwise, several groups hypothesized that might be other additional regulatory factors, as diverse neurotrophic factors or cytokines, required to facilitate gliotransmission. Indeed, it has been recently shown that tumor necrosis factor-alpha (TNFα) regulates glutamate gliotransmission and excitatory transmission in DG cells (Santello et al., 2011) and exogenous TNFα amplifies glutamate release from culture astrocytes (Bezzi et al., 2001). Since the levels of TNFα as well as other cytokines are subject to dramatic changes in conditions that involved neuroinflammation or microglia

activation as occurs the epileptic brain (Vezzani et al., 2008), TNFα may play a role in the astroglial signaling of the epileptic brain. Thus, an intriguing hypothesis is that in our conditions, in addition to the enhanced astroglial signaling, an increased level of proinflammatory molecules or cytokines may increase the astrocytic input to synapses, as the increased gliotransmission and upregulated synaptic efficacy we observed here.

Thus, these evidences suggest that the increase in ST incidence we recorded in kindled slices may facilitate the release of glutamate, being therefore a main factor in the elevated SICs frequency (Fig. 9A,B).

CHAPTER 3: THE IMPACT OF ALTERED ASTROGLIAL SIGNALING IN THE SYNAPTIC FUNCTION OF KINDLED-EPILEPTIC HIPPOCAMPUS

3.1 Increased astroglial Ca^{2+} signaling upregulate excitatory synaptic transmission in epileptic slices

CA3-CA1 synapses from epileptic slices showed an increased efficacy evident by the elevated mEPSC frequency, low PPR and elevated successful responses of meEPSC (Fig 9C-E and Fig 10). These findings are in accordance with the glutamatergic hypothesis of epilepsy since indicating an elevated excitatory tone of neuronal circuits. We did not observe differences in connectivity (i.e.: multiplicity index) or postsynaptic properties (i.e.: synaptic potency) between groups, which supports the idea that the main mechanism upregulating the synaptic strength in our epileptogenesis model operates mainly at the presynaptic level. The selective astroglial Ca^{2+}-signal blockade by intracellular-BAPTA experiments (Fig. 11) show that enhanced astroglial Ca^{2+} signaling is an important factor contributing for the maintenance of elevated synaptic efficacy in kindled hippocampal slices.

As described above, in control rats the basal glutamate gliotransmission set the basal Pr of hippocampal synapses (Bonansco et al., 2011), also determining the threshold for synaptic plasticity. Recent evidence suggests that actually astroglial Ca^{2+} signaling may also display a type of activity-dependent short-term plasticity in slices that also adjust synaptic transmission (Sibille et al., 2015). Interestingly, not only functional but also anatomical changes between astroglial processes and dendritic spines are produced by electrical stimulation, further showing that astrocytes display activity-dependent changes as much as neurons do. Thus, the fact that glutamate-mediated astrocyte-neuron pathway was increased in kindled slices, could suppose a kind of long-term plasticity in astrocyte-neuron signaling induced by electrical stimulation in the whole animal, which finally entails pathological consequences. This increased glutamate-gliotransmission and upregulated excitatory synaptic transmission could be involved in the glutamatergic imbalance described in epileptic networks, maintaining a high glutamatergic tone (Cavus et al., 2005) and setting excitatory transmission near to seizure threshold by taking part in the excitatory astrocyte-to-neuron

loop previously proposed in an acute ictogenesis models. This mechanism may also explain in part the higher basal levels of glutamate reported in the epileptic foci of patients with TLE (Seifert et al., 2006).

In parallel, at the postsynaptic level, due to the capability of astrocytes to influence the excitability of large groups of neighboring pyramidal cells (Fellin et al., 2004; Tian et al., 2005), the increase in SICs frequency may promote the hypersychronization of several CA1 neurons. This could be another mechanism contributing to the synchronic depolarization bringing neurons to the AP-firing threshold, thus decreasing the threshold for ictal activity (Crunelli and Carmignoto, 2013). However, our findings do not allow establishing a direct contribution of SICs to the excitability properties of CA1 pyramidal neurons in the epileptic network. Thus, further research in required for determining the effects of SICs in neuronal excitability.

CHAPTER 4: ASTROCYTE-NEURON PATHWAYS IN THE EPILEPTIC HIPPOCAMPUS

4.1 Role of group I mGluR

Our results show that two main receptors could be implicated in the abnormal astrocyte-neuron signaling: mGluR5 and P2Y1R. Selective blockade of mGluR5 reverses the upregulated synaptic strength decreasing the Pr, without affecting astroglial Ca^{2+} activity (Fig. 12 and Fig. 13). Accordingly, previous reports showed that glutamate gliotransmission modulates the Pr by activating group I mGluR at the presynaptic terminal (Perea and Araque, 2007; Bonansco et al., 2011), indicating that hyperexcitable astrocytes exert their abnormal modulation of synaptic transmission by using the same astrocyte-to-neuron signaling pathway than in healthy brain. This finding further support the idea that a long-term plastic change of this pathway occurs as a consequence of epileptogenic process. Despite group I mGluR may induce Ca^{2+} signals in astrocytes from younger rodents (Fellin et al., 2004; Panatier et al., 2011), recently it has been shown that mGluR5 expression is developmentally regulated, decreasing the level of mGluR mRNA and becoming undetectable in astrocytes during adulthood (Morel et al., 2014; Sun et al., 2013). Because our study was carried out in adult rats, this evidence corroborates the lack of effect of mGluR5 blockade on spontaneous astroglial Ca^{2+} activity, also observed in astrocytes from hippocampal *stratum lucidum* (Haustein et al., 2014); supporting the direct effect of this antagonist over presynaptic mGluR5 as previously shown. These data provide functional evidence to explain the inhibitory effect induced by group I mGluR antagonists on kindling progression (Nagaraja et al., 2005) as well as the pathophysiological role of the mGluR overexpression observed in hippocampal tissue obtained from TLE patients (Notenboom et al., 2006). Despite our data suggest that P2Y1R is involved in spontaneous Ca^{2+} transients, the remaining Ca^{2+} signals in P2Y1R antagonist presence (Fig.12 and Fig.13) suggest that other receptors or mechanisms may be involved. Interestingly, group II mGluR are found not only in neurons but also in astrocytes. Despite group II activation have linked to an inhibition of AMPc production, there is evidence that group II mGluR might also stimulate PLC and IP3 production then mediating Ca^{2+} signal in astrocytes (Nieden and Deitmer, 2006). Therefore,

it is likely that other receptors may be involved in astroglial Ca^{2+} signaling in epileptic hippocampus. Also, we cannot exclude alterations in the expression pattern of group I mGluRs from our model, since it has been reported in human and experimental epilepsy models by others (Aronica et al., 2000; Notenboom et al., 2006). However, we can confirm that under our conditions group I mGluR receptors are not involved in the generation of spontaneous astroglial Ca^{2+} signaling, but they are directly involved in the up-regulation of Pr in hippocampal synapses (Fig. 12 and Fig. 13). Even though an increased expression of mGluR5 in the presynaptic terminal could be enough to increase the basal Pr, the elevated SICs frequency suggests that increased Pr in our model is mainly due to elevated rate of gliotransmission (Fig. 9 and Fig.15).

We showed that bath application of DHGP for 5-10 minutes in presence of P2Y1R antagonist, which strongly reduced the mEPSC frequency; recovers the synaptic efficacy only in kindled slices (Fig. 14). Since no effect was observed in control slices after DHPG application, these findings strongly support that astrocyte-neuron signaling pathway is altered as a consequence of the epileptogenic process. According to that, long lasting functional enhancement of group I mGluR has been reported in amygdala-kindled rats, as well as in TLE and focal cortical dyplasia (Akiyama et al., 1992). Indeed, mGluRs have shown to be critically involved in plastic events (LTD and LTP) and changes particularly associated with epilepsy (Ure et al., 2006). Actually, stimulation of group I mGluRs elicits several forms of translation-dependent neuronal plasticity including epileptogenesis. Several works have shown that group I mGluRs are involved in the initiation of ictal discharges, and may play a crucial role in recruiting normal brain tissue into ictal synchronic discharges (Lee et al., 2002). Despite most of those works proposed a postsynaptic role of group I mGluR in the induction of prolonged epileptiform discharges (Zhao et al., 2015), since we blocked the intracellular pathway of postsynaptic metabotropic receptors by using GDPβS, our experiments strongly suggest that DHPG-mediated effects on synaptic transmission was mediated by presynaptic group I mGluRs. Another point we must report is that group I mGluR activation have been shown to promote epileptic-like discharges in CA3 neurons (Bianchi et al., 2009; Chuang et al., 2005). Because our experiments were performed in

TTX-containing ACSF –which blocked AP firing (Fig.13)- we can confirm that DHPG effects observed were restricted to CA3-CA1 synaptic area. Thus, we proposed that presynaptic group I mGluR activation by the glutamate released from astrocytes increase excitatory synaptic transmission, which could be another mechanism contributing to facilitate the propagation of seizure activity, therefore facilitating the epileptogenic process.

4.2 Role of purinergic P2YR1 in astroglial Ca^{2+} signaling

Astroglial Ca^{2+}-dependent hyperactivity was strongly reduced by P2YR antagonists in parallel with a decrease in the Pr (Fig.12 and Fig.13), replicating the effects observed in BAPTA experiments. MGluR5 inhibition eliminates any synaptic effect induced by P2Y1R inhibition (Fig. 13), suggesting that the modulation of Pr via mGluR occurs downstream of astroglial P2Y1R activation. Given that P2Y1R inhibition reduced the synaptic efficacy exclusively in kindled rats, we propose that increased purinergic signaling occur as a consequence of epileptogenesis induction.

Our results suggest that P2Y1Rs are directly implicated in the generation of astroglial Ca^{2+} signals responsible for the activation of the glutamate-mediated gliotransmission that contribute to the abnormal rise in the Pr. In agreement with this, it has been shown that P2Y1Rs activation, mainly expressed in astroglial processes (Jourdain et al., 2007), induces $[Ca^{2+}]i$ elevations and glutamate release from DG astrocytes (Domercq et al., 2006) that modulates synaptic strength through presynaptic mechanisms in physiological conditions (Jourdain et al., 2007; Pascual et al., 2012). Interestingly, in our control rats, P2Y1R did not produce any effect both in mEPSC frequency (Fig. 14) or astroglial Ca^{2+} signals (data not shown, manuscript in preparation). The animal's age and the hippocampal region of each study likely explain the disparity of the results, suggesting that astroglial Ca^{2+} signaling is both regional and developmentally regulated.

ATP-mediated signaling represents the main astrocyte-astrocyte communication pathway (Cotrina et al., 2000). Since in our experiments STs were TTX-insensitive in the kindled hippocampus, it is possible that astroglial hyperexcitability could arise from astrocytes

themselves, suggesting that an increase in astrocyte-to-astrocyte signaling could explain the exacerbated Ca^{2+} activity and gliotransmission. The molecular changes described in reactive astrocytes must be considered as a possible cause of the astroglial functional alteration observed here (Sofroniew, 2009). Altered expressions of several components involved in astrocyte-astrocyte signaling have been reported in gliotic conditions (Bennett et al., 2012), specifically, an upregulation of astroglial P2Y1R, P2Y2R (Franke et al., 2012) and connexin 43-containing hemichannels (Fonseca et al., 2002), as a result of a wide range of CNS injuries including epilepsy. Furthermore, blockade of hemichannels, the main non-vesicular pathway for astroglial ATP release in pathological conditions (Orellana et al., 2012), reduces the duration and severity of ictal events in experimental epilepsy models (Jahromi et al., 2002; Santiago et al., 2011). Hence, the above evidences support the idea that reactive astrocytes undergo structural changes that could alter Ca^{2+}-excitability and astrocyte-to-astrocyte signaling, which is in accordance with our observations in P2R blockade experiments (Fig. 12 and Fig. 13). However, we cannot rule out the possibility that microglia-derived purines, including ATP and ADP, could be also implicated (Pascual et al., 2012).

Another issue we have referred to is that in control conditions, P2Y1R activation produces an increased in excitatory synaptic transmission (Jourdain et al., 2007) but not the appearance of SICs (Shigetomi et al., 2008). Thus, since we observed both increased excitatory transmission and increased SICs frequency, there are two plausible possibilities to explain this matter. First, that P2Y1R expression is increased in kindled animal (Franke et al., 2012), leading to stronger Ca^{2+} signals therefore producing SICs appearance in CA1 neurons. Second, other receptors or mechanisms are involved in the increased SIC frequency from epileptic hippocampus, as the increased proinflammatory molecules or cytokines that occur in epileptic brain and that appears to play a role in the etiopathogenesis of several forms of epilepsy (Turrin and Rivest, 2004).

Nevertheless, we can conclude that the chronic or enhanced activation of P2Y1Rs in epileptic hippocampus may lead to increased extracellular glutamate able to activate mGluR5s present in presynaptic element, thereby increasing synaptic efficacy. Further

studies are needed to accurately determine the molecular mechanism underlying the aberrant astroglial function observed here.

4.3 Role of adenosine A_{2A}Rs

ATP is extracellularly degraded into adenosine, which could in turn participate in the Pr increase observed in kindled slices (Panatier et al., 2011). However, we did not observe a significant effect of the specific A_{2A}R antagonist, neither in the astroglial Ca^{2+} signal nor in Pr (Fig. 12). This finding could be explained at least in part by a lack of ecto-nucleotidase activity, responsible for catalyzing the breakdown of ATP into adenosine, as reported in epilepsy (de Paula Cognato et al., 2005). Moreover, the over-expression of ADK (i.e.: Adenosine kinasa) -an enzyme responsible for the conversion of adenosine into adenosine monophosphate (AMP)- reported in different epilepsy models (Boison et al., 2010) also supports our findings. However, we cannot rule out a probable compensatory mechanism in the expression of neuronal or glial adenosine receptors (D'Alimonte et al., 2009).

Taken together, these pieces of evidence suggest that the astrocyte-to-astrocyte purinergic signal seems to be the main source for astroglial hyperactivity that leads to upregulation of astrocyte-to-neuron signal, which could be responsible for maintaining high tone of glutamatergic neurotransmission in hippocampal epilepsy. Hence, this specific interplay between P2Y1 and mGluR5 can selectively activate astrocytes and neurons in a concerted way, which could account for the imbalance of neurotransmission in neuropathologies (model in Fig 15).

CONCLUDING REMARKS

In this work we have shown that astroglial physiology is chronically affected in kindled hippocampus. We successfully induced epileptogenesis in rats by using a new variant of RK protocol that produces severe alterations in the synaptic function of hippocampal formation. First, astrocytes from stratum radiatum showed both morphological and functional alterations, displaying hypertrophy, reactive astrogliosis and abnormal increase of spontaneous Ca^{2+} signaling namely astroglial hyperexcitability, which is likely mediated by an increase in purinergic astrocyte-astrocyte or neuron-astrocyte communication.

Second, as a consequence of such astroglial hyperexcitability, there is an increased astroglial glutamate release, which is able to activate presynaptic GPCRs, likely group I mGluR, upregulating the strengh of excitatory synaptic transmission by increasing the probability of neurotransmitter release. Both the increased neuronal and astroglial glutamate release contribute to maintain an elevated glutamatergic tone in the hippocampus of kindled rats. We resume this conclusion in the model presented in Figure 15.

These findings further suggest that astrocytes represent an important neuromodulatory system that works in parallel to neuronal one. Therefore here we show that, as alterations in neuromodulatory systems are related to diverse neuropathological processes, astroglial alteration strongly impact in synaptic function in the hippocampus of an epilepsy model. We proposed that pathological astrocyte-neuron communication could be the key mechanism that promotes seizure recurrence and epilepsy chronicity through the maintenance of an elevated glutamatergic tone.

This work provides direct evidence of the pathophysiological role of astrocytes, demonstrating that altered astroglial signaling has severe effects on synaptic transmission, and supporting the idea that astroglial hyper or hypoactivation could take part in a broad spectrum of neurological disorders, as previously suggested (Seifert et al., 2006; Halassa et al., 2007; Devinsky et al., 2013). Also, not only give us an idea about the impact of pathological astrocyte-neuron communication on brain physiology, but also open new fields to further consider astrocytes as therapeutic target other neuropathological conditions. Our findings support the pivotal role of astroglial Ca^{2+} signal and gliotransmission in pathophysiological conditions, providing a new perspective to advance toward understanding the biology of epilepsy and other brain diseases.

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