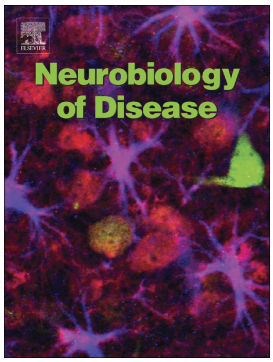

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P2Y1 receptor inhibition rescues impaired synaptic plasticity and astroglial Ca^{2+}-dependent activity in the epileptic hippocampus

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Abstract

Epilepsy is characterized by a progressive predisposition to suffer seizures due to neuronal hyperexcitability, and one of its most common co-morbidities is cognitive decline. In animal models of chronic epilepsy, such as kindling, electrically induced seizures impair long-term potentiation (LTP), deteriorating learning and memory performance. Astrocytes are known to actively modulate synaptic plasticity and neuronal excitability through Ca^{2+}-dependent gliotransmitter release. It is unclear, however, if astroglial Ca^{2+} signaling could contribute to the development of synaptic plasticity alterations in the epileptic hippocampus. By employing electrophysiological tools and Ca^{2+} imaging, we found that glutamatergic CA3-CA1 synapses from kindled rats exhibit an impairment in theta burst (TBS) and high frequency stimulation (HFS)-induced LTP, which is accompanied by an increased probability of neurotransmitter release (Pr) and an abnormal pattern of astroglial

Ca^{2+}-dependent transients. Both the impairment in LTP and the Pr were reversed by inhibiting purinergic P2Y1 receptors (P2Y1R) with the specific antagonist MRS2179, which also restored the spontaneous and TBS-induced pattern of astroglial Ca^{2+}-dependent signals. Two consecutive, spaced TBS protocols also failed to induce LTP in the kindled group, however, this impairment was reversed and a strong LTP was induced when the second TBS was applied in the presence of MRS2179, suggesting that the mechanisms underlying the alterations in TBS-induced LTP are likely associated with an aberrant modulation of the induction threshold for LTP. Altogether, these results indicate that P2Y1R inhibition rescues both the pattern of astroglial Ca^{2+}-activity and the plastic properties of CA3-CA1 synapses in the epileptic hippocampus, suggesting that astrocytes might take part in the mechanisms that deteriorate synaptic plasticity and thus cause cognitive decline in epileptic patients.

Keywords: gliotransmission, synaptic plasticity, epilepsy, astrocytes, purinergic receptors, long-term potentiation, kindling.

Introduction

Epilepsy is characterized by a progressive predisposition to suffer seizures as a consequence of an imbalance in neuronal excitability, which has been associated behavioral and cognitive alterations commonly exhibited, affecting about 70 million people worldwide (Fisher et al., 2005; Halgren et al., 1991; Helmstaedter, 2002). The difficulties that patients with epilepsy face extend far beyond seizures and include severe and disabling comorbidities as cognitive impairment, which has profound consequences in their life quality (Holmes, 2015). Several lines of evidence indicate that an exacerbated

glutamatergic activity, often referred to as the "glutamate hypothesis", is one of the key alterations in the pathophysiology of epilepsy (Soukupova et al., 2015). However, little is known about the cellular and molecular mechanisms that underlie cognitive decline in epileptic patients – which include deficits in attentional processes, working memory, and executive function –, and it is not fully understood how the aforementioned glutamatergic imbalance could contribute to its development (Campo et al., 2013; Celiker et al., 2019; Rai et al., 2015).

Kindling is the most employed experimental animal model of chronic epilepsy and closely resembles the development of epilepsy in humans (Loscher, 2011; McNamara et al., 1985; Morales et al., 2014; Morimoto et al., 2004). It consists in the presentation of repetitive, sub-convulsive electrical or chemical stimuli on a nervous structure (usually the amygdala or hippocampus) which elicits a gradual and progressive enhancement of electroencephalographic (EEG) activity and behavioral responses, culminating in generalized seizures. Numerous works suggest that the development of chronic epilepsy through electrical kindling and the induction of classical long-term potentiation (LTP) in glutamatergic synapses share a wide range of cellular and molecular mechanisms. Those mechanisms include the repetitive stimulation during the induction phase, the activity-dependent and input-specific strengthening of synaptic efficacy, the temporal coincidence between pre- and post-synaptic activity mediated by N-methyl-D-aspartate receptors (NMDARs; Larson and Munkacsy, 2015; Matsuzaki et al., 2004; Nicoll and Schmitz, 2005), among other mechanisms, all of which are also at the very basis of the plasticity processes that underlie learning and memory (Leung and Shen, 2006; Stasheff et al., 1989; Sutula et al., 1988). It has been suggested that repeated seizure activity causes an indiscriminate and widespread enhancement in synaptic efficacy, interfering with the

synaptic plasticity (i.e.: LTP) required for information processing and learning (Reid and Stewart, 1997). In fact, previous research indicates that LTP is markedly reduced at the epileptic focus (i.e.: hippocampus) in humans with temporal lobe epilepsy (Beck et al., 2000). In line with those results, experiments with animal models suggests that kindling can suppress LTP (Leung and Wu, 2003), whereas electrically-induced seizures can block (Hu et al., 2005) or reduce it and impair learning and memory (Esmaeilpour et al., 2017; Ghotbeddin et al., 2018; Moore et al., 1993). However, the cellular mechanisms by which epilepsy – like kindling – can alter LTP remain unclear.

During the last 15 years, it has been shown that astrocytes modulate both short- and long-term synaptic plasticity as well as neuronal excitability through Ca^{2+}-dependent gliotransmitter release (Bonansco et al., 2011; Perea and Araque, 2005; 2007). For instance, it has been proposed that spontaneously released glutamate from astrocytes actively regulate the basal Pr of glutamatergic terminals by activating presynaptic metabotropic glutamate receptors of the mGluR1/5 subtypes, operating as a gain control mechanism that modulates the induction threshold for LTP (Bonansco et al., 2011; Panatier et al., 2011). In addition, growing evidence indicates that astrocytes directly participate in the pathophysiology of several brain conditions, including epilepsy (Nikolic et al., 2019; Pirttimaki et al., 2013; Shigetomi et al., 2019; Verkhratsky et al., 2017). In previous reports, we showed that reactive astrocytes from kindled rats exhibit an abnormal pattern of Ca^{2+} transients (i.e.: Ca^{2+}-mediated hyperexcitability) that enhances glutamate-mediated gliotransmission and increases neuronal glutamate release via presynaptic mGluR1/5 receptor activation (Álvarez-Ferradas et al., 2015). This abnormal pattern of astroglial Ca^{2+} activity requires purinergic P2Y1 receptor (P2Y1R) and Pannexin-1 hemichannel activation (Wellmann et al., 2018). Those mechanisms could represent a form of astrocyte-mediated

metaplasticity and have been proposed as the basis for an astrocyte-to-neuron loop that chronically upregulate excitatory transmission, bringing epileptic circuits near seizure threshold (Álvarez-Ferradas et al., 2015; Cavus et al., 2005; Gómez-Gonzalo et al., 2010; Jones, 2015; Wellmann et al., 2018; Riquelme et al., 2020). Nonetheless, whether and how astroglial-mediated modulation of synaptic transmission contributes to the impairments in synaptic plasticity (i.e.: LTP) and thus to the development of cognitive decline in epileptic patients remains unknown.

Using hippocampal slices from fully kindled rats (Morales et al., 2014), we tested if different electrical stimulation protocols were able to induce LTP, assessing synaptic efficacy and astroglial Ca^{2+} activity before, during and after the induction of activity-dependent synaptic plasticity. We found that both theta-burst (TBS) and high frequency (HFS) stimulation-induced LTP are impaired in glutamatergic CA3-CA1 synapses from kindled animals. These impairments are associated with an anomalous pattern of astrocytic Ca^{2+} signals (i.e.: slow Ca^{2+}-transients: STs) that abnormally up-regulate the Pr of CA3-CA1 synapses. Both the impaired LTP and the increased Pr were reversed by inhibiting P2Y1 receptors with MRS2179, which also restored the pattern of astroglial Ca^{2+}-dependent activity. Altogether, our data suggest that hyper-excitable astrocytes are directly involved in the development of the glutamatergic dysfunction in the epileptic brain, likely impairing LTP by abnormally increasing the Pr in CA3-CA1 synapses. These results offer a new mechanism that explains the decreased plastic capacity of neural circuits and thus the cognitive decline in epileptic patients.

Methods

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

Stereotaxic surgery and kindling protocol

Epileptogenesis was induced using a rapid kindling protocol (RK) previously described (Álvarez-Ferradas et al., 2015; Morales et al., 2014; Wellmann et al., 2018). Briefly explained, male Sprague-Dawley rats (35-40 postnatal days) were anesthetized and subjected to stereotactic surgery procedures for electrode implantation. Following stereotaxic coordinates (Paxinos and Watson, 1998), two pairs of electrodes for cortical recording were bilaterally implanted in the primary motor cortex and the visual cortex, and one stimulation electrode in the right basolateral amygdala complex (BLA), plus an anchorage screw. Following surgery, rats had a recovery period of at least one week before the start of the kindling procedure.

The RK protocol consists of ten daily trains of biphasic rectangular current pulses at subthreshold after-discharge (ADs) intensity, applied for three consecutive days. Rats subjected to the RK protocol displayed progressive epileptic activity throughout the brain, accompanied by seizures that increased in severity as the protocol went on (Morales et al., 2014). ADs progression and seizure severity were assessed using EEG recordings and the Racine scale (De Jonge and Racine, 1987), respectively. Rats were considered fully kindled after having suffered at least five consecutive generalized epileptic seizures (i.e., Racine 4 and 5 states) accompanied by long-lasting repetitive ADs. Under these conditions, cortical and mesolimbic structures of fully kindled rats are considered epileptic tissue, including the hippocampal formation (Morales et al., 2014; Steward et al., 1991). The control group

consisted of rats that were subjected to electrode implantation surgery but remained non-stimulated (i.e., sham), and naïve animals; results were pooled together because no significant differences were found in this nor in previous reports (Alvarez-Ferradas et al., 2015; Morales et al., 2014; Wellmann et al., 2018).. All the experiments were performed at least 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.

Electrophysiology and hippocampal slice preparation

Acute slices from the dorsal hippocampus were obtained from control and kindled rats as previously described (Bonansco and Buño, 2003; Bonansco et al., 2011). Briefly, rats were anesthetized and decapitated, the brain was rapidly removed through craniotomy and placed in ice-cold (< 4ºC) artificial cerebrospinal fluid (ACSF), gassed with a mixture of 95% O2 and 5% CO2 (pH 7.4). Transversal brain slices (300-350 μm thick) were obtained 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 KH_2PO_4, 2.0 Mg_2SO_4, 26.0 $NaHCO_3$, 2.5 $CaCl_2$ and 10.0 glucose. 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 (5% CO_2 and 95% oxygen; 2ml/min) and maintained at room temperature (21-24ºC). All recordings were made in presence of the $GABA_AR$ antagonist picrotoxin (PTX, 10 μM).

Two recording methods were employed: extracellular field potentials and whole-cell patch-clamp recordings (Bonansco et al., 2007). Field excitatory postsynaptic potentials (fEPSP) were performed with a glass pipette (2–4 MΩ, filled with perfusion medium) placed in the middle of the CA1 *stratum radiatum* and connected to an AC amplifier (P-5 series; Grass) with a gain of 310.000x, a 3.0-kHz low pass filter, and a 0.30 Hz high pass filter. To evoke fEPSPs, a bipolar concentric electrode (concentric platinum/iridium electrode, 125 μm outer, FHC Inc., Bowdoin, ME, USA) was placed in the *stratum radiatum* within 100-200 μm from the recording site to activate the Schaeffer collaterals fibers using bipolar cathodic stimulation (50 μsec, 0.3 Hz, 20–100 μA) generated by a stimulator (A.M.P.I., Israel) connected to an isolation unit (Isoflex, AMPI), which elicited compound action potentials from the presynaptic axons (fiber volley; fV) followed by fEPSPs. Unless otherwise stated, whole-cell voltage-clamp recordings from CA1 pyramidal cells clamped at -60 mV were made using a EPC-7 amplifier (Heka Instruments, Germany), and patch-type pipette electrodes (~5 MΩ) containing (in mM): 130 KMeSO4, 10 HEPES, 4 ATP-Na2 and 20 KCl, 290 mOsm, adjusted to pH 7.3, (Zhang et al., 1994). Excitatory postsynaptic currents (EPSCs) were elicited every 5 s, filtered at 3.0 kHz and 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 the whole-cell configuration. Cells that exhibited a significant change in access resistance (>20%) were excluded from the analysis. We used a paired-pulse protocol and calculated the paired-pulse ratio (PPR) to estimate putative presynaptic changes. PPR was defined as the ratio between the peak amplitude of the second and the first EPSCs (80 ms apart). Relative changes in PPT throughout the recordings were

calculated as a percentage variation compared to baseline values. LTP was induced by using two well-known protocols delivered at the SCs: theta-burst stimulation (TBS) and high-frequency stimulation (HFS). TBS consisted in 10 trains of 5 pulses at 100 Hz, delivered at 200 ms intervals, containing 10-12 bursts 30 s apart; HFS consisted in 2 trains at 100 Hz (1-sec duration; 30 s apart). Off-line recording analysis was performed using the software pClamp by Molecular Devices (USA).

Ca^{2+} imaging in astrocytes

Intracellular Ca^{2+}-elevations from astrocytes were monitored by fluorescence microscopy using Fluo4-AM as cell-permeant Ca^{2+} indicator, as previously described (Wellmann et al., 2018). For Ca^{2+} imaging experiments, brain extraction and slice preparation were performed in sucrose enriched ACSF (215.0 mM). To confirm the specific recording of Ca^{2+} signals from astrocytes, slices were incubated first with the astroglial morphological marker sulforhodamine 101 (SR101; 0.5-1 μM; Kafitz et al., 2008) for ~30 minutes in low Ca^{2+}/high Mg^{2+} ACSF at 32-34 °C. Slices were then transferred to a maintenance chamber with regular ACSF for 30 min and later incubated with FLUO4-AM (1–2 μL of the dye dissolved in pluronic acid at 0.01% was dropped over the hippocampus, obtaining a final concentration of 5–10 μM) for ~75 minutes in regular ACSF at room temperature. Astrocytes were imaged using a CCD camera (Andor DR328G; Andor Technologies PLC, Ireland) attached to a fluorescence microscope (Nikon, Japan). The camera was controlled and synchronized by the Niss-Elements AR 3.2 software (Nikon, Japan), which was also used for offline analyses. Cells were illuminated with a Xenon lamp at 490 nm (200-400 ms exposure; 36,700 mm^2 area), and images were acquired at 1 Hz for 5 minutes, regulated by a shutter (Lambda SC-Smart shutter, Sutter Instrument Company).

Analyses of astroglial Ca^{2+} levels were restricted to the cell body and Ca^{2+}-transients were estimated as changes in the fluorescence signal over the baseline (ΔF/F0) after background subtraction. For every individual cell, the baseline was obtained by extracting and averaging the fluorescence values of at least 30 consecutive frames were the astrocyte exhibited no spontaneous Ca^{2+}-dependent activity. Changes in fluorescence were considered as events when the ΔF/F0 intensity exceeded the fluorescence of the baseline by at least two standard deviations for no less than five consecutive frames. For multi-peak astroglial Ca^{2+} activity, those events in which the ΔF/F0 dropped to a half of the maximum fluorescence intensity in relation to the baseline were considered as independent transients. As we previously described (Wellmann et al., 2018), the duration of astroglial Ca^{2+} transients was distributed among two populations starting from the 75th percentile of the cumulative distribution – which was 17 s –, being that value employed as a cut-off criterion from which random events in the same astrocyte were classified as slow (STs >17 s) or fast transients (FTs <17 s; Figure 1E-F). Also, we incorporated the FT/ST frequency ratio as an index of astroglial excitability (Wellmann et al., 2018), where a lower index would be associated to a greater Ca^{2+}-dependent excitability.

Statistical analysis

In all cases, a distribution analysis was performed first to determine if the data fitted a 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. For multiple comparisons, ANOVA or Kruskal-Wallis test was used as appropriate, both with *post hoc* Bonferroni correction. Unless otherwise stated, all data is expressed as mean ± SEM. Differences were considered statistically significant at $p<0.05$.

Results

TBS fails to induce LTP at CA3-CA1 synapses and causes an anomalous astroglial Ca^{2+}-dependent response in kindled hippocampal slices

The effect of epileptogenesis over synaptic plasticity paradigms such as LTP are often controversial, having previous reports described increases (Krug et al., 1997; Ruethrich et al., 1996; Schneiderman, 1997), decreases (De Jonge and Racine, 1987; Gilbert and Mack, 1990; Maru and Goddard, 1987) or even complete suppressions of LTP (Leung and Wu, 2003; Schubert et al., 2005; Suárez et al., 2012). Using hippocampal slices, we evaluated if the induction of LTP is altered in kindled rats (Morales et al., 2014). The application of a TBS protocol after a stable baseline (~10 min; Figure 1A) induced a robust LTP in control rats (at 30-40 min; 249.07 ±8.46 % of baseline, p = 0.016; n = 6), but failed to induce LTP in the kindled group (to 105.23 ±7.3 % of baseline, p = 0.073; n = 5; Figure 1A). In addition, kindled rats exhibited lower PPR values before and after the protocol compared to control animals (kindled: 1.25 ± 0.09; n = 6 vs control: 1.75 ± 0.05; n = 5; p = 0.03; Figure 1C, 1D). Since astrocytes from the epileptic hippocampus show an abnormal pattern of spontaneous Ca^{2+}-dependent activity (i.e.: increased frequency of slow transients, STs; Álvarez-Ferradas et al., 2015; Wellmann et al., 2018) that could alter astroglial-mediated mechanisms that regulate synaptic plasticity, we assessed if astrocytes in the kindled hippocampus respond with changes in their pattern of Ca^{2+}-dependent activity during and after the TBS protocol. In basal conditions, control slices exhibited both fast (i.e., FTs <17s) and slow (i.e., STs >17s) spontaneous astroglial Ca^{2+} transients, being FTs the most prevalent type of oscillations (Figure 1E). During the TBS protocol both FTs

and STs increased in number, but STs exhibited a greater relative increase compared to FTs (basal FT; 6.1 ± 0.5 vs TBS FT; 6.5 ± 0.5; $p < 0.05$ and basal ST; 1.4 ± 0.1 vs TBS ST; 3.1 ± 0.3; $p < 0.001$; n = 56; Figure 1E), which is reflected by the decrease in the FT/ST ratio (basal; 4.5 ± 0.60 vs during TBS; 2.3 ± 0.37, p = 0.021; Figure 1E). This shift in the overall Ca^{2+}-dependent activity pattern was quickly reversed after the protocol. In contrast, astrocytes from the kindling group showed a higher rate of basal activity (basal FT; 5.1 ± 0.4 vs basal ST; 4.5 ± 0.1; $p < 0.01$; n = 43), and a lower FT/ST ratio, which resembles the pattern of Ca^{2+}-dependent activity observed in the group control during the TBS protocol (Control TBS 2.3 ± 0.41, n = 57, vs Kindling baseline, 1.74 ± 0.40, n = 43, p = 0.29). During the protocol, STs augmented in number (Figure 1F; basal; 4.5 ± 0.36 vs during TBS; 6.3 ± 0.6; $p<0.05$, n = 61) but without fluctuations in the FT/ST ratio (Figure 1F; basal 1.74 ± 0.40, n = 43, vs TBS 1.76 ± 0.40, n = 52, p = 0.76), indicating that both FTs and STs increased together without changes in the global pattern of astroglial Ca^{2+}-dependent activity in response to TBS, which suggests that the abnormal pattern of Ca^{2+}-dependent activity emerges in an uncoupled, synaptic activity-independent fashion. These findings show that both neurons and astrocytes in the epileptic hippocampus exhibit an abnormal response to the TBS protocol, which is characterized by an impairment in TBS-induced LTP in CA3-CA1 synapses and the maintenance of an abnormal pattern of astroglial Ca^{2+}-dependent activity.

Consecutive, spaced TBS protocols failed to induce LTP in the kindled hippocampus

Since a single TBS protocol did not induce LTP, we next tested if a stronger protocol consisting in two spaced (30 min inter-stimulus interval), consecutive TBS can

induce a plastic change (Schubert et al., 2005). In control slices, both protocols (TBS1 and TBS2) induced a short period of post-tetanic facilitation followed by an increase in EPSC amplitude that reached a steady state after 30 min (159.19 ± 10.11 % of baseline; n = 5) for TBS1 and 50 min for TBS2 (208.88 ± 25.50 % of baseline; n = 5; Figure 2D). In kindled slices, although post-tetanic facilitation was observed after both TBS1 and TBS2, neither protocols were able to induce LTP (Figure 2D, kindled: TBS1; 109.41 ± 7.53 % versus TBS2; 117.55 ± 13.84 % respect to baseline; n = 6; $p > 0.05$). In addition, basal PPR differences between groups were maintained throughout the experiments (Figure 2C; basal, control: 1.55 ± 0.06 vs kindled: 1.24 ± 0.03; $p = 0.02$), without exhibiting any changes after TBS1 nor TBS2 (Figure 2D; after 50 min, control: 111.45 ± 9.03 % of baseline vs kindled: 103.18 ± 3.18 % of baseline; $p = 0.63$), which is consistent with the previous experiments. The fact that even repeated TBS protocols are not able to induce plastic changes in CA3-CA1 synapses from kindled rats further supports the previous results and suggests that there is likely no room for these synapses to be potentiated by TBS-dependent mechanisms.

Impairment in HFS-induced LTP in hippocampal glutamatergic synapses from kindled rats

It has been proposed that astrocytes modulate synaptic plasticity and neuronal excitability not only at a single-synapse level but also at a neural circuit level (Ma et al., 2016; Ben Haim and Rowitch, 2017). If that is the case, the abnormal pattern of astroglial Ca^{2+}-dependent activity observed in kindled slices should affect the excitability and plasticity of large neuronal populations, which would be reflected in extracellular field recordings. In order to address that, we recorded field excitatory postsynaptic potentials

(fEPSP) before and after the application of HFS – which is a stronger plasticity protocol compared to TBS – and population spikes. First, we compared fEPSP input-output relationships between control and kindled slices. Superimposed field responses showed that for a given fiber volley amplitude, the fEPSP is consistently larger in kindled rats, which is reflected by the input-output relationships (Figure 3A). Second, we evaluated the population spike threshold, which has been reliably used as an indicator of postsynaptic excitability (Andersen et al., 1980). The stimulation intensity required to induce a population spike in the kindled group was consistently lower compared to the control group (Figure 3B; 0.29 ± 0.17 μV, n = 10 in kindling vs 0.51 ± 0.01 μV, n = 10 in control; p = 0.032). We next evaluated the relative changes in fEPSP amplitude after the application of the HFS protocol. In control rats HFS induced a short period of post-tetanic facilitation followed by a progressive enhancement in synaptic transmission, which reached its peak after 40-50 min (167.82 ± 8.13 % of baseline, p<0.001; n= 8; Figure 3C-D). Kindled slices also exhibited a slight period of post tetanic facilitation, however, the subsequent LTP was of significantly lesser magnitude compared to the control group (125.51 ± 7.81 % of baseline, p < 0.001 n = 8; Figure 3C-D). Again, in both groups the PPR remained unchanged after the protocol (Control basal; 1.37 ± 0.14 and HFS; 1.32 ± 0.20; n = 10; P>0.05 vs Kindling basal; 1.10 ± 0.05 and HFS; 1.08 ± 0.04; n = 8; P>0.05; Figure 3D). Taken together, these findings suggest that the impairments in synaptic plasticity and excitability observed in the kindled group affects hippocampal neuronal circuits in a widespread manner, which could be associated with the abnormal pattern of Ca^{2+}-dependent activity in the astroglial network.

Rescuing the normal pattern of astrocytic Ca^{2+}-dependent activity restores TBS-induced LTP in the epileptic hippocampus

Since we recently showed that P2Y1R inhibition restores the FTs/STs relationship and thus the normal pattern of astroglial Ca^{2+} activity in the kindled hippocampus (Álvarez-Ferradas et al., 2015; Wellmann et al., 2018), we assessed if MRS2179 can also rescue the impairment in TBS-induced LTP in CA3-CA1 synapses. Control experiments were also performed in the presence of MRS2179 to rule out other potential effects of the purinergic antagonist over synaptic plasticity. As predicted, P2Y1R blockade rescued TBS-induced LTP in the kindled group, reaching a magnitude comparable to the observed in the control group (Figure 4D; kindled: 160.42 ± 12.01% of baseline; n = 6 vs control: 149.70 ± 23.51 % of baseline; n = 7; p = 0.72). MRS2179 also restored the basal PPR in the kindled group (Figure 4D; kindled: 1.47 ± 0.05; n = 4 vs control: 1.54 ± 0.07; n = 5; p = 0.26), which in both groups remained unchanged after the protocol (Figure 4; kindling: 106.33 ± 23.51 % of baseline; n = 7 vs control: 98.25 ± 6.31% of baseline; n = 6; p = 0.07). Concurrently, P2Y1R blockade restored the pattern of astroglial Ca^{2+}-dependent activity in the kindled group (Figure 4E), exhibiting a coupled, time-locked increase in STs oscillations (Figure 4F; 4.1 ± 0.88 before vs 6.33 ± 1.32 % after; n = 62; p = 0.02) in parallel to a decrease in the FT/ST ratio (Figure 4F; 3.91 ± 0.53 before vs 1.34 ± 0.25 after; p = 0.01), which was also observed in the control group with MRS2179 (Figure 4F). These findings indicate that the impairment in TBS-induced LTP observed in the kindling group could be directly associated with the abnormal pattern of Ca^{2+}-dependent signaling from astrocytes. To confirm if the impairment in LTP is linked to P2Y1R activation, we employed the protocol

consisting in two spaced (30 min inter-stimulus interval), consecutive TBSs again, but performed TBS2 in the presence of MRS2179. If P2Y1R blockade is sufficient condition to rescue TBS-induced LTP, pyramidal neurons from kindled slices should potentiate with TBS2 plus MRS2179 after TBS1 has failed to induce LTP. Indeed, while TBS1 alone had no effect, after 10 min incubation with MRS2179, TBS2 induced a strong LTP (Figure 5A-B: TBS1: 113.33 ± 5.88 % of baseline vs TBS2+MRS2179: 185.42 ± 16.19 % of baseline; n = 5; p = 0.015). As expected, P2Y1R blockade not only rescued LTP but also increased the PPR (Figure 5C-D: baseline: 1.21 ± 0.09; TBS1: 1.22 ± 0.06; TBS2 plus MRS2179: 1.54 ± 0.11; n = 5; p = 0.007). Altogether, these findings indicate that the inhibition of the purinergic signals (i.e.: P2Y1R) underlying astroglial Ca^{2+}-dependent hyper-excitability rescues TBS-induced LTP, suggesting that the overall pattern of spontaneous astroglial Ca^{2+} signals is crucial for the maintenance of the plastic properties of CA3-CA1 synapses.

Discussion

Here we have followed up on our previous studies showing that astrocytes in the epileptic hippocampus exhibit an abnormal pattern of Ca^{2+} activity that requires P2Y1R (Wellmann et al., 2018), which causes an upregulation in glutamate gliotransmission and thus enhances the Pr of CA3-CA1 synapses through presynaptic mGluR1/5 activation (Álvarez-Ferradas et al., 2015). Our current findings indicate that this anomalous purinergic-mediated pattern of astroglial Ca^{2+} signaling is also associated with an impairment in LTP at CA3-CA1 synapses, and could operate as an aberrant mechanism of astrocyte-dependent metaplasticity that interferes with synaptic plasticity in the hippocampus. This dysregulation in astrocyte-neuron crosstalk – which can be reversed by controlling astrocyte-to-astrocyte purinergic signaling – could work as one of the mechanisms

deteriorating synaptic plasticity in the epileptic brain and explain, at least partially, the development of cognitive symptoms in epileptic patients.

Synaptic plasticity impairments are associated with an abnormal pattern of astroglial Ca^{2+}-dependent signals in the epileptic hippocampus

Memory and learning alterations in epileptic patients have been associated with aberrant functional and structural neuroplasticity mechanisms, including changes in synaptic efficacy (i.e.: upregulated glutamatergic tone) as well as neuronal circuitries remodeling, both of which considered hallmarks of the epileptic brain (Chauvière, 2020; Mikkonen et al., 1998; Postnikova et al., 2019). Here we showed that neuroplasticity alterations in epilepsy extend beyond the neuronal domain and also affect Ca^{2+}-dependent activity in astrocytes, with neurons exhibiting an impairment in TBS-induced LTP and astrocytes showing an unresponsive, aberrant P2Y1R-mediated pattern of Ca^{2+} signals during the TBS protocol that is associated with the impairment in synaptic plasticity. In fact, several studies have revealed that astrocytes are actively involved in the pathophysiology of epilepsy, showing not only structural changes (i.e.: reactive astrogliosis, Fedele et al., 2005; Shigetomi et al., 2019) but also functional abnormalities in Ca^{2+}-dependent signaling and gliotransmission that affect synaptic transmission (Álvarez-Ferradas et al., 2015; Alves et al., 2017; Nikolic et al., 2019; Wellmann et al., 2018; see also Riquelme et al., 2020). Nonetheless, in spite that emerging evidence has shown that both spontaneous and evoked astroglial Ca^{2+}-dependent signals directly modulate several forms of short and long-term synaptic plasticity (Bonansco et al. 2011; Navarrete et al., 2012; Perea and Araque, 2007; Sherwood et al., 2017), it was not clear if and how

astrocytes could contribute to the alterations in synaptic plasticity and thus to the cognitive decline observed in epileptic patients. Previous works have shown that the electrical stimulation of the Schaffer collaterals – the main glutamatergic afferents of CA1 pyramidal neurons – induces stimuli-coupled, transient astroglial Ca^{2+} signals (Figure 1E; see also Perea and Araque, 2005; Honsek et al., 2012; Navarrete et al., 2012; Sibille et al., 2015) and gliotransmitter release, which in turn modulate synaptic plasticity in the healthy hippocampus (Covelo and Araque, 2018). Concordantly, our results indicate that astrocytes from both control and kindled slices respond to TBS with an increase in Ca^{2+}-dependent signals. However, astrocytes from control slices did not only exhibit an increase in Ca^{2+}-dependent activity but also a shift in the overall pattern of Ca^{2+}-signals, with STs showing a greater relative increment compared to FTs as revealed by the higher STs incidence accompanied by a decrease in the FT/ST ratio (Figure 1E). In contrast, astrocytes from kindled slices showed an aberrant pattern of spontaneous astroglial Ca^{2+} transients in basal conditions, characterized by a higher incidence of long-lasting Ca^{2+} events (STs) that persisted during the TBS protocol, without any modifications in the overall Ca^{2+} pattern as it is indicated by the unchanged FT/ST ratio. Moreover, the pattern of Ca^{2+}-dependent activity observed in the kindled group in basal conditions is comparable to the one exhibited by control astrocytes during the TBS protocol, suggesting that, at rest, astrocytes from the kindled group are as active as if they were subjected to an intense synaptic input. These observations are consistent with the lower PPR values exhibited here by the kindling group, since it has been repeatedly shown that astroglial Ca^{2+}-dependent activity regulate the Pr of C3-CA1 synapses through glutamate release and group I mGluRs activation at presynaptic terminals (Bonansco et al., 2011, Covelo and Araque, 2018; Panatier et al., 2011; Perea y Araque, 2007). In fact, astroglial-induced chronic modifications in the Pr and

group I mGluRs signaling could account for the alterations in synaptic plasticity observed here. It has been shown that mGluRs activation in hyperexcitable conditions (zero $Mg^{+}2$ or high concentrations of picrotoxin) can induce long-term depression by modulating voltage-dependent Ca^{2+} channels and Ca^{2+} release from intracellular stores in presynaptic terminals (Palmer et al., 1997; Schwartz and Alford, 2000). In addition, previous reports have shown that experimentally increasing the Pr by employing a high Ca^{2+} medium reduces the magnitude of LTP (Bolshakov and Siegelbaum, 1995; Schulz, 1997). Since astroglial Ca^{2+}-dependent hyperexcitability enhances glutamate gliotransmission and thus the Pr of CA3-CA1 synapses by tonically activating group I mGluRs (Álvarez-Ferradas et al., 2015), it is possible that stimulation protocols that normally induce LTP (TBS or HFS) trigger cellular and molecular mechanisms with opposing effects – depression vs potentiation – in the epileptic brain, canceling the induction of synaptic plasticity. Supporting this idea, our data shows that in kindled slices rescuing the normal pattern of astroglial Ca^{2+} activity restores the plastic properties of CA3-CA1. Although these results suggest that astroglial Ca^{2+}-dependent hyper-excitability could explain the impairment in synaptic plasticity by several mechanisms that include changes in glutamate gliotransmission, group I mGluRs activation, and Pr upregulation, we do not rule out other possible effects mediated by different gliotransmitters at the presynaptic or postsynaptic level.

Several works suggest that the inhibition of spontaneous astroglial Ca^{2+} transients with the gliotoxin fluorocitrate, intracellular BAPTA or the IP_3R blocker heparin increases the threshold for LTP, suggesting that the basal pattern of astroglial Ca^{2+} activity and gliotransmitter release tonically tunes the threshold for synaptic plasticity induction (Bonansco et al., 2011; Panatier et al., 2011; Sherwood et al., 2017). Our experiments here allow to suggest that, in addition to this basal astroglial-mediated modulation of synaptic

plasticity, the induction of LTP requires an activity-dependent, stimuli-coupled shift in the pattern of astroglial Ca^{2+}-dependent transients, which is absent in the hippocampus of epileptic rats. Supporting this idea, restoring the normal pattern of astroglial Ca^{2+}-dependent activity alone is enough for CA3-CA1 synapses to recover their plastic properties (Figure 4). Since it has been suggested that astrocytes actively process synaptic information by exhibiting different patterns of Ca^{2+}-dependent activity (Covelo and Araque, 2018; Perea et al., 2016), the unresponsive Ca^{2+} activity pattern exhibited by astrocytes in the epileptic hippocampus during the TBS protocol allows to suggests that its capacity to decode and adequately respond to synaptic information is impaired, which could interfere with its modulatory function over neural circuits.

The pattern of P2YRs-mediated astroglial Ca^{2+} signals modulates synaptic plasticity in the epileptic hippocampus

Our experiments here and previous evidence suggest that the abnormal pattern of astroglial Ca^{2+} activity observed in the hippocampus of epileptic rats is driven by purinergic astrocyte-to-astrocyte signals that require the activation of P2Y1R (Figure 4; Álvarez-Ferradas et al., 2015; Wellman et al 2018; see also Riquelme et al., 2020). It has been previously indicated that ATP-mediated purinergic signaling acts as the main pathway for the propagation of Ca^{2+}-dependent signals between astrocytes in several neuropathologies. In those conditions, ATP reaches the extracellular medium mostly through hemichannels and, by activating metabotropic P2Y1 and P2Y2 receptors (Bowser and Khakh, 2007; Cieślak et al., 2017; Nicolic et al., 2019; Pascual et al., 2005), allows for the generation of aberrant astrocyte-to-astrocyte Ca^{2+} signals in a wide range of neuropathologies, including

epilepsy (Shigetomi et al., 2019; Riquelme et al., 2020). These network-wide, purinergic-mediated alterations in astroglial Ca^{2+}-dependent activity could upregulate glutamate gliotransmission and not only abnormally modulate the Pr, as previously indicated, both also enhance synchronous activity and excitability of local neural circuits (Gómez-Gonzalo et al., 2010; Lee et al., 2013; Tian et al., 2005), which is one of the hallmarks of the epileptic brain.

As mentioned earlier, our results here showed that the synaptic activity triggered as prompted during TBS did not modify the global pattern of astrocytic Ca^{2+} transients in the kindled group. This specific finding suggests that Ca^{2+}-dependent gliotransmission could be increased and occur in a stimuli-uncoupled fashion in the epileptic hippocampus, meaning that is not elicited by activity-dependent neurotransmission. This could upregulate glutamate and ATP release from the astroglial network, modifying the induction threshold, degree, or direction of activity-dependent changes in synaptic efficacy (Jones, 2015; Notenboom et al., 2010; van Dam et al., 2004). In fact, in the kindled group, P2Y1Rs inhibition not only restored the basal pattern of astroglial Ca^{2+}-dependent activity and the PPR rate, but also the normal astroglial response to the TBS protocol and the plastic properties of CA3-CA1 synapses. Moreover, our results show that MRS2179 has an effect over the pattern of Ca^{2+}-dependent activity only in astrocytes from the kindled condition, suggesting that P2Y1R activation is a feature specifically associated with abnormal astroglial Ca^{2+} signals. In fact, recent evidence allows to suggest that the effects of MRS2179 are mostly mediated by the inhibition of astroglial and not neuronal P2Y1R: (i) ATP is released by astrocytes as a mechanism for autocrine and paracrine signaling, being P2Y1R activation one of the main pathways for astrocyte-to-astrocyte communication in pathological conditions (Riquelme et al., 2020; Shen et al., 2014); (ii) P2Y1R activation

induces slow astroglial Ca^{2+} transients (Álvarez-Ferradas et al., 2015; Wellmann et al., 2018; Shigetomi et al., 2019) ; (iii) P2Y1R are preferentially and mostly expressed by astrocytes in the hippocampus (Di Castro et al., 2011); (iv) P2Y1R are overexpressed in several animal models of experimental epilepsy and in brain tissue from epileptic patients (Alves et l., 2017); and (v) P2Y1R activation exacerbates epileptiform activity and seizure severity (Alves et al., 2020). Therefore, the dysregulation of the purinergic signals that mediate astrocyte-to-astrocyte communication alone could alter astroglial Ca^{2+}-dependent activity and thus profoundly disrupt not only the modulatory effect that astrocytes exert over basal synaptic transmission, but also modify the plastic properties of neighboring neural circuits.

Extracellular levels of adenosine, which acts as an endogenous anticonvulsant via adenosine type 1 receptor (A1Rs) activation, are also affected in the epileptic brain, exhibiting a marked decrease due to the overexpression of adenosine kinase (ADK) (Etherington and Frenguelli, 2004; Fedele et al., 2005). The decrease in A1Rs activation promotes glutamate release and postsynaptic depolarization, leading to an increase in neuronal excitability and epileptiform activity (Aronica and Crino, 2011; Shen et al., 2014). Since ATP/adenosine homeostasis is controlled exclusively by astrocytes, its alteration could chronically decrease adenosine availability and increase extracellular levels of ATP (Boison, 2012; Dossi et al., 2018), having a joint effect that could lower seizure threshold. These alterations in astroglial purinergic signaling and metabolism could also explain the reduced population spike threshold and larger fEPSPs exhibited by kindled rats (Figure 2A-D), which is consistent with the increased firing frequency exhibited by CA1 pyramidal neurons in our kindling model (Morales et al., 2014). Altogether, these findings suggest that the impairment in LTP observed in the epileptic hippocampus is associated with an

aberrant pattern of astroglial Ca^{2+}-dependent activity, which is reversed by the inhibition of P2Y1R mediated signals, restoring glutamatergic neurotransmission at rate that resembles physiological conditions. In this scenario, we propose that the increase in P2Y1R-mediated astrocyte-to-astrocyte purinergic signals – and probably the decrease in adenosinergic signals – causes an aberrant pattern of astroglial Ca^{2+}-dependent activity, which prevents astrocytes from decoding synaptic activity and normally modulate LTP (Covelo and Araque, 2018; Di Castro et al., 2011; Perea and Araque, 2005; Sibille et al., 2015). However, further studies are necessary to test whether and how different gliotransmitters, such as D-serine or ATP/adenosine (Henneberger et al., 2010), could alter astrocyte-mediated metaplasticity in the epileptic hippocampus.

Several evidence suggest that the repetitive activation of glutamatergic inputs can lead to LTP saturation (Morgan and Teyler, 2001; Schulz and Fitzgibbons, 1997), reaching the neural pathway a state in which no further potentiation is feasible and thus disrupting learning and memory encoding (Grutzendler, 2006; Leung and Shen, 2006; Moser, 1998). Chronic epilepsy induction – as in kindling models – requires the repeated activation of excitatory projections that arrive to the hippocampal formation (Morales et al., 2014; Morimoto et al., 2004), which resembles the process required to induce LTP saturation. The brief, low intensity trains of electrical stimulation employed in kindling protocols – delivered in a frequency similar to that used to induce LTP – could cause a permanent state of electroconvulsive seizures, which can also reduce the capacity to generate LTP (Reid and Stewart, 1997). We observed that the application of two consecutive TBS protocols failed to induce LTP in the kindled group, which could suggest that the impairment is indeed due to an on-demand state of synaptic saturation. However, after a first attempt that failed to induce LTP, the application of the second TBS in the presence of MRS2179

induced a robust LTP, suggesting that the mechanism that underlies the impairment in LTP is likely different from synaptic saturation.

In conclusion, our results here suggest that the abnormal pattern of astroglial Ca^{2+}-dependent signals in the epileptic hippocampus is associated with an impairment in TBS-induced LTP in CA3-CA1 synapses, and likely operates as an aberrant mechanism of astrocyte-dependent metaplasticity. The restoration of the normal pattern of astroglial Ca^{2+}-dependent activity by the blockade of P2Y1R-mediated signals rescued TBS-induced LTP, suggesting that the alterations in synaptic plasticity in epileptic circuits could be largely associated to an aberrant crosstalk between astrocytes. By means of an aberrant pattern of Ca^{2+}-dependent activity and an upregulation in glutamate gliotransmission, astrocytes could not only abnormally increase the Pr, neuronal synchrony and excitability, but also interfere with the induction of synaptic plasticity in epileptic circuits, which constitutes a novel, key mechanism that could explain the cognitive decline in epileptic patients.

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Figure 1. TBS fails to induce LTP at CA3-CA1 synapses and evokes an unresponsive pattern of astroglial Ca^{2+}-dependent activity in kindled hippocampal slices. (A) Average traces of excitatory postsynaptic currents (EPSC) before (black trace) and 40 min after TBS application (gray trace) in control (n = 5 cells; 4 slices) and kindled group (n = 6 cells; 5 slices). (B) (top) Time course of the changes in EPSC amplitude (%) before and after TBS and (bottom) normalized values of input resistance (Ri) for each cell, measured throughout the experiments. (C) Superimposed averaged EPSCs evoked by the PPR protocol recorded before (black trace) and after (gray trace) TBS in control and kindled condition. (D) Summary graphs for average TBS-induced potentiation (left), baseline PPR (middle) and PPR change (%; right), measured before and 40 min after the protocol. (E-F) Fluorescence traces of spontaneous Ca^{2+} elevations from five representative astrocytes, recorded before,

during and after the application of the TBS protocol in control (E) and kindled (F) groups. Blue boxes denote Ca^{2+}-dependent activity during TBS protocols. (Right) Summary graphs showing mean values of FT/ST ratio (fast Ca^{2+} transients/slow Ca^{2+} transients) and average number of slow Ca^{2+} transients (ST) and fast Ca^{2+} transients (FT) per astrocyte. Results expressed as mean ± SEM. * p <0.05.

Figure 2. Two consecutive, spaced TBS protocols were unable to induce LTP in kindled hippocampal slices. (A) Superimposed averaged EPSCs traces obtained in basal conditions (1), and after TBS1 (2) and TBS2 (3) in control (n = 5) and kindled (n = 6) groups. (B) (top) Time course of the changes in EPSC amplitude (%) and (bottom) normalized values of Ri for each cell, measured throughout the experiments. (C) Superimposed averaged EPSCs evoked by the PPR protocol recorded in basal condition (gray trace) and after TBS2 (black trace) from representative cells from control and kindled groups. (D) (left) Summary graphs for control and kindled condition for average TBS-induced potentiation after TBS1 and TBS2 (left), baseline PPR (middle) measured basal and after TBS2 and PPR change (%; right), measured before and 40 min after the protocol. Results expressed as mean ± SEM. * p <0.05.

Figure 3. Impairment in HFS-induced LTP in kindled hippocampal slices. (A) (left) Superimposed averaged fEPSPs traces with matching fiber volley amplitudes from control (thin trace) and kindled (thick trace) slices. (right) fEPSPs amplitudes plotted against the corresponding fiber volley amplitudes from control (open circles) and kindled (black circles) slices, showing the input-output relation. (B) (left) Superimposed, magnified representative fEPSPs traces showing the population spike (arrow) threshold in control and

kindled conditions. (right) Population spike threshold means values for both control and kindled groups. (C) (top) Superimposed representative fEPSP traces obtained before (1) and after (2) the HFS protocol in control and kindled condition. (bottom) Time course of the changes in fEPSPs amplitude (%) before (1) and after (2) HFS from control and kindled condition. (D)

(left) Summary graphs for average HFS-induced LTP (%), (middle) PPR absolute and (right) PPR change (%), measured before (1) and after HFS (2). Results expressed as mean ± SEM. * p <0.05.

Figure 4. Inhibition of P2Y1R-mediated purinergic signals rescues the normal pattern of astrocytic Ca^{2+}-activity and recovers LTP in kindled hippocampal slices. (A) Average EPSC traces before (black trace) and 40 min after TBS application (gray trace) recorded in the presence of the specific P2Y1R antagonist MRS2179 (100 μM) from control (n = 6) and kindled (n = 7) groups. (B) (top) Time course of the changes in EPSC amplitude (%) before and after TBS in the presence of MRS2179 and (bottom) normalized values of input resistance (Ri) for each cell, measured throughout the experiments. (C) Superimposed averaged EPSCs evoked by the PPR protocol recorded before (black trace) and after (gray trace) TBS performed with MRS2179 in control and kindled condition. (D) Summary graphs for average TBS-induced potentiation (left), baseline PPR (middle) and PPR change (%; right), measured before and 40 min after the protocol. (E) Fluorescence traces of spontaneous Ca^{2+} elevations from seven representative astrocytes recorded before, during and after the application of the TBS protocol in control perfused with MRS2179 (left), kindled in regular ASCF (middle) and kindled with MRS2179 (right). Blue boxes denote Ca^{2+}-dependent activity during TBS protocols. (F) Summary graphs showing mean values

of FT/ST ratio (fast Ca^{2+} transients/slow Ca^{2+} transients) and average number of slow Ca^{2+} transients (ST) per astrocyte for control plus MRS 2179 (left), kindled in regular ASCF (middle) and kindled perfused with MRS2179 (right). Results expressed as mean ± SEM. * p <0.05.

Figure 5. The inhibition of P2Y1R recovers TBS-induced LTP in pyramidal neurons from kindled slices that were unable to express LTP with TBS alone. (A) Average EPSC traces recorded at baseline (1), 30 min after the first TBS (2), and 30 min after the second TBS (3) in presence of MRS2179 (100 µM) from the kindled group (n = 5). (B) (top) Time course of the changes in EPSC amplitude (%) before TBS1 (1), after TBS1 (2) and after TBS2+MRS2179 in kindled slices, and (bottom) normalized values of input resistance (Ri) for each cell, measured throughout the experiments. (C) Superimposed averaged representative EPSC trances evoked by the PPR protocol recorded before (gray trace) and after TBS1 and TBS2+MRS2179 (black trace) in the kindled group. (D) (left) Summary graphs for average TBS-induced potentiation and (right) PPR measured at baseline, after TBS1 and after TBS2+MRS2179. Results expressed as mean ± SEM. * p <0.05.

Highlights

- Glutamatergic CA3-CA1 synapses fail in the LTP induction in a chronic epilepsy model.
- LTP impairment is accompanied by alterations of synaptic efficacy and astroglial Ca2+ -activity.
- P2Y1R inhibition rescues pattern of astroglial Ca2+ -activity and synaptic plasticity properties.
- Astrocytes could participate in the deteriorate synaptic that cause the cognitive decline in epilepsy.