RECIPROCAL NEURON-ASTROCYTE SIGNALING IN EPILEPTIC SEIZURE GENERATION AND PROPAGATION

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**INDEX**

1 INTRODUCTION..............................................................................................................2

1.1 NEURON TALK TO ASTROCYTES.................................................................3
Ca2+ signalling in astrocytes......................................................................................4
Astrocytes coding of neuronal input.........................................................................5

1.2 ASTROCYTES RESPOND TO NEURONS..................................................7
Modulation of synaptic activity and plasticity.........................................................8

1.3 THE ROLE OF ASTROCYTES IN EPILEPSY...............................................10
K+ buffering.............................................................................................................11
Glutamate uptake.....................................................................................................11
The role of gliotransmission (Glutamate)...............................................................12
The role of gliotransmission (ATP).........................................................................13

2 RESULTS..................................................................................................................15

2.1 A NEW EXPERIMENTAL MODEL OF FOCAL SEIZURE IN THE ENTHORINAL CORTEX. *Epilepsia* (2010).................................................................................16

2.2 AN EXCITATORY LOOP WITH ASTROCYTES CONTRIBUTES TO DRIVE NEURONS TO SEIZURE THRESHOLD. *PLoS Biology* (2010).................................26

2.3 COMPUTATIONAL MODEL OF NEURON-ASTROCYTES INTERACTIONS DURING FOCAL SEIZURE GENERATION. *Frontiers in Computational Neuroscience* (2012)..................................................................................45

2.4 FAST SPIKING INTERNEURON CONTROL OF SEIZURE PROPAGATION IN A CORTICAL SLICE MODEL OF FOCAL EPILEPSY. *Journal of Physiology* (2013).............................................................................................................58

2.5 APPENDIX: SELECTIVE STIMULATION OF INDIVIDUAL FAST SPIKING INTERNEURONS PREVENTS SEIZURE PROPAGATION IN A BRAIN SLICE MODEL OF FOCAL EPILEPSY..................................................................................74

2.6 WORK IN PROGRESS: A GABA MEDIATED Ca2+ SIGNALLING IN CORTICAL ASTROCYTES.................................................................78

3 CONCLUSION AND FUTURE PERSPECTIVES....................................................83

4 REFERENCES............................................................................................................84
INTRODUCTION

The idea that astrocytes – the main population of glial cells in the brain – are active partners of neurons in many aspects of brain functions represented a Copernican Revolution in neurobiology. Astrocytes, which were for many years considered just like the cement (from Greek glia i.e. glue) that keeps neuronal cells together, have now been moved from the periphery to the centre of the universe of information processing in the brain providing a radically different point of observation in the study of brain physiology. This new view of brain activity turns around the discovery of a bidirectional communication between neurons and astrocytes, a process called gliotransmission. Astrocytes respond to neurotransmitters and through a Ca$^{2+}$ dependent mechanism release neuroactive substances that induce functional changes in neurons. In spite of the resistances opposed against the desertion of the neuronal dogma, a large amount of evidence collected during the last three decades contributed to reshape the concept of synaptic communication, considering astrocytes - together with the pre- and post- synaptic membranes - a fundamental element of the tripartite synapse. In other words, astrocytes participate transversally to information processing in the brain by modulating both synaptic transmission and different forms of plasticity.

This new consciousness of astrocytes as active elements in brain physiology, naturally suggests that these glial cell can potentially be involved in the development of brain disorders. Indeed many studies revealed that dysfunctions in astrocyte-neuron signaling can be directly involved in many pathologies including Alzheimer’s disease, Parkinson disease, amyotrophic lateral sclerosis and epilepsy.

The main goal in my thesis was to understand how the release of gliotransmitters by astrocytes, in particular glutamate, may influence two distinct phases of epileptic activity: the generation and the propagation of a focal seizure.
NEURONS TALK TO ASTROCYTES

The information flow in the brain is conveyed by electrical signals. Astrocytes do not have a density of Na⁺ channels sufficient to generate an action potential and they respond to depolarizing steps with a linear current-tension characteristic. In other words, they are unable to elaborate the information using a language based on electrical signals. For this reason astrocytes have been segregated for many years at the periphery of the brain. Nevertheless, clues for a possible neuron-to-astrocyte signalling came from morphological studies, which revealed an intimate connection between the astrocytes processes and the synapse (see Figure 1; (Peters et al., 1991);(Dani et al., 1992);(Ventura and Harris, 1999).

In the early 90s, taking advantage of the novel Ca²⁺ imaging techniques, different groups provided the first evidence that astrocytes display a form of excitability mediated by Ca²⁺ signal changes in response to neuronal activity (see Figure 2; (Dani et al., 1992); (Porter and McCarthy, 1996); (Pasti et al., 1997). In both cell cultures and acutely isolated hippocampal slices, the stimulation of glutamatergic afferents evoked Ca²⁺ oscillations in astrocytic cytoplasm. In the following years, the pioneering observation of an astrocytic Ca²⁺ signaling was extended to different brain regions (Perea et al., 2009); for a review see (Volterra and Meldolesi, 2005), associated to many neurotransmitters and factors (for a review see (Haydon and Carmignoto, 2006), and, more recently, reported also in living animals in response to sensorial stimuli (Navarrete et al., 2012) and during locomotor activity (Nimmerjahn et al., 2009). Despite astrocytes speak a different language with respect to that of neurons - which is based on intracellular Ca²⁺ change and not on electrical signals - it seems,
however, that they can understand neurons. In the two following paragraphs I address
two important issues related to neuron-to-astrocyte signalling: i) what is the
mechanism that induces Ca\textsuperscript{2+} oscillations in astrocytes? ii) how Ca\textsuperscript{2+} oscillations are
codified in the astrocyte to convey signals to neurons?

**Ca\textsuperscript{2+} SIGNALLING IN ASTROCYTES**

A signaling based on Ca\textsuperscript{2+} dynamics is present in all eukaryotic cells and it generally
plays a crucial role in many cellular processes such as muscle contraction, gene
expression, metabolic reactions, etc... Generally speaking, Ca\textsuperscript{2+} is the fundamental
ion in cellular communication, providing the link between input signals coming to the
cells and output signals. In particular, Ca\textsuperscript{2+} is the key molecule for chemical
transmission in the nervous system: it couples the electrical excitability (input) with the
exocytosis of neurotransmitters from the pre-synaptic terminal (output). During the
action potential discharge at the pre-synaptic terminal, exocytosis requires that very
high concentrations of Ca\textsuperscript{2+} (10-50 \( \mu \text{M} \)) were reached in proximity of the compartment
specialized for releasing transmitters, the *active zones*, in order to induce the fusion of
the vesicles with pre-synaptic membrane. This process occurs at the time scale of a
few milliseconds and it thus implies a very fast mechanism and requires a huge Ca\textsuperscript{2+}
source. This source is constituted by voltage-gated Ca\textsuperscript{2+} channels (VGCCs) which are
abundantly expressed at the pre-synaptic membrane.

As we anticipated above, Ca\textsuperscript{2+} is the substrate for astrocytes excitability. Ca\textsuperscript{2+}
elevations in astrocytic cell body occur with slow kinetics (1-10 seconds time scale),
and are indeed very much slower compared with the kinetics which characterize Ca\textsuperscript{2+}
increase at the neuronal terminals. Not surprisingly astrocytes lack a fast source of Ca\(^{2+}\): VGCCs expressed by astrocytes have minor implications in cellular signalling (Carmignoto et al., 1998), and no evidence have been reported for the presence in astrocytes of other possible fast sources of Ca\(^{2+}\), like the Ca\(^{2+}\)-permeable glutamate NMDA receptor (Teichberg, 1991). The most important mechanism for Ca\(^{2+}\) elevations in astrocytes is the activation of G-protein-coupled receptors. When a neurotransmitter binds to a GPCR, Phospholipase C (PLC) hydrolyzes Phosphatidylinositol 4,5-bisphosphate (PIP2) generating Diacyl Glycerole (DAG) and Inositol 1,4,5-triphosphate (IP3) which binds to IP3 receptor inducing Ca\(^{2+}\) release from endoplasmatic reticulum. Astrocytes express many receptors that can be activated by different neurotransmitters, such as glutamate, D-serine, ATP, GABA, which are usually released by the pre-synaptic terminal. Also the post-synaptic neuron may activate astrocytes Ca\(^{2+}\) signal through the release of retrograde messenger such as endocannabinoid (Navarrete and Araque, 2008); (Navarrete and Araque, 2010).

A totally different mechanism of Ca\(^{2+}\) signaling in astrocytes was recently described by Shigetomi and colleagues (Shigetomi et al., 2011). Taking advantage of a membrane-tethered, genetically encoded Ca\(^{2+}\) indicator (Lck-GCaMP3), they observed Ca\(^{2+}\) signals which appeared as flashes at localized spots of the membrane. They were able to demonstrate that this type of Ca\(^{2+}\) signalling is mediated by Ca\(^{2+}\) influx through transient receptor potential A1 (TRPA1) channels.

**ASTROCYTES CODING OF NEURONAL INPUT**

The most compelling problem in the study of neuron-astrocyte interactions is our defective understanding of how the Ca\(^{2+}\) signals are codified by astrocytes. Differently from neurons, where the action potential represents a sort of basic unit of the electrical excitability in these cells, the language spoken by astrocytes lacks a similar hallmark. Ca\(^{2+}\) oscillations in astrocytes may have different patterns, in terms of frequencies and amplitude, and these differences may be translated into different functional outputs. A possible reason of our unsatisfactory understanding of the code used by astrocytes could be the fact that, at least until few years ago, scientists have monitored Ca\(^{2+}\) signals exclusively at the level of the astrocytic cell body. Astrocytes displays long lasting (one to ten seconds) Ca\(^{2+}\) elevations in their soma, mainly in response to a very intense neuronal activity. In other words, a somatic Ca\(^{2+}\) elevation
could occur only when neuronal activity reaches a certain threshold and the frequency of these oscillations increased according to an increasing intensity of the neuronal activity (Pasti et al., 1997); (Matyash et al., 2001).

A fundamental step forward in our understanding of Ca\(^{2+}\) signalling in astrocytes was given by two recent studies that by revealing that the spatio-temporal scale of the Ca\(^{2+}\) activity at the astrocyte processes unveil a totally new world previously hidden with features rather different from those of the Ca\(^{2+}\) signal at the level of the cellular body (Di Castro et al., 2011); (Panatier et al., 2011). Authors showed that low levels of neuronal activity, such as spontaneous synaptic events, may induce Ca\(^{2+}\) increases in tiny compartments (1-4 um) of the astrocytic processes. These signals occur randomly with a frequency significantly higher than that of similar events at the cell body), and are relatively short-lasting (less than a second rise time; \textit{focal Ca}^{2+} \textit{transients}). On the other hand, a single action potential generates a robust and long response (few seconds; \textit{Expanded Ca}^{2+} \textit{transients}) occurring synchronously in several of these compartments. The authors demonstrated that these Ca\(^{2+}\) signaling control a process of glutamate release which influences synaptic transmission at this spatial-temporal scale. Astrocytes are able to detect action potential-dependent synchronization of local synaptic activity, and to coordinate their response accordingly, thereby providing a very fine substrate for the detection and the modulation of local synaptic activity.

All in all, somatic Ca\(^{2+}\) signaling appears to be the tip of the iceberg of a very more complex and intricate network of signals. They represent a class of responses that can occur only above a fixed threshold and reflect a more intense and coordinated neuronal network activity.
ASTROCYTES RESPOND TO NEURONS

Although astrocytes can release gliotransmitters by different mechanisms including transporters and hemichannels, the great importance of Ca\(^{2+}\) signalling in the intercellular communication and the neuronal activity dependence of Ca\(^{2+}\) oscillations in astrocytes, strongly suggest that the output of astrocytes, i.e. the gliotransmission, might be fundamentally a Ca\(^{2+}\)-dependent process. First of all, researchers focused their attention on the possible similarities between the release of transmitters from astrocytes and the Ca\(^{2+}\)-dependent exocytosis of neurotransmitter from neurons. A major role in the regulated secretion of vesicles in neurons is played by the proteins of the SNARE complex, which are involved in the processes of docking and fusion of the vesicles. Astrocytes have been showed to express several proteins of SNARE complex, i.e., synaptobrevin II, the Ca\(^{2+}\) sensor synaptotagmin IV, syntaxin and the homolog of SNAP-25, SNAP-23, suggesting that a regulated Ca\(^{2+}\)-dependent exocytosis may occur in astrocytes too. Other kinds of evidence support the notion of Ca\(^{2+}\)-dependent exocytosis in astrocytes. The exocytosis process was directly monitored in cultured astrocytes using TIRF microscopy (Bezzi et al., 2004); (Bowser and Khakh, 2004); (Malarkey and Parpura, 2011) and the fusion of the vesicles with
the astrocytic membrane was measured as changes in the membrane capacitance (Kreft et al., 2004); (Zhang et al., 2004).

MODULATION OF SYNAPTIC ACTIVITY AND PLASTICITY
Do the transmitters released by astrocytes influence neuronal activity? Do they modulate synaptic transmission? Do they affect synaptic plasticity? Although until today we have not definitive answers to these questions, a huge amount of experimental data obtained transversally in all brain regions studied allow us to state that astrocytes has the potential to do release gliotransmitters that deeply influence synaptic transmission.

Astrocytic modulation of synaptic transmission is; however, a multifaceted world as it can result in both potentiation and depression of synaptic transmission. The overall nature of the gliotransmitter effects on neuronal activity depends on many different factors: the type of gliotransmitter released, the neuronal receptor activated, the type of neuron targeted, the brain region in which gliotransmission takes place. The release of glutamate acting on pre-synaptic mGluRs, for example, was demonstrated to potentiate synaptic transmission in hippocampal CA3-CA1 synapses (Perea and Araque, 2007), but the same effect can be provided by the activation of NMDARs in a different region of the hippocampus, the dentate gyrus (Jourdain et al., 2007). Analogously, the release of ATP by astrocytes, which is readily metabolized in adenine by specific enzymes in the extracellular space, has been showed to be linked to both inhibitory and excitatory effects activating A1 and A2A receptors respectively (Serrano et al., 2006); (Pascual et al., 2005); (Panatier et al., 2011). An additional factor of complexity is the spatial extensions of astrocytic processes. For example, a Ca^{2+} signal induced locally at an active synapse can travel intracellularly and triggers the release of gliotransmitters in different and distant sites. Astrocytes activated by endocannabinoids in the hippocampus have been shown to enhance synaptic transmission in relatively distant synapses, while neurotransmission was depressed by the direct effect of the endocannabinoid at the presynaptic membrane expressing CB1 cannabinoid receptors (Navarrete and Araque, 2008); (Navarrete and Araque, 2010). Similarly, the ATP released by astrocytes upon the stimulation of a high active synapse was observed to depress other synapses after its conversion to adenosine (heterosynaptic depression; (Serrano et al., 2006)).
Long lasting effects on synaptic efficacy were associated to astrocytes. Also in this context many variables determine the effect of astrocytes modulation of neuronal network. The gliotransmitter glutamate mediates a form of spike-timing dependent depression of excitatory transmission in neocortex (Min and Nevian, 2012), but, at the same time, was associated to an mGluR dependent and NMDA independent form of long term potentiation (LTP) in the CA1 region of the hippocampus. Here, this form of plasticity requires the coincidence between postsynaptic activity and astrocyte Ca$^{2+}$ signalling (Perea and Araque, 2007). A different form of LTP in the same hippocampal CA1 region was associated to the release of another gliotransmitters, D-serine, which acts as NMDAR co-agonist, and is necessary for the induction of this kind of NMDA dependent LTP (Henneberger et al., 2010). Also cholinergic activity evoked by sensory stimulation was shown to evoke an astrocytes dependent LTP. Authors showed that the activation of a Ca$^{2+}$ signaling in astrocytes, and the consequent gliotransmission is required to this form of plasticity (Takata et al., 2011); (Navarrete et al., 2012).

![Figure 4: Picture illustrating a model of the tripartite synapse. Transmitters released by presynaptic neurons can activate Ca$^{2+}$ rises in astrocytes which in turn release substances which act back to neurons to either inhibit or enhance neuronal activity. From Allen and Barnes, Nature 457, 675-677 (2009)](image-url)
THE ROLE OF ASTROCYTES IN EPILEPSY

The term epilepsy is referred to a chronic neuro-pathological condition characterized by recurrent seizures provoked by anomalous and hyper-synchronous neuronal discharges which originate from the hyperactivity of the neurons from a restricted cerebral region – the epileptogenic focus – and then propagate to other regions possibly involving the whole brain. Seizures can arise in a plenty of different ways, and a clinical classification has been made in 1981 by the Commission on classification and terminology of the international league against epilepsy. The 3% of the total world population is affected by this condition, that in a 30% percentage of cases is pharmacological untreatable, the only possible solution being the surgical ablation of the portion of tissue which generates the seizures. Epileptic activity is conventional characterized by three different phases (Pinto et al., 2005):

i) generation which consists in the transition from a local and asynchronous neuronal hyperactivity restricted to the epileptogenic focus, to a synchronous activity involving large neuronal populations

ii) propagation which consists in the recruitment to the epileptiform activity of neuronal population distant from the epileptogenic focus

iii) termination which consists in the sudden and synchronous cessation of the crisis

The increase in astrocytes number, morphological changes of their soma and processes, together with functional changes – a complex condition generally termed reactive astrogliosis – have been for a long time associated to brain disorders, and in particular to epilepsy. Although a causative role for astrogliosis in epileptogenesis has been never demonstrated, gliosis, which can be reveald from an increase in the expression of glial fibrillary acidic protein (GFAP), was observed in different epileptic tissues: both in patients (tissues obtained by surgical ablation or postmortem) and in animal models (such as pilocarpine or kainite induced epilepsy; (Seifert et al., 2006). More interestingly, was also shown that seizure often generate in proximity to gliotic brain tissue (McKhan et al., 2000). Thus, the hypothesis that astrocytes dysfunctions can contribute to epileptogenesis can be reasonably advanced.
How astrocytes can be connected to epileptic activity? In the next paragraphs I give a rapid sketch of two view of the astrocyte role in epilepsy. The first is related to $K^+$ buffering and glutamate uptake, while the second is emerging from recent results and it is directly related to the action of gliotransmission in epileptiform activities (for a review see (Losi et al., 2012)).

**$K^+$ BUFFERING**

Since even small elevations in extracellular $K^+$ concentration ($[K^+]_o$) might lead to a significant depolarization in neurons and to an increase in the activity of the neuronal network, the homeostasis of $K^+$ is considered to be both a fundamental aspect in the control of the general excitability in the neuronal network and a possible causal factor in epilepsy. The high and rapid permeability of astrocytes to $K^+$ makes them very important in the control of $[K^+]_o$ homeostasis. Astrocytes are able to spatially redistribute $K^+$ from areas characterized by an high $[K^+]_o$ to neighboring regions which have a lower $[K^+]_o$. Due to their ability of spatial buffering of $K^+$, astrocytes have been hypothesized to play a crucial role in epileptic activity in the clearance of the large $[K^+]_o$ increase due to intense action potential firing during epileptic activity (Kuffler et al., 1966). The $K^+$ channels responsible for the high astrocytic $K^+$ permeability are rectifying $K^+$ channels (Kir channels). These channels are characterized by high open probability at resting potential and by a channel conductance proportional to $[K^+]_o$ (Olsen and Sontheimer, 2008). For these reasons Kir channels can be effectively activated by large $[K^+]_o$ increases, and they mediate small $[K^+]_o$ efflux from depolarized cells and large influx at hyperpolarized potentials. Among the sixteen distinct Kir channel subunits, the most extensively studied is type 4.1, which is interestingly largely expressed in astrocytes in many brain regions (Takumi et al., 1995); (Li et al., 2001).

Altered Kir4.1 expression and activity has been observed in epileptic tissues from human patients and experimental animal models (Lenzen et al., 2005); (Inyushin et al., 2010). These observations do not dissect out if Kir4.1 dysfunctions were actively involved in epileptogenesis or if they were just a mere consequence of epileptic activity.
GLUTAMATE UPTAKE
Another important factor which can influence neuronal network excitability is the dynamics of glutamate concentrations in the extracellular space. A crucial role in taking up the glutamate released by excitatory neurons is played by five different excitatory amino acid transporters EAAT1-5. Among this group, EAAT1 and EAAT2 (GLAST and GLT-1 in rodents, respectively) are selectively expressed in astrocytes, they have the function of regulating the extracellular glutamate concentration (Tanaka et al., 1997). While elevated levels of glutamate have been reported in human epileptic tissues, there are no definitive results about the contribution of EAAT levels in epileptogenesis: some studies revealed a down regulation of astrocytic glutamate transporters level (Mathern et al., 1999); (Proper et al., 2002); (Sarac et al., 2009), others did not observed alterations (Tessler et al., 1999); (Eid et al., 2004).

THE ROLE OF GLIOTRANSMISSION (GLUTAMATE)
The hypersynchronzation which characterized epileptic activity has been considered for long time a pure neuronal phenomenon until glutamate released by astrocytes was revealed to be an extrasynaptic source that could synchronize groups of neurons from different brain regions. Two different studies demonstrated that glutamate released upon the stimulation of the metabotropic receptors in astrocytes is able to induced synchronous N-methyl-D-aspartic acid (NMDA) mediated slow inward currents (SICs) in pyramidal neurons (Fellin and Carmignoto, 2004); (Angulo et al., 2004). Using paired patch-clamp recordings from pyramidal neurons, authors showed that SICs occurred with a high level of synchrony when pair of neurons were located within a reciprocal distance of 100 μm. Furthermore, Ca^{2+} imaging experiments revealed that astrocyte Ca^{2+} elevations could be followed by the synchronous activation of spatially distinct domains of neurons. The power of astrocytes in synchronizing groups of neurons raises the hypothesis that astrocytes – and in particular glutamatergic gliotransmission – can be involved in the process of ictogenesis. In other words, the observation that Ca^{2+} oscillation in astrocytes could evoke synchronous response in groups of neurons, seems to sugges that this group of neurons could represent – in embryo - an epileptogenic focus. This thesis was strongly supported by a controversial study by Nedergaard’s group, which showed that in five different
experimental models of pharmacological induced epilepsy, 70-90% of paroxysmal depolarizing shift (PDSs), i.e. interictal spikes, were insensitive to tetrodotoxin (TTX) application (Tian et al., 2005). The interpretation given by the authors was that PDSs coincide with SICs and that seizure activity may have a pure astrocytic basis. However, the observation reported by Tian et al. was in contrast with previous and subsequent studies showing that TTX efficiently blocks PDS. Moreover, as was reviewed by Wetherington et al., (Wetherington et al., 2008) a huge amount of data supports the notion that SICs and PDSs are rather different cellular events. SICs have been demonstrated to be mediated exclusively by NMDA receptors, while PDSs were reported to be in large part D-AP5 insensitive. In spite of these possible contradictions against a hypothesis of an astrocytic basis of epilepsy, the possibility that glutamate released by astrocyte could be an important factor in epileptic discharge generation was never ruled out. In the Results we are going to report a recent work from our laboratory (Gomez-Gonzalo et al., 2010) showing how the contribute of astrocytic glutamate, although not sufficient, is very important to set the threshold for ictal discharge.

**THE ROLE OF GLIOTRANSMISSION (ATP)**

Glutamate is not the only gliotransmitter released by astrocytes which plays a relevant role in the control of epileptic discharges. ATP is released by astrocytes through different mechanisms, both Ca$^{2+}$ dependent and Ca$^{2+}$ independent (Coco et al., 2003); (Pascual et al., 2005). Although ATP can act directly on neurons and astrocytes respectively through the activation of P2 and P2Y receptors (for a review see (Kumaria et al., 2008)), the main effect of ATP on epileptic discharges is exerted by its metabolite adenosine (Ado). Ado is a strong suppressor of neurotransmitters release, activating A1 receptors on neuronal pre-synaptic terminals. Haydon’s group, taking advantage of a transgenic mouse characterized by impaired gliotransmission,
demonstrated that astrocytic ATP mediates an important form of synaptic transmission: ethosynaptic depression (Pascual et al., 2005); (Serrano et al., 2006). This result put astrocytes at the center of the stage of the purinergic signaling in synaptic transmission, opening the way to possible implications of this signaling in the control of epileptic discharges. Is today well established that Ado is a potent anticonvulsant, and astrocytes are very important regulators in Ado metabolism, since they are a major source of an enzyme that efficiently degrades Ado, adenosine kinase (ADK; (Gouder et al., 2004); (Boison, 2005)). The genetic reduction of ADK was shown to prevent seizure and reduce epileptogenesis in animal models of epilepsy (Hubert et al., 2001); (Li et al., 2007); (Li et al., 2008).

SUMMARY OF THE RESULTS

This brief introduction about gliotransmission and its role in epilepsy represents the background on which the experimental results obtained during my PhD program are built on. In the following section I am going to present these results. In the fist chapter (2.1) I am going to describe the experimental model developed in our lab and used for all the experiments described in this thesis. In cortical slices of young rodents perfused with 4-AP and low Mg²⁺, activation of layer V-VI by local NMDA applications evolved into an ictal discharge that propagated to the entire cortex. In this way we have the unique opportunity to repetitively evoke an ictal discharge from the same restricted site, and thus to study the cellular events at the basis of ictal discharge initiation. Using this model we next investigated the role of gliotransmission in ictal discharge generation. Chapters 2.2 and 2.3 present results about the contribution of astrocytic glutamate in setting the threshold for ictal discharge generation obtained using respectively an experimental and a computational approach. The next chapters are dedicated to ictal discharge propagation. Chapter 2.4 is a study in which we
demonstrated that Parvalbumin-expressing fast-spiking interneuron, the most abundant GABAergic interneurons population, are crucial in controlling the spatio-temporal features of the recruitment of pyramidal neurons during the propagation of ictal discharge. Starting from these results, in chapter 2.5, we show how the manipulation of this GABAergic signaling by the stimulation of an individual Parvalbumin-expressing fast-spiking interneuron can prevent ictal discharge propagation. Finally, chapter 2.6 present preliminary data about the interaction between this GABAergic signals and astrocytes.

RESULTS
A new experimental model of focal seizures in the entorhinal cortex

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SUMMARY

Purpose: Despite intensive studies, our understanding of the cellular and molecular mechanisms underlying epileptogenesis remains largely unsatisfactory. Our defective knowledge derives in part from the lack of adequate experimental models of the distinct phases that characterize the epileptic event, that is, initiation, propagation, and cessation. The aim of our study is the development of a new brain slice model in which a focal seizure can be repetitively evoked at a precise and predictable site.

Methods: Epileptiform activities were studied by fast Ca²⁺ imaging coupled with simultaneous single and double patch-clamp or extracellular recordings from neurons of entorhinal cortex (EC) slices from Wistar rats and C57BL/6J mice at postnatal days 13–17.

Results: In the presence of 4-aminopyridine (4-AP) and low Mg²⁺, activation of layer V–VI neurons by local N-methyl-D-aspartate (NMDA) applications evolved into an ictal discharge (ID) that propagated to the entire EC. NMDA-evoked IDs were similar to spontaneous events. IDs with similar pattern and duration could be repetitively triggered from the same site by successive NMDA stimulations. The high ID reproducibility is an important feature of the model that allowed testing of the effects of currently used antiepileptic drugs (AEDs) on initiation, propagation, and cessation of focal seizures.

Conclusions: By offering the unique opportunity to repetitively evoke an ID from the same restricted site, this model represents a powerful approach to study the cellular and molecular events at the basis of initiation, propagation, and cessation of focal seizures.

KEY WORDS: Epileptogenesis, Seizures, Entorhinal cortex, Calcium, NMDA.

Temporal lobe epilepsy (TLE) is the most frequent form of partial seizures in adults and is frequently resistant (30%) to the currently used antiepileptic therapies. In TLE, seizure discharges initiate at restricted epileptogenic foci in hippocampus, entorhinal cortex (EC), and amygdala before spreading to large portions of the brain. A crucial step in the pathophysiology of this type of epilepsy is thus to understand how the ictal discharge (ID) is generated at these restricted foci (Jefferys, 1990; McNamara et al., 2006; Baulac & Pitkanen, 2008). By using pharmacological tools to generate an imbalance between excitatory and inhibitory activities, previous studies in acute brain slices (Traub & Wong, 1982; Tsau et al., 1998; Avoli et al., 2002; Pinto et al., 2005; Trevelyan et al., 2006) and the whole guinea pig brain preparation (de Curtis et al., 1998; Uva et al., 2005; Gnatkovsky et al., 2008) provided important insights into the molecular mechanisms and signaling pathways that contribute to epileptiform activities, including focal seizures. In particular, the use of the proconvulsant 4-aminopyridine (4-AP) and reduced Mg²⁺ to induce IDs in brain slices of EC and hippocampus has been largely used in the last two decades (Voskayli & Albus, 1985; Rutecki et al., 1987; Perreault & Avoli, 1989; Avoli et al., 2002). However, the early cellular events that develop at a restricted brain site before ID generation and predispose neurons to seizures remain poorly defined. Indeed, in the currently used models, the epileptic discharge arises randomly from multiple foci (Demir et al., 1998; Tsau et al., 1998; Pitkänen et al., 2006). The inability of these models to predict both the site and the timing of ID generation hampers the study of the early cellular events that precede the initiation of a focal seizure.

We describe herein a model in EC slices from young rodents that allowed us to trigger at a precise timing repetitive IDs from the same restricted site. This model uses pressure pulses applied to an N-methyl-D-aspartate (NMDA)-containing glass pipette to stimulate the NMDA receptor of a limited number of layer V–VI neurons from young rat EC slices, in the presence of the proconvulsant
4-AP and reduced Mg$^{2+}$. Laser scanner microscopy, fast imaging of Ca$^{2+}$ signals from neurons and simultaneous patch-clamp recordings from pairs of neurons or field potentials, revealed that the NMDA-induced episode of local neuronal hyperactivity was regularly followed, with a few-second delay, by an ID that propagated to adjacent and distant neuronal populations. By generating a predictable ictogenic site, this model offers the opportunity to investigate the early events that at this site predispose neurons to seizures. The high reproducibility of the ID represents an additional important feature of this model that in an initial series of experiments allowed us to gain insights into the mechanisms of currently used antiepileptic drugs (AEDs).

**Materials and Methods**

**Brain slices and dye loading**

All experimental procedures were authorized by the Italian Ministry of Health; all efforts were made to minimize the number of animal used and their suffering. Coronal cortical-hippocampal slices were obtained from Wistar rats and C57BL/6J mice at postnatal days 13–17 as described previously (Pellin et al., 2004). Briefly, brain was removed and transferred to ice-cold cutting solution containing (in mM): 120 NaCl, 3.2 KCl, 1 KH$_2$PO$_4$, 26 NaHCO$_3$, 2 MgCl$_2$, 1 CaCl$_2$, 16 glucose, 5 Na-pyruvate, and 0.6 molaric acid at pH 7.4 (with 5% CO$_2$/95% O$_2$). Slices were obtained with a Leica Vibratome VT1000S (Mannheim, Germany) in the presence of the NMDA receptor inhibitor kynurenic acid (2 mM). Slices were recovered for 15 min at 37°C and then loaded with the Ca$^{2+}$ sensitive dye Oregon Green 488 DAPTAXA-1-acetoxyethyl ester (OGB-1 AM, 20 µM; Invitrogen, Carlsbad, CA, U.S.A.) for 60 min at 37°C. After loading was performed in the cutting solution containing sulfipyrazone (200 µM), pluronic (0.12%), and kynurenic acid (1 mM). After loading, slices were recovered and kept at room temperature in the presence of 200 µM sulfipyrazone.

**Electrophysiology and Ca$^{2+}$ imaging**

Brain slices were continuously perfused in a submerged chamber (Warner Instruments, Hamden, CT, U.S.A.) with (in mM): 120 NaCl, 3.2 KCl, 1 KH$_2$PO$_4$, 26 NaHCO$_3$, 1 MgCl$_2$, 2 CaCl$_2$, 10 glucose, and 0.2 sulfipyrazone at pH 7.4 (with O$_2$ 95%, CO$_2$ 5%). Whole-cell patch-clamp recordings in rat brain slices were performed using standard procedures with one or two Axopatch-200B amplifiers (Molecular Devices, Union City, CA, U.S.A.), as reported previously (Pellin et al., 2001). Typical pipette resistance was 3–4 MΩ. Data were filtered at 1 kHz and sampled at 5 kHz with a Digidata 1320 interface and pCLAMP8 software (Molecular Devices). Whole-cell intracellular pipette solution was (in mM): 140 K-gluconate, 2 MgCl$_2$, 0.3 ethylene glycol bis(2-aminoethyl) ether)-N,N,N',N”-tetraacetic acid (EGTA), 4 NaATP, 0.4 NaGTP, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10 sodium phosphocreatine at pH 7.2 with KOH and contained a low concentration (10–20 µM) of OGB1 cell impermanent (Invitrogen). All patch cells had the typical firing of regular spiking cells. The liquid junction potential at the pipette tip was −16 mV. This value should be added to all voltages to obtain the correct value of the membrane potential in whole-cell configuration. Ca$^{2+}$ signals were simultaneously acquired with a TCS-SPS-RS confocal microscope (Leica) equipped with a 20× objective (NA, 1.0) and a CCD camera for differential interference contrast images. Extracellular recordings were performed with an A-M System Model 1800 amplifier (=1 Hz and <1 KHz; Seimens, WA, U.S.A.) and glass pipettes filled with NaCl 0.9% (3–4 MΩ). Laser emission at 488 nm was used for stimulation of OGB-1. Time frame acquisitions from 314–491 ms (with 4–6 line averaging) were used. No background subtraction or other manipulations were applied to digitized Ca$^{2+}$ signal images that are reported as raw data, with the exception of the different images in Figs 1 and 3. A precise alignment of Ca$^{2+}$ and electrophysiological signals was achieved by using a synchronization signal produced by the confocal microscope.

**Drugs**

4-AP (100 µM) and kynurenic acid were obtained from Ascent Scientific (Avonmouth, Bristol, U.K.). NMDA (1 mg/mL Sigma-Aldrich, Milan, Italy) was prepared according (4–10 psi) for 400–600 ms) through a glass-pipette by a PDES Picospritzer (NPI, Tann, Germany). Lamotrigine (LIG), carbamazepine (CBZ), and valproic acid (VPA) were also purchased from Sigma-Aldrich.

**Data analysis**

Data analysis was performed with CLAMPFIT 8, Microsof Office 2003 (Microsoft, Seattle, WA, U.S.A.), ORIGIN 6.0 (Microcal software, Northampton, MA, U.S.A.), MATLAB (The MathWorks, Natick, MA, U.S.A.), LEICA LAS-AF (Leica), and MBF Imaging (NIH, Bethesda, MD, U.S.A.). The Ca$^{2+}$ signal is reported as G with FPD, where G is the fluorescence change and FPD is the baseline fluorescence. Data are shown as mean ± SEM (standard error of the mean) unless otherwise stated. Student’s t test was used, with p-values ≤0.05 taken as statistically significant. To evaluate the level of synchrony in large neuronal populations, MATLAB was used to calculate a mean cross-correlation function (CC) in neurons (N) by averaging cross-correlations between all pairs of neurons (N pairs) from the recording field, as follows:

\[ CC(t) = \frac{1}{N \cdot N_{pairs}} \sum_{i=1}^{N} \sum_{j=1, j \neq i}^{N_{pairs}} (X_i(t) - \bar{X}_i)(X_j(t + \tau) - \bar{X}_j(t + \tau)) \]

where CC(t) is the cross-correlation function between the ith and jth neuron normalized so that the autocorrelations at zero lag
potential from a neuron located ~150 µm apart from the NMDA pipette. In the presence of 100 µM 4-AP and 0.5 mM Mg²⁺, a single NMDA pulse (0.4–0.6 bar, 400–600 ms duration) evoked a transient Ca²⁺ elevation in the patched neuron as well as in other neurons from the region adjacent to the pipette tip, a region that we defined as “field A” (Fig. 1A, see also Movie S1). In the patched neuron, the Ca²⁺ elevation reflects a membrane depolarization with superimposed action potential (AP) firing (Fig. 1B). The transient Ca²⁺ response to NMDA is clearly illustrated by the difference images generated by subtracting the Ca²⁺ fluorescence image captured at basal conditions (t₀ or t₁) to that obtained after the NMDA stimulation (t₁; Fig. 1A). Neurons from the surrounding region, that we defined as “field B”, failed to respond to the single NMDA pulse (Fig. 1A,B).

The stimulation with two NMDA pulses, applied with a 3-s interval, evoked a Ca²⁺ elevation in neurons that was initially similar (Fig. 1C, t₁–t₃), but it soon evolved into a strikingly different response (Fig. 1C, t₃–t₅). In the patched neuron, the initial transient depolarization evoked by the double NMDA pulse was followed by a more sustained depolarization and by a sequence of AP bursts that is typical of the tetanic, seizure-like discharge, that is, the afterdischarges (Fig. 1D). This epileptic discharge was accompanied by a sustained Ca²⁺ increase followed by a sequence of Ca²⁺ peaks from the patched neuron as well as from other neurons in field A. In field B neurons, the initial response to NMDA was absent, but the sustained Ca²⁺ increase and the periodic Ca²⁺ elevations that we observed in field A neurons occurred simultaneously also in these neurons, suggesting that the ID involved all neurons in field A and field B (Fig. 1C,D). This sequence of events can be readily followed in the Movie S2. The difference images illustrate clearly the delayed recruitment of field B neurons into the massive Ca²⁺ rise of the ID (Fig. 1C, t₃–t₅). The activation of field B neurons was secondary to that of field A neurons, since it was totally abolished by tetrodotoxin (TTX). As shown by the cross-correlation plots, the Ca²⁺ signal of the patched neuron, both at the onset and during the afterdischarges, was highly correlated with the AP burst of the ID (Fig. 1D, inset 1 and 2) as well as with the Ca²⁺ signal of all neurons (Fig. 1D, insets 3 and 4). These data suggest that the Ca²⁺ signal can be a useful tool to monitor the onset of epileptic discharges, the extent of the underlying synchrony, and the spread to adjacent and distant neuronal populations.

These results also demonstrate that a local activation by NMDA of a group of EC neurons creates an epileptogenic focus that generated an ID, which propagated to adjacent neuronal populations. By dual patch-clamp recordings from two, layer VI neurons, one in field A and the other in field B (Fig. 2A,B, N₁ and N₂, respectively), we next confirmed that the Ca²⁺ elevation observed in field B neurons after a double NMDA pulse reflects a propagating ID. Indeed, field A and B neurons showed a simultaneous ID onset and synchronous afterdischarges (Fig. 2B,C). These experiments (n = 9) also confirmed that NMDA stimulation failed to activate directly field B neurons (Fig. 2D). Field potential recordings through two electrodes placed in layers V–VI at different distances from the focus of ID generation showed that the NMDA-evoked ID propagated laterally in the EC with the typical pattern of a seizure-like discharge. Notably, cross-correlation analysis revealed that the Ca²⁺ signal of neurons (Fig. 2D, gray traces) and the field potential recorded from the same region were highly correlated (Fig. 2D).

Spontaneous IDs were observed occasionally in 27 of 116 slices examined. As revealed by patch-clamp and Ca²⁺ signal recordings in slices from three different animals (Fig. 3), the spontaneous (n = 12) and evoked IDs (n = 16) were indistinguishable, as they showed similar AP discharge pattern, duration (102 ± 5%; p = 0.97), and amplitude (11 ± 17%; p = 0.55).

Threshold and reproducibility of the ID evoked by local NMDA stimulations

To find the threshold for ID generation, 10–15 min after the onset of slice perfusion with 4-AP and low Mg²⁺, we increased with small steps either the pressure or the duration of the two successive pulses applied with a 3-s interval to an NMDA-containing pipette until an ID was evoked. The application of a single NMDA pulse, even at high pressure and duration, was rarely capable of eliciting an ID, whereas a stimulation with two consecutive NMDA pulses (duration, 400–600 ms, pressure 0.4–0.6 bar), applied at a 3-s interval, effectively induced an ID. In a small percentage of slices (8 of 116), we failed to activate IDs, despite increasing the intensity and number of the NMDA pulses. Once the parameters of the pressure pulse were established, to study the reproducibility of our stimulation a double NMDA pulse was applied repetitively with 4–5 min intervals. As shown in the examples of control- and voltage-clamp recordings from neurons of two different slices, a double NMDA pulse regularly evoked an ID and no failures were observed even after stimulations were applied repetitively to the same slice (up to 20) over a long time period (Fig. 4A,B). Quantitative analysis of 145 IDs from 24 experiments reveals a mean delay of 11 ± 1 s between the first NMDA application and the ID onset (a period that we defined as a “transient phase”) and a mean ID duration of 42 ± 4 s. The ID duration from these experiments was variable (range 15–82 s; compare also the ID in Fig. 4A,B). However, within each experiment, the ID evoked by the successive NMDA stimulations had comparable durations (Fig. 4C). These results demonstrate that with the exception of a few slices in which neurons were completely refractory to ID generation, successive double NMDA pulses evoked comparable events with a high efficiency and over a long time period. IDs evoked in EC slices from mice (n = 3) were similar to those observed in rats slices, with an average
what was previously reported by Trevelyan et al. (2007).

Additional, specifically designed experiments are required to clarify this issue.

Our experimental approach can be also suited to investigate the distinct role of diverse signaling pathways, such as that of inhibitory interneurons, which is highly debated in epilepsy research (Avoli et al., 1996; Trevelyan et al., 2006, 2007; Ziburkus et al., 2006; Czirjakovsky et al., 2008). Our model applied to EC slices of transgenic mice expressing the green fluorescent protein in specific subsets of interneurons, has, indeed, the potential to become a powerful approach to clarify the role of interneurons in the initiation of a focal ID.

In previous studies that used brain slices in interface chambers and 4-AP perfusion, spontaneous IDs were commonly observed to originate from EC deep layers (Avoli et al., 2002). We thus applied NMDA to layer V–VI neurons, although we obtained similar results by stimulating layer II–III neurons (data not shown). The low spontaneous epileptic activity in our model may be linked to the submerged slice chambers that we used. However, given the large number of successive IDs evoked by NMDA stimulations in our slice preparations, the generation of spontaneous IDs may have been prevented by the postictal refractory period that follows each ID. Notably, the observation that the general features of IDs evoked by local NMDA stimulations, such as the pattern of AP bursts during the tonic and the clonic phases, and ID duration, were similar to spontaneous IDs, confirms the validity of our model to study focal seizure discharges.

Effects of AEDs on focal IDs

The results that we obtained with three AEDs that are currently used in the treatment of partial and generalized seizures, that is, LTG, CBZ, and VPA, validated our model for testing the efficacy of anticonvulsants. We found that these drugs affect differently focal epileptic activities. Although both LTG and CBZ inhibited ID generation, VPA, even after 30 min of slice perfusion, failed to prevent ID generation and reduced only the synchrony of neurons during the phase of the afterdischarges. Different mechanisms in the anticonvulsant action of these compounds may account for these data. LTG and CBZ, which both prolong Na+ channels inactivated state, similarly inhibited ID generation, whereas VPA, which has a different anticonvulsant mechanism, was poorly effective in our modelic model and affected only the late afterdischarges. VPA has been, indeed, reported to have variable effects on epileptiform activities in slice models (Loscher, 2002; Rogawski & Loscher, 2004). It will be of interest to investigate in future studies the nature of the effect of VPA on neuronal synchrony of the afterdischarges.

Limitations of our EC slice model of ictogenesis

Our experiments set the conditions for studying the generation of a focal seizure-like discharge in acute brain slice preparations. This preparation has unique advantages for investigating the cellular and molecular mechanism of epileptiform activities as well as the action of anticonvulsants. However, it also has several limitations that should be taken into account, such as the activation by slicing procedures of inflammatory signaling pathways, a reduced connectivity among neurons from the same and different regions, and the lack of blood-derived components. It will be thus important to test in future studies whether our model of ictogenesis can be replicated in chronic models of epilepsy, including genetically determined in vivo models of epilepsy, and in the close-to-in vivo whole guinea pig brain preparation (de Curtis et al., 1998; Uva et al., 2005) that more closely mimics the complex feature of seizures in epileptic patients.

CONCLUSIONS

We described a simple EC slice model of ictogenesis in which an ID can be repetitively evoked at a specific site and time, and its propagation to nearby and distant neuronal populations monitored by simultaneous dual patch-clamp field recordings and Ca2+ imaging. This model provides the unique opportunity to study the early events that at a restricted brain site contribute to the generation of a focal seizures-like discharge, as well as the mechanisms that at the surrounding regions favor or restrict the ID propagation. The reliability of ID generation renders our model particularly well suited to study the anticonvulsant action of drugs. Insights into the mechanism underlying the generation of seizure provided by this new model can be thus particularly relevant for developing new therapeutic strategies for human focal epilepsies.

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DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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G. Losi et al.


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Video clip S1. A single NMDA pulse evokes in VC neurons only a transient Ca2+ response.

Video clip S2. A double NMDA pulse evokes an ID.

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An Excitatory Loop with Astrocytes Contributes to Drive Neurons to Seizure Threshold

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Abstract

Seizures in focal epilepsies are sustained by a highly synchronous neuronal discharge that arises at restricted brain sites and subsequently spreads to large portions of the brain. Despite intense experimental research in this field, the cellular events that initiate and sustain a focal seizure are still not well defined. Their identification is central to understand the pathophysiology of focal epilepsies and to develop new pharmacological therapies for drug-resistant forms of epilepsy. The prominent involvement of astrocytes in pathogenesis was recently proposed. We test here whether a cooperation between astrocytes and neurons is a prerequisite to sustain focal seizures and interictal epileptiform discharges. To this end, patch-clamp recording and Ca2+ imaging techniques were performed in a new in vitro model of focal seizures induced by local applications of N-methyl-D-aspartic acid (NMDA) in rat entorhinal cortex slices. We found that a Ca2+ elevation in astrocytes correlates with both the initial development and the maintenance of a focal seizure-like discharge. A delayed astrocyte activation during ictal discharges was also observed in other models (including the whole in vitro isolated guinea pig brain) in which the site of generation of seizure activity cannot be precisely monitored. In contrast, interictal discharges were not associated with Ca2+ changes in astrocytes. Selective inhibition or stimulation of astrocyte Ca2+ signaling blocked or enhanced, respectively, ictal discharges, but did not affect interictal discharge generation. Our data reveal that neurons engage astrocytes in a recurrent excitatory loop (possibly involving glutamatergic transmission) that promotes seizure initiation and sustains the ictal discharge. This neuron-astrocyte interaction may represent a novel target to develop effective therapeutic strategies to control seizures.

Introduction

Focal epilepsies are characterized by a condition of neuronal hyperexcitability that is restricted to the epileptogenic regions. Focal seizures originate at this region and secondarily spread to distant cortical areas [1-5]. Several factors, from ion channel mutations to brain injury, may cause neuronal hyperexcitability changes that sustain an epileptic condition [6]. Yet, the earlier cellular events that initiate a seizure in the first place are still unclear. The understanding of pathogenesis is thus central to the pathophysiology of focal epilepsy and is a requirement to develop new pharmacological therapies for drug-resistant focal epilepsies [7].

In the present study, we specifically address the hypothesis that the activation of a loop between neurons and astrocytes is an early event that contributes to focal seizure initiation. This hypothesis stems from a series of recent studies that repositioned the role of neurons in epileptogenesis and hinted at a possible, direct contribution of astrocytes to the generation of an epileptic discharge. The first clue was the observation that the release of glutamate from astrocytes, elicited by Ca2+ oscillations, promotes local synchronous activities in hippocampal neurons by acting on extrasynaptic N-methyl-D-aspartic acid (NMDA) receptors [8]. Studies performed both on brain slices and in vivo showed that during epileptiform activity, the frequency of Ca2+ oscillations in astrocytes is significantly increased [9,10], and it is reduced by anticonvulsant drugs [9]. Moreover, the expression of metabotropic glutamate receptors (mGluRs, mediators of Ca2+ oscillations in these cells) in hippocampal astrocytes from animal models of temporal lobe epilepsy was found to be increased [11,12]. These observations suggest that the excessive neuronal synchronization that characterizes the epileptic discharge might be sustained, at least in part, by an astrocyte hyperexcitability. In support of an astrocyte role in epileptiform activities, it has been proposed that...
Author Summary

In focal epilepsy, seizures are generated by a localized, synchronous neuronal electrical discharge that may spread to large portions of the brain. Despite intense experimental research in this field, a key question relevant to the human epilepsy syndrome remains completely unanswered: what are the cellular events that lead to the onset of a seizure in the first place? In various in vitro models of seizures using rodent brain slices, we simultaneously recorded neuronal firing and Ca\(^{2+}\) signals both from neurons and from astrocytes, the principal population of glial cells in the brain. We found that activation of astrocytes by neuronal activity and signalling from astrocytes back to neurons contribute to the initiation of a focal seizure. This reciprocal excitatory loop between neurons and astrocytes represents a new mechanism in the pathophysiology of epilepsy that should be considered by those aiming to develop more effective therapies for epilepsies that are not controlled by currently available treatments.

the interictal events recorded between seizures might be in some conditions tetrodotoxin (TTX)-resistant and mediated by glutamate release from astrocytes [9]. These findings fuelled a controversial debate on the role of astrocytes in focal epileptogenesis and in the generation of epileptiform discharges [15,16].

In the present study, we used different models of epileptic seizures, including a new model of focal seizures, to define the role of astrocytes in the generation of epileptiform activities. We performed simultaneous Ca\(^{2+}\) imaging and electrophysiological recordings of epileptic discharges in brain slices and in isolated intact guinea pig brains, focusing on the entorhinal cortex. This experimental approach allowed us to define the timing of astrocytic Ca\(^{2+}\) excitability in relation to interictal and ictal discharges. By using different pharmacological tools to affect selectively the Ca\(^{2+}\) signal in astrocytes, we also investigated a possible causative role of astrocyte activation in the generation of these epileptic discharges.

We demonstrate here that a recurrent excitatory loop between neurons and astrocytes involving Ca\(^{2+}\) elevations in a large number of glial cells is an early event that contributes to the initiation of a focal seizure-like discharge.

Results

A Large Number of Astrocytes Are Activated by Ictal, but Not Intercital, Discharges

**Picrotoxin/zero-Mg\(^{2+}\)** entorhinal cortex slice model. In a first series of experiments, we investigated neuron and astrocyte activities in entorhinal cortex (EC) slices during interictal and ictal discharges induced by the gamma-aminobutyric acid (GABA\(_A\)) receptor antagonist picrotoxin applied in Mg\(^{2+}\)-free solution. Since incubation with the Ca\(^{2+}\)-free Oregon Green BAPTA-1 acetoxyethyl ester (OGB1-AM) allowed us to monitor Ca\(^{2+}\) signals from both neurons and astrocytes, identified according to morphological and functional criteria [16,18] (see also Materials and Methods). Patch-clamp recordings coupled to Ca\(^{2+}\) imaging revealed a clear correlation between action potential (AP) bursts and Ca\(^{2+}\) changes from the patched neurons during both the brief interictal and the prolonged ictal discharges (Figure 1A and 1B). Ca\(^{2+}\) elevations with similar onset and time course were also observed in unpatched neurons simultaneously monitored in the same field (Figure 1A, and other neurons in 1B). These observations demonstrate that the neuronal Ca\(^{2+}\) signal reflects faithfully the AP discharge during interictal and ictal discharges and represents a useful tool to i) detect ictal discharges; ii) mark ictal discharge initiation; and iii) evaluate the extension of underlying neuronal synchrony.

A Ca\(^{2+}\) rise was distinctly activated by ictal discharges in most astrocytes, whereas interictal discharges failed to evoke a similar astrocytic activation (Figure 1A and 1B) and Video S1), and it increased only the frequency of independent Ca\(^{2+}\) oscillations in single astrocytes (Figure S1). In a total of 15 experiments, 73.5% of the astrocytes present in the recording field (n = 227), Figure 1E, were activated by the ictal discharge, and in most of these (57.5%) a Ca\(^{2+}\) elevation occurred 1.8±0.2 s after the ictal discharge onset. A similar distinct activation of astrocytes during the ictal event evoked by picrotoxin/zero-Mg\(^{2+}\) was observed also in CA3 region from hippocampal slices of both rats (Figure 1E) and pGlutATP-EGFP transgenic mice in which astrocytes are labelled by the enhanced green fluorescent protein (EGFP) under the control of the human glial fibrillary acidic protein (GFAP) promoter (unpublished data).

**Ricurulinine-preferred, whole guinea pig brain model.** To validate in an intact brain the findings obtained in EC slices, we used the in vitro isolated whole brain from young adult guinea pigs [19], since imaging of the EC is impracticable in vivo. In this preparation, networks responsible for focal ictogenesis in the EC hippocampus have been analyzed in detail [20,21]. We simultaneously recorded the extracellular field potential and Ca\(^{2+}\) signals by two-photon laser scanning microscopy (2P-LSM) during epileptiform activities induced by intraventricular application of the GABA\(_A\) receptor antagonist bicuculline methiodide. Ca\(^{2+}\) signals in neurons were tightly correlated with the changes in the field potential observed during the seizure discharge and increased in parallel with the appearance of a fast activity at 20 30 Hz that accompanied the onset of the ictal discharge [21,20] (Figure 1D). In brain slices, in this close to in vivo condition, seizure-like events regularly evoked Ca\(^{2+}\) elevations in astrocytes (Figure 1E and 1D), whereas interictal events failed to activate astrocyte responses (Figure S1). A bar graph summarizes the different response of astrocytes to interictal and ictal discharges in the different models (Figure 1E).

Astrocyte Activation by the Ictal Discharge Involves Glutamate and ATP

The activation of astrocytes by neuronal activity is mainly mediated by synaptic neurotransmitter release, such as glutamate [16,53] and ATP [24]. We next asked whether these neuronal signals mediate Ca\(^{2+}\) elevations triggered in astrocytes by the ictal discharge. We found that the activation of astrocytes by the ictal discharge was significantly reduced by slice perfusion with either the antagonist of mGlur receptors 2-methyl-4-phenylthethylpyridine (MPEP), or the antagonist of purinergic (P2) receptors pyridoxal phosphate-6-azophenyl-2'4'-disulfonic acid (PPADS), Figure 2A). MPEP/PPADS co-perfusion abolished ictal discharges, thus hampering the possibility to clarify whether glutamate and ATP can entirely account for astrocyte activation by the ictal event.

We also found that after slice perfusion with either MPEP or PPADS, the duration and frequency of ictal episodes in neurons were significantly reduced with respect to controls (Figure 2B and 2C), whereas interictal discharges were either unaffected (PPADS and MPEP/PPADS) or increased in frequency (MPEP, Figure 2D). These results clearly show that Ca\(^{2+}\) elevations mediated by mGlur and P2 receptors in astrocytes (and neurons) do not have a role in the generation of interictal discharges. Given that MPEP and PPADS block receptors in both neurons and astrocytes, there
Figure 1. The large majority of astrocytes respond with a Ca²⁺ elevation to ictal, but not interictal, discharges. (A) Ca²⁺ changes, with respect to the basal level (t₀), in neurons (white arrows) and astrocytes (black arrowsheads) during an interictal (t₁) or an ictal (t₂) event after EC slice perfusion with picrotoxin/zine-Mg++. Scale bar represents 20 µm. (B) AP bursts and Ca²⁺ change (green trace) from the patched neuron, and Ca²⁺ changes from other neurons and from astrocytes indicated in (A). Note the large Ca²⁺ rise evoked in astrocyte by ictal (t₂) and not by interictal (t₁) events. (C) 2P-LSM Ca²⁺ imaging in guinea pig EC before (t₀) and during the development (t₁ and t₂) of the ictal discharge induced by arterial perfusion with bicucullines. Scale bar represents 20 µm. (D) Field potential and Ca²⁺ signal changes from neuropile (dashed circle in (C)) and astrocytes (arrowheads in (C)) during the ictal discharge. (E) Mean percentage of interictal and ictal-activated astrocytes. Numbers over each bar indicate the total number of astrocytes examined. The interictal and ictal events analyzed were, respectively, 130 and 21 in rat CA3, 69 and 15 in rat EC, 347 and 31 in guinea pig EC. Error bars in this and other figures indicate SEM.

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results also suggest that Ca\textsuperscript{2+} signals activated by these receptors, on one or both cells, may have a role in ictal discharge generation.

Selective Activation of Astrocytes Favours Ictal Discharge Generation

We next asked whether astrocyte Ca\textsuperscript{2+} elevations may have a specific role in ictal discharge generation. To investigate this hypothesis, an agonist able to selectively trigger a Ca\textsuperscript{2+} increase in astrocytes should be used. The peptide TFLLR, a PAR-1 thrombin receptor agonist, is preferentially expressed in astrocytes and is known to activate glutamate release in astrocytes [25,26]. We found that PAR-1 immunoreactivity in the EC was largely associated with the soma and the processes of GFAP-positive astrocytes (Figure 3A). Noteworthy, GFAP-negative PAR-1 puncta appeared in continuity with distal portions of astrocyte processes, where GFAP is hardly expressed [27] (Figure 3B). Following TFLLR (10 μM) bath perfusion in the presence of both TTX and D-2-amino-5-phosphonomopentanoic acid (D-AP5), which blocks NMDAR-mediated astrocyte-to-neuron signalling [8,28], we could not detect any Ca\textsuperscript{2+} change in EC neurons, whereas large Ca\textsuperscript{2+} elevations were observed in astrocytes (Figure 3C).

We next asked whether PAR-1 receptor activation could simulate the release of glutamate from EC astrocytes, as previously reported for hippocampal astrocytes [25,26]. We found that Ca\textsuperscript{2+} elevations triggered in EC astrocyte by short pressure pulses applied to a TFLLR-containing pipette (1 nM) were followed by slow inward currents (ICIs) in adjacent patched neurons (Figure 3D). Most of the ICIs recorded in six of 12 neurons occurred within 10 s (mean delay ± SEM, 1.2 ± 0.3 s) after the TFLLR-induced Ca\textsuperscript{2+} elevations in astrocytes (Figure 3D and 3E). Unlike fast spontaneous synaptic currents (i.e., spikes in Figure 3D), ICIs have typical slow kinetics (τ: 13.6 ± 3.8 ms, decay time, 85 ± 17 ms; n = 15), are insensitive to TTX, and are sensitive to the NMDAR blocker D-AP5 (Figure 3E), as demonstrated in neurons from other brain regions [8,25,26].

In the presence of zero Mg\textsuperscript{2+} coronal slice model, we then investigated whether selective astrocyte activation enhanced ictal discharge generation. We found that Ca\textsuperscript{2+} elevations triggered in astrocytes by local TFLLR applications were sufficient to shift neurons towards the ictal discharge threshold (Figure 3F; Video S2). To demonstrate the causal link between the ictal discharge and the immediately preceding TFLLR-induced Ca\textsuperscript{2+} increase in astrocytes, we simulated the ictal occurrence by a Monte Carlo procedure. Results from this analysis revealed that in six experiments in which 30 TFLLR applications were performed, 10 of the 15 observed ictal events were correlated at the 0.05 confidence level with a preceding astrocyte Ca\textsuperscript{2+} increase (Figure 3G).

These results suggest that when the level of basal excitability and the predisposition of neurons to generate epileptic discharges is high, as in the picrotoxin/zero Mg\textsuperscript{2+} model, activation of the NMDAR by astrocytic glutamate could trigger neuronal hyperactivity that is sufficient to generate an ictal discharge. Compelling, although indirect, support for this hypothesis derives from the observation that a short pressure-pulse application of NMDA via an NMDA-containing pipette could also evoke a ictal discharge (Figure 3H).

In the Presence of 4-AP, a Local Application of NMDA Evokes a Focal Ictal Discharge

To further investigate the possible role of astrocytes in seizure initiation, we developed a model of focal excitotoxic damage based on the picrotoxin/zero Mg\textsuperscript{2+} model. In this latter model, indeed, epileptic activities arise spontaneously and at unpredictable foci [31,32], and therefore the cellular mechanism of seizure initiation cannot be analyzed accurately.

In our new model, ictal discharges are reproducibly generated at discrete sites of the EC by focal NMDA applications. Figure 4A reports schematically the positions of the NMDA-containing pipette and an OGB1-containing patch pipette in layer V of the EC. A confocal image of this region under basal conditions (b) is also presented. Focal episodes of neuronal hyperactivity are induced in the presence of 100 μM 4-aminopyridine (4-AP) and 0.5 mM Mg\textsuperscript{2+} by short pressure-pulse applications of NMDA via the NMDA-containing pipette. The effect of the NMDA pulse was monitored by simultaneously recording Ca\textsuperscript{2+} signals from neurons and the AP firing from one of the neurons close to the NMDA pipette. Notably, in the submersed chamber experiment used in our study, no spontaneous ictal discharges were observed during 4-AP slice perfusion, whereas under different experimental conditions, such as in interface chamber experiments, epileptic activities arise spontaneously [2]. As illustrated by the fluorescence change, a single NMDA pulse induced a transient Ca\textsuperscript{2+} increase in a limited number of layers V-VI neurons from the region close to the pipette tip, that we termed the field I (f1, Figure 4B; Video S3). This local response is clearly illustrated by the difference image generated by subtracting the fluorescence image captured at basal conditions to that obtained after the NMDA stimulation (f1 - b1, Figure 4B).
Figure 3. PAR-1 receptor activation in astrocytes favours ictal discharge generation. (A) Images showing PAR-1 (red) and GFAP (green) immunoreactivity and the merged image in EC layers V-VI. (B) High magnification of a confocal image showing a merged image of PAR-1 and GFAP immunoreactivity from EC layer V-VI. Note the continuity of PAR-1 punctuate signals that are presumably associated with GFAP-negative distal portions of GFAP-positive astrocyte processes (white arrowheads). Scale bar represents 20 μm. (C) Bar graph showing that bath perfusion with TLLR (10 μM) in the presence of D-AP5 and TTX failed to trigger any Ca²⁺ response in 20 neurons analyzed from three different experiments. (D) Ca²⁺ elevations in astrocytes (top traces) and whole-cell recordings of an adjacent neuron (lower traces) after a single TLLR pulse (indicated by an open red arrowhead) applied in the presence of TTX. The Ca²⁺ elevation in astrocytes is followed by the activation of SICS in the nearby patched neurons. Note the slower kinetics of SICS (arrows) with respect to those of a spontaneously occurring fast synaptic miniature event (>). (E) Bar graph reporting the frequency of SICS before and after TLLR pulses. The large majority of SICS (n of 31) occurred within 1/2 s of the TLLR-evoked Ca²⁺ increases in astrocytes. No SICS were observed after TLLR-induced Ca²⁺ elevations in astrocytes in the presence of D-AP5. (F) In the picrotoxin/zero-Mg²⁺ model, astrocyte stimulation by TLLR (open red arrowhead) was sufficient to evoke an ictal discharge.

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Simultaneous patch-clamp recording and Ca²⁺ imaging revealed that the NMDA stimulus leads to AP burst firing in the patched neuron coupled with a Ca²⁺ elevation in this and the other neurons from field A, but it failed to activate neurons of the surrounding region, which we termed field B (Figure 4D).

Two-pulse NMDA stimulation with a 5-s interval evoked a stronger activation of neurons and a transient Ca²⁺ elevation in some of the previously unresponsive neurons from the surrounding field B (t₁ and t₂ = 65, Figure 4C). The response to the double NMDA pulse evolved into a sustained plateau with superimposed Ca²⁺ spikes correlated with AP bursts typical of an ictal discharge, i.e., the cellular equivalent of a seizure [2] (Figure 4D, t₃). The ictal discharge was characterized by Ca²⁺ spikes from unpatched neurons in both field A and field B, highly synchronized with the AP bursts (Figure 4D; Video S4). The recruitment of neurons in field B that underlies the spreading to this region of the ictal discharge is also clearly illustrated by the difference image t₄ − t₃ (Figure 4C). The time window between

the double NMDA pulse and the Ca\textsuperscript{2+} elevation that occurs synchronously in both field A and B neurons represents a transition phase during which the ictal discharge develops in field A. In the presence of TTX, the ictal discharge in both field A and B neurons was abolished, whereas the initial response of field A neurons was unaffected (Figure 4E). The size of the cortical region occupied by neurons that responded directly with a transient Ca\textsuperscript{2+} rise to a double NMDA pulse applied in the presence of TTX was 369±17 μm. Notably, the number of neurons in this response (56.5±12) is underestimated since it comprises only neurons activated by NMDA in a single focal plane. These results demonstrated that 1) AP-mediated events secondary to the initial activation of field A neurons are crucial for ictal discharge modulation; and 2) the activation of neurons from field B and the generation of the ictal discharge was not due to a delayed diffusion of NMDA. Paired recordings from two pyramidal neurons (one in field A and the other in field B) confirmed that similar ictal discharges were regularly evoked in field A and B by successive double NMDA pulses (Figure 5A).

According to results obtained from 14 experiments, no failures were observed in a total of 31 double NMDA pulse stimulations, and the mean duration of the ictal discharge repetitively evoked by these stimulations was reproducible over long time periods (up to 60 min, Figure 5B). By applying successive double NMDA pulse in the presence of TTX, no NMDA-mediated Ca\textsuperscript{2+} elevations were detected in field B neurons, whereas the number of field A neurons activated directly by NMDA and the amplitude of their Ca\textsuperscript{2+} response were found to be unchanged over the same time period (Figure 5A).

Ictal discharges could be evoked also by two single NMDA pulses applied at two different sites, either simultaneously or in succession. Intervals of 3 or 5 s were successful, but not an interval of 10 s. To be effective, the two pipette tips should be positioned close enough to allow a large superficial overlapping of the two pulses. Only in this overlapping region were neurons strongly activated by the two NMDA pulses. Notably, if the distance between the two pipette tips was 172±30.2 μm (n = 5) (a value similar to the mean radius of the field A directly activated by double NMDA pulses), the two single NMDA pulses regularly evoked an ictal discharge. If the distance of the two pipette tips was 230±35.5 μm, no ictal discharges could be evoked.

Alternatively, these data show that an episode of activity evoked in a group of neurons by local NMDA applications creates an initiation site for a seizure-like discharge that secondarily involves adjacent neuronal populations. They also demonstrate that our model is highly reliable since comparable ictal discharges can be evoked by repetitive stimulations applied to the same restricted site. Notably, in contrast to the picrotoxin/-zero-Mg\textsuperscript{2+} model, in the 4-AP model, single NMDA pulses failed to trigger focal ictal discharges, suggesting different thresholds for seizure generation in these two models (see Discussion).

The Development of the Ictal Discharge Is Accompanied by Astrocyte Activation

We next investigated astrocyte activities during the development of focal ictal discharges. We observed that shortly after the initial neuronal response to a double NMDA pulse, a large Ca\textsuperscript{2+} elevation occurred almost simultaneously in the large majority of field A astrocytes (Figure 5A, red traces; Video S4). Similar Ca\textsuperscript{2+} elevations in these astrocytes were never observed during the neuronal response to a single NMDA pulse. In 13 experiments, a mean of 17.4±5.5 out of 20±5.1 responsive astrocytes in field A displayed an early Ca\textsuperscript{2+} elevation during the transition phase. As a mean, astrocyte activation in field A occurred 4.8±1.1 s before field B neurons were recruited into the ictal discharge. Most of the astrocytes in field B were activated later, i.e., after the invasion of the ictal discharge into this region (Figure 5A, blue traces; Video S4). High-magnification images in Figure 5B illustrate "early" and "late" Ca\textsuperscript{2+} changes of astrocytes from field A and B, respectively. The mean percentage of astrocytes from field A and B displaying "early" and "late" responses is reported in Figure 5C. Notably, when the ictal discharge was evoked by two single NMDA pulses applied at two distinct sites (Figure 5D), most astrocytes from both the field of spatial overlapping of the two pulses and the immediately surrounding regions (fields A and B) displayed a similar early Ca\textsuperscript{2+} elevation (35.6±5.4%), whereas most astrocytes from the surrounding regions (the fields A) and B) showed a late activation (7.6±5.4%). Noteworthy is that astrocytes failed to be similarly activated by each single NMDA pulse alone (Figure 5D).

We next asked whether the initial Ca\textsuperscript{2+} elevation in astrocytes (and neurons) from field A spread to other astrocytes (and neurons) in the surrounding regions through a concentric wave of activation centred on the NMDA pipette. We found that the Ca\textsuperscript{2+} response of astrocytes as well as the recruitment of neurons into the ictal discharge is more consistent with a process of modular recruitment rather than with a propagation of a concentric wave of activity (Figure 5S).

Astrocyte activation was largely due to AP-mediated neurotransmitter release since 70.4±5.3% (n = 143, 5 experiments) of the field A astrocytes, activated by a first double NMDA pulse, failed to respond to a second double NMDA pulse applied in the presence of TTX. The Ca\textsuperscript{2+} rise in still-responsive astrocytes displayed slow kinetics and were of small amplitude (AF/Fo 64.1±5.6 before and 29.0±2.2 after TTX, p<0.001). This residual astrocyte response in TTX could be due either to neurotransmitter release mediated by activation of pyramidal NMDA receptors [33] or to the direct activation by NMDA of NMDA receptors that may be present on astrocytes [34,35]. The results from these experiments indicate that the development of a focal ictal discharge is accompanied by Ca\textsuperscript{2+} elevations in astrocytes.
Figure 5. The ictal discharge generation is accompanied by Ca\(^{2+}\) elevations in astrocytes. (A) Ca\(^{2+}\) changes of a field A neuron, a field B neuron, and astrocytes in field A and field B from the same organism illustrated in Figure 4A–4D. The single NMDA pulse fails to activate astrocytes (red and blue traces, left), whereas the double NMDA pulse evokes a large astrocyte Ca\(^{2+}\) rise that in field A (red traces, right) is associated with the initial development of the ictal discharge, whereas in field B (blue traces, right), the rise follows it. Vertical dashed lines mark the transition phase.

*Trace from Figure 4A is shown for comparison. (B) Imaging at high magnifications (dashed boxes in left panel) illustrating the early Ca\(^{2+}\) increase that in field A astrocytes occurs during the transition phase (t1) and the late Ca\(^{2+}\) increase that in field B astrocytes occurs after the ictal discharge (t2). Asterisks and arrowheads mark neurones and astrocytes, respectively. Scale bars represent 20 μm. (C) Percentage of field A astrocytes (15 experiments; 262 responsive astrocytes) and field B astrocytes (12 experiments, 187 responsive astrocytes) that displayed an early or a late Ca\(^{2+}\) increase. (D) Left,
Selective Inhibition of Astrocytes Impairs Ictal Discharge Generation

If this early Ca²⁺ elevation in astrocytes is not a mere consequence of neuronal activity and has, instead, a causative role in ictal discharge generation, its inhibition should reduce the ability of NMDA to trigger an ictal discharge. To address this hypothesis, we first bath applied MPEP and PPADS (n = 4) and found that the direct activation of neurons by a double NMDA pulse was unchanged, but early activated astrocytes were reduced to 4.6±2.6% of controls. Under these conditions, the generation of the ictal discharge in field A and the subsequent recruitment of neurons into the epileptic discharge in field B were inhibited (Figure 6A). The ictal discharge recovered after washout of the antagonists and the reappearance of the associated Ca²⁺ elevation in astrocytes. Interestingly, a stronger neuronal stimulation obtained by increasing the number of successive NMDA puffs evoked an ictal discharge, although of short duration, even in the presence of MPEP/PPADS and without a recovery of astrocyte Ca²⁺ signals (Figure 6A).

We also found that the NMDA-induced ictal discharge was blocked after inhibition of the early responsive astrocytes in field A by MPEP/PPADS applied locally to this region (Figure 6B; n = 4). Ictal discharge recovery was regularly observed 5–10 min after cessation of MPEP/PPADS pulses. In contrast, applications of MPEP/PPADS to a limited sector of field B failed to affect the spread to field B of the ictal discharge generated in field A (n = 4). However, it is noteworthy that the Ca²⁺ elevations in astrocytes from this sector were poorly affected (Figure 6B).

Given that MPEP and PPADS are not selective antagonists of Ca²⁺ signals in astrocytes, to provide a direct evidence for a causal link between Ca²⁺ elevations in field A astrocytes and ictal discharge generation, we inhibited Ca²⁺ signals in these astrocytes selectively by introducing the Ca²⁺ chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetracetic acid (BAPTA; 50 μM) into individual astrocytes through a patch pipette [60].

First, we indirectly evaluated BAPTA spreading in the astrocyte syncytium by patching single EC astrocytes with a Texas Red-containing pipette. We counted 31±7 red-labelled astrocytes in an area of 242±50 μm in diameter (Figure 7A). This value is close to the size of the cortical region occupied by neurons that respond directly with a transient Ca²⁺ rise to a double NMDA pulse applied in the presence of TTX (Figure 7A). In subsequent experiments, before patching a field A astrocyte with a BAPTA-containing pipette, a double NMDA pulse was applied to trigger an ictal discharge (Figure 7B and 7C). In five out of nine BAPTA experiments, a double NMDA pulse applied 50 min after BAPTA diffusion in the astrocyte syncytium failed to activate both the Ca²⁺ elevations in astrocytes and the ictal discharge (Figure 7B and 7C).

Notably, in these five experiments, the response of early activated field A astrocytes was strongly reduced with respect to that observed before BAPTA (Figure 7D). In these experiments, we addressed the contribution of astrocytes in the activation of neurons during the transition phase. In the presence of BAPTA, which specifically inhibited Ca²⁺ signals in field A astrocytes, the number of recruited neurons upon the double NMDA pulse was 33±2.2% lower than in controls (p<0.05). Such a reduction is unlikely due to experimental variability in the intensity of the NMDA stimulation since the number of neurons activated and the amplitude of their Ca²⁺ responses to successive double NMDA pulse stimulations (as measured in the presence of TTX) were unchanged over a 50-min period (Figure 8). These observations indicate that the recruitment of neurons into the ictal discharge is also mediated by the early activated astrocytes that signal back to neurons.

In the four experiments with BAPTA in which the ictal discharge was preserved, most of the astrocytes in field A still displayed an early Ca²⁺ response, suggesting a defective diffusion of BAPTA in the astrocyte syncytium in these experiments (Figure 7D). These data provide a plausible explanation for the lack of inhibition of the ictal discharge in these BAPTA experiments.

In a number of different control experiments, we found that two subsequent double NMDA pulses applied before and 50 min after patching either a neuron (n = 8) or an astrocyte (n = 4) with a pipette not containing BAPTA always evoked comparable ictal discharges, indicating that such a long time interval does not affect the ability of a double NMDA pulse to trigger an ictal discharge; double NMDA pulses regularly evoked an ictal discharge even after 50 mM BAPTA was applied directly over the neurons for 7 min via a pipette (n = 8), indicating that a leakage of BAPTA, putatively occurring during astrocyte seal formation, cannot account for the ictal discharge inhibition observed in the BAPTA experiments; successive double NMDA pulses applied in the presence of TTX over a period of 50 min, while patching single astrocytes with a BAPTA-containing pipette, evoked an unchanged response in neurons (Figure 7E), demonstrating that the direct response of neurons to NMDA is not affected after BAPTA-mediated inhibition of astrocyte Ca²⁺ signals.

We next asked whether the late activation of astrocytes in field B contributes to the spreading of the ictal discharge. After patching individual field B astrocytes with a BAPTA-containing pipette, we observed that the ictal discharge evoked in field A by a double NMDA pulse still invaded field B and further propagated to the adjacent region, whereas the activation of field B astrocytes was drastically affected both in term of Ca²⁺ signal amplitude (~56.6±2.4%, p<0.001) and kinetics (time to peak, 2.6±0.4 s and 5.2±3.3 s, before and after BAPTA, respectively; p<0.001; Figure 8B).

As a further control for the specificity of the BAPTA effect, we demonstrated that the ictal discharge inhibition by BAPTA was spatially restricted. After the astrocyte syncytium in region 1 was loaded with BAPTA, a double NMDA pulse stimulation close to the BAPTA-loaded region failed to trigger an ictal discharge, whereas the same NMDA stimulation applied ~500 μm away from region 1 readily evoked an ictal discharge (region 2; Figure 7F and 7H).

The ictal discharge blocked after the BAPTA-mediated inhibition of field A astrocytes was recovered in two of three experiments by applying a stronger stimulation of neurons, such as a triple NMDA pulse (Figure 7); white arrowheads). Notably is that under these conditions, astrocytes recovered a Ca²⁺ response that was, however, delayed and of reduced amplitude with respect to that without BAPTA. These results are consistent with the hypothesis that the astrocyte contribution to ictal discharge generation is not an absolute requirement and can be bypassed by a stronger stimulation of neurons, as already suggested by the results obtained in MPEP/PPADS experiments.
Figure 6. Inhibition of field A astrocytes by MPEP/PPADS impairs ictal discharge generation. (A) Ca\(^{2+}\) signal from a field A neuron, a field B neuron, and field A astrocytes in response to repetitive episodes of NMDA stimulation (black arrowheads). The NMDA stimulation that evoked an ictal discharge became ineffective after blocking the astrocyte response by bath perfusion with MPEP and PPADS. An ictal discharge could be recovered by increasing the number of NMDA pulses (white arrowheads). A double NMDA pulse evoked both astrocyte activation and the ictal discharge after inhibition washout. (B) Top, drawings of field A and B illustrating different experimental conditions. The three black arrows symbolize the spreading of a focal ictal discharge. The red spots indicate the region of local MPEP/PPADS applications. Bottom, Ca\(^{2+}\) signal from a field A neuron, a field B neuron, field A astrocytes, and field B astrocytes in response to repetitive episodes of NMDA stimulation (black arrowheads). The double NMDA pulse that evoked an ictal discharge became ineffective after local MPEP/PPADS application to field A, but not to field B. Note also the absence of “early” responsive astrocytes after MPEP/PPADS applications to field A, whereas “late” responsive astrocytes are only slight changed after MPEP/PPADS applications to field B.

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Fig. 7. Selective inhibition of astrocytes impairs ictal discharge generation. (A) Maximal projection of a Texas Red–labelled astrocyte in the ictal discharge (n = 3) and of the region of neurons directly activated by NMDA (n = 6). Scale bar represents 20 μm. (B) Images from an EC slice illustrating the Ca²⁺ signal in neurons and astrocytes from Field A before and after BaPPA spreading in the astrocyte synaptocyst, at rest (A1), and after stimulation (A2). Scale bar represents 10 μm. (C) Number of neurons and amplitude of the Ca²⁺ signal after repetitive double NMDA pulses applied in the presence of TTX during BaPPA spreading in the astrocyte synaptocyst (n = 3), expressed as relative values with respect to measurements performed before BaPPA spreading. (D) Differential interference contrast image of an EC slice showing the BaPPA-containing pipette (arrowhead) and the first location of the NMDA pipette (asterisk) in region 1 that was used to trigger the first ictal discharge. The NMDA pipette was then moved to region 2 and its tip is indicated by the two asterisks. Dashed circle indicates the field A in each region. The small circles mark the position of a field B neuron in region 1 (A1) and in region 2 (A2). Scale bar represents 100 μm. (E) Double NMDA pulse–induced ictal discharge in Field A and B neurons in region 1 and its failure 50 min after BaPPA spreading in the astrocyte synaptocyst. (F) Recovery of the ictal discharge evoked by a double NMDA pulse and moving the NMDA pipette to region 2. (G) Ca²⁺ changes in Field A and B neurons and Field A astrocytes illustrating the inhibition of the ictal discharge by BaPPA in a different EC slice and its recovery after increasing the NMDA stimulation to three pulses (white arrowhead). Note that after increasing the NMDA stimulation, astrocyte activation partially recovers, but the response is delayed and develops after the emergence of the ictal discharge in Field B neurons.

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Tolled together, the results of these series of experiments confirm the reliability of the double NMDA pulse paradigm in evoking an ictal discharge over long time periods and, on the other hand, validate the selective inhibition of astrocyte Ca²⁺ signals by intracellular BaPPA application.

Selective Activation of Astrocytes Favors the Generation of Focal Ictal Discharges.

If inhibition of Ca²⁺ signals in astrocytes can block the generation of focal ictal discharges, it would be expected that direct astrocyte stimulation promotes ictal discharges. In the experiments that addressed this hypothesis, we took advantage of the finding that none of the 48 single NMDA pulses performed in the 4-AP ictogenic model could produce a focal event. Single NMDA pulses were collectively used to trigger an ictal discharge, which was effective when they were coupled with TFLLR (Fig. 6). We found that a single NMDA pulse coupled with TFLLR, evoked an ictal event in six of nine trials from a total of 15 experiments. In these experiments, we also found that the number of neurons activated by the NMDA/TFLLR coapplication during the transition phase was higher than that activated by NMDA alone (mean increase, ±119.32±16.3%; n = 6; p < 0.001). These data confirm that the contribution of astrocytes in the recruitment of neurons can be critical for the generation of the ictal discharge.

Discussion

In brain slice models of seizures, the ictal discharge is proposed to initiate at local brain sites by asynchronous neuronal hyperactivity that progressively recruit adjacent neurons into a synchronous discharge [1,3,4]. In our study, we found that neuronal hyperactivities at these restricted brain sites are accompanied by Ca²⁺ elevations in a large number of astrocytes that contribute to drive neurons towards seizure threshold.

The ictogenic focus in our model is schematically illustrated in Fig. 9. This process starts with an isolated episode of local neuronal hyperactivity that triggers a large and synchronous Ca²⁺ elevation in closely associated astrocytes (N1). These activated astrocytes signal back to neurons (A1) favouring the recruitment of neurons into a coherent activity that underlines the hyper-

![Figure 8](image8.png)

**Figure 8.** Selective astrocyte activation favours focal ictal discharges. In the 4-AP model, single NMDA pulse evokes the ictal discharge only when an astrocyte Ca²⁺ increase is induced by TFLLR.

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![Figure 9](image9.png)

**Figure 9.** Neuron-astrocyte loop in ictal discharge generation. Schematic of the sequence of events in the recurrent neuron-astrocyte excitatory loop that develops at a restricted brain site to generate seizures. N1 and N2, neuron-to-astrocyte signalling; A1 and A2, astrocyte-to-neuron signalling.

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chronous ictal discharge. This event, in turn, triggers a second activation of astrocytes (N2). The secondary astrocyte activation may then contribute to sustain the ictal discharge (A2). This sequence of events represents a recurrent neuronal-astrocyte excitatory loop that drives neurons towards the ictal discharge threshold.

Since our slice experiments were performed mainly in young animals, the role of astrocytes in seizure generation may be restricted to the immature brain. Although additional experiments are necessary to clarify this important issue, the ability of astrocytes to release glutamate and activate neuronal SGs in slices from young adult rats [33,37,38] suggests that astrocyte-to-neuron signalling may contribute to seizure initiation also in the adult brain.

The Early Ca²⁺ Elevation in Astrocytes Has a Causative Role in Ictal Discharge Initiation

In EC slices perfused with the proconvulsant agent 4-AP in low Mg²⁺ conditions, we found that a synchronous Ca²⁺ elevation in a high number of astrocytes occurred along with the development of the ictal discharge evoked by a local NMDA application. This response was largely TTX sensitive, indicating that astrocytes were activated by AP-mediated neurotransmitter release. Most importantly, the early astrocyte activation was a crucial step in the generation of ictal discharges. Indeed, when Ca²⁺ elevations in field A astrocytes were inhibited by BAPTA, the episode of neuronal hyperactivity induced by NMDA failed to generate an ictal discharge. According to results obtained from different control experiments, the effect of BAPTA on ictal discharge generation was specifically linked to the inhibition of astrocyte Ca²⁺ signals.

The Ca²⁺ elevations in astrocytes are associated with the release of glutamotransmitters, such as glutamate [39,41] and D-serine [42], that modulates neurotransmitter release [24,43,44], triggers AP firing in neurons [10], and promotes local neuronal synchrony [33,38]. Ca²⁺-dependent release of D-serine from astrocyte activated by Schaffer collateral stimulation has been also recently shown to be responsible for the potentiation of synaptic transmission in the CA1 hippocampal region [45]. As previously reported in the hippocampus [25,26], we show here that Ca²⁺ elevations stimulated in EC astrocytes by the PAR-1 receptor agonist, TFLLR, triggers glutamate release in these cells and, in turn, NMDA receptor mediated SGs in neurons. The activation of neurons by glutamotransmission can thus account for the finding that a single NMDA pulse, ineffective per se, was able to trigger the ictal discharge if coupled with the direct stimulation of a Ca²⁺ rise in astrocytes by TFLLR. Data analysis of these experiments revealed that the number of astrocytes activated after NMDA/TFLLR coapplication was higher than that activated after NMDA alone.

These results suggest that when an episode of hyperactivity in a group of neurons consistently engages nearby astrocytes, a larger population of neurons is recruited into a coherent activity. If this feedback signal operates on a brain network prone to seizures, it contributes to drive neurons towards the ictal discharge threshold. The initiation site is thus represented, not only by the neurons activated by NMDA, but also by those that are secondarily activated in a recruitment process that involves astrocytes. Consistent with this view is our finding that when a double NMDA pulse (that successfully evoked an ictal discharge) was applied either after BAPTA was introduced in the astrocyte synctium or after local applications of MPEP/PDADS to the site of activation, astrocytes were poorly activated, fewer neurons were recruited and the ictal discharge was evoked. Furthermore, the conclusion derived from the experiments with a single NMDA pulse delivered from two pipettes positioned at different distances. These experiments revealed that an ictal discharge could be evoked when astrocytes from the region of overlapping neuronal activation were activated. When the pipette tips were closer, i.e., the overlapping region was reduced, astrocytes were poorly activated, and no ictal discharge was evoked.

Distinct subpopulations of astrocytes might differentially contribute to modulate neuronal hyperactivity in the epileptogenic region, possibly by releasing in addition to glutamate, ATP, and other neuroactive signals, e.g., GABA, through a Ca²⁺-dependent or -independent mechanism [40,47]. Given that inhibitory interneurons have been reported to restrain the recruitment of neurons during the development of the ictal discharge [4,48], the opposite action of astrocytes in this process might involve a distinct inhibition of interneurons by GABA released from astrocytes. Indirect support for this possible astrocyte action derives from the observation that GABA released from astrocytes can, indeed, result in a long-lasting inhibition of inhibitory gamma cell activity in the olfactory bulb [37]. Whether a similar signalling between a subpopulation of GABA-releasing astrocytes and interneurons may be involved in ictal discharge initiation in the EC represents an interesting question to be addressed in future studies.

Episodes of focal seizures can arise in a nonepileptic tissue due to genetic causes or as a consequence of various brain damage. These may lead to status epilepticus (SE), a condition of persistent seizures, and evolve into chronic epilepsy after a latent period of epileptogenesis. Our results were obtained in nonepileptic brain tissue and provide evidence for the contribution of astrocytes in the initiation of seizure during SE. Therefore, whether astrocytes contribute also to seizure initiation in chronic epilepsy is, at present, unknown and should be appropriately investigated in chronic epilepsy models. However, results from a recent in vivo study showed that astrocytes, which exhibited long-lasting Ca²⁺ oscillations during SE, contributed to the neuronal death that characterizes chronic epilepsy [35]. This effect was due to astrocytic glutamate that activated neuronal NMDARs, possibly favouring seizure generation. It is also worth underlining that in the epileptic brain tissue, astrocytes undergo significant changes in their physiological properties that may result in decreased glutamate uptake, altered extracellular K⁺ buffering capacities, and activation of inflammatory pathways [49,50]. All these changes may contribute to the increased neuronal network excitability that characterizes the epileptic brain.

The Astrocyte Contribution to Ictal Discharge Generation Depends on Neuronal Excitability Levels

The efficacy of astrocyte stimulation in evoking an epileptic discharge was different in the two models used in the present study, probably because of differences in their intrinsic neuronal predisposition to ictal discharge generation. As suggested by the presence of recurrent spontaneous epileptic discharges, the picrotoxin/zro-Mg²⁺ model can be considered, indeed, a model with a low-threshold for epileptic discharges. In this model, a single NMDA pulse triggered synchronous activity in a number of neurons sufficient to reach the ictal discharge threshold, and a single stimulation of astrocytes was also sufficient to trigger an ictal discharge. As suggested by the absence of spontaneous epileptic events, the 4-AP model has a higher threshold for epileptic phenomena. In this model, seizure discharges could be triggered by a more prolonged and intense episode of neuronal activity induced by a double NMDA pulse, and not by single NMDA or TFLLR pulses. An ictal discharge could be also evoked when a single NMDA application (infective per se) was coupled with TFLLR-mediated astrocyte activation. Furthermore, the reduc-
tion in astrocyte Ca\(^{2+}\) signals blocked the ictal discharge in the 4-AP model, but not in the picrotoxin/zero-Mg\(^{2+}\) model. These data demonstrate that experimental manipulations of the astrocyte Ca\(^{2+}\) signal can influence neuronal recruitment and this, in concert with the level of neuronal activity, the likelihood of ictal events.

As revealed by results from both BAPTA and MPEP/PPADS experiments, when the astrocyte contribution was reduced by inhibiting Ca\(^{2+}\) signals in these cells and the double NMDA pulse consequently failed to evoke an ictal discharge, we could recover an ictal discharge by applying a more intense NMDA stimulation. By activating directly a larger number of neurons, this higher stimulus evokes a level of correlated activity that is sufficient for seizure-like discharge generation, bypassing the astrocyte contribution in the recruitment process. Thus, astrocyte activation is not an absolute requirement for ictal discharge generation.

However, astrocytes respond readily to synaptic activity with Ca\(^{2+}\) oscillations [14,23,31], and the frequency of these oscillations increases in parallel with an increased neuronal activity [16]. In vivo studies also revealed that sensory stimuli can evoke distinct Ca\(^{2+}\) elevations in astrocytes confirming the strict association between neuron and astrocyte activities [52-55]. Thus, pathological hyperexcitability in neurons [6] should be regularly accompanied by an increased astrocyte activity. In support of this view, studies in brain slices showed that chemically induced epileptiform activity causes a sustained increase in astrocyte Ca\(^{2+}\) signalling [9,10], and in vivo studies reported a long-lasting hyperexcitability of astrocytes after pilocarpine-induced SE [38]. It is conceivable that a pathological hyperexcitability that predisposes neurons to seizure discharges may originate from abnormalities in the network activity, whatever the origin of the initial dysfunction might be. As we showed here, depending on the different level of excitability in neurons, the astrocyte contribution varies, but it can even be crucial for ictal discharge generation.

Ictal, but Not Intercitial, Discharges Activate a Secondary Astrocyte Ca\(^{2+}\) Elevation

In our 4-AP dice model, a second Ca\(^{2+}\) elevation even of larger amplitude than that early evoked by the double NMDA pulse, occurred in astrocytes in both field A and field B. This delayed activation of astrocytes was observed also after the spontaneously occurring ictal discharges in the picrotoxin/zero-Mg\(^{2+}\) model in both rats and pGFAP-EGFP transgenic mice, as well as in other models such as the 4-aminopyridine/picrotoxin and high-potassium models (unpublished data). Most importantly, this observation was validated in the intact guinea pig brain preparation, a well-characterized model of EC hippocampal focal ictogenesis [30,31]. In this close-to-in vivo preparation, the development of the ictal discharge was regularly accompanied by a Ca\(^{2+}\) elevation in virtually all astrocytes present in the recording field, whereas large-amplitude interictal discharges were never associated with a significant Ca\(^{2+}\) change in astrocytes. This Ca\(^{2+}\) elevation and the following release of gliotransmitters may contribute to the maintenance of AP bursts and to the process of neuronal recruitment that characterize seizure discharge propagation. Our finding that the duration of the ictal discharge was significantly reduced upon inhibition of the astrocyte Ca\(^{2+}\) signal by bath perfusion with MPEP or PPADS is consistent with this hypothesis, which needs, however, to be specifically addressed in future experiments.

In the present study, we also addressed a possible role of the late astrocyte response in the propagation of the ictal discharge outside the foci region. After BAPTA introduction in field B astrocyte synctium, the ictal discharge still propagated to this region and further, suggesting that Ca\(^{2+}\) elevations in field B astrocytes may have no role in this process. Given that initiation, propagation and cessation of the ictal discharge are likely governed by distinct mechanisms [3], it would be surprising these astrocytes have, indeed, a role in ictal discharge initiation but not in propagation. This conclusion is, however, reasonable, but it is not proven beyond all doubt. Indeed, the inhibition by BAPTA could be exerted only in astrocytes from a small sector of the large field B, whereas astrocytes outside this sector were totally unaffected. Their activation might thus be sufficient to sustain the propagation of the ictal discharge even to the small sector of field B, whereas astrocytes were inhibited by BAPTA. As to MPEP/PPADS, when locally applied to field B, these competitive receptor antagonists failed to inhibit the ictal discharge propagating to this region. These results, however, do not allow us to draw any conclusions since the ictal discharge involving field B still activated a significant response in astrocytes even in the presence of MPEP/PPADS. To clarify this point, another experimental approach is thus required.

It is unclear why MPEP/PPADS failed to inhibit the Ca\(^{2+}\) elevation evoked by the ictal discharge in field B astrocytes. It is likely that, with respect to the NMDA pulse, the ictal discharge represents a more powerful stimulus that triggers the release of glutamate and ATP. Accordingly, the extracellular concentration of MPEP/PPADS reached after local applications might have been insufficient to inhibit the large activation of astrocyte mGlu and P2 receptors upon the ictal discharge. However, mechanisms other than mGlu and P2 receptor activation may be also involved in this astrocyte response.

Intercitellar discharges failed to activate significantly a Ca\(^{2+}\) elevation in astrocytes. Recently, it has been reported that glutamate release triggered by Ca\(^{2+}\) elevations in astrocytes plays a predominant, if not obligatory role in the generation of epileptic activity in the hippocampus and, in particular, in the slow depolarization shift associated with interictal discharges [9]. This conclusion is, however, at variance with a number of studies showing that both interictal and ictal seizure-like discharges from different brain regions, including the hippocampus, are strongly linked to neuronal activity being efficiently prevented or blocked, depending on the time of application, by TTX [0,30,56,58]. In the present study, we observed that i) the interictal activity was not blocked after Ca\(^{2+}\) elevations in astrocytes were drastically reduced; and ii) synchronous astrocyte Ca\(^{2+}\) elevations were never observed to accompany an interictal discharge in the different models. We thus failed to confirm the role of astrocytic glutamate in interictal discharge generation. The reasons for this discrepancy are, at present, unknown.

Conclusions and Perspectives

The present study reveals a crucial role of neuron astrocyte interactions in sculpting activity at the epileptogenic zone. When a group of neurons is abnormally active (due to acquired or genetic causes), interictal epileptiform events may occur through the activation of astrocytes. Astrocytes can thus play a key role in seizure initiation in a nonneoplastic brain tissue and, in contrast to previous observations [9], do not appear to be involved in the generation of the interictal events. This peculiarly makes the astrocyte neuron unit a primary target for novel drug development aimed at interfering selectively with ictogenesis, without affecting the interictal activity that, by preventing seizure precipitation, may have a beneficial role in focal epilepsy [59,60].

The high reproducibility in the generation of comparable ictal discharges represents an important advantage of our new EC slice model of ictogenesis. This model allowed us to investigate the early events that, at a restricted brain site, predispose neurons to seizure
and to obtain some insights into the mechanism of focal ictal generation that involves astrocytes. Other aspects that were not addressed in the present study, such as the neuronal recruitment processes during the discharge of the ictal discharge to region distant from the site of ictal discharge generation, could be investigated in this model. These acute experiments set the conditions for validating the mechanisms here described in future studies in chronic models of epilepsy, including genetically determined in vivo models of epilepsy, that more closely mimic the complex feature of seizures in epileptic patients. A validation of the astrocyte role in seizures generation in these models is fundamental to provide further arguments in favour of astrocytes as targets for developing new therapeutic strategies for epilepsy.

Materials and Methods

Ethics Statement
All experimental procedures were authorized by the Italian Ministry of Health.

Brain Slice, Guinea Pig Brain Preparations, and Dye Loading

Transverse cortico-hippocampal slices were prepared from postnatal day 14-18 water rats or pGFAP-EGFP transgenic mice [61], and loaded with OGB1-AM (excited at 488 nm) or Rhod-2 (excited at 543 nm), respectively, as previously described [6]. Briefly, brain was removed and transferred to ice-cold cutting solution containing (in mM): NaCl, 120; KCl, 3.2; KH2PO4, 1; NaHCO3, 26; MgCl2, 2; CaCl2, 1; glucose, 10; Na-pyruvate, 2; and ascorbic acid 0.6% at pH 7.4 with 5% CO2/95% O2. Coronal slices were obtained by cutting with a Leica vibratome VT1000S in the presence of the ionotropic glutamate receptor inhibitor kynurenic acid (2 mM). Slices were recovered for 15 min at 37°C and then loaded with the Ca2+-sensitive dye OGB1-AM (Invitrogen) for 60 min at 37°C. Loading was performed in the cutting solution containing sulfonylpyrazine (200 μM), luminous (0.1%), and kynurenic acid (1 mM). After loading, slices were recovered and kept at room temperature in the presence of 200 μM sulfonylpyrazine. Brains from postnatal day 14-20 guinea pigs were isolated and perfused at a rate of 5.5 ml/min through the basilar artery [19,62] with a solution containing (in mM): NaCl, 126; KCl, 3; KH2PO4, 1.2; MgSO4, 1.3; CaCl2, 2.4; NaHCO3, 26; glucose, 15; and 3% dextrose M.W. 70,000; oxygenated with a 95% O2/5% CO2 gas mixture (pH 7.3). The dye OGB1-AM (50 μg) was diluted in 5 μl of standard perfusate/DMSO solution and 75 μl of saline, and filtered through a 0.2-μm microfilter (Millipore). A patch pipette (3.4 MΩ) was used to pressure inject (1-2 min at 4 PSI) the Ca2+-dye into the EC at a depth of about 200 μm via a picopipette (NIPEN Electronics). Following this procedure, the Ca2+ signal from astrocytes, neurons, and neuropile was monitored. All experiments were performed at 33°C ± 0.3°C.

Ca2+ Imaging

In slice experiments, we used a TCS-SP5RS or a TCS-SP5-RS confocal microscope (Leica) equipped with a 50× objective (NA: 1.0) and a CCD camera for differential interference contrast images. For experiments on isolated guinea pig brains, we used a Bioview 300 scanning head customized for two-photon microscopy equipped with a SW Verdi-Mira laser (Coherent) tuned at 830 nm and external photomultipliers (Hamamatsu). Time frame acquisitions from 3-4 ms to 1.2 s (with one to six line averaging) were used. No background subtraction or other manipulations were applied to digitized Ca2+ signal images that are reported as raw data, with the exception of the difference images in Figure 4 that were obtained by subtracting the prestimulation image from the poststimulation image. In brain slice preparations, neurons and astrocytes were distinguished on the basis of the distinct kinetics of their Ca2+ response to a stimulation with high K+ extracellular solution (40 mM) obtained by isometric replacement of Na+ with K+ [16], applied at the end of the recording session in the presence of 1 μM TTX (Figure 7). Due to the lack of voltage-dependent Ca2+ channels in astrocytes, the Ca2+ elevation in these cells upon high K+ stimulation occurs with a delay of several seconds with respect to the response in neurons, and appears to be mediated by glutamate release from depolarizing neurons [17]. In the present study, the presence of TTX was necessary to block the epileptic discharges and the underlying Ca2+ changes in neurons and astrocytes that would have hampered the possibility to distinguish these cells from their different responses to high K+ stimulation. Astrocytes were identified also by their small size, low membrane potentials (−74 ± 0.4 mV) without the correction for the liquid junction potential at the pipette tip, which was 15 mV; n = 9), and passive responses to a series of depolarizing steps. In slices from pGFAP-EGFP mice, astrocytes were identified by their green GFP fluorescence. In the guinea pig brain, astrocytes were identified using the astrocyte-specific marker sulfatedodehydroxybamine 101 (Invitrogen) applied at 100 μM to the cortical surface [63]. The onset of the slow Ca2+ elevation in astrocytes was determined on the basis of a threshold criterion. The onset was identified by the change in AF/F0 that should be more than two standard deviations over the average baseline and remained above this value in the successive frames for at least 3 s (two to six frames, depending on the frame acquisition rate).

Electrophysiology and NMDA Pulse Applications

Krat brain slices in a submerged chamber (Warner Instruments) were continuously perfused at a rate of 2 ml/min with (in mM): NaCl, 128; KCl, 3.2; KH2PO4, 1; NaHCO3, 26; MgCl2, 1; CaCl2, 2; glucose, 10; sulfonylpyrazine, 0.2; at pH 7.4 with 5% O2/95% CO2. Whole-cell patch-clamp recordings in cat brain slices were performed using standard procedures and one or two Axopatch-200B amplifiers (Molecular Devices), as previously reported [8]. Typical pipette resistance was 3–4 MΩ for neurons. Data were filtered at 1 kHz and sampled at 5 kHz with a Digidesign (1320 interface and pClamp8) software (Molecular Devices). Whole-cell intracellular pipette solution was (in mM): K-glutamate, 145; MgCl2, 2; EGTA-NA, 9.5; NaATP, 2; NaGTP, 0.2; HEPES, 10; to pH 7.2 with KOH, and contained a low concentration (10 μM) of OGB1 (Invitrogen); composition, 305 ± 5.5 mOsm. Data analysis was performed with Clampfit 8 and Origin 6.0 (Microcal Software) software. SKG with an amplitude greater than 20 pA and a rise time slower than 10 ms are classified as SKG, as described previously. SKG rise time was calculated with the 20%–80% criterion and the decay time as the time constant of a single exponential fit. The delay of each SKG activated in neurons after astrocyte stimulation with TLLR was calculated with respect to the peak of the immediately preceding astrocyte Ca2+ increase.

Intertial and icat sciare-like events resembling those recorded at the electrophographic recordings from patient’s brain [21], at a cellular level manifest as intense and hyperpolarizing discharges that involve large neuronal population and fundamentally differ in their duration. Despite this common characteristic, they have radically different durations. Indeed, the duration of the epileptic event was an important criterion for classifying interictal and ictal events in slice and the isolated whole guinea pig brain preparations. In Ca2+ imaging experiments, interictal events lasted less than 3 s, whereas ictal discharges were sustained for tens of
seconds with a final pattern of highly synchronous afterdischarges. The duration of ictal events varied between 15 and 110 s in brain slices and between 2 and 13 s in the guinea pig brain. Postictal depression was also consistently observed after an ictal event, whereas it was not present after an interictal spike [21]. A pressure ejection unit (PE8, NPI Electronik) was used to apply pressure pulses (40 μl/pulse, 300 μm duration) to NMDA-containing pipettes. Pressure pulse duration was increased until a double NMDA pulse elicited an ictal discharge. The stimulus parameters for successive stimulations remained unchanged over the entire recording session, except in the BAPTA experiments in which they were changed to increase the stimulation of neurons by NMDA and thus to recover the ictal discharge after inhibition of Ca²⁺ signals in astrocytes. In the double NMDA pulse, the interval between the two pulses was 5 s. NMDA pulses applied with intervals of 5 s, but not 20 s, also triggered an ictal discharge (unpublished data). For BAPTA dialysis into the astrocyte somatodendritic area, we used a patch pipette (external diameter 5–6 MΩ; 300–310 μm) containing (in mM): K£-methylsulfate, 50; ATP, 2; GTP, 0.4; HEPES, 50; BAPTA, 50. To avoid leakage of BAPTA from the pipette during seal formation, the BAPTA solution was backfilled after loading the tip with a standard intracellular solution. Texas Red dye (excited at 543 nm) was included at 0.2 mM in a patch pipette containing standard solution and monitored 50–60 min after the whole-cell configuration. For the BAPTA and Texas Red experiments, only GluT (coupled) astrocytes were included. GluR (uncoupled) astrocytes were distinguished according to their different responses to hyperpolarizing and depolarizing current pulses of increasing amplitude and 750 ms duration. Field potentials were recorded from the guinea pig brain with saline-filled micropipettes used to deliver OGB-1 AM via a multichannel differential amplifier (NPI Elektronik). A precise alignment of Ca²⁺ and electrophysiological signals was achieved by acquiring with a synchronization signal produced by the confocal microscope. Tip potential was measured against a ground reference placed in the recording chamber by means of a voltage follower coupled to an amplifier.

Drugs

MPEP (50 μM), PPADS (10 μM), TTX (1 μM), D-APV (50 μM), 4-AP (100 μM; Ascent Scientific), TFLKR (10 μM; Toeris), and picotoin (50 μM; Sigma-Aldrich) were bath applied. TFLKR (1 μM) and NMDA (1 mM; Sigma-Aldrich) were pressure applied. To induce local astrocyte inhibition, we applied pressure pulses of 2 s at 5 min at a frequency of 0.1 Hz to a pipette containing MPEP (500 μM) and PPADS (5 μM). Bisacodyl-methylsulfate (50 μM; Sigma-Aldrich) was applied by arterial perfusion to guinea pig brains.

Monte Carlo Simulation Procedures

The Monte Carlo simulation was designed to test whether the observed series of stimuli and ictal episodes were compatible with a random distribution. Each simulation run generated randomly distributed stimuli and ictal events based on 1) recording duration; ii) number of stimuli and ictal events; iii) minimum interval between stimuli; iv) minimum interval between two successive ictal events; and v) minimum interval between a stimulus and an ictal event (ictal events seem to be followed by at least 20 s of refractory period). These rules imply that the occurrence of ictal and pulses are not completely independent. The random generator produced 30,000 temporal series for each experimental run, using experiment-specific parameters. Figure S/A shows an experiment and three simulated runs. The distance between each stimulus and the first following ictal events were computed for each simulation. The datasets were used to compute the density probability p(i) of observing one ictal at time i after an astrocyte activation (Figure S/B). The cumulative probability CP(i) is obtained by the integration of the density probability and yields the probability of observing an ictal at a time ≤i under the hypothesis that stimuli and ictals are not causally related (Figure S/C). Each ictal event in the experiment was associated with the delay from the immediately preceding stimulus, and the probability of observing the ictal was calculated. If the cumulative probability was less than 0.05, the event was deemed as not satisfying the null hypothesis. Results are presented in Figure S/D.

Immunocytochemistry

Coronal slices (100-μm thick) of rat brains were cut with a VT 1000S vibratome (Leica) and directly fixed for 1 h in ice-cold 4% paraformaldehyde in phosphate-buffered solution (PBS). Floating sections were first preincubated in a blocking solution (BS: 1% BSA, 2% horse serum, and 2% goat serum) containing 0.3% triton X-100 and subsequently incubated with the primary mouse anti-thrombin receptor PAR-1 antibody (1:300, Zymed Laboratories, Invitrogen) and rabbit anti-GFAP (1:500, Dako) diluted in BS. After 24 h, slices were washed at 4°C in PBS and incubated with the secondary antibodies (Cy3 conjugated donkey anti-mouse IgG, and Cy2 conjugated goat anti-rabbit Fab’2 fragments; Chemicon International) for 2 h at room temperature. Slices were extensively washed in PBS, mounted in Elvanol, and observed with a Leica SP2 laser scanning confocal microscope. Negative controls were performed in the absence of the primary antibodies. Images were assembled using CS Adobé Photoshop software.

Data Analysis

The Ca²⁺ signal is reported as ΔF/F₀, where F₀ is the baseline fluorescence. Data are shown as mean ± standard error of the mean (S.E.M). Under stated otherwise, the Student's t-test was used, with p values ≤0.05 taken as statistically significant.

Supporting Information

Figure S1. Interictal events activate in astrocytes only an increase in Ca²⁺ oscillation frequency. (A) Representative experiment from a rat hippocampal slice showing the Ca²⁺ elevations in CA3 neurons (black trace, averaged signal from all 30 neurons monitored) corresponding to interictal discharges in the picrotoxin/xenon-Mg²⁺ model. This interictal discharge activity is accompanied by an increase in Ca²⁺ oscillation frequency in astrocytes (blue traces). (B) Bar graphs reporting the mean astrocyte Ca²⁺ oscillation frequency in control and during interictal activity. *p<0.05. (C) 2P-LSM images from the EC of a guinea pig brain before (b), during (t), and after (a) an interictal discharge induced by arterial perfusion with bicuculline. Astrocytes (white arrowheads), neuropsilin (dashed circle), and a neuron (yellow arrow) are indicated. Scale bar represents 20 μm. (D) Field potential recording of two interictal discharges and correlated Ca²⁺ changes in the neuropil and the neuron indicated in (C). No correlated Ca²⁺ changes were observed from the two astrocytes (arrowheads in (C)).

Found at: doi:10.1371/journal.pbio.0000552.s001 (0.72 MB TIF)

Figure S2. Analysis by Monte Carlo simulation. (A) Diagram representing the entire length of the recording of the experiment partially reported, in terms of Ca²⁺ signal changes in neurons and astrocytes, in Figure 3F. Horizontal bar on the top marks the timing of the traces shown in Figure 3F. Black bars indicate the peak of the astrocyte Ca²⁺ response triggered by TFLKR stimuli, and the green boxes correspond to the ictal
events. The three underlying rows represent three results from the Monte Carlo simulation procedure (see Materials and Methods). (B) and (C) Density probability (B) and cumulative probability (C) computed from the Monte Carlo simulation for the depicted experiment in (A). Green arrowheads indicate the p value for the five t-test events occurring during the recording. These are the probabilities for each t-test to be independent from the astrocyte activation. The value p = 0.05 (reported in [B]) is the probability of correlation of the timing of the t-test discharge with the astrocyte Ca\textsuperscript{2+} increase. (D) Graphs showing the probability that the 15 t-test events (black dots) observed in six experiments are correlated positively with an astrocyte Ca\textsuperscript{2+} increase induced by TFFLR in the picrotoxin/zero-Mg\textsuperscript{2+} model. Found at: doi:10.1371/journal.pbio.0000352.e002 (0.35 MB TIF)

**Figure 83** In the picrotoxin/zero-Mg\textsuperscript{2+} model, a single NMNDA application is sufficient to trigger epileptiform discharges. Representative experiment showing the effect of single local NMNDA stimulation (arrowheads) on neurons from an EC slice perfused with picrotoxin/zero-Mg\textsuperscript{2+}. The Ca\textsuperscript{2+} signal from a neuron in the region close to the NMNDA pipette tip (blue trace) and the current-clamp recording from a neuron located in a region distant from the NMNDA pipette (black trace) revealed that NMNDA puffs could induce a local response that either remained restricted (open arrowheads) or triggered a response (black arrowheads) that evolved into an interictal (second and third puff) or an ictal event (fifth puff). Spontaneous and evoked interictal and spontaneous and evoked ictal discharges recorded from the patched neurons are undistinguishable (see lower panels). This observation validates our model since it suggests that both events are sustained by a similar number and subtype of active cells and it relies on a common basic mechanism. Found at: doi:10.1371/journal.pbio.0000352.e003 (0.46 MB TIF)

**Figure 84** The ictal discharge triggered by a local neuronal stimulation is highly reproducible. (A) Pair recording showing that successive ictal discharges occur in both field A and B neurons, whereas only field A neuron showed a direct NMNDA effect. (B) Mean duration of successive ictal discharges evoked by repetitive double NMNDA puffs expressed as relative values with respect to the first ictal episode. (C) Number of responsive neurons and mean Ca\textsuperscript{2+} change after repetitive double NMNDA puffs applied in the presence of TTX (n = 3). Data are expressed as percentage of the first response. Found at: doi:10.1371/journal.pbio.0000352.e004 (0.53 MB TIF)

**Figure 85** Spatiotemporal profile of the Ca\textsuperscript{2+} signal in neurons and astrocytes during a focal ictal discharge. (A) Pseudocolor raster plots showing the time derivative of the smoothed fluorescence signal (by averaging over five points) for individual neurons and astrocytes from the region within 200 \mu m of the NMNDA pipette tip (field A) and from the surrounding region (field B) during an ictal discharge evoked by a double NMNDA pulse applied at t = 0. If the activation were propagating as a concentric wave originating at the focus, the time of ictal onset in each cell would increase proportionally with the distance from the focus. Therefore, the raster plots would show a diagonal band of activation. Instead, the development and spreading of the ictal discharge is more similar to a process of modular recruitment of groups of neurons (and astrocytes): cells in the field A enter in the ictal phase more or less simultaneously. Cells in field B are recruited simultaneously, but at a later time than cells at the focal site of activation. (B) Bar graphs showing the distribution of the Ca\textsuperscript{2+} elevation onset in neurons and astrocytes. The onset of the Ca\textsuperscript{2+} signal for each neuron was defined as the time of the absolute maximum derivative value (that better reflects the large Ca\textsuperscript{2+} rise of the recruitment of neurons into the ictal discharge), whereas for astrocytes, it was the time of the first local maximum derivative value (that reflects the initial Ca\textsuperscript{2+} rise in these cells). Found at: doi:10.1371/journal.pbio.0000352.e005 (1.76 MB TIF)

**Figure 86** BAPTA-containing astrocyte syncytium in field B does not impair ictal discharge generation. The introduction of BAPTA into astrocytes from a sector of field B does not impair either the generation of the ictal discharge in field A or the engagement of distant neurons from fields B and C into the ictal discharge. Note, however, that in the BAPTA-containing regions, field B astrocytes are still activated by the ictal discharge, although their Ca\textsuperscript{2+} response has reduced amplitude and slower kinetics. Found at: doi:10.1371/journal.pbio.0000352.e006 (0.27 MB TIF)

**Figure 87** High-potassium stimulation as a tool for cell classification in brain slices. (A) Pseudocolor image from an EC slice loaded with OGB1-AM. Scale bar represents 30 \mu m. (B) Ca\textsuperscript{2+} signal from the eight cells indicated in (A) during an ictal event (in the 4-AP model [left traces]) and during the perfusion with a 40 mM K\textsuperscript{+} solution in 1 \mu M TTX (right traces). TTX was perfused for 5 min to block the epileptic activity before perfusion with the high K\textsuperscript{+} solution. Note that presumed neurons, i.e., large cells 1-4, in response to high K\textsuperscript{+} displayed a Ca\textsuperscript{2+} elevation largely before that of the presumed astrocytes, i.e., small cells 5-8. This delayed Ca\textsuperscript{2+} elevation is due to the lack of voltage-dependent Ca\textsuperscript{2+} channels in these cells. (C) Pseudocolor image from an EC slice loaded with OGB1-AM from a different experiment on the picrotoxin/zero-Mg\textsuperscript{2+} model. Scale bar represents 20 \mu m. (D) Ca\textsuperscript{2+} signal from the eight cells indicated in (C) during an ictal event that arose spontaneously (left traces) and during the perfusion with a 40 mM K\textsuperscript{+} solution and TTX (1 \mu M, right traces). Note that similar to what observed in (B), presumed astrocytes, i.e., small cells 5-8, displayed a delayed Ca\textsuperscript{2+} response to the high-potassium stimulation with respect to that in the large cells, presumed neurons. Note the high synchronous Ca\textsuperscript{2+} peaks in neurons from both experiments that reflect the afterdischarges of the seizure-like event. Found at: doi:10.1371/journal.pbio.0000352.e007 (1.80 MB TIF)

**Video 81** Response of astrocytes to ictal and interictal discharges in EC slices. This video shows the synchronous Ca\textsuperscript{2+} increase in EC neurons after slice perfusion with picrotoxin/zero-Mg\textsuperscript{2+} that reflects an interictal and an ictal discharge (as reported in Figure 8A and B). Note that at the beginning of the video there is an interictal discharge that activates only one astrocyte. A subsequent ictal discharge evokes a large and synchronous Ca\textsuperscript{2+} response in most astrocytes (indicated by the arrowheads in the right panels). In the final part of the video, synchronous, repetitive Ca\textsuperscript{2+} elevations in neurons reflect the afterdischarges typical of the late ictal phase. Time frame: 314 ms. Found at: doi:10.1371/journal.pbio.0000352.s001 (3.34 MB MOV)

**Video 82** Selective stimulation of astrocytes with TFFLR triggers an ictal discharge. This video shows the Ca\textsuperscript{2+} change in neurons and in astrocytes (indicated in the first frame by the orange arrowheads) evoked by a local application of TFFLR in an EC slice during perfusion with picrotoxin/zero-Mg\textsuperscript{2+} (as reported in Figure 8F). TFFLR induces a Ca\textsuperscript{2+} increase in astrocytes that is followed by an ictal discharge. Time frame, 1 s. Found at: doi:10.1371/journal.pbio.0000352.s002 (5.49 MB MOV)
Video 83 A single NMDA stimulation triggers only a local Ca²⁺ response. This video shows the Ca²⁺ increase that is restricted to a group of layer V-IV EC neurons that follows a single NMDA stimulation (as reported in Figure 4). The orange circle marks the timing of the NMDA puff and the position of the NMDA pipette. Note the absence of astrocyte responses. Time frame: 356 ms. Found at: doi:10.1371/journal.pbio.0003522.s010 (0.78 MB MOV)

Video 84 Two successive NMDA applications trigger an ictal discharge. This video shows an ictal event generated by an NMDA stimulation composed of two successive puffs (as reported in Figure 4). The orange circle in field A marks the timing of the NMDA puff and the position of the NMDA pipette, whereas the yellow circle in field B marks the engagement of neurons from this region in the ictal discharge. The orange arrowsheads indicate astrocytes in field A at the onset of their Ca²⁺ increase. Note that Ca²⁺ elevations in these astrocytes accompany the development of the ictal discharge. Yellow arrowsheads indicate astrocytes in field B (at the onset of their Ca²⁺ increase) that are activated after the emergence of the ictal discharge in field B. Time frame: 356 ms. Found at: doi:10.1371/journal.pbio.0003522.s011 (2.39 MB MOV)

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Author Contributions
The authors have made the following declarations about their contributions: Conceived and designed the experiments: MOV GC. Performed the experiments: MGG GL AC MZ MB PV LU MGC GRC. Analyzed the data: MGG GL AC MZ MC MB MGC GRC. Wrote the paper: TP MGC GRC GC.

References
Astrocyte Role in Isotogenesis


Computational model of neuron-astrocyte interactions during focal seizure generation

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INTRODUCTION

The intracellular Ca²⁺ elevations occurring in cultured astrocytes in response to a glutamate challenge (Cornell-Bell et al., 1990) was the initial observation that hinted at the existence of a form of excitability in astrocytes based on cytosolic Ca²⁺ concentration changes. A few years later, Ca²⁺ elevations in astrocytes from both cell cultures (Parpura et al., 1994) and brain slices (Paxi et al., 1997) were observed to result in Ca²⁺ increases in nearby neurons mediated by astrocytic glutamate. Considering that astrocytes occupy non-overlapping spatial territories (Busch et al., 2002; Hallwa et al., 2007) and that the processes of a single astrocyte can contact hundreds of synapses (Ventura and Harris, 1999), it was suggested that astrocyte-to-neuron communication may play a fundamental functional role in the brain. It was also found that astrocytes establish extensive contacts with cerebral blood vessels (Simard et al., 2003), which added further complexity to the functional role of neuron-to-astrocyte signaling. This neuron-astrocyte blood-vessel signaling pathway was revealed to be central in neurovascular coupling, the process by which episodes of intense neuronal activity at restricted brain regions trigger local increases in cerebral blood flow to satisfy the energy demand of active neurons (Zonts et al., 2003; Mulligan and MacVicar, 2004; Gordon et al., 2008).

These pioneering results lead to the idea that astrocytes and neurons establish a bidirectional communication in the brain which may play fundamental roles in the modulation of synaptic transmission and plasticity (Carmignoto, 2005; Hayden, 2001).

Over the last decade numerous studies provided evidence for the ability of astrocytes to listen and talk to the synapse by exerting both excitatory and inhibitory actions on neurons (Arache et al., 1999; Braddock and Deitmer, 2002; Zhang et al., 2003; Pascual et al., 2005; Passier et al., 2006; Serrace et al., 2006; Joudain et al., 2007; Perez and Arache, 2007). These studies revolutionized our view of how the brain works. The processing of sensory information in the brain, which has been for many years considered to be based exclusively on neuronal communication, is now viewed as a product of the dynamic signals that neurons and astrocytes constantly exchange in the brain network.
Such a bidirectional communication between neurons and astrocytes was conceptualized in the tripartite synapse in which the astrocyte composes with the pre-synaptic terminal and the post-synaptic target neuron, a third functional element of the synapse (Anique et al., 1999; Carrington, 2000; Halassa et al., 2007a; Peskind et al., 2009).

The discovery that astrocytes are crucially involved in normal brain function raised the intriguing possibility that these cells may be involved also in brain disorders. The observation that glutamate released by astrocytes evokes episodes of synchronous activity in small groups of nearby neurons (Fellin et al., 2004; 2006), was the first clue suggesting that glutamatergic astrocytes may represent a relevant non-neuronal mechanism for neural synchrony, which may ultimately favor the generation of focal epileptiform activity (Kang et al., 2005; Tian et al., 2009). A new experimental protocol was recently developed by our group in rat entorhinal cortex (EC) slices in order to reproduce the spatial and temporal features of focal epileptiform discharges (Gómez-Gonzalo et al., 2010; Losi et al., 2010). In this model, a pharmacological stimulation of neurons from a restricted cortical region induces a propagating seizure-like ictal discharge (ID). The ability to emulate an epileptogenic focus allows us to study the early cellular events that take place during the generation of epileptiform activity as it arises at a focal site and propagates to the surrounding brain tissue. By using this experimental protocol we recently provided evidence that neurons engage astrocytes into an excitatory loop that pushes the neuronal network toward the ID generation threshold (Gómez-Gonzalo et al., 2010).

There are currently many computational models of seizures generation, development and cessation (Piklás et al., 2006). The level of description ranges from mean field models (Wendling et al., 2002; Stiefczyński et al., 2004) to biophysically detailed models (Destexhe, 1998; Bauchere et al., 2004; Trinh et al., 2005). We used here a simplified approximation in the description of the dynamics of single neurons and astrocytes. With this simplified dynamics we implemented a computational network model that allowed us to investigate the network mechanisms of focal ID generation and the role of astrocytes at the onset of the ID.

We found that the beneficial feedback provided by the astrocytes influences the dynamics of the system and favors the generation of epileptiform activities. The computational model quantitatively reproduces the spatial and temporal features of ID generation and propagation and provides mechanistic insights into the astrocyte contribution.

METHODS

NEURON MODEL

The computational model aims to reproduce the behavior of a brain network that in response to NMDA pulse stimulation generates a focal ID (Losi et al., 2010). The network consists of 320 excitatory and 80 inhibitory neurons randomly disposed and synaptically connected in a 2D configuration. As in our previous work (Reato et al., 2010a), we used Izhikevich’s model (Izhikevich, 2003) to describe the dynamics of single neurons. Briefly, the voltage dynamics of single neurons is characterized by four parameters a, b, c, d as follows:

\[
\frac{dv}{dt} = 0.04 v^2 + 5 v + 140 - u + I = f(v, u) + I
\]

\[
\frac{du}{dt} = (\beta v - u)
\]

With a reset of the dynamic variables u, v when a spike is generated:

\[
\begin{align*}
    \text{if } \nu \geq 50 \text{ mV, then} & \\
    v & \leftarrow c \\
    u & \leftarrow u + d
\end{align*}
\]

The choice of values for the four parameters defines different spiking behaviors. The parameters were chosen to reproduce the behavior of a regular spiking neuron for excitatory neurons (a = 0.02, b = 0.2, c = −65, d = 10) and of a fast spiking neuron for inhibitory neurons (a = 0.2, b = 0.26, c = −65, d = 0.5). The variable I represents the sum of the synaptic current and the external stimulation.

The synaptic currents mimic AMPA, NMDA, GABA_A and GABA_B receptor activation following (Izhikevich and Edelstein, 2009). Briefly, the synaptic conductances are described by first-order linear kinetics,

\[
\tau_g \frac{dg}{dt} = \alpha g + \gamma (g - g_n)
\]

(1)

where \( \gamma \) is the reversal potential for glutamate, \( g_n \) is the resting conductance, \( \alpha \) is the receptor sensitivity constant, \( \beta \) is the receptor desensitization constant, and \( \gamma \) is the conductance. Every time a pre-synaptic neuron fires an action potential the conductance of the post-synaptic neuron increases instantaneously by \( \gamma \) for the duration of the action potential (Becker et al., 2006). The ratio of NMDA to AMPA receptors was set to be uniform at a value of 2, while GABA_A to GABA_B equal to 0.34 (Izhikevich, 2009).

The synaptic current of a post-synaptic neuron is then given by:

\[
I_{syn} = I_{syn}^e + I_{syn}^i
\]

\[
I_{syn}^e = g_{AMPA} (v_{syn} - \nu)
\]

\[
+ g_{NMDA} \frac{(v + 80)/60)^2}{1 + (v + 80)/60} (v_{syn} - \nu)
\]

(3)

\[
I_{syn}^i = g_{GABA}\frac{(v_{syn} - \nu) + g_{GABA_B} (v_{syn} - \nu)}
\]

Where \( \nu \) (function of time, Equation 1) is the voltage of the post-synaptic neuron and \( v_{syn} \) and \( v_{syn}^i \) are the reversal potentials for excitatory and inhibitory synapses. Here we chose \( v_{syn}^i = −90 \text{ mV}, v_{syn} = 0 \text{ mV} \). Each neuron receives excitatory inputs from a square of maximum 30 neighbors, while inhibition from maximum eight neurons. Using these parameters a single excitatory pre-synaptic spike induces a depolarization of maximum ~0.1 mV, while an inhibitory pre-synaptic spike leads to maximum ~0.5 mV hyperpolarization. All the main parameters of the simulations (the a, b, c, d parameters describing the dynamics of single neurons for both excitatory and inhibitory neurons and the s parameters for synaptic connections) were selected from a normal distribution with standard deviation equal to 1% of the average value. To mimic the onset of an ID, a few parameters of the network were chosen in order to place the network in a hyperexcitable state. The
excitability of excitatory neurons was slightly increased by injecting depolarizing currents (amplitude equal to 2), that could mimic the effects of 4-AP (a K⁺ channel blocker) used in the slice preparation. The high values chosen for both the conductance and the time constant of NMMA currents aim to reproduce the low Mg²⁺ experimental conditions. Without stimulation, both excitatory and inhibitory neurons are completely silent.

The NMMA stimulation that in experimental slice preparations evoked an ID was simulated in the model by depolarizing a set of neurons within a 7 x 7 square area above threshold (500 ms 60 neurons). We refer to this as a stimulated pulse (SimP). Alternatively, the NMMA pulses could have been simulated by activating NMMA currents. However, since we are interested in analyzing the effects on NMMA currents during the ID onset, this would have resulted in “stimulation artifacts” (the NMMA current induced by the pulse). Since we were also interested in studying the mechanisms leading to ID generation, the intensity of the stimulation was set to a value that not necessarily induced an ID in all the simulations (see Figure 3D).

In all simulations, nine SimPs were applied. In unsuccessful simulations, the average firing rate in the network increases during each SimP, but it suddenly drops to zero between successive SimPs. An ID was considered to be successfully generated when the firing rate in neurons remains sustained above 1 Hz. The ID onset was then defined as the number of SimPs which starts this process.

The cessation of the ID was obtained by a modification of the parameter b in a firing specific way. More specifically, we assumed that an elevated spiking activity decreases the excitability of single neurons. Possible physiological correlates of this event are the inactivation of Na⁺ channels (Bazhenov et al., 2004), the activation of Ca²⁺ or Na⁺ dependent K⁺ channels (Alger and Nicoll, 1980; Schwindk et al., 1989; Bazhenov et al., 2004; Timofeev et al., 2004) or the exhaustion of metabolic support (Yamada et al., 2001; Kirchner et al., 2000).

The equation used is:

\[
\frac{db}{dt} = -mR(t) + (b_0 - b)
\]

Where \( R(t) \) is the spike train of a single neuron low pass filtered (time constant equal to 150 s), \( m \) is the coupling constant between the spiking activity and \( b \) (chosen here to be 15), and \( b_0 \) the value of \( b \) in resting condition (no spiking activity). The second term in the equation can be thought as a driving force to recover the normal neuronal functionality of the neuron, for example the metabolic support.

Because of the hyperexcitability of the network, i.e., neurons are firing intensively at ID onset, we had to integrate Izhikevich’s equations with the method proposed in Izhikevich (2010) assuming the time step to be 1 ms:

\[
v(t + 1) = v(t) + \frac{f(v(t), u(t)) + g(t)R(t) + I}{1 + g(t)}
\]

Where \( E(t) = \sum (g(i)E_i)g(t) \) with \( g(t) = \sum g(i) \) (the total sum of conductances) and \( E_i = \text{exc}, \text{inh} \) for excitatory and inhibitory connections, respectively. This method is efficient and stable even for large synaptic currents.

“Excitation” refers to the sum of excitatory currents (AMPA and NMDA) averaged across neurons, and similarly “inhibition” refers to the average summed inhibitory currents (GABAA and GABAG). Excitatory and inhibitory firing rate indicate the firing rate of excitatory and inhibitory neurons, respectively. Where otherwise indicated, excitation, inhibition and firing rates of single simulations were always filtered with a moving average filter using a 50 ms time window for better visualizations. Postictal refractory period was estimated as the time between the end of the seizure (average firing rate back to zero) and the time at which the \( b \) variable recovers to the 95% of the initial value.

Under the conditions described above, our computational model is able to generate a neuronal network activity which resembles several characteristics of experimental focal IDs (see later in the “Results”):

1. (i) the simulated ID originates from a small number of neurons in the network and propagates outside the focal area with a delay (Trabucco et al., 1982; Avidon et al., 2002);
2. (ii) the simulated ID arises from an imbalance between inhibitory and excitatory activity at the focal area (Bradford, 1995; Rein Ai, 2002);
3. (iii) the simulated IDs have a cessation and a similar average duration (Jefferys, 1990; Trabucco et al., 1993; Pinto et al., 2005);
4. (iv) the network enters into a period of postictal refractoriness (Jefferys, 1990);
5. (v) the peak in the firing rate of the excitatory and inhibitory neurons during simulated IDs is compatible with that measured in the in vivo experimental models.

Our model failed to reproduce the bursting behavior which characterizes the firing discharges in individual neurons and the two main phases in ID development, i.e., the initial tonic and the delayed clonic activity. However, the main focus in this computational model was to include astrocytes in the neuronal network and gain insights into how these non-neuronal cells can affect the equilibrium between excitation and inhibition in the network.

ASTROCYTE MODEL

We introduce here a simple representation of astrocytes interacting with a neuronal network. The parameters related to the ability of astrocytes to respond to neuronal activity with cytosolic Ca²⁺ elevations were captured from results obtained in experiments performed both in brain slices (Posti et al., 1997; Porter and McCarthy, 1997) and in the living brain (Hirase et al., 2001; Wang et al., 2006; Kuga et al., 2011). To simulate the Ca²⁺ dynamics of a single astrocyte we used a model similar to the Izhikevich neuronal model. The equations represent a dynamical system of two variables \( \text{[Ca}^{2+}\text{]} \) and \( q \), without non-linear action potential or reset. The set of equation describing the Ca²⁺ concentration
has the following form:

\[
\frac{d (\text{Ca}^{2+})}{dt} = -\psi + \sum_j \gamma_j (\psi - \eta_j) \\
\frac{d \psi}{dt} = \alpha (\text{Ca}^{2+} - \psi)
\]

where \(\psi\) is a recovery variable and \(\psi_j\) is assumed here to be the neuronal input when an action potential is generated by the neuron \(j\), since astrocytes respond to neuronal releases of glutamate (Pasti et al., 1997; Porter and McCarthy, 1997). Although the equations are dimensionless, the values \(\psi_j\) where chosen to reproduce the pattern and amplitude of the Ca\(^{2+}\) elevations that are experimentally observed in astrocytes in response to neuronal activity (Porter and McCarthy, 1986; Pasti et al., 1997). The value of \(\psi\) was chosen as being normally distributed with mean 0.0003 and standard deviation equal to 1% of the mean. Ca\(^{2+}\) concentration was restricted to be non-negative. Similarly to the dynamics described by the Hodgkin-Huxley's single neuron, different values of \(\alpha\) and \(\beta\) determine different behaviors (time constant of changes and coupling with the recovery variable). Here we chose \(\alpha = 0.001\) and \(\beta = 0.01\). This choice was made to reproduce the slow time course of Ca\(^{2+}\) changes in astrocytes (Kasai et al., 1996). When astrocytes were included in the whole network, these values were chosen to be normally distributed with a standard deviation equal to 1% of the mean.

To describe the release of astrocytic glutamate triggered by Ca\(^{2+}\) elevations, we considered a first order dynamics (low pass filters), with a release of glutamate that can occur only when Ca\(^{2+}\) reach a threshold (Pasti et al., 1997; Puspura and Hayden, 2000; Pasti et al., 2001):

\[
\frac{d [\text{glu}]}{dt} = \begin{cases} 
- [\text{glu}] + [\text{Ca}^{2+}] & \text{if } [\text{Ca}^{2+}] < [\text{Ca}^{2+}]_{\text{th}} \\
- [\text{glu}] - \kappa \psi & \text{otherwise}
\end{cases}
\]

\[
\frac{d \psi}{dt} = -\lambda + [\text{glu}]
\]

Where \([\text{Ca}^{2+}]_{\text{th}} = 0.0018\) mM is the threshold for glutamate release, \(\kappa = 200\) describes the coupling between the glutamate concentration \([\text{glu}]\) and the recovery variable \(\psi\). Glutamate concentration was imposed to be non-negative. The time constants for the two variables were \(\mu = 0.5\) s and \(\eta = 10\) s. The value of \([\text{Ca}^{2+}]_{\text{th}}\) was set based on available data showing that an increase in astrocytic Ca\(^{2+}\) of a few hundreds of nM was able to trigger glutamate release (Puspura and Hayden, 2000). Assuming a value of 200 nM for a single synapse (Nadkarni and Jung, 2002) and considering that astrocytes in our model receive inputs from a maximum of nine neurons, the threshold value can be determined by multiplying the threshold for the single synapse by the number of inputs, as considered in other studies (Wade et al., 2011).

The set of parameters used for a single astrocyte reproduces basic features of Ca\(^{2+}\) dynamics and glutamate release in astrocytes. Increasing the input to an astrocyte, simulated as Poisson-distributed spike trains of increasing frequencies, leads to increasing Ca\(^{2+}\) concentrations (Figures 1A–1M). The Ca\(^{2+}\) increase due to a single spike is less than 100 nM and lasts for about half a second (inset in Figure 1A). These results are compatible with experimental evidences (Pasti et al., 1997; James et al., 2011) and previous computational models (Jefferys, 1990; Nadkarni and Jung, 2002, 2007; Wade et al., 2011). The linear dependence of Ca\(^{2+}\) increase as a function of simulated firing rate is reported in Figure 1B. Increasing the number of inputs by summing up Poisson-distributed spike trains (color scale from red to blue) also elevated Ca\(^{2+}\) concentrations. Since the release of glutamate, due to the Ca\(^{2+}\) increases occurs only when Ca\(^{2+}\) is above a threshold, only strong activation can drive the release. As an example, nine spike trains at 10 Hz induced transient releases of glutamate (Figures 1C, 1D, Ca\(^{2+}\) threshold in red). Figure 1D summarizes the dependence of glutamate released by the astrocyte as a function of the firing rate and the number of inputs. For very low firing rates, there is no astrocytic glutamate release independent on the number of inputs. In case of high firing rates, the release is linearly dependent on both the number of inputs and the firing rate. To further validate the parameters that we choose, we stimulated single astrocytes with a spike train from nine neurons from a simulated 1D (see later in the ‘Results’). The neuronal activity leads to Ca\(^{2+}\) increases in the astrocyte (Figure 1E) that caused a glutamate release (Figure 1F) only after the second pulse (see also below). Interestingly, when the 1D was fully developed, Ca\(^{2+}\) elevations reached a steady state value and glutamate was no longer released. It is known from experiments in culture and in brain slices (Pasti et al., 1997; James et al., 2011) that upon intense stimulation the Ca\(^{2+}\) level in astrocytes increases rapidly and remains at an elevated steady-state value for tens of seconds (Figure 1E1). A single episode of glutamate release is experimentally observed only after the initial Ca\(^{2+}\) rise.

Astrocytes were included in the network with a 1:1 ratio with neurons. The ratio of glia to neurons increases in phylogenesis and is 1.65 in the human frontal cortex (Oberheim et al., 2006; Sherwood et al., 2006). Given that astrocytes account for about 50% of the total number of glial cells, a 1:1 ratio seems to represent an acceptable approximation. The input from neuronal activity, \(\theta\) for each neuron, was considered as the excitatory input from neurons firing rate \(\text{pm}(m)\) (the excitatory component) in a 3 x 3 square inputs from nine excitatory neurons). This choice was made considering that the feedback of astrocytes on neuronal activity is thought to be local with four to eight neuronal somata enveloped by a single astrocyte (Halassa et al., 2007b), but with the processes from a single astrocyte associated with up to 600 dendrites and many thousands of synapses (Bushong et al., 2002; Oberheim et al., 2006). The glutamate released by astrocytes was used as input to the same neurons to which the astrocyte is exposed. This glutamate generated NMDA currents in these neurons by activating the NMDA channel (in the same way than \(\text{pm}(m)\)). In some simulations (see ‘Results’) we considered the effects of inhibitory inputs from astrocytes. This was done by considering that the astrocytic response at the GABA receptors instead of NMDA (so simulating the effect of GABA release).
All the simulations were performed using MATLAB (Mathworks), and the code is available at www.neuralengr.com/code.

RESULTS
FOCAL ID GENERATION IN ENTORHINAL CORTEX SLICES
As we previously reported (Losi et al., 2010), an episode of neuronal hyperactivity can generate a focal ID in EC slices perfused with the K⁺ channel blocker 4-aminopyridine (4-AP) and low Mg²⁺. Figure 2 illustrates a typical ID that was generated in cortical layer V-VI by a double brief pressure pulse applied to an NMDA-containing glass pipette (Figures 2A,B).

Dual patch-clamp recordings revealed that the firing in neurons located within the focus (Figure 2C, neuron 1) evolved into a focal ID with some delay after the NMDA double pulse. Following the ID generation at the focus (Figure 2A, gray circle), neurons outside the focus (~400 μm from the NMDA pipette tip) were also recruited and exhibited a similar pattern of action potential firing (Figure 2C, neuron 2). Given that the somatic Ca²⁺ change in neurons reflects faithfully the action potential firing in these cells, in slices loaded with the Ca²⁺ sensitive dye Oregon Green BAPTA1-AM (OGB-1-AM) we could monitor the activity of tens of neurons and follow how a focal ID is generated in the neuronal network. These experiments revealed that the NMDA stimulation...
evoked a rapid Ca\(^{2+}\) elevation in neurons located within the focal area, while neurons from the surrounding network were recruited into the ID only after a delay of 10.9 ± 0.8 s (30 IDs from 15 slices).

FOCAL ID GENERATION IN THE NEURONAL NETWORK MODEL

In the model we first examined how the neuronal network responds to a sequence of simulated NMDA pulses in the absence of astrocytes. To mimic the NMDA pulses at the focus, a depolarizing current pulse was injected for 500 ms in an area of 7 × 7 neurons (see “Methods”). The first SimP evoked robust spiking activity that remained restricted to neurons of the focus (Figure 3A1). Upon successive SimPs the firing activity spread from the focus to the surrounding neurons approximately 10 s after the SimP onset (Figures 3A1–A4, B1). The neuronal firing discharge remained high thereafter for tens of seconds (61 ± 2 s) before a sudden cessation (Figure 3B1). A postictal refractory period was observed with an average duration of 266 ± 1 s (see “Methods”). This pattern of activity resembles the focally evoked ID in slice preparations (see Figure 2D). A raster plot of the activity in a subpopulation of excitatory and inhibitory neurons within and outside the focus revealed that inhibitory neurons fire more intensively as compared to excitatory neurons during the SimPs, while the spiking activity in excitatory neurons increases with successive SimPs (Figure 3B2). The peak of the activity in the whole network was reached
during the ID (Figure 3B3) and its spectrogram clearly revealed two main components corresponding to the different activity in excitatory and inhibitory neurons that fire at about 15 and 60 Hz, respectively (Figure 3B4). This pattern of activity in the two neuronal populations is consistent with experimental observations (Ziburkus et al., 2006). While both excitatory and inhibitory neurons at the focus were activated upon the initial stimulation (representative traces in Figures 3C1,C2), neurons outside the focus were recruited into the propagating ID with some delay (representative traces in Figures 3C3,C4).
ID GENERATION THRESHOLD

We consider an ID threshold the number of NMDA pulses that are needed to evoke an ID. This value is constant for a given slice (Gomez-Gonzalez et al., 2010), but it can vary for different slices. Simulations with different parameters (see "Methods") showed that an ID could be generated in average by five SimPs and in more than 25% of cases no IDs could be evoked regardless the number of applied SimPs (Figure 3D, n = 250 simulations). The successive SimPs induced excitatory responses at the focus with increasing amplitude (Figure 3E1). The excitatory and inhibitory neurons outside the focus were not directly activated by the SimPs and induced their firing activity simultaneously, but with a marked delay (Figures 3E1, 3E2).

DYNAMICS OF EXCITATION AND INHIBITION AT THE FOCUS EXPLAIN ID GENERATION

We next investigated the interplay between excitation and inhibition in the genesis of the ID. We compared the simulations which successfully evoked an ID with those that failed to evoke an ID (in the different simulations excitatory and inhibitory neurons were randomly located within or outside the focus while maintaining their total number). For the cases in which an ID was successfully generated, we find that the ratios between the number of excitatory and inhibitory neurons, the average inhibitory and excitatory currents during the first SimP and the firing rate of inhibitory and excitatory neurons in the same time interval were lower compared to cases where an ID was not successfully generated (Figures 4A1–A3).

We next tested whether a different strength in either excitation or inhibition at the focus changed the efficacy of the SimPs in generating an ID. We analyzed the time course of excitation and inhibition at the focus and the average firing rate in the whole network. We examined three sets of network parameters chosen at random, but leading to different ID thresholds, i.e., no ID generation, high ID threshold (five SimPs) and low ID threshold (three SimPs) (Figures 4B–D respectively). In all cases, excitatory and inhibitory drive increased the firing rate (Figures 4B2.B3, C2, C3, D2, D3). A detailed analysis of the dynamics revealed that after each SimP both excitation and inhibition were strongly but transiently activated (Figures 4B1, C1, D1). An important difference is that, in contrast to inhibition, excitation failed to recover the initial basal conditions, including the simulations in which no ID is generated (Figure 4B). An additional striking difference between the three examples is the maximal inhibition level provided by the inhibitory neurons (the dynamic range). Inhibition reached its highest value in the high ID threshold condition. These results support the view that inhibition strength is a critical factor for local ID onset. Notably, excitation rose faster than inhibition (slope > 1) driving the growth in firing rate forward. However, the ID occurred only after inhibition reached its maximal value (all inhibitory neurons were active). Therefore the ratio of excitatory versus inhibitory drive and the limiting dynamic range of inhibition are the two critical factors in ID generation. As a summary of results obtained, we report the distribution of points in the excitation-inhibition plane at the focus during the first seven SimPs in 250 Monte-Carlo simulations for the cases that evoked or failed to evoke an ID (normalized by the total area Figures 4E1, 4E2). When inhibition at the focus reached high values, no IDs were generated and the ratio between excitation and inhibition remained low. This stands in contrast to the cases which lead to IDs, further supporting the notion that the relationship between excitation and inhibition determines not only the threshold for ID generation, but also whether or not an ID could be evoked. Data obtained from 250 runs also showed a clear correlation between the ID threshold and the average excitation and inhibition in the network during the first SimP (Figures 4F1, 4F2). This indicates that the overall response of the network, in terms of excitation and inhibition levels, is a good predictor of ID thresholds: an increased excitation results in the lowering of the ID threshold and an opposite relationship holds for inhibition.

ASTROCYTE-TO-NEURON SIGNALING DECREASES THE ID THRESHOLD

The model of the single astrocyte (see "Methods") was incorporated into the network to test how astrocytes may affect ID threshold. Specifically, 400 astrocytes were added to the network model in a parallel 2D sheet of cells (see "Methods"). Astrocytes provide an excitatory feedback to neuronal activity in a Ca^{2+}-dependent way (Figure 5A). As illustrated in Figure 5B, in the presence of the astrocyte feedback signal, the ID was evoked by two SimPs, while in its absence a more intense stimulation of neurons was necessary. As illustrated in Figures 5F1, 5F2 the Ca^{2+} change from a representative astrocyte at the focus was observed to follow rapidly the spiking activity in neurons (Figures 5C1, 5C1), and astrocytic glutamate release occurred upon the second SimP (Figure 5C2). The average astrocytic Ca^{2+} follow the neuronal activity (example in Figure 5D1) while the average glutamate release occurs transiently (Figure 5D2). The Ca^{2+} change and the release of glutamate from astrocytes outside the focus failed to affect local ID threshold. The results from 250 Monte-Carlo runs show that the ID threshold was lowered after including the astrocytic feedback signal to neurons (Figure 5E). Once the ID was fully evoked, both the Ca^{2+} change and the release of glutamate from astrocytes within and outside of the focus did not differ significantly (Figures 5F1, 5F2). However, the initially dominant activity of astrocytes at the focus was followed by an activity of the astrocytes outside the focus that became dominant immediately after the ID onset. These results are consistent with those from slice experiments which showed that when Ca^{2+} elevation in astrocytes from the focus were selectively blocked (by the Ca^{2+} chelator BAPTA) or stimulated (by TFLK, a peptide agonist of thrombin PAR-1 receptors), the threshold of ID generation increased or decreased, respectively (Gomez-Gonzalez et al., 2010).

DOES AN ASTROCYTE INHIBITORY FEEDBACK SIGNAL TO NEURONS AFFECT ID THRESHOLD?

The ID threshold is mainly affected by the interplay between excitation and inhibition. Indeed, as we reported above, ID threshold can be increased by increasing the overall value of the inhibitory activity. The bar graph in Figure 6A reports the results from 250 simulations in different simulation settings, with and without an astrocytic contribution (mean and error represents repeated a Poisson fit to the ID threshold distributions), while Figure 6B
is the cumulative sum of the ID threshold distributions corresponding to the analyzed cases. Blue and red bars show that the ID threshold can be increased by increasing the overall strength of inhibitory connections (in this case from 0.01 to 0.015) in a purely neuronal network (no astrocytes). With higher inhibition, the simulated stimulation failed to induce an ID in 40% of simulations (Figure 6B). As already shown, the introduction of an astrocytic excitatory feedback lowers the ID threshold (green...
and decreases the number of failures to about 10%. In slice experiments we observed that the inhibition of Ca\(^{2+}\) signals in astrocytes at the focus, but not outside the focus, increased the threshold of ID generation. These observations were fully reproduced in the computational model (dark blue and magenta bars) without further manipulations of the model over the results from the previous section. Astrocytic excitatory feedback in a network with stronger inhibitory connections (0.015 as for the red bar) brings back to baseline the ID threshold (yellow).

To explore other factors that may affect ID threshold, we considered the possibility that the activation of astrocytes, or of a subpopulation of astrocytes, results in a release of GABA that can lead to an overall increase of the inhibition strength in the neuronal network. Astrocytes can, indeed, release GABA.
As expected, when we included an astrocytic GABA release in the model, the threshold for ID generation increased (Figure 6, dark green). Note that this was an inhibitory-only feedback involving only GABA release and no glutamate. Surprisingly, however, the threshold for ID generation did not rise over the baseline condition with no astrocytic feedback (blue). A possible explanation for this could be the synchronizing action of an inhibitory GABA signal. Alternatively, the inhibitory feedback signal from astrocytes could be more effective in suppressing inhibitory than excitatory neurons. This action may ultimately generate a new level of complexity in the mechanism that governs the inhibition/excitation balance in the neuron-astrocyte network.

**DISCUSSION**

Increasing experimental evidence highlights the physiological significance of the tripartite synapse in which the astrocyte senses neurotransmitter release and, in turn, releases through a Ca\(^{2+}\)-dependent mechanism for neurotransmitters that have feedback modulatory actions on neurons. A number of recent studies in vitro and in vivo showed that the release of glutamate from astrocytes can, indeed, control both the basal excitability of neurons and some forms of long-term potentiation of synaptic strength (Serrano et al., 2006; Jourdain et al., 2007; Navarrete and Araque, 2010; Sautelle et al., 2011; Min and Nevian, 2012) and long-term depression (Zhang et al., 2003; Serrano et al., 2006; Han et al., 2012; Min and Nevian, 2012). The contribution of glutamate to brain dysfunctions remains, however, poorly understood. A model composed of a network of interacting neurons and astrocytes represents a useful tool in which the spatial-temporal features of focal seizure generation observed in slice models can be replicated and new mechanistic hypotheses can be tested.

Over the last 10 years, different models have been advanced to describe the Ca\(^{2+}\) dynamics of astrocytes in response to neuronal signals (Nadjari and Jung, 2003; 2007; Silchenko and Tass, 2008; Griffo, 2009). These biophysical approaches described not only the astrocytic Ca\(^{2+}\) response (Li and Rinzel, 1994), but also the possible feedback to neurons. More recently, the possible contribution of astrocytes in events related to the plasticity of synaptic transmission were also included in models (Nadjari et al., 2008; Fau Pits et al., 2013; Wu et al., 2011). While biophysical models are very useful to simulate basic units, like the tripartite synapse, they are hardly suitable for large scale simulations. In contrast, simplified models that include only the basic features of neuron-astrocyte interactions (Pitom et al., 2009) appear more appropriate to describe network dynamics and to investigate the role of astrocytes in epilepsy (Amiri et al., 2012).

In our model we did not include any distinct biophysical features that characterize the physiological actions of either neurons or astrocytes. We rather describe the activity of a single astrocyte in terms of the specific input-output signals with which astrocyte and neurons interact. This simplified astrocyte model was then embedded in a neuronal network model of IDs based on the Izhikevich’s single neuron model.

The slow kinetics of the astrocyte Ca\(^{2+}\) response to neuronal activity in the model reflect those of the mGluR-mediated Ca\(^{2+}\) elevations that were evoked in astrocytes by axonal afferent stimulation in young rat hippocampal slices (Porter and McCarthy, 1995; Pasti et al., 1997; Perea and Araque, 2005). Indeed, the intracellular Ca\(^{2+}\) variations in astrocytes depend primarily, although not exclusively, on activation of metabotropic receptors, phospholipase C-dependent inositol(1,4,5)-trisphosphate (IP3) production and, finally, stimulation of Ca\(^{2+}\) release from IP3-sensitive internal Ca\(^{2+}\) stores (Kawahata et al., 1996). Glutamate release at the synapse triggers a Ca\(^{2+}\) response in astrocytes that increases in both amplitude and frequency of oscillations according to increased levels of the neuronal activity (Pasti et al., 1997). These Ca\(^{2+}\) changes trigger a SNARE-dependent exocytosis of glutamate that signals back to affect the excitability of neurons (Araque et al., 2000; Pasti et al., 2001; Pambruna et al., 2004). Accordingly, in the model we reproduced the most essential features of glutamate release in response to Ca\(^{2+}\) elevations in astrocytes. The release of glutamate is pulsatile and depends on the frequency of Ca\(^{2+}\) oscillations (Pasti et al., 2001), while its
efficacy is controlled by the amplitude of the Ca2+ increase (Papadopoulos and Haydon, 2000). In addition, a steady state Ca2+ elevation may trigger only a single episode of release (Pasit et al., 2001).

Some approximations were applied to describe two features that characterize astrocyte signaling in our model. Firstly, we restricted our analysis to somatic Ca2+ signals. These Ca2+ increases cannot be intended to fully represent the synaptic to astrocyte signaling occurring functionally at the proximal and the distal processes that are in contact with the synapse. Indeed, somatic Ca2+ increases exhibit a lower frequency and slower kinetics with respect to those at the processes (Tan et al., 2011; Panzer et al., 2011). While these recent studies also showed that Ca2+ elevations at the astrocytic processes can have a distinct functional role, it is noteworthy that the Ca2+ elevation at the soma may represent a response that integrates the signals from the processes where astrocytes sense neurotransmitter release. Accordingly, Ca2+ signals at the soma may adequately reflect the overall firing activity of surrounding neurons. Amplitude, frequency and general pattern of somatic Ca2+ changes are, indeed, observed to vary among different levels of neuronal activity (Pasit et al., 1997; Porter and McCarthy, 1997). Secondly, while glutamate (Papapetropoulos et al., 1994; Pasit et al., 1997; Beccari et al., 1998) and D-serine (Mohter et al., 2003; Henningsen et al., 2010; ATP (Arciuc et al., 2002; Serrano et al., 2006; Bovier and Khakh, 2007), and GABA (Kozlov et al., 2006; Lee et al., 2011) [for a review see Haydon and Carmignoto (2006)] are the main astrocyte-to-neuron signaling pathways in our model we fundamentally focused on glutamate because a large body of information is available about its modulatory action on both central synaptic transmission (Fellin et al., 2004; Di Castro et al., 2011; Panzer et al., 2011) and long-term plasticity (Zhang et al., 2003; Panzer et al., 2006; Serrano et al., 2006; Joucla et al., 2010; Santello et al., 2011; Han et al., 2012; Min and Navarrete, 2012). In addition, the contribution of astrocytic glutamate in some forms of long-term potentiation of synaptic transmission has been recently confirmed in in vivo experiments (Takata et al., 2011; Navarrete et al., 2012). The potential role in focal seizure generation of ATP, D-serine and GABA will be the subject of future investigations. It is worth mentioning here that D-serine, and not glycine, is most likely the physiological co-agonist of the synaptic NMDA receptor in the brain (Mohter et al., 2003; Panzer et al., 2006; Fagg et al., 2012; Pagouli et al., 2012). Given that D-serine is mainly, although not exclusively (Ding et al., 2011), synthesized in astrocytes and released through a Ca2+-dependent mechanism (Welsher et al., 1999; Welsher, 2011), astrocytic D-serine may cooperate with glutamate to enhance NMDA receptor openings and through this action favor neuronal excitability ultimately promoting epileptic discharges.

The pathological increase in brain network excitability that eventually leads to focal seizure generation is believed to derive from the activity of excitatory and inhibitory neurons as well as of astrocytes. The cellular events that favor or oppose seizure initiation and propagation remain, however, poorly defined. Our model offers the opportunity to study ID generation in simulated networks composed of either only neurons or interactive astrocytes and neurons. The results that we obtained are summarized in Figure 6 and can be, in our opinion, useful to understand how distinct signaling pathways may govern focal ID generation. Figure 6 plots the average threshold for ID generation in different conditions (mean ± SD, Figure 6A) and the cumulative sum of the threshold histograms (Figure 6B) showing failures. We found that in a network composed exclusively of neurons an ID can be generated by applying an intense stimulation of a group of neurons. The introduction of astrocytes into the network lowered ID threshold, while the inhibition of astrocyte signaling to neurons within, but not outside the focus, increased ID threshold. These results are fully consistent with those obtained in slice experiments (Gomez-Gonzalez et al., 2010) and demonstrate that focal IDs can be faithfully reproduced in our computational model. Accordingly, our model can be used to make predictions on the distinct contribution of different signaling pathways to ID generation. We present here some results regarding inhibitory signaling pathways. The ID threshold was observed to increase upon procedures that increase the strength of inhibition onto the principal neurons. This was achieved by either increasing the strength of the inhibitory transmission or by including in the model a distinct inhibitory feedback signal from astrocytes to neurons via GABAA receptors. These observations will be useful when addressing in future slice experiments the role of inhibitory signaling in ID generation.

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Fast spiking interneuron control of seizure propagation in a cortical slice model of focal epilepsy

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Key points

• In focal epilepsy the propagation of seizure discharges arising at restricted brain sites is opposed by feedforward inhibition. Failure of this inhibition marks focal seizure propagation to distant neurons.
• The cellular source of inhibition and the mechanism of inhibition failure are, however, undefined.
• Here we reveal that a subclass of GABAergic interneurons, i.e. the parvalbumin-expressing, fast-spiking interneurons, are a main source of the inhibitory signal that locally restrains seizures. Furthermore, a firing impairment in these interneurons, probably due to a drastic membrane depolarization, is an important event that by reducing the overall strength of local inhibition allows seizures to propagate across the cortex.
• Our data suggest that modulation of fast-spiking interneuron activity may represent a new therapeutic strategy to prevent generalization of focal epilepsies.

Abstract In different animal models of focal epilepsy, seizure-like ictal discharge propagation is transiently opposed by feedforward inhibition. The specific cellular source of this signal and the mechanism by which inhibition ultimately becomes ineffective are, however, undefined. We used a brain slice model to study how focal ictal discharges that were repetitively evoked from the same site, and at precise times, propagate across the cortex. We used Ca²⁺ imaging and simultaneous single/dual cell recordings from pyramidal neurons (PyNs) and different classes of interneurons in rodents, including GABA and GIN transgenic mice expressing the green fluorescence protein in parvalbumin (PV)-fast spiking (FS) and somatostatin (SOM) interneurons, respectively. We found that these two classes of interneurons fired intensively shortly after ictal discharge generation at the focus. The inhibitory barrage that were recorded in PyNs occurred in coincidence with PV-FS, but not with SOM interneuron burst discharges. Furthermore, the strength of inhibitory barrage increased or decreased in parallel with increased or decreased firing in PV-FS interneurons but not in SOM interneurons. A firing impairment of PV-FS Interneurons caused by a membrane depolarization was found to precede ictal discharge onset in neighbouring pyramidal neurons. This event may account for the collapse of local inhibition that allows spatially defined clusters of PyNs to be recruited into propagating ictal discharges. Our study demonstrates that PV-FS interneurons are a major source of the inhibitory barrages that oppose ictal discharge propagation and raises the possibility that targeting PV-FS interneurons represents a new therapeutic strategy to prevent the generalization of human focal seizures.

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Introduction

Focal epilepsies arise at restricted brain sites of abnormally high neuronal network activities and secondarily involve adjacent regions, eventually spreading to distant neuronal populations (Jefferys, 1999; Traub et al. 1993). Over the last decade, experimental research in this field has made significant advances. We know now that various synaptic and non-synaptic factors contribute to form a hyperactive network that, in turn, promotes seizure initiation and favours seizure propagation (Stanton et al. 1987; Jones & Lambert, 1990; Fare et al. 1992; McNamara, 1990, 2000; Avoli et al. 2002). Among the synaptic factors, GABA mediated inhibitory synaptic transmission is proposed to control the pathogenesis and propagation of epileptic discharges by regulating the general excitability in the neuronal network. Consistent with this hypothesis, recent studies in occipital cortex slice preparations revealed that a powerful feedforward inhibition controls the propagation speed of epileptic discharges arising spontaneously in the low-Mg2+ model (Trevelyan et al. 2006, 2007). Inhibition ultimately fails and surrenders to the wave of excitation of the propagating seizure-like, ictal discharge (Trevelyan et al. 2006). The mechanism at the basis of this failure is, however, unclear.

The puzzle of adequate experimental models in which we can specifically investigate how a focal ictal discharge propagates across the neuronal network accounts, at least in part, for our defective knowledge. We recently developed in rat entorhinal cortex (EC) slices a new model of focal ictal discharge in which we can control when and where a focal epileptic discharge will occur (Gomez-Gonzalo et al. 2010; Losi et al. 2010). The precise information on the timing and site of ictal discharge generation gives us the unique opportunity to study in this model the delay in the propagation of focal ictal discharges to regions that are at increasing distances from the focus as well as the local cellular events that govern this delay by favouring or opposing the progression of the ictal discharge. Our most relevant aims were (i) to identify the cellular source of the inhibitory signal that transiently opposes focal ictal discharge propagation and (ii) to gain insights into the cellular events that, by reducing the strength of inhibition (Trevelyan et al. 2007; Duraschansky et al. 2008), cause the neuronal network to give up their resistance to the propagating focal seizure.

We used single and dual cell patch-clamp recordings from pyramidal neurons and interneurons coupled with simultaneous fast laser scanning microscopy imaging of the Ca2+ signal from tens of cells. By using, in addition, transgenic mice in which distinct subsets of interneurons are distinguished by the enhanced green fluorescence protein (GFP), we found that the inhibitory barrages opposing focal ictal discharge propagation were largely generated by local parvalbumin (PV)-fast-spiking (FS) interneurons and not by somatostatin (Som) interneurons. After PV-FS interneurons entered into a depolarization block phase, the local inhibitory barrier collapsed and spatially distinct groups of neurons were recruited into the epileptiform discharge.

Methods

All experimental procedures were in strict accordance with the Italian and EU regulations on animal welfare and had prior authorization from the Italian Ministry of Health. The experiments included in these studies comply with the policies and regulations described by Drummond (2009). The number of animals used in our study was reduced to the minimum necessary to allow an adequate statistical analysis.

Brain slice preparations and dye loading

Coronal cortico-hippocampal slices were prepared from postnatal day 14–20 Wistar rats or mice, i.e. O42 (Chattopadhyaya et al. 2004) and GIN mice (Oliva et al. 2009). Briefly, mice were deeply anesthetized with intraperitoneal-injected Zoletil (40 mg kg⁻¹; Virbac, Milan, Italy), a combination of a benzodiazepine-like molecule (zolazepam) and a dissociative anesthetic (etomidate). After decapitation, the brain was removed and transferred to ice-cold cutting solution containing (in mM): NaCl, 126; KCl, 3.2; KH₂PO₄, 1; NaHCO₃, 26; MgCl₂, 2; CaCl₂, 1; glucose, 10; sodium pyruvate, 2; and ascorbic acid, 0.6; at pH 7.4 (with 5% CO₂–95% O₂). Coronal slices were obtained by using a Leica vibratome VT1000S in the presence of the ionotropic glutamate receptor inhibitor kynurenic acid (2 mM). Slices were recovered for 15 min at 34°C and then loaded with either a green fluorescence dye (OGB1-AM or Fluo-4, 10 μM, excited at 488 nm) or a
red dye (Rhod-2, 10 μM excited at 543 nm) for 50–60 min at 34°C, as previously described (Gómez-Gonzalo et al. 2010).

Although Rhod-2 is known to accumulate in mitochondria (Kovacic et al. 2005) with this loading protocol, it was highly present in the cytosol. Dye loading was performed in the cutting solution containing sulfinpyrazone (200 μM), pluronic (0.12%) and l-lysine acid (1 mM). After loading, slices were recovered and kept at room temperature and experiments performed at 32°C.

**Ca²⁺ imaging**

Images were acquired with a single- (TCS-SP5-RS, Leica) and two-photon (UltimaV, Prairie Technologies) laser scanning microscope with a time frame acquisition from 351 ms to 491 ms (five to seven linear averaging), and from 300 to 900 ms, respectively. Both systems were equipped with a CCD camera for differential interference contrast images. All experiments were performed in layer V–VI. With two-photon excitation we could easily distinguish GFP-expressing cells in Fluo 4-loaded slices from G42 mice because two-photon excitation spectra of GFP and Fluo 4 differ significantly. The wavelengths used for Fluo 4 and GFP were 570 and 920 nm, respectively.

**Electrophysiology and induction of focal ictal discharges**

Brain slices were continuously perfused in a submerged chamber (Warner Instruments) at a rate of 3–4 ml min⁻¹ with (in mM): NaCl, 120; KCl, 5.2; KH₂PO₄, 1; NaH₂CO₃, 26; MgCl₂, 1; CaCl₂, 2; glucose, 10; at pH 7.4 (with 95% O₂, 5% CO₂). Whole-cell patch-clamp recordings were performed using standard procedures and one or two Axopatch-200B amplifiers or Multiclamp 700B (Molecular Devices, USA), as previously reported (Fellin & Carragiotto, 2004). Typical pipette resistance was 3–4 MΩ. Data were filtered at 1–2 kHz and sampled at 5–10 kHz with a Digidata 1320 or 1440 interface and pCLAMP10 software (Molecular Devices). The whole-cell intracellular pipette solution was (in mM): potassium gluconate, 145; MgCl₂, 1; EGTA, 0.5; Na₂ATP, 2; Na₂GTP, 0.2; Hapes, 10; to pH 7.2 with KOH and, when needed, contained a low concentration (10 μM) of QNB1 (Invitrogen); osmolality, 305–315 mosmol kg⁻¹. Liquid junction potentials for all solutions were measured, and all voltages reported are corrected values. Experiments were performed in the presence of 4-AP (50–100 μM) unless otherwise specified. Patched neurons were classified according to their response to hyperpolarizing and depolarizing 750 ms current steps. Neurons with no spike amplitude accommodation (except for the second action potential in some cells), small after-hyperpolarization (AHP) and low steady-state frequency (15–23 Hz with 200 pA current injection) were considered as regular-spiking pyramidal neurons (PyNs). Cells with high steady-state frequency (50–60 Hz with 200 pA current injection), spike amplitude accommodation or frequency adaptation and large AHPs were considered as FS interneurons; cells with strong spike amplitude accommodation and spike frequency adaptation were considered as adapting non-pyramidal (ANP) interneurons; cells with clear sign, one or more rebound action potentials, spike amplitude accommodation and frequency adaptation were considered as low-threshold (LTS) interneurons; cells with bursts of spikes with irregular frequency were considered as irregular-spiking (IS) interneurons. In GIN mice we discarded cells that did not show the typical features of LTS interneurons. All patched neurons in rat and mouse slices, i.e. principal PyNs and different classes of interneurons, were from cortical layer V–VI. Juxtaposed recordings were performed in cell-attached mode, without rupture of the membrane patch, with pipettes filled with the bath perfusing solution. A pressure ejection unit (PDE5, NPI Electronics) was used to apply a double pulse to NMDA (1 mM)–containing pipettes with a 3 s interval, a pressure of 4–10 p.s.i., and a duration of 200–600 ms. In the bicuculline experiments, three pressure pulses (4 p.s.i., 200 ms duration) with a 3 s time interval were applied to a 1 mM bicuculline-containing pipette. To test for a possible inhibitory synaptic connection between a PV–FS interneuron and PyN action potential firing at 50–70 Hz was stimulated in the PV–FS interneuron by current injections. In the pairs in which we applied this test (4 of 11 pairs) we failed to find evidence of a direct synaptic connection.

**Neuron reconstruction**

PV–FS interneurons and PyNs were patched with a bicuculline-containing (0.5%) pipette (Sigma-Aldrich, Italy). Potassium gluconate was reduced to achieve a final osmolarity of 275 mosmol kg⁻¹. Patched neurons were kept in whole-cell configuration for 20–30 min before gently detaching the pipette tip to preserve membrane integrity. Slices were then maintained in the standard oxygenated extracellular solution for 1 h to allow bicuculline intracellular diffusion and then fixed in 4% paraformaldehyde and 0.15 M phosphate buffer (PB) at 4°C for 1–4 days. Slices were then rinsed in PB and incubated in 1% hydrogen peroxide in PB for 5 min, permeabilized in 2% Triton X 100 for 1 h and kept overnight at 4°C in the avidin–biotin–peroxidase complex solution (RTU, Vectastain Kit PK 7100, Vector Laboratories, CA, USA). Slice were then placed in DAB-enhanced liquid substrate solution 1 × (Sigma Aldrich, D3959) for a few minutes.
until the slice turned light brown and then immediately transferred again to fresh PB to block the reaction. Each slice was mounted (Fluoromount mounting medium) and viewed with ×20 to ×40 objectives on a DMR light microscope (Leica, Wetzlar, Germany). Images at different focal plates were acquired by a Leica DC300 digital photocamera and then reconstructed using Neuroulida software (MicroBrightField). Single ×10 optical images of pyramidal and fast-spiking neurons were reconstructed by Adobe Photoshop CS5.

Data analysis
Data analysis was performed with Clampfit 10, Origin 8.0 (Microcal Software), Microsoft Office and MATLAB 7.6.0 (R2008A). In parallel recording experiments the increase in interneuron spiking activity was considered statistically significant when the instantaneous frequency, defined as the reciprocal of the interspike interval, exceeded by three standard deviations the mean of the instantaneous frequency after 4-AP slice perfusion. The relationship between inhibitory events in PyNs and the firing activity in PV-FS and Som-ETS interneurons reported in Figs 6 and 7 was studied comparing the charge transferred to a weighted frequency (\( f_w \)) in all the inhibitory barrages before transition from inhibition to excitation (\( t_{in} \)). The charge transferred was computed as the area of each inhibitory event and \( f_w \) was defined as:

\[
\hat{f}_w = \sum_{i=1}^{N} \frac{A_i}{t_{dur}(i)}
\]

where \( N \) is the number of action potentials within each inhibitory burst, \( A_i \) is the duration of the burst, \( A_i \) is the amplitude of each action potential and \( A \) is the mean amplitude of the action potentials of the same neuron before NMDA stimulation. \( f_w \) was calculated taking into account both action potential frequency and amplitude because a reduced action potential amplitude can affect neurotransmitter release by causing a failure in propagation through axonal branch points (Strett et al. 1992; Brody & Yue, 2000) and by reducing Ca\(^{2+}\) influx at synaptic terminals (Katz & Miledi, 1970; Nehér & Sakaba, 2008). Data were normalized from each experiment to the maximal values. A burst was classified as a train of action potentials superimposed on a depolarization with an instant frequency higher than the basal one. To estimate the timing of the period of depolarization block in PV-FS interneurons, we considered the first time in which, after NMDA stimulation, PV-FS interneuron firing activity was abolished for at least 400 ms in the presence of marked membrane depolarization.

Ca\(^{2+}\) imaging experiments. The Ca\(^{2+}\) signal is reported as \( \Delta F/F_0 \), where \( F_0 \) is the baseline fluorescence. Each row of the recruitment diagrams in Figs 1, 2 and 5 is the pseudocolour plot of the temporal derivative of the Ca\(^{2+}\) signals in neurons ordered by the time of the maximum time derivative which marked the recruitment of neurons into ictal discharges. No background subtraction or other manipulations were applied to digitized Ca\(^{2+}\) signal images that are reported as raw data. Ca\(^{2+}\) signals from neurons were obtained after the semi-automatic detection of regions of interest (ROIs) performed using a home-made algorithm written in MATLAB.

Definition of the timing of recruitment into propagating ictal discharges. We defined the timing of recruitment of each neuron as the timing of the maximum temporal derivative of the Ca\(^{2+}\) signal. In voltage-clamp recordings, illustrated in Figs 2, 6, 7 and 8, the recruitment of a neuron into ictal discharge is marked by the transition from predominantly inhibitory to the predominantly excitatory phase (\( t_{in} \)), which is defined as the timing in which the ratio between the inhibition and the excitation index is for the first time less than 0.1. The inhibition and the excitation indices were calculated, respectively, as the part of the positive and the negative component of the time derivative of the trace which exceeded 3 times the mean SD value evaluated during the baseline. Finally, the timing for the propagating ictal discharge in the current-clamp recordings was defined as the time of occurrence of the first excitatory burst with a duration of at least 400 ms. In Ca\(^{2+}\) imaging experiments performed in GF2 mice, we compared the timing of the activation of each GFP-expressing PV-FS interneuron (or GFP negative, early activated neurons) and the timing of the recruitment of PyNs belonging to the cluster in which each PV-FS interneuron was localized. In this way, we provide an average measurement of the delay between PV-FS interneuron activation and the recruitment of the surrounding PyNs.

Cluster size measurements. The radius of the clusters defined in Ca\(^{2+}\) imaging experiments was calculated as the maximum Euclidean distance between the cells belonging to the same subgroup and the centre of gravity of each group calculated as (\( X_{GC}, Y_{GC} \)) = \( \sum_{i=1}^{N} (x_i, y_i)/N \), where \( x_i \) and \( y_i \) are, respectively, the coordinates of each neuron in the cluster and \( N \) is the number of neurons in the cluster.

Drugs
4-Aminopyridine (4-AP; Ascent Scientific) was both applied. NMDA (Sigma-Aldrich) and bicuculline methiodide (Sigma Aldrich) were pressure applied.
**Statistical analysis**

Data are shown as mean ± standard error of the mean (SEM). We analysed quantitative results by Student’s t test, setting the statistical significance at *P* < 0.05. To statistically compare the distributions of the latencies between the timing of depolarization block in the different classes of interneurons and that of AP in PyNs, we applied the Fischer’s exact test to data binned into 300 ms groups (Fig. 6).

**Results**

**Propagation of ictal discharges from the focus to distant regions**

Current-clamp recordings from layer V–VI PyNs in rat EC slices revealed that in the presence of the procainamide 4-AP (50–100 μM) (Rutecki et al. 1987; Peresault & Ardu, 1989, 1991; but see Steer et al. 2011; Zahn et al. 2012) and 0.5 mm Mg²⁺, a local NMDA stimulation evokes a focal ictal discharge (mean duration, 32.9 ± 0.2 s, *n* = 89; Fig. 1A; Gomez-Gonzalez et al. 2010; Losi et al. 2010). As previously reported (Losi et al. 2010), spontaneous ictal discharges were only occasionally observed. The ictal discharge propagated to neurons surrounding the site of NMDA applications (Fig. 1A and B, grey circle) with a delay that increased significantly with distance from the focus (Fig. 1B and C). Previous studies reported a much faster propagation of seizure-like discharges upon 4-AP (Weissinger et al. 2005) or low Mg²⁺ slice perfusion (Bachheimer et al. 2000). These were, however, spontaneously occurring seizures and they were observed under experimental conditions that may guarantee a better preservation of synaptic connectivity (horizontal vs. coronal slices and interface chambers vs. submerged). The ictal discharge in neurons from regions located >700 μm from the focus was regularly preceded by a series of hyperpolarizing events (Fig. 1B). As revealed by the recruitment diagram (Fig. 1D, see also Methods), derived from the analysis of the Ca²⁺ change in mice brain with the Ca²⁺ sensitive slice Oregon Green BAPTA-1 AM (OG-01 AM; blue box in Fig. 1A), neurons from the region immediately surrounding the site of NMDA application were homogeneously recruited into the ictal discharge. In contrast, more distant neurons were recruited in spatially distinct domains at different times (Fig. 1E and F). These data suggest that ictal discharges spread across the EC through neither a rapid homogeneous process nor an avalanche-like wave, but rather through a process in which clusters of neurons are intermittently recruited.

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**Figure 1. Modular propagation of focally evoked ictal discharges**

A and B: current-clamp recordings from a PyN close (A) or distant (B) from the site where NMDA was applied to evoke an ictal discharge. In this and the other figures the black arrowheads mark the timing of the double NMDA pulse. The drawing reports the pulse and the NMDA-containing pipette, the epileptogenic focus (grey circle) and the region of neuronal Ca²⁺ imaging (blue box). C: Intrahippocampal. The lower trace in B illustrates at expanded time scale the hyperpolarizing events that preceded ictal discharge propagation to a PyN. C, means delay after the NMDA pulse of ictal discharge propagation to a PyN located close (<400 μm), middle (400–700 μm) or distant (>700 μm). D, ictal discharges from 6 experiments or distant (>700 μm), 19 ictal discharges from 10 experiments; *P* < 0.001 from the focus. D and E: recruitment diagrams reporting in a standardized scale the temporal centroid of the Ca²⁺ signal in each neuron (see Methods) from two different regions (<500 μm in D, >700 μm in E) outlined by the blue boxes in A and B. Lower traces in E show average Ca²⁺ changes from the three main clusters of neurons. F: OGB-1 fluorescence image illustrating in different colours the three main clusters. Scale bar, 100 μm.
A GABAergic inhibitory signal restrains and shapes ictal discharge propagation to PyNs distant from the focus

To investigate the role of the hyperpolarizing events in ictal discharge propagation to distant regions, in rat EC slices we next performed voltage-clamp recordings from layer V–VI PyNs. A holding potential of −50 mV was applied to monitor both excitatory and inhibitory currents. We confirmed previous observations (Tevelyan et al., 2006, 2007) that in propagating ictal discharges, PyNs faced an initial phase dominated by inhibitory currents before entering into a phase dominated by excitatory currents (Fig. 2A, black trace). In the majority of PyNs the inhibitory barrages initiated at the time of ictal discharge generation at the focus (Supplemental Fig. S1, available online only) and lasted for a mean of 10.5 ± 0.9 s (n = 20). The transition from inhibition to excitation (t_i; see Methods) occurred 20.9 ± 2.3 s (n = 12) after the NMDA pulse, at a time similar to that of the ictal discharge onset as measured in current-clamp recordings from neurons of the same region (Fig. 1C). Simultaneous recordings of the Ca^{2+} signal also revealed that in a number of neurons the Ca^{2+} level remained unchanged during the phase of dominant inhibition and increased at the time of t_i in the patched neuron (Fig. 2A, blue trace). These neurons were grouped in clusters with a mean radius of 190 ± 14 µm (n = 67 from 6 experiments; see Methods), and represented 75 ± 2% of the total number of responsive neurons in the clusters (Fig. 2B). The patched PyN was always localized within the cluster. These data suggest that groups of neurons share a common inhibitory signal that locally opposes the recruitment of neurons into propagating ictal discharges. In dual recordings from two PyNs located at different distances from the focus we occasionally observed that in the distant neuron only inhibitory barrages were observed, suggesting an effective block of ictal discharge propagation (Fig. 2C).

The inhibitory currents in patched PyNs were blocked by a local application of the GABA_A receptor antagonist bicuculline. Consequently, the delay was reduced significantly (Fig. 2D; mean, t_i = 7.5 ± 0.8 s; n = 7, P < 0.001) and 90% of the total number of the neurons present in the field of view were more rapidly and homogeneously recruited into the propagating ictal discharge (Fig. 2E). In controls and after bicuculline applications, neurons were recruited in a time interval of 11.6 ± 0.93 s (n = 8) and 5.57 ± 0.44 s (n = 5, P < 0.001), respectively. These results demonstrate that the delay in the recruitment of PyNs into the propagating ictal discharge was due to local inhibitory GABAergic events in PyNs. Notably,

![Image of Figure 2: GABAergic inhibitory barrages restrain propagating ictal discharges. A voltage-clamp recording in a PyN (black trace); V_h = −50 mV and averaged Ca^{2+} signal (blue trace) from the putative PyNs of the same region in (A). B: Schematic diagram of the experiment and OCT imaging from the same experiment showing in red the cluster of neurons recruited at the time of t_i in the patched neuron. Scale bar, 100 µm. C: Voltage-clamp recordings from a PyN pair showing a full ictal discharge in the PyN close to the focus and only inhibitory barrages in the more distant neuron. D: Voltage-clamp recordings from a distant PyN before and after bicuculline applied locally before the NMDA pulse. E: Recruitment diagrams from a region distant from the focus (upper panel) and after bicuculline application (lower panel).]
after inhibition was massively blocked by bath-applied bicuculline, ictal discharges could be evoked by a single NMDA pulse and the modular recruitment was totally abolished (Fig. 2E).

**Source of the GABAergic inhibitory barrages**

To identify among the large variety of interneurons (Markram et al. 2004; Ascoli et al. 2008) the distinct type(s) that generate the inhibitory events in PyNs during ictal discharge propagation in rat EC, we monitored the activity of different classes of interneurons while evoking an ictal discharge. We found that interneurons classified as fast spiking (FS) interneurons, but not the other interneurons recorded (see Methods), fired at low-frequency during 4-AP slice perfusion. After the NMDA pulse all the FS interneurons recorded ($n = 7$) fired high frequency action potential bursts (Fig. 3A) before entering into a depolarization block phase of spike generation. The increase in spiking discharge occurred at the same time as the inhibitory barrages recorded from PyNs (Fig. 3B). We also monitored the activity in a number of non-FS interneurons subtypes ($n = 14$). Three of four low-threshold (LTS) interneurons showed a firing increase soon after the NMDA stimulation, while putative adapting non-pyramidal (ANP) interneurons ($n = 7$) and irregular spiking interneurons (IS interneurons; $n = 8$) fired at about the same time as PyN recruitment (Fig. 3A and B) and they never exhibited a spiking activity at the time of the inhibitory barrages in PyNs.

**Pv-FS Interneuron hyperactivity and block in propagating ictal discharges**

To provide further evidence for the increased activity in FS interneurons after focal ictal discharge generation, we next took advantage of a transgenic mouse line (G42 mice) (Chattopadhyay et al. 2005) expressing the enhanced GFP in a PV-positive subset of FS interneurons. Similarly to putative FS interneurons from rat slices, PV-FS interneurons from the EC of G42 mouse slices ($n = 5$) exhibited an early activation after ictal discharge generation (Fig. 4A) followed by a depolarization block of spike generation (white arrowhead). Cells that co-expressed Pv and GFP were only rarely found in EC, so instead, further experiments were performed in the temporal cortex (TC) where these cells were more numerous (Fig. 4B and C). We first confirmed that GFP-expressing PV-FS interneurons from TC slices also showed an early increase in firing activity after ictal discharge generation followed by a depolarization block ($n = 29$, Fig. 4D). In Ca$^{2+}$ imaging experiments we then observed that after the spiking impairment in the patched PV-FS interneurons, a number of surrounding neurons exhibited Ca$^{2+}$ elevations typical of the ictal discharge (Fig. 4E, blue trace). These neurons were grouped in clusters with a mean radius of 205 ± 19 μm ($n = 32$ neurons from 3 experiments; see Methods) and they represented an average of 73 ± 5% of the total number of responsive neurons in the cluster.

Figure 3. Activity of different classes of interneurons during propagating ictal discharges. A: Representative response to hyperpolarizing and depolarizing current steps (scale bars: 20 ms and 200 nA) and current-clamp recordings from different classes of layer I-IV interneurons during propagating ictal discharges in the EC. The enlarged trace also reports the firing instant (blue, thin dots) of the FS interneuron. B, Bar histogram reporting the mean delay of the inhibitory barrages in PyNs and of the increase in firing activity from the different interneuron types after the NMDA pulse (22 ictal discharges from 12 PyNs; 11 ictal discharges from 7 FS interneurons; 14 ictal discharges from 7 ANP interneurons; 13 ictal discharges from 3 IS interneurons and 6 ictal discharges from 3 LTS interneurons; **P < 0.001).
Notably, the size of these clusters is comparable to that of the clusters recruited into ictal discharges in rat EC slices. Biocytin-filled GFP-expressing Pv-FS interneurons in layer V–VI of the TC in G42 mouse slices show the typical morphology of basket cells with distinct axonal arborizations on and around the soma of target neurons (Kawaguchi & Kondo, 2002; Fig. 4E). Axon projections are mainly confined to their layer of origin and display...
an extensive arborization around the soma (mean radius, 268 ± 39 μm, n = 7).

As Pz-FS interneurons exhibited an early increase in their bursting discharge after the NMDA pulse, they should exhibit a Ca²⁺ increase before that of nearby PyNs. However, due to the low frequency firing activity evolved in these cells by 4-AP, the Ca²⁺ signal in Pz-FS interneurons is already high before the application of the NMDA pulse. To circumvent this caveat we perfused slices with a low Mg²⁺ solution (0-0.2 mM) without 4-AP. Also under these conditions NMDA pulses reliably evolved a propagating ictal discharge (see Fig. 6A, middle traces). Figure 5 illustrates a representative experiment in a Rhod-2-loaded slice in which four GFP-expressing Pz-FS interneurons in TC from a region distant >700 μm from the focus were early activated after the NMDA pulse (Fig. 5A). The Ca²⁺ signal reveals that the GFP-expressing cells (Fig. 5B, green cells in panel 1 and green traces) and two GFP-negative cells (orange labelled cells in panel 1 and orange traces) increased their Ca²⁺ before two clusters of putative PyNs were recruited into the propagating ictal discharge (Fig. 5B, cyan labelled cells in panels 2 and 3 and blue traces; see also online Supplemental Movie 1). Early activated GFP-negative cells may belong either to the subset of Pz-positive, GFP-negative cells, that are present in the TC of G42 mice, or to a different class of interneurons, such as the LFS interneurons that in rat slice experiments increased their firing discharge soon after the NMDA pulse. The early Ca²⁺ elevation in GFP-positive Pz-FS interneurons and in a subset of GFP-negative neurons preceded the recruitment of PyN clusters into the propagating ictal discharge with a similar average interval (Fig. 5C, see Methods). In the five experiments performed in TC with the low Mg²⁺ solution, these neurons were 9.8 ± 1.5% of the total number of neurons present in the field of view, and the GFP-positive Pz-FS interneurons (n = 52) were 61.6 ± 3.7% of the early activated neurons. These data provide further evidence for the early activation of Pz-FS interneurons during ictal discharge propagation.

Relationship between FS interneuron firing activity and PyN recruitment into propagating ictal discharges

We next performed a series of paired recordings from a Pz-FS interneuron and a nearby PyN in TC slices from G42 mice. The inhibitory barrages in the PyN of the pair occurred in coincidence with a burst in the Pz-FS interneuron in both the 4-AP (Fig. 6A, upper trace) and the low Mg²⁺ model (Fig. 6A, middle trace). The analysis of the relationship between the amplitude of the inhibitory barrages and the spiking discharge intensity in Pz-FS interneurons revealed that the strength of the inhibitory barrages onto PyNs, as measured by the charge

Figure 5: Early recruitment into propagating ictal discharges of GFP-expressing Pz-FS interneurons in G42 mice

A. Fluorescence image of a layer V-VI region distant from the focus in a slice loaded with Rhod-2 showing GFP-expressing cells (white arrows; bar, 50 μm). B. A fifth GFP-expressing Pz-FS interneuron present in the field of view was not considered because it was not loaded with the Ca²⁺ dye. C, Recruitment diagram from the region imaged in A. Lower traces, four GFP-expressing green traces and green cells in panel 1) and two GFP-negative neurons (orange traces and orange cells in panel 1) exhibited a Ca²⁺ increase soon after NMDA stimulation and before two different clusters of putative PyNs were recruited (blue traces and cyan cells in panels 2 and 3). The lower right traces show the Ca²⁺ change in these cells during the visible ictal discharge. D, bar histogram of the mean interval (μs) between the timing of PyN recruitment (n = 135) and the Ca²⁺ increase in GFP-expressing Pz-FS Interneurons (n = 52, from 5 experiments) and GFP-negative neurons (n = 20).

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Figure 6. Hyperactivity and block in PV-S interneurons precede the transition to ictal discharge.

PV-S interneurons are a main source of the inhibitory barrage. A and B: dual current-clamp recordings from a PV-FS (A) or a Som-S (B) interneuron and voltage-clamp recording from a neighbouring PN in a TC slice from a G42 mouse in the 4-AP or low Mg²⁺ model (A, middle panel). Dual recording from a PV-FS and a PN (both in current-clamp mode) is also presented in A (lower panel). Response of the L5-Som interneuron to current injection is also reported (A, inset): acute bars, 20 mV and 500 ms. C: linear regression showing the correlation between the number of PV-FS interneuron firings and the normalized inhibitory charge transferred in the PNs in the 4-AP model \( R = 0.79, P < 0.001, 39 \) inhibitory barrages from \( 9 \) ictal discharges, \( 5 \) pairs \( 3 \) ictal discharges, \( 3 \) pairs. D: linear regression showing the absence of correlation between the number of PV-FS interneuron firings and the normalized inhibition \( R = 0.23, P = 0.08, 50 \) inhibitory barrages from \( 9 \) ictal discharges, \( 3 \) pairs. E: distribution of the latency of depolarization block in the PV-FS interneurons (blue circles, \( 25 \) ictal discharges, \( 10 \) pairs) and in the Som interneurons (red diamonds, \( 15 \) ictal discharges, \( 3 \) pairs) with respect to the \( t_f \) in the PNs. Distribution of values in a time window of \( \pm 100 \) ms are also reported.
transferred, were linearly correlated with the change in the firing intensity of Pv-FS interneurons in both the 4-AP (Fig. 6C; circles, 39 inhibitory barrages in 9 ictal discharges from five pairs) and the low Mg²⁺ model (Fig. 6C, circles, 39 inhibitory barrages in six ictal discharges from three pairs). Furthermore, the time in the PyN (Fig. 6A, black arrows) occurred when the PV-FS interneuron entered into a depolarization block phase (Fig. 6A, white arrowheads). Detailed quantitative analysis from paired recordings confirmed that in 23 of 25 events the depolarization block in Pv-FS interneurons occurred before (mean ± SEM, 105 ± 48 ms; n = 25; Fig. 6E, blue circles; 25 ictal discharges from ten pairs, pooled data from 4-AP and low Mg²⁺ experiments) the PyN recruitment (i.e. the time 0 in Fig. 6E corresponding to the tₜ). Similar results were obtained in rat EC slices (online Supplemental Fig. S2). Paired recordings in current-clamp configuration from Pv-FS interneurons and PyNs in close proximity to each other and at a similar distance from the focus confirmed that the PyN exhibited the intense firing of the ictal discharge during the depolarization block phase in the Py-FS interneuron (Fig. 6A, lower traces).

While results described above suggest that the inhibitory barrages which oppose ictal discharge propagation are generated by Pv-FS interneurons, an additional putative source of the inhibitory barrages in PyNs are the LTS interneurons. In rat slices these cells were, indeed, observed to fire shortly after the double NMDA pulse. To address this hypothesis, we performed paired recordings in EC slices from GIN mice in which the enhanced GEP is selectively expressed in a subset of Som interneurons (Oliva et al. 2009). Only cells that exhibited the typical firing pattern of LTS interneurons were selected (see Methods) (Oliva et al. 2000; McGarry et al. 2010). In the 4-AP model we confirmed that layer V–VI Som-LTS interneurons fired intensively before the recruitment of PyNs into the ictal discharge (Fig. 6F). However, the inhibitory currents in PyNs were poorly correlated with a bursting discharge in Som-LTS interneuron (Fig. 6F; 50 inhibitory barrages from 9 ictal discharges, three pairs) and some events (11 of 50) rather coincided with a hyperpolarization in Som-LTS interneurons (Fig. 6F, dashed line bars). Indeed, recordings from these PyNs/Som interneuron pairs, with both cells in voltage-clamp configuration, revealed that 29 of 41 inhibitory events recorded in the PyN during ictal discharges also occurred synchronously in the Som-LTS interneurons (online Supplemental Fig. S3). The depolarization block in the Som-LTS interneurons was poorly associated with the tₜ in the PyN of the pairs (Fig. 6F, red diamonds; 15 ictal discharges from three pairs). All of these results are consistent with the hypothesis that PV-FS interneurons are a main source of the inhibitory barrages recorded in PyNs.

To rule out the possibility that the depolarization block in Pv-FS interneurons was an artifact of the whole-cell recording mode, we recorded the firing discharges in cell-attached mode. Paired recordings confirmed that the firing activity in GEP-expressing Pv-FS interneurons was strictly correlated with the inhibitory barrages in the PyN of the pairs (Figs. 7A and B) and that in 7 of 9 events the impairment of the firing discharges in Pv-FS interneurons preceded (32.6 ± 50.7 ms; n = 9) the ictal discharge onset in the PyNs (Fig. 7C).

Single and dual cell recordings from Pv-FS interneuron pairs (Figs. 8A and B) confirmed that with respect to the NMDA stimulation, the depolarization block in Pv-FS interneurons close to the focus (< 400 μm) occurred with a significantly shorter delay (10.9 ± 1.3 s; n = 6) than that in Py-FS interneurons distant from the focus (> 760 μm, 23.0 ± 1.6 s; n = 15; P < 0.001). Notably, these delays are comparable to those of the ictal discharge propagation to PyNs of these regions. In PyN–Pv-FS interneuron pairs (Fig. 8C) the depolarization block in the Pv-FS interneuron close to the focus occurred largely before the recruitment into the ictal discharge of the more distant PyN.

**Discussion**

In the present study, we gained two fundamental insights into the events that govern local ictal discharge propagation. First, that the inhibitory barrages which temporarily oppose the spreading of local ictal discharges are largely generated by local Pv-FS interneurons, and second, that a functional impairment in these interneurons contributes to the collapse of the local inhibition that allows focal ictal discharges to propagate further across the cortex.

The conclusions summarized above are based on the results that we obtained in a number of different experiments. We first found that electrophysiologically classified FS interneurons from rat slices exhibited an early bursting activity during ictal discharge propagation. We then found that GEP-expressing Pv-FS interneurons in G12 mice exhibited intense bursting discharges and correlated Ca²⁺ elevations before nearby PyNs were recruited into propagating ictal discharges. In addition, dual recordings from a PyN and a nearby Pv-FS interneuron revealed that the inhibitory barrages in PyNs (i) occurred in strict coincidence with the firing discharge in Pv-FS interneurons, (ii) their strength was determined by the firing discharge intensity in PV-FS interneurons, and (iii) they ceased after a depolarization block impaired the firing activity in PV-FS interneurons. In our paired recordings from a PyN and a nearby Pv-FS interneuron, the latter being in either whole-cell or cell-attached mode, we also found that the depolarization block in the Pv-FS
interneuron preceded the recruitment of the PyNs into the ictal discharge. This conclusion was confirmed in recordings from Pvn-FS interneuron pairs and Pyn/Pv-FS interneuron pairs located at different distances from the focus as well as in Ca$^{2+}$ imaging experiments. Results from these latter experiments revealed that clusters of Pyns surrounding the patched Pvn-FS interneuron were recruited into the propagating ictal discharge after the depolarization block in the Pvn-FS interneuron. Neurons from a cluster may share a common inhibitory input that, while it is fully operative, allows these cells to resist the incoming excitatory volley of the propagating ictal discharge. Our results support the view that the recruitment of neurons can be a local process governed mainly by Pvn-FS interneurons from the same local network, although they cannot rule out the possibility that different classes of interneurons may also provide a significant contribution (see below).

Previous findings described an important role of the feedforward inhibition in the spreading of epileptiform discharges (Prince & Wilder, 1967; Schwarz & Bornhoeffer, 2001; Trewelahan et al., 2006, 2007). In occipital cortex slices a reduction in the strength of inhibition was also described as a crucial step in the recruitment process during ictal discharge propagation (Trewelahan et al., 2006, 2007). Our study provides evidence that a presynaptic block of Pvn-FS interneuron activity is an important factor in this process. However, we cannot rule out that different events, such as a depletion of GABA vesicular stores (Liang et al., 2006; Ortsinski et al., 2010; Zhang et al., 2012), a reduced release probability (Bear, 2005), modifications of GABA receptors (Thompson & Gahwiler, 1989; Whittington et al., 1995; Naylor et al., 2005) and a depolarizing GABA action (Staley et al., 1995) resulting from a Cl$^{-}$ intracellular accumulation during an intense activity at GABAergic synapses (Fujiwara-Tsuchimato et al., 2004; Ribeiro et al., 2004) may contribute. A recent study reported, however, that intensively active Pvn-FS interneuron synapses onto Pyns can be protected from a Cl$^{-}$ accumulation since an efficient mechanism of Cl$^{-}$ extrusion, based on voltage and intracellular Cl$^{-}$-dependent Cl$^{-}$ channels, appears to be expressed specifically at these synapses (Foldy et al., 2010).

Non-FS spiking interneurons, such as putative ANP and IS-Interneurons (Ascoll et al., 2000) that we picked in our recordings from rat BG slices, appeared not to contribute to the inhibitory barrage since they were all quiescent while this inhibitory activity was recorded in Pyns. In contrast, Som- LTS interneurons in GIN mice (and also putative LTS-interneurons in rats) fired intensively prior to the ictal discharge, but significant correlation was found neither between the firing discharges in Som-LTS interneurons and the inhibitory currents in Pyns nor between the depolarization block in these interneurons and the ict in Pyns. However, due to defective space clamp at distal dendrites, the inhibition by Som interneurons on distal synapses may be poorly visible in our Pyn soma recordings. Therefore, we cannot rule

![Figure 7. Hyperactivity and block in Pvn-FS interneurons precede the transition to ictal discharge (cell-attached mode)](image-url)

**Figure 7.** Hyperactivity and block in Pvn-FS interneurons precede the transition to ictal discharge (cell-attached mode)

A, dual patch-clamp recordings from a GFP-expressing Pvn-FS interneuron firing activity in the cell-attached mode and a neighboring Pyn (in whole-cell, voltage-clamp mode) in a TC slice from a G62 mouse in the A-AP model.  
B, linear regression showing the correlation between the f_{r} of GFP-expressing Pvn-FS interneuron firing and the normalized inhibitory charge transferred in the Pyns in the A-AP model (r = 0.83; p < 0.001; dotted lines indicate the mean f_{r} of 3 patches).  
C, distribution of the latency of depolarization block in the Pvn-FS interneurons (9 ictal discharges, 3 patches with respect to the ict in the Pyns).
out the possibility that dendrites targeting Som interneurons contribute to reduce the overall excitation onto Pyns during propagating ictal discharges. Consistent with this view, a recent study showed that the inhibition by Som interneurons contributes to control the burst spiking response of hippocampal CA1 Pyns to Schaffer collateral stimulation (Lewitt-Barron et al. 2012).

An interplay similar to that investigated here between Pyns and Pv-FS interneurons has been previously observed between hippocampal CA1 Pyns and unidentified interneurons during spontaneous seizure-like events (Zerbus et al. 2006). Our results extend this observation to a different brain region, such as the EC and the TC, and to a different epileptic activity, such as focally evoked seizures, and they also provide evidence that Pv-FS interneurons are the specific interneuron subset involved. Because FS interneurons, and in particular Pv-FS interneurons, represent a major source of perisomatic inhibition onto Pyns, they are ideally positioned to exert a strict control on the output and synchrony of these neurons (Cobb et al. 1995; Freund & Buzsáki, 1996; Miles et al. 1996; Freund, 2003; Freund & Katona, 2007). The efficiency of Pv-FS interneurons in opposing ictal discharge propagation may rely also on their intrinsic properties that promote high frequency of action potential firing, a highly synchronous release (Bacc et al. 2005; Hefti & Jonas, 2005) and a fast recruitment by glutamatergic input (Aradi & Maccioni, 2004).

Our data also provide a plausible cellular mechanism for the enhanced susceptibility to epileptic seizures observed in mice in which firing activity in FS interneurons was affected due to a loss in these interneurons of either K3.2 channels (Lau et al. 2006) or Na1.1 channels (Coulth et al. 2007). Consistent with an important role of Pv-FS interneurons in seizure generation are also recent data showing that Pv-FS interneuron excitability, modulated by NRG1-ErbB4 signalling, contributes to limbic seizure activity (Li et al. 2011; Tan et al. 2011).

In our study we have not addressed the mechanism of the depolarization block in Pv-FS interneurons. This may rely on the extracellular K+ increase that is recognized to accompany seizure discharges in different in vitro and in vivo models (Lux et al. 1986; Drew & Heine, 1991; Perreault & Avoli, 1992; Durand et al. 2010; Frodlch et al. 2010), and to represent a possible causal factor for epileptic seizures (Gnatovsky et al. 2008; Ulus & Schiff,

Figure 8. The timing of the depolarization block in Pv-FS interneurons depends on the distance of these cells from the focus. A, dual current-clamp recording from two adjacent Pv-FS interneurons showing simultaneous depolarization block. B, two Pv-FS interneurons located at different distances from the focus entered into the depolarization block at different times. C, dual recording from a Pyn and a Pv-FS interneuron located in EC layer V-VI at different distances from the focus. The depolarization block in the Pv-FS interneuron closer to the focus occurred several seconds before the one in the Pyn. The recording periods outlined by the dashed boxes are expanded on the right.
2010). Consistent with this hypothesis, an extracellular K+ increase to 12.5 mM was reported to result in a depolarization block in hippocampal CA3 interneurons (Shin et al. 2010). Additional, specifically designed experiments are necessary to clarify this important issue.

Our data on the intense activity of Pv-FS interneurons and Som-LTS interneurons during seizure propagation are consistent with the intense firing described in interneurons in vivo just before seizure discharges during the paroxysmal depolarizing shifts (Timofeev et al. 2002) and in all types of experimental models in vivo (Avoli et al. 2002; Derckasisky et al. 2008; Gnatkovsky et al. 2008). High frequency EEG activities, possibly reflecting interneuron discharges (Treluyer, 2009), are also recorded in patients at the initiation of temporal lobe seizures (Fisher et al. 1992; de Curtis & Gnatkovsky, 2009; Engel et al. 2009). Interestingly, high activity aligned with standard EEG recordings from seizure-onset areas in patients revealed that seizure spread was either delayed for several seconds, or in some cases failed to take hold, providing further evidence for an inhibitory restraint mechanism in naturally occurring chronic epilepsy (Schevon et al. 2012).

In conclusion, our data point to Pv-FS interneurons as a major source of the feedforward inhibitory activity that locally restrains focal ictal discharge propagation. Although our results do not rule out the possibility that other classes of Interneurons, such as Som interneurons, can contribute, they suggest that an initial drastic increase and a subsequent block of the firing discharge are two opposing events in the activity of local Pv-FS interneurons that shape ictal discharge propagation. Additional experiments are, however, necessary to provide direct evidence for a causal relationship between the collapse of inhibition and the depolarization block in the Pv-FS interneurons. Our study suggests that the development of pharmacological tools capable of modulating Pv-FS interneuron activity may represent a new therapeutic strategy to prevent the generalization of focal epilepsies.

References


Fast-spiking interneurons in focal seizure-propagation


Author contributions
The experiments were performed in the CNR Institute of Neuroscience, Padova section, Padova Italy. M.C., G.L. and G.C. conceived and designed the experiments, collected, analysed and interpreted the data, drafted the article or revised it critically for important intellectual content; A.C. and M.Z. collected and analysed the data. All authors approved the final version of the manuscript.

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APPENDIX:

SELECTIVE STIMULATION OF INDIVIDUAL FAST SPIKING INTERNEURONS PREVENTS SEIZURE PROPAGATION IN A BRAIN SLICE MODEL OF FOCAL EPILEPSY

Mario Cammarota, Gabriele Losi and Giorgio Carmignoto

ADDITIONAL METHODS:

Analysis of afterdischarges. To evaluate in voltage-clamp recordings the delay between excitatory bursts from two neurons located at different distance from the epileptogenic focus, we calculated the cross-correlation between the time derivative of both signals, in time bins of 1000 ms separated by 30 ms and we represented the cross-correlation values in a pseudo-colors plot. We measured the lag corresponding to the maximal value of the cross-correlation before and after the change in direction of the afterdischarge.

Preventing depolarization block in Pv-FS-INs, but not in Som-INs, arrests ID propagation

If the depolarization block in Pv-FS-INs is a necessary condition to the local recruitment of PyNs into propagating IDs, one expects that ID propagation would be either delayed or blocked if the functional impairment in Pv-FS-INs is prevented. To test this possibility, an intense firing discharge was evoked in individual Pv-FS-INs by a sequence of depolarizing current pulses (500 ms duration at 0.5 or 1 Hz) applied through a patch pipette. Under these conditions, the double NMDA pulse failed to induce a depolarization block in the Pv-FS-IN and no ID was observed in 11 of a total of 12 NMDA stimulations (Figure 1 A,B). In the same neurons, when no current pulses were applied, the NMDA stimulation evoked both a depolarization block and a full ID
Similar intracellular current pulses applied to Som-INs always failed to prevent the depolarization block and the ID in these cells (n = 7 IDs from 3 experiments; Figure 1 A,B). The results reported above were further validated in Ca$^{2+}$ imaging experiments. In all the performed experiments (n = 3), the sustained firing discharge evoked in individual Pv-FS-INs drastically reduced the recruitment of nearby neurons into propagating IDs. The experiment illustrated in Figure 1C shows the activity of a patched Pv-FS-IN distant from the focus and the Ca$^{2+}$ change in neurons from the same recording field (Figure 1C) in response to double NMDA pulses applied in the absence or presence of a Pv-FS-IN stimulation. When the Pv-FS-IN was not stimulated (Figure 1C, left and right traces), an ID was successfully evoked and two neuronal clusters were subsequently recruited (red and blue traces). When current pulses were delivered to evoke an intense firing in the Pv-FS-IN (for details, see Methods), the ID propagated to neurons of the first cluster (red), but not to neurons of the second cluster (blue) surrounding the patched Pv-FS-IN (Figure 1C, middle traces). Noteworthy is that under these conditions neurons from the first cluster that were still recruited into the propagating epileptic discharge, but the ID duration in these neurons was reduced (-57 ± 5 %; n=24 neurons). Such an ID reduction might be linked to the existence of multiple foci of the afterdischarges that sustain the late ID phase. Indeed, during spontaneous IDs recorded in neocortical slices, afterdischarges have been described to travel also in the opposite direction with respect to that of ID propagation (Trevelyan et al., 2007). If neurons become sources of the afterdischarges as they are recruited into the ID wavefront, then the block of the recruitment process would reduce the afterdischarges in already recruited regions and shorten the overall ID duration, as we observed. To address this hypothesis we analyzed the timing of the afterdischarge in paired voltage-clamp recordings from two PyNs located at different distances from the
focus. We found that the ID wavefront propagated firstly to the neuron closer to the focus (Figure 1D, upper trace) and then to the more distant neuron (lower trace). During the clonic phase, however, the afterdischarges that initially propagated in the same direction as the ID wavefront, changed direction (Figure 1E). As revealed by the cross correlation diagram, after the switch in direction all the afterdischarges propagated from a distant region to the original site of ID generation (Figure 1D,E, lower panels). Similar results were obtained in 4 IDs from 3 PyN pairs. On the average, the direction switch occurred 7.97 ± 15 s after the tIE in the more distant neuron. Before the direction switch, the afterdischarge in the neuron closer to the focus preceded by 30.5 ± 12.18 ms that in the more distant neuron, while after the switch it followed with a mean delay of 18.11 ± 6.4 ms (see Additional Methods).
Figure 1: Preventing depolarization block in PV-FS-INs arrests ID propagation in layer V-VI of TeCx. A, Single current-clamp recordings from a PV-FS-IN and a Som-IN. Steps of current were repeatedly injected to evoke firing activity (insets; see methods) that in the PV-FS-IN, but not in Som-IN, prevented the depolarization block and the occurrence of a full ID. B, Summary of the average percentage of NMDA evoked IDs without (-) or during (+) stimulated firing of Som and PV-FS INs. C, Current-clamp recording from a PV-FS-IN and simultaneous mean Ca2+ signal from two neuronal clusters (red and blue traces, their soma position is reported in right panel) according to their different recruitment time. D, Dual voltage-clamp recordings from two PyNs and pseudocolor plot of the cross-correlation (see methods) during a focal ID showing the switch of afterdischarge direction. E, Two afterdischarges at enlarged time scale with cross-correlation diagrams of the relative bin.
WORK IN PROGRESS:
A GABA MEDIATED Ca\textsuperscript{2+} SIGNALLING IN CORTICAL ASTROCYTES
Gabriele Losi, Mario Cammarota, Letizia Mariotti and Giorgio Carmignoto

INTRODUCTION
Astrocytes can respond to the neuronal release of excitatory neurotransmitters such as glutamate with G-coupled protein receptor-mediated intracellular Ca\textsuperscript{2+} elevations (Porter and McCarthy, 1996, Pasti et al., 1997). The Ca\textsuperscript{2+} change triggers the release of diverse gliotransmitters, including glutamate, that affect neuronal excitability and modulate synaptic transmission. This discovery revolutionized the study of the brain and changed the basic concept that the function of the brain relies exclusively on neurons and neuron-to-neuron synaptic communication. While the vast majority of the studies on gliotransmission describe the interactions of astrocytes with excitatory neuronal network (for a review see Haydon and Carmignoto, 2006), little is known about the hypothesis that astrocytes can be also activated by synaptically released GABA and signal back to interneurons and/or principal neurons to modulate inhibitory and excitatory synaptic transmission.

It is noteworthy that feed-forward inhibition is highly active during ictal discharge propagation in the cortical network. A large amount of GABA is, indeed, released by early activated interneurons to oppose ictal discharge propagation. This raised the question of whether such an intense activity of GABAergic interneurons can result in a strong activation of astrocytes.

METHODS:
Cortical slices from Enthorinal (EC), Temporal (TC) or Somatosensory (SCC) cortex of P15 wild type mice were obtained as described in the methods sections of the previous chapters. Slices were incubated with Sulforodamine 101 (SR101) to selectively labeled astrocytes (Nihemermaier et al., non mi ricordo come si scrive e data). We developed a new protocol in order to load slices with Fluo4-AM: slices were incubated with the Ca\textsuperscript{2+} indicator for 15 minutes only instead of 1h as commonly done. Under these conditions only a few neurons (5% of the total labeled cells) were loaded. Ca\textsuperscript{2+}-evoked fluorescence changes were measured by a confocal or two-photon microscope during either local pressure-pulses applied to a GABA (or Baclofen) - containing glass pipette or perfusion with the same agents alone or in conjunction with...
a selective GABA<sub>B</sub> receptor antagonist (CGP or SCH). In the experiments performed on Tomato-floxed mice crossed with PV-CRE mice, the total number within the recording field of potentially responsive astrocytes was evaluated as the number of astrocytes responsive to the application of the mGluR agonist DHPG (50 μM). Only astrocytes that showed a Ca<sup>2+</sup> increase greater than 3 standard deviation of their basal trace were considered responsive.

RESULTS
We first clarify whether GABA can evoke Ca<sup>2+</sup> elevations in astrocytes. In Entorinal Cortex (EC) slices loaded with both SR101 to selectively mark astrocytes (Nimmerjahn, 2004) and the Ca<sup>2+</sup> sensitive dye Fluo4-AM (Figure 1A), we found that bath application of GABA (25 μM) activated a Ca<sup>2+</sup> rise in a group of astrocytes (18 ± 6 astrocytes per recording field; Figure 1B). The GABA<sub>B</sub> antagonist CGP55485 (25 μM) almost completely abolished GABA effect on astrocytic Ca<sup>2+</sup> (Figure 1B; n=3 slices). The GABA<sub>B</sub> agonist baclofen (25 μM) evoked a Ca<sup>2+</sup> response similar to that evoked by GABA (19 ± 3 astrocytes, 11 experiments), and also in this case CGP55485 drastically reduced the number of activated astrocytes. (Figure 1B; see Methods). In temporal Cortex (TeCx) slices we also found that local baclofen applications (500 μm) evoked Ca<sup>2+</sup> elevations in a large group of astrocytes. On the average, Baclofen local applications activated 10.1 ± 1.5 astrocytes (n = 8 experiments) that were located within a radius of approximately 150 μm from the Baclofen-containing pipette tip, inducing a mean fluorescence increase (ΔF/F<sub>0</sub>) of 171 ± 16% (n = 60 astrocytes from 6 experiments; Figure 1C). This response was prevented by the GABA<sub>B</sub> antagonist SCH90911 (500 μm) locally applied 2 s before Baclofen applications (Figure 1C). These results clearly indicate that astrocyte respond to GABA through GABAB-mediated Ca<sup>2+</sup> changes. We next tested whether GABA synaptically released by GABAergic interneurons activates astrocytic Ca<sup>2+</sup> changes. Since we recently observed that parvalbumin positive fast spiking (Pv-FS) interneurons are a major source of the feed-forward inhibition that restrains focal ictal discharge propagation (Cammarota, 2013), we focussed on this interneuron subtype. We used a mouse line in which Pv-FS interneurons express a red fluorescent protein (Tomato-floxed mice crossed with PV-CRE mice) which allowed us to identify these cells in slices loaded with the green fluorescence dye Fluo-4. In order to preferentially
load astrocytes with the Ca\textsuperscript{2+} sensor we reduced the loading protocol duration (see Methods). We then performed patch-clamp recordings of a single Pv-FS interneuron and simultaneous Ca\textsuperscript{2+} imaging of nearby astrocytes before and during induction of high frequency action potential firing in the patched interneuron (trains of 80 Hz action potentials for 1000 ms, train frequency 0.5 Hz, induced by current injection through the patch pipette). Out of 74 astrocytes monitored, 24 displayed spontaneous activity. Twenty of these showed a significant increase in the frequency of Ca\textsuperscript{2+} oscillations after Pv-FS interneuron stimulation (p<0.02; Figure 2) (whereas amplitude did not increase significantly, the remaining 4 did not show any Ca\textsuperscript{2+} oscillations). In the remaining 50 astrocytes studied - which were not spontaneously active - we found that 19 cells displayed intense Ca\textsuperscript{2+} oscillations after Pv-FS interneuron stimulation. Notably in the presence of bath applied GABA\textsubscript{B} receptor antagonist SCH90911 (25 \(\mu\text{M}\)) almost all the responses of astrocytes were prevented. Altogether, these data show that firing activity in a single Pv-FS interneuron can evoke a GABA\textsubscript{B}-mediated Ca\textsuperscript{2+} elevation in a group of neighbouring astrocytes.
Figure 1: Astrocytes display a GABA$_A$-mediated Ca$^{2+}$ signalling. A: 2-photon image of an EntCx slice loaded with SR101 and Fluo4-AM. B-C: Left: Ca$^{2+}$ changes of astrocytes in response to GABA bath application (B) and to Baclofen local application (C), in control condition and during the application of GABA$_A$ antagonist CGP55485 (B) or SCH (C). Right: histograms summarizing the results.
What are the consequences of GABAB-mediated Ca\(^{2+}\) increases in astrocytes? In presence of TTX to block all neuronal activity, in TeCx slices we found that Baclofen (BAC) local applications evoked slow inward currents (SICs) in 5 out of 9 Pv-FS interneurons tested. Similar experiments were performed in somatosensory cortex slices from Pv-Cre/Tomato-floxed mice where numerous Pv interneurons express the tomato fluorescent protein. In this area, in 4 out of 5 Pv interneurons and in 5 out of 5 pyramidal neurons Baclofen induced a significant increase in SIC amplitude and frequency (Figure 3A-B). Interestingly, as a mean SICs in Pyramidal neurons were significantly larger than in FS interneurons, both in control and after GABAB activation.
(Figure 3B). This result raises the possibility that GABA$_B$-mediated Ca$^{2+}$ elevations in astrocytes result in a different effect in Pyr vs FS-interneurons.

**Figure 3:** Ca$^{2+}$ oscillations in astrocytes stimulated with GABAB receptor agonist induce SICs in Py-FS interneurons and in pyramidal neurons. A: upper traces: Ca$^{2+}$ oscillations in astrocytes before and after the application of baclofen (black triangles). Central black trace: voltage-clamp recording from a Py-FS interneuron. In the lower trace the boxed SIC is reported with higher magnification.
CONCLUSIONS AND FUTURE PERSPECTIVES

Full understanding of the cellular mechanisms at the basis of generation, propagation and cessation of epileptic seizures represents one of the most complex and challenging problem in the pathophysiology of the brain. A seizure is comparable to a big electric tilt in the brain where thousands of neurons fire synchronous action potentials and release multiple inhibitory and excitatory transmitters. The problem became even more intricate when we consider the emerging concept that the function of the brain, and perhaps also the dysfunction of the brain, is based on a network of intensively interacting neurons and astrocytes rather than a network of only neurons. Exciting results have been published on the hidden capabilities of these cells to communicate with neurons in fundamental phenomena in brain function.

In my thesis I used a multidisciplinary experimental approach that was based on patch-clamp recordings coupled with confocal and 2-photon Ca$^{2+}$ imaging in a newly developed model of focal ictal discharge in rodents brain slices. All our results indicate that a neuron-astrocyte reciprocal signaling can be deeply involved in the different phases of epileptiform activity. We showed that a release of glutamate from astrocytes – that were activated after an intense firing discharge was induced in a group on neurons by a local NMDA application - is a crucial event that generates a local hyperactivity of neurons and ultimately an epileptogenic focus that predisposes the neuronal network to seizure initiation. Seizure generation is, therefore, triggered by a concerted action of neurons and astrocytes, connected each other in an excitatory loop. The role of astrocytes in determining the threshold of ictal discharge generation was further corroborated by data obtained in a computational model of a neuron-astrocyte network. As regards the propagation of epileptic activity, we firstly investigated the role of GABAergic transmission in governing the spatial-temporal features of the recruitment of new neuronal territories into the propagating seizure. We found that one of the most abundant GABAergic interneurons in the grain, i.e., the parvalbumin expressing fast-spiking interneurons, play a crucial role in governing the timing of the recruitment of pyramidal neurons to the propagating ictal discharge. Preliminary results described in the last chapter of this thesis, raised the possibility that in areas distant from the focus astrocytes may establish with parvalbumin fast-spiking interneurons a series of reciprocal interactions that contribute to control
seizure propagation. This hypothesis will be specifically address in future experiments.

REFERENCES


