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NEURONAL MECHANISMS OF EPILEPTOGENESIS

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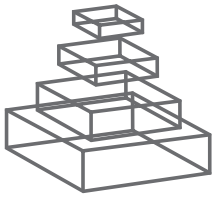

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ISSN 1664-8714
ISBN 978-2-88919-382-0
DOI 10.3389/978-2-88919-382-0

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NEURONAL MECHANISMS OF EPILEPTOGENESIS

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frontiers in
CELLULAR NEUROSCIENCE

EDITORIAL
published: 21 February 2014
doi: 10.3389/fncel.2014.00029

Neuronal mechanisms of epileptogenesis

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Keywords: epileptogenesis, neuronal epileptic damage, hippocampal damage, epilepsy, Temporal Lobe Epilepsy (TLE), TLE prevention

The primary purpose of this topic is to collect scientific contributions providing novel insights in the cellular and molecular mechanisms of epileptogenesis as potential targets for innovative therapeutic approaches aimed at preventing the chronic epileptic disorder.

Prevention of chronic epileptic disorder with an appropriate intervention might represent the most ambitious goal in the clinical treatment of this epileptic disorder, but has been largely unsuccessful to this point. Clinical trials aimed at prevention of chronic epilepsy have often produced negative, disappointing results. However, in most cases, these studies ultimately evaluated the downstream clinical manifestations, failing to monitor early, specific molecular epileptogenic events. Therefore, elucidation of the underlying mechanisms of epileptogenesis, are essential.

Several types of brain injuries are causes of acquired epilepsy, including brain trauma, one of the most common causes of idiopathic epilepsy (Hunt et al., 2013; Timofeev et al., 2013). Genetic mutations enhancing structural and functional alterations of key proteins including pre-synaptic complexes (Toader et al., 2013) and potassium channels (D'Adamo et al., 2013) are also related to the occurrence of epileptic disorders. Consistently with these findings obtained in genetic animal models of epilepsy, studies conducted in animal models of acquired epilepsy addressed the critical role of vesicular neurotransmitters transporters (VNTs) (Van Liefferinge et al., 2013) and non-neuronal potassium channel (Kir4.1) (Nagao et al., 2013) expression during epileptogenesis.

Temporal Lobe Epilepsy (TLE) is the most common form of refractory epileptic disorder often related to childhood seizures. The symptomatic manifestations of TLE appear only after a widespread irreversible damage of entorhinal cortex (Bartolomei et al., 2005), hippocampus (Mathern et al., 2002) and perirhinal cortex, which has a major role in the spread of limbic seizures (Biagini et al., 2013), These pathological features of TLE reduce the possibility of successful therapeutic approaches, often rendering the disease refractory. The difficult clinical management of chronic TLE and the limited success rate of surgical approaches, increase the incapacitating nature of this specific epileptic disorder.

Despite its complex etiology, a common feature of the epileptic disorders is a paroxysmal excitatory activity, which is able to produce the same pathological features that are ultimately recognized clinically as epileptic disease.

Only recently the role of oxidative stress in epilepsies has begun to be recognized. Neuronal hyper-excitability is associated with a calcium-dependent activation of intracellular oxidant systems, including NOX2, which is the major NMDAR-regulated source of superoxide (Di Maio et al., 2011). This early phenomenon occurring during the epileptic onset might be responsible for the long-term neuronal dysfunction leading to the chronic epileptic disorder (Di Maio et al., 2012).

Excitatory/inhibitory unbalance and oxidative-related events might be determinant in the epileptic pathogenesis of neuronal networks mediating a complex disruption of self-regulatory homeostatic mechanisms such as the bioenergetics systems (Boison et al., 2013).

Epileptic neurons may develop short and long-term adaptive changes in sensitivity to GABA-ergic neurotransmission by means of $GABA_A$ receptor (Cifelli et al., 2013), worsening the excitatory/inhibitory unbalance and reducing the possibility of successful therapeutic approaches with the conventional Antiepileptic Drugs. Interesting insights have been recently provided on this regard. Epileptogenic changes of $GABA_A$ receptor may be caused by altered expression of scaffolding proteins involved in the trafficking and anchoring of $GABA_A$ receptors. This phenomenon could directly impact the stability of GABA-ergic synapses and promote impairment of the neuronal response to the inhibitory GABA-ergic input. These findings offer novel potential therapeutic targets to prevent the development of epilepsy.

Dopaminergic projections to limbic system play also a critical role in the control of seizures. Dopaminergic activity in limbic structure exerts a complex neuromodulation of neuronal excitability mainly through D1 and D2 receptors subtypes. Impairment of the fine tuning mediated by dopamine (DA) receptors activity can contribute to spread of seizures in the limbic system. Recent evidences on the identification of intracellular signaling pathways activated by DA receptors activity are leading to promising studies aimed at the identification of novel targets for the treatment of epilepsy (Bozzi and Borrelli, 2013).

An increasing number of experimental evidences suggest a major involvement of inflammation in epileptogenesis. Seizure activity elicits release of pro-inflammatory cytokines and activates immune responses. These phenomena have been widely related to an increased brain susceptibility to seizure, synaptic reorganization and neuronal death (Xu et al., 2013).

Inflammatory processes in brain can affect the extracellular neuronal matrix (ECM) integrity. ECM plays a critical role in the modulation of AMPA receptor mobility, paired-pulse depression, L-type voltage-dependent Ca^{2+} channel activity and LTP processes. Noteworthy, an original study published in this topic, suggests that changes in the expression of Hyaluronic acid, the

major component of neuronal ECM, can lead to neuronal hyperexcitability and calcium dysregulation (Vedunova et al., 2013).

Neuronal cell death has been implicated as a causal factor leading to the development of the epileptic disorder. The findings reported in this topic support the idea that repeated seizures mediate neuronal necrosis and apoptosis prevalently associated to the activation of certain distinct anti/pro-apoptotic Bcl-2 family factors. Thus, epileptogenesis elicits apoptotic events by means of a specific pattern of Bcl-2 family proteins, which might represent a possible target of intervention to protect against the epileptic damage (Henshall and Engel, 2013).

Hormones play an important role in the epileptic disorders. Corticosteroids, progesterone, estrogens, and neurosteroids have been shown to affect seizure activity in animal models and in human. However, the impact of hormones on epileptogenesis is still underexplored and controversial. Further studies are required in the field to generate evidences on the therapeutic potential of hormonal agents in epileptogenesis (Reddy, 2013).

The circadian pattern of seizures is one of the first phenomena described in the epileptic disorders. However, due to the lack of promising hypotheses, has not attracted enough scientific attention. Recent findings provide novel insights in the implication of circadian rhythm in modulating transcription factors governing clock genes expression, and the mTOR signaling pathway, one of the most relevant signaling pathway in epilepsy (Cho, 2012).

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Received: 09 December 2013; accepted: 03 February 2014; published online: 21 February 2014.

Citation: Di Maio R (2014) Neuronal mechanisms of epileptogenesis. Front. Cell. Neurosci. ***8****:29. doi: 10.3389/fncel.2014.00029*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 29 August 2013
doi: 10.3389/fncel.2013.00130

Perirhinal cortex and temporal lobe epilepsy

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The perirhinal cortex—which is interconnected with several limbic structures and is intimately involved in learning and memory—plays major roles in pathological processes such as the kindling phenomenon of epileptogenesis and the spread of limbic seizures. Both features may be relevant to the pathophysiology of mesial temporal lobe epilepsy that represents the most refractory adult form of epilepsy with up to 30% of patients not achieving adequate seizure control. Compared to other limbic structures such as the hippocampus or the entorhinal cortex, the perirhinal area remains understudied and, in particular, detailed information on its dysfunctional characteristics remains scarce; this lack of information may be due to the fact that the perirhinal cortex is not grossly damaged in mesial temporal lobe epilepsy and in models mimicking this epileptic disorder. However, we have recently identified in pilocarpine-treated epileptic rats the presence of selective losses of interneuron subtypes along with increased synaptic excitability. In this review we: (i) highlight the fundamental electrophysiological properties of perirhinal cortex neurons; (ii) briefly stress the mechanisms underlying epileptiform synchronization in perirhinal cortex networks following epileptogenic pharmacological manipulations; and (iii) focus on the changes in neuronal excitability and cytoarchitecture of the perirhinal cortex occurring in the pilocarpine model of mesial temporal lobe epilepsy. Overall, these data indicate that perirhinal cortex networks are hyperexcitable in an animal model of temporal lobe epilepsy, and that this condition is associated with a selective cellular damage that is characterized by an age-dependent sensitivity of interneurons to precipitating injuries, such as *status epilepticus*.

Keywords: cholecystokinin, hippocampal formation, interneurons, neuropeptide Y, parvalbumin, perirhinal cortex, pilocarpine, temporal lobe epilepsy

BACKGROUND

The perirhinal cortex is a limbic structure that is closely interconnected with the lateral entorhinal cortex, the amygdala, and with unimodal and polymodal association cortices (Suzuki and Amaral, 1994; Burwell et al., 1995; Kealy and Commins, 2011). Hippocampal networks exchange information with the neocortex through the rhinal cortices (Van Hoesen, 1982; Naber et al., 1999; Kealy and Commins, 2011) (**Figure 1**), and it has been consistently demonstrated that the perirhinal cortex is intimately involved in learning and memory (Zola-Morgan et al., 1989; 1993; Murray et al., 1993; Suzuki et al., 1993; Suzuki, 1996; Weintrob et al., 2007; Kealy and Commins, 2011).

Knowledge on the memory functions of the perirhinal cortex has been obtained from patients presenting with temporal lobe epilepsy. Initial observations in patients undergoing epilepsy neurosurgery reported vivid recollection or sensation of familiarity known as *déjà vu* and *déjà vécu* when the temporal lobe was electrically stimulated (Penfield and Perrot, 1963; Bancaud et al., 1994). In addition, Bartolomei et al. (2004) found that similar experiential phenomena were elicited more frequently by stimulating the rhinal cortices than the amygdala or the hippocampus. Specifically, they reported that *déjà vu* was obtained following stimulation of the entorhinal cortex, whereas reminiscence of memories occurred during perirhinal cortex stimulation.

The perirhinal cortex has also been investigated for the potential contribution of this region to ictogenesis in the limbic system (McIntyre and Plant, 1989; Kelly and McIntyre, 1996). Pioneering investigations based on the kindling protocol identified the amygdala and the piriform cortex as major epileptogenic areas (Kelly and McIntyre, 1996). For this reason, McIntyre and his collaborators proposed an *in vitro* amygdala-piriform slice preparation to characterize the properties of these limbic areas. Because of the limited spontaneous epileptiform activity observed in the slice preparation, they challenged neuronal networks with a modified bathing medium, devoid of magnesium; this experimental procedure revealed a prominent epileptiform activity that was generated in the perirhinal cortex (McIntyre and Plant, 1989). These findings gave rise to a series of *in vivo* experiments demonstrating that: (i) the piriform cortex is not crucial in the spread of seizures originated in the hippocampus; (ii) the perirhinal cortex is kindled in a faster manner compared to other limbic regions and, above all, presents with the lowest latency to seizure spread

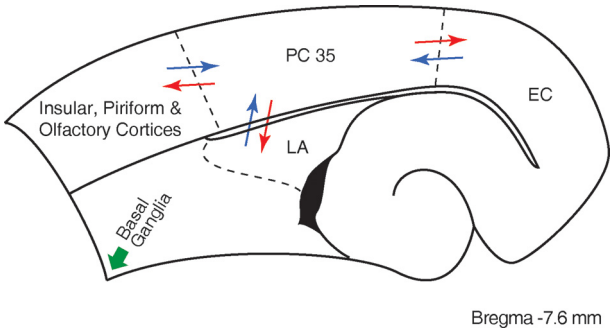

FIGURE 1 | Scheme of the main afferent/efferent connections of the perirhinal cortex (PC, area 35) under physiological conditions. The drawing corresponds to a section taken at 7.6 mm from the bregma according to the Paxinos and Watson (2007) atlas. Major afferents projections to the perirhinal cortex (blue arrows) originate from olfactory insular and piriform cortices, lateral amygdala (LA) and entorhinal cortex (EC); conspicuous efferent projections of the perirhinal cortex (red arrows) are directed to these areas as well. Note that subcortical efferents from the perirhinal cortex (green arrows) terminate in several brain regions, including the basal ganglia (cf., Furtak et al., 2007).

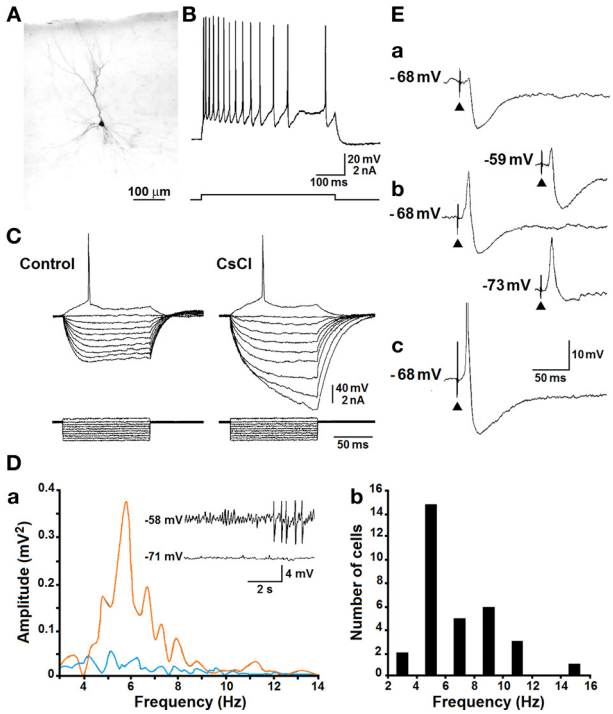

FIGURE 2 | (A) Photomicrograph of a neurobiotin-filled perirhinal cortex cell that corresponds to an upright pyramidal neuron (cf., Furtak et al., 2007). Scale bar, 100 µm. **(B)** Regular repetitive firing with adaptation is generated by a pyramidal-like neuron recorded in the perirhinal cortex during injection of a pulse of depolarizing current. **(C)** Effects induced by bath application of CsCl (3 mM) on the voltage responses to intracellular current pulses; note that under control conditions stepwise hyperpolarization of the membrane leads to the appearance of a sag toward the resting level as well as that addition of CsCl to the bath increases the neuronal input resistance and abolishes the sag. Bottom traces represent current monitor. **(D)** Power spectra of the intracellular signals recorded in the cell shown in the insets at two different levels of depolarization are illustrated in **(a)**; note that the spectrum obtained from the signal recorded at −58 mV (orange line) is characterized by a peak at ∼5.5 Hz. In **(b)**, histogram of the peak frequencies of membrane oscillations recorded in 29 perirhinal cortical neurons. **(E)** Responses recorded intracellularly from a perirhinal cortex neuron following local single-shock extracellular stimuli of progressively increasing strength (from **a** to **c**). Inserts in panel **(b)** illustrate the intracellular responses recorded during injection of depolarizing (−59 mV trace) or hyperpolarizing (−73 mV trace) current.

to frontal cortex motor areas; and (iii) the posterior region of the perirhinal cortex is critical to the propagation of hippocampal seizures (Kelly and McIntyre, 1996).

Compared to other limbic areas, the perirhinal cortex remains overlooked, and in particular detailed information on its dysfunctional characteristics are scarce. Over the last decade, however, some studies have begun to unveil the fundamental electrophysiological properties and the morphological features of perirhinal cortex cells (Bilkey and Heinemann, 1999; Faulkner and Brown, 1999; Beggs et al., 2000; D'Antuono et al., 2001; Furtak et al., 2007). In addition, new pathophysiological roles for this limbic structure in epileptogenesis and ictogenesis are emerging. Our paper is aimed at: (i) reviewing the electrophysiological characteristics of neurons that are recorded in the perirhinal cortex in an *in vitro* slice preparation; (ii) summarizing data regarding the ability of perirhinal cortex neuronal networks to generate epileptiform discharges when challenged with acute epileptogenic pharmacological procedures; (iii) highlighting the changes in neuronal excitability that occur in the pilocarpine model of temporal lobe epilepsy; and (iv) elucidating the contribution of selective interneuron subtype damage in promoting epileptogenesis.

FUNDAMENTAL INTRINSIC AND SYNAPTIC PROPERTIES

Intracellular studies performed in the perirhinal cortex have shown that neurons include fast-spiking, burst-spiking and regular-spiking cells (Kelly and McIntyre, 1996; Faulkner and Brown, 1999; Kealy and Commins, 2011). In addition, Beggs et al. (2000) have described late-spiking pyramidal cells that are capable of generating delayed action potential discharges, and proposed that these neurons may play a role in encoding "long-time intervals" during associative learning. By employing sharp intracellular recordings (D'Antuono et al., 2001; Benini et al., 2011), we found that most of the neurons recorded in the perirhinal cortex correspond morphologically to spiny pyramidal cells and are regularly firing (**Figures 2A,B**). These neurons generate several types of sub-threshold responses during injection of intracellular current pulses including: (i) tetrodotoxin-sensitive inward rectification in the depolarizing direction (not illustrated) and (ii) Cs^+-sensitive inward rectification during injection of hyperpolarizing current pulses (**Figure 2C**). In addition, the repetitive firing generated by these neurons is characterized by adaptation and is followed by a slow after-hyperpolarization upon termination of the depolarizing current pulse (**Figure 2B**). We have also found that both phenomena are greatly reduced by application of Ca^{2+} channel blockers, indicating that Ca^{2+}-activated K^+ conductances play an important role in controlling the intrinsic excitability of pyramidal cells in the perirhinal cortex. These intrinsic properties are indeed similar to those demonstrated in

principal cells recorded intracellularly in several cortical structures (Constanti and Galvan, 1983; Stafstrom et al., 1985; Spain et al., 1987; Mattia et al., 1997).

Pyramidal neurons in the perirhinal cortex are also capable of generating voltage-gated, subthreshold membrane oscillations at 5–12 Hz during steady injection of depolarizing current (Bilkey and Heinemann, 1999). As illustrated in **Figure 2Da** (inserts), when neurons were recorded at resting membrane potential (more negative than −70 mV), no significant oscillatory activity was observed; however, when they were depolarized with injection of steady intracellular current, sinusoidal-like oscillations became evident along with "clustered" or "tonic" action potential firing. This phenomenon is further identifiable in the power spectrum of the intracellular signals recorded at −70 and −58 mV (**Figure 2Da**), while the plot histogram in **Figure 2Db** summarizes the peak frequencies of the subthreshold membrane oscillations recorded from several perirhinal cortical cells. It should be emphasized that as reported in entorhinal cortex or subicular cells (Alonso and Llinas, 1989; Mattia et al., 1997), this voltage-dependent oscillatory activity persisted during blockade of glutamatergic and γ-aminobutyric acid (GABA)ergic transmission with specific receptor antagonists as well as during application of Ca^{2+} channel blockers. However, it disappeared during application of tetrodotoxin suggesting that voltage-gated Na^{+} electrogenesis contributes to this oscillatory phenomenon.

As shown in **Figure 2E**, perirhinal principal cells generate synaptic potentials with polarity and amplitude that depend on the intensity of the extracellular stimulus; thus, stimuli at threshold strength (**Figure 2Ea**) often induced a hyperpolarizing inhibitory postsynaptic potential (IPSP) while, at progressively higher intensities, an excitatory postsynaptic potential (EPSP)-IPSP sequence (**Figure 2Eb**) and eventually an EPSP-single action potential (**Figure 2Ec**) occurred. Moreover, these responses changed in amplitude during injection of depolarizing or hyperpolarizing current (**Figure 2Eb**) and the early hyperpolarizing component of the IPSP was characterized by reversal potential values at approximately −80 mV (not illustrated).

Overall these findings indicate that the intrinsic properties of principal cells in the perirhinal cortex reproduce those reported for cortical pyramidal cells in several areas of the brain. The presence of fast-spiking cells (Faulkner and Brown, 1999) that are known to release GABA is mirrored by the ability of principal neurons in the perirhinal cortex to generate robust inhibitory responses both spontaneously and following electrical stimuli (Benini et al., 2011).

EPILEPTIFORM SYNCHRONIZATION *in vitro*

Experiments performed *in vitro* in extended brain slices comprising the hippocampus along with the entorhinal and perirhinal cortices have shown that interictal and ictal discharges are generated during bath application of the convulsant drug 4-aminopyridine or Mg^{2+}-free medium (de Guzman et al., 2004). These epileptiform patterns were only identified after severing the connections between these parahippocampal areas and the hippocampus; such a procedure abolished the propagation of CA3-driven fast interictal discharges that controlled the propensity of parahippocampal neuronal networks to generate "slow" interictal events along with prolonged ictal discharges (see for review, Avoli and de Curtis, 2011). As illustrated in **Figure 3A**, the epileptiform events recorded under control conditions from the entorhinal and perirhinal cortices occurred synchronously in these two areas, could initiate from any of them, and propagated to the neighboring structure with delays ranging from 8 to 66 ms. However, cutting the connections between entorhinal and perirhinal cortices generated independent epileptiform activity in both structures (**Figure 3A**, EC/PC cut); interestingly, these procedures shortened ictal discharge duration in the entorhinal but not in the perirhinal cortex. These experiments have also demonstrated that network synchronization underlying ictogenesis in the perirhinal cortex is N-Methyl-D-aspartate (NMDA) receptor-dependent (de Guzman et al., 2004).

We have recently reported that 4-aminopyridine-induced ictal discharges in the rat entorhinal cortex are preceded by an isolated "slow" interictal discharge or suddenly initiate from a pattern of frequent polyspike interictal discharges; only rarely ictal discharge onset was characterized by an acceleration of interictal event rates (Avoli et al., 2013). These findings contrast with what has been observed in the perirhinal cortex since retrospective analysis of the experiments published by de Guzman et al. (2004) indicates that in this area approximately half of the slices treated with 4-aminopyridine presented with ictal discharge onset characterized by acceleration of interictal events (**Figure 3Ba**) while in the remaining experiments ictal discharges are preceded by a "slow" interictal discharge (**Figure 3Bb**). These electrographic

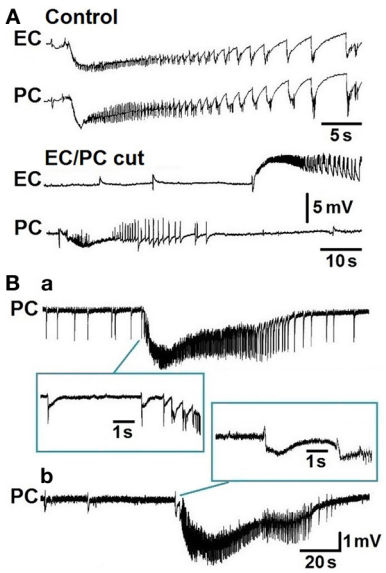

FIGURE 3 | (A) Ictal discharges occur synchronously in entorhinal (EC) and perirhinal (PC) cortices in an *in vitro* brain slice treated with 4-aminopyridine (Control panel). Note that cutting the connections between entorhinal and perirhinal areas causes the occurrence of independent ictal activity in these two structures (EC/PC cut panel). **(B)** Two types of ictal discharge initiation can be recorded from the perirhinal cortex during bath application of 4-aminopyridine. Note that the ictal discharge shown in **(a)** is characterized by acceleration of the interictal events preceding its onset while that in **(b)** is characterized by a "slow" interictal event.

characteristics are reminiscent of the hypersynchronous onset and of the low-voltage, fast activity onset patterns, respectively, that have been reported to occur *in vivo* in both epileptic patients (Velasco et al., 2000; Ogren et al., 2009) and animal models (Bragin et al., 1999, 2005; Lévesque et al., 2012, 2013).

Overall, these *in vitro* data indicate that the perirhinal cortex may be more prone to generate ictal discharges as compared with the entorhinal cortex. In line with this view, *in vivo* studies have shown that kindling within the perirhinal cortex promotes seizure activity more rapidly than stimulation of the piriform cortex, amygdala or dorsal hippocampus (McIntyre et al., 1993, 1999; Sato et al., 1998). Moreover, lesioning the perirhinal cortex (Kelly and McIntyre, 1996; Fukumoto et al., 2002) or applying glutamatergic receptor antagonists (Tortorella et al., 1997) or adenosine A1 receptor agonists (Mirnajafi-Zadeh et al., 1999) to the perirhinal cortex attenuated and even prevented the appearance of seizure activity following amygdala kindling.

CHANGES IN EXCITABILITY IN PILOCARPINE-TREATED EPILEPTIC RATS

By using *in vitro* electrophysiological recordings we have recently reported that brain slices obtained from pilocarpine-treated epileptic rats present with remarkable changes in synaptic excitability when compared to age-matched, non-epileptic controls (Benini et al., 2011). The pilocarpine model of temporal lobe epilepsy—which consists of an initial *status epilepticus* induced by i.p. injection of this cholinergic agonist that is followed 1–4 weeks later by a chronic condition of recurrent limbic seizures—is presumably the most commonly used model for studying this epileptic disorder (Curia et al., 2008). It provides the opportunity of controlling epilepsy severity and associated brain damage by pharmacologically regulating the duration of the initial *status epilepticus*. Moreover, in contrast to other chronic epilepsy models, spontaneous seizures recur frequently and consistently in virtually all pilocarpine-treated rats.

Neurons recorded intracellularly from the deep layers of the perirhinal cortex of non-epileptic control and pilocarpine-treated animals had similar intrinsic and firing properties (Benini et al., 2011). Moreover, they generated spontaneous depolarizing and hyperpolarizing postsynaptic potentials with comparable duration and amplitude. However, spontaneous and stimulus-induced epileptiform discharges could be recorded with field potential and intracellular recordings in over one-fifth of pilocarpine-treated slices but never in control tissue (**Figures 4A,B**). These network events were reduced in duration by antagonizing NMDA receptors, and abolished by concomitant application of NMDA and non-NMDA glutamatergic receptor antagonists (Benini et al., 2011).

As illustrated in **Figure 4C**, electrical stimuli delivered during blockade of glutamatergic transmission induced IPSPs in perirhinal neurons recorded in both control and pilocarpine-treated brain slices. However, analysis of these stimulus-induced IPSPs revealed that the reversal potential of the early, $GABA_A$-receptor-mediated component was significantly more depolarized in pilocarpine-treated vs. control cells (**Figure 4D**) while no difference in peak conductance was identified (Benini et al., 2011). These differences are presumably caused by a decrease in the expression of the potassium-chloride cotransporter 2 that leads to a dysfunction in the balance of intracellular chloride. Indeed, we have found that immunoreactivity for the potassium-chloride cotransporter 2 is consistently lower in pilocarpine-treated epileptic rats, both in the perirhinal cortex (Benini et al., 2011) and in other parahippocampal regions (de Guzman et al., 2006).

INTERNEURONS ARE SELECTIVELY DAMAGED IN THE PILOCARPINE MODEL OF TEMPORAL LOBE EPILEPSY

Substantial damage to perirhinal cortex has been reported in an animal model based on electrically induced *status epilepticus* (Bumanglag and Sloviter, 2008). However, injury to this limbic area has rarely been documented in rats treated with lithium-pilocarpine, in which neuronal cell counts were similar to control animals or non-significantly decreased by ∼ 10% (André et al., 2000); in this study, neuronal damage in the perirhinal cortex became evident only when rats were exposed to electroshocks preceding the lithium-pilocarpine treatment (André et al., 2000). We were also unable to demonstrate any consistent damage to the perirhinal cortex in rats exposed to various durations of pilocarpine-induced *status epilepticus* (Benini et al., 2011; Gualtieri et al., 2012), a finding further confirmed by staining necrotic cells with Fluoro-Jade (**Figure 5**) (Biagini et al., 2005, 2008). These experimental findings are at odds with clinical data showing that the perirhinal cortex presents with consistent asymmetries when the region ipsilateral to the sclerotic hippocampus is compared with the contralateral (Bernasconi et al., 2000, 2003; Salmenperä et al., 2000; Jutila et al., 2001; O'Brien et al., 2003; Alessio et al., 2006; Guedj et al., 2010). These discrepancies may have several explanations. For instance, it should be considered that the time span between the precipitating injury and histopathological analyses is much shorter in experimental models than in clinical studies; hence, if cell damage in the perirhinal cortex requires more time than in the hippocampus or entorhinal cortex, most animal models would probably fail in detecting these changes.

Interestingly, a different scenario emerges when specific cell types are analyzed in the perirhinal cortex of pilocarpine-treated epileptic rats. We have reported that ∼ 20 weeks after pilocarpine treatment parvalbumin (PV), neuropeptide Y (NPY) and cholecystokinin (CCK) immunopositive interneurons are significantly decreased in adult rats (Benini et al., 2011). The loss of interneurons in absence of a corresponding reduction in principal cells may lead to a profound alteration in the functional characteristics of this brain structure (de Guzman et al., 2006, 2008; Benini et al., 2011). Whereas in control tissue cells immunopositive for PV were clearly prevalent (68% of all stained interneurons) when compared with other putative interneurons (25 and 7% of all stained interneurons were NPY or CCK immunopositive, respectively), in pilocarpine-treated rats PV immunopositive cells decreased to 56% of all stained interneurons, whereas NPY and CCK immunopositive cells increased to 32 and 12%, respectively (Benini et al., 2011). Further evaluation of these interneuronal subpopulations also revealed that the superficial perirhinal cortical layers of pilocarpine-treated rats contain more interneurons than analogous layers in control non-epileptic rats

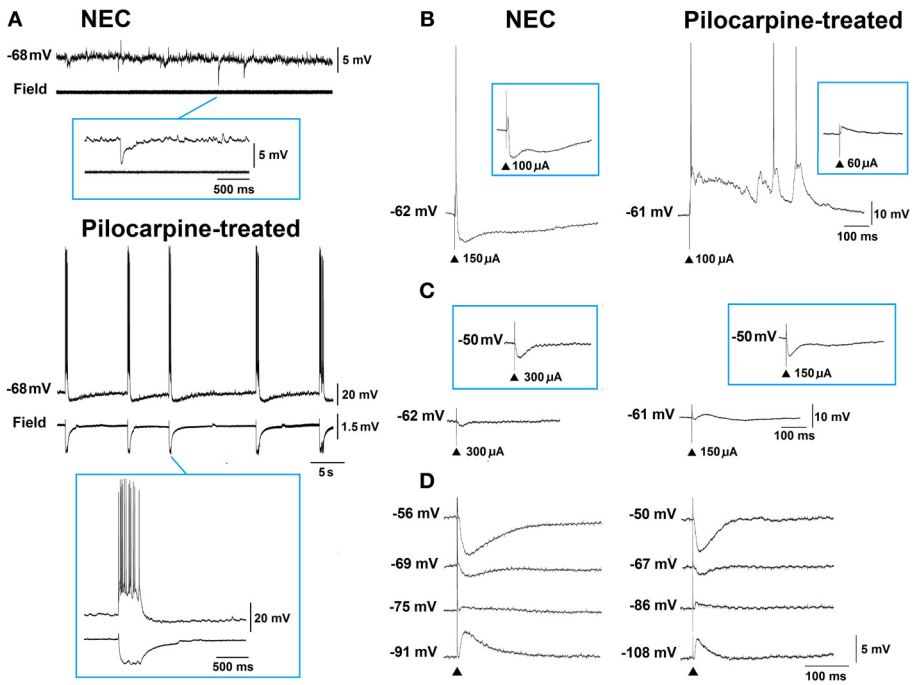

FIGURE 4 | (A) Simultaneous field and intracellular recordings (−68 mV) in brain slices obtained from non-epileptic control (NEC) and pilocarpine-treated epileptic animals. Note in the NEC recording the presence of depolarizing and hyperpolarizing postsynaptic potentials (PSPs) while robust epileptiform activity occurs in the pilocarpine-treated experiment; expansion of the events is shown in the inserts. **(B)** and **(C)** Intracellular responses generated by perirhinal cortex neurons to local single-shock stimulation under control conditions and during blockade of glutamatergic transmission, respectively. Inserts illustrate the responses obtained by stimuli of lower strength **(B)** or during intracellular injection of steady depolarizing current **(C)**. **(D)** Intracellular responses induced by local single-shock stimulation in the presence of glutamatergic receptor antagonists. In this experiment the early γ-aminobutyric acid type A ($GABA_A$) receptor-mediated component of the evoked inhibitory PSP was found to have a more depolarized reversal potential in the pilocarpine-treated neuron (−69.5 mV) as compared to the non-epileptic control cell (−74.7 mV).

(53% vs. ∼ 48% of all stained interneurons) (Benini et al., 2011). These data are in line with those obtained from other limbic regions of epileptic rats, such as the hippocampal CA1 subfield (André et al., 2001) and the dentate hilus (Gorter et al., 2001). Interestingly, PV immunopositive cells are also decreased in the neocortex (DeFelipe et al., 1993) and hippocampus (Arellano et al., 2004) of epileptic patients presenting with intractable seizures. Inhibitory networks within the perirhinal and entorhinal cortices confer these structures with the ability to actively gate signal transmission between the neocortex and the hippocampus (Biella et al., 2002; de Curtis and Paré, 2004; Pelletier et al., 2004). These functional characteristics may be relevant for controlling the spread of epileptiform activity within the limbic system and for understanding the role played by decreased inhibition in the perirhinal cortex of epileptic patients and animals.

Recently, we have also studied interneuron damage in rats exposed to pilocarpine-induced *status epilepticus* at 3 weeks of age; these animals are more resistant to damage and have a tendency to develop chronic seizures of lower severity, compared with 8-week-old rats (Biagini et al., 2008). Results obtained from adult rats confirmed our previous findings regarding a decrease in PV-positive cells (**Figure 6**; cf., Benini et al., 2011). In addition, although rats exposed to pilocarpine-induced *status epilepticus* at 3 weeks of age were less prone to develop neuronal damage (Biagini et al., 2008), we found approximately a 50% decrease in PV interneurons 3 days after the pilocarpine administration; such reduction was maintained in the following time intervals of 7 and 14 days (**Figures 6A–D**), suggesting that this interneuron subtype is very sensitive to damage and that this occurs independently of brain maturation.

At variance with PV-immunopositive cells, CCK interneurons in the perirhinal cortex showed transitory changes in 3-week-old rats. This phenomenon, which may reflect functional adaptation to *status epilepticus* rather than cell damage, was limited to young rats whereas adult rats presented merely with loss of interneurons. As shown in **Figures 7A–D**, interneurons stained by an antibody against CCK (Benini et al., 2011; Gualtieri et al., 2013) were significantly ($p < 0.01$) reduced in 3-week-old rats at day 3 after pilocarpine treatment, but counts of these interneurons were comparable to control values at days 7 and 14 after *status epilepticus*. This finding could be related to a transient impairment in CCK synthesis or to an increased release. In contrast with the time course observed in the young group of animals, 8-week-old rats presented a strong reduction in CCK immunopositive cells to ∼ 20% of control values, which was found at every considered time point (**Figure 7D**). Interestingly, these results highlight a different age-related sensitivity of CCK interneurons to

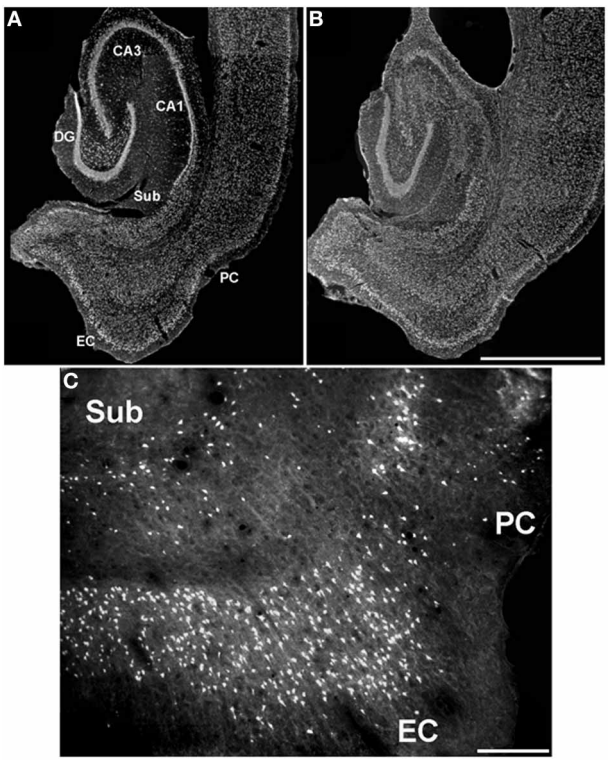

FIGURE 5 | Characterization of the neuronal damage occurring in the hippocampus and in parahippocampal structures in pilocarpine-treated rats. Rats were treated and processed as described in Biagini et al. (2005). **(A** and **B)** dark field photomicrographs of Nissl-stained brain sections obtained from a non-epileptic control rat and a pilocarpine-treated rat, respectively. Note that remarkable damage is localized to the Cornu Ammonis (CA) subfield 1 (CA1), hilus of the dentate gyrus (DG), layer III of medial entorhinal cortex (EC), whereas the subiculum (Sub) and perirhinal cortex (PC) are apparently spared. **(C)** Section stained by Fluoro-Jade to identify dying cells shows the presence of necrotic neurons also in the perirhinal cortex, but to a lower extent than in the entorhinal cortex. Scale bars, 1,900 μm for **(A)** and **(B)**, 200 μm for **(C)**.

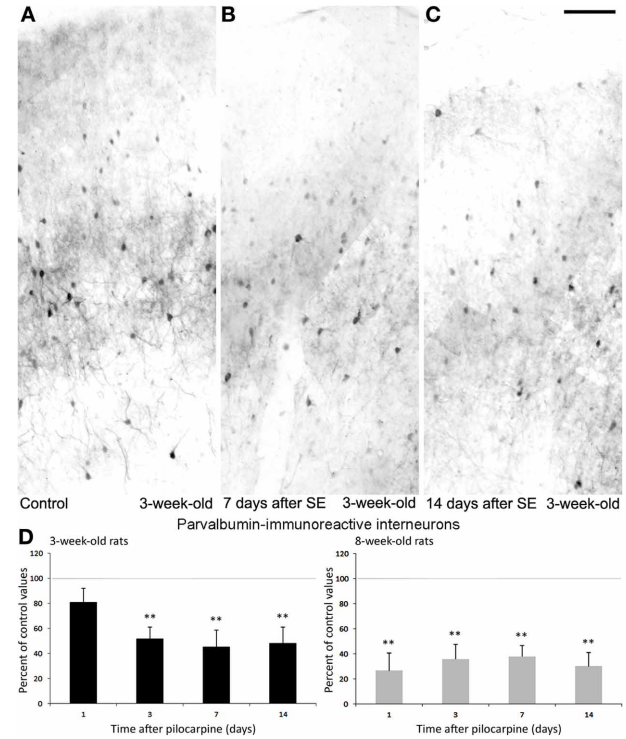

FIGURE 6 | Parvalbumin (PV)-immunopositive interneurons in the rat perirhinal cortex. Photomicrographs showing interneurons stained with an antibody against PV in the perirhinal cortex of 3-week-old rats **(A–C)**. Specifically, PV immunostaining is shown in a control, non-epileptic rat **(A)**, and in pilocarpine-treated rats 7 **(B)** and 14 days **(C)** after pilocarpine treatment. **(D)** Normalized (respect to control) quantification of PV immunostained neurons in 3 and 8-week-old rats following pilocarpine treatment. Note in the 3-week-old animals ($n = 3$–6 for each time interval) that PV immunostained neurons decrease significantly 3 days as well as 7 and 14 days later. Note that similar findings were observed in 8-week-old rats ($n = 4$–5 for each time interval). $^{**}p < 0.01$, analysis of variance followed by Tukey's test for multiple comparisons. Scale bar: 100 μm. Animal treatment is described in Biagini et al. (2008). Details of the immunostaining procedure and cell counts are in de Guzman et al. (2006, 2008); Bortel et al. (2010) and Benini et al. (2011).

pilocarpine-induced *status epilepticus* by confirming an enhanced resilience to damage in young animals. It remains to be established, however, whether this difference could be related to the lower propensity of young rats to develop recurrent generalized seizures when exposed to *status epilepticus*, in contrast to what observed in adult animals (Biagini et al., 2008).

As illustrated in **Figures 8A–D**, interneurons expressing NPY transiently increased 1 day after *status epilepticus* induction both in young and adult rats, but in the latter group the changes were not large enough to be statistically significant. In young rats, counts of NPY interneurons decreased to basal values a week later and were maintained at normal levels also 14 days after pilocarpine treatment (**Figure 8C**). Thus, the transient, functional changes occurring in the counts of NPY interneurons may be related to an increased synthesis of this neuropeptide, or to a block of its release. In contrast to these findings, adult rats presented a steady decrease of NPY interneurons to less than 50% of basal values (**Figure 8D**), thus confirming our previous observations (Benini et al., 2011). These age-dependent discrepancies further support the hypothesis that acute functional changes occur, as in the case of CCK interneurons, also for other interneuronal subpopulations. In addition, this evidence underscores age-related differences; specifically, interneurons are more preserved after exposure to *status epilepticus* in young rats than in adult animals.

Changes in the ratio of interneurons and principal cells may play a role in epileptogenesis, as suggested by Tuunanen et al. (1997). However, we recently obtained evidence of a similar selective alteration in subpopulations of interneurons with limited changes in neuronal excitability in the insular cortex (Bortel et al., 2010). The differences found in the various subpopulations of interneurons between the two groups of young, 3-week-old rats and adult animals may also confirm the involvement of interneuron loss in epileptogenesis (Tuunanen et al., 1997; Gorter et al., 2001). Young animals with preserved CCK and NPY

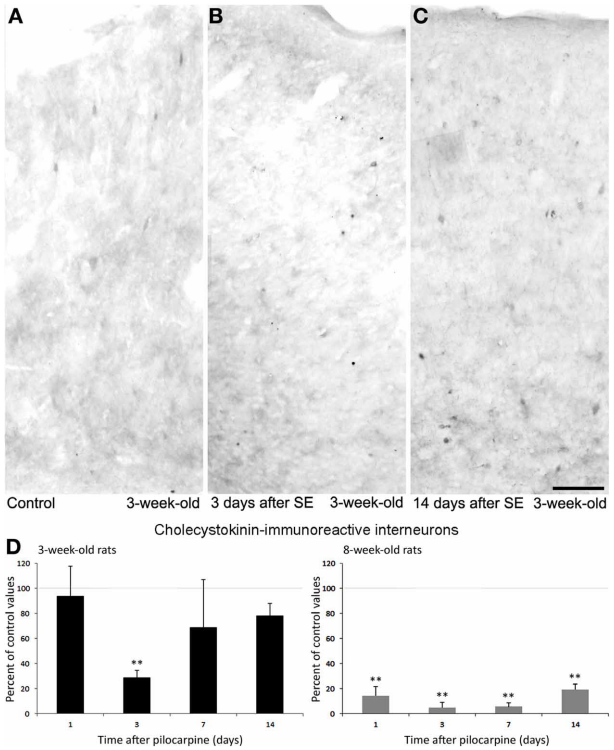

FIGURE 7 | Cholecystokinin (CCK)-immunopositive interneurons in the rat perirhinal cortex. Photomicrographs showing interneurons stained with an antibody against CCK in the perirhinal cortex of 3-week-old rats **(A–C)**. Specifically, CCK immunostaining in a control, non-epileptic rat, **(A)** and in pilocarpine-treated rats 3 **(B)** and 14 days **(C)** after pilocarpine treatment are respectively shown. **(D)** Normalized (respect to control) quantification of CCK immunostained neurons in 3 and 8-week-old rats following pilocarpine treatment. Note in the 3-week-old animals ($n = 3$–6 for each time interval) that CCK immunostained neurons decrease significantly 3 days after pilocarpine but recovered 7 and 14 days later. Note also that a consistent decrease in CCK-positive neurons was observed in 8-week-old rats ($n = 4$–5 for each time interval). $^{**}p < 0.01$, analysis of variance followed by Tukey's test for multiple comparisons. Scale bar: 100 μm. Animal treatment is described in Biagini et al. (2008). Details of the immunostaining procedure and cell counts are as in Benini et al. (2011) and Gualtieri et al. (2013).

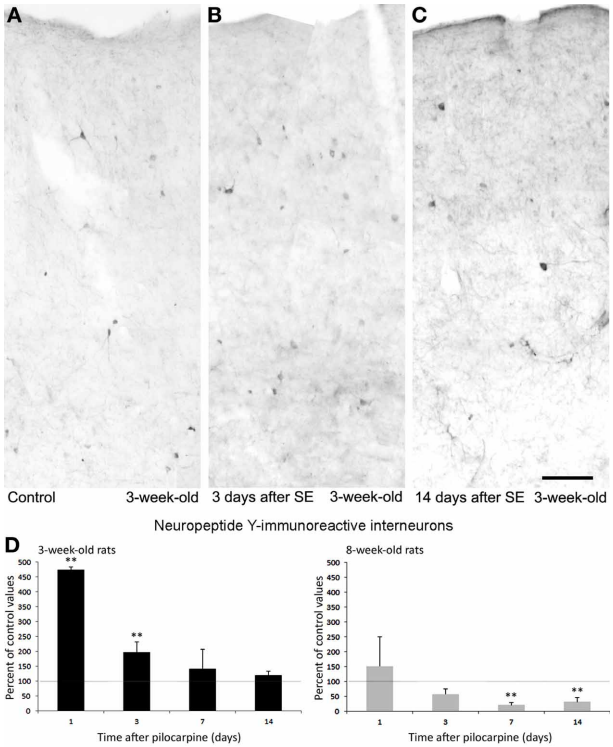

FIGURE 8 | Neuropeptide Y (NPY)-immunopositive interneurons in the rat perirhinal cortex. Photomicrographs showing interneurons stained with an antibody against NPY in the perirhinal cortex of 3-week-old rats **(A–C)**. Specifically, NPY immunostaining is shown in a control, non-epileptic rat **(A)** and in pilocarpine-treated rats 3 **(B)** and 14 days **(C)** after pilocarpine treatment. **(D)** Normalized (respect to control) quantification of NPY immunostained neurons in 3 and 8-week-old rats following pilocarpine treatment. Note in the 3-week-old animals ($n = 3$–6 for each time interval) that NPY immunostained neurons increased significantly 1 and 3 days after pilocarpine, but recovered 7 and 14 days later. Note that a consistent decrease in NPY interneurons was instead observed in 8-week-old rats ($n = 4$–5 for each time interval). $^{**}p < 0.01$, analysis of variance followed by Tukey's test for multiple comparisons. Scale bar: 100 μm. Animal treatment is described in Biagini et al. (2008). Details of the immunostaining procedure and cell counts are as in Bortel et al. (2010); Benini et al. (2011) and Gualtieri et al. (2013).

interneurons (**Figures 7**, **8**) were less prone to develop generalized convulsive seizures than adult rats (Biagini et al., 2008), in which the same interneurons were markedly decreased. Therefore, it is likely that these interneuronal subclasses contribute to maintain the perirhinal cortex under a rather physiological condition. In contrast, since similar levels of PV interneuron loss were found in young and adult rats exposed to pilocarpine-induced *status epilepticus*, we are inclined to hypothesize a less critical role of these interneurons in modulating the propensity of perirhinal cortex neuronal networks to generate chronic seizure following the initial *status epilepticus* induced by pilocarpine.

CONCLUSIVE REMARKS

Perhaps, one of the most essential developments in temporal lobe epilepsy research in the last few years has been the recognition that the pathophysiological substrates underlying this neurological disorder extend beyond the hippocampus to involve not only extrahippocampal but extratemporal structures as well (De Carli et al., 1998; Lee et al., 1998; Sandok et al., 2000; Dreifuss et al., 2001; Moran et al., 2001; Natsume et al., 2003; Seidenberg et al., 2005). Advances in neuroimaging techniques have revealed that volumetric reductions of the amygdala, entorhinal and perirhinal cortices do occur in a subset of patients affected by temporal lobe epilepsy in spite of normal hippocampal volumes (Cendes et al., 1993; Bernasconi et al., 1999, 2001, 2003; Salmenperä et al., 2000; Jutila et al., 2001). Histopathological examination of human epileptic tissue have also corroborated these findings by demonstrating the presence of selective neuronal loss and synaptic reorganization within these structures even in the absence of hippocampal sclerosis (Du et al., 1993; Hudson et al., 1993; Miller et al., 1994; Wolf et al., 1997; Mikkonen et al., 1998; Yilmazer-Hanke et al., 2000; Aliashkevich et al., 2003).

The data reported in this review provide evidence that supports the view that the perirhinal cortex may be implicated in the processes of epileptogenesis and ictogenesis. Our data showing a clear role of the perirhinal cortex in the onset of seizure-like discharges *in vitro*, indicate that this structure has the capacity to generate ictal events and to reproduce in several cases the so-called hypersynchronous seizure onset pattern. In addition, the decrease of specific interneuronal subclasses in the perirhinal cortex of pilocarpine-treated rats suggests that the impairment of perirhinal functions may have been overlooked by simply considering the gross anatomical changes that occur in this region in temporal lobe epilepsy patients. In this respect, it is enticing to propose that the main network function of the perirhinal cortex may consist of a strong inhibitory control exerted on the entorhinal cortex, presumably mediated by GABAergic neurons located in layers III and IV of areas 35 and 36 (Apergis-Schoute et al., 2007). The prominent decrease of interneurons stained by PV, CCK and NPY antibodies observed in the perirhinal cortex of epileptic rats, including its superficial layers (Benini et al., 2011), could have a very important role in determining the hyperexcitability consistently observed in the entorhinal cortex of pilocarpine-treated rodents (D'Antuono et al., 2002; de Guzman et al., 2008; Panuccio et al., 2010). Further exploration of these brain regions is necessary for identifying their specific roles in the initiation and spread of seizures in temporal lobe epilepsy. Finally, it is worthwhile to mention that a detailed assessment of extrahippocampal structures in temporal lobe epilepsy might help to increase our understanding of the mechanisms underlying the pathophysiology of this neurological disorder as well as the functional changes that occur within these limbic areas during epileptogenesis.

ACKNOWLEDGMENTS

This investigation has been supported by the Canadian Institutes of Health Research (Grant MOP-8109) and the Mariani Foundation (R-06-50; Milan, Italy; http://www.fondazione-mariani.org).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 April 2013; accepted: 01 August 2013; published online: 29 August 2013.

Citation: Biagini G, D'Antuono M, Benini R, de Guzman P, Longo D and Avoli M (2013) Perirhinal cortex and temporal lobe epilepsy. Front. Cell. Neurosci. 7:130. doi: 10.3389/fncel.2013.00130

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 16 July 2013
doi: 10.3389/fncel.2013.00109

Homeostatic control of brain function – new approaches to understand epileptogenesis

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Neuronal excitability of the brain and ongoing homeostasis depend not only on intrinsic neuronal properties, but also on external environmental factors; together these determine the functionality of neuronal networks. Homeostatic factors become critically important during epileptogenesis, a process that involves complex disruption of self-regulatory mechanisms. Here we focus on the bioenergetic homeostatic network regulator adenosine, a purine nucleoside whose availability is largely regulated by astrocytes. Endogenous adenosine modulates complex network function through multiple mechanisms including adenosine receptor-mediated pathways, mitochondrial bioenergetics, and adenosine receptor-independent changes to the epigenome. Accumulating evidence from our laboratories shows that disruption of adenosine homeostasis plays a major role in epileptogenesis. Conversely, we have found that reconstruction of adenosine's homeostatic functions provides new hope for the prevention of epileptogenesis. We will discuss how adenosine-based therapeutic approaches may interfere with epileptogenesis on an epigenetic level, and how dietary interventions can be used to restore network homeostasis in the brain. We conclude that reconstruction of homeostatic functions in the brain offers a new conceptual advance for the treatment of neurological conditions which goes far beyond current target-centric treatment approaches.

Keywords: adenosine, glial cells, ketogenic diet, mitochondrial bioenergetics and physiology, DNA methylation, transmethylation pathway, epileptogenesis, homeostasis

INTRODUCTION

Epileptogenesis is a complex process that not only involves changes in neuronal excitability and circuitry, but also changes in glial physiology and in the homeostatic environment in which neurons need to survive and to function properly (Kunz, 2002; Borges et al., 2003; David et al., 2009; Ravizza et al., 2011; Devinsky et al., 2013). Characterized by abnormal and excessive neuronal firing, each seizure represents a rapid loss of homeostatic equilibrium, with altered energy and molecular gradients, and a corresponding interruption of normal behavior and consciousness. Because having a seizure can increase the likelihood of future seizures, seizures themselves contribute to epileptogenesis. Similarly, conditions that can precipitate epilepsy – such as traumatic brain injury, and diseases in which epilepsy can be comorbid – such as Alzheimer's disease, are accompanied by a chronic loss of homeostatic function. Therefore, the loss of homeostasis associated with epilepsy is found acutely during the seizure or precipitating event, and also during the chronic process of epileptogenesis.

Unfortunately, the pursuit of neurocentric therapeutic targets did not yield any antiepileptogenic therapies to date (Loscher and Brandt, 2010). In contrast, a revised understanding of epilepsy as a complex syndrome of disrupted network homeostasis may yield novel therapeutic avenues to halt, disrupt, or even reverse the process of epileptogenesis. Key elements to consider with the goal of restoring network homeostasis are glial function and metabolism. Akin to seizures themselves, which have negative acute and chronic effects, restoring homeostasis can benefit acute brain function and avert the progressive process of epileptogenesis.

Glial cells play a major role in the homeostatic state of the brain by regulating the ambient concentration of synaptic neurotransmitters; modulating the permeability of the blood brain barrier (BBB) through astrocyte–endothelial interactions; regulating cerebral blood flow; and microglial control of brain immunity. Thereby, glial cells directly influence brain function on multiple levels including neuronal excitability and synaptic transmission; delivering energy substrates from the periphery; and recovery from injury or infection (Eulenburg and Gomeza, 2010; Kofler and Wiley, 2011; Petzold and Murthy, 2011; Santello et al., 2012). As a consequence, disruptions to normal glial cell function as observed in neurological disorders with a gliotic pathology has widespread deleterious ramifications that contribute to disease progression and maintenance through changes in synaptic activity, BBB permeability, brain immunity, and inflammation (Carmignoto and Haydon, 2012; Coulter and Eid, 2012; Kovacs et al., 2012).

Within human epileptic foci the most prominent pathological finding is gliosis, with reports of reactive astrocytes, microglia, glial scars, and/or gliomas being present (Kallioinen et al., 1987; Kurzwelly et al., 2010; Butler et al., 2013). Pathological glial cells

have been associated with a spectrum of different neurological diseases that result in epilepsy including mesial temporal lobe epilepsy with hippocampal sclerosis (mTLE), focal cortical dysplasia (FCD), tuberous sclerosis complex (TSC), and Rasmussen's encephalitis (Sosunov et al., 2008; Malmgren and Thom, 2012; Butler et al., 2013). Concurrent with a gliotic pathology, studies from both epileptic patients and rodent models for epilepsy have identified abnormal glial cell activity as a contributing factor to seizures and/or epileptogenesis.

Alongside gliosis, it is increasingly appreciated that epilepsy is a global dysregulation involving metabolic dysfunction (DiMauro et al., 2002; Kunz, 2002), and, furthermore, that metabolic dysfunction is common in neurological disorders including neurodegenerative (Sas et al., 2007) and psychiatric disorders (Rezin et al., 2009). The ketogenic diet, developed as a treatment for epilepsy nearly 100 years ago, is a highly successful metabolic strategy now moving broadly into translational work for a variety of neurological disorders. Multiple lines of evidence suggest that key mechanisms underlying the acute anticonvulsant effects of a ketogenic diet may be adenosine acting via K_{ATP} channels. These findings highlight the potential for altered metabolism restoring and maintaining homeostasis in the central nervous system (CNS), and have implications for exploring the prevention and treatment of neurological disorders using strategies other that traditional neurocentric approaches.

Here we outline homeostatic therapeutic strategies with a focus on adenosine, an endogenous bioenergetic homeostatic regulator; multiple lines of evidence suggest that adenosine homeostasis is a key factor in preventing and stopping seizures. This review will first discuss glial mechanisms of epileptogenesis with emphasis on disruptions of adenosine homeostasis. Based on these mechanisms, we identify glia-centric therapeutic strategies that treat epilepsy through restoring normal brain homeostasis. We then highlight recent evidence regarding the role of adenosine in the process of epileptogenesis and describe metabolic therapy via a ketogenic diet, which may restore adenosine homeostasis.

GLIAL MECHANISMS IN EPILEPTOGENESIS

Historically epilepsy research has predominantly focused on disruptions to normal neuronal function as the primary etiology. However, there is a substantial amount of evidence that implicate glial dysfunction as a major contributing factor to epileptogenesis (**Figure 1**). Astrocytes regulate or modulate a number of neuronal functions including excitability, synaptic transmission, and plasticity. As a consequence, the presence of reactive astrocytes, as found in patients with mTLE, FCD, and TSC, disrupts normal neuronal activity that either promotes epileptogenesis or decreases seizure threshold (Sosunov et al., 2008; Miyata et al., 2013). Multiple mechanisms by which reactive astrocytes may directly modulate neuronal activity at the synaptic cleft have been proposed. These include, but are not limited to (i) increases in neuronal excitability caused by decreased adenosine tone; increased synaptic glutamate levels; changes in the extracellular space (ECS) volume and K^+ ion concentration; and (ii) modulation of synaptic transmission through glutamate, adenosine triphosphate (ATP), adenosine, gamma-aminobutyric acid (GABA), or D-serine from astrocytes (Devinsky et al., 2013). While multiple glial mechanisms of epileptogenesis have been investigated, disruption of adenosine homeostasis has consistently been identified as sufficient for seizure generation and has proven to be an effective therapeutic target for seizure suppression and stopping disease progression (Boison, 2013). Here we will further review the supporting data from human epilepsy and rodent models of epilepsy that pertain to astroglial-mediated disruptions in synaptic transmission as a mechanism for epileptogenesis, with a more thorough discussion of adenosine homeostasis in epilepsy to follow.

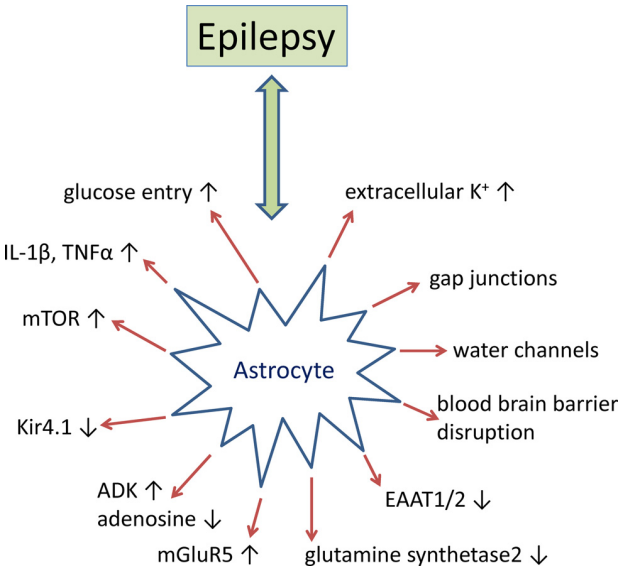

FIGURE 1 | Epilepsy-associated alteration of astrocyte-based homeostatic functions. Summary of key pathophysiological alterations of astrocytes as found in animal models of epilepsy and/or in human epilepsy. For details, please refer to main text.

GLUTAMATE HOMEOSTASIS

Reactive astrocytes cause neuronal hyperexcitability through increased synaptic glutamate and K^+ levels and decreased ECS volume. Decreased astrocyte-mediated glutamate uptake and glutamate to glutamine conversion have been proposed to increase synaptic glutamate in the gliotic hippocampus (Cavus et al., 2005). Increased levels of synaptic glutamate may in part be attributed to reduced expression of glutamate transporters within reactive astrocytes. High affinity glutamate transporters (2–90 μM) are concentrated on the astrocyte membrane and are integral to maintaining a low glutamate tone within the synaptic cleft. Thus, a decrease in astrocyte glutamate transporters may increase neuronal hyperexcitability and decrease seizure threshold. A patient diagnosed with spontaneous seizures was found to have a mutation in the human gene SLC1A3, resulting in decreased EAAT-1 protein expression and reduced capacity for glutamate uptake (Jen et al., 2005). A substantial decrease in both astrocyte glutamate transporters, EAAT-1 and EAAT-2, has also been identified in resected mTLE hippocampi (Sarac et al., 2009). However, this finding has not been reproduced in other studies (Tessler et al., 1999; Eid et al.,

2004). Research with transgenic mice further implicates deregulation of astrocyte-mediated glutamate uptake as a contributing factor to epilepsy. A mouse model of TSC with progressive epilepsy was found to have decreased glutamate/aspartate transporter (GLAST) and glial glutamate transporter 1 (GLT-1) protein levels (genetic equivalents to EEAT-1 an EAAT-2, respectively) and glutamate transporter currents (Wong et al., 2003). In addition, GLT-1 and GLAST knockout mice have a decreased pentylenetetrazol (PTZ) seizure threshold. The GLT-1 knockouts also exhibit spontaneous lethal seizures and have increased levels of synaptic glutamate (Tanaka et al., 1997; Watanabe et al., 1999). Downregulation of glutamine synthetase (GS) within reactive astrocytes has also been postulated as a potential cause for the increased synaptic glutamate tone observed in the epileptic hippocampus (Eid et al., 2004). GS primarily resides in the astrocyte cytoplasm and is responsible for the ATP-dependent conversion of glutamate to glutamine. GS protein and enzymatic activity are profoundly decreased, 40 and 38%, respectively, in the hippocampus of mTLE patients with the greatest reduction observed in proliferating astrocytes (Eid et al., 2004). Causative evidence that reduced GS activity is sufficient for epileptogenesis is from a pharmacological study with rats that developed seizures and neuropathology reminiscent of mTLE when chronically infused with the GS in inhibitor methionine sulfoximine (Wang et al., 2009). Furthermore, a mutation in the gene encoding GS has been linked to child with epilepsy (Haberle et al., 2011).

WATER AND POTASSIUM HOMEOSTASIS

The state of neuron excitability is also tightly coupled to the ECS volume and associated K^+ homeostasis (Schwartzkroin et al., 1998). More specifically, hypoosmolarity treatment reduces the ECS volume and increases neuron excitability and epileptiform activity; while hyperosmolarity treatment has the reverse effect. Aquaporin 4 (AQP4) is a water transport channel that is expressed within glial cells and which is integral to regulating ECS volume and implicated in epileptogenesis (Binder et al., 2012). AQP4 is normally localized to both the perivascular endfeet and within perisynaptic processes of astrocytes where it permits the bidirectional flow of water from the ECS to the blood (Nielsen et al., 1997; Rash et al., 1998; Nagelhus et al., 2004). In human mTLE brain specimens, AQP4 is redistributed primarily to the perisynaptic processes, which has been hypothesized to be a contributing factor of hyperexcitability through dysregulating water and K^+ homeostasis (Eid et al., 2005). Research with transgenic AQP4 knockout mice support this hypothesis as they have increased ECS volume and are less susceptible to PTZ-induced seizures (Binder et al., 2004a,b). Glial-mediated water flow is also tightly coupled to K^+ transport from the ECS through the inward rectifying K^+ channel, Kir4.1, that is colocalized with AQP4 on the astrocyte membrane (Hsu et al., 2011). Similar to ECS volume, changes in the K^+ concentration influence neuronal excitability with millimolar increases in ECS K^+ exacerbating epileptiform activity (Feng and Durand, 2006). Human polymorphisms in KCNJ10, the gene that encodes Kir4.1, are associated with epilepsy and a glial specific deletion of Kir4.1 in mice reduces K^+ clearance from the synaptic cleft (Heinemann et al., 2000; Haj-Yasein et al., 2011). Dysregulation of AQP4 might also be linked to cholinergic imbalances in epilepsy, since overexpression of synaptic acetylcholinesterase has been associated with overexpression of AQP4 (Meshorer et al., 2005)

GLIOTRANSMITTER HOMEOSTASIS

Aside from increasing neuronal excitability, astrogliosis disrupts synapse homeostasis through dysregulation of transmitter release from astrocytes. The list of transmitters proposed to be released by astrocytes includes glutamate, D-serine, ATP, adenosine, and GABA (Devinsky et al., 2013). In regards to adenosine homeostasis, astrocytes express two types of equilibrative nucleoside transporters, which mediate transport based on the concentration gradient of adenosine (Baldwin et al., 2004; Gray et al., 2004; Guillen-Gomez et al., 2004; Peng et al., 2005; Alanko et al., 2006). Adenosine in synapses of CA1 pyramidal neurons can be generated in response to high frequency stimulation that induces a Ca^{2+}-mediated release of ATP from astrocytes through either vesicular transport or hemichannels (Cotrina et al., 1998; Zhang et al., 2003; Pascual et al., 2005; Kang et al., 2008) or the direct release of adenosine from neurons (Lovatt et al., 2012). Once in the synaptic cleft ATP is rapidly converted to adenosine by a series of ectonucleotidases (Zimmermann, 2000). Ca^{2+} waves within astrocytes have also been linked to glutamate and D-serine release. Within the epileptic brain Ca^{2+} signaling may regulate the glutamate-induced paroxysmal deporalization shift, which is the intracellular analog to the interictal spike (Tian et al., 2005). However, results from a separate study suggest that astrocytes may initiate seizures and not contribute to interictal activity (Gomez-Gonzalo et al., 2010).

GROWTH FACTORS

Status epilepticus (SE) can induce a wide range of growth factors, neurotrophins, and transcription factors (Grabenstatter et al., 2012). Increased brain-derived neurotrophic factor (BDNF) in particular has been linked to epileptogenesis (Grabenstatter et al., 2012). In neurons, BDNF was shown to activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), inducible cAMP early repressor (ICER), and early growth response factors (Egrf) that induce a shift in the expression of specific subunits of the $GABA_AR$ as well as the expression levels of *N*-methyl-D-aspartate receptors (NMDARs; Roberts et al., 2006; Lund et al., 2008; Kim et al., 2012). In astrocytes, activation of the trkB receptors by BDNF has been linked to the development of astrogliosis, a mechanism that is also influenced by transactivation of the TrkB receptor through the adenosine $A_{2A}R$ (Brambilla et al., 2003). Whereas the role of growth factors on neuronal function is well documented, growth factor-dependent mechanisms that contribute to epileptogenesis via the disruption of glial homeostatic functions are not well established.

BLOOD BRAIN BARRIER

The breakdown of the BBB leading to albumin extravasation has directly been linked to epileptogenesis (Heinemann et al., 2012). Albumin is a potent astroglial activator through stimulation of transforming growth factor beta (TGF-β) signaling and activation of the SMAD-2/5 pathway (Ivens et al., 2007; Cacheaux

et al., 2009). BBB disruption also triggered expression changes of genes associated with the TGF-β pathway, early astrocyte activation, inflammation, and reduced buffering for glutamate and K^+ (Cacheaux et al., 2009; David et al., 2009). The reduced buffering capacity of transformed astrocytes for glutamate and K^+ appears to be most critical during repetitive activation. Experimental blockade of TGF-β signaling following BBB disruption decreased those transcriptional responses and prevented epileptogenesis. BBB disruption has been demonstrated in patients with posttraumatic epilepsy (Tomkins et al., 2008) and in patients with brain tumors who developed epilepsy (Marchi et al., 2007). Thus, pathogenetic neurovascular interactions which involve astroglial dysfunction, changes in the immune response, and gene expression changes that promote neuronal hyperexcitability may play a critical role in epileptogenesis. Consequently, BBB disruption might constitute a valuable biomarker for the prediction of epileptogenesis following an insult to the brain.

IMMUNOLOGICAL RESPONSES

Inflammatory processes play important roles in the pathogenesis of epilepsy (Aronica et al., 2012). Molecules linked to inflammatory reactions, such as TNF-α or prostaglandins, control the release of glutamate from astrocytes (Rossi and Volterra, 2009). The activation of pro-inflammatory pathways, such as the interleukin-1/Toll-like receptor (IL-1R/TLR) pathway, appear to be involved in the precipitation and recurrence of seizures in rodent models of epilepsy (Vezzani et al., 2011a). Importantly, components of these pathways were found to be overexpressed in surgically resected specimens from human TLE (Ravizza et al., 2008). Activation of the IL-1R/TLR pathway can increase excitability of the brain by the induction of post-translational changes in voltage- and ligand-gated ion channels. Among the endogenous ligands are danger signals, such as high mobility group box 1 (HMGB1), which can be released from injured or activated cells (Vezzani et al., 2011b). HMGB1 is the endogenous ligand of TLR4 and normally bound to chromatin. However, HMGB1 can be released into the ECS following either cell damage or neuronal hyperexcitability. The pro-epileptogenic role of HMGB1 is supported by recent data showing that blockade of the TLR4 pathway significantly delays seizure onset (Maroso et al., 2010). Likewise, engineered mice with defects in the IL-1R/TLR signaling pathway are intrinsically resistant to seizures (Vezzani et al., 2011b). Intriguingly, activation of the IL-1R/TLR pathway may alter the permeability properties of the BBB via the production of cytokines and prostaglandins, promoting brain extravasation of albumin (Cacheaux et al., 2009). Thus, inflammatory processes and disruption of the BBB might form a self-perpetuating vicious cycle supporting chronic hyperexcitability of the brain via compromised astrocyte function on multiple levels.

ADENOSINE – A HOMEOSTATIC NETWORK REGULATOR

The purine ribonucleoside adenosine has early evolutionary origins and likely played already a role in prebiotic evolution (Oro and Kimball, 1961). Importantly, adenosine is not only part of the energy metabolite ATP but also of RNA, the nucleic acid thought to be at the origin of life (Lahav, 1993; Dworkin et al., 2003; Robertson and Joyce, 2012). While ATP reflects the energy pool in the environment, RNA reflects the metabolic activities of a cell. Thus, adenosine assumes a central place between energy availability and metabolic demands and has therefore been termed a retaliatory metabolite (Newby et al., 1985). It is fair to assume that adenosine played an early evolutionary role as key bioenergetic network regulator central to the energy homeostasis of a cell. The early evolutionary principle to conserve energy was likely a rise in adenosine as a consequence to ATP depletion and to use the increase in adenosine as a negative feedback regulator to attenuate all cellular activities that consume energy. This early evolutionary principle is omnipresent in all living systems and in every human organ. In the brain, epileptic seizures cause a rapid drop in energy, which results in the generation of adenosine levels that can exceed the baseline level more than 40 times (During and Spencer, 1992); it is this rise in adenosine that acts as endogenous terminator of seizures and which is responsible for the postictal refractoriness that normally follows a seizure (Lado and Moshe, 2008). Consequently, adenosine augmentation therapies constitute a promising avenue for seizure control (Boison, 2007). Seizure suppression by adenosine depends on the activation of G-protein coupled adenosine A_1 receptors (Fredholm et al., 2005); however, new evidence suggests that adenosine retains important adenosine receptor-independent regulatory functions, which are based on interactions with mitochondrial bioenergetics, interference with biochemical enzyme reactions, and epigenetic functions. Thereby adenosine assumes a unique role as homeostatic network regulator.

ADENOSINE RECEPTOR-DEPENDENT PATHWAYS

A number of adenosine's actions are mediated by a group of specific receptors, G protein-linked transmembrane proteins of the P1 family, distinguished from the P2 ATP receptor family. Four members of the P1 class have been cloned in mammals: A_1R, $A_{2a}R$, $A_{2b}R$, and A_3R (Fredholm et al., 2011), and not surprisingly, given the ancient biological origin of adenosine, homologous genes have been found in numerous other animal groups (Sazanov et al., 2000; Petersen et al., 2003; Dolezelova et al., 2007; Boehmler et al., 2009; Malik and Buck, 2010). These receptors have biochemical specificity as each acts through a particular set of G proteins to influence second messengers: for the classical second messenger cAMP, A_1R and A_3R activation inhibits its production, whereas $A_{2a}R$ and $A_{2b}R$ activation are stimulatory (Fredholm et al., 2011). Other second messengers such as diacylglycerol, inositol triphosphate, and Ca^{2+} are also modulated. Each receptor presents a distinct pharmacology, and each has a particular distribution in tissues and cell types. For instance, A_1Rs are expressed most highly in brain, whereas $A_{2b}Rs$ and A_3Rs have their highest expression in the periphery (Dixon et al., 1996). Within the brain, A_1Rs are widespread with particularly high levels in the limbic system, whereas $A_{2a}Rs$ are expressed mostly in the basal ganglia (Dixon et al., 1996).

Adenosine can have powerful receptor-mediated effects on synaptic transmission in the brain (Fredholm et al., 2011). Presynaptic A_1Rs inhibit synaptic release of most, if not all, neurotransmitters, with an apparently greater effect on excitatory transmission. Thus, if adenosine levels are raised sufficiently, synaptic transmission can be blocked altogether. On the postsynaptic side,

A_1Rs hyperpolarize membranes by opening inwardly rectifying K^+ channels. These combined A_1R effects strongly dampen the synaptic network, and undoubtedly play a major role in the efficacious anticonvulsant effect of adenosine and A_1R agonists (Boison, 2007). The effect of A_{2a}Rs on network excitability is less clear, and more anatomically restricted, but if seizures reflect brain network imbalance, then one seizure model suggests that A_1Rs and A_{2a}Rs may cooperate to promote homeostasis (De Sarro et al., 1999).

MITOCHONDRIAL BIOENERGETICS

Mitochondria generate ATP via oxidative phosphorylation, and this is the main pathway for generating this critical cell energy molecule. Regarding the relationship between adenosine and ATP, intracellular adenosine is dephosphorylated from AMP by cytosolic 5′-nucleotidase and is converted back to AMP via adenosine kinase (ADK). The adenosine-AMP cycle is linked to ADP and ATP with adenylate kinase. Thus, adenosine is linked tightly to energy metabolism. Whereas mitochondrial uncouplers decrease ATP and increase adenosine (via net dephosphorylation of ATP), mitochondrial enhancers, or other strategies, which enhance ATP also appear to increase adenosine. Therefore, improving mitochondrial bioenergetics has the potential to offer dual benefits of improving metabolic dysfunction and restoring adenosine homeostasis.

Adenosine triphosphate is released from various pathways including vesicular release (Coco et al., 2003; Pascual et al., 2005), gap junction hemichannels (Kang et al., 2008) and chloride channels (Anderson et al., 2004), and hydrolyzed to adenosine by a series of ectonucleotidases (Zimmermann, 2000). Interestingly astrocytes express all types of ATP-releasing proteins and are capable of releasing ATP from these pathways simultaneously (Garre et al., 2010). After ATP is dephosphorylated, extracellular adenosine is salvaged into the intracellular space by equilibrative nucleoside transporters and/or concentrative nucleoside transporters (Latini and Pedata, 2001). However, it has also been reported that these nucleoside transporters release adenosine with various types of metabolic stress (Lloyd et al., 1993; Frenguelli et al., 2007). Adenosine-AMP cycles and bidirectional adenosine uptake and release via nucleoside transporters maintain adenosine homeostasis. Therefore, changes in extracellular adenosine due to adenosine and/or ATP release can alter adenosine receptor signaling described above, and experimentally increasing intracellular adenosine or ATP concentration can increase the activity of adenosine receptors (Brundege and Dunwiddie, 1996; Kawamura et al., 2010).

Taken together, adenosine's role in maintaining homeostasis interacts directly with mitochondrial bioenergetics and energy metabolism (Newby, 1984; Newby et al., 1985; Sommerschild and Kirkeboen, 2000). The intracellular concentration of ATP is nearly 50 times higher than that of AMP (Arch and Newsholme, 1978) and about 10,000 times higher than that of adenosine (Pazzagli et al., 1995; Delaney and Geiger, 1996). Thus, minor decreases in intracellular ATP leads to a large rise of intracellular adenosine level. Thus, various excitatory stimuli cause decreased brain energy and a subsequent increase in adenosine (Shepel et al., 2005). It has been reported that increases in intramitochondrial AMP cause adenosine production in the purified mitochondria, and thus extramitochondrial adenosine levels increase in a time-dependent manner, suggesting a concentration-dependent adenosine output from mitochondria by diffusion or facilitated diffusion (Raatikainen et al., 1992). Conversely, the cytosolic adenosine formation with a balance of cytoplasmic ADK and cytosolic 5′-nucleotidase might influence mitochondrial adenosine production and affect mitochondrial bioenergetics. This interpretation is supported by a severe mitochondrial pathology in ADK knockout mice (Boison et al., 2002). As a "retaliatory metabolite" adenosine is thought to be one of the key links between neuronal network homeostasis and mitochondrial bioenergetics with both adenosine receptor-dependent and -independent pathways.

EPIGENETICS

Modifications to the epigenome that include changes in DNA methylation, histone tail modifications, and incorporation of histone variants are mechanisms by which network homeostasis can be dramatically altered and consequently change the entire gene expression profile of a tissue. There are a number of epilepsy-associated neurological diseases that are directly attributed to primary genetic mutations and result in secondary deregulation of the epigenome (Kobow and Blumcke, 2011). Gene promoters from mTLE patients are characterized by altered DNA methylation patterns and decreased DNA methyltransferase (Dnmt) gene expression (Kobow et al., 2009; Zhu et al., 2012).

Biochemically, DNA methylation is intricately linked to *S*-adenosylmethionine (SAM)-dependent transmethylation reactions (**Figure 2**). SAM donates a methyl group to unmethylated cytosines in DNA. Following methyl group donation, SAM is converted to *S*-adenosylhomocysteine (SAH). SAH is further hydrolyzed to adenosine and homocysteine (HCY). Adenosine is cleared by ADK-mediated phosphorylation to AMP, and HCY is converted to methionine in a folate-dependent manner. Importantly, DNA methylation is dependent on the continuous removal and subsequent equilibrium constants of SAH, adenosine and HCY (Lu, 2000; Boison et al., 2002). Thus, an accumulation of adenosine prevents the biochemical conversion of SAM to SAH and therefore inhibits DNA methylation.

Humans with ADK deficiency caused by a missense mutation in the ADK gene have disruptions to the transmethylation pathway with increased methionine and SAH levels. Furthermore, they have abnormal liver function; encephalopathy; and severe progressive neurological deficits (Bjursell et al., 2011). Transgenic mice with an ADK knockout also have disruptions in the transmethylation pathway with decreased blood adenine levels and increased HCY levels and the fatal liver disease neonatal hepatic steatosis (Boison et al., 2002). Peripheral changes in the transmethylation pathway are conserved within the brain. We recently found that hippocampal adenosine levels regulate the global DNA methylation status by shifting the equilibrium constant of the transmethylation pathway; thereby either increasing (high ADK and low adenosine) or decreasing (low ADK and high adenosine) methylation. These adenosine dependent changes in DNA methylation are receptor-independent and can be evoked by either a single pharmacological bolus of adenosine (icv) or in

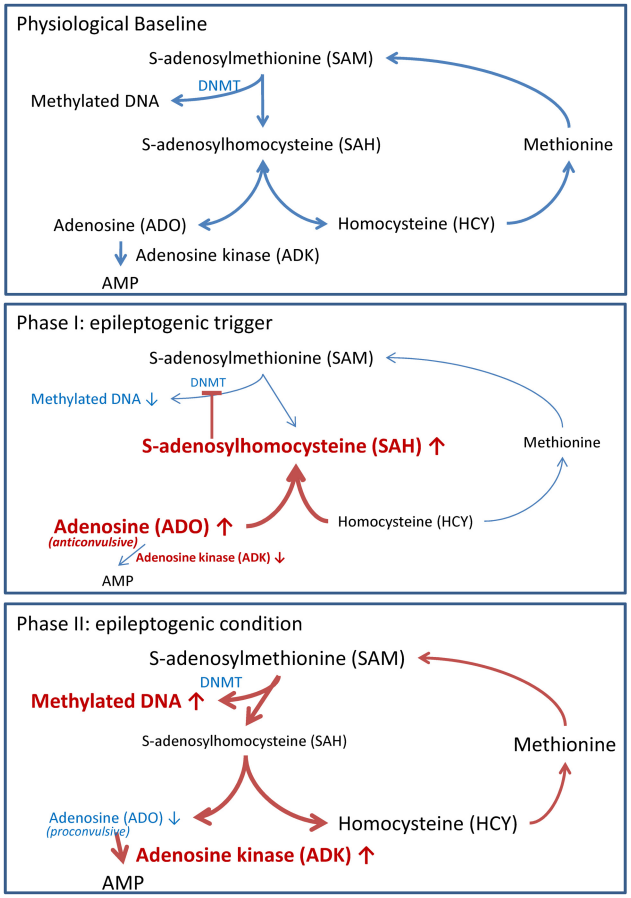

FIGURE 2 | Adenosine tone regulates the transmethylation pathway. Pathways in blue reflect steady state pathways, whereas pathways in red show pathway shifts induced by alteration of adenosine homeostasis. Physiological baseline: DNA methylation of cytosine residues is mediated by the transmethylation pathway. *S*-adenosylmethionine (SAM) donates a methyl group, which is added to cytosine residues by DNMT. In the process SAM is converted to *S*-adenosylhomocysteine (SAH). SAH is further converted to adenosine (ADO) and homocysteine (HCY) by the enzyme *S*-adenosylhomocysteine hydrolase. ADO is phosphorylated to AMP by the enzyme adenosine kinase (ADK). HCY is converted to methionine and subsequently back to SAM. DNA methylation is dependent on the constant removal of the obligatory endproducts ADO and HCY. Phase I (epileptogenic trigger): we hypothesize that the injury and/or SE induced decrease of ADK and surge of ADK (Clark et al., 1997; Gouder et al., 2004; Pignataro et al., 2008) shifts the equilibrium constant of the transmethylation pathway to SAH. The increased SAH prevents SAM donation of a methyl group to DNA. Reduced DNA methylation permits the transcription of early epileptogenesis genes. Phase II (epileptogenic condition): increased ADK within reactive astrocytes reduces adenosine tone to pathologically low levels. Low adenosine tone shifts the biochemical pathway to favor SAM conversion to SAH; thereby, DNA methylation will be increased. Pathological hypermethylation of DNA is present in resected hippocampi of mTLE with hippocampal sclerosis patients (Kobow et al., 2009).

response to endogenous changes in ADK expression or activity (Williams-Karnesky et al., 2013). As an obligatory endproduct of transmethylation, the adenosine tone non-specifically drives the transmethylation pathway by regulating substrate availability; with site specific DNA methylation mediated by DNMT1, 3a or 3b complexes (Goll and Bestor, 2005; Caiafa et al., 2009; Feng et al., 2010; Zampieri et al., 2012). Consequently, the adenosine tone does not regulate site specific DNA methylation, but instead the homeostasis of the DNA-methylome.

THE ADENOSINE KINASE HYPOTHESIS OF EPILEPTOGENESIS

As outlined above, glial pathologies play important roles in epileptogenesis. Astrocytes form the major metabolic reuptake route for synaptic adenosine and control the availability of extracellular adenosine via expression changes of the astrocyte-based enzyme ADK, which phosphorylates adenosine to AMP and thereby drives the influx of adenosine into the astrocyte through equilibrative transporters (Boison, 2013). Using transgenic approaches and adenosine microelectrode biosensors we previously demonstrated that ADK expression levels in astrocytes directly control the levels of tissue adenosine under baseline conditions (Etherington et al., 2009). During epileptogenesis the adenosine/ADK system undergoes biphasic changes that might be instrumental in epileptogenesis and seizure generation (**Figure 2**).

PHASE I OF ADENOSINE DYSREGULATION

Injuries to the brain such as trauma, stroke, or SE trigger an acute surge in adenosine, which is accompanied by transient downregulation of ADK (Clark et al., 1997; Pignataro et al., 2008). The initial injury and the associated surge in adenosine can trigger several mechanisms possibly implicated in epileptogenesis, among which the induction of $A_{2A}R$ expression in glial cells appears to play a prominent role. In primary cultures of glial cells lipopolysaccharide (LPS) was found to induce $A_{2A}R$ mRNA and protein expression with a peak at 48 h after treatment (Saura et al., 2005). Likewise, in microglial cells and astrocytes of the mouse substantia nigra 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced the expression of $A_{2A}Rs$ within 24 h after intoxication. The proliferation of astrocytes and thereby the development of astrogliosis is in part regulated by the ratio of the different adenosine receptors expressed on the astrocyte membrane. Importantly, the increased activation of $A_{2A}Rs$ by an injury-associated surge in adenosine can increase astrocyte proliferation and activation, whereas the blockade of $A_{2A}Rs$ prevented the induction of astrogliosis by BDNF, which is a known transactivator of the $A_{2A}R$ (Hindley et al., 1994; Brambilla et al., 2003; Rajagopal et al., 2004). In addition, important immune functions of the brain are under the control of adenosine homeostasis (Hasko et al., 2005, 2008). Via simultaneous activation of A_1 and A_{2A} receptors adenosine was shown to stimulate the proliferation of naïve microglial cells (Gebicke-Haerter et al., 1996), whereas the A_{2A} receptor-dependent upregulation of cyclooxygenase 2 (COX-2) and the release of prostaglandin E2 (PGE2) were shown to mediate additional pro-inflammatory effects of adenosine (Fiebich et al., 1996). Multiple inflammatory processes, which have been linked to epileptogenesis (Ravizza et al., 2011) could be triggered by an injury-induced surge in adenosine. Recent findings also demonstrate that increased levels of adenosine induce hypomethylation of hippocampal DNA as described previously by shifting the equilibrium constant of the transmethylation pathway (Williams-Karnesky et al., 2013). The reduced methylation of CpG rich promoter regions could induce the transcription of epileptogenesis genes, suggesting a novel mechanism

whereby an acute injury-induced surge in adenosine could trigger epileptogenesis.

PHASE II OF ADENOSINE DYSREGULATION

Astrogliosis is a pathological hallmark of the human and experimental epileptic brain and has consistently been associated with overexpression of ADK resulting in adenosine deficiency (Li et al., 2008; Aronica et al., 2013). Adenosine deficiency in human epilepsy has directly been identified via the analysis of microdialysis samples. A 25% reduction of adenosine in the epileptogenic versus the contralateral control hippocampus was found (During and Spencer, 1992). In our prior work we provided the following evidence linking expression levels of ADK to seizure propensity: during epileptogenesis increased ADK expression and emergence of spontaneous electrographic seizures coincided both temporally as well as spatially (Li et al., 2008). The transgenic overexpression of ADK (40% increase) in the brain of mice triggered spontaneous electrographic seizures (Li et al., 2008), whereas a transgenic approach that reduced ADK expression in cortex and hippocampus of mice (40% reduction) rendered those animals resistant to seizures and resistant to epileptogenesis (Li et al., 2008). These data demonstrate that ADK provides a molecular link between astrogliosis and increased neuronal excitability.

In addition, the epileptogenic hippocampus is characterized by increased DNA methylation. This hypermethylated state forms the basis of the methylation hypothesis of epileptogenesis, which suggests that seizures by themselves can induce epigenetic chromatin modifications and thereby aggravate the epileptogenic condition (Kobow and Blumcke, 2011). Hypermethylation of DNA can be triggered by a variety of mechanisms, however, the mechanisms underlying the gradual increase in DNA methylation status during the course of epileptogenesis are not well characterized and largely the subject of speculation (Kobow and Blumcke, 2012). The epigenetic drift hypothesis suggests that a gradual shift in the ratio of active DNA demethylation and *de novo* methylation, triggered by a precipitating injury and modified by environmental and intrinsic factors leads to increased DNA methylation, altered gene expression, and an altered (e.g., seizure) phenotype (Feil and Fraga, 2011). We propose that overexpression of ADK in the epileptogenic hippocampus and resulting adenosine deficiency drives the biochemical transmethylation pathway and thereby increases the methylation rate of the hippocampal DNA. It is important to note that adenosine affects DNA methylation in a non-cell-autonomous manner and thereby is uniquely positioned to effect homeostasis of the DNA-methylome on a global scale within the hippocampal formation (Williams-Karnesky et al., 2013). Through this mechanism, astrogliosis and associated overexpression of ADK could contribute to continued epileptogenesis through maintenance of a hypermethylated state of hippocampal DNA. Conversely, reduction of DNA methylation through therapeutic adenosine augmentation may provide a rational therapeutic approach for the prevention of epileptogenesis.

ANTIEPILEPTOGENIC THERAPIES

Several lines of evidence suggest that adenosine might prevent epileptogenesis. Transgenic mice with an engineered reduction of ADK expression in forebrain were found to be resistant to the development of epilepsy, even when the epileptogenesis-triggering SE was coupled with transient blockade of the A_1R (Li et al., 2008). Similarly, adenosine-releasing stem cells – implanted into the hippocampal formation after triggering epileptogenesis – dose-dependently attenuated astrogliosis, suppressed ADK increases, and attenuated development of spontaneous seizures (Li et al., 2008). Using an independent therapeutic approach, the transient delivery of adenosine by intraventricular silk for only 10 days provided long-lasting (beyond adenosine release) antiepileptogenic effects in the rat kindling model of epilepsy (Szybala et al., 2009). More recent findings, as will be discussed in more detail below, suggest that the antiepileptogenic effects of adenosine are based on an epigenetic mechanism. Since dietary interventions have been shown to increase adenosine signaling in the brain (Masino et al., 2011), dietary manipulations such as the ketogenic diet might likewise hold promising therapeutic potential for the prevention of epileptogenesis.

EPIGENETIC THERAPIES

As mentioned previously, DNA methylation has been highlighted as a component of the methylation hypothesis of epileptogenesis (Kobow and Blumcke, 2011). Consequently, DNA methylation inhibitors might be of therapeutic value to either treat epilepsy by restoring non-pathological epigenetic homeostasis. Unfortunately, the use of DNMT inhibitors for treating epileptic patients must be approached with caution due to target related complications or side effects. As an alternative to conventional pharmacological DNMT inhibitors focal adenosine therapy may serve as an effective epigenetic medicine. Recently, we described a novel antiepileptogenic role for adenosine; whereby a transient adenosine augmentation therapy administered to epileptic rats after the onset of spontaneous recurrent seizures not only suppressed seizures during active adenosine release, but also prevented further disease progression that lasted long after the therapy was suspended. Adenosine-dependent changes in DNA methylation were pinpointed as an underlying mechanism for the antiepileptogenic properties of this adenosine therapy. Adenosine treatment was found to restore normal DNA methylation levels in the otherwise hypermethylated hippocampus of the epileptic rat. More specifically, genome wide analysis using a methylated DNA immunoprecipitation (MeDIP) array revealed that out of the 125 genes which showed increased DNA methylation in epilepsy, 66 also showed reduced DNA methylation after adenosine therapy in treated epileptic rats. Interestingly, multiple targets that function to either interact with DNA or play a role in gene transcription and translation (*PolD1, Polr1e, Rps6kl1, Snrpn, Znf524, Znf541, Znf710*) responded to adenosine therapy with a decrease in the DNA methylation status of their respective promoters. Consequently, these targets are poised as likely candidates to mediate adenosine-dependent changes in major homeostatic functions (Williams-Karnesky et al., 2013).

DIETARY INTERVENTIONS

Dietary therapies for epilepsy can be useful in cases where medications and other treatments are ineffective. The most obvious application is for epilepsies associated with specific inborn errors

of metabolism that can be treated by removing or adding specific dietary components. Such disorders include dysfunctions of the enzymes phenylalanine hydroxylase, argininosuccinate synthetase, guanidinoacetate methyltransferase, 3-methylcrotonyl-CoA carboxylase, antiquitin, or various enzymes and transporters for metabolizing different classes of lipids (Papetti et al., 2012). Such disorders range from somewhat to extremely rare.

For more common forms of epilepsy, one effective dietary treatment is also the simplest: no diet at all. Fasting has been known to alleviate convulsions since antiquity. Some of the first work in the modern era on this topic was done by Geyelin in the 1910s (Geyelin, 1921). In working with grand mal and petit mal patients of various ages, he found that 22 out of 26 subjects were seizure-free after 10 days of fasting, with many experiencing beneficial effects after just 2 days of fasting. Furthermore, two subjects remained seizure-free for a year after the end of fasting, suggesting the potential for epigenetic and antiepileptogenic effects. Yet fasting has a number of problems, including the difficulty of maintaining a fast and being necessarily time-limited. Also, fasting would be contraindicated in patients that have other health problems besides epilepsy, growing children, and adults with very low body mass index.

Fasting forces a metabolic shift in which the liver metabolizes fatty acids into ketone bodies, which in the absence of sufficient glucose can be used as fuel by other tissues, particularly the brain, which is glucose-dependent. Hypothesizing that the antiseizure effects of fasting were due to this state of ketosis, in the 1920s Wilder began to use in his epileptic patients a strict modified diet which also produces ketosis (Wilder, 1921; Wilder and Winter, 1922). This ketogenic diet, low in carbohydrate, high in fat, with moderate-to-low protein, thus reproduces a major metabolic effect of fasting while still allowing food and was found to be an effective anticonvulsant in children and adults (McQuarrie and Keith, 1927; Baborka, 1930). Similar to fasting, initial reports also suggested an antiepileptogenic potential of a dietary approach, but there has been little research to follow up on using controlled studies.

The ketogenic diet was a fairly common epileptic treatment until the synthesis of phenytoin and other effective anticonvulsant drugs in the 1930s, which were easier to implement than a strict diet. Notably, anticonvulsant drugs often have sedative and cognitive side effects that the ketogenic diet lacks (Gupta et al., 2001; Drane and Meador, 2002). Thus, the ketogenic diet became a rare treatment, until a slow resurgence began in the 1990s in its use mostly for drug-refractory pediatric epilepsy. Often the diet now is supplemented with medium-chain triglycerides, which are absorbed quickly and metabolized easily to ketones. Good success rates were reported in retrospective (Kinsman et al., 1992; Hassan et al., 1999) and prospective studies (Freeman et al., 1998; Vining et al., 1998).

The first randomized, controlled study of the ketogenic diet clearly demonstrated a beneficial effect (Neal et al., 2008). These studies often showed efficacy equal to anticonvulsant drugs and while seizures worsened as a whole in patients on anticonvulsant medications they improved as a whole in the group on the diet. While a subset of the children on the ketogenic diet became seizure-free, this did not occur in the group receiving standard therapy with anticonvulsants. Even though this study was randomized and controlled, blinded studies of the ketogenic diet present obvious difficulties; one attempt, with mixed results, has been published (Freeman et al., 2009).

Multiple lines of evidence suggest that a ketogenic diet can increase ATP or mitochondrial biogenesis (Bough et al., 2006), and that it may exert anticonvulsant effects via adenosine (Masino et al., 2011). Further validation of these findings could identify the metabolic shift, which accompanies a ketogenic diet (or fasting), as a key strategy to increase adenosine and restore homeostasis. Along those lines, and as noted above, the potential for the ketogenic diet to restore homeostasis and offer antiepileptogenic activity deserves additional research, particularly as this dietary therapy is pursued for diverse neurological indications in which epilepsy is often comorbid.

CONCLUSIONS AND OUTLOOK

Homeostasis has long been recognized as a core physiological principle, and the CNS depends critically on maintaining its milieu, including ion gradients, temperature, pH, and cell energy, as well as also regulating transcription and translation to ensure proper function. Chronic disruption of either its environment (the milieu) or its adaptive response to its environment (transcription and translation) results in a loss of CNS homeostasis and pathological dysfunction. It becomes clear that complex neurological syndromes, such as epilepsy, which are not only defined by a dominant symptom (i.e., a seizure), but also by a growing number of associated comorbidities, can best be explained by the disruption of network homeostasis. Disruption of network homeostasis will lead to the dysregulation of several molecular pathways (e.g., those dependent on K^+, glutamate, and adenosine homeostasis) simultaneously. It becomes clear that conventional drugs with a mode of action that is restricted to only one target or pathway might be sufficient to block a symptom (e.g., a seizure), but are unlikely to affect a neurological condition on the network level. Novel therapeutic interventions based on adenosine, epigenetic mechanisms, or dietary interventions might hold promise to affect network homeostasis as a novel conceptual strategy to treat and prevent epilepsy on the network level. For future therapy development it is important to note that adenosine augmentation has no known adverse effects. In preclinical toxicity studies of intrathecal adenosine in dogs, no side effects were observed with intrathecal adenosine infused chronically for 26 days (Chiari et al., 1999). Likewise, intrathecal adenosine was tested in humans in escalating doses of up to 2 mg without any adverse effects (Eisenach et al., 2002a,b). Importantly, suprahippocampal implants of adenosine-releasing cells demonstrated a robust pro-cognitive effect in mice (Shen et al., 2012), suggesting that therapeutic adenosine augmentation might combine anticonvulsant with cognition-enhancing effects. Whereas seizure suppression in chronic epilepsy would require continuous long-term augmentation of adenosine, e.g., by gene therapy, cell grafts, or dietary intervention, preventing disease progression in the early stages of epilepsy might require only the transient delivery of adenosine. Adenosine-releasing silk might be an attractive therapeutic candidate due to the bioresorbable properties of this biopolymer. Those and related approaches are currently in preclinical development and molecular pathways stimulated by adenosine augmentation are currently under intense investigation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2013; accepted: 22 June 2013; published online: 16 July 2013.

Citation: Boison D, Sandau US, Ruskin DN, Kawamura M Jr and Masino SA (2013) Homeostatic control of brain function – new approaches to understand epileptogenesis. Front. Cell. Neurosci. 7:109. doi: 10.3389/fncel.2013.00109

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 17 September 2013
doi: 10.3389/fncel.2013.00157

The role of dopamine signaling in epileptogenesis

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Clinical and experimental studies implicate most neuromodulatory systems in epileptogenesis. The dopaminergic system has a seizure-modulating effect that crucially depends on the different subtypes of dopamine (DA) receptors involved and the brain regions in which they are activated. Specifically, DA plays a major role in the control of seizures arising in the limbic system. Studies performed in a wide variety of animal models contributed to illustrate the opposite actions of D1-like and D2-like receptor signaling in limbic epileptogenesis. Indeed, signaling from D1-like receptors is generally pro-epileptogenic, whereas D2-like receptor signaling exerts an anti-epileptogenic effect. However, this view might appear quite simplistic as the complex neuromodulatory action of DA in the control of epileptogenesis likely requires a physiological balance in the activation of circuits modulated by these two major DA receptor subtypes, which determines the response to seizure-promoting stimuli. Here we will review recent evidences on the identification of molecules activated by DA transduction pathways in the generation and spread of seizures in the limbic system. We will discuss the intracellular signaling pathways triggered by activation of different DA receptors in relation to their role in limbic epileptogenesis, which lead to the activation of neuronal death/survival cascades. A deep understanding of the signaling pathways involved in epileptogenesis is crucial for the identification of novel targets for the treatment of epilepsy.

Keywords: dopamine receptor, seizure, limbic system, temporal lobe epilepsy

INTRODUCTION

Epilepsy is a chronic neurological disorder, characterized by spontaneous and recurrent bursts of neuronal hyperactivity (seizures) generally arising in restricted regions of the brain. Seizures may remain confined to their area of origin ("focal" or "partial" seizures) or spread to the whole cerebral hemispheres ("generalized" seizures). The behavioral outcome of seizures strictly depends on the brain regions that are affected by hyperactivity. Seizures have been traditionally characterized as an imbalance between excitatory (glutamatergic) and inhibitory (GABAergic) transmission. The role of glutamate and GABA in epileptogenesis (i.e., the process by which a normal brain develops epilepsy) has been extensively addressed elsewhere (see for example McNamara et al., 2006; Ben-Ari et al., 2012) and will not be further discussed here. Clinical and experimental studies investigated the role of the major neuromodulatory systems in epilepsy (Kurian et al., 2011). Acetylcholine (Friedman et al., 2007; Steinlein and Bertrand, 2010), serotonin (Bagdy et al., 2007), noradrenaline (Weinshenker and Szot, 2002; Giorgi et al., 2004), and dopamine (DA; Starr, 1993, 1996; Bozzi et al., 2011) are all known to regulate seizure activity. In this review, we will focus on the role of DA in seizure onset and spread discussing evidence obtained in human and animal studies. We present a unifying hypothesis on the intracellular signaling cascades triggered by DA and involved in long-term epileptogenesis.

Molecules that stimulate the dopaminergic (DAergic) system such as apomorphine, amphetamines, L-DOPA (L-3,4-dihydroxyphenylalanine), and anti-parkinsonian drugs (e.g., pergolide and bromocriptine) have anti-epileptic action and anti-convulsant effects. Seizures involving the limbic system appear to be the most critically affected by modulation of DA signaling. Brain areas receiving afferents from the mesolimbic DAergic pathway express different types of DA receptors (Bozzi and Borrelli, 2006; Bozzi et al., 2011). Interestingly, while pharmacological studies using animal models support the anti-convulsant effects of DA on limbic seizures (Starr, 1996; Clinckers et al., 2005), contrasting biochemical evidence has been obtained for the presence of DAergic dysfunctions either in the brain of epileptic patients or in animal models of seizure and epilepsy. This suggests that the involvement of DA in seizure and epilepsy is likely due to a dysfunctional control of DA levels or an alteration in the expression of specific receptors. Indeed, levels of DA and its metabolites markedly vary depending on the type of epilepsy and animal models considered (Starr, 1996). However, it is interesting to note that increased levels of DA (Meurs et al., 2008) as well as increased firing of DA neurons (Cifelli and Grace, 2012) were detected in rodent models of temporal lobe epilepsy (TLE). These findings suggest also that variations of DA levels very likely alter the neuromodulatory action of DA on brain circuits of the limbic system.

For instance, a glutamate–DA interaction has been proposed to explain individual susceptibility to epilepsy in limbic areas (Starr, 1996). According to this hypothesis, paroxysmal activity of the cerebral cortex in the epileptic brain would increase

the tonic excitation of DA neurons by glutamate. This would then induce phasic release of DA, possibly leading to downregulation or desensitization of DA receptors and subsequently decreased phasic responses. Indeed, DA exerts a marked inhibitory effect on hippocampal excitability through activation of DA D2 receptors (D2Rs). Anti-psychotics (i.e., DAergic D2-like antagonists) lower seizure threshold in epileptic patients and promote seizures in patients with no previous history of the disease. Conversely, seizure inhibition occurs in patients administered anti-parkinsonian drugs such as pergolide and bromocriptine, which both act by stimulating D2Rs (Starr, 1996). Further observations supported the anti-convulsant effect of a low dose treatment with bromocriptine. According to the Starr's hypothesis, a low dose of a D2R agonist would act through stimulation of presynaptic D2 autoreceptors leading to decreased DA release, while preventing the downregulation of postsynaptic D2R (Chen, 2006). Based on our results using mice lacking D2R ($D2R^{-/-}$ mice), we postulated that D2R activation might exert a neuroprotective action on hippocampal and DAergic neurons against excitotoxicity (Bozzi et al., 2000; Bozzi and Borrelli, 2006). Conversely, activation of DA D1 receptors (D1Rs) has a proconvulsant effect, lowering seizure threshold (Starr, 1993, 1996). The opposite action of D2R and D1R signaling might also be explained by the glutamate–DA interaction hypothesis for limbic epileptogenesis. Indeed, the activation of D1R in cortical tissue samples obtained from children undergoing epilepsy surgery has been shown to induce glutamate receptor-mediated neuronal hyperexcitability (Cepeda et al., 1999). More recent studies performed in animal models during seizures support these results showing a D1R-mediated activation of glutamatergic neurons (Gangarossa et al., 2011; see also below).

These data clearly point to a prominent role of the DAergic circuits in limbic epileptogenesis. Classical pharmacological studies supporting this view have been extensively reviewed by Starr (1993, 1996), to which the reader is referred for a more detailed description. In the next paragraphs, we will summarize recent human studies in support of this hypothesis. Animal studies will then be discussed to highlight the role of specific DA receptor signaling pathways in seizure onset and spread. We will also describe the mechanisms by which DA receptor signaling may affect neuronal excitability and epileptogenesis in the long-term. The potential importance of DA receptor-based drugs for the treatment of epilepsy will be finally discussed.

HUMAN STUDIES

Recent studies performed on human epileptic patients (**Table 1**) confirm the role of DA-mediated neurotransmission in epilepsy. The role of DA in epilepsy is most likely mediated by the neuromodulatory effect of this molecule on structures belonging to the basal ganglia and elements of the limbic system. These structures are strongly interconnected and defective DA signaling either in the basal ganglia or in the limbic system might affect the electric properties of neurons located at distal sites through either direct interactions or through feedback mechanisms connecting the cortex to the striatum or other areas. In agreement, it has been postulated that the DAergic transmission in the basal ganglia may provide an inhibitory role (Rektor et al., 2012). Indeed, the basal ganglia are not able to generate specific epileptic activity, as detected by electroencephalographic (EEG) recordings. However, seizures originating in the mesiotemporal lobe of TLE patients can induce EEG changes in the basal ganglia, that may act as filter to the further spread of ictal activity (Rektor et al., 2012).

IMAGING

Dopaminergic pathway arising from the ventral mesencephalon [substantia nigra and ventral tegmental area (VTA)] innervate the basal ganglia, the limbic system, and the cerebral cortex (Cooper et al., 1996). Several imaging studies demonstrate that reduced DAergic activity is present in various forms of epilepsy. Reduced [^{18}F]-fluoro-L-DOPA uptake (indicating a reduced binding to the DA transporter, DAT) was detected in the basal ganglia of patients suffering of ring20 epilepsy (Biraben et al., 2004; Del Sole et al., 2010), resistant generalized "absence-like" epilepsy and drug-resistant TLE with hippocampal sclerosis (Bouilleret et al., 2005). In TLE patients, [^{18}F]-fluoro-L-DOPA uptake was reduced in the caudate, putamen, and substantia nigra (Bouilleret et al., 2008). Reduced DAT has also been detected in patients with juvenile myoclonic epilepsy (Ciumas et al., 2008; Odano et al., 2012) and epilepsy with tonic–clonic seizures only (GTCS; Ciumas et al., 2010).

Alterations of both D1-like and D2-like receptors have been associated to different forms of epilepsy. For example, positron emission tomography (PET) with [^{11}C]-SCH23390 revealed a reduced striatal D1R binding in patients with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE; Fedi et al., 2008), suggesting that neurotransmitter alterations in nigrostriatal DA circuits may contribute to nocturnal paroxysmal motor activity in ADNFLE. A reduced D2R/D3R density (as evaluated by PET using the high-affinity DA D2R/D3R ligand [^{18}F]fallypride) was instead found in the temporal lobe of TLE with hippocampal sclerosis. Interestingly, the reduction of [^{18}F]fallypride binding did not correlate with hippocampal atrophy, indicating that reduced D2R/D3R density is not just a consequence of the degenerative process, but might play a specific role in the pathophysiology of mesial TLE (Werhahn et al., 2006). The same authors also detected a reduction in D2R/D3R binding in the putamen of patients with juvenile myoclonic epilepsy (Landvogt et al., 2010). A recent study evaluated the expression and binding of both D1R and D2R cerebral cortex samples from surgically treated patients with TLE associated with mesial sclerosis (MTLE). As compared to control samples, higher D1R expression and binding and decreased D2R expression were detected in the neocortex of MTLE patients, whereas D2R binding was unaffected. MTLE samples also presented elevated DAT binding and low tissue content of DA (Rocha et al., 2012). It is interesting to note that in this study, D1R binding negatively correlated with seizure onset age and frequency, and positively with epilepsy duration; conversely, D2R binding positively correlated with seizure onset age and negatively with epilepsy duration (Rocha et al., 2012). These results are in agreement with several data from animal models of TLE (see below), respectively, showing a pro-epileptic and anti-epileptic role of D1R and D2R, and confirm that an altered function of the DAergic system might contribute to TLE.

Table 1 | Dopamine signaling in epilepsy: human studies.

Epilepsy type	DA target	Analysis/findings	Reference
Ring20	DAT	Reduced [^{18}F]-fluoro-L-DOPA binding in basal ganglia	Biraben et al. (2004); Del Sole et al. (2010)
"Absence-like" TLE with sclerosis	DAT	Reduced [^{18}F]-fluoro-L-DOPA binding in basal ganglia	Bouilleret et al. (2005)
TLE	DAT	Reduced [^{18}F]-fluoro-L-DOPA binding in substantia nigra	Bouilleret et al. (2008)
JME	DAT	Reduced [^{11}C]PE2I binding in substantia nigra	Ciumas et al. (2008); Odano et al. (2012)
GTCS	DAT	Reduced [^{11}C]PE2I binding in putamen	Ciumas et al. (2010)
ADNFLE	D1R	Reduced [11C]-SCH23390 binding in striatum	Fedi et al. (2008)
TLE with sclerosis	D2R/D3R	Reduced [18F]fallypride binding in hippocampus	Werhahn et al. (2006)
JME	D2R/D3R	Reduced [18F]fallypride binding in putamen	Landvogt et al. (2010)
MTLE	D1R	Increased expression and binding in cortex	Rocha et al. (2012)
MTLE	D2R	Reduced expression in cortex	Rocha et al. (2012)
MTLE	DAT	Increased binding in cortex	Rocha et al. (2012)

See text for abbreviations.

GENETICS

Despite the notion that several genetic factors are predisposing to epilepsy, little evidence is available in favor of a direct link between epilepsy and variation of genes coding for protein involved in DAergic neurotransmission. Two studies reported the association of DNA polymorphisms in the DAT gene and idiopathic absence epilepsy (Sander et al., 2000) and alcohol-withdrawal seizures (Gorwood et al., 2003), indicating that genetic variation of the DAT gene may modulate neuronal network excitability and contribute to epileptogenesis. More recently, the genetic variation in DAergic function has been associated with the risk of adverse effect of anti-epileptic drug treatment. Specifically, chronic epileptic patients carrying genetic variants associated with decreased DAergic activity (DA-β-hydroxylase, DBH; catechol-*O*-methyltransferase, COMT and D2R) showed a higher susceptibility to adverse psychotropic effects of levetiracetam (Wood, 2012; Helmstaedter et al., 2013). This suggests that reduced DAergic transmission in epileptic patients might contribute to worsen the outcome of specific anti-epileptic medications.

ANIMAL STUDIES

Pharmacological studies demonstrated that the activation of different DA receptor subtypes plays distinct roles in the onset and spread of limbic seizures (Starr, 1996). DA acts through two different types of G-protein-coupled receptors (GPCRs), named D1-like and D2-like (Beaulieu and Gainetdinov, 2011). Activation of D1-like (D1 and D5) receptors results in reduction of seizure threshold and increased seizure severity (DeNinno et al., 1991; Starr and Starr, 1993). Conversely, the effect of D2-like (including D2, D3, and D4) receptors on seizure modulation is mainly inhibitory. Administration of D2-like receptor agonists lowers seizure activity, whereas blockade of these receptors has proconvulsant effects (Starr, 1993, 1996). More recently, studies performed on DA receptor knockout mice (**Table 2**) and the use of compounds acting on specific DA receptor subtypes contributed to dissect the intracellular pathways activated by different DA receptors in response to seizures (**Figure 1**).

D1R

The D1R agonist SKF38393 has a proconvulsant action (Starr, 1996); D1-like receptors (D1R and D5R)-mediated signaling increases cAMP levels and protein kinase A (PKA) activity via the stimulation of adenylyl cyclase (AC) by stimulatory G-proteins (Beaulieu and Gainetdinov, 2011). DA and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) is a critical downstream target of D1R- and D5R-mediated signaling. PKA-catalyzed phosphorylation activates DARPP-32, and converts it into an inhibitor of protein phosphatase-1 (PP-1). Phosphorylated DARPP-32, by inhibiting PP-1, activates a series of signaling cascades that are important in regulating neuronal excitability (Greengard et al., 1999; Greengard, 2001). In mice, D1-like receptor agonist administration induces seizures and DARPP-32 phosphorylation. Accordingly, seizure behavior is absent or greatly reduced in both D1R and DARPP-32 knockout mice, thus highlighting the crucial role of this signaling pathway in mediating DAergic control of seizures (O'Sullivan et al., 2008). In addition to its direct effect on DARPP-32, D1R-dependent activation of PKA signaling also leads to phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2). Accordingly, seizure-induced ERK activation in the granule cell layer of the dentate gyrus is absent in D1R knockout mice (Gangarossa et al., 2011). Seizures resulting from D1R activation depend on the specific coupling of D1R to the PKA–DARPP-32–ERK pathway. D1-type receptor agonists stimulating the AC pathway increase the levels of Zif268 and Arc/Arg3.1 [two immediate early genes (IEGs) involved in transcriptional regulation and synaptic plasticity] in the dentate gyrus, with a time-course that parallels that of ERK phosphorylation (Gangarossa et al., 2011). Conversely, D1 agonists that stimulate

Table 2 | Dopamine signaling in epilepsy: knockout mouse studies.

Mouse	Seizure model	Phenotype	Reference
D1R$^{-/-}$	SKF38393-induced seizures	No seizures	O'Sullivan et al. (2008)
Mice lacking D1R neurons	None	Spontaneous seizures	Gantois et al. (2007)
DARPP-32$^{-/-}$	SKF38393-induced seizures	No seizures	O'Sullivan et al. (2008)
		No ERK activation	Gangarossa et al. (2011)
D5R$^{-/-}$	SKF38393-induced seizures	Increased seizure latency, reduced total EEG seizures	O'Sullivan et al. (2008)
D2R$^{-/-}$	KA seizures	Lower seizure threshold, increased c-fos induction, KA-induced CA3 neuronal apoptosis	Bozzi et al. (2000)
D2R$^{-/-}$	Pilocarpine seizures	Lower seizure threshold	Bozzi and Borrelli (2002)
D2R$^{-/-}$	KA seizures	Increased caspase-3 and GSK-3b activation	Tripathi et al. (2010)
D2R$^{-/-}$	KA seizures	Reduced pAkt(Ser473) in CA3	Dunleavy et al. (2013)
D4R$^{-/-}$	4-Aminopiridine or bicuculline on cortical slices	Increased excitability	Rubinstein et al. (2001)

See text for abbreviations.

phospholipase C (PLC) but not AC do not induce seizure behaviors (Clifford et al., 1999; O'Sullivan et al., 2008). These results clearly indicate that activation of D1R-dependent signaling has a proconvulsant activity. However, it must be pointed out that postnatal ablation of D1R-expressing striatal neurons results in spontaneous seizures in mice (Gantois et al., 2007), suggesting seizure control may depend on the anatomical integrity of DAergic striatal pathways.

D5R

D5R activation triggers both cAMP and PLC signaling (Sahu et al., 2009; Beaulieu and Gainetdinov, 2011). Similarly to D1R, D5R-mediated signaling through the cAMP pathway seems to be mainly involved in seizure control. D5R$^{-/-}$ mice treated with the proconvulsant D1R agonist SKF83822, showed an increased latency to first seizure and a reduced total time spent in EEG seizures when compared to wild-type (WT) mice (O'Sullivan et al., 2008). However, it must be pointed out that D5R seems to have less pronounced effects than D1R in regulating synaptic activity (O'Sullivan et al., 2008), as also confirmed by other studies (Granado et al., 2008).

D2R

Several pharmacological lines of evidence indicate that D2R is the major DA receptor subtype involved in the anti-epileptogenic action of DA in limbic areas. In accordance with imaging studies performed in epileptic patients (**Table 1**), animal studies confirmed that reduced levels of D2R expression are detected in epileptogenic areas in seizing rodents. For example, D2-like binding sites were reduced in the caudate–putamen (CP) of pilocarpine-treated rats (Yakushev et al., 2010) and genetically epileptic GAERS (genetic absence epilepsy rat from Strasbourg; Jones et al., 2010) and WAG/Rij (Wistar Albino Glaxo rats from Rijswijk; Birioukova et al., 2005) rats. Interestingly, WAG/Rij rats also showed a reduced D2-like binding in the CA3 region, confirming a prominent role of D2R signaling in limbic epileptogenesis (Birioukova et al., 2005). The crucial role of D2R signaling in the prevention of hippocampal epileptogenesis is highlighted by the observation that intra-hippocampal administration of remoxipride (a selective D2R antagonist) completely abolished the protective effects of DA against limbic seizures induced by pilocarpine in adult rats (Clinckers et al., 2004).

D2R-mediated cAMP-dependent "canonical" pathway

D2-like receptor stimulation has an antagonistic effect to D1-like stimulation. D2-like receptors are coupled to Gi proteins that inhibit AC activity. Gi protein activation following DA binding to D2R leads to a decrease of cAMP production (Beaulieu and Gainetdinov, 2011) and subsequent modulation of PKA/ERK signaling (Bozzi et al., 2011). Accordingly, D2R activation is able to counterbalance DARPP-32 activity (Nishi et al., 1997). In the hippocampal kindling model, an increased activation of Gi protein coupled to D2-like receptors was detected in the hippocampus and other brain areas, as evaluated by increased [^{35}S]GTPγS *in situ* binding (Alcantara-Gonzalez et al., 2013). Ligand stimulation of G-protein-coupled receptors results into the activation of the associated G-protein and binding of GTP to the catalytic α-subunit. Measuring the binding of [^{35}S]GTPγS (a radiolabeled GTP analog) is therefore considered a reliable tool to quantify GPCR activation (Harrison and Traynor, 2003). Increased activation of Gi signaling downstream of D2R autoreceptors might explain the lower DA release detected in the hippocampus of fully kindled animals, which might facilitate seizures (Alcantara-Gonzalez et al., 2013). Accordingly, hippocampal administration of the D2-like receptor antagonist sulpiride induces enhanced DA release and longer seizure duration in kindled animals (Alcantara-Gonzalez et al., 2013). Genetic inactivation of the D2R gene and the consequent impairment of D2R-mediated signaling results

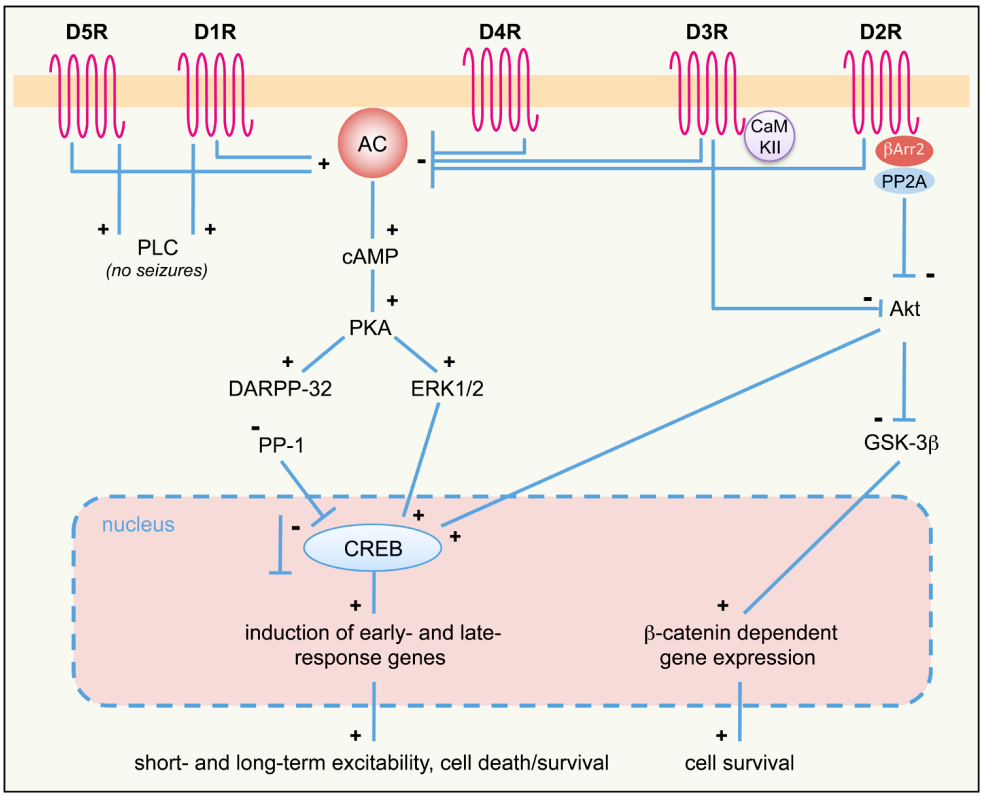

FIGURE 1 | DA receptor signaling pathways activated in response to seizures. D1-like (D1R, D5R) and D2-like (D2R; D3R and D4R) DA receptor differently regulate the AC–PKA–ERK pathway. ERK-regulated gene transcription modulates both short- and long-term responses, including neuronal excitability, survival, and cell death. PLC and Akt pathways are also regulated by D1R/D5R and D2R/D3R, respectively. The proposed scheme is a general (though not complete) summary of the intracellular pathways induced by seizures in the limbic system, where all DA receptor subtypes are expressed. Differences may occur, however, in different limbic areas, due to different expression levels of specific DA receptors. See text for details. +, activation; −, inhibition; AC, adenylyl cyclase; βArr, β-arrestin; cAMP, cyclic AMP; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; CREB, cAMP response element-binding protein DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kDa; D1-5R, dopamine receptors (D1 to D5 subtypes); ERK, extracellular-regulated kinase; GSK-3β, glycogen synthase kinase 3β; PKA, protein kinase A; PLC, phospholipase C; PP-1, protein phosphatase 1; PP2A, protein-phosphatase 2A.

in more severe limbic seizures. $D2R^{-/-}$ mice have an increased susceptibility to seizures induced by kainic acid (KA; Bozzi et al., 2000) and pilocarpine (Bozzi and Borrelli, 2002): $D2R^{-/-}$ mice experience generalized limbic motor seizures at doses that are not convulsant in WT mice. The canonical D2R-mediated signaling pathway negatively regulates ERK activity through reduction of cAMP levels and PKA activity, thereby modulating the expression of cAMP-responsive IEGs (Beaulieu and Gainetdinov, 2011). Accordingly, KA administration in $D2R^{-/-}$ mice induces a massive c-*fos* expression (a typical cAMP-responsive gene; West et al., 2002), at a dose lower than in WT mice (**Figure 2A**). KA-induced c-*fos* mRNA upregulation mainly involves the DG-CA3 hippocampal circuit (Bozzi et al., 2000, and **Figure 2A**), thus indicating that the D2R-mediated seizure control mainly involves this limbic circuit. A more rapid and longer-lasting ERK phosphorylation (consistent with the time-course of c-*fos* mRNA induction; **Figure 2A** and Bozzi et al., 2000) is detectable in the hippocampus of $D2R^{-/-}$ mice, as compared to WT controls (**Figure 2B**). In addition, KA-induced seizures result in a stronger and longer-lasting c-Fos protein upregulation in the $D2R^{-/-}$ hippocampus as compared to WT (**Figure 2C**; see also Bozzi et al., 2000). Taken together, these data confirm the critical role of the D2R cAMP-dependent signaling in mediating the first steps of DAergic control of hippocampal activity during seizures.

D2R-mediated cAMP-independent pathway

In addition to their increased susceptibility to KA-induced seizures, $D2R^{-/-}$ mice also display increased susceptibility to KA-induced CA3 hippocampal cell death (Bozzi et al., 2000; Bozzi and Borrelli, 2006). This death occurs by apoptosis, as indicated by Bax (Bozzi et al., 2000) and Caspase-3 (Tripathi et al., 2010) upregulation in the hippocampus of KA-treated $D2R^{-/-}$ mice. We recently investigated the intracellular pathways involved in D2R-mediated control of seizure-induced CA3 hippocampal cell death. Several studies show that D2R may also trigger a cAMP-independent pathway. Activation of this pathway following DA binding to D2R results in the inhibition of Akt activity, by dephosphorylation of the threonine 308 (Thr308) residue, leading to activation of glycogen synthase kinase 3β (GSK-3β; Beaulieu et al.,

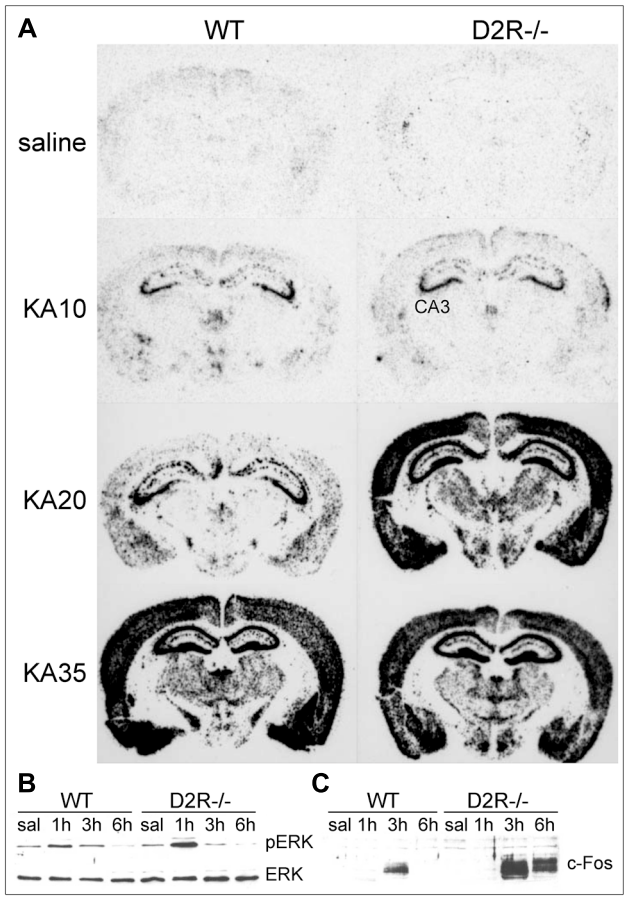

FIGURE 2 | D2R signaling pathways activated in response to seizures. **(A)** Pattern of c-*fos* mRNA induction (dark staining) in the brain of WT and D2R$^{-/-}$ mice treated with 10, 20, and 35 mg/kg kainic acid (KA), as indicated. Brains were dissected 3 h after KA administration and coronal sections were processed by mRNA *in situ* hybridization with a c-*fos* specific anti-sense riboprobe (see Bozzi et al., 2000 for experimental details). Administration of 20 mg/kg KA induced limbic motor seizures in D2R$^{-/-}$ but not WT mice (Bozzi et al., 2000; Tripathi et al., 2010; Dunleavy et al., 2013), whereas generalized seizures were observed in both genotypes at 35 mg/kg KA (Bozzi et al., 2000). **(B,C)** Induction of ERK phosphorylation (pERK) and c-Fos protein synthesis in the hippocampus of WT and D2R$^{-/-}$ mice following KA-induced seizures. Mice received a single systemic dose of KA (20 mg/kg) and ERK/pERK **(B)** and c-Fos **(C)** induction were analyzed by immunoblotting on total hippocampal protein extracts at different times after KA (1, 3, and 6 h, as indicated). CA3, pyramidal cell layer of the hippocampus; D2R$^{-/-}$, D2R knockout mice; KA, kainic acid; (p)ERK, (phosphorylated) extracellular-regulated kinase; saline, saline-treated mice; WT, wild-type mice.

2005, 2007a). Accordingly, we observed GSK-3β activation in the D2R$^{-/-}$ hippocampus after KA (Tripathi et al., 2010), suggesting that upregulation of GSK-3β activity might contribute to increased susceptibility to seizure-induced cell death observed in these mice. However, GSK-3β upregulation in KA-treated D2R$^{-/-}$ mice was independent of Akt phosphorylation at Thr308 (Tripathi et al., 2010), implicating that alternative pathways might contribute to modulate GSK-3β in the hippocampus during epileptic activity. The p38 mitogen-activated protein kinase (MAPK) and Wnt pathways, which have been implicated as potential alternative pathways in regulating GSK-3β activity (Thornton et al., 2008; Inestrosa and Arenas, 2010), are not affected in KA-treated D2R$^{-/-}$ mice (Dunleavy et al., 2013). We were able to show that following KA, phosphorylation of Akt occurs at the serine 473 residue (Ser473) in the CA3 region of WT but not of D2R$^{-/-}$ mice (Dunleavy et al., 2013; CA3 neuron loss following KA is detected in D2R$^{-/-}$ but not WT mice; Bozzi et al., 2000; Bozzi and Borrelli, 2006). Conversely, a strong induction of Akt (Ser473) phosphorylation after KA was detected in the CA1 subregion (where no neuronal cell loss is detected after KA) of both WT and D2R$^{-/-}$ mice (Dunleavy et al., 2013). We therefore proposed that loss of D2R signaling results in reduced Akt (Ser473) phosphorylation, rendering CA3 neurons more vulnerable to apoptosis. Further investigation is required to fully elucidate the Akt/GSK-3β targets involved in D2R-mediated response to excitotoxicity (see also below).

D3R

Contrasting results were obtained about the role of D3R signaling in seizure modulation. D3R are mainly expressed in the limbic forebrain (Beaulieu and Gainetdinov, 2011; see also below). However, stimulation of D3R has a minimal inhibitory effect on limbic seizures: intra-accumbens pretreatment with D3 agonists delayed the onset of limbic seizures induced by pilocarpine, without any effect on their frequency and severity. In the same model, D2R agonists exerted an anti-convulsant action (Alam and Starr, 1994). Thus, the protective effect of DA on seizure propagation through the limbic forebrain is predominantly mediated by D2R rather than D3R. It has been proposed that D3R participates in D2R cAMP-independent pathway by enhancing D2R-mediated Akt (Thr308) phosphorylation (Beaulieu et al., 2007b). Signaling cascades downstream of D3R also involve Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), that binds to the N-terminal region of the third intracellular loop of D3R (Liu et al., 2009), as well as ERK (Collo et al., 2012) and CREB (cAMP response element-binding protein; Karasinska et al., 2005) phosphorylation, whose activity is negatively modulated by DR3.

D4R

As the other members of the D2-like receptor family, D4R have a prominent inhibitory role on neuronal hyperexcitability. The frequency of spontaneous synaptic activity and the frequency and duration of epileptic discharges induced by 4-aminopyridine or bicuculline were increased in cortical slices from D4R$^{-/-}$ mice, as well as in brain slices from WT mice treated with a selective D4R antagonist (Rubinstein et al., 2001). *In vivo*, D4R$^{-/-}$ mice showed a reduction of SKF83822-induced seizures, indicating that D4R interacts with AC-coupled D1R to positively regulate D1R-mediated seizures (O'Sullivan et al., 2006).

BUILDING A UNIFYING VIEW OF DA SIGNALING IN EPILEPTOGENESIS

The results reported in the previous sections clearly highlight the opposite neuromodulatory role of D1- and D2-like receptors on seizures arising in the limbic system. However, most of (if

not all) these studies investigated the role of DA signaling on the modulation of *acute* seizures (such as those occurring during an experimentally induced *status epilepticus*). Few data are available suggesting a direct link between DA signaling and *epileptogenesis*, i.e., the establishment of a chronic epileptic condition following an initial precipitating injury. In the next paragraphs we will present current understanding of how altered DA signaling might contribute to a chronic epileptic condition. In the attempt to build up a common signaling network for DA in epileptogenesis, we will first consider (i) the (co)expression of DA receptors in epileptogenic brain areas and (ii) what we know about DAergic modulation of chronic seizures in appropriate animal models of epileptogenesis. Then we will try to explain how altered DA neurotransmission in epileptogenic brain areas might interfere with intracellular pathways involved in long-term hyperexcitability. Finally, we will propose a novel, testable hypothesis on the role of DA receptor signaling in epileptogenesis.

EXPRESSION OF DA RECEPTORS IN EPILEPTOGENIC AREAS

All DA receptor subtypes are expressed in epileptogenic brain areas. D1Rs are expressed at high levels in the CP, nucleus accumbens (NAc), substantia nigra pars reticulata (SNr), amygdala, and cerebral cortex, and to a lower level in the hippocampus. D5Rs are expressed in the entorhinal cortex, SNr, and hippocampus mainly in the dentate gyrus). Lower levels of expression are found in the NAc and CP neurons. D2Rs are mainly expressed in the CP, NAc, SN pars compacta (SNc), in the ventral tegmental area, cerebral cortex, amygdala, and hippocampus. D3R expression is mainly restricted to areas of the limbic system (NAc, islands of Calleja), but is also present in the SNc, VTA, hippocampus, and cerebral cortex. Finally, D4R expression in epileptogenic areas is limited to the frontal cortex, amygdala, hippocampus, and SN (Beaulieu and Gainetdinov, 2011).

This brief summary clearly shows that most DA receptor subtypes are present in epileptogenic areas within the limbic system. In these areas, DA receptors are generally expressed in different subsets of neurons, but co-expression of different subtypes has also been detected in restricted neuronal populations. For example, D1R and D2R are generally expressed in distinct subpopulations of striatal medium spiny neurons, but a small percentage (5–15%) of these neurons has been shown to co-express both receptors; similarly, 20–25% of pyramidal neurons of the prefrontal cortex do co-express D1R and D2R (Valjent et al., 2009; Beaulieu and Gainetdinov, 2011). In the hippocampus, D1R mRNA is predominantly expressed in the granule cell layer of dentate gyrus, whereas the protein is localized in the molecular layer (Fremeau et al., 1991). D2R mRNA is also expressed in granule cells of the dentate gyrus, but its expression is also detectable in CA1–CA3 pyramidal layers; D2R protein is instead localized in the hilus and stratum lacunosum moleculare (Martres et al., 1985; Bouthenet et al., 1987). According to the expression profile of DA receptor subtypes, it is therefore likely that the signaling cascades depicted in **Figure 1** may cooperate at least in some, restricted neuronal subpopulations within the limbic system, such as dentate granule cells in the hippocampal formation. Indeed, DA has been shown to markedly regulate neuronal excitability in the dentate gyrus (Hamilton et al., 2010), as well as other limbic regions (Tseng and O'Donnell, 2004; Hammad and Wagner, 2006; Rosenkranz and Johnston, 2006) via D1-like and D2-like signaling pathways.

INVESTIGATING DOPAMINERGIC MODULATION OF CHRONIC SEIZURES IN ANIMAL MODELS OF EPILEPTOGENESIS

The vast majority of studies demonstrating a DAergic modulation of seizure onset and spread were performed on animal models of acute but not chronic seizures (Starr, 1996). Thus, a direct demonstration of a neuromodulatory effect of DA in epileptogenesis is substantially lacking. However, some important indications may be obtained from the limbic kindling model. Limbic kindling consists in the repeated, subthreshold electrical stimulation of the amygdala or hippocampus, that ultimately leads to the expression of chronic seizures (Morimoto et al., 2004). Kindling has been extensively used for the preclinical evaluation of antiepileptic drugs; many studies demonstrated that drugs showing anti-epileptic effects against limbic kindling also have an antiepileptic efficacy in clinical TLE (Morimoto et al., 2004). The effect of DAergic drugs on kindled seizures is well-documented. Non-selective DA agonists (such as amphetamines) have an antiepileptic action. Interestingly, while the prototypical D1R agonist SKF38393 has no effect in this model, D2R-selective compounds do modify seizure threshold. D2R agonists (lisuride) are protective, whereas D2R blockers (haloperidol) exacerbate kindled seizures (Starr, 1996).

The advantages of the limbic kindling model of epileptogenesis are multiple: a precise, focal activation of specific brain areas; a reliable development of chronic epileptogenesis; and a rapid and consistent pattern of seizure propagation and generalization. However, the kindling procedure is labor intensive, and spontaneous seizures develop only after a very large number of stimulations. For these reasons, DAergic modulation of seizure onset and spread has been more extensively studied in pharmacological models of limbic epileptogenesis, namely seizures induced by the muscarinic agonist pilocarpine and glutamatergic agonist KA. These two drugs induce very similar epileptic activity despite their distinct mechanism of action. Pilocarpine and KA initially provoke signs of focal epilepsy (stereotyped pre-convulsive behaviors), due to the activation of limbic areas (dentate gyrus, hippocampal formation, amygdala, entorhinal cortex). From these areas, epileptic activity rapidly propagates to the whole cerebral cortex, culminating in acute motor seizures and *status epilepticus*. Most importantly, pilocarpine- and KA-induced seizures result in extensive neurodegeneration in specific regions of the brain and may lead to the occurrence of spontaneous chronic seizures in the long-term (Turski et al., 1983, 1984; Ben-Ari, 1985; Leite et al., 2002). Using these models, a clear effect of D1R and D2R signaling on the genesis of limbic seizures has been observed, as described in previous paragraphs. However, several questions remain open. Specifically, do DA drugs (namely, D1R antagonists and D2R agonists) have a disease-modifying effect? Do they reduce or stop the occurrence of chronic seizures? The first issue could be addressed by administering DA compounds *after* pilocarpine- or KA-induced *status epilepticus*, and recording the occurrence of spontaneous chronic seizures. To test the anti-convulsant effect of DA drugs onto chronic

seizures, D1R- and D2R-selective compounds should instead be administered during the occurrence of spontaneous seizures in appropriate models of chronic epilepsy (such as that resulting from intrahippocampal administration of KA; Antonucci et al., 2008).

DA SIGNALING AND EPILEPTOGENESIS: A TESTABLE HYPOTHESIS

Evidence discussed above supports a neuromodulatory role of DA signaling in limbic epileptogenesis. However, the mechanisms by which DA signaling affects neuronal excitability and epileptogenesis in the long-term remain largely unknown. Here we propose that activation of neuronal cell death pathways (a well-known causal factor of limbic epileptogenesis; Bozzi et al., 2011; Henshall and Engel, 2013) following altered DA signaling might contribute to chronic epilepsy. As summarized in **Figures 1** and **3**, stimulation of D1R and blockade of D2R signaling can lead to the activation of neuronal cell death pathways. This phenomenon essentially involves two intracellular cascades: the PKA/ERK/Fos/Jun pathway and the mammalian target of rapamycin (mTOR) pathway.

Canonical, cAMP-dependent signaling through D1R and D2R activates the expression of the IEGs c-*fos* and c-*jun*. Treatment with D1R agonists results in a robust Fos-like immunoreactivity in basal ganglia and limbic structures of rats undergoing pilocarpine-induced generalized seizures (Barone et al., 1993). Recent studies indicate that D1R signaling through the G protein $\alpha_{olf}\beta_2\gamma_7$ might contribute to seizure-induced neuropathology (Schwindinger et al., 2012). Similarly, D2R receptor blockade by haloperidol induces Fos and Jun B expression during status epilepticus in the hippocampus and striatum (Dragunow et al., 1993); accordingly, KA seizures in $D2R^{-/-}$ mice markedly induce c-*fos* and c-*jun* expression (Bozzi et al., 2000; **Figure 2**). The protein products of c-*fos*/c-*jun* form the AP-1 transcription factor, whose activation regulates the expression of a wide number of cell death genes. The prolonged activation of c-*fos* after acute seizures was proposed as one of the crucial steps that trigger long-term neuronal death (Smeyne et al., 1993; Kasof et al., 1995). Jun phosphorylation (mediated by the c-Jun N-terminal kinase, JNK) activates Jun transcriptional activity and triggers apoptotic neuronal cell death after seizures (Schauwecker, 2000; Bozzi et al., 2000; Spigolon et al., 2010).

D2R signaling also occurs through a cAMP-independent, Akt/GSK-3β-dependent pathway (Beaulieu and Gainetdinov, 2011; see also **Figure 1** and references above). Loss of D2R signaling in $D2R^{-/-}$ mice results in reduced Akt (Ser473) phosphorylation and subsequent overactivity of GSK-3β (Tripathi

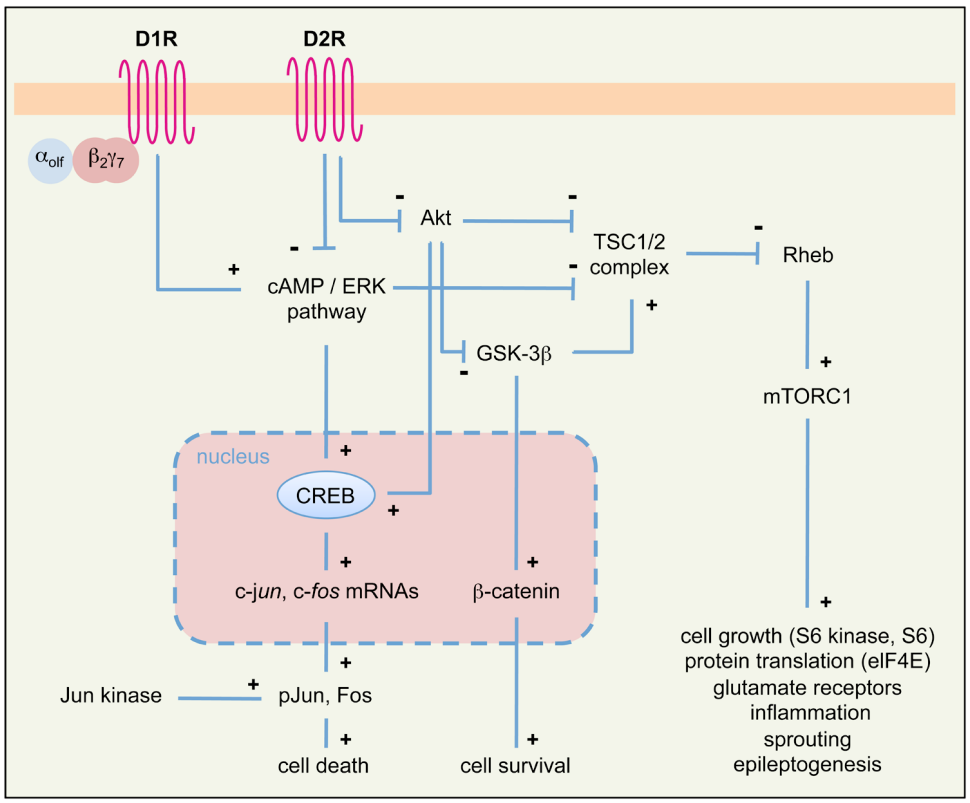

FIGURE 3 | Simplified diagram of intracellular pathways downstream of DA receptors, potentially involved in seizure-induced cell death and epileptogenesis in the limbic system. We propose that signaling cascades downstream of D1R and D2R may converge on two principal intracellular pathways (PKA/ERK/Fos/Jun and Akt/GSK-3β/mTOR) to regulate seizure-induced cell death and epileptogenesis. See text for details. $\alpha_{olf}\beta_2\gamma_7$, trimeric G_{olf} protein; eIF4E, elongation factor 4E; mTORC1, mammalian target of rapamycin complex 1; pJun, phosphorylated Jun; Rheb, Ras homolog enriched in brain; TSC1/2, tuberous sclerosis complex 1/2; see also **Figure 2** for other symbols and abbreviations.

et al., 2010; Dunleavy et al., 2013), thus rendering CA3 neurons more susceptible to apoptosis. GSK-3β hyperactivity is known to induce hippocampal neurodegeneration (Lucas et al., 2001; Sirerol-Piquer et al., 2011; Llorens-Martín et al., 2013), through mechanisms involving the blockade of the pro-survival β-catenin pathway (De Ferrari and Inestrosa, 2000) as well as the activation of the mTOR pathway (Beaulieu and Gainetdinov, 2011). KA seizures do not alter the β-catenin pathway in the $D2R^{-/-}$ hippocampus (Dunleavy et al., 2013). Thus, it is likely that GSK-3β hyperactivity in KA-treated $D2R^{-/-}$ mice results in the activation of the mTOR pathway. Several evidences support the crucial role of this pathway in epileptogenesis (Cho, 2011; Galanopoulou et al., 2012). For example, the components of the mTOR pathway are upregulated after seizures (Macias et al., 2013) and, most importantly, inhibition of mTOR by rapamycin may ameliorate the development of epilepsy-related pathology and reduce the expression of spontaneous seizures in TLE models (Zeng et al., 2009; Huang et al., 2010). In addition, there is strong evidence that rapamycin may prevent epilepsy and ameliorate its progression in mice lacking the tuberous sclerosis complex genes 1 and 2 (TSC1/2), which act as negative regulators of mTOR (Zeng et al., 2008, 2011). The mechanisms through which mTOR overactivity promotes epileptogenesis and neurodegeneration remain to be understood; according to the multiple action of the mTOR targets, these might involve altered cell growth and morphology, dysregulation of glutamatergic neurotransmission, inflammation, axonal sprouting, and remodeling of epileptogenic circuits (Galanopoulou et al., 2012; see also **Figure 3**).

Taken together, these data lead us to propose that loss of D2R signaling (induced by pharmacological blockade or genetic inactivation of D2R) might contribute to epileptogenesis via the activation of the mTOR pathway. This hypothesis might be tested by checking whether targets of the mTOR complex are upregulated in $D2R^{-/-}$ mice following KA- or pilocarpine-induced seizures. It would be then possible to investigate whether the mTOR inhibitor rapamycin is able to prevent seizures in KA or pilocarpine-treated $D2R^{-/-}$ mice. It is interesting to point out that seizure induction by activation of D1R might also converge onto the mTOR pathway; indeed, this pathway is also activated via PKA/ERK signaling (**Figure 3**), and the D1R agonist SKF81297 was shown to increase the phosphorylation of the mTOR target ribosomal protein S6 in the dentate gyrus, in an ERK-dependent manner (Gangarossa and Valjent, 2012). However, SKF81297 administration did not activate (but rather suppressed) the mTORC1/S6 kinase pathway, suggesting that S6 phosphorylation occurs independently of mTOR (Gangarossa and Valjent, 2012). Further investigation is therefore needed to understand whether stimulation of D1R signaling may promote epileptogenesis via activation of the mTOR pathway.

EXPLORING THE CLINICAL USE OF DOPAMINERGIC DRUGS IN EPILEPSY

Modulation of limbic seizures by DAergic drugs, as detected in the kindling, pilocarpine and KA models (see above) might predict a similar effect of these drugs on clinical epileptogenesis. However, no known DAergic drug is currently used to treat epilepsy (Beaulieu and Gainetdinov, 2011), despite anti-epileptic effects of DA agonists have been reported in epileptic patients (Starr, 1996). The lack of a systematic investigation of the anti-epileptic efficacy of DA agonists is certainly due to their severe neurological and neuropsychiatric side effects. However, some studies investigated the potential use of the D2R-selective agonist bromocriptine in some forms of epilepsy. Bromocriptine was originally reported to have an anti-epileptic effect in a case of self-induced, drug-resistant epilepsy (Clemens, 1988). Other studies subsequently confirmed that bromocriptine was highly effective in reducing seizure frequency in TLE patients affected by pituitary prolactinomas (Gatterau et al., 1990; Saie and Sills, 2005; Deepak et al., 2007). It is important to observe that these studies did not report severe side effects of prolonged bromocriptine treatment (see also Chen, 2006). Interestingly, in $D2R^{-/-}$ mice, increased seizure susceptibility (Bozzi et al., 2000; Bozzi and Borrelli, 2002) is accompanied by the progressive development of pituitary prolactinomas (Saiardi et al., 1997; Iaccarino et al., 2002; Radl et al., 2013), suggesting that altered D2R signaling might be a common cause of these two conditions. These observations definitely prompt for a better investigation of the anti-epileptogenic efficacy of D2R-selective agonists. Indeed, different D2R agonists (including bromocriptine) have neuroprotective efficacy against KA-induced brain damage (Micale et al., 2006), and recent studies promisingly show that lisuride may reduce seizures occurring after traumatic brain injury (Zweckberger et al., 2010). Further investigation in both animal models and clinical settings is needed to establish the anti-epileptogenic efficacy of D2R agonists.

CONCLUSION

In this review, we described recent evidence from both human and animal studies supporting the opposite role of D1-like and D2-like receptor signaling in limbic epilepsy. These studies indicate that increased D1R and decreased D2R function might be involved in limbic epilepsy. We propose that altered D1R and D2R signaling might contribute to epileptogenesis via the activation of the neuronal cell death cascades, activated by the PKA/ERK and mTOR pathways. The possible therapeutic application of these findings has been long disregarded, mainly due the severe side effects of DAergic drugs. However, the beneficial effects of selective D2R agonists observed in both animal and human epilepsy would deserve more attention.

ACKNOWLEDGMENTS

Data reported in this review were identified by searches of PubMed (as of August 6, 2013) with the terms "dopamine AND seizure," "dopamine AND epilepsy," "dopamine AND epileptogenesis," "dopamine agonist AND seizure," "dopamine AND mTOR," "mTOR AND epilepsy," and "glycogen synthase kinase AND epilepsy." Abstracts and reports from meetings were not included, and only papers published in English were reviewed. However, due to the large amount of bibliographic material available on this subject, we apologize with those authors whose studies have not been cited in this review. This work was funded by grants from NIH (DA024689;DA033554) to Emiliana Borrelli, the Italian Ministry of Health (RF-TAA-2008-1141282) and the University of Trento (CIBIO start-up) to Yuri Bozzi.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 May 2013; accepted: 29 August 2013; published online: 17 September 2013.

Citation: Bozzi Y and Borrelli E (2013) The role of dopamine signaling in epileptogenesis. Front. Cell. Neurosci. ***7****:157. doi: 10.3389/fncel.2013.00157*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 11 July 2013
doi: 10.3389/fncel.2013.00108

Changes in the sensitivity of $GABA_A$ current rundown to drug treatments in a model of temporal lobe epilepsy

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The pharmacological treatment of mesial temporal lobe epilepsy (mTLE), the most common epileptic syndrome in adults, is still unsatisfactory, as one-third of the patients are or become refractory to antiepileptic agents. Refractoriness may depend upon drug-induced alterations, but the disease *per se* may also undergo a progressive evolution that affects the sensitivity to drugs. mTLE has been shown to be associated with a dysfunction of the inhibitory signaling mediated by $GABA_A$ receptors. In particular, the repetitive activation of $GABA_A$ receptors produces a use-dependent decrease (rundown) of the evoked currents (I_{GABA}), which is markedly enhanced in the hippocampus and cortex of drug-resistant mTLE patients. This phenomenon has been also observed in the pilocarpine model, where the increased I_{GABA} rundown is observed in the hippocampus at the time of the first spontaneous seizure, then extends to the cortex and remains constant in the chronic phase of the disease. Here, we examined the sensitivity of I_{GABA} to pharmacological modulation. We focused on the antiepileptic agent levetiracetam (LEV) and on the neurotrophin brain-derived neurotrophic factor (BDNF), which were previously reported to attenuate mTLE-induced increased rundown in the chronic human tissue. In the pilocarpine model, BDNF displayed a paramount effect, decreasing rundown in the hippocampus at the time of the first seizure, as well as in the hippocampus and cortex in the chronic period. In contrast, LEV did not affect rundown in the hippocampus, but attenuated it in the cortex. Interestingly, this effect of LEV was also observed on the still unaltered rundown observed in the cortex at the time of the first spontaneous seizure. These data suggest that the sensitivity of $GABA_A$ receptors to pharmacological interventions undergoes changes during the natural history of mTLE, implicating that the site of seizure initiation and the timing of treatment may highly affect the therapeutic outcome.

Keywords: pilocarpine, GABA, hippocampus, neocortex, BDNF, levetiracetam

INTRODUCTION

Mesial temporal lobe epilepsy (mTLE) is the most common form of epilepsy of adulthood. In mTLE an initial "epileptogenic event" (head trauma, stroke, brain infection or tumor) is often identifiable which is followed, after a latent period of weeks to years, by the spontaneous occurrence of seizures. Multiple pathological and physio-pathological alterations have been identified that may be responsible for the transformation of a normal brain in an epileptic one (Pitkänen and Lukasiuk, 2011). In particular, we have focused on alterations in the GABA system, and found that $GABA_A$ receptors from epileptic tissue (hippocampus and neocortex) become less responsive to repeated activation (as detected by current rundown) than those from healthy tissue (Palma et al., 2004, 2007a,b; Ragozzino et al., 2005). This use-dependent $GABA_A$ receptor desensitization may imply hyper-excitability and favor the occurrence of spontaneous seizures. This phenomenon occurs both in human tissue and in animal models (pilocarpine), becomes detectable in the hippocampus at the time of the first spontaneous seizure and may depend upon alteration in the molecular composition of the $GABA_A$ receptor (Mazzuferi et al., 2010).

Once spontaneous seizures begin to occur and the diagnosis of epilepsy is made, the disease often continues to progress, with increasing severity of seizures; neurological decline and appearance of co-morbidities; development of resistance to pharmacological treatments (Pitkänen and Sutula, 2002). Many studies have been performed that provided mechanistic interpretations for the development of pharmaco-resistance. The best-described mechanisms are drug-related, i.e., drug-induced alterations in transport to the CNS (blood–brain barrier crossing) or in pharmacodynamics, which lead to attenuation or loss of therapeutic effects (Schmidt and Löscher, 2005). However, the progression of the disease *per se* may also implicate alterations in the responsiveness to pharmacological agents. Identifying these disease-induced alterations in the response to drugs may provide the basis

for more effective treatment strategies in the different phases of mTLE.

To challenge the hypothesis that the disease progression affects drug responsiveness, we explored the sensitivity to pharmacological treatments of the increased rundown of the GABA current (I_{GABA}) observed in epileptic tissue at different stages of experimental mTLE, namely at the time of the first spontaneous seizure and in the chronic period. We employed two structurally unrelated agents, levetiracetam (LEV) and brain-derived neurotrophic factor (BDNF), because both have been previously demonstrated to be capable of reducing the increased I_{GABA} rundown in the human and also in the rat epileptic brain (Palma et al., 2005b, 2007a,b). Whereas LEV is a clinically employed anti-epileptic drug, BDNF has been reported to provide anti-epileptic effects under some (Paradiso et al., 2009) but not all (He et al., 2004) experimental conditions and is not in clinical use.

MATERIALS AND METHODS

ANIMALS

Male Sprague-Dawley rats (240–260 g; Harlan, Italy) were used for all experiments. Animals were housed under standard conditions: constant temperature (22–24°C) and humidity (55–65%), 12-h dark–light cycle, and free access to food and water. All efforts were made to minimize animal suffering. Procedures involving animals and their care were carried out in accordance with European Community and national laws and policies (authorization number: D.M. 83/2009-B; 246/2012-13).

PILOCARPINE

Pilocarpine was administered i.p. (300 mg/kg), and behavior was observed for several hours thereafter. Within the first hour after injection, all animals developed seizures evolving into recurrent generalized convulsions [status epilepticus (SE); average time between pilocarpine administration and onset of convulsive SE: 19 ± 2]. SE was interrupted 3 h after onset by administration of diazepam (10 mg/kg i.p). The animals were then assigned to two experimental groups representing different phases of the natural history of the disease: a subgroup was sacrificed 6 h after the first spontaneous seizure; the other subgroup was sacrificed 1 month after SE, i.e., in the chronic period when animals were experiencing an average of 5.3 ± 1.2 spontaneous seizures per day.

Seizures were assessed by 24/24-h, 7/7-day video monitoring, performed using a digital video surveillance system DSS1000 (AverMedia Technologies, USA). Recording electrodes were implanted in the hippocampus and cortex for identification of the first spontaneous seizure [continuous video-EEG (electroencephalogram) monitoring from day 4 after SE until the day of the first spontaneous seizure]. EEG seizure were categorized as paroxysmal activity of high frequency (>5 Hz) characterized by a >3-fold amplitude increment over baseline (Williams et al., 2009; Paradiso et al., 2011). Seizure severity was scored using the scale of Racine (1972): (1) chewing or mouth and facial movements; (2) head nodding; (3) forelimb clonus; (4) generalized seizure with rearing; (5) generalized seizure with rearing and falling. Analysis was performed by two independent investigators that were blind for the group to which the rats belonged. In case of differential evaluation, data were reviewed together to reach a consensus (Paradiso et al., 2011). In the chronic period, animals were continuously video recorded for a week before being killed (i.e., 23–30 days after SE), to identify frequency and duration of generalized seizures.

OOCYTES

Membranes were prepared from the hippocampus and the fronto-temporal cortex. Preparation of *Xenopus laevis* oocytes and injection procedures were as previously described in detail (Miledi et al., 2006). Briefly, tissues were homogenized using a Teflon glass homogenizer with 2 ml of assay buffer of the following composition (in mM): 200 glycine, 150 NaCl, 50 ethylene glycol tetraacetic acid (EGTA), 50 ethylenediaminetetraacetic acid (EDTA), 300 sucrose; 20 μl protease inhibitors (Sigma Aldrich Inc., USA); pH 9 (adjusted using NaOH). The homogenate was centrifuged for 15 min at 9,500 *g*. The supernatant was collected and centrifuged for 2 h at 105 *g* at 4°C. The pellet was washed, re-suspended in 5 mM glycine and used directly, or aliquoted and stored at −80°C for later use. From 12 to 48 h after injection, membrane currents were recorded from voltage-clamped oocytes using two microelectrodes filled with 3 M KCl. The oocytes were placed in a recording chamber (0.1 ml) perfused continuously (9–10 ml/min) with oocyte's Ringer solution (OR) at room temperature (20–22°C). OR had the following composition (in mM): NaCl 82.5, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 1, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5, pH 7.4 (adjusted using NaOH). GABA current rundown was defined as the decrease (in percentage) of the current peak amplitude after six 10-s applications of 1 mM GABA at 40-s intervals (Palma et al., 2007a). The fast I_{GABA} desensitization was defined as the time taken for the current to decay from its peak to half-peak value ($T_{0.5}$).

Levetiracetam was dissolved in H_2O and stored as frozen stock solutions (100 mM). BDNF (Sigma) was dissolved in H_2O, stored as frozen stock solutions (50 μg/ml). Both LEV and BDNF were diluted to working concentrations shortly before the experiments and applied to oocytes for 2 h. In all experiments the holding potential was −60 mV. In some experiments, 3 h washout with OR was performed before initiation of a new rundown protocol.

All drugs were purchased from Sigma except GABA, which was purchased from Tocris (UK). Data in **Figure 1** were analyzed for fitting a single exponential curve; data in **Figures 2** and **3** were statistically analyzed using analysis of variance (ANOVA) and *post hoc* the Holm–Sidak test (SigmaPlot Software, USA).

RESULTS

In agreement with previous reports (Palma et al., 2007b; Mazzuferi et al., 2010) applications of 1 mM GABA to oocytes injected with membranes from the cortex and hippocampus elicited inward currents that were sensitive to 100 μM bicuculline (not shown). Depending on the oocytes, the frogs and the rats, I_{GABA} currents had variable amplitudes: some were as large as −250 nA, others as small as −10 nA. These currents exhibited rundown after repetitive GABA applications: I_{GABA} elicited by the sixth GABA application fell to 69.4 ± 3 and 68.6 ± 4% of the one elicited by the first GABA application in oocytes injected with cortical and

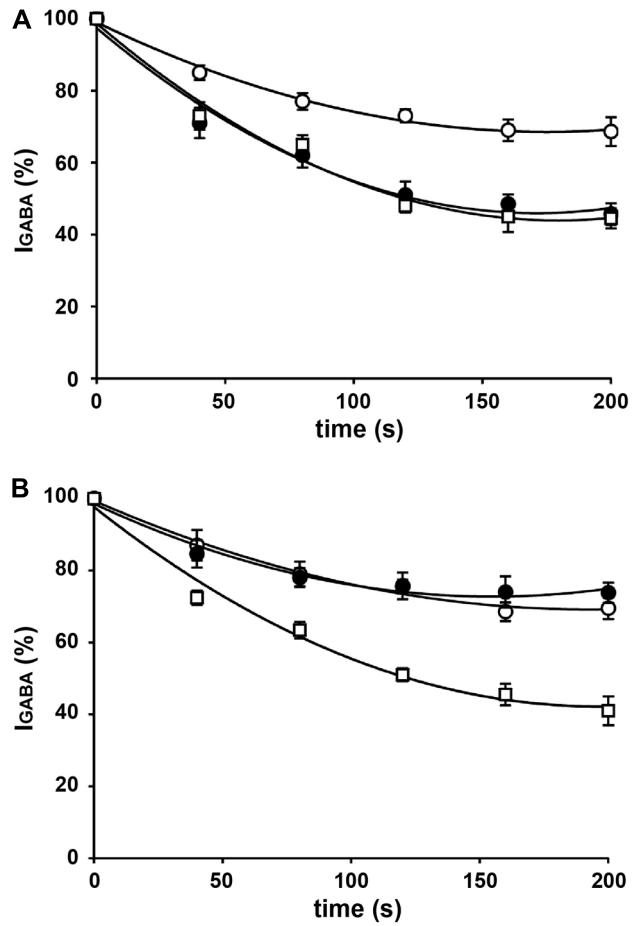

FIGURE 1 | I_{GABA} rundown in oocytes injected with rat brain membranes. (A) Time course of current rundown in oocytes injected with hippocampal membranes form control rats (○), rats killed after the first spontaneous seizure (●), and rats killed in the chronic period, 30 days after SE (□). Peak amplitudes of I_{GABA} were normalized to those elicited by the first GABA application (95 ± 20 nA in the control group; 115 ± 21 nA in the first seizure group; 98 ± 18 nA in the chronic group). **(B)** Time course of current rundown in oocytes injected with cortical membranes form control rats (○), rats killed after the first spontaneous seizure (●), and rats killed in the chronic period, 30 days after SE (□). Peak amplitudes of I_{GABA} were normalized to those elicited by the first application (190 ± 25 nA in control; 230 ± 25 nA in first seizure; 201 ± 30 nA in chronic). GABA pulses were 1 mM, 10-s duration every 40 s. Data are means ± SEM ($n = 12$). All data sets fitted to an exponential curve.

hippocampal membranes, respectively (mean ± SEM of 3 rats, 9 frogs, 49 oocytes). As previously described (Mazzuferi et al., 2010), I_{GABA} rundown was increased in membranes prepared from epileptic rats at the time of the first spontaneous seizure in the hippocampus (fall to 45.7 ± 3%; range 14–62%; $P < 0.01$) but not in the cortex (fall to 73.8 ± 4%; range 43–113%; **Figure 1**). Again consistent with previous reports (Mazzuferi et al., 2010), I_{GABA} rundown was significantly increased both in the hippocampus (44.5 ± 3%; range 20–62%; $P < 0.01$) and in the cortex (41 ± 4%; range 15–54%; $P < 0.01$) of in membranes prepared from chronic animals (**Figure 1**). This current rundown was not accompanied by a significant change in current decay and it was

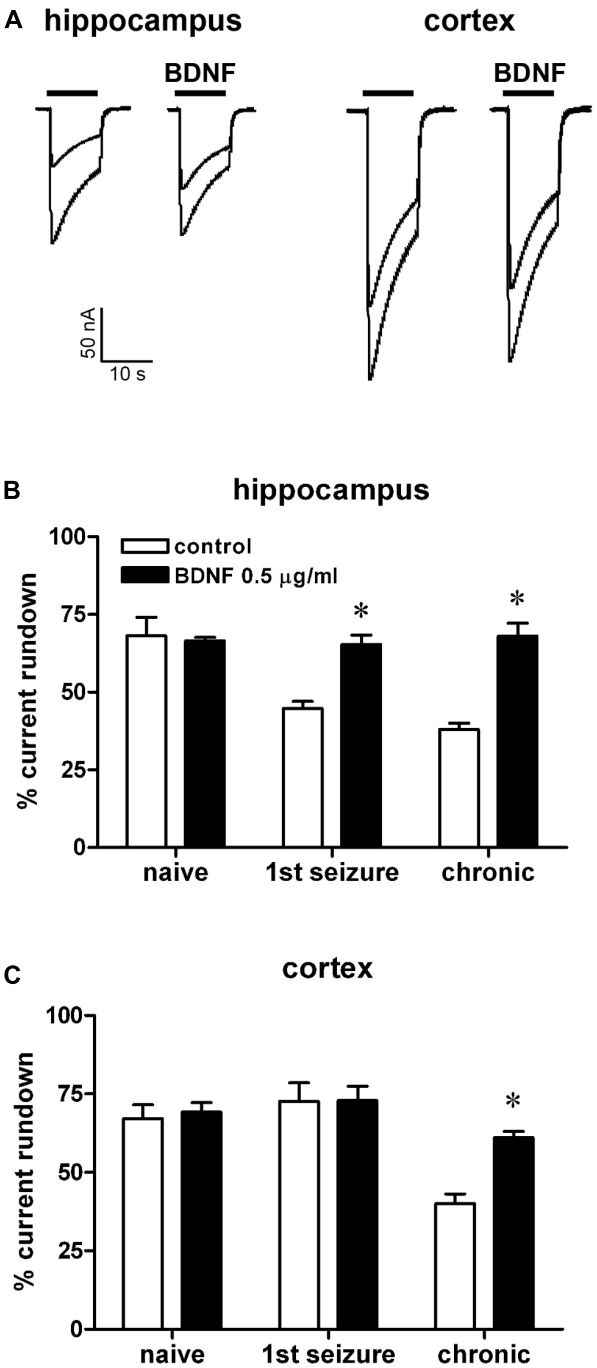

FIGURE 2 | Effect of BDNF on I_{GABA} run-down from oocytes injected with membranes prepared from rats killed at various time points after pilocarpine-induced SE. (A) Representative superimposed currents elicited by the first and sixth GABA application (1 mM, horizontal bar) in oocytes injected with hippocampal or cortical membranes prepared from rats killed 6 h after the first spontaneous seizure, in the presence or absence of 0.5 μg/ml BDNF, as indicated. I_{GABA} rundown in oocytes injected with hippocampal **(B)** or cortical **(C)** membranes, in the absence or in the presence of BDNF, as indicated. Data in **(B,C)** are the means ± SEM of 15–36 oocytes per group (three to four rats; nine frogs). I_{GABA} peak values were normalized to the first I_{GABA} peak current amplitude. Holding potential, −60 mV. $^*P < 0.05$ vs. control values, ANOVA and *post hoc* Holm–Sidak test.

partially reversible after 15–20 min of washout (not shown), as previously shown in human brain tissue (Palma et al., 2004; Ragozzino et al., 2005).

Application of the neurotrophic factor BDNF abolished the increase in I_{GABA} rundown associated with epilepsy. Indeed, in oocytes injected with hippocampal membranes, 2-h incubation with 0.5 μg/ml BDNF decreased I_{GABA} rundown both in the first seizure (65.3 ± 3%) and in the chronic epilepsy group (68 ± 4%; **Figures 2A,B**). Moreover, BDNF abolished the increased I_{GABA} rundown in oocytes injected with cortical membranes from chronic animals (61 ± 2%), whereas it did not influence the small I_{GABA} rundown in first seizure animals (72.6 ± 5% **Figures 2A,C**).

The pattern of LEV effects dramatically differed from the one of BDNF. LEV (1 μM) did not affect rundown in the hippocampus, neither in control nor in epileptic tissues (48.8 ± 3 and 49.0 ± 7%, first seizure and chronic animals, respectively; **Figures 3A,B**), but significantly attenuated it in the epileptic cortex (91.0 ± 5 and 78.0 ± 4%, first seizure and chronic animals, respectively; **Figures 3A,C**). It is noteworthy that BDNF and LEV shared the same effect in decreasing I_{GABA} rundown only in the cortex from chronic animals. This effect was not linked to a change in the current decay ($T_{0.5}$ = 9.0 ± 2.0 s in untreated cortical membranes; 8.4 ± 1.5 s with BDNF; 8.7 ± 1.0 s with LEV; $P > 0.05$).

DISCUSSION

MAIN FINDINGS

We found here that: (1) an increased I_{GABA} rundown is observed in the hippocampus but not in the cortex at the time of the first spontaneous seizure, whereas it is observed in both brain areas in the chronic period; (2) the neurotrophic factor BDNF abolishes this increased rundown in the hippocampus at the time of the first seizure, as well as in the hippocampus and cortex in the chronic period; (3) LEV does not affect rundown in the hippocampus, but attenuates it in the cortex. Below, we will discuss the possible mechanisms by which BDNF and LEV may affect I_{GABA} current rundown, that is, use-dependent $GABA_A$ receptor desensitization; we will propose mechanisms that may underlie the alterations in rundown intensity and sensitivity to drugs during the progression of epilepsy; we will examine the implications of these findings.

BRAIN-DERIVED NEUROTROPHIC FACTOR

The effects of BDNF in epilepsy are still controversial (Simonato et al., 2006). Whereas some studies support a proepileptogenic role (He et al., 2004), BDNF has also been reported to exert beneficial effects based on its neuroprotective and/or neurogenic actions (Paradiso et al., 2009). The anti-rundown effects of BDNF reported here confirm a previous report (Palma et al., 2007b) and suggest an anti-seizure potential. However, it is unclear why BDNF, at variance with LEV, can prevent increased $GABA_A$ receptor rundown in all epileptic tissue that exhibit it, hippocampus or cortex, but does not affect rundown in normal tissue (importantly, this has been also observed in the human tissue; Palma et al., 2005b). A working hypothesis may be that this is due to modulatory effects on $GABA_A$ receptor subunits expressed in

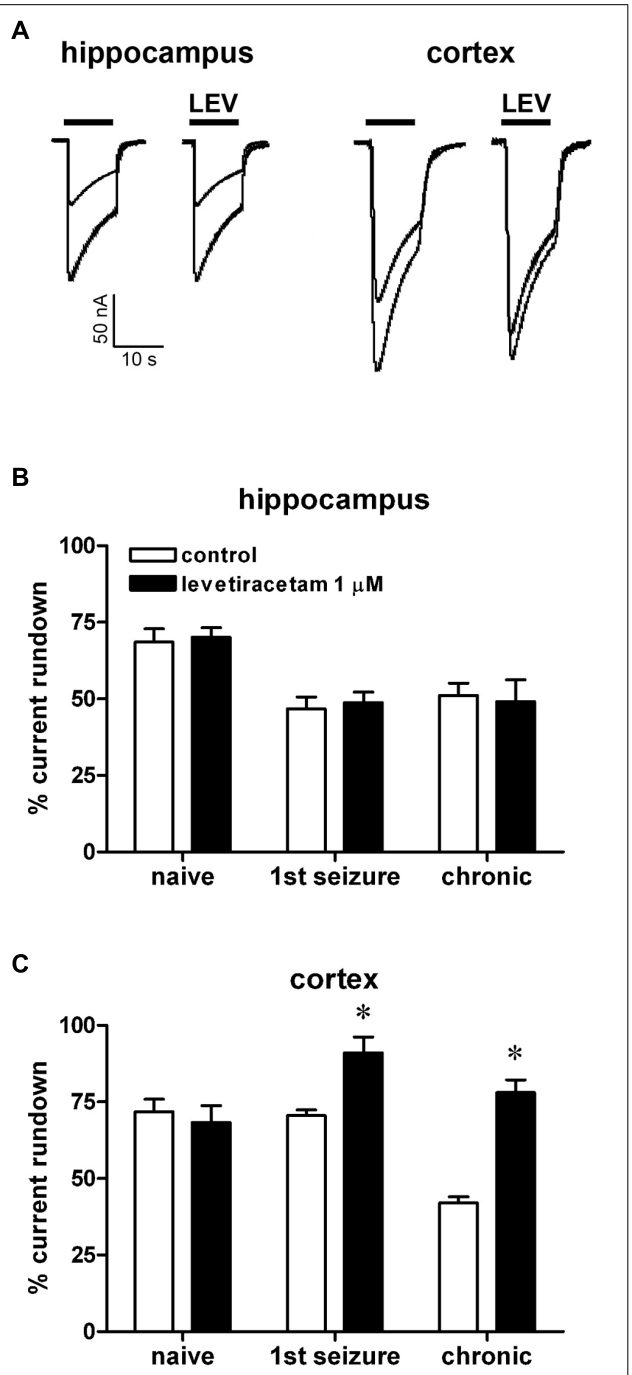

FIGURE 3 | Effect of levetiracetam (LEV) on I_{GABA} run-down from oocytes injected with membranes prepared from rats killed at various time points after pilocarpine-induced SE. (A) Representative superimposed currents elicited by the first and sixth GABA application (1 mM, horizontal bar) in oocytes injected with hippocampal or cortical membranes prepared from rats killed 6 h after the first spontaneous seizure, in the presence or absence of 1 μM LEV, as indicated. I_{GABA} rundown in oocytes injected with hippocampal **(B)** or cortical **(C)** membranes, in the absence or in the presence of LEV, as indicated. Data in **(B,C)** are the means ± SEM of 9–25 oocytes per group (three to four rats; nine frogs). I_{GABA} peak values were normalized to the first I_{GABA} peak current amplitude. Holding potential, −60 mV. *$P < 0.05$ vs. control values, ANOVA and *post hoc* Holm–Sidak test.

the epileptic (but not as much in the normal) hippocampus and cortex.

The molecular mechanisms underlying the increased rundown in the epileptic tissue are still unknown. However, it has been hypothesized that they depend on alterations in $GABA_A$ receptor subunit composition (Mazzuferi et al., 2010). Indeed, changes in the expression levels (thus, in the expected molecular composition) of $GABA_A$ receptors have been described in epilepsy models and in the human epileptic tissue. Increased expression of the α4 subunit has been reported in many studies, whereas the α1 subunit has been reported to be slightly increased, unaltered, or even decreased (Brooks-Kayal et al., 1998; Sperk et al., 2004; Peng and Houser, 2005; Sperk, 2007). Therefore, a shift in balance toward an increase in the relative representation of α4- compared with α1-containing $GABA_A$ receptors has been proposed, and is supported by initial immunohistochemical evidence (Mazzuferi et al., 2010). This alteration should be proepileptic because (1) the α1 subunit is significantly more expressed in animals less susceptible to seizures, like immature (Zhang et al., 2004) or slow kindling rats (Poulter et al., 1999); (2) viral vector-mediated correction of the reduced α1/α4 ratio inhibits epilepsy development (Raol et al., 2006). Moreover, α4-containing $GABA_A$ receptors exhibit reduced response to repetitive GABA application, i.e., increased rundown (Lagrange et al., 2007).

It has been suggested that BDNF may favor increased α4 gene expression and/or decreased α1 gene expression (Brooks-Kayal and Russek, 2012). However, these effects should be pro-epileptic and, therefore, could not account for those observed in the present study. One alternative hypothesis may be based on protein kinase C (PKC) activation. It has been proposed that the abnormal GABA current run-down is caused by receptor dephosphorylation (Palma et al., 2004) and that BDNF modulation of GABA rundown is PKC-dependent (Palma et al., 2005a). Based on these data, it may be hypothesized that BDNF corrects $GABA_A$ receptor malfunction phosphorylating GABA subunits whose expression is altered in epilepsy, like the α1 and the α4, but also the δ or the γ2. Expression of the δ subunit has been reported to be consistently reduced in granule cell dendrites (Schwarzer et al., 1997; Sperk et al., 2004; Nishimura et al., 2005), and the δ subunits may be replaced by γ2 , resulting in impairment of both tonic and phasic GABA transmission (Zhang et al., 2007).

LEVETIRACETAM

Levetiracetam is a widely used antiepileptic drug that also has utility in migraine prophylaxis (Lewis et al., 2004; Glauser et al., 2006). Despite its efficacy, there is no well-accepted mechanism that explains the antiepileptic action of LEV. It is well known that LEV binds to the presynaptic protein SV2A, indicating a role in vesicle exocytosis (Lynch et al., 2004). Because SV2A is implicated in maintaining the size of the readily releasable pool of synaptic vesicles (Custer et al., 2006), LEV has been suggested to directly inhibit presynaptic neurotransmitter release (Yang et al., 2007). In addition, however, PKC inhibitors have been found to block LEV effects on GABA rundown, indicating a role for PKC in LEV action (Palma et al., 2007a). LEV has been also reported to increase ROMK1 channel activity in a PKA-dependent manner (Lee et al., 2008). PKC-mediated phosphorylation of $GABA_A$ receptors (with decreased rundown) and PKA-mediated phosphorylation of the ROMK1 channels (with stabilization of the resting membrane potential) may both contribute to the anti-epileptic effects of LEV, which would therefore include both a pre-synaptic (SV2A) and a post-synaptic (PKC- and PKA-dependent) component. Of course the latter and not the former may be implicated in the effect on I_{GABA} current rundown observed in this study.

It still remains to be determined why LEV does not reduce $GABA_A$ receptor rundown in the hippocampus, whereas it reduces it in the epileptic neocortex even when it is not yet increased by the disease progression, i.e., at the time of the first spontaneous seizure. A working hypothesis may be that this is due to phosphorylation of one or more $GABA_A$ subunits differentially expressed between the epileptic cortex and hippocampus. These subunit(s) should be expressed even before increased rundown is detectable in the cortex and should be different from the one that is putatively targeted by BDNF. *Ad hoc* studies should be performed to challenge this hypothesis. In any event, it is noteworthy that, in a previous work in human mTLE, LEV did not affect subicular $GABA_A$ receptors whereas it profoundly influenced the cortical ones (Palma et al., 2007a), supporting the present finding that LEV effects are brain region specific.

In summary, it may be hypothesized that BDNF exerts its effects by phosphorylation of GABA subunits specifically expressed in the epileptic brain, while LEV may act on other subunit(s) that are specific to the epileptic cortex. Moreover, LEV reduces rundown in the cortex even before it is increased in the chronic epileptic period, whereas BDNF can only abolish disease-associated increases in I_{GABA} rundown. These observations implicate differences in efficacy on the control of seizures of different anatomical origin or occurring at different stages in the natural history of mTLE.

CONCLUSION

In this study, we challenged the hypothesis that the disease progression affects drug responsiveness by examining the sensitivity to pharmacological treatments of the increased I_{GABA} rundown in the epileptic hippocampus and cortex at different stages of experimental mTLE. The data suggest that the sensitivity of $GABA_A$ receptors to pharmacological interventions undergoes changes during the natural history of mTLE, implicating that site of seizure initiation and the timing of treatment may highly affect the therapeutic outcome. Further studies will be needed to better validate this hypothesis and to characterize its mechanism. These will include testing other drugs for their ability to modulate rundown in the different regions and at the different time-points, as well as analyzing the alterations in GABA receptor subunit composition during epilepsy development and correlating it with rundown. Importantly, part of these experiments is amenable to verification in the human tissue. If successful, these studies may lead to new and more effective therapies.

ACKNOWLEDGMENTS

We thank UCB Pharma (Dr. Bruno Ferrò) for supplying LEV. This work was supported by grants from the Ri.MED Foundation (to Pierangelo Cifelli) and from the Italian Ministry for University and Research (PRIN 2009, to Eleonora Palma).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 April 2013; accepted: 21 June 2013; published online: 11 July 2013.
*Citation: Cifelli P, Palma E, Roseti C, Verlengia G and Simonato M (2013) Changes in the sensitivity of $GABA_A$ current rundown to drug treatments in a model of temporal lobe epilepsy. Front. Cell. Neurosci. **7**:108. doi: 10.3389/fncel.2013.00108*

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 25 April 2013
doi: 10.3389/fncel.2013.00046

Resilience to audiogenic seizures is associated with p-ERK1/2 dephosphorylation in the subiculum of *Fmr1* knockout mice

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Young, but not adult, fragile X mental retardation gene (*Fmr1*) knockout (KO) mice display audiogenic seizures (AGS) that can be prevented by inhibiting extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation. In order to identify the cerebral regions involved in these phenomena, we characterized the response to AGS in *Fmr1* KO mice and wild type (WT) controls at postnatal day (P) 45 and P90. To characterize the diverse response to AGS in various cerebral regions, we evaluated the activity markers FosB/ΔFosB and phosphorylated ERK1/2 (p-ERK1/2). Wild running (100% of tested mice) followed by clonic/tonic seizures (30%) were observed in P45 *Fmr1* KO mice, but not in WT mice. In P90 *Fmr1* KO mice, wild running was only present in 25% of tested animals. Basal FosB/ΔFosB immunoreactivity was higher ($P < 0.01$ vs. WT) in the CA1 and subiculum of P45 *Fmr1* KO mice. Following the AGS test, FosB/ΔFosB expression consistently increased in most of the analyzed regions in both groups at P45, but not at P90. Interestingly, FosB/ΔFosB immunoreactivity was significantly higher in P45 *Fmr1* KO mice in the medial geniculate body ($P < 0.05$ vs. WT) and CA3 ($P < 0.01$). Neurons presenting with immunopositivity to p-ERK1/2 were more abundant in the subiculum of *Fmr1* KO mice in control condition ($P < 0.05$ vs. WT, in both age groups). In this region, p-ERK1/2-immunopositive cells significantly decreased (–75%, $P < 0.01$) in P90 *Fmr1* KO mice exposed to the AGS test, but no changes were found in P45 mice or in other brain regions. In both age groups of WT mice, p-ERK1/2-immunopositive cells increased in the subiculum after exposure to the acoustic test. Our findings illustrate that FosB/ΔFosB markers are overexpressed in the medial geniculate body and CA3 in *Fmr1* KO mice experiencing AGS, and that p-ERK1/2 is markedly decreased in the subiculum of *Fmr1* KO mice resistant to AGS induction. These findings suggest that resilience to AGS is associated with dephosphorylation of p-ERK1/2 in the subiculum of mature *Fmr1* KO mice.

Keywords: acoustic stimulus, epilepsy, extracellular signal-regulated kinase (ERK), FosB, Fragile X Syndrome, hippocampus, geniculate body, subiculum

INTRODUCTION

The Fragile X Syndrome (FXS) is one of the leading causes of mental retardation (Rousseau et al., 1995; Dombrowski et al., 2002). In FXS, CGG triplet expansion in the fragile X mental retardation gene (*Fmr1*) prevents the synthesis of the fragile X mental retardation protein (FMRP) (De Rubeis and Bagni, 2010), causing anatomical and functional alterations, such as abnormal dendrite spines morphology and dysfunctions in synaptic plasticity (Zalfa et al., 2006). Individuals affected by FXS suffer from mental retardation, learning disabilities, and attention deficit. They also show behavioral problems including anxiety, autism, hyperactivity, and aggression (Hagerman, 1996). FXS patients may also respond to olfactory, tactile, visual, and auditory stimuli with hyper-reactivity and convulsions (Ferri et al., 1994; Miller et al., 1999; Berry-Kravis et al., 2010). The prevalence of epilepsy in FXS is larger than in the normal population, ranging from 14 to 50% of FXS patients (Berry-Kravis, 2002).

Epilepsy associated with FXS is generally benign, spontaneously remitting during or immediately after adolescence (Singh et al., 1999). Rolandic epileptiform potentials during hand tapping suggest neurophysiological similarities between FXS and benign childhood epilepsy with centrotemporal spikes (Musumeci et al., 1994). In absence of external stimulation, epileptic condition in FXS patients has been extensively investigated (Musumeci et al., 1988, 1991, 1999; Sabaratnam et al., 2001; Berry-Kravis et al., 2010; Gauthey et al., 2010). Seizures in FXS individuals appear after the age of 2 years with some cases of late onset. They are frequently complex partial seizures, but simple partial seizures, generalized tonic-clonic seizures, febrile convulsions, and *status epilepticus* have been observed as well. Seizure foci are commonly located in frontal or temporal lobes. Although in most of the epileptic FXS patients seizures are well controlled by antiepileptic drugs (Hagerman and Stafstrom, 2009), sometimes they may be frequent, severe and unresponsive

to treatments (Sabaratnam et al., 2001; Incorpora et al., 2002). Even when very mild and well controlled pharmacologically, seizures may still be dangerous for FXS patients. Indeed, recently it has been demonstrated that in an animal model of temporal lobe epilepsy (TLE), pilocarpine-treated rats experiencing seizures show an increase in dopamine neuron activity and an increase in amphetamine-stimulated locomotor activity, suggesting that TLE-associated psychosis is probably due to abnormal hippocampal overdrive of dopamine neuron activity (Cifelli and Grace, 2012). In addition, a number of well-known genetic disorders, including FXS, but also tuberous sclerosis complex and Rett Syndrome, shares epilepsy, intellectual disability and autism (Brooks-Kayal, 2011), suggesting a possible link between epilepsy and psychiatric disorders. In recent studies it has been found an increased incidence of seizures in individuals with FXS also diagnosed with autism compared to FXS patients without autism (Garcia-Nonell et al., 2008; Berry-Kravis et al., 2010). In addition, it has been shown that in another form of mental retardation, the Down Syndrome, cognitive deficit is more pronounced in patients presenting epilepsy than in Down Syndrome patients without epilepsy (Eisermann et al., 2003). These observations suggest that early life seizures can result in cellular and molecular changes that could contribute to learning and behavioral disabilities and that, similarly to Down Syndrome, also in FXS, epilepsy may play a crucial role in worsening cognitive functions. As electroencephalographic (EEG) abnormalities have been observed also in non-epileptic FXS patients (Berry-Kravis, 2002), and short non-spreading events not associated with obvious clinical manifestations (subclinical seizures) have been demonstrated in other forms of partial epilepsy (D'Ambrosio et al., 2009), abnormal brain activity can actually be a problem not restricted to the 23% of FXS population presenting with spontaneous motor seizures. Therefore, a better understanding of epileptic activity in FXS is crucial for improving quality of life of all FXS patients.

Fmr1 knockout (KO) mice provide a suitable animal model for studying FXS because they reproduce the FXS phenotype (The Dutch-Belgian Fragile X Consorthium, 1994). Although they have not been evaluated for epilepsy prospectively by video-EEG, the presence of age-dependent epilepsy was reported in these mice (Musumeci et al., 1999, 2000). Similarly to FXS patients, they are characterized by an anomalous reaction to sensory stimuli (Musumeci et al., 2000) and show audiogenic seizures (AGS), characterized by wild running followed by clonic, tonic-clonic, and/or tonic convulsions in response to loud sounds (Henry, 1967; Musumeci et al., 2000; Chen and Toth, 2001). Recent studies have demonstrated that inhibitors of extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation prevent AGS induction in *Fmr1* KO mice (Osterweil et al., 2010; Michalon et al., 2012; Wang et al., 2012). In order to further investigate the role of phosphorylated ERK1/2 (p-ERK1/2) in AGS in the FXS mouse model, we considered two different age groups of *Fmr1* KO mice and compared them with age-matched wild type (WT) control animals. Both genotypes were exposed to the test for AGS induction at postnatal day (P) 45 or P90. In order to verify the effects of the testing procedure and AGS induction on neuronal networks, we also investigated the expression of the activity markers FosB/ΔFosB (Biagini et al., 2005, 2008), the ideal tool available now to investigate network activity in epileptic animals (Chen et al., 1997; Biagini et al., 2005; Madsen et al., 2006) since products of the *fosB* gene family are stable and tend to accumulate in repeatedly activated neurons (Chen et al., 1997; Kelz and Nestler, 2000). Instead, c-Fos, another tool being used for many years to track acute changes in neuronal network activity (Chen and Toth, 2001), is extremely short living making c-Fos reliability questionable in cases of recurrent neuronal synchronization. In line with the previous findings in other models of epilepsy (Biagini et al., 2005, 2008), we found that FosB/ΔFosB immunoreactivity was significantly correlated with seizure induction in P45 *Fmr1* KO mice, especially in acoustic regions (medial geniculate body) and the hippocampus proper (CA3). Interestingly, p-ERK1/2 was not significantly changed in *Fmr1* KO mice experiencing seizures, but it was instead markedly decreased in the subiculum of P90 *Fmr1* KO mice that were resistant to AGS induction. These findings suggest that the disappearance of sensitivity to AGS during development is associated with dephosphorylation of p-ERK1/2 in the subiculum.

MATERIALS AND METHODS

ANIMALS

Male C57BL/6 WT ($n = 22$) and C57BL/6 *Fmr1* KO mice ($n = 25$) were studied at P45 ($n = 25$) or P90 ($n = 22$). Mice were housed at the animal facility of the Montreal Neurological Institute and sacrificed after the testing procedure. All the procedures were approved by the Canadian Council of Animal Care and were in accordance with the European Communities Council Directive 2010/63/EU.

AUDIOGENIC TESTS

The experimental chamber consisted in a plastic cage 25 × 25 × 47 cm in which the doorbell (Electrical bell Heath Zenith, model 172C-A) was mounted on the cage roof. Mice were taken from their housing room one by one, transferred into the experimental chamber and allowed to explore the novel environment (basal noise ∼65 dB) for a period of 30 s, then the bell was rung (122 dB), while concomitant behavior was video-recorded. The motor responses were classified using a scale modified from the one originally described by Jobe et al. (1973): no response (NR, in **Figure 1**, consisting of pause or continuous exploration), wild running (WR, in **Figure 1**), clonic seizure (CS, in **Figure 1**), tonic seizure (TS, in **Figure 1**), respiratory arrest and/or death (RA, in **Figure 1**). In order to define the intensity of the behavioral response we used the seizure severity score (SSS, in **Figure 1**) (Musumeci et al., 2000), consisting of a score assigned to each animal depending on its behavioral response (NR = 0, WR = 1, CS = 2, TS = 3, RA = 4). Mice of WT ($n = 6$ at P45, $n = 4$ at P90) and *Fmr1* KO ($n = 9$ at P45, $n = 8$ at P90) groups were exposed to a single 60 s continuous stimulus. As controls, WT ($n = 6$ at P45, $n = 6$ at P90) and *Fmr1* KO mice ($n = 4$ at P45, $n = 4$ at P90) were placed in the chamber but the bell was not rung.

IMMUNOHISTOCHEMISTRY AND DENSITOMETRIC ANALYSIS

Tested WT and *Fmr1* KO mice, and untested WT and *Fmr1* KO mice were decapitated under deep isoflurane anesthesia 14–17 h after the behavioral test. Brains were extracted and cut

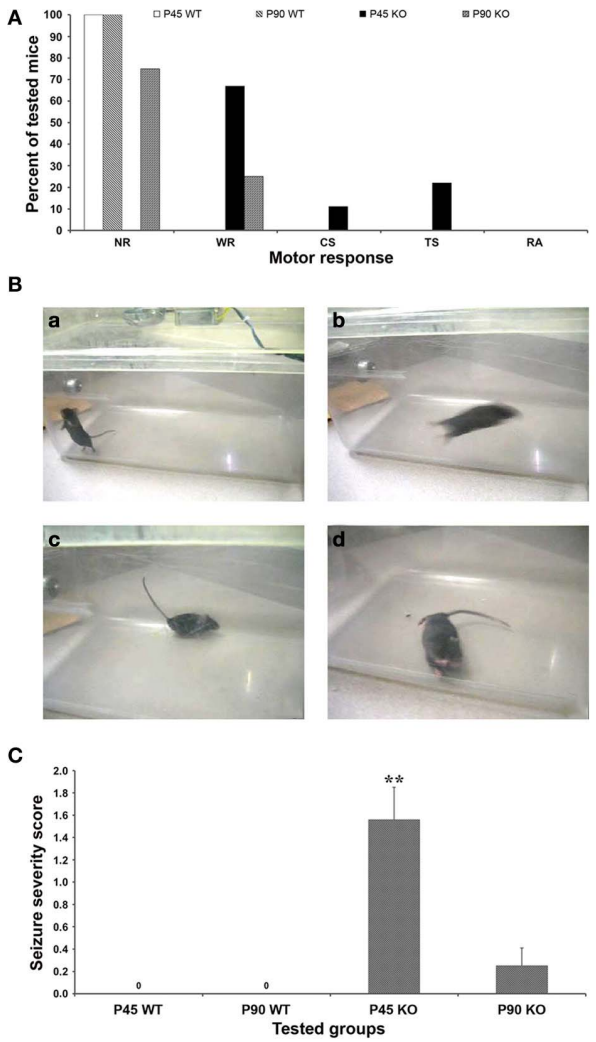

FIGURE 1 | Behavioral response to audiogenic stimuli. (A) *Fmr1* KO mice studied at postnatal day (P) 90 showed a less intense motor response to the audiogenic stimuli (6 NR, 2 WR; $n = 8$) than P45 group (6 WR, 1 CS, 2 TS; $n = 9$), both higher than WT mice that did not respond behaviorally to the test either at P45 nor at P90. **(B)** Frames obtained from the video recordings of a P45 WT mouse during exploratory behavior (a), and of P45 *Fmr1* KO mice during WR (b), Straub tail (c), TS (d). **(C)** The seizure severity score (SSS) shows that the intensity of seizing in P45 *Fmr1* KO mice is 6-fold higher than in P90 group, both higher than WT mice (no response). $^{**}P < 0.01$, Kruskal–Wallis test followed by Dunn's test. NR, no response; WR, wild running; CS, clonic seizures; TS, tonic seizures; RA, respiratory arrest.

in 1 mm-thick horizontal sections using a vibratome (VT1000S Leica, Germany); slices were fixed overnight in 4% formaldehyde, cryoprotected in 15 and 30% sucrose-buffered solutions, and then stored at −80°C. Forty μm-thick sections were obtained at the cryostat (Leica, Jung CM 3000, Germany) and processed for immunohistochemistry using a rabbit polyclonal anti-FosB/ΔFosB (H75) antibody (sc-7203, Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:250) (Biagini et al., 2005, 2008) or a mouse monoclonal antibody against p-ERK1/2 (Thr202/Tyr204, cat-9106, Cell Signal Technology, Beverly, MA, USA; dilution 1:1500), according to the avidin-biotin-peroxidase complex technique and using diaminobenzidine as a chromogen (Biagini et al., 2005, 2008). Briefly, sections were treated with 3% H_2O_2 in phosphate buffered saline (PBS) to block endogenous peroxidase and then incubated with the primary antibody for 48 h at 4°C. Slices were then incubated with the secondary antibody (dilution 1:200) for 1 h at room temperature and finally incubated with streptavidin biotinylated horseradish peroxidase complex (dilution 1:300) for 45 min, always at room temperature.

Sections mounted on gelatin-coated slides were analyzed using the image analysis software KS300 (Zeiss-Kontron, Munich, Germany). Background values in stained sections were obtained from areas that did not contain any stained cell (i.e., the angular bundle). Stained profiles were discriminated from background throughout every sampled region (Biagini et al., 1993, 1998, 2005, 2008). Cell profile counts were determined in each field as the number of immunopositive profiles after transformation in D-circles (i.e., the diameter of circles having the same area as measured) by considering a minimum cutoff value of 7 μm. Cell counts were then divided by the sampled field area and expressed as cell densities. Sampled areas were the hippocampal regions CA1 and CA3, dentate gyrus, presubiculum, subiculum, entorhinal cortex, perirhinal cortex, lateral amygdala, primary auditory cortex, and medial geniculate body.

IMMUNOFLUORESCENCE

Double-immunostaining experiments were performed on free floating sections washed in PBS at room temperature and permeabilized for 1 h in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin. For double immunolabeling, sections were incubated overnight in a mixture of the mouse monoclonal anti-p-ERK1/2 (1:500) and the rabbit polyclonal anti-FosB/ΔFosB antibody (1:250). Further co-labeling experiments were designed using the mouse monoclonal anti-p-ERK (1:500) and, respectively, a rabbit polyclonal antibody against parvalbumin (no. 235, Swant, Bellinzona, CH; diluted at 1:2000), neuropeptide Y (no. IHC 7180, Peninsula, San Carlos, CA, USA; diluted at 1:800), and somatostatin (no. 20089, Immunostar, Hudson, WI, USA; diluted at 1:1000). After washing, sections were incubated for 90 min at room temperature in a 1:200 dilution of goat anti-mouse AlexaFluor546® and goat anti-rabbit AlexaFluor488® (Invitrogen, Carlsbad, California). Sections were counterstained with 4′,6 diamidino-2-phenylindole (DAPI, Vector Laboratories, USA) to assess nuclear morphology. Images were visualized using a Leica TCS SP2 confocal microscope, equipped with Argon (488 nm) and Helium/Neon (543 nm).

STATISTICAL ANALYSIS

Data on behavioral score were analyzed with the Fisher's test. The seizure severity score was analyzed with Kruskal–Wallis nonparametric analysis of variance (ANOVA), followed by *post-hoc* Dunn's test for multiple comparisons. Cell counts underwent a Three-Way ANOVA, using as factors the genotype (WT or *Fmr1* KO), the acoustic test (yes or no) and the age (P45 or P90).

Post-hoc test for multiple comparisons was the Fisher's Least-Significant-Difference (LSD). Data were analyzed with Sigmaplot 11 (Systat Software, San Jose, CA, USA). Results are shown as mean ± standard error of the mean (SEM), and $P < 0.05$ was considered statistically significant.

RESULTS

MOTOR RESPONSE ANALYSIS

Audiogenic stimulus was given once and concomitant animal behavior was observed and scored (**Figure 1**). The test for AGS did not induce anomalous behaviors in WT mice ($n = 6$ at P45 and $n = 4$ at P90; **Figure 1A**), which continued to explore the novel environment (**Figure 1Ba**) or, sometimes, paused. In contrast, a motor response was triggered in P45 *Fmr1* KO mice ($n = 9$), 6 of which developed wild running episodes (**Figure 1Bb**) and often presented with the Straub tail (**Figure 1Bc**); one progressed from wild running to clonic seizures, and 2 further progressed to tonic seizures (**Figure 1Bd**). In P90 *Fmr1* KO mice ($n = 8$) the response was less marked than in P45 mice: only 2 out of 8 developed wild running episodes (**Figure 1A**). All mice resumed normal behavior before the acoustic stimulation was over, and death due to respiratory arrest was never observed neither in P45 nor in P90 group. Motor response rate was 100% in P45 *Fmr1* KO group (9 out of 9), 25% in P90 *Fmr1* KO mice (2 out of 8) and 0% (no response) in P45 and P90 WT groups ($P < 0.01$, Fisher's test).

Using a scale created for AGS (see Materials and Methods section for details), the seizure severity score was calculated for each group: it was 1.56 ± 0.29 in P45 *Fmr1* KO mice, 0.25 ± 0.16 in P90 *Fmr1* KO mice, and 0.0 in P45 and P90 WT mice (**Figure 1C**). The Kruskal–Wallis test revealed a significant difference between seizure severity score at P45 and P90 *Fmr1* KO mice ($P < 0.01$). These findings confirm that FXS mice are more susceptible to AGS compared to WT mice and that this susceptibility is age-dependent, resulting more pronounced in young adult FXS mice than in older subjects (Musumeci et al., 2000, 2007; Yan et al., 2005).

FosB/ΔFosB IMMUNOSTAINING

In order to disclose the neuronal networks mediating AGS, we mapped FosB/ΔFosB expression in activated neurons in hippocampal (Sub, CA1, CA3, DG in **Figures 2A,B**), parahippocampal (preSub, EC in **Figures 2A,C**) and extrahippocampal regions (LA in **Figures 2A,C**), including the auditory pathways (MGB, Au1 in **Figures 3A,B**). FosB/ΔFosB immunoreactivity was barely detectable in P45 WT mice in control condition

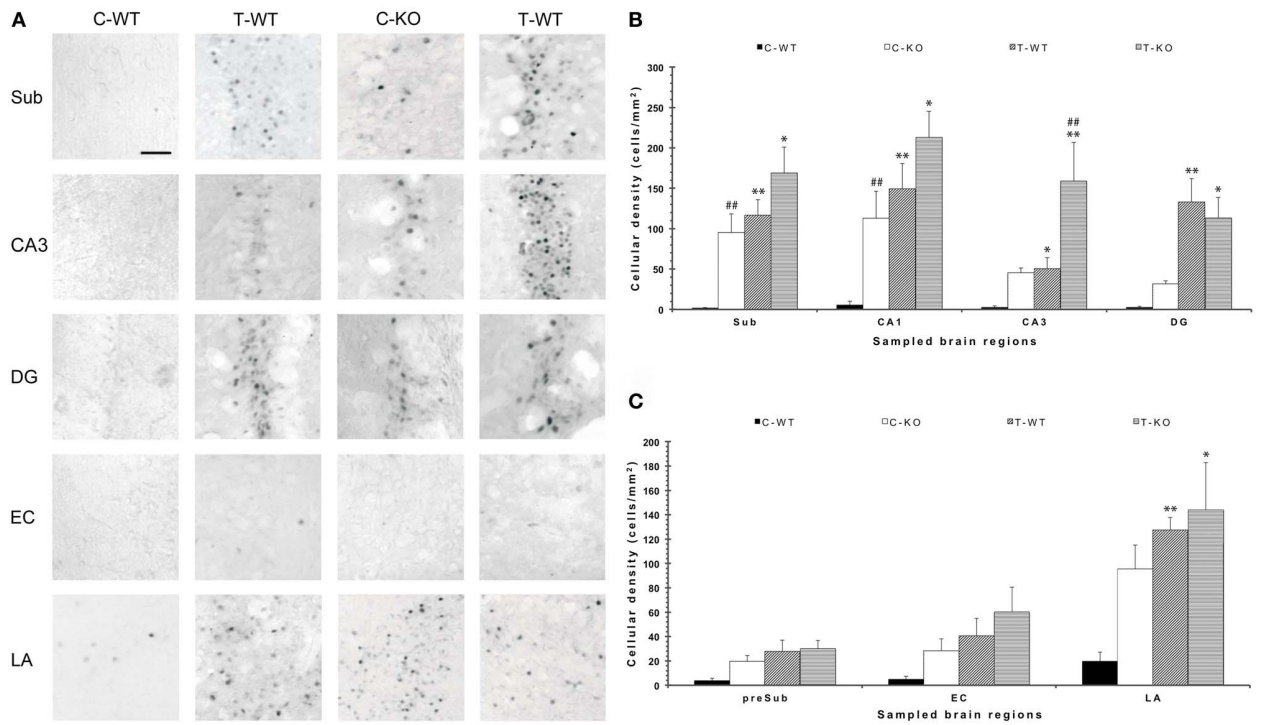

FIGURE 2 | Changes in limbic FosB/ΔFosB immunoreactivity determined by exposure to a test for audiogenic seizure induction and in relation with the mouse genotype (WT *vs.* *Fmr1* KO) at P45. **(A)** Photomicrographs of FosB/ΔFosB-immunopositive cell nuclei in the Sub, CA3, DG, EC, and LA. Scale bar: 25 μm. **(B)** Bar graphs of cellular density based on counts of FosB/ΔFosB-immunopositive cell nuclei per mm^2 in Sub, CA1, CA3 and DG. **(C)** Bar graphs of cellular density based on count of FosB/ΔFosB-immunopositive cell nuclei per mm^2 in preSub, EC and LA. $^*P < 0.05$, $^{**}P < 0.01$, acoustically stimulated group vs. the respective unstimulated group; $^{\#\#}P < 0.01$, *Fmr1* KO vs. WT mice with similar treatment (tested or not tested). Three-way ANOVA followed by LSD test. C-WT, WT mice not exposed to auditory stimulus; T-WT, WT mice exposed to the audiogenic test, C-KO, *Fmr1* KO mice not exposed to auditory stimulus; T-KO, *Fmr1* KO mice tested; Sub, subiculum; CA, cornu Ammonis; DG, dentate gyrus; EC, entorhinal cortex; LA, lateral amygdala.

(C-WT; **Figure 2A**), while it increased after the acoustic test (T-WT) in several brain regions and it reached statistical significance ($P < 0.01$, LSD test) in the subiculum, CA1, dentate gyrus, lateral amygdala (**Figures 2B,C**) and primary acoustic area (**Figures 3A,B**). A significant ($P < 0.05$) increase was present also in the CA3 hippocampal region (**Figures 2A,B**). Interestingly, in non-stimulated P45 *Fmr1* KO mice, FosB/ΔFosB values were significantly higher than in WT mice already in basal conditions (C-KO in **Figures 2A,B**), particularly in the hippocampus proper ($P < 0.01$ for CA1) and in the subiculum ($P < 0.01$; **Figure 2B**). Following exposure to the test for AGS, FosB/ΔFosB immunoreactivity was significantly increased in P45 *Fmr1* KO mice (T-KO in **Figures 2A–C**) in the hippocampus ($P < 0.05$ for CA1 and $P < 0.01$ for CA3), subiculum ($P < 0.05$), dentate gyrus ($P < 0.05$), lateral amygdala ($P < 0.05$), medial geniculate body ($P < 0.05$, **Figure 3A**), and primary acoustic cortex ($P < 0.05$, **Figures 3A,B**) compared with unstimulated *Fmr1* KO mice, while it was unchanged in the presubiculum and entorhinal cortex (**Figures 2A,C**). In addition, in the CA3 region the density of immunopositive neurons was significantly ($P < 0.01$) higher in P45 *Fmr1* KO mice after the test compared with age-matched WT also exposed to the testing procedure. A significant difference ($P < 0.05$) between the two genotypes, after the auditory test, was also found in the medial geniculate body ($P < 0.05$, **Figure 3A**), but not in other sampled areas (**Figures 2B**,**C**, **3A**).

Following exposure to the test for AGS of P90 mice, FosB/ΔFosB immunoreactivity was not increased in *Fmr1* KO mice (T-KO; **Table 1**) compared with unstimulated P90 *Fmr1* KO mice (C-KO; **Table 1**) in none of the sampled areas, including acoustic regions. In addition, no differences were found in age-matched WT mice (T-WT; **Table 1**) also exposed to the testing procedure.

The statistical analysis (Three-Way ANOVA) revealed main effects of test exposure in the subiculum ($P < 0.05$), CA3 ($P < 0.05$), dentate gyrus ($P < 0.01$) and amygdala ($P < 0.05$). The different genotype did not affect the level of FosB/ΔFosB immunoreactivity in any of the sampled regions, whereas age-related changes were found in the subiculum ($P < 0.05$) and CA3 ($P < 0.05$). Interestingly, a significant interaction of all the 3 main factors (genotype × test × age) was present only in the subiculum ($P < 0.05$). In the hippocampal CA1 and CA3 subfields, as well as in the subiculum, significant ($P < 0.01$ for all regions) interactions between test exposure and age, as well as between genotype and age ($P < 0.01$ for all regions) were present. Significant interactions between test exposure and age were present in the dentate gyrus ($P < 0.01$) and entorhinal cortex ($P < 0.01$); in the latter we also found a significant interaction between age and

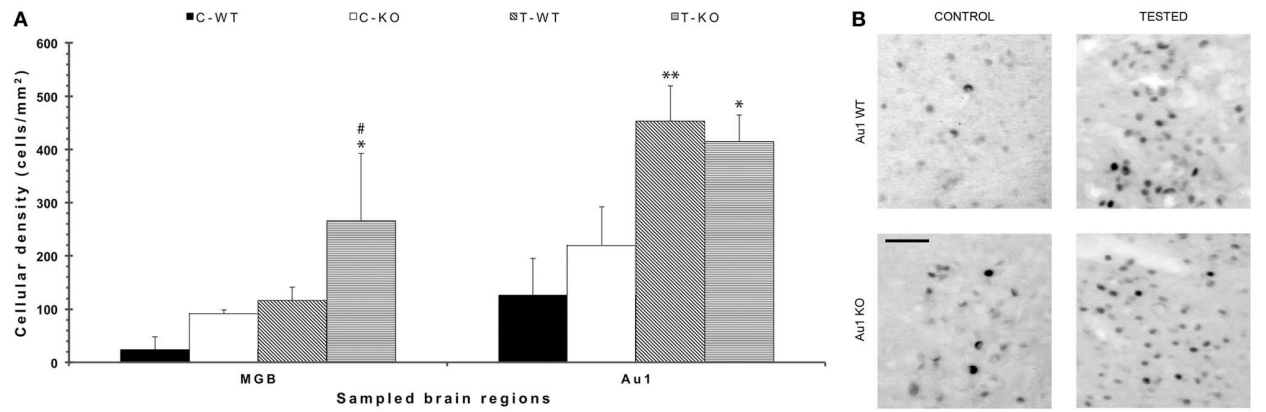

FIGURE 3 | FosB/ΔFosB immunostaining in acoustic structures after exposure to the test for audiogenic seizure induction and in relation with the mouse genotype (WT *vs. Fmr1* KO) at P45. (A) Bar graphs of cellular density based on counts of FosB/ΔFosB-immunopositive cell nuclei per mm^2 in the MGB and Au1. *$P < 0.05$, **$P < 0.01$, acoustically stimulated group vs. the respective unstimulated group; #$P < 0.05$, *Fmr1* KO vs. WT mice with similar treatment (tested or not tested). Three-way ANOVA followed by LSD test. **(B)** Photomicrographs of FosB/ΔFosB-immunopositive cell nuclei in Au1. Scale bar: 25 μm. C-WT, WT mice not exposed to auditory stimulus; T-WT, WT mice exposed to the audiogenic test; C-KO, *Fmr1* KO mice not exposed to auditory stimulus; T-KO, *Fmr1* KO mice tested; MGB, medial geniculate body; Au1, primary acoustic cortex.

Table 1 | Cellular density based on count of FosB/ΔFosB-immunopositive cell nuclei per mm^2 in *Fmr1* KO and WT mice studied at age P90.

	Sub	CA1	CA3	DG	preSub	EC	LA	MGB	Au1
C-WT	117 ± 20	87 ± 40	45 ± 60	89 ± 40	25 ± 90	27 ± 60	ND	13 ± 20	47 ± 70
T-WT	67 ± 11	34 ± 21	29 ± 20	71 ± 12	25 ± 22	12 ± 50	ND	25 ± 50	32 ± 70
C-KO	76 ± 42	59 ± 37	20 ± 10	35 ± 16	12 ± 11	10 ± 70	ND	36 ± 16	33 ± 80
T-KO	79 ± 45	42 ± 20	14 ± 80	79 ± 45	14 ± 80	12 ± 10	ND	25 ± 80	78 ± 13

No significant differences were found. It was not possible to sample a sufficient number of animals for lateral amygdala (LA).

C-WT, WT mice not exposed to auditory stimulus; T-WT, WT mice exposed to the acoustic test; C-KO, Fmr1 KO mice not exposed to auditory stimulus; T-KO, Fmr1 KO mice tested; Sub, subiculum; CA, cornu Ammonis; DG, dentate gyrus; preSub, presubiculum; EC, entorhinal cortex; LA, lateral amygdala; MGB, medial geniculate body; Au1, primary acoustic area; ND, not determined.

genotype ($P < 0.05$). No significant interactions were found in the amygdala. The analyzed acoustic regions presented with significant effects of test ($P < 0.05$ for the medial geniculate body; $P < 0.01$ for the primary acoustic region) and age ($P < 0.05$ for the medial geniculate body; $P < 0.01$ for the primary acoustic region), as well as significant interactions between test exposure and age ($P < 0.05$ for the medial geniculate body; $P < 0.01$ for the primary acoustic region).

CHANGES OF ERK1/2 ACTIVATION IN RELATION TO SEIZURES AND AGE

As further marker of neuronal activation, we evaluated p-ERK1/2 immunoreactivity in basal conditions and after the test (**Figures 4** and **5**). In limbic structures, p-ERK1/2-immunoreactive cells were consistently observed in the subiculum and perirhinal cortex of both strains. Sparse immunopositive cells were also present in other regions in few mice. Thus, we focused on the subiculum and perirhinal cortex to analyze changes in p-ERK1/2-immunoreactive cell counts due to test exposure. In the subiculum, p-ERK1/2-immunoreactive cells were located in the pyramidal cell layer, as in the case of FosB/ΔFosB-immunopositive cells (cf. **Figure 2A**). The Three-way ANOVA revealed a significant ($P < 0.05$) effect of age and a significant ($P < 0.01$) interaction of genotype and test. Notably, a significant ($P < 0.01$) interaction among genotype, age and test was also present. Interestingly, a significant large number of p-ERK1/2-immunopositive cells was already detectable in basal conditions

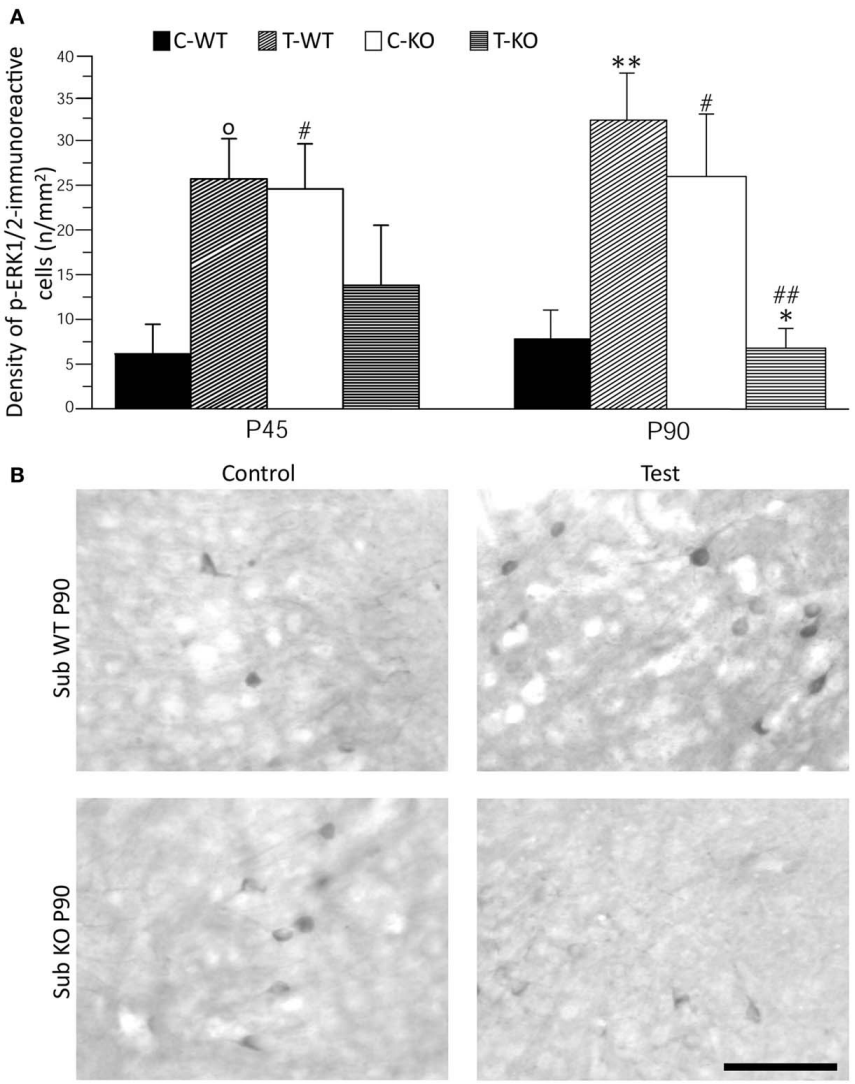

FIGURE 4 | Immunostaining of p-ERK1/2 in subiculum (Sub) after exposure to the test for audiogenic seizure induction and in relation with the mouse genotype (WT *vs. Fmr1* KO) and the age (P45 and P90). **(A)** Bar graphs of cellular density based on counts of p-ERK1/2-immunopositive cell nuclei per mm^2 in Sub. **(B)** Photomicrographs of p-ERK1/2-immunopositive cells in the Sub of P90 mice of both genotypes. Scale bar: 100 μm. C-WT, WT mice not exposed to auditory stimulus; T-WT, WT mice exposed to the audiogenic test; C-KO, *Fmr1* KO mice not exposed to auditory stimulus; T-KO, *Fmr1* KO mice tested. $^{o}0.1 < P < 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, acoustically stimulated group vs. the respective unstimulated group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *Fmr1* KO vs. WT mice with similar treatment (tested or not tested). Three-way ANOVA followed by LSD test.

in *Fmr1* KO (**Figure 4B**), both in P45 and P90 groups of age ($P < 0.05$, Fisher's LSD test; **Figure 4A**). After exposure to the AGS test, p-ERK1/2-immunoreactive cells did not change significantly at P45 in both genotypes (but a trend toward increase was present in P45 WT mice; $P = 0.06$ vs. basal values), whereas a remarkable decrease (−75%) was observed in *Fmr1* KO mice at P90 ($P < 0.05$, **Figure 4**). This change was at variance with the significant ($P < 0.01$ vs. basal levels in WT) increase in p-ERK1/2 levels observed in WT rats exposed to AGS induction. Thus, at P90, after exposure to the AGS test, levels of p-ERK1/2-immunopositive cells in *Fmr1* KO mice were significantly less than in tested WT ($P < 0.01$; **Figure 4**).

Neuronal immunolabelling with the anti-p-ERK1/2 antibody was consistently observed also in the superficial layers of perirhinal cortex. We analyzed the pattern of p-ERK1/2 immunoreactivity and found them to be similar both in P45 and P90 groups and independent of exposure to the AGS test (**Figure 5**).

CHARACTERIZATION OF p-ERK1/2-IMMUNOPOSITIVE CELLS

We investigated whether neurons identified by FosB/ΔFosB and p-ERK1/2 antibodies were the same cells or, instead, distinct elements. Experiments of co-labeling with FosB/ΔFosB and p-ERK1/2 antibodies in P45 mice revealed that approximately 50% of p-ERK1/2-immunopositive cells expressed FosB/ΔFosB antigens, whereas the other 50% was composed by distinct neuronal elements, both in subiculum (**Figures 6A–C**) and perirhinal cortex (**Figures 6D–I**), independently of the genotype.

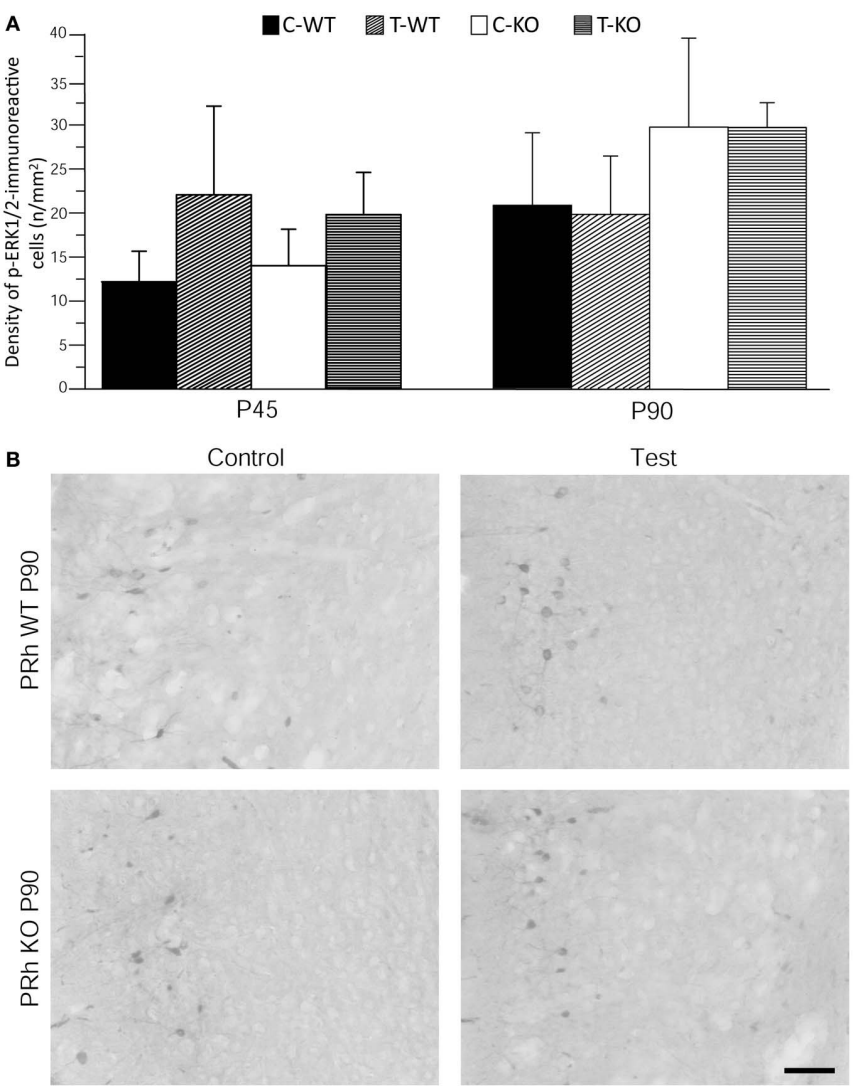

FIGURE 5 | Immunostaining of p-ERK1/2 in perirhinal cortex (PRh) after exposure to the test for audiogenic seizure induction and in relation with the mouse genotype (WT *vs. Fmr1* KO) and the age (P45 and P90). (A) Bar graphs of cellular density based on counts of p-ERK1/2-immunopositive cell nuclei per mm^2 in PRh. **(B)** Photomicrographs of p-ERK1/2-immunopositive cells in the PRh of P90 mice of both genotypes. Scale bar: 100 μm. C-WT, WT mice not exposed to auditory stimulus; T-WT, WT mice exposed to the audiogenic test; C-KO, *Fmr1* KO mice not exposed to auditory stimulus; T-KO, *Fmr1* KO mice tested.

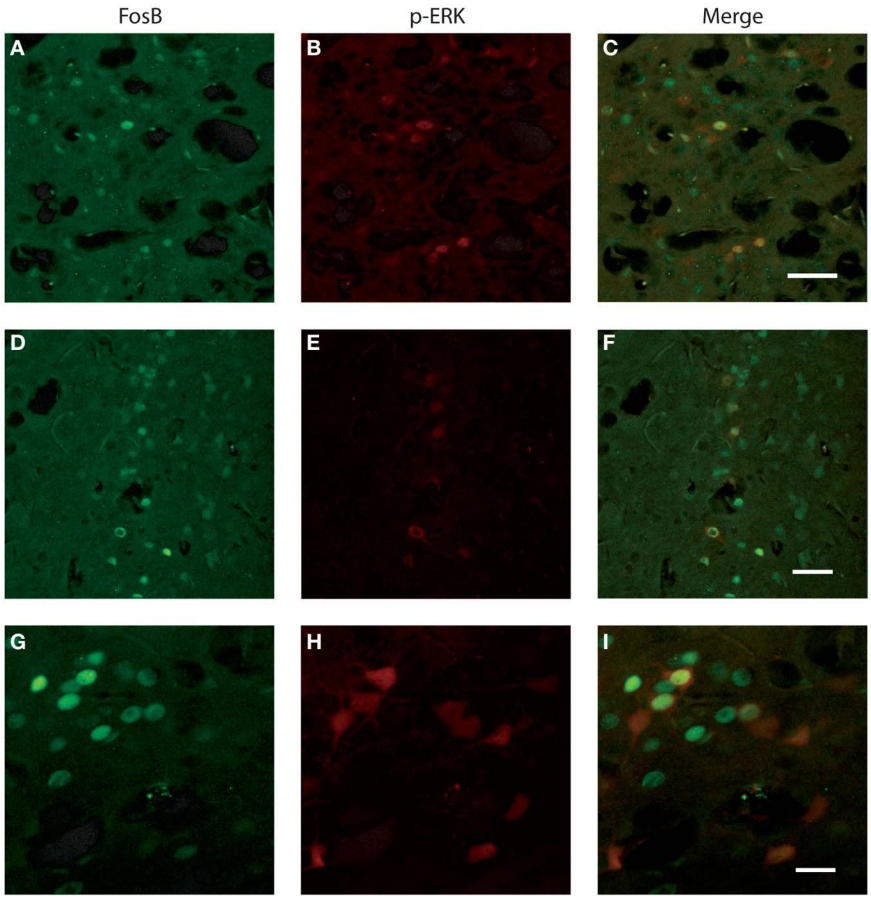

FIGURE 6 | Photomicrographs illustrating double immunofluorescence experiments with antibodies against p-ERK1/2 and FosB/ΔFosB in three exampled mice. (A–C) illustrate the co-labeling of subicular neurons in a *Fmr1* KO mouse at P45. **(D–F)** demonstrate the co-labeling found in the perirhinal cortex of a WT mouse at P45. **(G–I)** Neurons co-labeled in the perirhinal cortex of a WT mouse showed at higher magnification. Scale bars, 50 μm for **(A–F)**, 25 μm for **(G–I)**.

We also explored the possibility that p-ERK1/2-immunopositive cells could be interneurons. Experiments of co-labeling with p-ERK1/2 and markers for specific interneuron subclasses were performed in the subiculum (**Figure 7**) and perirhinal cortex (not shown). These experiments demonstrated that parvalbumin, neuropeptide Y and somatostatin antibodies, respectively, did not co-localize with cells stained with p-ERK1/2 antibodies (**Figure 7**).

DISCUSSION

We investigated the response to the AGS test at two different ages in mice characterized by the *Fmr1* KO genotype, compared with age-matched control WT mice. As previously reported, FXS patients (Ferri et al., 1994; Miller et al., 1999) and mice (Musumeci et al., 2000) show anomalous reaction to sensory stimuli and our experiments confirm the hyper-reaction of *Fmr1* KO mice to loud acoustic stimulus (∼122 dB), given using a doorbell mounted inside the experimental cage. This experimental design gave us the possibility to test either the reaction to sensory stimuli, either the susceptibility to AGS. Reportedly, seizure susceptibility in the FXS is age-dependent: C57BL/6 *Fmr1* KO mice showed higher susceptibility between P15 and P47, while it is between P14 and P94 in FVB *Fmr1* KO mice (Musumeci et al., 2000, 2007; Yan et al., 2005). This is confirmed in our experiments where P45 C57BL/6 *Fmr1* KO mice showed higher susceptibility to AGS than P90 mice. Clinical investigations reported that seizures are found in a small but significant subpopulation of FXS patients during the infancy and usually disappear with maturation (Hagerman and Stafstrom, 2009; Gauthey et al., 2010). The presently reported findings and previous works in the FXS animal model suggest that high homology exists between *Fmr1* KO mice and the humans with FXS.

We also analyzed the neuronal networks participating in the seizure activity in *Fmr1* KO mice. In particular, we analyzed FosB/ΔFosB expression as an alternative to previous studies based on c-Fos immunoreactivity (Chen and Toth, 2001), another tool being used for many years to track acute changes in neuronal network activity. The analysis of FosB/ΔFosB expression presents several advantages, since c-Fos reliability has been questioned in cases of recurrent neuronal synchronization, like the one typical of epileptic animals (Mello et al., 1996). Moreover, the turnover of c-Fos is very rapid when compared to that of other markers, such

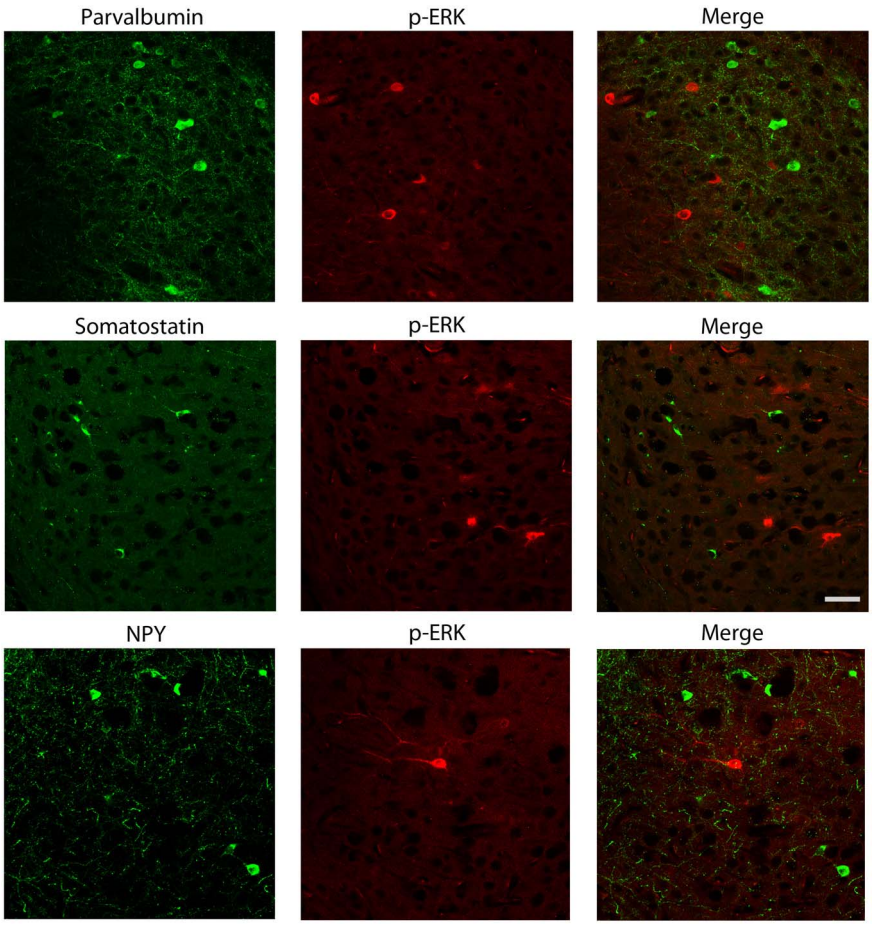

FIGURE 7 | Photomicrographs illustrating double immunofluorescence experiments with antibodies against p-ERK1/2 and markers of interneurons. In particular, antibodies to parvalbumin (**top** panels), somatostatin (**middle** panels) or neuropeptide Y (NPY; **bottom** panels) did not co-localize with p-ERK1/2-immunopositive cells. Scale bar: 25 μm.

as FosB and ΔFosB (Madsen et al., 2006), making c-Fos expression extremely short living. In contrast, thanks to their stability, products of the *fosB* gene family tend to accumulate in repeatedly activated neurons (Chen et al., 1997; Kelz and Nestler, 2000), being the ideal tool available now to investigate network activity in epileptic animals (Chen et al., 1997; Biagini et al., 2005; Madsen et al., 2006). As expected, the acoustic stimulus induced an increased labeling for FosB/ΔFosB in regions involved in processing auditory information, especially in the medial geniculate body and primary acoustic area, both in WT and *Fmr1* KO mice, indicating that acoustic pathways had been activated. These findings are consistent with those by Chen and Toth (2001), demonstrating increased c-Fos expression after acoustic stimulus in specific thalamic regions of the FXS genotype, such as the medial geniculate body, compared to stimulated WT mice. We also observed enhanced FosB/ΔFosB immunoreactivity in the medial geniculate body of *Fmr1* KO compared with WT stimulated mice. At variance, no differences between the two genotypes were present in FosB/ΔFosB expression in the primary acoustic area, a result that is also in agreement with c-Fos immunodetection (Chen and Toth, 2001).

Basal levels of FosB/ΔFosB immunoreactivity in the medial geniculate body and primary acoustic area were similar in both genotypes, but a different scenario emerged in the hippocampal formation, in which FosB/ΔFosB immunoreactivity was unexpectedly higher in unstimulated P45 *Fmr1* KO mice compared to age-matched WTs, suggesting that regions notoriously involved in cognitive and emotional physiological functions (Koe et al., 2009; Herry et al., 2010) were hyperactive in our animal model of FXS. These differences were previously unreported in experiments based on antibodies to c-Fos (Chen and Toth, 2001; Li et al., 2002). However, the hippocampus is responsive to acoustic stimuli (Miller and Freedman, 1995) and, as shown in other models, there is a good evidence that some hippocampal regions are strictly related to the formation of memory traces of acoustic information (Huang et al., 2010). The link among acoustic information, seizures and the hippocampus has also been confirmed by evaluating patients affected by TLE (Boutros et al., 2008). In TLE, the hippocampus is consistently involved in epileptic neuronal synchronization (Avoli et al., 2002), thus the enhanced immunopositivity for FosB/ΔFosB found in the CA1 region and in the subiculum of *Fmr1* KO mice not exposed to the acoustic

test could be interpreted as caused by unobserved spontaneous seizures. Alternatively, differences in the response to environmental stimuli or to stress could be taken into account (Miller et al., 1999; Lauterborn, 2004). The CA1 subfield is involved in the termination of the stress response by regulating the neuroendocrine axis that controls corticosterone levels, a function that strictly integrates the effects of stress with memory processing (Joëls et al., 2009). This hippocampal region is especially sensitive to stressful stimuli and can be markedly altered by chronic stress exposure both during development (Biagini et al., 1998) and in the adulthood (Biagini et al., 1993), circumstances that result in the impairment of stress response termination. Even with slightly different results, most likely due to the different type and duration of stress and the different time after stress when tissue was collected, others also demonstrated that changes in *c-fos* gene expression induced by stress are greater in *Fmr1* KO compared to WT mice (Lauterborn, 2004). In addition, the increase in corticosterone levels is greater in FXS mice compared to WT subjected to the same type of stress (Lauterborn, 2004). In analogy to patients affected by FXS, *Fmr1* KO mice presented with a prolonged return to basal corticosterone levels in response to acute stress, which could be related to an altered hippocampal feedback regulation (Markham et al., 2006).

We have evaluated a second marker of neuronal cell activity by characterizing p-ERK1/2-immunopositive cells. ERK is member of the mammalian mitogen-activated protein kinase (MAPK) family of serine/threonine kinases and regulates a variety of physiological and pathophysiological cellular activities (Subramaniam et al., 2004). This is a marker alternative to FosB. ERK1/2 phosphorylation is known to be followed by c-Fos activation. c-Fos phosphorylation is catalyzed by ERK1/2 at Ser374 and, furthermore, the further downstream reaction at Ser362 stabilizes c-Fos for several hours (Okazaki and Sagata, 1995; Murphy et al., 2002). No regulatory effects on FosB are instead reported by ERK1/2 activation (Miller et al., 1998). Accordingly, transactivation by c-Fos is mediated by two C-terminal motifs named HOB1 and HOB2, of which HOB1, absent in FosB (Herdegen and Leah, 1998), can be phosphorylated by ERK1/2. Consistently, our findings on FosB and p-ERK1/2 co-labeling demonstrate only a partial superimposition of these markers, suggesting that activated neurons were indeed responsive to different molecular pathways. It has been proposed a critical role for ERK1/2 in regulating social behaviors, and it has been suggested that it may be an important factor in human psychiatric disorders (Engel et al., 2009; Satoh et al., 2011). Interestingly, Hou et al. (2006) and Price et al. (2007) showed that the basal p-ERK1/2 levels in hippocampal synaptosomes were elevated in *Fmr1* KO mice compared to WTs, but p-ERK1/2 could not be increased by stimulating metabotropic glutamate receptor (mGluR) as instead in WT mice. There is, however, some controversy in the literature regarding ERK1/2 activation in *Fmr1* KO tissue (Hu et al., 2008; Gross et al., 2010) and given its critical role in synaptic plasticity, as well as in neurodevelopment and regulation of social behaviors, Wang and colleagues (2012) decided to investigate further this question using human brain tissue obtained from FXS patients. For the first time, they demonstrated that ERK1/2 phosphorylation is altered in neocortex and hippocampus of FXS patients, suggesting that there is a chronic activation of the MAPK/ERK kinase (MEK)-ERK1/2 pathway; in addition they confirmed these results in cortical tissue obtained from *Fmr1* KO mice (Wang et al., 2012). Similar upregulation of p-ERK1/2 in *Fmr1* KO mice was also found by Michalon et al. (2012). In line with these data, we observed a higher level of p-ERK1/2-immunopositive cells in basal conditions, but this difference was specifically found in the subiculum of *Fmr1* KO mice. This finding does not exclude that in fresh tissue, studied with different techniques (Michalon et al., 2012; Wang et al., 2012), enhanced ERK1/2 phosphorylation is found in other brain regions, but the subiculum could anyway represent an area of prominent ERK1/2 activation in the FXS model.

Activation of ERK1/2 by phosphorylation is strongly promoted by glutamate release during seizures and by stimulation of glutamate receptors (Jeon et al., 2000; Otani et al., 2003; Merlo et al., 2004; Houser et al., 2008). Interestingly, inhibition of ERK1/2 phosphorylation was shown to decrease *in vitro* ictogenesis induced by 4-aminopyridine (Merlo et al., 2004). Consistently with these data, a constitutively active form of MEK1 induced ERK1/2 activation and caused spontaneous epileptic seizures when conditionally expressed in the murine brain (Nateri et al., 2007). Recent experiments have cleared the relationship between ERK1/2 phosphorylation and the occurrence of AGS. Using the inhibitor of the MEK-ERK1/2 kinase cascade U0126, three different laboratories were able to block AGS in *Fmr1* KO mice (Osterweil et al., 2010; Michalon et al., 2012; Wang et al., 2012). To this regard, we found that p-ERK1/2 immunoreactivity is decreased in the subiculum, but not in perirhinal cortex, of *Fmr1* KO mice not manifesting AGS in response to acoustic stimulation. At variance, no significant changes of p-ERK1/2 immunoreactivity were observed in mice presenting with AGS induction. On the other hand, p-ERK1/2 levels were upregulated by the acoustic test in WT mice, in which we did not observe any epileptic response. This discrepancy suggests a differential regulation of ERK1/2 activity in normal and epilepsy-prone animals. Using pilocarpine to induce seizures, Houser and colleagues (2008) actually observed a decrease in p-ERK1/2 levels in naïve mice, whereas ERK1/2 phosphorylation increased immediately before the appearance of recurrent spontaneous seizures in the animals that experienced the pilocarpine-induced *status epilepticus*. Thus, it is possible that the enhanced phosphorylation of ERK1/2 observed in WT mice in the subiculum represents a molecular fingerprint of the exposure to subthreshold proconvulsive stimuli in presence of a normal genotype, whereas the increase of p-ERK1/2 basal levels found in *Fmr1* KO mice could be related to a proconvulsive genotype. In addition, an inefficient p-ERK1/2 dephosphorylation in the subiculum of *Fmr1* KO mice appears to be responsible for AGS induction, whereas enhanced phosphatase activity, acquired during maturation, could explain the resilience to proconvulsive stimuli in older *Fmr1* KO mice. This overall evidence suggests that abnormally elevated and timely maintained p-ERK1/2 levels in the subiculum are required for AGS induction in *Fmr1* KO mice. Notably, the changes we found in p-ERK1/2 levels were age-dependent, suggesting that this mechanism could be involved in the modification of seizure susceptibility during maturation in the FXS.

Contrary to results obtained in the subiculum, no differences at all were observed in the perirhinal cortex of WT and *Fmr1* KO mice exposed to the test for AGS induction. This result is consistent with previous findings that showed defective neurotransmission in the subiculum of *Fmr1* KO mice (D'Antuono et al., 2003; Curia et al., 2009). In particular, p-ERK1/2 was shown to display regulatory properties on γ-aminobutyric acid (GABA) type A receptors by decreasing peak currents generated by α1β2γ2 combination of subunits, an effect prevented by UO126 treatment (Bell-Horner et al., 2006). This effect on GABA peak current could be particularly important in a background of decreased GABA tonic current, such as that found in *Fmr1* KO mice (Curia et al., 2009). The subiculum from patients affected by TLE, due to hippocampal sclerosis, was found to generate spontaneous epileptic activity *in vitro* (Cohen et al., 2002). Consistently, enhanced neuronal excitability was demonstrated in the subiculum of pilocarpine-treated rats, a model of TLE associated with brain damage, in which GABAergic neurons were significantly decreased (De Guzman et al., 2006). Thus, the present findings on markedly enhanced FosB/ΔFosB levels and p-ERK1/2 expression in the subiculum of *Fmr1* KO mice further support the view of a critical role of this limbic region in controlling the spread of seizure activity in neuronal networks also in models of genetic epilepsy.

ACKNOWLEDGMENTS

This investigation has been supported by the Italian Ministry for Education, University and Research (PRIN2007, grant 2007CX2R77_002 to Giuseppe Biagini; "Rientro Cervelli" project 17DZE8RZEA to Giulia Curia), the University of Modena and Reggio Emilia (UNIMORE), the Fondazione Cassa di Risparmio di Modena (FCRM to Giuseppe Biagini), the Pierfranco and Luisa Mariani Foundation (R-12-94 to Giulia Curia and Giuseppe Biagini). We acknowledge the generous collaboration to this project of Dr. Massimo Avoli, who allowed Dr. Fabio Gualtieri to perform the AGS test on colonies hosted at the Animal Facility of the Montreal Neurological Institute during his sabbatical leave, granted by the PhD School in Clinical and Experimental Medicine of UNIMORE.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 February 2013; paper pending published: 08 March 2013; accepted: 03 April 2013; published online: 25 April 2013.

Citation: Curia G, Gualtieri F, Bartolomeo R, Vezzali R and Biagini G (2013) Resilience to audiogenic seizures is associated with p-ERK1/2 dephosphorylation in the subiculum of Fmr1 knockout mice. Front. Cell. Neurosci. 7:46. doi: 10.3389/fncel.2013.00046

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 13 September 2013
doi: 10.3389/fncel.2013.00134

K^+ channelepsy: progress in the neurobiology of potassium channels and epilepsy

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K^+ channels are important determinants of seizure susceptibility. These membrane proteins, encoded by more than 70 genes, make the largest group of ion channels that fine-tune the electrical activity of neuronal and non-neuronal cells in the brain. Their ubiquity and extremely high genetic and functional diversity, unmatched by any other ion channel type, place K^+ channels as primary targets of genetic variations or perturbations in K^+-dependent homeostasis, even in the absence of a primary channel defect. It is therefore not surprising that numerous inherited or acquired K^+ channels dysfunctions have been associated with several neurologic syndromes, including epilepsy, which often generate confusion in the classification of the associated diseases. Therefore, we propose to name the K^+ channels defects underlying distinct epilepsies as "*K^+ channelepsies*," and introduce a new nomenclature (e.g., K*x.y-channelepsy*), following the widely used K^+ channel classification, which could be also adopted to easily identify other *channelopathies* involving Na^+ (e.g., Na_V*x.y-phenotype*), Ca^{2+} (e.g., Ca_V*x.y-phenotype*), and Cl^- channels. Furthermore, we discuss novel genetic defects in K^+ channels and associated proteins that underlie distinct epileptic phenotypes in humans, and analyze critically the recent progress in the neurobiology of this disease that has also been provided by investigations on valuable animal models of epilepsy. The abundant and varied lines of evidence discussed here strongly foster assessments for variations in genes encoding for K^+ channels and associated proteins in patients with idiopathic epilepsy, provide new avenues for future investigations, and highlight these proteins as critical pharmacological targets.

Keywords: Potassium channels: [Kv1, Kv2, Kv3, Kv4, Kv8, Kv11(HERG), $K_{Ca1.1}$, Kvβ1, Kvβ2, KChIP LGI1, Kir1-Kir7 (GIRK, K_{ATP})], temporal lobe epilepsy, autism–epilepsy, channelopathies

INTRODUCTION

Epilepsy is a brain disorder due to abnormal firing of neuronal networks in the brain that often causes convulsions, muscle spasms, and loss of consciousness. Seizures sometimes cause brain damage, particularly if they are severe. More than 2 million people in the United States—*about 1 percent*—have experienced an unprovoked seizure or been diagnosed with epilepsy. For about 80 percent of those diagnosed with epilepsy, seizures can be controlled pharmacologically, or treated surgically. However, the rest of people with epilepsy—*intractable epilepsy*—will continue to experience seizures even with the best available treatment. Although the causes of epilepsy are numerous, the fundamental disorder is secondary to abnormal synchronous discharges of a network of neurons, either due to neuronal membrane instability, or an imbalance between excitatory and inhibitory influences. Neurons store and convey information in the form of electrical impulses generated by ion channels. Neuronal excitability can be controlled by both the intrinsic activity of K^+ channels and the receptors-mediated modulation of their activity (Pessia et al., 1994; Imbrici et al., 2000; Pessia, 2004; D'Adamo et al., 2013). Their opening and resulting outward K^+ flux dampen neuronal excitability and therefore they are viewed as inhibitory channels. However, contrary to this general notion, increased K^+ channel activity may also result in enhanced cell excitability. Hence, *K^+ channels are critical for neuronal excitability*, as they control the resting membrane potentials and enable rapid repolarization of the action potentials. Moreover, they are essential effectors of neurotransmitter-mediated signaling, regulator of Ca^{2+} homeostasis and cell survival. To fulfill these pivotal functions efficiently, K^+ channels are found in virtually every cell of the human body, are distinguished by being the largest and most diverse class of ion channels, and are encoded by more than 70 genes (http://www.genenames.org/genefamily/kcn.php). An additional reason for their large diversity resides in the fact that they form macromolecular complexes, involving several proteins. The need for such a large number of K^+ channels remains unclear.

In the past few years, several types of epilepsies have been associated to dysfunction of K^+ channels, resulting from mutations in their encoding genes (**Table 1**), which appear prime

elements potentially underlying idiopathic epilepsy. Indeed, the extremely high molecular and functional diversity of K^+ channels, unmatched by any other types of channels, places them (*by statistical probability alone*) as primary targets of genetic variations. Besides the intrinsic K^+ channels' gene defects associated with epilepsy, it is increasingly clear that also disruption/modification in K^+ channel properties, even in the absence of a primary channel defect, may underlie increased susceptibility to seizures. The association between K^+ channel dysfunctions and epileptic phenotypes is also confirmed by a multitude of animal models of epilepsy, that is, animals carrying K^+ channel mutations or genetic manipulations and displaying spontaneous seizures or increased susceptibility to stimulus-induced seizure (**Table 2**). The extremely high diversity of K^+ channels and the numerous variations identified in their genes often generate confusion in the classification of the associated diseases. Therefore, we propose to name the K^+ channels defects underlying distinct epilepsies as "K^+ channelepsies," and offer a new classification according to a widely used K^+ channel nomenclature (e.g., K_Vx.y). Moreover, here we discuss the different aspects of K^+ channels dysfunctions underlying distinct epileptic phenotypes and describe the recent progress in the neurobiology of seizure susceptibility in animal models of epilepsy. Comprehensive knowledge of the neurobiological processes altered by K^+ channel defects is a pivotal step to identify original therapeutic solutions for this devastating disease. Full understanding of how mutations in K^+ channels give rise to distinct human and animal epileptic phenotypes requires a basic knowledge of their molecular features, expression pattern, and physiological roles. Thus, brief overviews on these topics for each K^+ channel subfamily have been included.

VOLTAGE-GATED K^+ CHANNELS AND CHANNELEPSY

Voltage-gated K^+ channels (Kv) are generally closed at the resting membrane potential of nerve cells (ca.–70 mV) and open following membrane depolarization. At a single channel level, membrane depolarizations elicit channel opening and closing (a process named *gating*) visible as upwards and downwards deflections of current trace (Pessia, 2004). The first Kv channel was cloned from the *Shaker* mutant of *Drosophila melanogaster* in 1987 (Tempel et al., 1987). The human ortholog of *Shaker* K^+ channel is encoded by the gene *KCNA1*(Kv1.1). Since the first cloning, several other genes encoding for Kv channels have been identified from many different species. Based on sequence relatedness, Kv channels have been classified in subfamilies by using the abbreviation *Kvy.x* (Chandy and Gutman, 1993). According to this standardized nomenclature *Shaker*-related channels have been classified in the subfamily *Kv1.x* and each member numbered Kv1.1 through Kv1.8. The same criteria have been used to classify channels related to the *Drosophila* subfamilies *Shab* (Kv2.1 and Kv2.2), *Shaw* (Kv3.1 to Kv3.4), and *Shal* (Kv4.1 to Kv4.3). These channels may exist as homomers, whenever four identical α-subunits are assembled. However, different types of α-subunits may heteropolymerize to form channels with functional and pharmacological properties that are different from the parental homomeric channels (Isacoff et al., 1990; Ruppersberg et al., 1990).

The predicted 496 amino acids of the Kv1.1 α subunit form six transmembrane segments (TM) with the N- and C-termini residing inside the cell. TM5, TM6, and the H5 loop linking them contribute to the ion-conducting pore, and the *GYG* residues, that reside within the loop, control the K^+ selectivity of the channel. The TM4 segment of each Kv1.1 α subunit is made of regularly spaced positively charged arginines and lysines and embodies the main voltage-sensor region that opens the channel by undergoing a conformational rearrangement upon membrane depolarization (Pessia, 2004). The full crystal structure, provided for a Kv channel, confirmed that this channel is composed of four homologous pore-forming α subunits (Jiang et al., 2003a,b). The description of the membrane-delimited Kv channel structure, T1 domain and β subunits allowed elucidation of many biophysical mechanisms controlling channel function. The Kv1 family members exhibit diverse expression patterns in the central and peripheral nervous system and are found tightly clustered within distinct neuronal compartments (Trimmer and Rhodes, 2004). Kv channels regulate the duration of action potentials, modulate the release of neurotransmitters, control the excitability, electrical properties, and firing pattern of central and peripheral neurons (Pessia, 2004). Moreover, the activity of Kv channels can be dynamically modulated by several events, including neurotransmitter-stimulated biochemical cascades (Imbrici et al., 2000; D'Adamo et al., 2013). Knowledge of their precise targeting and neurophysiological functions has important implications for defining the roles played by each Kv channel type in the pathophysiology of epilepsy.

Kv1.1 CHANNELEPSY

Episodic ataxia type 1 (EA1) [OMIM 160120] is a *Shaker*-like K^+ channels disease characterized by constant myokymia and dramatic episodes of spastic contractions of the skeletal muscles of the head, arms, and legs with loss of both motor coordination and balance (D'Adamo et al., 2012). EA1 was clearly described during the mid '70s by van Dyke and colleagues who first reported electroencephalographic (EEG) recordings characterized by runs of paroxysmal slow waves and generalized motor seizures in the proband's mother (van Dyke et al., 1975). The subsequent genetic analysis revealed that the individuals displaying epilepsy carried the F184C mutation in their *KCNA1* gene that profoundly altered the channel's properties (Browne et al., 1994; Adelman et al., 1995). Confirmations of an increased susceptibility to seizures in EA1 came from several subsequent studies (**Table 1**). Isolated photosensitive generalized tonic–clonic seizure (Imbrici et al., 2008) and abnormal EEGs have been observed in individuals with EA1 (Zuberi et al., 1999). EEGs may be characterized by intermittent and generalized slow activity, frequently intermingled with spikes. Zuberi et al. (1999) described a 3-year-old boy who presented with an ictal EEG with rhythmical slow-wave activity over the right hemisphere, becoming spike-and-wave complexes that then spread to the left hemisphere. Neuronal circuit dysfunctions within the hippocampus have been postulated to play a role in seizures and cognitive dysfunctions associated with EA1. Indeed, the hippocampus is a major brain region of the limbic system which plays an important role in the consolidation of information and in spatial memory, and it

Table 1 | Human K^+ channelepsies.

Channel	Gene/protein	Expression in brain regions relevant to epilepsy	Epilepsy type	Gene mutation/channel dysfunction	References
Voltage-gated K^+ channels	*KCNA1*/Kv1.1 (pore forming α subunit)	Axons/terminals of hippocampal neurons (*Shaffer* collateral axons and mossy fibers contacting CA3 neurons); hippocampal interneurons of hilus and CA1; neocortical pyramidal neurons	Generalized and partial seizures associated to EA1	*Loss-of-function* mutations generally associated with reduced current amplitudes; positive shift of the activation $V_{1/2}$; increased sensitivity to Zn^{2+} inhibition	Assaf and Chung, 1984; Browne et al., 1994; Adelman et al., 1995; D'Adamo et al., 1998, 1999; Zuberi et al., 1999; Geiger and Jonas, 2000; Cusimano et al., 2004; Imbrici et al., 2006, 2007, 2008; Guan et al., 2006
	KCNAB2/Kvβ2 (β accessory subunit for Kv1 channels)	Widely expressed in cerebral cortex and hippocampus	Severe epilepsy including infantil spasms	Allele deletion/ haploinsufficiency	Heilstedt et al., 2001
	LGI1 (Accessory protein for Kv1 channels)	Neocortex and hippocampus	Autosomal dominant lateral temporal lobe epilepsy	E383A, frameshift with protein truncation/Mutated LGI1 does not prevent Kvβ1-mediated Kv1 channel inactivation, a function performed by the WT protein	Kalachikov et al., 2002; Morante-Redolat et al., 2002
	KCND2/Kv4.2 (I_A pore forming α subunit)	Dendrites of hippocampal neurons	Temporal lobe epilepsy	Truncated Kv4.2 subunit/attenuated I_A current density	Singh et al., 2006
	KCNV2/Kv8.2 (silent subunit associating with Kv2 channels)	Pyramidal neurons and principal excitatory neurons of the pyramidal cell layers and the dentate gyrus; cortex, with high levels of transcript in Layers 2/3 and 5	Febrile and afebrile partial seizures; epileptic encephalopathy	R7K, M285K/Reduction of Kv2.1 mediated current; M285K impairs the voltage-dependence of the channel	Jorge et al., 2011
	KCNQ2-3/ Kv7.2-3 (M current pore forming α subunit)	Widely expressed in brain at neuronal cell bodies	Benign familial neonatal convulsions	Five–base pair insertion deleting more than 300 amino acids from the *KCNQ2*; missense mutations in critical regions for *KCNQ3* channel function / Reduced *KCNQ* current amplitudes	Biervert et al., 1998; Charlier et al., 1998; Schroeder et al., 1998; Singh et al., 1998
	KCNH2/Kv11.2 (HERG channel pore forming α subunit)	Widely expressed in brain	Epilepsy associated with type 2 long QT syndrome	*Loss-of-function* mutations	Keller et al., 2009; Omichi et al., 2010; Tu et al., 2011; Zamorano-León et al., 2012

(Continued)

Table 1 | Continued

Channel	Gene/protein	Expression in brain regions relevant to epilepsy	Epilepsy type	Gene mutation/channel dysfunction	References
Ca^{2+}-dependent K^+ channels	*KCNMA1*/$K_{Ca1.1}$ (BK channel pore forming α subunit)	Axons and pre-synaptic terminals of excitatory neurons of cortex and hippocampus	Generalized epilepsy and paroxysmal diskynesia	D434G/ Increase of channel open probability and calcium dependence of $K_{Ca1.1}$ expressed alone or with $K_{Caβ1}$, $K_{Caβ2}$, or $K_{Caβ4}$; *loss-of-function* of $K_{Ca1.1}$/$K_{Caβ3}$–mediated currents	Du et al., 2005; Díez-Sampedro et al., 2006; Lee and Cui, 2009; Yang et al., 2010
	KCNMB3/$K_{Caβ3}$ (accessory protein for $K_{Ca1.1}$ channels)	Widely expressed at low levels in brain	Idiopathic generalized epilepsy	*Loss-of-function* of $K_{Ca1.1}$/$K_{Caβ3}$-mediated currents	Behrens et al., 2000; Hu et al., 2003; Lorenz et al., 2007
Inwardly-rectifying K^+ channels	*KCNJ2*/Kir2.1 (pore forming α subunit)	Hippocampus, caudate, putamen, nucleus accumbens; to lower levels in habenula and amygdala	Seizures associated to the Andersen Tawil Syndrome (ATS)	*Loss-of-function* mutations with dominant-negative effects	Haruna et al., 2007; Chan et al., 2010
	KCNJ10/Kir4.1 (pore forming α subunit)	Oligodendrocytes and astrocytes surrounding synapses and blood vessels, mainly in the cortex, thalamus, hippocampus, and brainstem	Seizure susceptibility	R271C missense variation; no alteration in the biophysical properties of the channel when heterologously expressed	Buono et al., 2004; Connors et al., 2004; Shang et al., 2005
			Epilepsy associated to EAST syndrome	*Loss-of-function* recessive mutations	Bockenhauer et al., 2009; Scholl et al., 2009
			Epilepsy associated to autism spectrum disorders (ASDs)	R18Q, V84M, *gain-of-function* of Kir4.1 and Kir4.1/Kir5.1-mediated current	Sicca et al., 2011
	KCNJ11/Kir6.2 (K_{ATP} channel pore forming α subunit) *ABCC8*/SUR1 (K_{ATP} channel accessory regulatory subunit)	Hippocampus (principal neurons, interneurons, and glial cells); neocortex, entorhinal and piriform cortex	Developmental delay, epilepsy and neonatal diabetes mellitus (DEND syndrome)	*Gain-of-function* mutations leading to decreased channel inhibition by ATP, or enhanced Mg^{2+}-nucleotide-induced activation	Karschin et al., 1997; Hattersley and Ashcroft, 2005

The table depicts the principal inherited human channelepsies caused by mutations in K^+ channel α subunits or associated accessory proteins. For each channelepsy, information about the name of the mutated gene/protein, its expression in brain regions relevant to the pathology, type of epilepsy and association with other known syndromes, and dysfunction in channel behavior caused by the mutations are reported.

is often the focus of epileptic seizures. In rodent hippocampus, Kv1.1, Kv1.2, and Kv1.4 are found in *Schaffer* collateral axons and are highly expressed in axons and terminals of the medial perforant path in the middle third of the molecular layer of the dentate gyrus. In particular, Kv1.1, Kv1.4, and Kvβ1.1 subunits are expressed in mossy fiber boutons (*swellings of mossy fiber axons*) that form *en passant* synapses with pyramidal neurons in CA3. The macromolecular channel complex, composed of these subunits, regulates the activity-dependent spike broadening of hippocampal mossy fiber boutons and, consequently, the amount of neurotransmitter released during high-frequency stimuli (Geiger and Jonas, 2000). Mutations associated with EA1 profoundly alter the function of heteromeric channels composed of Kv1.1, Kv1.2, Kv1.4, and Kvβ1.1 subunits (D'Adamo et al., 1999; Imbrici et al., 2006) that likely contributes to seizures susceptibility and cognitive symptoms (Imbrici et al., 2006) in EA1.

Table 2 | Animal models of K^+ channelepsies.

Channel	Gene/protein	Animal model/channel disfunction	Epilepsy phenotype	Functional effects of mutation on neurons relevant to epilepsy	References
Voltage-gated K^+ channels	*Kcna1*/Kv1.1 (pore forming α subunit)	Kv1.1$^{V408A/+}$ mice/ EA-1 mutation that alters the biophysical properties of the channel	unknown	Cf. **Table 1** for WT expression; unknown	
		Kv1.1$^{-/-}$ knockout mice	Spontaneous seizures resembling human temporal lobe epilepsy	Hippocampus with neural loss, astrocytosis, and mossy fiber sprouting; mossy fiber stimulation mediates long-latency epileptiform burst discharges; mossy fibers and medial perforant path axons were hyperexcitable and produced greater pre- and post-synaptic responses with reduced paired-pulse ratios	Smart et al., 1998; Rho et al., 1999
		Kv1.1$^{S309T/+}$ rats/80% smaller current amplitudes with dominant-negative effects	neuromyotonia and spontaneous convulsive seizures aggravated by stress	Cortical and hippocampal EEG with aberrant large spike activity associated with falling-down behavior, low-voltage fast wave discharges during the tonic stage, spike-and-wave discharges (2 Hz) during the clonic convulsive stage. Behavioral phenotypes and abnormal discharge patterns similar to other rodent models of temporal lobe epilepsy	Ishida et al., 2012
		mceph/mceph mice, carrying a 11-basepair deletion in the *Kcna1* gene. The mutation leads to a frame shift and to a premature stop codon	Running seizures, complex partial seizures and post-anesthetic tonic-clonic seizures	Increased brain volume and hypertrophic brain cells; hippocampal hyperexcitability consistent with limbic status epilepticus	Donahue et al., 1996; Petersson et al., 2003
		Adar2$^{-/-}$ mice	Increased susceptibility to epileptic seizure	Adenosine deaminase (*Adar2*) acting on Kv1.1 mRNA and leading to a *gain-of-function* of the resulting current (increased amplitude and faster recovery from inactivation). *Adar2* also edits the mRNA of AMPA glutamate receptors	Higuchi et al., 2000; Bhalla et al., 2004

(Continued)

Table 2 | Continued

Channel	Gene/protein	Animal model/channel disfunction	Epilepsy type	Functional effects of mutation on neurons relevant to epilepsy	References
	Kcna2/Kv1.2 (pore forming α subunit)	Kv1.2$^{-/-}$ knockout mice	Spontaneous generalized seizures	WT Expression: mainly overlapping with that of Kv1.1 channels; fibers and neuropil, but not somata, neocortex; synaptic terminals of entorhinal afferents. Strongly expressed in axon initial segment, where they partecipate to action potential generation	Brew et al., 2007
	Kcnab2/Kvβ2 (β subunit for Kv1 channels)	Kvβ2$^{-/-}$ knockout mice/ β2 promotes the trafficking of Kv1.1 and Kv1.2 to the membrane surface	Increased neuronal excitability, occasional seizures	Cf. **Table 1** for WT expression; deficits in associative learning and memory; reduction in the slow afterhyperpolarization and concomitant increase in excitability of projection neurons in the lateral nucleus of the amygdala	McCormack et al., 2002; Connor et al., 2005; Perkowski and Murphy, 2011
	Kchip2 (Accessory subunit for Kv4 channels)	*Kchip2*$^{-/-}$ knockout mice/ Reduced I_A current density and slowed recovery from inactivation in hyppocampal neurons	Increased susceptibility to seizure induced by kindling	WT Expression: apical dendrities of hyppocampal pyramidal cells Chronic hyperexcitability of hyppocampal pyramidal neurons	Wang et al., 2013
	Lgi1 (Accessory protein for Kv1 channels)	*Lgi1*$^{-/-}$ knockout mice/ Kvβ1-mediated Kv1 channel inactivation is not prevented, a function performed in WT mice	Lethal epilepsy; the heterozygous has a lowered seizure thresholds	Cf. **Table 1** for WT expression. A lack of *Lgi1* disrupts synaptic protein connection and selectively reduces AMPA receptor-mediated synaptic transmission in the hippocampus	Fukata et al., 2010
	Kcnd2/Kv4.2 (I_A pore forming α subunit)	Kv4.2$^{-/-}$ knockout mice	Enhanced susceptibility to kainate-induced seizure, but see Hu et al., 2006	Cf. **Table 1** for WT expression. Increased epileptiform bursting in area CA1	Hu et al., 2006; Nerbonne et al., 2008; Barnwell et al., 2009
Ca^{2+}-dependent K^+ channels	*Kcnb4*/KCaβ4 (accessory subunit for K_{Ca}1.1 channels)	$K^{-/-}_{Caβ4}$ mice/Gain-of-function of $K_{Ca1.1}$-mediated current	Temporal cortex seizures	WT expression: axons and presynaptic terminals of shaffer collaterals and CA3 hippocalpal neurons. Higher firing rate of dental gyrus neurons	Jin et al., 2000; Raffaelli et al., 2004; Brenner et al., 2005; Shruti et al., 2008; Sheehan et al., 2009

(Continued)

Table 2 | Continued

Channel	Gene/protein	Animal model/channel disfunction	Epilepsy type	Functional effects of mutation on neurons relevant to epilepsy	References
Inwardly-rectifying K^+ channels	*Kcnj6*/Kir3.2 (GIRK2 channel pore forming α subunit)	Kir3.2$^{-/-}$ knockout mice	Spontaneous convulsions and increased propensity for generalized seizures; more susceptible to pharmacologically-induced seizure	WT expression: cortex, hippocampus, weaker signal in thalamic nuclei and amygdaloid nuclei; reduced GIRK1 expression in brain	Signorini et al., 1997
		Weaver (w/w) mice, G156S/alteration of the K^+ selectivity of the channel	epileptic seizures	Neurodegeneration; calcium oveload within cells, reduced GIRK1 expression in brain	Patil et al., 1995; Slesinger et al., 1996
	Kcnj10/Kir4.1 (pore forming α subunit)	Kir4.1$^{-/-}$ knockout mice	Stress-induced seizures	WT expression: oligodendrocytes and astrocytes sourronding synapses and blood vessels in cortex, thalamus, hyppocampus, braistem No membrane depolarization is observed in astrocytes following $[K^+]_o$ increase by neuronal activity	Neusch et al., 2001; Djukic et al., 2007
		DBA/2 mouse strain, T262S missense variation resulting in a barium-sensitive Kir currents in astrocytes substantially reduced; No alteration in the biophysical properties of the channel	Greater susceptibility to induced seizures compared to the C57BL/6 strain	Potassium and glutamate buffering by cortical astrocytes is impaired	Ferraro et al., 2004; Inyushin et al., 2010
	Kcnj11/Kir6.2 (K_{ATP} channel pore forming α subunit)	Kir6.2$^{-/-}$ knockout mice	High-voltage sharp-wave bursts EEG	Cf. **Table 1** for WT expression; Substantia nigra neurons are depolarized by hypoxia (WT neurons are instead hyperpolarized)	Yamada et al., 2001

The table depicts the principal animal models of epilepsy caused by mutations in K^+ channel α subunits or associated accessory proteins. For each animal model, information about the name of the mutated gene/protein, type of epileptic phenotype, dysfunction in channel behavior caused by the mutation, and functional effects of the mutation are reported.

Notably, epileptiform brain activity has also been associated with intracranial administration of Zn^{2+} salts, and changes in Zn^{2+} modulation of GABA receptors have been implicated in the etiology of epilepsy. Zn^{2+} is released from mossy fiber terminals in the hippocampus, and from the basket cell terminals of the cerebellum (Assaf and Chung, 1984) where Kv1 channel activity likely is subjected to Zn^{2+} modulation. Indeed, homomeric and heteromeric channels containing Kv1 subunits are inhibited by extracellular Zn^{2+}, and a distinct EA1 mutation increases several folds the Zn^{2+} sensitivity of these channels (Cusimano et al., 2004; Imbrici et al., 2007). Whether Zn^{2+} plays a role in triggering epilepsy-like symptoms in EA1 remains an intriguing hypothesis. A murine model that recapitulates the EA1 phenotype (*mKv1.1*$^{V408A/+}$) has been generated by inserting in the mouse *Kcna1*(mKv1.1) a very conservative *valine* to *alanine* substitution (V408A) previously identified in patients (Herson et al., 2003; Brunetti et al., 2012, **Table 2**). Although data concerning the *mKv1.1*$^{V408A/+}$ are not yet available, the role of Kv1.1

channels in the neurobiology of epilepsy has been investigated by using Kv1.1 knockout mice (*mKv1.1*$^{-/-}$). The hippocampus of these animals displays morphological characteristics typical of epilepsy, with neural loss, astrocytosis, and mossy fiber sprouting (Rho et al., 1999). Moreover, *mKv1.1*$^{-/-}$ also exhibits frequent spontaneous seizures throughout adult life, although the intrinsic passive properties of CA3 pyramidal cells are normal (**Table 2**). Antidromic action potentials were recruited at lower thresholds in *mKv1.1*$^{-/-}$ slices, and mossy fiber stimulation triggered synaptically-mediated long-latency epileptiform burst discharges. These data indicate that loss of Kv1.1 results in increased excitability in the CA3 recurrent axon collateral system, perhaps contributing to the limbic and tonic–clonic components of the observed epileptic phenotype of EA1 (Smart et al., 1998). Recently, *in vitro* extracellular recordings were performed by using a multielectrode array to characterize spontaneous sharp waves and high frequency oscillations in *mKv1.1*$^{-/-}$ hippocampi. This study showed that the mossy fibers and medial perforant path axons of *mKv1.1*$^{-/-}$ were hyperexcitable and produced greater pre- and post-synaptic responses with reduced paired-pulse ratios. Microdissection of mossy fibers and perforant path in *mKv1.1*$^{-/-}$ hippocampal slices ameliorated the abnormal oscillatory pattern and improved spike timing. In contrast, blockade of Kv1.1 channels with *dendrotoxin-K* reproduced these effects in WT slices. These findings suggest that loss of Kv1.1 enhances synaptic neurotransmitter release in the CA3 region, which reduces spike timing precision of individual neurons, leading to disorganization of network oscillatory activity and promoting the emergence of fast ripples (Simeone et al., 2013).

N-ethyl-N-nitrosourea (ENU) mutagenesis has been widely used to generate animal models of human diseases. An ENU-mutagenized rat strain has been recently generated and named "*autosomal dominant myokymia and seizures*" (ADMS) rats (Ishida et al., 2012). Genetic analysis of these animals resulted in the identification of the missense mutation S309T in the voltage-sensor domain of Kv1.1 channels (*rKv1.1*$^{S309T/+}$). This heterozygous mutation resulted in 80% smaller current amplitudes with dominant-negative effects (**Table 2**). From 16 weeks of age severe periodic seizures were observed, and by 30 weeks of age, 84% of *rKv1.1*$^{S309T/+}$ had died. Cooling induced severe neuromyotonia, ataxia and aberrant spike-and-wave discharges (2–3 Hz) associated with clonus behaviors. Spontaneous convulsive seizures from 10 to 16 weeks of age were aggravated by stress (*cage changing or animal handling*). Cortical and hippocampal EEG recordings identified aberrant large spike activity associated with falling-down behavior, low-voltage fast wave discharges detected during the tonic stage, and spike-and-wave discharges (2 Hz) detected during the clonic convulsive stage (Ishida et al., 2012). The behavioral phenotypes and abnormal discharge patterns in *rKv1.1*$^{S309T/+}$ are similar to other rodent models of temporal lobe epilepsy (TLE). Carbamazepine (CBZ) administration ameliorated seizures. The videos related to this animal model of EA1 are available online at doi:10.1016/j.brainres.2011.11.023.

The megencephaly mice, *mceph/mceph*, are characterized by increased brain volume, hypertrophic brain cells and slight hippocampal astrocytosis. They display a complex behavioral phenotype including running seizures, complex partial seizures and postanaesthetic tonic-clonic seizures (Donahue et al., 1996, **Table 2**). Remarkably, an 11-basepair deletion in the *Kcna1* gene of *mceph/mceph* mice has been identified (Petersson et al., 2003). The mutation leads to a frame shift and a premature stop codon that was predicted to truncate the protein at amino acid 230 (out of 495). Therefore, *mceph/mceph* mice express Kv1.1 subunits lacking the last five TMs and the C-terminal domain: The absence of Kv1.1 channels with intact C-terminal domains was confirmed by Western blotting analysis of whole protein extracts from brain. Electrophysiological investigations from *mceph/mceph* brain slices revealed hippocampal *mceph/mceph* brain slices revealed hippocampal hyperexcitability consistent with limbic status epilepticus (SE) C-teminal domain of Kv1.1 channels (R417stop) has also been found in a EA1 proband displaying episodes of ataxia precipitated by exercise, stress, startle or high temperature occurring after a hot bath or when using a hairdryer but, absence of epilepsy (Eunson et al., 2000).

Kv1.2 knockout mice display increased seizure susceptibility (Brew et al., 2007, **Table 2**). It should be recalled that Kv1.1 and Kv1.2 are closely coupled K^+ channel subunits, as they form heteromeric channels in several brain regions. Indeed, biochemical and electrophysiological studies have shown that Kv1.1/Kv1.2 channels control neuronal excitability, action potentials propagation and synaptic transmission. Notably, EA1 mutations alter the function of heteromeric channels composed of Kv1.1 and Kv1.2 subunits (D'Adamo et al., 1999). In conclusion, these investigations with animal models of Kv1.1 channelepsy highlighted the crucial brain regions that are likely the site of origin of abnormal discharges in EA1 and the relevant mechanisms underlying their susceptibility to seizures.

ROLE OF RNA EDITING IN Kv1.1 CHANNELEPSY

Kv1.1 mRNA is target for enzymatic deamination by adenosine deaminase acting on RNA (ADAR2). Knockout mice for the ADAR2 gene are prone to epileptic seizures and die within a few weeks after birth (Higuchi et al., 2000, **Table 2**). Kv1.1 editing by ADAR2 results in channels with an I400V exchange in the S6 segment (*Kv1.1*^{I400V}). *In vitro*, an increase in increase in *Kv1.1*^{I400V} editing increases K^+ outward current upon membrane depolarization and accelerates recovery from inactivation at negative membrane potentials (Bhalla et al., 2004). Interestingly, increased levels of *Kv1.1*^{I400V} editing were found in chronic epileptic rats. It has also been reported a reduced ability of 4-AP to trigger seizure-like effects in brain slices dissected from a kainic acid rat model of chronic epilepsy (Zahn et al., 2008; Streit et al., 2011). A similar phenomenon was observed in human brain slices of patients with pharmacoresistant TLE (Gabriel et al., 2004). The postulated ictiogenic mechanism of 4-AP action is its ability to block *Kv1* and *Kv3* channels, which results in increased transmitter release. *Kv1.1*^{I400V} editing results in 4-AP–insensitive homomeric *Kv1.1* channels, and alters the pharmacology of heteromeric channels (Decher et al., 2010; Streit et al., 2011). These findings suggest that mRNA editing of Kv1.1 in chronic epilepsy may to the reorganization of the entorhinal cortex and have anticonvulsive effects. The editing of Kv1.1 mRNA could therefore represent a compensatory mechanism in brain areas where epileptic seizures

originate. TLE, namely spontaneous seizures involving the hippocampal formation, is the most prevalent refractory epilepsy. TLE can be sub-classified into mesial, lateral and neocortical. Mesial TLE (MTLE) is defined by clinic-anatomical observations and characterized by seizures arising from the hippocampus or parahippocampal structures, and frequent pharmacotherapy resistance. A recent screening study identified a significant negative correlation between epilepsy duration in patients with MTLE plus hippocampal sclerosis and the I/V editing site of Kv1.1 channels. This may be the result of the epileptic process itself, together with the medication history of the patient, and may thus reflect compensatory mechanisms to either of these factors (Krestel et al., 2013).

Kv4 CHANNELEPSY

Kv4 channels underlie the main dendritic A-type Kv currents in hippocampal neurons and play a critical role in regulating the extent to which back-propagating action potentials invade the dendritic tree. They also impact the propagation of synaptic potentials from the dendritic arbor to the soma (Jerng et al., 2004). The relevance of Kv4.2 to epilepsy comes from the identification of a mutation in a patient with TLE, which results in the expression of a truncated Kv4.2 subunit (Singh et al., 2006, **Table 1**), and the observation that pharmacological blockade of Kv4 channels is epileptogenic (Avoli, 2001). These studies support the concept that Kv4.2 deficiency may contribute to aberrant network excitability and regulate seizure threshold. Intriguingly, it has been reported that Kv4.2 knockout mice do not exhibit an overt seizure phenotype (Hu et al., 2006), perhaps due to post-translational *up-regulation* of other Kv currents (Nerbonne et al., 2008). A more recent study, however, suggests that loss of Kv4.2 channels is associated with enhanced susceptibility to seizures after kainate injection (Barnwell et al., 2009, **Table 2**).

Systemic administration of the muscarinic agonist pilocarpine to rats recapitulates the features of human limbic seizures and SE. The expression of a number of different K^+ channels, including Kv (Monaghan et al., 2008) and K_{Ca} (BK) channels (Pacheco Otalora et al., 2008) is altered in response to pilocarpine-induced SE. The amplitude of A-type currents in the dendrites of hippocampal CA1 pyramidal neurons is reduced in response to pilocarpine-induced seizures (Bernard et al., 2004). In addition to Kv4.2, Kv4.3 and KChIP2 current decrease, pilocarpine-treated rats exhibited staining changes for these proteins in the molecular layer of the dentate gyrus from being uniformly distributed across the molecular layer to become concentrated in just the outer two-thirds (Monaghan et al., 2008). As a consequence, an increased dendritic excitability was found in CA1 pyramidal cell dendrites in response to the same pilocarpine model of TLE (Bernard et al., 2004). *In situ* hybridization studies revealed that generalized seizures induced by pentylenetetrazol (Tsaur et al., 1992) or kainic acid (Francis et al., 1997) also altered the regional hippocampal gene expression of rat Kv4.2. A decrease of A-current density in this proximal region of the granule cell dendrite—a location that receives massive aberrant excitatory mossy fiber input following induction of SE and as spontaneous seizures develop—would lower the firing threshold and thus contribute to the development of spontaneous recurrent seizures. On the other hand, seizures *in vivo* and glutamate *in vitro* induce a rapid surface recruitment of Kv4.2 channels in neurons. Thus, seizure would induce dampening of phasic firing, generated by glutamatergic synaptic transmission, by enhancing the surface expression of Kv4.2 channels. Interestingly, mutant LGI1 blocks this homeostatic neuronal response (Smith et al., 2012). Thus, it seems that convulsants may affect Kv4 currents in different ways and compensatory pathways may be recruited to dampen hyper-excitability.

One of the most pronounced anatomical effects of TLE, exhibited in the pilocarpine animal model, is mossy fiber sprouting. The expansion of Kv1.4 staining in stratum lucidum of CA3 in pilocarpine-SE animals suggests that expansion of the mossy fiber terminal field, due to SE-induced sprouting, is accompanied by parallel increases in targeting of Kv1.4 to the newly sprouted mossy fiber axons and terminals. Changes in Kv1.4 immunoreactivity were observed also in the molecular layer of the dentate gyrus. In conclusion, variations in A-type Kv1.4-containing channels in mossy fiber presynaptic terminals, and Kv4.2-containing channels in dentate granule cell and CA1 dendrites, represent important mechanisms intervening in the acquisition of the permanent epileptic phenotype in this animal model of human TLE and attractive therapeutic targets. Moreover, these studies highlighted the essential nature of Kv4.2 and the specific contributions of its auxiliary KChIP subunits (*see below*) in regulating the seizure susceptibility associated with epileptogenesis.

Kv7-M-CHANNELEPSY

Five members of the *KCNQ* gene family have been identified (*KCNQ1-5*) which form homomeric or heteromeric K^+ channels. In many brain regions, heteromeric *KCNQ2*(Kv7.2)/*KCNQ3*(Kv7.3) channels seem the major determinant of "*M*" currents that are inhibited by several neurotransmitters, including acetylcholine (ACh) through the muscarinic receptors (Devaux et al., 2004; Pan et al., 2006). Functionally, these slow-gating K^+ channels that are open at subthreshold voltages, contribute to setting the resting membrane potential of neurons, prevent repetitive firing, and control spike-frequency adaptation. Indeed, blockade of M-currents is associated with depolarization of the resting membrane potential, and generation of trains of action potentials (Brown and Adams, 1980; Delmas and Brown, 2005). Mutations in *KCNQ2* or *KCNQ3* cause a form of juvenile epilepsy called *benign familial neonatal convulsions* (Biervert et al., 1998; Charlier et al., 1998; Schroeder et al., 1998; Singh et al., 1998, **Table 1**). The clinical features of *KCNQ2*-related benign familial neonatal epilepsy (*KCNQ2-BFNE*) are characterized by tonic or apneic episodes, focal clonic activity, or autonomic changes that start between the second and eighth day of life and spontaneously disappear between the first and the sixth to 12th month. The *KCNQ2*-related epileptic encephalopathy (*KCNQ2-NEE*) is characterized by multiple daily seizures that begin the first week of life, are mostly tonic, with motor and autonomic features, and cease after 9 months to 4 years. Most affected individuals are intellectually impaired (Bellini et al., 1993-2013). Just a 25% decrease in M-current amplitude is sufficient to drive human

neurons to epileptogenic levels, and causes neonatal epilepsy (Schroeder et al., 1998). The clinical severity of the disease may be related to the extent of mutation-induced functional K^+ channel impairment (Miceli et al., 2013). Drugs that enhance neuronal M-currents, such as retigabine, represent a valuable therapeutic treatment for certain hyperexcitatory diseases, including epilepsy (Porter et al., 2012). Flupirtine, a structural analogue of retigabine, which also activates channels formed by neuronal Kv7 subunits, has been shown to be effective in animal models of neonatal convulsion (Raol et al., 2009). Kv7.1 (*KCNQ1*) channels are mainly expressed in the heart where they contribute to termination of the action potential. Mutations in *KCNQ1*(Kv7.1) are responsible for one form of long QT syndrome (LQT1) (Wang et al., 1996). However, Kv7.1 channels are also expressed in the brain. Intriguingly, LQT1 patients exhibit an increased risk of epilepsy, suggesting a relationship between these two diseases (*see below*). Notably, M-channels are also highly sensitive to intracellular Ca^{2+} variations, being inhibited by Ca^{2+} with an IC50 of ∼100 nM in sympathetic neurons (Selyanko and Brown, 1996; Gamper and Shapiro, 2003). Recently, it has been proposed that abnormal $[Ca^{2+}]i$ transients induced by Kv1.1 channel dysfunction may result in repetitive discharges in myelinated nerves (Brunetti et al., 2012). This mechanism may underlie not only the neuromyotonic/myokymic discharges typically observed in EA1 individuals, but also their susceptibility to seizures.

The neuronal serum- and glucocorticoid-regulated kinase 1 (SGK1.1) appears to be a physiological M-current regulator, as it enhances Kv7.2/3 current levels. Transgenic mice expressing a constitutively active form of SGK1.1 are resistant to kainic acid-induced seizures which occur mainly in the temporal lobe. These findings indicate that SGK1.1 activity can regulate neuronal excitability through M-current modulation and protects against seizures (Miranda et al., 2013).

Kv8 CHANNELEPSY: ROLE OF THE SILENT MODIFIERS OF K^+ CHANNELS IN EPILEPSY

A number of subunits have been cloned and classified in the K^+ channel subfamilies Kv6, Kv8, and Kv9. Although they possess the hallmarks of Kv subunits they do not appear to form functional homomeric K^+ channels when expressed alone and, therefore, have been named "*silent*" subunits. However, these proteins do co-assemble with other Kv subunits (e.g., Kv2), and confer distinct biophysical properties to heteromeric channels Kv2/Kv6, Kv2/Kv8, or Kv2/Kv9. Thus, these gene products are also known as "*silent modifiers*" of Kv channels. In particular, Kv8.2 (*KCNV2*) co-assembles with Kv2.1 as a heterotetramer, significantly reducing the surface expression of the resulting channels and influencing their biophysical properties. Within the hippocampus, Kv2.1 and Kv8.2 co-localize in pyramidal neurons and in the principal excitatory neurons of the pyramidal cell layers and the dentate gyrus. Moreover, both are expressed in the cortex, with high levels of transcript in layers 2/3 and 5 (Allen brain atlas; http://www.brain-map.org). These regions are critically involved in seizure generation and propagation. It has been shown that Kv2.1 channels contribute significantly to the delayed-rectifier K^+ currents in hippocampal neurons (Murakoshi and Trimmer, 1999). Importantly, knockdown of Kv2.1 in hippocampal slices resulted in increased CA1 pyramidal neuron excitability under conditions of high-frequency stimulation (Du et al., 2000). Thus, the Kv2.1 current reduction, mediated by the inclusion of Kv8.2 subunits in heterotetramers, could regulate the membrane repolarization and excitability of hippocampal neurons, contributing to seizure susceptibility. Indeed, mutations in two related silent subunits have been associated with neurological disorders (**Table 1**). In particular Kv8.2 (*KCNV2*) with epilepsy (Jorge et al., 2011) and Kv9.1 (*KCNS1*) with chronic pain (Costigan et al., 2010). Recently, an individual affected by febrile and afebrile partial seizures has been reported to carry a genetic variation in *KCNV2*, inherited from his unaffected mother. This resulted in the substitution of a highly conserved lysine for an arginine (R7K) in the cytoplasmic amino terminus of Kv8.2 subunits. An additional patient who inherited a methionine to arginine mutation (M285R) in Kv8.2 subunits from his unaffected mother was likewise identified with an epileptic encephalopathy and with severe refractory epilepsy (Jorge et al., 2011). The pathogenic relevance of these variants was assessed by electrophysiological recordings from cells co-expressing the human Kv2.1 with Kv8.2 wild-type or its mutated subunit (R7K or M285R). This assay showed that both variants suppressed Kv2.1-mediated current more than the wild-type; in addition, the M285R impaired the voltage-dependence of the channel. These results suggest that both variants enhance seizure susceptibility of affected patients by reducing neuronal delayed-rectifier K^+ channel function in brain regions critically involved in seizure generation and propagation (Jorge et al., 2011).

The severity of seizures in mouse models of epilepsy is highly dependent on their genetic background. In transgenic mouse models of sodium channel-dependent epilepsy, the phenotype is more severe in SJL/J compared with the C57BL/6J strain (Bergren et al., 2005). Interestingly, it has been shown that the hippocampal Kv8.2 (*Kcnv2*) transcript is ∼3-fold greater in SJL/J compared with the C57BL/6J strain (Jorge et al., 2011). The enhanced availability of Kv8.2 subunits for co-assembling with Kv2.1 would remarkably reduce the surface expression of the resulting channels in hippocampal neurons of SJL/J mice contributing to their greater susceptibility to seizures.

In conclusion, these studies implicate Kv8.2 (*Kcnv2*) as an epilepsy gene in rodents and humans. Moreover, although silent subunits have been mostly ignored since they were first cloned a decade or more ago, their potential clinical relevance is now becoming fully evident and therefore represents an attractive new avenue of investigation in neurologic channelopathies research.

Kv11-HERG-CHANNELEPSY

The human ether-a-go-go-related gene (HERG) encodes for voltage-gated K^+ channels. HERG channels exhibit functional properties remarkably different from other K^+ channels (Tristani-Firouzi and Sanguinetti, 2003). They are widely expressed in the brain where they contribute to setting the frequency and the discharge stability of neurons, and to adapting their intrinsic properties to signal processing (Pessia et al., 2008). They also modulate the excitability of dopaminergic and GABAergic neurons (Nedergaard, 2004; Canavier et al., 2007). HERG channels are expressed in the heart where they control the repolarization of ventricular action potentials. *Loss-of-function*

mutations in *KCNH2* (HERG) cause type 2 long QT syndrome (LQT2), a condition in which the induced delayed repolarization of the heart following a heartbeat increases the risk of episodes of sudden death due to ventricular fibrillation. However, LQT syndrome is closely associated with seizure and frequently it is misdiagnosed as epilepsy (**Table 1**). Sudden unexpected death in epilepsy is the most frequent epilepsy-related cause of death for which an underlying arrhythmogenic predisposition has been suggested. Several clinical reports have recently described seizures and arrhythmic events in LQT2 triggered by visual or acoustic stimuli (Keller et al., 2009; Omichi et al., 2010; Tu et al., 2011; Zamorano-León et al., 2012). Considering that HERG channels control several neuronal electrical features, including discharge dynamics (Pessia et al., 2008), these clinical findings raise the possibility that alteration in *KCNH2*-encoded K^+ channels may confer susceptibility for epilepsy and cardiac LQT2 arrhythmia.

AUXILIARY SUBUNITS OF K^+ CHANNELS AND CHANNELEPSIES

K^+ channels expressed by distinct cell types may be formed by unique hetero-oligomeric complexes comprising auxiliary subunits. Several types of these subunits have been identified including beta-subunits (Kvβ), minK (minimal K^+ channel peptide), MiRP (minK-related peptide), KChAP (K^+ channel-associated protein), KChIP (K^+ channel-interacting protein) and neuronal calcium sensor (NCS). Each type of auxiliary subunit modulates the activity of the associated K^+ channel in distinct ways. Defects in these subunits may alter the function of the channel and result in increased seizure susceptibility.

Kvβ CHANNELEPSY

Auxiliary subunits such as Kvβ1.1 and Kvβ1.2 confer fast *N-type* or *A-type* inactivation to non-inactivating Kv1 channels by means of a "*ball-and-chain*" mechanism of pore occlusion whereby the tethered (*chain*) positively charged inactivation particle (*ball*) in the amino-terminus of the Kvβ1 subunit binds to the intracellular entrance of the pore discontinuing the outflow of K^+ ions. The fast inactivation of delayed-rectifier K^+ channels is a physiologically relevant process, as it controls the firing properties of neurons and their response to input stimuli (Pessia, 2004). Surprisingly, Kvβ1.1 knockout mice do not display an overt epileptic phenotype. On the other hand, Kvβ2 knockout and Kvβ1/Kvβ2 double-knockout mice are characterized by an increased neuronal excitability, occasional seizures, cold swim-induced tremors and a reduced life span (McCormack et al., 2002; Connor et al., 2005, **Table 2**). Clinical investigations have found an association between the severity of seizures, including infantile spasms and the loss of the Kvβ2 gene. Moreover, the hemizygosity of this gene in epileptic patients suggests that haploinsufficiency for *KCNAB2* is a significant risk factor for epilepsy (Heilstedt et al., 2001). Notably, Kvβ2 subunits do not confer fast inactivation properties to Kv1 channels, since they lack the inactivation particles. Thus, a chaperone-like function has been proposed for these subunits. Although these findings are consistent with a role for accessory subunits in regulating central nervous system excitability, further functional assays are necessary to determine thoroughly how loss or haploinsufficiency of the Kvβ2 gene affects distinct network excitability and causes Kvβ2 channelepsy.

Kv_{LGI1} CHANNELEPSY

The *leucine-rich glioma-inactivated-1* (*LGI1*) is a secreted neuronal protein, complexed with Kv channels, and highly expressed in neocortex and hippocampus. *LGI1* mutations—e.g., *the point mutation E383A that prevents the neuronal secretion of LGI1*—have been found in patients with autosomal dominant lateral temporal lobe epilepsy (ADLTE), a syndrome characterized by partial seizures with acoustic or other sensory hallucinations (Kalachikov et al., 2002; Morante-Redolat et al., 2002; Fukata et al., 2010, **Table 1**). Moreover, loss of *Lgi1* in mice causes lethal epilepsy (Fukata et al., 2010, **Table 2**). In the hippocampus, both Kv1.1 and *Lgi1* appear co-assembled with Kv1.4 and Kvβ1 in axonal terminals. In A-type channels composed of these subunits, *Lgi1* prevents N-type inactivation mediated by the Kvβ1 subunit. In contrast, defective *LGI1* molecules identified in ADLTE patients fail to exert this effect, which results in channels with rapid inactivation kinetics. These data suggest that these changes in inactivation gating of presynaptic A-type channels may promote epileptic activity (Schulte et al., 2006).

Specific auto-antibodies underlie an emerging class of seizures named "autoimmune epilepsy" that involves K^+ channels. Indeed, several of these auto-antibodies do not bind directly with Kv1.1, Kv1.2, or Kv1.6 channels, as previously believed, but rather to associated proteins such as *LGI1*, contactin-associated protein 2 (*CASPR2*), contactin-2, or others to be identified (Irani et al., 2010; Lai et al., 2010; Lancaster et al., 2011).

Kv_{KChIP1} CHANNELEPSY

Cytosolic Kv channel-interacting proteins KChIP1–KChIP4 (An et al., 2000), which belong to the NCS family of calcium binding EF-hand proteins, co-assemble with the N-terminus of Kv4 subunits (Zhou et al., 2004) to form a native complex that encodes major components of neuronal somatodendritic A-type K^+ current (I_A). KChIP2 expression is high in the hippocampus, particularly within the apical dendrites of pyramidal cells. *KChIP2* gene deletion in mice (*Kchip2*$^{-/-}$) affected the I_A in hippocampus, namely reduced current density, decreased channel availability, and slowed recovery from inactivation. This results in chronic hyper-excitability in hippocampal pyramidal neurons and *Kchip2*$^{-/-}$ mice exhibited increased susceptibility to seizures induced by kindling (**Table 2**). However, a compensatory up-regulation of inhibitory synaptic activity (*up-regulation of GABA currents*) was also observed (Wang et al., 2013). These findings indicated that *KChIP2* is essential for homeostasis in hippocampal neurons and mutations in these K^+ channel auxiliary subunits may be loci for epilepsy (Wang et al., 2013). Interestingly, *Kchip2*$^{-/-}$ mice are highly susceptible to cardiac arrhythmias (Kuo et al., 2001). Thus, this evidence suggests that *loss-of-function* mutations in *KChIP2* could confer an increased susceptibility to both seizures and cardiac arrhythmias, increasing the risk to sudden unexpected death in epileptic patients.

In conclusion, these studies demonstrated that the auxiliary subunits play important roles in the pathogenesis of epilepsy by

affecting K^+ channel function and network excitability in distinct ways (*see also* $K_{Ca1.1}$ *channelepsy*).

CALCIUM-ACTIVATED K^+ CHANNELS AND CHANNELEPSY

The calcium-activated K^+ (K_{Ca}) channels are highly conserved across species, and widely expressed in the human brain. The phylogenetic tree of the K_{Ca} channels shows that they are made of two genetically well-distinct groups (Wei et al., 2005), the large conductance (BK; $K_{Ca1.1}$), and the small/intermediate-conductance (SK/IK; $K_{Ca2.1}$, $K_{Ca2.2}$, $K_{Ca2.3}$, $K_{Ca3.1}$) K_{Ca} channels. With regard to gating mechanism, the Ca^{2+} sensitivity of SK/IK channels is provided by tightly bound calmodulin (Xia et al., 1998; Fanger et al., 1999), in contrast to the direct binding of Ca^{2+} at specific internal sites on the channel protein of $K_{Ca1.1}$ channels (Lee and Cui, 2010). Moreover, unlike the SK/IK channels, $K_{Ca1.1}$ channels are also activated by voltage.

In brain neurons K_{Ca} channels are widely distributed in the axons plasma membrane and at the presynaptic terminals (Knaus et al., 1996; Blank et al., 2004), and often located close to voltage-gated Ca^{2+} channels (Ca_v; Marrion and Tavalin, 1998). The Ca^{2+} influx that follows neuronal excitation activates K_{Ca} channels whose outward K^+ flux contributes to terminate the action potential and establish the afterhyperpolarization (AHP) that closes Ca_v channels. This negative feedback control has been generally assumed to make K_{Ca} channels critical players in opposing repetitive firing and hyperexcitability typical of epileptic disorders. To date only mutations in the $K_{Ca1.1}$ channel have been clearly associated to epilepsy.

$K_{Ca1.1}$ CHANNELEPSY

The $K_{Ca1.1}$ channel, originally cloned from the *Drosophila slowpoke* locus, thus the name *slo* (Atkinson et al., 1991), is coded by one single gene, the *KCNMA1* (Wei et al., 2005). The variety observed in biophysical and pharmacologic properties of the channel derives from the extensive alternative splicing of its mRNA (Tseng-Crank et al., 1994), and the type of accessory β subunit ($K_{Caβ}$) that associates with the channel (Jiang et al., 1999). The $K_{Ca1.1}$ channel is composed of four identical pore-forming α subunits, each displaying a transmembrane portion very much like the Kv channel, with the voltage sensing domain made by segments S1–S4, and the permeating pore domain by the S5, P, and S6 segments. Unlike Kv channels, the $K_{Ca1.1}$ channel has an additional transmembrane domain (S0), thought to subserve for ($K_{Caβ}$) subunit interaction and modulation of the channel (Morrow et al., 2006). The channel has also a large C-terminal cytosolic domain that confers Ca^{2+} sensitivity to the channel. Notably, the amino-acid sequence of the C-terminal domain contains no conventional Ca^{2+} binding motif such as EF hands or C2 domains. The two putative high affinity Ca^{2+} binding sites of the channel are formed, respectively, by two closely located (five amino acids apart) aspartate residues (Xia et al., 2002) on the RCK1 (regulator of K^+ conductance) domain, and by a series of aspartate residues in a region known as the Ca^{2+} bowl, located in the RCK2 domain (Wei et al., 1994; Schreiber and Salkoff, 1997).

$K_{Ca1.1}$ channels expression predominates in axons and presynaptic terminals of excitatory neurons located in epileptic relevant structures, such as cortex and hippocampus (Knaus et al., 1996; Hu et al., 2001; Misonou et al., 2006; Martire et al., 2010). In brain neurons, the Ca^{2+} that activates $K_{Ca1.1}$ channels enters primarily through Ca_v channels (Berkefeld et al., 2006), with which $K_{Ca1.1}$ channels strictly co-localize in order to be activated during an action potential by the Ca^{2+} microdomains that form around the Ca^{2+} source (Müller et al., 2007). The $K_{Ca1.1}$ channel activity is thus limited by the duration of the action potential-evoked Ca^{2+} transients, and consequently restricted to the action potential repolarization phase and the fast portion of the after-hyperpolarization (fAHP; Sah and Faber, 2002), and generally assumed to reduce neuronal excitability. Recent findings point, however, to a role of $K_{Ca1.1}$ channels in promoting high frequency firing, an effect likely attributed to fast spike repolarization, fAHP generation and the consequent reduction in the activation of other slower Kv channels and of the inactivation of Na^+ channels (Storm, 1987a,b; Gu et al., 2007). Whereas they appear to be virtually uninfluential to synaptic release modulation under physiologic conditions (Hu et al., 2001; Raffaelli et al., 2004; Shruti et al., 2008; Martire et al., 2010).

Given the role of $K_{Ca1.1}$ channels in promoting high neuronal firing frequency and their predominant expression in excitatory neurons of cortex and hippocampus, it is no surprising that several lines of evidence from animal models point to a pro-epileptic role for $K_{Ca1.1}$ channels. For example, spontaneous cortical bursting in mice with high susceptibility to convulsions was completely inhibited by the $K_{Ca1.1}$ channel blocker *iberiotoxin* (IbTX) (Jin et al., 2000). A similar inhibitory effect of $K_{Ca1.1}$ channel antagonists was reported on chemoconvulsant-induced seizures *in vitro* and *in vivo* (Jin et al., 2000; Shruti et al., 2008; Sheehan et al., 2009). Finally, $K_{Caβ4}$ knockout mice displayed temporal cortex seizures and a *gain-of-function* of $K_{Ca1.1}$ channels in dentate gyrus slices, resulting in higher firing rate (Brenner et al., 2005, **Table 2**).

Evidence for an association of human epilepsy with the $K_{Ca1.1}$ channel has also been found. A missense mutation (D434G) in the *KCNMA1* gene coding for the α subunit has been found in family patients suffering from generalized epilepsy and paroxysmal dyskinesia (Du et al., 2005, **Table 1**). Expression studies indicated that the D434G mutant channel displayed markedly greater macroscopic currents and single channel open probability, resulting from a 5-fold increase in Ca^{2+} sensitivity (Du et al., 2005). In accordance, the mutation appeared to be located in the RCK1 domain, close to the putative Ca^{2+} binding site and the segments subserving the allosteric coupling between Ca^{2+} binding site and the activation gate (Yang et al., 2010). Subsequent studies showed that the effects of the D434G mutation on the $K_{Ca1.1}$ channel properties were similar or even more pronounced in the presence of the $K_{Caβ1}$, $K_{Caβ2}$, or $K_{Caβ4}$ subunit, highly expressed in the brain (Díez-Sampedro et al., 2006; Lee and Cui, 2009). Notably, a polymorphism in the $K_{Caβ4}$ subunit has been associated to human MTLE in an Irish cohort, but this has not been confirmed in other populations (Cavalleri et al., 2007; Manna et al., 2013). By contrast, the α subunit coexpressed with $K_{Caβ3b}$ (a splicing variant of $K_{Caβ3}$) was modified by the mutation with a slowing of the activation, a reduction in the voltage-dependence, but no change in Ca^{2+}-dependence, suggesting a *loss-of-function* of the $K_{Ca1.1}$ current (Lee and Cui, 2009). Interestingly, a $K_{Caβ3}$ single-nucleotide

mutation, causing a *loss-of-function* of $K_{Ca1.1}$ current containing the $K_{Caβ3b}$ subunit, displays a small but significant association with idiopathic generalized epilepsy (Hu et al., 2003; Lorenz et al., 2007, **Table 1**). Taken together, these data suggest that both a *loss-of-function* of $K_{Caβ3b}$-containing $K_{Ca1.1}$ channels and a *gain-of-function* of $K_{Caβ1}$, $K_{Caβ2}$, or $K_{Caβ4}$-containing $K_{Ca1.1}$ channels would favor the epileptic phenotype, but more information on the location and function of $K_{Caβ3b}$ subunits is needed to clarify this point.

A modulation of $K_{Ca1.1}$ channel expression in epilepsy models has also been found. A *gain-of-function* of $K_{Ca1.1}$ currents associated with increased spontaneous and evoked firing rates occurs in mouse neocortical pyramidal neurons 24 h after chemoconvulsant-induced generalized tonic-clonic seizures (Shruti et al., 2008). Conversely, in a model of pilocarpine-induced MTLE $K_{Ca1.1}$ channel α subunit was down-regulated at the protein and mRNA level in hyppocampal mossy fibers originating from the dentate gyrus (Ermolinsky et al., 2008; Miceli et al., 2013). Notably, $K_{Ca1.1}$ channel proteins remaining after seizure induction were mostly changed to the STREX splicing isoform, displaying an increased Ca^{2+}-sensitivity with respect to the ZERO splice variant normally present (Ermolinsky et al., 2008). The functional consequence of the observed changes is thus not clear.

INWARDLY-RECTIFYING K^+ CHANNELS AND CHANNELEPSIES

Members of the inwardly-rectifying family of K^+ channels (Kir) are found in virtually every cell type where they are major regulators of K^+ fluxes across membranes (Hibino et al., 2010). The principal role of most Kir channels is the maintenance of the resting membrane potential and thereby the control of cell excitability, while others subserve the transport and recycling of K^+ across membranes. Like other K^+ channels, Kir subunits assemble as tetramers, and their ability to heteromultimerise adds functional diversity to a limited number of gene products. Kir subunits possess two transmembrane domains and approximately 15 distinct Kir clones have been identified so far, forming seven major subfamilies: Kir1–Kir7 (Bond et al., 1994; Hibino et al., 2010). Important physiological roles have been established for nearly all of these subfamilies. Generally, a Kir channel acts as a diode whereby the inward current through these channels is greater at potentials more negative than the E_K, as compared to more positive values, where the outward flow is inhibited and the membrane potential free to change. The rectifying nature of Kir channels is due to a voltage-dependent block of the intracellular side of the pore by cytoplasmic polyamines and Mg^{2+} ions (Matsuda et al., 1987; Lopatin et al., 1994; Lu and MacKinnon, 1994; Stanfield et al., 1994). Several studies highlighted the role of inwardly-rectifying K^+ channels' dysfunction in neuropsychiatric disorders and epilepsy although the relevant mechanisms in some instances await clarification.

Kir2 CHANNELEPSY

Kir2.1 channels are highly expressed in brain, particularly in hippocampus, caudate, putamen, nucleus accumbens, and to lower levels in habenula and amygdala (Karschin et al., 1996), where they contribute to control neuronal excitability. In particular, the amplitude of Kir2.1 currents is small in young dentate granule neurons (DGCs), and increases ∼3-fold in mature DGCs to optimize their excitability. Thus, Kir2.1 channels play an important role in DGCs firing properties during development (Mongiat et al., 2009). Moreover, Kir2.1 channels in combination with Kir4.1 control the astrocyte-mediated K^+ buffering (Bordey and Sontheimer, 1998; Jabs et al., 2008; Chever et al., 2010). It has been proposed that *up-regulation* of Kir2.1 in DGCs would counterbalance the hyper-excitability observed in TLE, thus functioning as an anti-convulsant (Young et al., 2009). Individuals harboring *loss-of-function* mutations in *KCNJ2* (e.g., Andersen-Tawil syndrome; OMIM 170390) may present with mood disorders and seizures (Haruna et al., 2007; Chan et al., 2010, **Table 1**), suggesting a possible role for Kir2.1 channels in the pathogenesis of neuropsychiatric disorders and epilepsy (Haruna et al., 2007; Chan et al., 2010).

Kir3-GIRK-CHANNELEPSY

Several neurotransmitters, including dopamine, opioid, somatostatin, acetylcholine, serotonin, adenosine, and GABA exert their actions by modulating the activity of G protein-coupled Kir channels (GIRK) belonging to the subfamily 3 (Kir3). Four subunits have been cloned: GIRK1-GIRK4, also known as Kir3.1–Kir3.4, that may heteropolimerize. Generally, receptors activation of intracellular heterotrimeric G proteins αβγ leads to stimulation of heteromeric Kir3 channels activity, resulting in an outward flux of K^+ ions that causes membrane hyperpolarization and inhibition of cell excitability (Krapivinsky et al., 1995; Slesinger et al., 1995; Tucker et al., 1996a). The crystal structure of this channel type has been resolved, recently (Whorton and Mackinnon, 2013). *Gain-of-function* of Kir3 channels can considerably reduce neuronal activity, whereas *loss-of-function* can lead to excessive neuronal excitability and epilepsy. Indeed, ablation of the gene encoding for Kir3.2 channels (GIRK2) results in spontaneous convulsions and increased susceptibility for generalized seizures in rodents (Signorini et al., 1997, **Table 2**). The principal phenotype of *weaver* mice (*wv/wv*) is an ataxic gait, due to severe hypoplasia of the cerebellum, learning deficits and epileptic seizures. *Weaver* mice carry a deleterious mutation in the pore of Kir3.2 channels (G156S; Patil et al., 1995, **Table 2**). This mutation alters the K^+ selectivity of the channel, induces calcium overload in cells, and reduces channel availability (Slesinger et al., 1996; Tucker et al., 1996b) with a mechanism different from heteromeric subunit degradation (Tucker et al., 1996c). It is likely that these molecular defects, induced by the G156S mutation, would lead to neurodegeneration and seizures susceptibility that characterize the phenotype of *weaver* mice.

Kir3 channel inhibition, induced by intrathecal administration of tertiapin, is pro-convulsant (Mazarati et al., 2006). Moreover, several drugs used in clinics—desimipramine, fluoxetine, haloperidol, thioridazine, pimozide and clozapine—inhibit Kir3 channel activity, and cause seizures as side effect. Conversely, electroconvulsive shock leads to increased expression of Kir3 channels (Pei et al., 1999), which may provide compensatory mechanisms against excessive electrical activity leading to

neuroprotection. In support of this hypothesis, stimulation of galanin type 2 receptors that activate GIRK channels prevents kindled epileptogenesis in rats (Mazarati et al., 2006). In conclusion, these studies point out that distinct changes in Kir3 channel activity or availability throughout the brain may result in pro-convulsant or anti-convulsant effects.

Kir4/Kir5 CHANNELEPSY

Kir4.1 subunits (*KCNJ10*; BIR10; Bond et al., 1994; Lagrutta et al., 1996) may form homomeric channels or may polymerize with Kir5.1 (*KCNJ16*) to form heterotetramers (Pessia et al., 1996) highly sensitive to pH (Tucker et al., 2000; Pessia et al., 2001; Casamassima et al., 2003; D'Adamo et al., 2011b). Kir4.1 channels are expressed primarily in oligodendrocytes and astrocytes surrounding synapses and blood vessels, mainly in the cortex, thalamus, hippocampus, and brainstem (Takumi et al., 1995; Higashi et al., 2001). Kir4.1 channel activity shows a profound developmental regulation, which correlates with both cell differentiation and the developmental regulation of extracellular K^+ dynamics (Connors et al., 1982; MacFarlane and Sontheimer, 2000; Neusch et al., 2001). Kir4.1 controls primarily the resting membrane potential of astrocytes, and maintains the extracellular ionic and osmotic environment by promoting K^+ transport from regions of high $[K^+]_o$, which results from synaptic excitation, to those of low $[K^+]_o$. This polarized transport of K^+ in astrocytes, referred to as "*spatial buffering of* K^+" is essential for normal neuronal activity, excitability, and synaptic functions. Among the genes associated with different forms of epilepsy Kir4.1 is receiving increasing interest. Genetic studies have indicated a linkage between missense variations in Kir4.1 and seizure susceptibility (Buono et al., 2004; Connors et al., 2004, **Table 1**). The DBA/2 mouse strain exhibits a greater susceptibility to induced seizures compared to the C57BL/6 strain. Previous QTL mapping identified the seizure susceptibility locus (*Szs1*) on the distal region of mouse chromosome 1 and further fine mapping studies suggested that a missense variation (T262S) in *Kcnj10* was the likely candidate for this linkage (Ferraro et al., 2004, **Table 2**). In a second linkage study, a variation in the human *KCNJ10* gene (R271C) was associated with seizure resistance in groups of patients with either focal or generalized epilepsy (Buono et al., 2004). However, a functional study demonstrated that these variations (T262S and R271C) do not produce any observable change in channel function or in predicted channel structure (Shang et al., 2005). It is therefore unlikely that the seizure susceptibility phenotypes associated with these missense variations are caused by changes in the intrinsic functional properties of Kir4.1. However, this study was unable to comprehensively disprove this association, and alterations in Kir4.1 channel activity remain an attractive mechanistic hypothesis. Future investigations willing to prove the association between these variants and seizure susceptibility phenotypes should include examination of how these variants could produce subtle changes in their interaction with cell-specific trafficking or regulatory proteins, or other possible pathways.

Recordings from surgical specimens of patients with intractable epilepsies have demonstrated a reduction of Kir conductance in astrocytes (Bordey and Sontheimer, 1998) and potassium clearance (Jauch et al., 2002). Moreover, *loss-of-function* recessive mutations of *KCNJ10* (Kir4.1) have been recently associated with a disease, named EAST syndrome or SeSAME syndrome, consisting of seizures, ataxia, sensorineural deafness, mental retardation, and renal salt-losing tubulopathy (Bockenhauer et al., 2009; Scholl et al., 2009, **Table 1**). A number of additional elements substantiate the hypothesis that variants in *KCNJ10* might contribute to brain dysfunction and seizures susceptibility. Conditional knockout mice lacking Kir4.1 exhibit stress-induced seizures, severe ataxia, spongiform vacuolation, axonal swellings, and degeneration, in addition to hearing loss and premature lethality (Neusch et al., 2001; Djukic et al., 2007, **Table 2**). In Kir4.1 knockout glial cells, no variations in membrane potential were observed during increases in $[K^+]_o$ induced by nerve stimulations (Chever et al., 2010). Hence, it has been proposed that the *loss-of-function* of glial K^+ conductance would favor extracellular K^+ accumulation, contributing to neuronal hyperexcitability and epilepsy (Orkand et al., 1966; Chever et al., 2010).

Epilepsy and autism spectrum disorders (ASD) are strongly associated. The prevalence of seizures is highly represented in ASD (5–46%) (Bryson et al., 1988; Hughes and Melyn, 2005), compared with the general population (0.5–1%). The prevalence of autism in the epilepsy population is ∼32%, which is about 50 times higher than in the general population (Clarke et al., 2005). An "autism–epilepsy phenotype" has been identified (Tuchman et al., 2009). Recently we reported a mutational screening of *KCNJ10* in 52 children with cryptogenic epilepsy that resulted in the identification of two heterozygous *KCNJ10* mutations in two identical twins (R18Q) and in a 14-year-old child (V84M; Sicca et al., 2011, **Table 1**). Clinically, the two 8-year-old identical twins showed impaired social interaction, sleep difficulties, hypotonia and both exhibited epileptic spasms within the same 24 h period. Other symptoms, typical of ASD, included clumsiness, absence of speech, severe disorder of social interaction, stereotypies, repetitive behaviors, symptoms of anxiety, depression, obsessive compulsive disorder and intellectual disability (IQ: 58). The 14-year-old child showed normal psychomotor development until 12 months of age, when ASD symptoms such as poor social gaze, no response to name, absence of language development, and withdrawal behaviors became evident. At the age of 6, he experienced complex partial seizures. EEG recordings showed synchronous and asynchronous paroxysmal abnormalities over frontal regions in both hemispheres tending to spread. The functional consequences of these heterozygous mutations were *gain-of-function* of either Kir4.1 or Kir4.1/Kir5.1 channels (Sicca et al., 2011). To date, we have identified several new probands displaying an autism–epilepsy phenotype who carry mutations in *KCNJ10* that cause *gain-of-function* effects, assessed by using astrocytoma cell lines. Collectively, our findings point to a new class of genes that should be examined in autism-epilepsy patients, and disclose novel molecular mechanisms that may increase the susceptibility to this distinct neuropsychiatric phenotype by altering the K^+ homeostasis in the brain (D'Adamo et al., 2011a; Sicca et al., 2011). Indeed, Kir4.1 is the main glial inward conductance, astrocytes make up 90% of all human brain cells, and each astrocyte controls the activity of many thousands

of synapses (about 140,000 in the hippocampus; Benarroch, 2009). Co-occurrence of epilepsy and ASD in patients harboring *KCNJ10 gain-of-function* mutations suggests that dysfunction in the astrocytic-dependent K^+ buffering may be a common mechanism contributing to seizures as well as the core behavioral features of ASD. It has been shown that an isolated episode of local neuronal hyperactivity triggers a large and synchronous Ca^{2+} elevation in closely associated astrocytes. Activated astrocytes signal back to neurons favoring their recruitment into a coherent activity that underlines the hypersynchronous ictal discharge (Gómez-Gonzalo et al., 2010). It is possible that an increased and faster influx of K^+ into astrocytes through *high functioning* Kir4.1-containing channels may lead, during intense neuronal activity, to larger membrane depolarization and higher intracellular Ca^{2+} elevations in these cells. Ca^{2+} elevations in astrocytes are associated with the release of gliotransmitters, such as glutamate and D-serine, which trigger discharges in neurons, promote local neuronal synchrony and epileptic activity (Parpura et al., 1994; Bezzi et al., 1998; Pasti et al., 2001; Angulo et al., 2004; Fellin et al., 2004; Mothet et al., 2005; Tian et al., 2005). Speculatively, a recurrent neuron-astrocyte-neuron excitatory loop may develop at a restricted brain site, as a consequence of *gain-of-function* of Kir4.1 channels, and contribute to initiation of seizures. These mutations may alter the noradrenergic (NA) system of the brain as well, since Kir4.1/Kir5.1 channels control the excitability of *locus coeruleus* (LC) neurons (D'Adamo et al., 2011b). Indeed, a developmental dysregulation of this LC-NA network (Samuels and Szabadi, 2008) has been suggested to underlie epilepsy. From a therapeutic perspective, these studies indicate that, alike neurons, astrocytes may represent a crucial target for the pharmacological control of abnormal electrical discharge and synaptic function.

Kir6-K_{ATP}-CHANNELEPSY

The adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels are octamers composed of four pore-forming subunits, consisting of Kir6.1 or Kir6.2, with four regulatory sulfonylurea receptors such as SUR1, SUR2A, or SUR2B, which are members of the ATP-binding-cassette transporter family (Aguilar-Bryan et al., 1995; Inagaki et al., 1995; Nichols et al., 1996). The secretion of insulin from pancreatic β-cells is mediated by the closure of these channels caused by increased levels of cytoplasmic ATP. Neuronal K_{ATP} channels are predominantly composed of Kir6.2/SUR1, although Kir6.1/SUR2B and Kir6.2/SUR2B are also found. These channels link the metabolic state of neurons to their excitability by sensing changes in intracellular phosphate potential (i.e., ATP/ADP ratio). DEND syndrome (OMIM 606176) is an inherited disease characterized by **d**evelopmental delay, **e**pilepsy and **n**eonatal **d**iabetes mellitus. Twenty percent of these patients have associated neurologic defects, the most severe of which are generalized epilepsy, marked delay of motor and social development, including late development of speech, and learning difficulties. Numerous *gain-of-function* mutations have been identified in the genes encoding Kir6.2 (*KCNJ11*) or the associated regulatory SUR1 subunit (*ABCC8*) of patients affected by DEND syndrome (**Table 1**). To date, all mutations in *KCNJ11* that have been characterized functionally, produce marked decrease in the ability of ATP to inhibit the K_{ATP} channel when expressed in heterologous systems or enhance the activatory effects of Mg^{2+}-nucleotides. This reduction in ATP sensitivity translates in more fully openings of the channel at physiologically relevant concentrations of ATP, increased K_{ATP} current, hyperpolarization of the β-cell plasma membrane, and consequent suppression of Ca^{2+} influx and insulin secretion (Hattersley and Ashcroft, 2005). Clinical severity of the disorder correlates with the magnitude of shift in the ATP affinity. Sulfonylureas, which block opened channels and restore glucose homeostasis, ameliorate some of the neurological symptoms of DEND syndrome. How K_{ATP} channel over-activity in the central nervous system results in epilepsy is unclear. Whereas insights have been provided on the mechanisms linking loss of K_{ATP} channel function to increased seizure susceptibility. Notably, generalized seizures can be evoked by metabolic stresses such as hypoxia and hypoglycemia. Kir6.2 knockout mice exhibited high-voltage sharp-wave bursts in the EEG recordings, myoclonic jerks followed by severe tonic-clonic convulsion and death upon exposure to hypoxia (**Table 2**). However, the wild-type mice remained sedated during this challenge and revived normally (Yamada et al., 2001). Substantia nigra pars reticulata (SNr) and its efferents act as a central gating system in the propagation of seizure activity (Iadarola and Gale, 1982). Remarkably, wild-type neurons in brain slices from the substantia nigra pars reticulata (SNr) were hyperpolarized by hypoxia, whereas the membrane potential of Kir6.2 knockout neurons were depolarized by the perfusion with hypoxic solutions. The SNr and its efferents act as a central gating system in the propagation of seizure activity (Iadarola and Gale, 1982). Therefore, hyperpolarization of SNr neurons upon opening of K_{ATP} channels has been proposed to protect against seizure propagation during metabolic stress, although other brain regions could be involved in this process (Yamada et al., 2001).

CONCLUDING REMARKS

Solving the puzzle of epilepsy is an extremely difficult task. Here we have put several new pieces in the puzzle by describing novel genetic defects in K^+ channels and related proteins that underlie distinct human epileptic phenotypes. We have also analyzed critically the new insights in the neurobiology of this disease that have been provided by investigations on valuable animal models of epilepsy. It is becoming increasingly clear that mutations in K^+ channel genes or perturbations in K^+ channel function, even in the absence of a primary channel defect, underlie an increased susceptibility to epilepsy. Despite the abundance of genes encoding for K^+ channels and associated subunits, and their established crucial functional roles, these genes have been greatly overlooked in the search for the causes underlying idiopathic epilepsy. The extremely high diversity of K^+ channels and the numerous mutations identified in their genes often generate confusion in the classification of the associated diseases. Therefore, we proposed to name the K^+ channels defects underlying distinct epilepsies as "*K^+ channelepsies*" and suggested a new classification according to a widely used K^+ channel nomenclature (Chandy and Gutman, 1993; Kubo et al., 2005; Wei et al., 2005). This original classification could be also adopted to easily unify, identify and describe multiple organ dysfunction related to a

single ion channel gene defect (*e.g.*, K*x.y-phenotype;* Na_v*x.y-phenotype*; Ca_v*x.y-phenotype*) (Catterall et al., 2005a,b). K^+ channels represent crucial targets for novel pharmacological control of abnormal electrical discharges and synaptic function in the brain. Much greater efforts should thus be made to find new K^+ channel modulators and gene therapies to ameliorate the symptoms of this devastating disease. On the other hand, the effects of newly developed drugs on the activity of most K^+ channel types should be tested in order to predict their pro-convulsant side effects. Research on *K^+ channelepsies* is clearly providing important knowledge on the signaling pathways and circuits involved in epilepsy. The understanding of these "experiments" of *Nature* will also help us to uncover, in a much broader sense, the physiological workings of the human body.

ACKNOWLEDGMENTS

This work was supported by Telethon (GGP11188), Ministero della Salute (GR-2009-1580433); MIUR-PRIN 20108WT59Y_004; COMPAGNIA di San Paolo (Turin) "*Programma Neuroscienze*," and Fondazione Cassa di Risparmio di Perugia.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 June 2013; accepted: 06 August 2013; published online: 13 September 2013.

Citation: D'Adamo MC, Catacuzzeno L, Di Giovanni G, Franciolini F and Pessia M (2013) K^+ channelepsy: progress in the neurobiology of potassium channels and epilepsy. Front. Cell. Neurosci. 7:134. doi: 10.3389/fncel.2013.00134

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 22 July 2013
doi: 10.3389/fncel.2013.00113

The possible role of $GABA_A$ receptors and gephyrin in epileptogenesis

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The term epileptogenesis refers to a dynamic alteration in neuronal excitability that promotes the appearance of spontaneous seizures. Temporal lobe epilepsy, the most common type of acquired epilepsy, often develops after an insult to the brain such as trauma, febrile seizures, encephalitis, or status epilepticus. During the pre-epileptic state (also referred as latent or silent period) there is a plethora of molecular, biochemical, and structural changes that lead to the generation of recurrent spontaneous seizures (or epilepsy). The specific contribution of these alterations to epilepsy development is unclear, but a loss of inhibition has been associated with the increased excitability detected in the latent period. A rapid increase in neuronal hyperexcitability could be due, at least in part, to a decline in the number of physiologically active $GABA_A$ receptors ($GABA_AR$). Altered expression of scaffolding proteins involved in the trafficking and anchoring of $GABA_AR$ could directly impact the stability of GABAergic synapses and promote a deficiency in inhibitory neurotransmission. Uncovering the molecular mechanisms operating during epileptogenesis and its possible impact on the regulation of $GABA_AR$ and scaffolding proteins may offer new targets to prevent the development of epilepsy.

Keywords: epilepsy, epileptogenesis, GABA receptors, gephyrin, status epilepticus

This review centers on the possible role of $GABA_A$ receptors ($GABA_AR$) and the scaffolding protein gephyrin in epileptogenesis. The basic premise is that disruption of the network of proteins involved in the trafficking and anchoring of $GABA_AR$ might result in decreased inhibitory drive, which may promote the development of spontaneous seizures. A brief introduction to epilepsy and epileptogenesis is provided along with some of the fundamental aspects of the regulation of $GABA_AR$ by gephyrin. Finally an overview of the alterations in gephyrin and $GABA_AR$ function observed during epileptogenesis is included.

EPILEPTOGENESIS AND EPILEPSY

In the majority of patients, temporal lobe epilepsy (TLE) appears to be the result of an injury to the brain caused by trauma, febrile seizures, encephalitis, or status epilepticus (SE; Duncan et al., 2006; Sharma et al., 2007). The precise point in time when spontaneous seizures appear is unknown but it is suspected that following a brain injury there is a pre-epileptic state when many changes occur and transform a normal brain into an epileptic one. Since in many cases, after a brain injury, patients recover from a triggering injury without presenting overt spontaneous seizures, the period that precedes the appearance of recurrent epileptic seizures is known as latent or silent period (Sharma et al., 2007; O'Dell et al., 2012). During the early stages of the chronic period spontaneous seizures are more likely to be controlled with medication but as the disease progress seizures become less responsive to medication and might become intractable (Wieser, 2004). So far there is a consensus that acquired epilepsy results from a triggering insult to the brain and that the cellular and molecular alterations resulting from such insult play a key role in the development of epilepsy, but a direct link between the brain abnormalities detected in epileptic tissue and the generation of recurrent spontaneous seizures remains to be firmly established.

Due to the inherent or obvious limitations associated with the acquisition of human samples, the mechanisms responsible for the appearance of spontaneous seizures are being aggressively studied in experimental models of epilepsy. Post-SE models of chronic epilepsy closely mimic the clinical manifestations and tissue abnormalities observed in humans affected with TLE (Sharma et al., 2007; Curia et al., 2008; Loscher and Brandt, 2010). Induction of SE in rodents by systemic or local administration of a chemoconvulsant is usually followed by a silent (latent) period lasting days or weeks when no obvious seizure activity is observed. During the latent period brain abnormalities develop and interictal activity becomes more frequent, and then suddenly with no apparent cause overt spontaneous seizures manifest (Curia et al., 2008; Scorza et al., 2009). To capitalize the methodological advantages provided by the experimental models, several laboratories have attempted to characterize and establish the duration of the latent (silent) period, unfortunately these attempts have yielded a wide range of measures (Williams et al., 2007). It appears that despite the advantages provided by a well-controlled experimental setting, the animals used in the experiments and the type and severity of injury being tested might impact the length of the latent period. More importantly, some methodological limitations (i.e., accuracy of seizure detection, continuous vs. intermittent monitoring, authentication of when the first seizure occurred, etc.) directly impact the accuracy of the measurements and need to be overcome in order to obtain a definitive characterization of the latent period (Williams et al., 2007).

The dynamic process characterized by progressive alterations in neuronal excitability that promotes appearance of spontaneous seizures and its associated structural lesions is known as epileptogenesis (Pitkanen and Lukasiuk, 2011). Currently, the terms epileptogenesis and latent (or silent) period are used interchangeably to describe the period that encompass the occurrence of an insult to the brain and the appearance epileptic seizures. However, recent findings in patients and experimental models suggest that the alterations resulting from an injury to the brain might progress beyond the appearance of the first spontaneous seizure. Accordingly, it has been suggested that during the natural evolution of epilepsy, each new episode of spontaneous seizures (an insult itself) produces new damage that compounds the damage produced by the original brain injury (Williams et al., 2007; Sloviter, 2008; Pitkanen and Lukasiuk, 2009; O'Dell et al., 2012). Many cellular and molecular alterations in both neuronal and non-neuronal cells have been observed during epileptogenesis. Neuronal alterations include neurodegeneration, neurogenesis, and axonal damage in addition to the architectural reorganization of neuronal processes that result from abnormal sprouting and altered dendritic plasticity. Astrocytes become hypertrophic, develop longer and thicker processes and increase the expression of glial fibrillary acidic protein all as part of a process known as reactive astrogliosis (Gibbons et al., 2012; Heinemann et al., 2012; Kovacs et al., 2012). During epileptogenesis there are many pathological processes that affect brain excitability including the breakdown of the blood–brain barrier (BBB) and inflammation. Ultrastructural analysis of epileptic tissue revealed significant abnormalities in the BBB components and focal opening of the BBB by direct application of albumin can lead to the generation of an epileptic focus (Ivens et al., 2007; Vezzani et al., 2011; Heinemann et al., 2012). Further, the magnitude of BBB leakage occurring during epileptogenesis has been shown to directly correlate with the seizure frequency detected during the chronic period (van Vliet et al., 2007; Vezzani et al., 2013). Inflammatory response due to activation of microglia and astrocytes is associated with brain damage and increased BBB permeability in the tissue adjacent to the region of injury (Vezzani et al., 2011; Marchi et al., 2012). SE-induced inflammation is observed during the epileptogenic period and appears to be reactivated by the occurrence of spontaneous seizures, suggesting that inflammation might have a role in epileptogenesis and promote epileptic activity during the chronic period due to the continuous presence of inflammation intermediaries (Vezzani et al., 2011, 2013).

GABA$_A$ RECEPTORS AND ITS ASSOCIATED PROTEINS

The majority of fast inhibitory neurotransmission in the mature brain is mediated by anion-selective GABA$_A$R that are assembled as pentamers from an array of multiple subunit subtypes including α1–2, β1–3, γ1–3, δ, ε, π, θ, and σ1–3 (Fritschy, 2008; Jacob et al., 2008; Luscher et al., 2011; Brickley and Mody, 2012). The subunit composition of GABA$_A$R governs the intrinsic properties of the channel such as affinity for GABA, receptor kinetics, conductance, and allosteric modulation. In addition, intracellular loops of each subunit have the potential to interact with scaffolding proteins and affect the cellular distribution and clustering (synaptic or extrasynaptic) of the channels (Jacob et al., 2008; Luscher et al., 2011; Brickley and Mody, 2012). GABA$_A$R assembled by combining γ2 and α1–3 subunits (α1–3,βx,γ2) are more commonly located at synaptic sites and mostly responsible for phasic inhibition, whereas receptors located at perisynaptic or extrasynaptic sites are primarily composed of α4 or α6 subunits combined with δ subunits (α4/α6,βx,δ) and mediate most tonic inhibition (Fritschy, 2008; Jacob et al., 2008; Luscher et al., 2011; Hines et al., 2012). Notably, tonic currents in pyramidal neurons of the hippocampus can also be generated by receptors containing α5 subunits (α5βγ2) that are located at extrasynaptic locations (Brickley and Mody, 2012; Hines et al., 2012).

Typically, following synthesis in the endoplasmic reticulum, GABA$_A$R are delivered to extrasynaptic compartments within the plasma membrane and then diffuse toward its final destination in either synaptic or extrasynaptic locations. GABA$_A$R located at the plasma membrane also transit among different cellular compartments due to internalization and recycling events. Thus, the final number of receptors located at the cell surface is determined by continuous insertion of *de novo* synthesized and recycled receptors (Michels and Moss, 2007; Leidenheimer, 2008). There are a number of accessory proteins that facilitate the transit of GABA$_A$R along the biosynthetic pathway and the different recycling compartments within the cell (Chen and Olsen, 2007; Jacob et al., 2008; Leidenheimer, 2008). Early on, during GABA$_A$R oligomerization, accessory proteins like BIP (heavy chain binding protein), calnexin and BIG2 (Brefeldin-A-inhibited GDP/GTP exchange factor 2) form interactions with the nascent receptors within the membranous compartments of the endoplasmic reticulum and help with the translocation of the receptors into the Golgi apparatus. During the vesicular trafficking of receptors toward the plasma membrane, GABA$_A$R containing γ subunits are linked to tubulin and the microtubules by GABARAP (GABA$_A$R-associated protein) that acts as a bridge between vesicles containing GABA$_A$R and the machinery that moves those vesicles toward the plasma membrane. GABARAP can also bind to NSF (*N*-ethylmaleimide-sensitive factor), PRIPs (phospholipase-C-related catalytically inactive proteins) and GRIF (GABA$_A$R-interacting factors also known as TRAK), and together these proteins promote the interaction of intracellular vesicles containing GABA$_A$R with the cytoskeleton and facilitate the motor-dependent transport of receptors toward the plasma membrane (Kneussel et al., 2000; Wang and Olsen, 2000; Charych et al., 2004). The final destination of GABA$_A$R into synaptic or extrasynaptic sites is intrinsically determined by the subunits forming the channels and extrinsically by protein–protein interactions with scaffolding proteins (Luscher and Keller, 2004; Chen and Olsen, 2007; Jacob et al., 2008; Leidenheimer, 2008).

Gephyrin is the main structural scaffold that links proteins located at the subsynaptic compartment with the cytoskeleton and it is required for the organization and clustering of GABA$_A$R at inhibitory synapses (Michels and Moss, 2007; Fritschy et al., 2008). During development, increased concentration of gephyrin precedes the accumulation of GABA$_A$R at synaptic sites and facilitates the formation and stabilization of inhibitory synapses (Christie

et al., 2002; Danglot et al., 2003; Swanwick et al., 2006; Fritschy et al., 2008). Removal of gephyrin by gene targeting or siRNA interference strongly affects GABA$_A$R clustering and reduces inhibitory post-synaptic currents, a reciprocal effect on gephyrin clustering and GABAergic innervations has been observed following elimination of γ2 subunits (Essrich et al., 1998; Kneussel et al., 1999; Li et al., 2005; Yu et al., 2008). GABA$_A$R clustering also occurs in neurons lacking gephyrin, but the clusters formed in these conditions have increased mobility and show less accumulation at inhibitory synapses, which reinforces the notion that gephyrin enhance anchoring of receptors at synaptic sites (Levi et al., 2004; Jacob et al., 2005; Yu et al., 2007). GABA$_A$R containing α5 subunits are responsible for tonic inhibition and can also be found forming extrasynaptic clusters (Brunig et al., 2002; Loebrich et al., 2006). A crucial player in α5-containing GABA$_A$R clustering is radixin. Both radixin antisense and genetic knockout causes a loss of α5 clusters in hippocampal neurons and hippocampal tissue (Loebrich et al., 2006; Kneussel and Loebrich, 2007). Radixin and gephyrin do not interact or colocalize with each other, suggesting that the mechanisms behind radixin and gephyrin-dependent clustering are independent (Loebrich et al., 2006; Kneussel and Loebrich, 2007).

The structural and molecular details underlying the regulation of GABA$_A$R by gephyrin are starting to emerge. Gephyrin consist of three major domains, a 20-kDa N-terminal domain (G-domain), a 43-kDa C-terminal domain (E-domain), and an 18–21 kDa central domain (C-domain; Saiyed et al., 2007; Fritschy et al., 2008). Trimerization of the G-domain and dimerization of the E-domain appear to be the oligomerization pattern involved in the formation of the hexagonal lattice required for proper formation of inhibitory synapses (Sola et al., 2004; Saiyed et al., 2007; Fritschy et al., 2008; Tyagarajan and Fritschy, 2010; Herweg and Schwarz, 2012). In addition to its role in the formation of the gephyrin lattice, the E-domain is the binding site for GABA$_A$R (Tyagarajan and Fritschy, 2010) whereas the C-domain binds to several accessory proteins like Pin1 (Zita et al., 2007), dynein light chain 1 and 2 (Fuhrmann et al., 2002; Maas et al., 2006) and collybistin (Kins et al., 2000). Experiments in recombinant expression systems have demonstrated that collybistin is essential for the distribution and stabilization of gephyrin at post-synaptic sites and that overexpression of selected collybistin domains increases the size and density of gephyrin clusters (Kins et al., 2000; Chiou et al., 2011; Tyagarajan et al., 2011), suggesting that collybistin aids with the proper clustering of GABA$_A$R at synaptic sites by forming a partnership with gephyrin (Yu et al., 2008). The small GTPase Cdc42 also binds to the C-domain of gephyrin and works in collaboration with collybistin to help with the translocation of gephyrin toward the plasma membrane (Tyagarajan et al., 2011). Another protein that interacts with gephyrin and indirectly regulates the post-synaptic trafficking and/or accumulation of GABA$_A$R is termed GRIP1 (glutamate receptor interacting protein 1). *In vivo* and *in vitro* evidence suggest that GRIP1 is located at GABAergic synapses and physically interacts with gephyrin to directly modulate gephyrin clustering and indirectly regulate the post-synaptic distribution of GABA$_A$R (Yu et al., 2008).

ALTERATIONS IN GABA$_A$R AND SCAFFOLDING PROTEINS DURING EPILEPTOGENESIS

The belief that following a brain injury there is a quiescent, pre-epileptic state in which there is gradual changes at the molecular, cellular, and circuit levels that ultimately results in the manifestation of spontaneous seizures, has led to the search for mechanisms underlying epileptogenesis. Induction of SE using the chemoconvulsant pilocarpine produces a transient decrease in GABAergic drive readily detectable during the latent period. Abnormal electroencephalogram (EEG) patterns, such as large amplitude spikes and sharp waves can be detected as early as 3–5 days following SE, which overtime culminate with the appearance of full-blown electrographic seizures (El-Hassar et al., 2007). During SE there is a rapid increase in neuronal hyperexcitability due to a quick decline in the number of physiologically active GABA$_A$R at the plasma membrane (Goodkin et al., 2005; Naylor et al., 2005). SE triggers a rapid loss of synaptic GABA$_A$R containing β and γ subunits while extrasynaptic receptors containing α5 and δ subunits remain unaffected (Goodkin et al., 2008; Terunuma et al., 2008). A decrease in the phosphorylation of β3 subunits allows the interaction of β3-containing GABA$_A$R with the clathrin-adaptor protein 2 and the recruitment of GABA$_A$R into clathrin-coated pits promotes a faster removal of these receptors from the cell surface, suggesting that a decrease in the phosphorylation of β3 subunits may account for the selective loss of synaptic GABA$_A$R observed following SE (Goodkin et al., 2008; Terunuma et al., 2008). These biochemical observations directly link the decrease in miniature inhibitory post-synaptic currents observed after induction of SE with the selective internalization of synaptic GABA$_A$R containing β and γ subunits and explain why the currents mediated by extrasynaptic receptors are spared (Goodkin et al., 2005; Naylor et al., 2005).

The fate of internalized receptors following induction of SE is more likely to be determined by the cellular compartment where they are transiently stored (**Figure 1**). Receptors present in endosomal compartments can be reincorporated into the active pool of receptors at the plasma membrane or they can be relocated to the lysosomes for degradation (Chen et al., 2007; Wasterlain and Chen, 2008). Recent studies suggest that the network of proteins required for the proper trafficking and anchoring of GABA$_A$R might be disrupted following SE. During the latent period there is a reduction in the total expression of gephyrin that appears to translate into a reduction in the number of gephyrin clusters (Knuesel et al., 2001; Thind et al., 2010; Fang et al., 2011; González et al., 2013). The pattern of gephyrin loss observed during the silent period parallels the changes in excitability previously observed, and suggests that the loss of scaffolding proteins directly impact the function of GABA$_A$R during epileptogenesis. Intriguingly, during the chronic period there is an increase in both the total expression and the number of gephyrin clusters (Thind et al., 2010; Fang et al., 2011), but it is unclear if this rebound in gephyrin expression results in fully functional inhibitory synapses.

Alterations in the expression of GABA$_A$R during the epileptogenic period include rapid down-regulation of α4, β2/3, γ2, and δ subunits (Schwarzer et al., 1997; Houser and Esclapez, 2003; Peng et al., 2004). Our recent characterization of the expression of several GABA$_A$R subunits in microdissected CA1

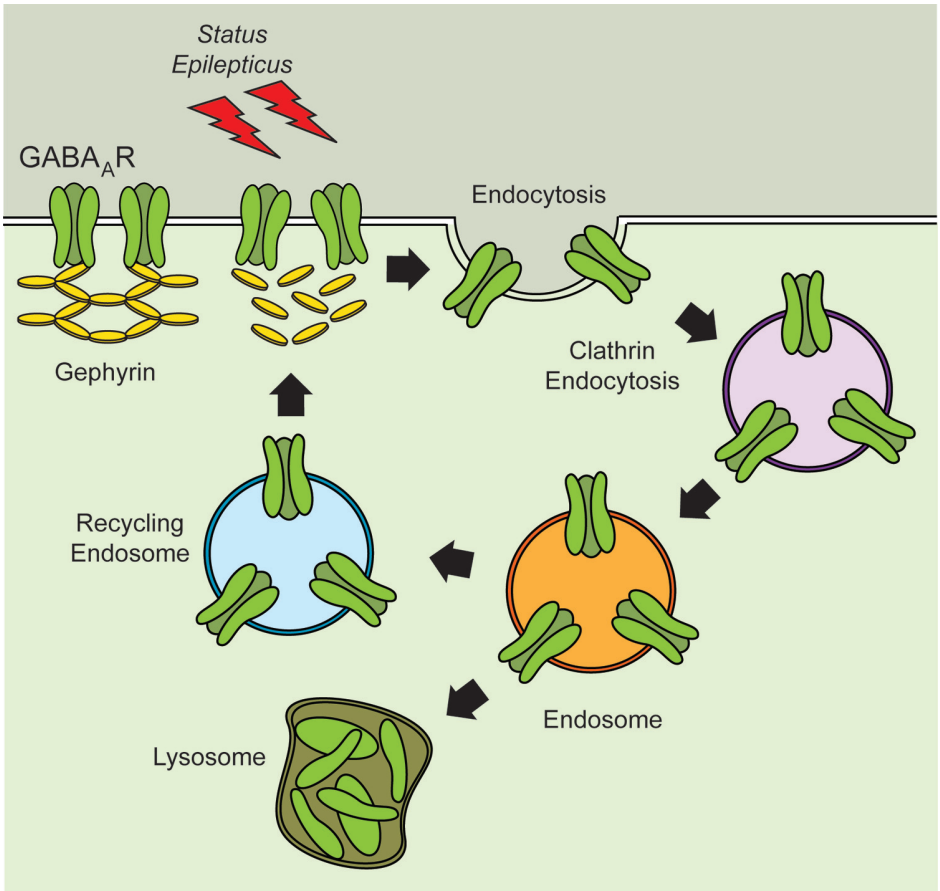

FIGURE 1 | Altered stability of GABA$_A$R and scaffolding proteins during epileptogenesis. Induction of *status epilepticus* produces alterations in the expression of gephyrin and might disrupt GABA$_A$R anchoring. Decreased expression of gephyrin might compromise the recycling of GABA$_A$R and reduce the stability of receptors present at the plasma membrane. Reduced expression of GABA$_A$R at the plasma membrane may contribute to the increased excitability observed during epileptogenesis.

also showed a reduction in the levels of α4, β2/3, and γ2 (but not α1) subunits as early as 4 days after SE (González et al., 2013). The loss of these subunits correlated with the down-regulation of gephyrin, suggesting that the loss of GABA$_A$R might result from the lack of proper receptor anchoring and clustering (González et al., 2013). Accordingly, analysis of the cell surface levels of GABA$_A$R revealed a time-dependent reduction in the plasma membrane levels of α4 and γ2 subunits that correlated with the down-regulation of gephyrin (González et al., 2013). These observations hint to the possibility that during the epileptogenic period, the stability of the GABA$_A$R receptors that recycle back to the plasma membrane might be compromised because they cannot be properly anchored (González et al., 2013). They also suggest that the loss of inhibition and increased inter-ictal activity observed during the latent period might result from the persistent dysregulation of GABA$_A$R trafficking and anchoring (Wasterlain and Chen, 2008). However, whether a loss of scaffolding proteins is a factor contributing to the hyperexcitability observed during the epileptogenic period remains to be fully characterized.

Additional support for hypothesis that a lack of GABA$_A$R stability contributes to the hyperexcitability observed during the latent period comes from studies in *Xenopus* oocytes transplanted with cell membranes isolated from epileptic tissue (Palma et al., 2007; Mazzuferi et al., 2010). Repetitive stimulation of microtransplanted receptors induces a characteristic run-down of GABA$_A$R-mediated currents that is independent of changes in receptor affinity or membrane potential (Palma et al., 2007). The run-down in GABA$_A$R currents can be readily detected in receptors isolated following the manifestation of the first spontaneous seizure and its appearance has been associated with the transition from the latent to the chronic stage of epilepsy (Mazzuferi et al., 2010). Initial exploration of the molecular mechanisms behind this phenomenon revealed a switch in the composition of GABA$_A$R and uncovered an increase in the ratio of α4/α1 subunits incorporated into receptors. More importantly, the switch in GABA$_A$R assembly occurs at the same time that the current run-down appears, underscoring previous findings showing alterations in the expression of α4 and α1 subunits that affect the assembly, localization, and function of GABA$_A$R and results in the impairment of tonic and phasic inhibition (Brooks-Kayal et al., 1998; Peng et al., 2004; Mazzuferi et al., 2010).

The specific mechanisms involved in the regulation of gephyrin during epileptogenesis and epilepsy remain to be fully characterized, but some clues are starting to emerge. Analysis of samples obtained from epileptic patients show a reduction in gephyrin expression, which correlates with the appearance of protein fragments probably resulting from gephyrin degradation (Forstera et al., 2010; Fang et al., 2011). The process involved in the generation of gephyrin fragments remains unclear but a favored hypothesis is that cellular stress (alkalosis and hyperthermia) might be sufficient to induce the skipping of exons in gephyrin messenger RNA resulting in the production of abnormally spliced variants of gephyrin. These abnormal variants may then interact with normal gephyrin molecules and act as dominant-negative mutants and promote the accumulation of gephyrin in ubiquitin positive inclusions (Forstera et al., 2010). The impact abnormally spliced variants of gephyrin has been associated with oligomerization deficits and aberrant clustering of GABA$_A$R containing α2 subunits (Forstera et al., 2010). Induction of mild seizures or inflammatory events triggers the generation of adult-born hippocampal neurons and increases the expression of gephyrin, mostly in the newly generated neurons (Jakubs et al., 2006, 2008; Jackson et al., 2012). Electrophysiological recordings revealed that newborn neurons produced after these injuries have reduced excitatory and increased inhibitory drive partially associated with the increase in gephyrin expression. Together, these observations point out to the possibility that newly generated cells might increase gephyrin expression as a compensatory mechanism to mitigate the hippocampal hyperexcitability observed after an insult to the brain (Jakubs et al., 2006; Jackson et al., 2012).

Another line of evidence implicating gephyrin dysfunction as a key element in the generation of epileptic seizures includes recent genetic evidence found in individuals affected by pathologies associated with a seizure phenotype. Rare hemizygous microdeletions in the chromosome 14q23.3 that encompass exons 3–5 in the coding region the G-domain were found in individuals from six unrelated families presenting autism, schizophrenia, and seizures (Lionel et al., 2013). Other mutations that interfere with collybistin function have also been associated with abnormalities observed in patients with epilepsy and mental retardation (Harvey et al., 2004; Marco et al., 2008; Kalscheuer et al., 2009; Lesca et al., 2011). These mutations found in the coding sequence of collybistin interfere with the somatic and synaptic localization of gephyrin via dominant-negative mechanisms that indirectly affect the distribution and synaptic clustering of GABA$_A$R (Harvey et al., 2004; Kalscheuer et al., 2009).

The impact that disruption of gephyrin and other scaffolding proteins might have on GABA$_A$R function during epileptogenesis remains to be elucidated. If a loss of gephyrin directly impacts the number and function of GABA$_A$R at inhibitory synapses, interventions to promote the stability of gephyrin and GABA$_A$R might ameliorate the deleterious changes in excitability observed during epileptogenesis and epilepsy. Altered expression of gephyrin has been observed in several pathologies presenting symptomatic seizures, but it is unclear if changes in gephyrin are beneficial or pathologic (Jakubs et al., 2008; Thind et al., 2010; Jackson et al., 2012). Understanding the molecular mechanism(s) behind the dysregulation of scaffolding proteins involved in the regulation of GABA$_A$R might provide new insights into the pathologic events that contribute to the generation of spontaneous seizures and might offer new targets to disrupt epileptogenesis and prevent epilepsy.

ACKNOWLEDGMENT

Marco I. González is supported by the National Institutes of Health Grant K01-NS069583.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 April 2013; accepted: 26 June 2013; published online: 22 July 2013.

Citation: González MI (2013) The possible role of GABA$_A$ receptors and gephyrin in epileptogenesis. Front. Cell. Neurosci. 7:113. doi: 10.3389/fncel.2013.00113

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 16 July 2013
doi: 10.3389/fncel.2013.00110

Contribution of apoptosis-associated signaling pathways to epileptogenesis: lessons from Bcl-2 family knockouts

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Neuronal cell death is a pathophysiological consequence of many brain insults that trigger epilepsy and has been implicated as a causal factor in epileptogenesis. Seizure-induced neuronal death features excitotoxic necrosis and apoptosis-associated signaling pathways, including activation of multiple members of the Bcl-2 gene family. The availability of mice in which individual Bcl-2 family members have been deleted has provided the means to determine whether they have causal roles in neuronal death and epileptogenesis *in vivo*. Studies show that multiple members of the Bcl-2 family are activated following status epilepticus and the seizure and damage phenotypes of eight different knockouts of the Bcl-2 family have now been characterized. Loss of certain pro-apoptotic members, including Puma, protected against seizure-induced neuronal death whereas loss of anti-apoptotic Mcl-1 and Bcl-w enhanced hippocampal damage. Notably, loss of two putatively pro-apoptotic members, Bak and Bmf, resulted in more seizure-damage while deletion of Bid had no effect, indicating the role of certain Bcl-2 family proteins in epileptic brain injury is distinct from their contributions following other stressors or in non-CNS tissue. Notably, Puma-deficient mice develop fewer spontaneous seizures after status epilepticus suggesting neuroprotection may preserve functional inhibition, either directly by preserving neuronal networks or indirectly, for example by limiting reactive gliosis and pro-inflammatory responses to neuronal death. Together, these studies support apoptosis-associated molecular mechanisms controlling neuronal death as a component of epileptogenesis which might be targetable to protect against seizure-damage, cognitive deficits and mitigate the severity of syndrome following epilepsy-precipitating injuries to the brain.

Keywords: apoptosis, bcl-2, brain, hippocampus, mitochondria, neuron, stroke, temporal lobe epilepsy

INTRODUCTION

Epileptogenesis is the process by which a normal brain is transformed into one capable of generating recurrent spontaneous seizures (Pitkanen and Lukasiuk, 2011; Goldberg and Coulter, 2013). The process can be triggered by injuries to the brain such as trauma, stroke, infection and acute symptomatic seizures such as status epilepticus. Interrupting this process would offer the possibility of preventing the development of epilepsy, a chronic and often life-long neurologic disorder characterized by an enduring predisposition to seizures (Chang and Lowenstein, 2003). If complete prevention is not possible, a secondary goal would be to modify the severity of the condition, for example resulting in fewer or less severe seizures (e.g., partial seizures rather than generalized tonic-clonic) (Pitkanen and Lukasiuk, 2011).

Current anti-epileptic drugs (AEDs) do not appear to significantly modify the underlying pathophysiology, with the possible exception of levetiracetam and ethosuximide in immature models (Loscher and Brandt, 2010; Pitkanen and Lukasiuk, 2011). This implies that the mechanisms of ictogenesis and epileptogenesis are not the same (Sloviter and Bumanglag, 2013). This underscores the need to understand the cell and molecular mechanisms underlying epileptogenesis and then identify potentially novel approaches and targets with which to design anti-epileptogenic treatments.

There has been major progress in characterizing the cell and molecular processes that occur during epileptogenesis [for review, see Mcnamara et al. (2006); Boison (2008); Engel and Henshall (2009); Pitkanen and Lukasiuk (2011); Vezzani et al. (2011); Goldberg and Coulter (2013)]. Epileptogenesis is associated with acute and delayed neuronal death, gliosis (particularly astrocytes and microglia), changes to synaptic and circuit function (morphology, channelopathies, firing properties, γ-amino butyric acid (GABA)-ergic tone), neuroinflammation, aberrant neurogenesis and extracellular matrix remodeling. We remain uncertain which individual processes or combination of processes are important and some undoubtedly reflect repair processes that are beneficial (Loscher and Brandt, 2010; Sloviter, 2011). Efforts to target epileptogenesis and prevent the subsequent emergence of epilepsy have been largely unsuccessful to date (Loscher and Brandt, 2010; Pitkanen and Lukasiuk, 2011).

NEURONAL DEATH AS A CONSEQUENCE OF SEIZURES AND CAUSAL FACTOR IN EPILEPTOGENESIS

Neuron loss within the hippocampus is a common pathologic hallmark of mesial temporal lobe epilepsy (mTLE) in humans.

The most frequent finding is end folium sclerosis (loss of neurons within the hilus) and loss of CA1 and CA3 pyramidal neurons. Area CA2 and dentate granule neurons are usually spared, although some neurons may also be lost from these regions (Chang and Lowenstein, 2003; Thom, 2009). The neuron loss in mTLE is mainly thought to be a result of an initial precipitating insult such as prolonged febrile seizures, infection, trauma or status epilepticus. Prolonged or repeated epileptic seizures, however, may contribute to further neuron loss (Mathern et al., 2002; Henshall and Meldrum, 2012).

Animal models of mTLE produced by repeated brief seizures or status epilepticus are also associated with significant neuron loss within the hippocampus, as well as various other pathologic changes including gliosis, inflammation, blood brain barrier damage and circuit reorganization. How might neuronal death contribute to epileptogenesis or shape the epileptic phenotype? Both direct and indirect mechanisms are possible. Neuron loss may cause an imbalance between excitation and inhibition with brain networks, for example by removing inhibitory neurons or by removing excitatory neurons that function to activate inhibitory neurons (Pitkanen, 2002; Loscher and Brandt, 2010; Sloviter, 2011). The immediacy of epilepsy in some models supports a direct pro-epileptogenic effect of neuronal death. For example, spontaneous seizures are detected with minimal delay after intense hippocampal activation via perforant path stimulation that destroys neurons in the dentate hilus (Bumanglag and Sloviter, 2008). CA3-restricted lesions of the hippocampus also produce epilepsy with minimal latency suggesting neuronal death in this region is also epileptogenic (Li et al., 2008; Mouri et al., 2008). Neuron loss is also implicated as an epileptogenic mechanism following injury to thalamocortical circuitry (Paz et al., 2010). Epilepsy has also been reported to develop with no significant latent period following status epilepticus-induced brain injury in humans (Mikaeloff et al., 2006). Other supporting evidence includes the strong association found in some studies between epileptic seizure rates and the extent of damage to the neocortex (Kharatishvili and Pitkanen, 2010) and hippocampus (Jimenez-Mateos et al., 2010). Neuronal death may have pro-epileptogenic effects through indirect mechanisms; reactive gliosis and inflammation accompany neuronal damage and can promote hyperexcitability (Maroso et al., 2010; Vezzani et al., 2011). Recent transcriptome analysis of epileptogenesis identified genes regulating apoptosis as common to the epileptogenic process (Okamoto et al., 2010) and mice lacking the apoptosis-regulating gene *Chop* exhibit increased hippocampal damage and cognitive deficits following status epilepticus and develop more frequent spontaneous seizures (Engel et al., 2013).

If neuronal death is important for epileptogenesis and/or shaping the emergent phenotype then neuroprotection should have anti-epileptogenic effects. There are, however, relatively few studies which have provided direct evidence. A common problem is that drugs have been given before or during status epilepticus and this has modified the initial precipitating injury (Acharya et al., 2008; Loscher and Brandt, 2010; Sloviter, 2011). Excluding such studies, we are left with some examples where neuroprotection was found to be anti-epileptogenic. Injection of rats with a neuroprotective dose of a caspase-3 inhibitor 3 h after status epilepticus strongly reduced the number of spontaneous seizures and the proportion of rats developing epilepsy (Narkilahti et al., 2003). Neuroprotection through seizure preconditioning is also associated with reduced spontaneous seizures (Jimenez-Mateos et al., 2008). Neuroprotection also protects against cognitive deficits such as memory impairment as well as behavioral changes (Loscher and Brandt, 2010).

Not all studies, however, have found a convincing link between neuronal death and epileptogenesis. For example, glutamate receptor antagonist and valproate treatment after status epilepticus protected against cell death but did not prevent epileptogenesis (Acharya et al., 2008; Loscher and Brandt, 2010). Genetic deletion of p53 was recently shown to result in a more severe epilepsy phenotype despite animals displaying smaller hippocampal lesions after status epilepticus (Engel et al., 2010d). This may reflect possible "neuro-overprotection" or circumstances where loss of neurons, particular irrevocably damaged cells, aids the tissue repair process and leads to improved functional recovery (Andre et al., 2000; Gilby et al., 2005). Last, the role of neuronal death may be less important in the developing brain where epileptogenesis has been reported without overt cell death (Raol et al., 2003; Dube et al., 2006).

GENETIC TOOLS TO ADDRESS THE ROLE OF NEURONAL DEATH IN EPILEPTOGENESIS

Pharmacological neuroprotection is rarely complete in status epilepticus models, leaving open the question of whether full protection, if feasible, would have a stronger anti-epileptogenic effect. Pharmacological tools also have limitations, including the question of when to deliver, how much, for how long and the duration of action (Sloviter, 2011). We also lack specific drugs to many of the pathways implicated in the control of neuronal death. Genetic models enable an assessment to be made based on the complete absence of a particular protein during the injury. Such tools also enable us to understand the causal roles of the genes in the signaling pathways that coordinate cell death.

Which genes to target? Genes involved in apoptosis-associated signaling are attractive because (1) apoptosis-associated signaling is a molecular feature of seizure-induced neuronal death; (2) extensive mouse lines have been developed to study the role of these genes in other diseases (mainly cancer), including targeting entire families; and (3) the genes are highly conserved between mice and humans (**Figure 1**). The Bcl-2 family represents a large group of genes for which multiple knockout mice exist and is the focus of the present article. In the past decade, researchers have characterized seizures and the damage phenotype in mice lacking eight different members of the family. Together, the findings reveal a select role for members of the Bcl-2 family in seizure-induced neuronal death and evidence that cell death controlled by Bcl-2 family proteins is functionally important in epileptogenesis (**Table 1**). Reviews on the broader topic of apoptosis and epilepsy can be found elsewhere [see Henshall and Simon (2005); Engel and Henshall (2009); Bozzi et al. (2011)].

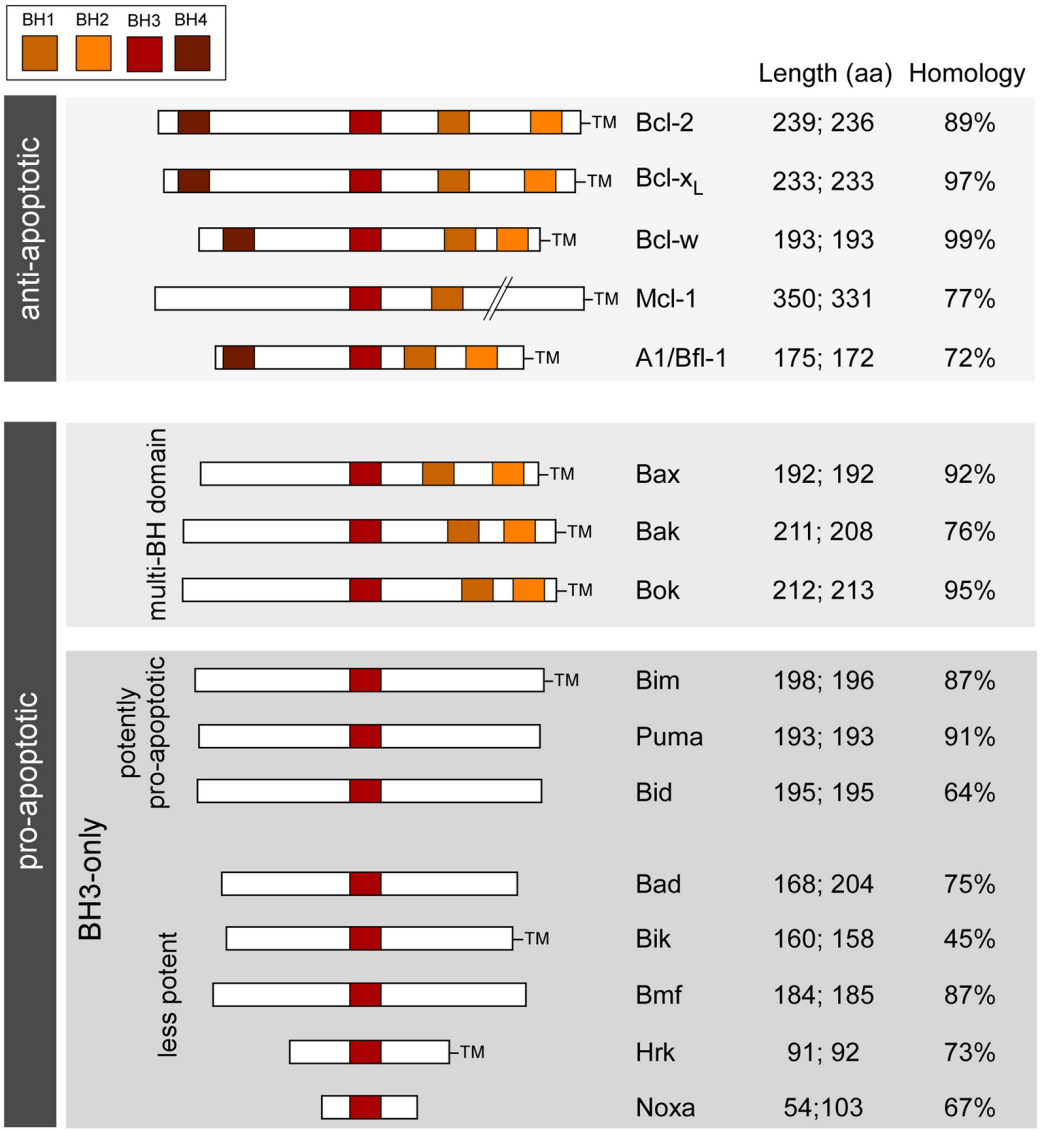

FIGURE 1 | Bcl-2 family; organization, functions, and characteristics. Schematic shows the main Bcl-2 family proteins in humans and mice along with the main conserved structural motifs (BH domains), grouped according to function and depicting amino acid length and homology between species. TM, transmembrane domain. Length refers to amino acid number in (left side) *homo sapiens* and (right side) *mus musculus* (splice variants not included). Homology is between human and mouse amino acid sequences (BLAST).

Bcl-2 FAMILY PROTEINS

Bcl-2 family proteins are the gatekeepers of the intrinsic (mitochondrial) pathway of apoptosis (Youle and Strasser, 2008; Hotchkiss et al., 2009; Chipuk et al., 2010). The intrinsic pathway of apoptosis is triggered by intracellular disturbances such as DNA damage, hypoxia, calcium overload, growth factor withdrawal, oxidative stress and misfolded proteins (Galluzzi et al., 2009; Tait and Green, 2010). Mitochondria are central to the convergence of these signals, and in the molecular processes which result in either restitution of cell homeostasis or execution of cell death. Loss of mitochondrial membrane potential constitutes a critical, irreversible step in cell death with subsequent release of pro-apoptogenic proteins (cytochrome c, apoptosis inducing factor), bioenergetic failure and downstream activation of caspase-dependent and caspase-independent cell death (Hotchkiss et al., 2009).

The Bcl-2 family are pivotal to the initiation, integration and execution of the intrinsic apoptosis pathway. About twenty Bcl-2 family members are recognized in mammals, based on sequence and function and they are typically organized into three groups (**Figure 1**). The anti-apoptotic members include Bcl-2, Bcl-xL, Bcl-w, and Mcl-1. Each member shares four Bcl-2 homology (BH) domains and a transmembrane domain that anchors them to intracellular membranes, particularly to

Table 1 | Summary of effects of genetic disruption of Bcl-2 family proteins on status epilepticus-induced neuronal death and post-status epilepsy in mice.

Gene	Expected role	SE model	Chemoconvulsant/SE threshold in knockout	Hippocampal pathology in knockout	Effect on post-SE epilepsy	References
Mcl-1[a]	Anti-apoptotic	Pilocarpine	Lower threshold for convulsions	Increased CA3 damage	Not tested	Mori et al., 2004
Bcl-w	Anti-apoptotic	i.a. KA	Earlier onset of SE	Increased CA3 damage	Not tested	Murphy et al., 2007
Bak	Pro-apoptotic	Systemic KA PTZ	Increased seizures[b] Same as wt	Increased CA3 damage	Not tested	Fannjiang et al., 2003
Bim	Pro-apoptotic	i.h. KA i.a. KA Systemic KA	Same as wt Same as wt Same as wt	Same as wt Reduced CA3 damage Not reported	Not tested Not tested Not tested	Theofilas et al., 2009 Murphy et al., 2010 Gimenez-Cassina et al., 2012
Bid	Pro-apoptotic	i.a. KA Systemic KA	Same as wt Same as wt	Same as wt Not reported	Not tested Not tested	Engel et al., 2010a Gimenez-Cassina et al., 2012
Puma	Pro-apoptotic	i.a. KA i.a. KA (high)[c]	Same as wt Same as wt	Reduced CA3 damage Reduced CA3 damage	Reduced epileptic seizures Not tested	Engel et al., 2010c Engel et al., 2010b
Bad	Pro-apoptotic	Systemic KA, PTZ	Reduced seizures	Not reported	Not tested	Gimenez-Cassina et al., 2012
Bmf	Pro-apoptotic	i.a. KA	Same as wt	Increased CA3, CA1 and hilar damage	Increased epileptic seizures[d]	Moran et al., 2013

[a] *Studies performed using heterozygous, not knockout mice.*

[b] *Behavioral assessment, not by EEG.*

[c] *Higher dose (1 μg) of KA was used to produce more severe SE.*

[d] *Mice displayed ~30% more seizures although effect did not reach statistical significance.*

Abbreviations: KA, kainic acid; i.a., intra-amygdala; i.h., intra-hippocampal; PTZ, pentylenetetrazole; SE, status epilepticus; SzPC, seizure preconditioning; wt, wild-type.

mitochondria (Chipuk et al., 2010). Pro-apoptotic members are divided into the multi-BH domain "effectors" and the BH3-only proteins. The effectors comprise Bax and Bak. They share BH1-3 domains and have a transmembrane domain. Bok, a third putative member of this sub-group based on structural similarities, appears to be less important and cannot promote apoptosis in the absence of Bax/Bak (Echeverry et al., 2013). Activation of Bax/Bak involves oligomerization and pore formation in mitochondrial membranes that triggers mitochondrial outer membrane permeabilization and release of apoptogenic proteins (Galluzzi et al., 2009; Chipuk et al., 2010).

BH3-only proteins are a highly heterogeneous group of structurally unrelated proteins with the exception that they share a BH3 domain in common (see **Figure 1**). The most accepted model is that BH3-only proteins function either as "sensitizers," including Bad, or "direct activators," such as Bim and Bid. Sensitizers bind and neutralize anti-apoptotic Bcl-2 family proteins lowering the threshold for apoptosis, but not directly causing mitochondrial outer membrane permeabilization or apoptosis. Direct activators are capable of interacting with multi-domain Bax/Bak and triggering their insertion into mitochondria (Galluzzi et al., 2009; Chipuk et al., 2010).

SEIZURES ACTIVATE MULTIPLE Bcl-2 FAMILY PROTEINS

Multiple members of the Bcl-2 family have been found to undergo transcriptional and/or post-translational responses following status epilepticus in rat and mouse models. Seizures cause up-regulation of anti-apoptotic Bcl-2 (Graham et al., 1996), Bcl-w (Henshall et al., 2001b; Murphy et al., 2007) and Mcl-1 (Mori et al., 2004). Seizures also upregulate and activate pro-apoptotic Bax (Graham et al., 1996; Ananth et al., 2001; Henshall et al., 2002; Moran et al., 2013), trigger Bid cleavage to its truncated (most active) form (Henshall et al., 2001a; Engel et al., 2010a), induce dissociation of Bad from its cytoplasmic chaperone 14-3-3 (Henshall et al., 2002), and upregulate at a transcript and/or protein level, Bim (Shinoda et al., 2004; Murphy et al., 2010), Puma (Engel et al., 2010c), and Bmf (Moran et al., 2013).

How do Bcl-2 family proteins regulate seizure-induced neuronal death? Co-immunoprecipitation and mitochondrial enrichment analysis shows pro-apoptotic Bcl-2 family proteins functionally target anti-apoptotic members and/or cluster at mitochondria during seizure-induced neuronal death (Henshall et al., 2002; Shinoda et al., 2004; Engel et al., 2010a). This is predicted to cause release of apoptogenic proteins from mitochondria that activate downstream caspase-dependent and

–independent cell death. The timing of the activation of pro-apoptotic Bcl-2 family proteins (1–4 h post status epilepticus) is broadly consistent with the release of apoptogenic molecules from mitochondria; cytochrome c and apoptosis-inducing factor are released between 1 and 4 h after status epilepticus (Henshall et al., 2000; Murphy et al., 2007; Engel et al., 2010a; Zhao et al., 2010). Nevertheless, there is no direct evidence that blocking Bcl-2 family proteins alters apoptosis signaling down-stream of mitochondria after status epilepticus.

There is also evidence that levels of Bcl-2 family members are altered in brain tissue from patients with pharmacoresistant temporal lobe epilepsy. Analysis of hippocampus and neocortex removed for the treatment of intractable seizures has found higher levels of Bcl-2, Bcl-xL, Bcl-w, and lower levels of Bim. The molecular repertoire appears shifted toward an anti-apoptotic balance which may serve to resist further neuron loss in patients experiencing frequent seizures (Henshall and Meldrum, 2012).

Several members of the Bcl-2 family have yet to be characterized in seizure models. These include anti-apoptotic Bcl-b and pro-apoptotic multi-domain Bok and BH3-only proteins Bik [the first BH3-only protein for which the mechanism of action was specifically linked to the BH3 domain (Boyd et al., 1995)], BNip3 and Noxa, among others.

SEIZURES AND SEIZURE-INDUCED NEURONAL DEATH IN MICE LACKING Bcl-2 FAMILY PROTEINS

Convulsive thresholds, seizure severity and/or seizure-induced neuronal death have been assessed in knockouts of eight of the Bcl-2 family members using status epilepticus as the trigger (see **Table 1**). Chemoconvulsive thresholds and/or status epilepticus were found to be altered in mice lacking Bak, Mcl-1, Bcl-w and Bad, whereas loss of Bim, Bid, Puma and Bmf does not affect evoked seizures. We do not yet understand why convulsant thresholds differ in some of these mice and assessment of the neuroanatomy of these mice has in most cases ruled out any significant structural abnormalities accounting for phenotypes. Possible compensatory up-/down-regulation of other members of the Bcl-2 family have also been excluded in some, but not all, studies. For some (e.g., Bim and Bid knockout mice) assessments have been made by independent laboratories and for two members of the family, Puma and Bmf, long-term recordings have been performed to assess the impact of the loss of the gene on spontaneous (i.e., epileptic) seizures. Several members of the Bcl-2 family have not been tested as yet or their assessment remains unfeasible at present because of brain abnormalities [e.g., $Bax^{-/-}$ mice, (Moran et al., 2013)]. A brief chronological review of the seizure and damage phenotypes in these mice follows and results are further summarized in **Figure 2**.

Bak

Bak is predominantly expressed in neurons in the CNS (Krajewska et al., 2002) and was the first Bcl-2 family member for which mice lacking the gene were subjected to a functional assessment in status epilepticus. Contrary to expectation, mice lacking Bak displayed increased seizure-induced neuronal death following systemic kainate injection, supporting an anti-apoptotic rather than pro-apoptotic function (Fannjiang et al., 2003) (**Table 1**). Bak deficiency had no effect on pentylenetetrazol-induced convulsions, however, and was protective in a model of stroke (Fannjiang et al., 2003). This suggests that Bak may switch between pro- and anti-apoptotic functions according to the nature of the neurological insult. This may be possible because neurons can express a Bak splice variant that serves an anti-apoptotic function (Sun et al., 2001; Fannjiang et al., 2003). Bak deficiency was also found to result in more severe behavioral seizures (Fannjiang et al., 2003). Whether this relates to subtle differences in brain development or other effects of the loss of Bak on neuronal excitability is uncertain. It complicates, however, conclusions on whether differences in damage relate to an altered initial insult or an effect specific to signaling pathways. Verification of the impact of Bak loss in other models (e.g., pilocarpine) and direct evidence for activation *in vivo* may be warranted.

Mcl-1

Mcl-1, first cloned in 1993 (Kozopas et al., 1993), is constitutively expressed at low levels in the adult brain, including in the main neuronal populations of the hippocampus, and Mcl-1 is strongly up-regulated in the areas that survive status epilepticus (Mori et al., 2004). A neuroprotective role for Mcl-1 was supported by the finding that heterozygous mice ($Mcl1^{+/-}$) (knockout is embryonic lethal) displayed a four-fold increase in seizure-induced neuronal death following pilocarpine-induced status epilepticus (Mori et al., 2004). The study also found that heterozygotes were more sensitive to chemoconvulsant, necessitating the use of a lower dose of pilocarpine to produce comparable status epilepticus (Mori et al., 2004). As with Bak-deficient mice, this makes it somewhat difficult to conclude that effects are only due to altered cell death signaling. Nevertheless, Mcl-1 appears to be among the most important anti-apoptotic members of the family in seizure models.

Bcl-w

Bcl-w was cloned in 1996 (Gibson et al., 1996) and is expressed in many tissues, including the brain (Hamner et al., 1999; O'Reilly et al., 2001). Protein levels of Bcl-w are bi-directionally altered in the hippocampus after seizures (Henshall et al., 2001b; Murphy et al., 2007). In damaged subfields after status epilepticus, Bcl-w appears to be targeted by Bim and becomes integrated, possibly inactivated, in the mitochondrial compartment (Shinoda et al., 2004; Murphy et al., 2007). In contrast, Bcl-w is up-regulated following brief electroshock seizures, a model of epileptic tolerance (Murphy et al., 2007). Hippocampal damage is significantly increased in Bcl-w-deficient mice following status epilepticus (Murphy et al., 2007). The mice also display a shorter time period between injection of the chemoconvulsant and their first paroxysmal seizure discharge. As a result, Bcl-w deficient mice experience a longer period of status epilepticus during monitoring (Murphy et al., 2007). A statistical analysis of the relationship between seizure duration and damage showed this could not account for all the additional damage (Murphy et al., 2007). Thus, the

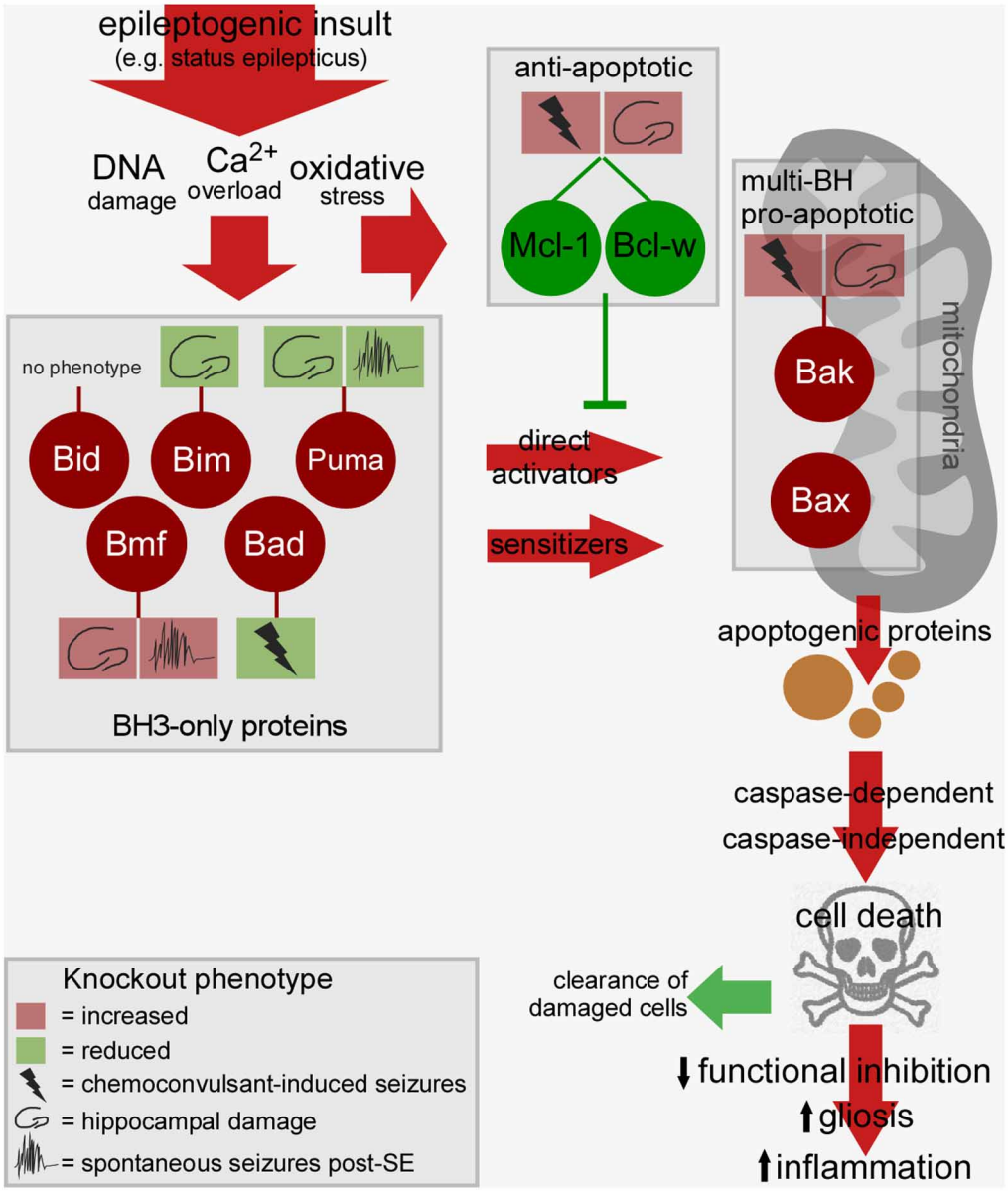

FIGURE 2 | Bcl-2 family-regulated pathway and phenotypes in Bcl-2 knockout mice after status epilepticus. Cartoon depicts the relationship between the activated Bcl-2 family proteins after status epilepticus, the signaling pathways they drive toward cell death, and possible consequences of such cell death on epileptogenesis. Multiple BH3-only proteins are activated by epileptogenic insults which may promote mitochondrial dysfunction via Bax or other mechanisms, a step that can be blocked by anti-apoptotic members of the Bcl-2 family. The cell death controlled by this pathway may promote tissue repair or drive epileptogenesis via disruption of neuronal networks. The phenotype of Bcl-2 family protein knockout mice is indicted in the boxes linked by a stalk. For each, the key shows how deletion of the gene impacts convulsive thresholds (e.g., to kainate), hippocampal injury after status epilepticus or emergent spontaneous seizures. Not represented: The figure does not include other intracellular compartments in which Bcl-2 family proteins are commonly found such as the endoplasmic reticulum. SE, status epilepticus.

exacerbated hippocampal injury in Bcl-w-deficient mice is at least partly the result of losing the neuroprotective properties of the protein. As with Mcl-1 and Bak, we do not know why these knockout mice display altered responses to chemoconvulsants but it is assumed to relate to subtle effects of gene deficiency on brain development or non-cell death related functions of the proteins that impact on excitability.

Bim

Bim was the first of the BH3-only proteins studied for a causal role in seizure-induced neuronal death. Mice lacking Bim display select abnormalities of apoptosis control (Bouillet et al., 1999) but the brain appears normal (Murphy et al., 2010). Bim is upregulated in a Foxo- or JNK-dependent manner following status epilepticus, and has been shown to co-immunoprecipitate

with anti-apoptotic Bcl-2 family proteins, suggesting it is active (Shinoda et al., 2004; Murphy et al., 2010). Three studies have investigated the effect of loss of Bim, all in kainate models. In the first, kainate was directly injected into the hippocampus and in that study mice lacking Bim showed no difference in seizures or neuronal death (Theofilas et al., 2009). In contrast, a significant reduction in hippocampal damage was found in mice lacking Bim in which seizures were triggered by intra-amygdala kainate, although cortical injury was not affected (Murphy et al., 2010). Again, seizures were normal in $Bim^{-/-}$ mice. The authors suggested that deficiency in Bim protects only those regions in which Bim was normally induced by seizures (Murphy et al., 2010). Other data support causal roles for Bim in excitotoxic neuronal death (Concannon et al., 2010). A third study that used systemic kainate to trigger seizures in Bim-deficient mice, in this case forebrain-specific knockouts, also found the mice underwent normal seizures (Gimenez-Cassina et al., 2012). Thus, loss of Bim has no effect on seizures although has a model- and brain region-specific contribution to seizure-induced neuronal death.

Puma

Puma (Bbc3) is present at very low levels in the normal adult brain but undergoes rapid up-regulation after status epilepticus, driven by p53 (Engel et al., 2010c). Seizure-induced neuronal death was found to be strongly reduced in mice lacking Puma (Engel et al., 2010c). Lack of Puma also protected in a model of more severe status epilepticus, suggesting that Puma deficiency protects against necrosis (Engel et al., 2010b). The development of spontaneous seizures was also investigated in Puma-deficient and Puma-expressing mice subject to status epilepticus. Remarkably, mice lacking Puma had over 60% less epileptic seizures during the two week recordings. Also, only 63% of these mice developed any spontaneous seizures (vs. 89% of the heterozygous animals used as seizure-controls). When epileptic seizures did occur, they were of similar severity and duration (Engel et al., 2010c). The mechanism of this anti-epileptic effect is uncertain. The reduced neuronal death may have resulted in diminished gliosis and reduced inflammation. Neuroprotection may also have preserved some functional inhibition although electrophysiological studies were not performed and the type of protected neurons (e.g., glutamatergic or GABAergic) remain unidentified.

Bid

Bid is another member of the subgroup of potently pro-apoptotic BH3-only proteins that is robustly expressed in the adult brain, including in hippocampal neurons (Krajewska et al., 2002). Previous work had shown the loss of Bid to protect against focal cerebral ischemia *in vivo* and excitotoxicity *in vitro* (Plesnila et al., 2001; Yin et al., 2002); the expectation was that Bid loss would also prevent seizure-induced neuronal death. Although Bid was activated by status epilepticus, seizure-induced neuronal death in $Bid^{-/-}$ mice was similar to wild-type animals (Engel et al., 2010a). In this and another recent study, seizures were not different, indicating that Bid probably does not have a critical role in neurodevelopment or neurophysiological functions (Engel et al., 2010a; Gimenez-Cassina et al., 2012). Thus, in contrast to established pro-apoptotic roles in ischemic brain injury *in vivo*, loss of Bid does not affect seizure-induced neuronal death. This suggests the contribution of individual BH3-only proteins is dependent on the brain insult which may be because of differences in the nature of the molecular stress and how this interacts with the prevailing expression of the protein at the time of the insult (Engel et al., 2011).

Bad

While hippocampal damage after status epilepticus in mice lacking Bad has not been reported, convulsive thresholds have been investigated. Mice lacking Bad display significantly reduced seizure severity in response to kainate and other chemoconvulsants (Gimenez-Cassina et al., 2012). No other mouse lacking a BH3-only protein has been found to have such a phenotype. The cause of the anticonvulsant effect of Bad deficiency may relate to reduced mitochondrial use of glucose and a shift to use of ketone bodies; possibly the same mechanism that underlies the anti-epileptic effect of the ketogenic diet (Danial et al., 2013) (see also below).

Bmf

The most recently studied Bcl-2 family protein in seizure-induced neuronal death is Bmf. Transcript levels of Bmf are increased rapidly following status epilepticus in mice in a AMP kinase dependent manner, although the paucity of specific antibodies has prevented an assessment of where the protein is within the hippocampus and whether this is affected by seizures (Moran et al., 2013). Bmf-deficient mice display normal neuroanatomy and undergo normal seizures in the intra-amygdala kainate model (Moran et al., 2013). Remarkably, however, mice lacking Bmf displayed significantly more, not less, seizure-induced neuronal death than wild-types. Increased damage was mainly found in the CA3 subfield but was also evident in CA1 and the hilus and even in the contralateral hippocampus, an area normally spared in this model (Moran et al., 2013). This is the first example of a BH3-only protein apparently protecting against seizure-induced neuronal death. Nevertheless, the mechanism of this protection is unknown. Long-term EEG recordings in $Bmf^{-/-}$ mice after status epilepticus found ∼30% higher rate of spontaneous seizures, suggesting exacerbated hippocampal damage may worsen the form of developed epilepsy (Moran et al., 2013). Notably, the severity of individual seizures was not different in the $Bmf^{-/-}$ animals supporting findings with Puma-deficient mice that modulating neuronal cell death affects the occurrence/threshold of the seizures but not the severity of the seizures once they occur (Engel et al., 2010c).

ARE THERE COMPENSATORY RESPONSES IN KNOCKOUT MICE THAT DO NOT DISPLAY A PHENOTYPE?

It might be possible that compensatory responses in knockout mice rather than functional redundancy explain the lack of seizure phenotype in some models. This may be the case for Bid, where increased Bim is observed (Engel et al., 2010a), although this is not direct evidence of compensation. In other studies, compensatory up- or down-regulation of other Bcl-2

family proteins has not been found (Fannjiang et al., 2003; Moran et al., 2013) although the same level of a related protein may take over functions, especially in a group of proteins that are highly dependent on subcellular distribution, as is the case with the Bcl-2 family.

CONSTITUTIVE AND NON-CANONICAL FUNCTIONS OF Bcl-2 FAMILY PROTEINS IN THE BRAIN

The presence of quite high levels of several pro-apoptotic Bcl-2 family proteins in the normal adult mammalian brain is at odds with the need for neurons to survive for many years or decades; possessing high levels of proteins whose only role is to kill cells is both energy-expensive and risky. Moreover, several non-Bcl-2 family-related components of the apoptotic machinery are down-regulated in the adult brain including the apoptotic protease activating factor-1 (Apaf-1) and caspase-3 (Yakovlev et al., 2001). We now know that particular Bcl-2 family proteins have non-canonical roles, reviews of which can be found elsewhere (Lamb and Hardwick, 2010; Hardwick et al., 2012). In some cases this involves a switch to anti-apoptotic rather than pro-apoptotic function, as for Bak (Fannjiang et al., 2003) and perhaps Bmf (Moran et al., 2013). For others, however, Bcl-2 family proteins serve completely different functions in the normal brain, although remain capable of being co-opted to regulate cell death when called upon (e.g. after a severe stressor).

Bad has previously been implicated in the control of glycolysis in non-neuronal cells (Danial et al., 2003). Recent work showed that Bad is also involved in metabolism of glucose in neurons and glia (astrocytes) (Gimenez-Cassina et al., 2012). Loss of Bad resulted in a switch to ketone-based metabolism in neurons. This may phenocopy the ketogenic diet and the study reported that mice lacking Bad displayed strongly reduced seizure susceptibility to kainate and pentylenetetrazole (Gimenez-Cassina et al., 2012).

Bcl-xl contributes to mitochondrial outer membrane conductance which in turn may enhance synaptic transmission (Jonas et al., 2003; Chen et al., 2011). Bcl-xL also potentiates ATP production in neurons in the resting and active state, which appears to result from a more efficient use of oxygen in ATP production and effects on the F_1F_0 ATPase (Alavian et al., 2011). Whether these effects are independent of the canonical role of Bcl-xL in inhibiting apoptosis is unclear. Bcl-xL as well as several other members of the Bcl-2 family are linked to the control of intracellular calcium homeostasis (Hetz and Glimcher, 2008).

A seizure phenotype was also found in Bcl-w-deficient mice. Although the mechanism is not understood, electrophysiology and binding studies showed that Bcl-w can augment GABA-evoked currents in neurons (Murphy et al., 2007).

These emerging, non-cannonical functions of Bcl-2 family proteins in the brain are an exciting focus of research that may yield mechanisms relevant to epileptogenesis independent of their traditional roles in controlling cell death. Modulating certain Bcl-2 family proteins that have direct effects on excitability is an alternative tactic that may complement the neuroprotective therapeutic opportunities of targeting this gene family.

SUMMARY AND FUTURE DIRECTIONS

Neuronal loss is a potential consequence of prolonged or repeated brief seizures and may contribute to epileptogenesis. The availability of Bcl-2 family knockout mice has enabled epilepsy researchers to learn about the functional contributions of this gene family to seizure-induced neuronal death. Eight members of this family have been assessed *in vivo* using genetic models, representing one of the most comprehensive analyses of any single gene family in the epilepsy field. The studies have significantly expanded our understanding of the influence of apoptosis-associated signaling pathways in epileptogenesis, revealing effects on convulsant thresholds and neuronal death. For two members, altered spontaneous seizures were also reported in mice lacking a Bcl-2 family member supporting neuronal death controlled by these genes as having direct or indirect effects on functional inhibition in the brain.

What are some of the next challenges? Studies are needed to test network function and explore the underlying direct and indirect mechanisms by which modulation of Bcl-2 family proteins influences cell death and excitability. Bcl-2 family proteins have important non-cell death functions in neurons, some of which are directly relevant to the excitability of the brain and thus may be novel treatment targets in epilepsy. We know relatively little about which hippocampal cell type(s) make these proteins. There is an assumption that all effects are neuronally-mediated but this needs to be established. All work to date has used knockouts and there is a need to investigate whether transgenic overexpression of an anti-apoptotic Bcl-2 family protein such as Mcl-1 can reduce seizure damage and/or have an anti-epileptogenic effect. There remain important members of the family for which we have little or no data on their influence on seizures and damage in a genetic model *in vivo*, including Bcl-xL. The possibility that some Bcl-2 family proteins can compensate for others could be tested using double (or even triple) knockout mice, which are now available (Ren et al., 2010). This may be particularly apt for Bid where the absence of a damage phenotype in the knockout was unexpected. Another key experiment is to prove that seizure-induced cell death is really Bax-dependent *in vivo*. Bax-deficient mice display brain abnormalities that are presumably a result of deficiencies in developmental cell death (Moran et al., 2013), necessitating the use of conditional Bax knockout mice. Conditional knockouts or transgenic mice would also provide a means to test whether the pro/anti-convulsive seizure thresholds in certain Bcl-2 family knockouts (see **Table 1**) are simply an effect of the loss of the gene during brain development or instead represent an effect relating to neurophysiologic functions of the protein in the adult brain. Finally, can the knowledge be applied therapeutically? We know the transcriptional and post-transcriptional control mechanisms for many of the Bcl-2 family proteins and these provide potential drug targets. The availability of BH3 mimetics, developed for use in cancer, offer tools to probe the contribution of Bcl-2 family proteins in seizure models but we await small molecules that enhance or mimic the actions of anti-apoptotic members of the Bcl-2 family that might be put to use to protect the brain against injury caused by status epilepticus and, perhaps, epileptogenesis.

ACKNOWLEDGMENTS

The authors would like to thank their funding sources, including supports from grant NS47622 from the National Institutes of Health, RP/2005/24 from the Health Research Board (Ireland), GR076576MA from the Wellcome Trust, and post-doctoral fellowships (to Tobias Engel) from the Irish Research Council and Health Research Board.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 April 2013; accepted: 22 June 2013; published online: 16 July 2013.

Citation: Henshall DC and Engel T (2013) Contribution of apoptosis-associated signaling pathways to epileptogenesis: lessons from Bcl-2 family knockouts. Front. Cell. Neurosci. 7:110. doi: 10.3389/fncel.2013.00110

frontiers in
CELLULAR NEUROSCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 23 November 2012
doi: 10.3389/fncel.2012.00055

Molecular mechanism of circadian rhythmicity of seizures in temporal lobe epilepsy

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The circadian pattern of seizures in people with epilepsy (PWE) was first described two millennia ago. However, these phenomena have not received enough scientific attention, possibly due to the lack of promising hypotheses to address the interaction between seizure generation and a physiological clock. To propose testable hypotheses at the molecular level, interactions between circadian rhythm, especially transcription factors governing clock genes expression, and the mTOR (mammalian target of rapamycin) signaling pathway, the major signaling pathway in epilepsy, will be reviewed. Then, two closely related hypotheses will be proposed: (1) Rhythmic activity of hyperactivated mTOR signaling molecules results in rhythmic increases in neuronal excitability. These rhythmic increases in excitability periodically exceed the seizure threshold, displaying the behavioral seizures. (2) Oscillation of neuronal excitability in SCN modulates the rhythmic excitability in the hippocampus through subiculum via long-range projections. Findings from published results, their implications, and proposals for new experiments will be discussed. These attempts may ignite further discussion on what we still need to learn about the rhythmicity of spontaneous seizures.

Keywords: epilepsy, circadian, rhythmicity, mTOR, CLOCK, hippocampus, SCN, subiculum

INTRODUCTION

Circadian rhythmicity of epileptic seizures was described over 2000 years ago, and modern scientific studies were conducted in the late nineteenth century (Gowers, 1885; Wilson and Reynolds, 1990). In these early studies, approximately two thirds of people with epilepsy (PWE) showed circadian patterns of epileptic episodes (diurnal, nocturnal, and the rest categorized as "diffuse" type—seizures occur randomly without a certain pattern). Diurnal seizures were known to cluster in wakefulness or in the late afternoon, while nocturnal seizures occurred frequently at bedtime and in the early morning before awakening (Langdon-Down and Brain, 1929; Gfiffiths and Fox, 1938). This circadian pattern of seizures in PWE tends to be well preserved. In sleep-related studies, non-rapid eye movement is typically associated with the increase in epileptiform discharges and seizures in nocturnal cases (Shouse et al., 2000). Circadian rhythm has a tremendous influence on sleep (and vice versa) and the relationship between epilepsy and the sleep-wake cycle has been actively studied (Bazil and Walczak, 1997; Matos et al., 2011; Zarowski et al., 2011). Therefore, this article will focus mainly on the relationship between intrinsic circadian rhythm and epilepsy.

Abbreviations: 4E-BP1, eukaryotic initiation factor 4 binding protein 1; AMPK, AMP protein kinase; CCG, clock controlled gene; CK1/2, Casein Kinase1/2; DBP, albumin D-site-binding protein; EEG, eletroencephalography; EPSP, excitatory postsynaptic potential; GABA, gamma-aminobutyric acid; GFAP, glial fibrillary acidic protein; IRS, insulin receptor substrate; LTP, long term potentiation; mTOR, mammalian target of rapamycin; NSE, neuron specific enolase; PI3K, phosphoinositide 3-kinase; STAT3, signal transducer and activator of transcription-3.

CIRCADIAN RHYTHMS

Circadian rhythms are endogenously controlled 24 h (approximately) cycles of behavioral and physiological processes such as sleep-wake cycle, hormonal production (e.g., cortisol, glucocorticoid, and melatonin), and regulation of body temperature and blood pressure (Hastings et al., 2007; Albrecht, 2012). The circadian clock of most organisms is controlled by both photic (light–dark cycle of the environment) and non-photic (such as daily feeding or behavioral activities) stimuli (Reebs and Mrosovsky, 1989; Rosenwasser and Dwyer, 2001; van Oosterhout et al., 2012).

MOLECULAR MECHANISM OF CIRCADIAN GENE REGULATION

Maintenance of the circadian clock involves coordinated feedback regulation of transcription and translation of CLOCK genes to achieve the oscillatory levels of activators and repressors (**Figure 1**; for the review, see Albrecht, 2012; Zheng and Sehgal, 2012). In a primary loop, CLOCK and BMAL1 (also known as ARNTL) form a large complex in the cytoplasm and translocate to the nucleus after being phosphorylated by protein kinases (e.g., CK1ε/δ) to activate the transcription of PERIOD (PER1, PER2, and PER3) and CRYPTOCHROME (CRY1 and CRY2) genes. The PER-CRY complex then subsequently binds to CLOCK-BMAL1 complex to repress their transcriptions. PER and CRY are degraded through the ubiquitin-proteosomal pathway (e.g., FBXL3-dependent) and this whole process takes about a 24 h cycle. An additional feedback loop is at work with nuclear hormone receptors such as ROR (RORa, RORb, and RORc) and REV-ERBα/β to modulate the expression of clock-controlled genes

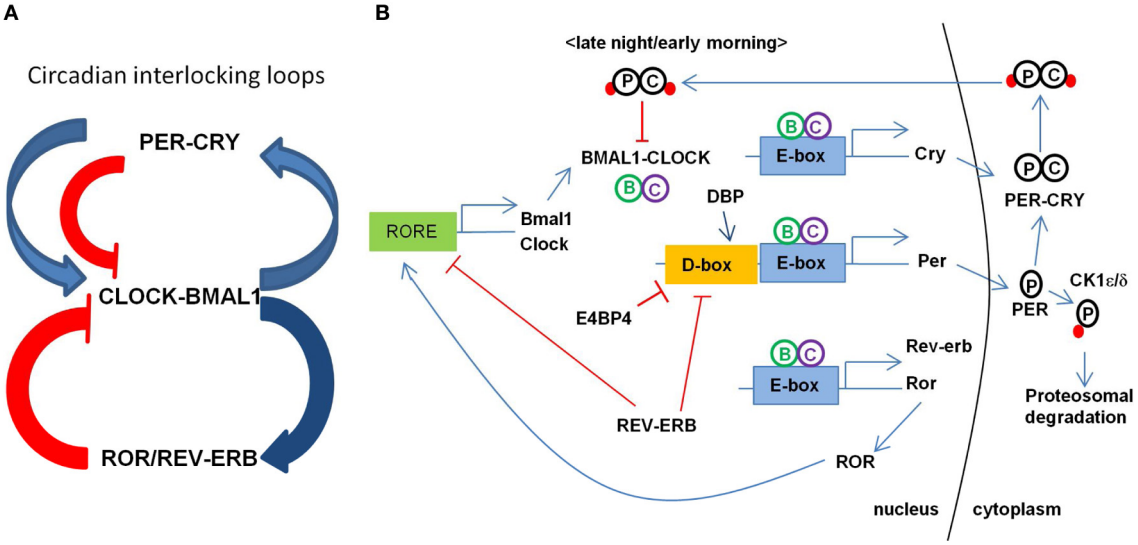

FIGURE 1 | Transcriptional regulation circuit of clock genes in mammals. (A) Circadian interlocking loops show that a primary loop of CLOCK-BMAL1 and PER-CRY complexes and an additional feedback loop of ROR/REV-ERB, conferring a tight transcriptional regulation. The blue arrows indicate the transcriptional activation and the red lines indicate the inhibiting activity of the targets. ROR as an activator and REV-ERB as a repressor regulate the expression of BMAL1. **(B)** Transcription of BMAL1 and CLOCK is regulated by ROR and REV-ERB through binding RORE elements at their promoters. CLOCK and BMAL1 activates the expression of CRY, PER, REV-ERB, ROR, and other CCGs (clock controlled genes) through binding to E-box element at their promoters. CRY-PER complex is phosphorylated and transported back to the nucleus inhibiting the CLOCK-BMAL1 activity. PER is phosphorylated to degrade through proteosomal pathway via CK1ε/δ.

(CCGs) and clock-modulated genes. Several circadian modulators such as DEC1/2 (also called BHLHE40/41), DBP, and E4BP4 (also called NFIL3) provide the additional level of circadian regulation. In the promoter region of core CLOCK genes and CCGs, E-box elements are recognized by BMAL1-CLOCK, D-box elements by DBP-E4BP4, and REV-ERBα/ROR-regulatory elements (RORE) by ROR. CLOCK, BMAL1, and PER1 are acetylated in response to the environmental stimuli to adjust the activity of core clock proteins. Changes or disruption in this multi-step regulation influences the 24-h period by shortening or lengthening it.

CIRCADIAN REGULATION OF ION CHANNELS AND MEMBRANE EXCITABILITY

Neurotransmitter receptors and ion channels have been shown to have rhythmic expression and activity under circadian regulation (Kafka et al., 1986; Ko et al., 2009). Radioactive ligand binding assay of several neurotransmitter receptors in rat brains showed that the cortex has the highest variation and that the cerebellum has the lowest. Hippocampus has circadian patterns of ligand binding activities of α1 adrenergic and benzodiazepine receptors (Kafka et al., 1986). Although the studies have been limited mostly to the visual system (photoreceptors, retinal neurons, and suprachiasmatic nucleus), cGMP-gated ion channel, T- and L-type Ca channels, and voltage-gated K channels have been shown to be under circadian control (Ko et al., 2009). Clock gene products are involved in rhythmicity of membrane excitability and electrical activities mostly due to changes in potassium conductance (Belle et al., 2009; Itri et al., 2010). The expression of pyridoxal kinase, an enzyme involved in metabolism of pyridoxal phosphate and neurotransmitters (e.g., serotonin and dopamine), has shown to be regulated by circadian PAR bZIP transcription factors (Gachon et al., 2004). Thus, circadian rhythm modulates neuronal excitability at the multiple levels, may trigger the hyperexcitability out of delicate control.

EPILEPSY

Neuronal excitability is homeostatically controlled between excitatory and inhibitory drives in the nervous system. Hyperexcitability, caused by the disruption of this delicate balance at the microcircuit level, may trigger the excessively synchronized electrical discharges of neurons in the brain which can manifest as epileptic seizures (Bertram, 2008). As a global health issue, epilepsy affects ∼1% of the general population (World Health Organization, 2005). Temporal lobe epilepsy (TLE), especially, is often pharmacologically refractory and is the most common type of acquired epilepsy that involves the hippocampus, entorhinal cortex, and amygdala (Bertram, 2008).

CIRCADIAN PATTERN OF EPILEPTIC SEIZURES IN HUMAN AND ANIMAL MODELS

The circadian pattern of seizures tends to be well preserved over the years in individuals and some PWE experience the episodes at the certain time of the day. However, the majority of one type over the other (nocturnal vs. diurnal) in the epileptic population is not always consistent in the literature (Méndez and Radtke, 2001). This may be the result of heterogeneity between the cohorts recruited for each study. For instance, Gowers and Patry described independently that seizures are more frequent during the daytime than the night among the PWE they have observed (Gowers, 1885; Patry, 1931). On the contrary, Janz and

Hopkins independently found that nocturnal seizures are more prevalent than the diurnal ones (Hopkins, 1933; Janz, 1962). It is not straightforward to compare their findings because PWE were not classified based on seizure type, age, or other possibly important factors (e.g., comorbidity) of PWE in individual study. Additional studies, possibly collaborations at multiple epilepsy clinics, with a standardized protocol to recruit PWE, a clear classification of epilepsy/seizure types, and continuous monitoring and data analysis, are needed in order to provide a better picture of the phenomena.

In studies with a small cohort, epileptic seizures with circadian rhythmicity seem to be dependent on the origin and type of seizures (Hofstra et al., 2009a,b; Zarowski et al., 2011). For example, de Weerd and colleagues used the video-EEG monitoring to describe that complex partial and temporal seizures in adults have the peak activity during 11:00–17:00 h period, and parietal seizures occurs more frequently during 17:00–23:00 period. In addition, frontal seizures showed the age-specific peak activities during 23:00–5:00 period in adults and 17:00–23:00 in children (Hofstra et al., 2009a,b). Children with generalized seizures showed that tonic and tonic-clonic seizures were more frequently observed during sleep, whereas clonic, absence, atonic, and myoclonic types of seizures have various peak times in wakefulness (Zarowski et al., 2011).

Animal models of epilepsy also display circadian patterns of seizures (Fenoglio-Simeone et al., 2009; Tchekalarova et al., 2010; Matzen et al., 2012). Chronically epileptic KCNA1 null mice have peak seizure occurrence early in the morning (at Zeitgeber 2.3), and seizure occurrence and rest-activity rhythm are inversely correlated. KCNA1 null mice have a longer circadian period than wild-type mice, and they are either phase-delayed or -advanced (Fenoglio-Simeone et al., 2009). A kainate rat model of TLE showed the higher seizure prevalence during the day and those placed in constant darkness (light-deprived) displayed spontaneous seizures that still followed a circadian pattern, suggesting that there is an endogenously mediated circadian pattern (Quigg, 2000; Tchekalarova et al., 2010). This diurnal tendency has been also found in several different epilepsy models (Quigg et al., 1998; Arida et al., 1999; Hellier and Dudek, 1999; Nissinen et al., 2000; Stewart and Leung, 2003; Raedt et al., 2009). Human and rodent models of TLE showed higher seizure prevalence during the day regardless of the species difference in the sleep-wake cycle. No direct association has been established between abnormalities (e.g., mutation) of major CLOCK gene products and epilepsy.

CHRONOTHERAPY FOR EPILEPSY

Circadian influence on the dynamics and kinetics of medications in individuals is important in drug efficacy, and it needs to be monitored for improved treatment (Ohdo et al., 2010; Paschos et al., 2010). Differential dosing of medication for patients with cancer, asthma, hypertension, or diabetes based on individuals' circadian patterns have been shown effective (Lévi et al., 2010; Gimble et al., 2011; Hermida et al., 2011). Differential dosing of anticonvulsants to relieve the seizure has been reported to be more effective when the timing of drug intake is adjusted to the day-night shift (Yegnanarayan et al., 2006; Guilhoto et al., 2011).

THE mTOR PATHWAY IN EPILEPSY AND CIRCADIAN REGULATION

The mTOR signaling pathway play major roles in regulating gene transcription and protein translation and it has been deeply involved in several physiological and pathological conditions (Laplante and Sabatini, 2012). This pathway has also been recognized as a major signaling pathway in acquired epilepsies as well as a few mutation-based epilepsies (see Cho, 2011 for the detail). Rapamycin, an mTORC1 kinase inhibitor, blocks epileptogenesis and reduces the seizure frequency in the pilocarpine/kainate-injected rats when repeatedly administrated (Buckmaster et al., 2009; Zeng et al., 2009; Huang et al., 2010). Rapamycin also suppresses axonal sprouting of somatostatin-positive interneurons in the dentate/hilus (Buckmaster and Wen, 2011). A study shows that the sclerotic hippocampi of human specimen with refractory TLE, as well as kainate mouse model, have over-activated mTOR markers in reactive astrocytes (Sha et al., 2012; Sosunov et al., 2012).

Relatively high levels of basal mTOR activity have been reported in SCN. Its maximal activity occurs during the subjective day and minimal activity during the late subjective night (Cao and Obrietan, 2010; Cao et al., 2010). Phosphorylated (activated) S6, a ribosomal protein important in protein synthesis and a downstream target of mTORC1, oscillates synchronously with PER1 expression, and photic stimulation elicits a coordinate upregulation of PER1 and mTOR activation in SCN (Cao et al., 2010). Interestingly, some of the key molecules in the mTOR pathway have been shown to be regulated in circadian manner (Zhang et al., 2009). By genome-wide RNAi screening in a model cell line, 17 gene products have been identified as strong circadian clock modifiers in period length and amplitude. These proteins showed a "network effect"—leading to dynamic changes in protein-protein interaction, phosphorylation, trans-activation, or trans-repression when affected. An insulin signaling pathway (mTOR-dependent) has been shown to regulate the circadian clock (Zhang et al., 2009).

In addition, by genetically manipulating signaling molecules in *Drosophila in vivo*, PTEN-AKT-Rheb-TOR-S6K pathway has been shown to affect the circadian period (Zheng and Sehgal, 2010). SGG (*Drosophila* homolog of GSK3β) is phosphorylated by AKT and S6K1 and it phosphorylates TIMELESS (*Drosophila* homolog of CRYPTOCHROME), modulating its nuclear translocation with PERIOD (**Figure 2**; Martinek et al., 2001; Papadopoulou et al., 2004; Zhang et al., 2006). GSK3β may also modulate CLOCK, *BMAL1*, and REV-ERBα (Yin et al., 2006; Spengler et al., 2009; Sahar et al., 2010). Conditional knockout PTEN mice driven by the NSE-Cre promoter have a lengthened period (Ogawa et al., 2007). PI3K and mTOR are periodic and cyclic, and IRS and 4EBP1 are cyclic (Zhang et al., 2009). High-fat diet lengthened the locomotor activity rhythm and modulated CLOCK genes at the molecular level in mice (Kohsaka et al., 2007).

The catalytic subunits (α1 and α2) of AMP protein kinase (AMPK), which is an upstream regulator of mTOR kinase, regulate circadian rhythms (Um et al., 2011). AMPK phosphorylates and modulates the activity of CRYPTOCHROME (Lamia et al., 2009). Ketogenic diet (KD), a strict dietary plan to reduce the

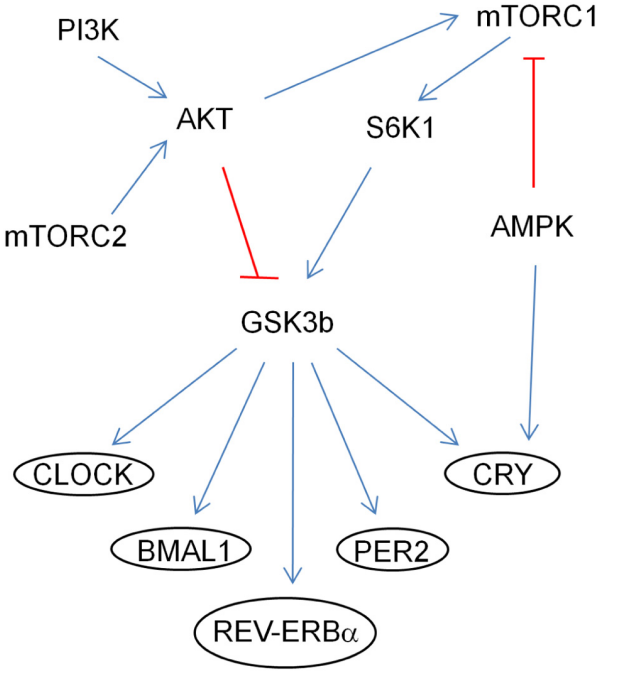

FIGURE 2 | Regulation of CLOCK proteins by the mTOR pathway through GSK3β. The arrows (in blue) indicate activation of the targets and the ones (in red) indicate inhibition by phosphorylation.

frequency and severity of seizure episodes in some population of epileptic patients, has been shown to be mTOR-dependent (McDaniel et al., 2011). In epileptic KCNA1 null mice, KD reduces frequency and periodicity of seizures, and it also improves diurnal rhythmicity (Fenoglio-Simeone et al., 2009). Since KD works through mTOR pathway, it will be interesting to see if mTOR inhibitors will have a beneficial effect on this mouse model. Therefore, it is a plausible that the circadian rhythmicity of seizure episode is mediated by the fluctuation in activity of the mTOR signaling molecules. However, there is no direct evidence so far to support this hypothesis. Examining the circadian pattern of activity and expression of mTOR signaling molecules in epilepsy models, and studying the behavioral rhythm of null mice of those molecules will be valuable.

TRANSCRIPTION FACTORS GOVERNING THE CIRCADIAN CLOCK AS MOLECULAR LINKS TO EPILEPSY

There are over 2000 CCGs reported in mammals from the public microarray studies, and more than 20 transcription factors are found or suggested to be important in circadian expression patterns of CCGs via a large scale promoter analysis (Bozek et al., 2009). There are several findings to suggest that these transcription factors have been involved in epilepsy.

A GC-rich motif, EGR is significantly overrepresented in the promoter region of CCGs (Bozek et al., 2009). Increased levels of EGR-1 and EGR-2, which bind to the EGR element, have been reported in the neocortex of epileptic patients (Rakhade et al., 2005). AP1, a promising circadian regulator, has the high level in cerebral cortex and hippocampus of epileptic E1 mice (Yoneda et al., 1993). STAT3, which regulates the expression of GABAα1 receptor subunit, has been shown to be activated (phosphorylated) in GFAP-positive astrocytes in the hippocampus in pilocarpine-induced model (Lund et al., 2008; Xu et al., 2011). SP1 has a long-lasting increased activity in kainate-induced epilepsy model, and neonatal epilepsy-associated KCNQ2 and KCNQ3 genes are activated by SP1 (Feng et al., 1999; Mucha et al., 2010). DBP has the increased level in cerebrospinal fluid has been found in TLE patients and its overepxression in mice increased the seizure susceptibility (Klugmann et al., 2006; Xiao et al., 2009). In contrast, triple knockout mice of circadian PAR bZIP transcription factors (DBP, HLF, and TEF) exhibit epileptic symptoms (Gachon et al., 2004).

XBP1 (X-box-binding proteins 1), a basic leucine zipper family transcription factor, is recently identified as one of light-inducible genes in chicken pineal gland, and its spliced form has circadian pattern of gene expression (Hatori et al., 2011). The splicing and expression of XBP1 is increased when the mTOR pathway is activated, affecting XBP1-targeted genes (Pfaffenbach et al., 2010). Its increased expression and activation has been shown in hippocampi of epileptic patients (Liu et al., 2011). SREBP1 (Sterol regulatory element binding protein) is a transcription factor controlling expression of genes involved in lipid and cholesterol biosynthesis (Laplante and Sabatini, 2009; Porstmann et al., 2009). The mTORC1 phosphorylates SREBP1 to upregulate the expression of its target genes (Porstmann et al., 2008). Its expression follows the circadian pattern as it is XBP1 and mTOR-dependent (Hatori et al., 2011). One of SREBP1 downstream targets, stearoyl-CoA desaturase 1 has been shown to be upregulated in human cortical specimen of TLE (Arion et al., 2006).

In addition to the proteins that were mentioned above, Oligophrenin-1, PAM, and the $GABA_A$ receptor β2 subunit are linked to epilepsy and circadian behavior (Tentler et al., 1999; Bergmann et al., 2003; Arion et al., 2006; Yin et al., 2010). Specifically, Oligophrenin-1 interacts with Rev-erbα, a nuclear receptor involved in regulation of the circadian clock, and regulates the oscillatory expression of a clock gene BMAL1 in the hippocampus (Valnegri et al., 2011). Therefore, abnormal activity of these transcription factors controlling circadian rhythm is also deeply involved in epilepsy. Several questions remain. Is the increased expression of these transcription factors sufficient to lower the seizure threshold and/or make the neurons hyperexcitable? Will reducing these factors in the epileptic animals (e.g., using siRNA technique) decrease the seizure frequency or even change the circadian pattern of seizures? Are the expression and/or activation of these factors mTOR pathway dependent? Will the altered activity of these factors be reversed when the rapamycin or anticonvulsants are administered?

FUNCTIONAL CONNECTION BETWEEN HIPPOCAMPUS AND SCN

In the hippocampus, the activity (and/or the expression level) of several memory-related proteins has been shown to oscillate in the circadian manner (e.g., adenylyl cyclases, ERK/MAPK, Ras, MEK, and CREB) (Eckel-Mahan et al., 2008). LTP, field EPSP slope, and population spike in the dentate are greater during the dark phase than the light phase when medial perforant path

was stimulated (Harris and Teyler, 1983; Bowden et al., 2011). PER2 is highly expressed in pyramidal cell layers in the hippocampus and its expression fluctuate in circadian manner. Expression of PER2 in the hippocampus is out-of-phase with that in SCN, and PER2 null mice showed abnormal LTP (**Figure 3A**; Wang et al., 2009). Circadian patterns of expression of CLOCK in the DG and BMAL1 in CA1 and CA3 have been reported in the mouse hippocampus (Wyse and Coogan, 2010). The findings of oscillation of PER1 in the hippocampus are not consistent. The expression of PER1, high in the DG of hippocampus, has not shown to oscillate (Yamamoto et al., 2001; Abe et al., 2002). However, PER1 and BMAL1 in the hippocampus have been shown to oscillate depending on SCN (Wang et al., 2009; Jilg et al., 2010). Interestingly, PER1 has been reported to be upregulated in the mouse hippocampus and cerebral cortex by electroconvulsive shock or kainate injection (Eun et al., 2011). In electrically induced rat model of chronic epilepsy, the excitability of DG shows two distinct phases (high and low) of seizures (Matzen et al., 2012). In the same model, hippocampal CA1 region during latent period of epileptogenesis shows a phase shift between two types of population spikes which follow the circadian rhythm (Talathi et al., 2009).

Suprachiasmatic nucleus (SCN) in the hypothalamus is the central circadian pacemaker to coordinate and synchronize local clocks throughout the body (Welsh et al., 2010). SCN receives direct inputs from tens of different regions, and projects to more than dozen regions which includes three major afferent connections—retinohypothalamic and geniculohypothalamic projections, and median raphe serotonertic pathway (for the detail, see Morin, 2012). A direct neural pathway from the hippocampus to SCN is known, however, the SCN output to hippocampus is still unclear (Krout et al., 2002). It has been reported that the indirect pathways through multiple synaptic connections and hormonal influence (e.g., hypocretin and melatonin) onto the hippocampus confer the circadian rhythmicity (Monnet, 2002; Perreau-Lenz et al., 2003). Subiculum to SCN connection has been reported, and long-range GABAergic projections may be able to synchronize the oscillatory activity between these two areas (Meibach and Siegel, 1977; Canteras and Swanson, 1992; Jinno et al., 2007). As shown in paraventricular nucleus, the strength of the GABAergic input from the SCN to subiculum can be rhythmic (Kalsbeek et al., 2008). Either strong excitatory input or weak inhibitory input from SCN to subiculum/hippocampus, with or without synchronization to the rhythmic excitability of the hippocampus, may overcome the seizure threshold (**Figure 3B**). Interestingly, hippocampus-dependent spatial and contextual fear memories were compromised when the SCN is lesioned after training

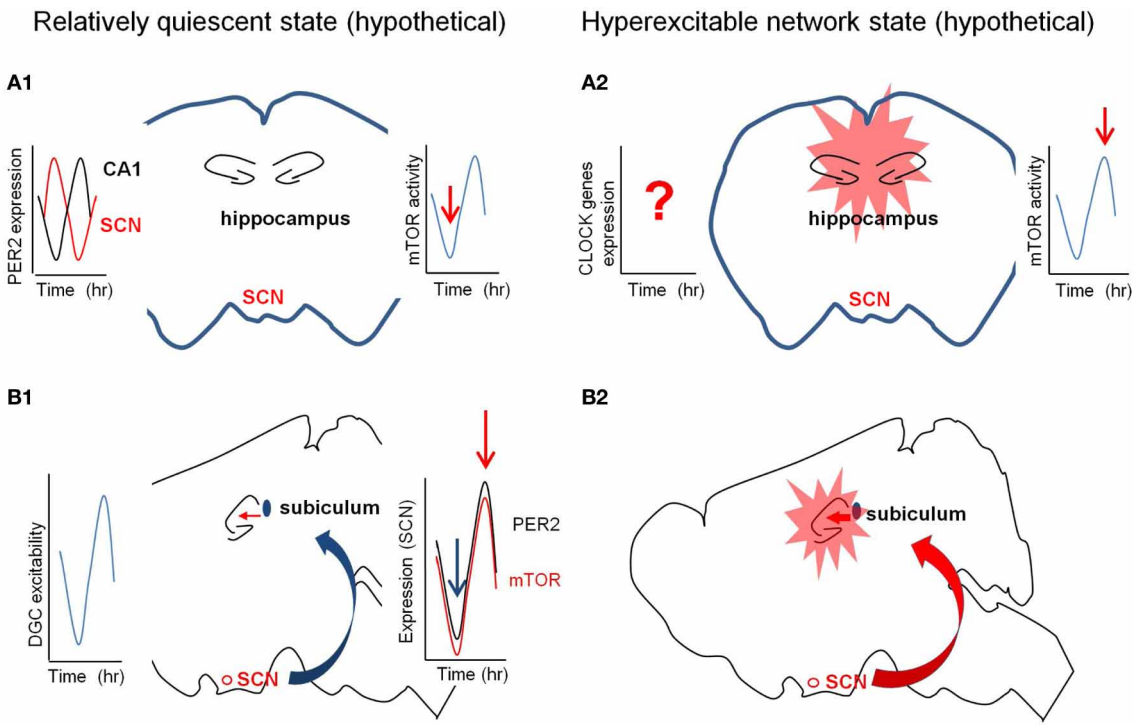

FIGURE 3 | Hypothetical diagrams of two different network excitability states. The left panel (in **A1** and **B1**) is in quiescent state and the right (in **A2** and **B2**) is in hyperexcitable state. **(A)** The circadian fluctuation of the expression and/or activity of mTOR signaling molecules in the hippocampus may determine the quiescent or hyperexcitable states of the hippocampus prone to epileptic seizures. The red arrows in the right graphs from the cartoon of the coronal section indicate the hypothetical activation states of mTOR pathway (**A1** and **A2**). The left graph in **(A1)** indicates the out-of-phase rhythmic expression of PER2 in the SCN and CA1. **(B)** The circadian fluctuation of the SCN inputs to hippocampus via subiculum may regulate the excitability states of the hippocampus. The left graph in **(B1)** shows the rhythmic excitability of the dentate gyrus and the right graph in B2 signifies the synchronized rhythmicity of PER2 and mTOR signaling molecules. SCN, suprachiasmatic nucleus.

(Phan et al., 2011). It will be interesting to see if the circadian episode of seizures will be altered when the SCN of the epileptic animals is lesioned. To study this SCN output pathway to hippocampus transgenic mice with fluorescence labeling of identifiable neuronal population (e.g., GAD-GFP mice) and optogenetic approaches (e.g., Channelrhodopsin2 and Halorhodopsin) may be useful (Adamantidis et al., 2010; Kokaia et al., 2012).

INVOLVEMENT OF EPIGENETICS IN CIRCADIAN RHYTHM AND EPILEPSY

Epigenetic regulation should be considered in this type of study because individual organisms show the differential response in both circadian rhythm and epilepsy to environmental stimuli (Bellet and Sassone-Corsi, 2010; Qureshi and Mehler, 2010). It should be noted that the CLOCK protein has a histone acetyltransferease activity (Doi et al., 2006). There are circadian changes in histone acetylation at the promoter of CLOCK genes (Etchegaray et al., 2003). MLL1, a H3K4 methyltransferase, is associates with CLOCK and recruited to promoters of CCGs in a circadian manner, and null mice of SMCX/JARID1c, a H3K4 demethylase, develops epilepsy (Tahiliani et al., 2007; Katada and Sassone-Corsi, 2010; DiTacchio et al., 2011). Even in one type of animal model of epilepsy, epileptic animals may not show the single circadian pattern of epileptic episodes (e.g., diurnal vs. diffuse types). Therefore, there is room to improve or develop better models. Examining the circadian behaviors of existing mutant mice with epileptic seizures to find a suitable model is highly desirable (Yoneda et al., 1993).

CONCLUSION

The phenomena of circadian rhythmicity of spontaneous epileptic seizures are evident in human and animal models although there are inconsistency and studies yet to be done in detail. Findings from the literature regarding the circadian regulation and epilepsy were reviewed to formulate the rationale for its molecular mechanism. As one may notice there is no strong evidence to support some premises for the hypothesis proposed here, and there are many more questions than answers on the subject of this article. By testing the hypotheses proposed here; (1) fluctuating activity of activated mTOR signaling molecules and their targets increase the neuronal excitability in the epileptic brain, raising beyond the seizure threshold to display the behavioral seizures. (2) The rhythmic input strength from SCN to hippocampus contributes to synchronizing hyperexcitability which manifests with the epileptic seizures. By addressing this question, hopefully we can have the opportunity to address another mysterious side of epilepsy.

ACKNOWLEDGMENTS

I greatly appreciate the comments of Drs. M. McCartney and J. Jackson during the preparation of this manuscript. This article was supported by the NIH grant NINDS RO1 NS38572.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 August 2012; accepted: 05 November 2012; published online: 23 November 2012.

Citation: Cho C-H (2012) Molecular mechanism of circadian rhythmicity of seizures in temporal lobe epilepsy. Front. Cell. Neurosci. ***6****:55. doi: 10.3389/fncel.2012.00055*

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 18 June 2013
doi: 10.3389/fncel.2013.00089

Neural circuit mechanisms of post-traumatic epilepsy

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Traumatic brain injury (TBI) greatly increases the risk for a number of mental health problems and is one of the most common causes of medically intractable epilepsy in humans. Several models of TBI have been developed to investigate the relationship between trauma, seizures, and epilepsy-related changes in neural circuit function. These studies have shown that the brain initiates immediate neuronal and glial responses following an injury, usually leading to significant cell loss in areas of the injured brain. Over time, long-term changes in the organization of neural circuits, particularly in neocortex and hippocampus, lead to an imbalance between excitatory and inhibitory neurotransmission and increased risk for spontaneous seizures. These include alterations to inhibitory interneurons and formation of new, excessive recurrent excitatory synaptic connectivity. Here, we review *in vivo* models of TBI as well as key cellular mechanisms of synaptic reorganization associated with post-traumatic epilepsy (PTE). The potential role of inflammation and increased blood–brain barrier permeability in the pathophysiology of PTE is also discussed. A better understanding of mechanisms that promote the generation of epileptic activity versus those that promote compensatory brain repair and functional recovery should aid development of successful new therapies for PTE.

Keywords: epilepsy, epileptogenesis, neuroinflammation, pilocarpine, seizures, synapse, traumatic brain injury

INTRODUCTION

Post-traumatic epilepsy (PTE) is a common long-term consequence of traumatic brain injury (TBI), for which there are few effective therapies. More than one million people are presented for medical care each year in the United States after sustaining a head injury (Faul et al., 2010). While the incidence of epilepsy in the general population is about 1–2% (Hauser and Hesdorffer, 1990), the overall incidence of epilepsy is 7–39% after severe closed-head injury and as high as 57% after penetrating injury (Caveness et al., 1979; Salazar et al., 1985; Annegers et al., 1998; Asikainen et al., 1999; Herman, 2002; Englander et al., 2003), and approximately 20% of all acquired epilepsies are the result of TBI (Hauser et al., 1991). Injury severity is generally considered the major determining factor for developing a seizure disorder after brain trauma (Annegers et al., 1998; Herman, 2002; Englander et al., 2003). Focal brain damage and cortical contusions to the frontal, temporal, or parietal lobes are also important risk factor for human PTE (D'Alessandro et al., 1982; Englander et al., 2003). Several other risk factors have been identified, many of which are associated with focal lesions: dural penetration, depressed skull fracture, intraparenchymal hemorrhage, epidural and/or subdural hematomas, reduced brain volume, prolonged impaired consciousness, presence of injury-induced seizures, age (young children and the elderly), and gender (male; Salazar et al., 1985; Annegers et al., 1998; Asikainen et al., 1999; Englander et al., 2003; Frey, 2003). PTE occurs as temporal lobe epilepsy (TLE) in 35–62% of trauma patients (Diaz-Arrastia et al., 2000; Hudak et al., 2004).

Here, we review experimental animal models used in PTE research, including the neurophysiological and structural abnormalities believed to underlie the increased propensity of the injured brain to generate spontaneous seizures. Emphasis is placed on modifications of synaptic networks in the injured dentate gyrus that are associated with post-traumatic epileptogenesis. The hippocampus has long been recognized as an important structure in epilepsy (Lothman et al., 1991). The dentate gyrus is particularly susceptible to injury, often undergoes time-dependent structural reorganization, is a widely used model system for altered synaptic circuitry of cortical structures in epilepsy, and is one of the brain regions that has been best characterized in terms of changes in structure–function relationships after TBI (Dudek and Spitz, 1997; Nadler, 2003; Dudek and Sutula, 2007; Sutula and Dudek, 2007).

ANIMAL MODELS OF PTE

Most information about cellular mechanisms of epileptogenesis has been derived from animal models. Although there is ongoing debate over which experimental paradigms make for appropriate models of PTE (Pitkänen and McIntosh, 2006; Dichter, 2009), the presence of spontaneous burst discharges, preferably in the form of spontaneous seizures *in vivo*, that develop following a sudden, mechanical injury (i.e., TBI) seems to be necessary minimal criteria for a "post-traumatic epilepsy" model. The presence of relatively high numbers of animals in which the behavioral, anatomical, and physiological characteristics of epileptic pathology in humans are reproduced, including short but distinct latencies between initial injury and spontaneous seizure onset, also has obvious advantages. Ideally, a model will effectively reproduce the range of tissue deformation and damage observed in mild, moderate, and severe TBI in a controllable way so that the dual

pathology of cellular and molecular factors associated with injury dynamics and epileptogenesis might be dissociated. Using these selection criteria, many traditional experimental epilepsy models, such as pharmacologically induced status epilepticus, do not qualify as models of PTE, because they do not induce spontaneous seizures by a mechanical insult. This is not to negate these models, because they have provided valuable information about cellular mechanisms of epileptogenesis. Rather, these criteria provide a more restrictive and specific basis for differentiating between epileptogenesis mediated by a mechanically induced lesion versus brain insults by other causes (e.g., pharmacological agents, electrical stimulation, or models of neurodegenerative diseases). It is important to make this distinction, because there are currently few effective treatments for PTE (Temkin, 2009; discussed below). Therefore, it is important to understand how the epileptogenic cellular processes in experimental neurotrauma compare with human PTE and other experimental epilepsy models (e.g., TLE induced by status epilepticus).

FLUID PERCUSSION INJURY

The fluid percussion injury (FPI) model is one of the best characterized in terms of epilepsy development after closed-head TBI (Pitkänen and McIntosh, 2006). In this model, injury is delivered through a craniotomy by rapid fluid injection, accomplished by a hammer swung on a pendulum. The fluid pulse first strikes the intact dura and then moves into the epidural space (Lifshitz, 2009). The height from which the pendulum hammer is dropped determines the pressure of the fluid pulse transmitted through a fluid-filled cylinder. This pressure can be used to estimate injury severity. Injury severities that have been investigated with regard to long-term changes in excitability can be categorized into two groups: severe injuries, with fluid pressure typically >3 atm; and moderate injuries, with impact forces of 2–2.2 atm. Craniotomies can be applied to the midline to produce more diffuse injuries or laterally to produce mixed focal and diffuse injury (Lifshitz, 2009).

Several studies have reported electrographic seizures in rats and more recently in mice after *severe* FPI (D'Ambrosio et al., 2004, 2005, 2009; Kharatishvili et al., 2006; Bolkvadze and Pitkänen, 2012). Susceptibility to pharmacologically induced seizures is also increased after more moderate injuries, but clear evidence for spontaneous seizures after *moderate* FPI has not been demonstrated (Kharatishvili et al., 2007; Echegoyen et al., 2009; Gurkoff et al., 2009). After severe lateral FPI, spontaneous seizures are present in up to 50% of rats (Kharatishvili et al., 2006) and 3% of mice (Bolkvadze and Pitkänen, 2012) by 12 months post-injury. Depth-electrode recordings have inferred that spontaneous electrographic seizures involve hippocampal structures, and these electrographic events are accompanied by obvious behavioral abnormalities defined by a widely used Racine rating scale for rodent seizures (Racine, 1979; Kharatishvili et al., 2006). However, seizure frequencies are typically quite low and time to first seizure is long in these animals, making it somewhat difficult to definitively attribute cellular mechanisms that occur following an injury to epileptogenesis.

In addition to lateral FPI, rats injured by rostral parasagittal FPI develop seizure-like epileptiform activity and seizures that are accompanied by more subtle changes in behavior (e.g., behavioral arrest), but do not generally develop tonic–clonic convulsive seizures (D'Ambrosio et al., 2004, 2005, 2009). Similar findings were also reported for injuries to parietal and occipital cortices, but fewer animals developed spontaneous epileptiform activity (Curia et al., 2011). Originally, behavioral seizures in this model were examined based on the traditionally used Racine rating scale (Racine, 1979; D'Ambrosio et al., 2004), but later studies developed a new seizure classification scale to describe the subtle behavioral abnormalities associated with electrographic activity in this model (D'Ambrosio et al., 2005, 2009). The authors reasoned that post-traumatic seizures after rostral parasagittal FPI did not fit well with the Racine scale and proposed that electrographic abnormalities with $\geq$2-s duration represent ictal events (D'Ambrosio et al., 2009). These "epileptiform" events were also observed in nearly 40% of sham-control rats by 21 weeks post-injury (D'Ambrosio et al., 2005). It is unclear why control rats also sometimes have epileptiform activity, but this may be due to how a seizure is defined (D'Ambrosio et al., 2009). Thus, it appears that the majority of electrographic abnormalities in rats injured by rostral parasagittal FPI represent relatively brief events that are associated with behavioral inactivity or crouching, compared to robust convulsive seizures observed after lateral FPI, but they may not have been considered "seizure" activity in other PTE studies (Kharatishvili et al., 2006, 2007; Hunt et al., 2009, 2010; Statler et al., 2009). Future studies that combine long-term EEG monitoring with electromyogram (EMG) and electro-oculogram (EOG) may be useful to better distinguish ictal activity from interictal events or benign variants of normal electrographic patterns that can morphologically reflect epileptiform activity but are not epileptic (Santoshkumar et al., 2009).

WEIGHT DROP INJURY

The weight drop model, also referred to as an impact-acceleration injury, has been examined as a model of closed-head post-traumatic hyper-excitability (Golarai et al., 2001). Trauma is delivered to the neocortex by dropping a large blunt weight through a tube to impact the skull. Injury severity is managed by adjusting the height at which the weight is dropped (Marmarou et al., 2009). This injury produces large and extensive damage to cortical and subcortical structures, including the dentate gyrus and hippocampus in rats (Golarai et al., 2001). Seizures have not been reported in this model. However, increased seizure susceptibility to pentylenetetrazol (PTZ) is observed 15 weeks after injury (Golarai et al., 2001). The lack of demonstrated spontaneous seizures after weight drop is an obvious limiting factor in using this injury to model PTE. Impact is delivered to the intact skull, not directly to the brain via craniotomy as in FPI. This is sometimes considered a limitation of the model, due to increased risk for skull fracture; and injury dynamics after weight drop can depend somewhat on skull thickness (Marmarou et al., 2009). On the other hand, this characteristic might better reflect the range of variability expected in human closed-head TBI, which is unlikely to occur by craniotomy. However, by using gravitational forces to produce head injury, there can also be a risk for secondary "rebound" injury. Weight drop injury can also be difficult to perform on mice, limiting

the use of transgenic animals and genetic manipulation with this model (Marmarou et al., 2009).

CONTROLLED CORTICAL IMPACT INJURY

Controlled cortical impact (CCI) injury is a widely used experimental model of closed-head injury that was recently identified as a model of injury-induced epilepsy (Hunt et al., 2009). First developed by Lighthall (1988), this model often utilizes an electronically controlled pneumatic impactor to apply a focal contusion injury to the brain surface through a craniotomy (Dixon et al., 1991; Scheff et al., 1997; Hall et al., 2005, 2008; Saatman et al., 2006; Dixon and Kline, 2009). Injury severity is primarily managed by adjusting the depth of tissue compression, but other external injury parameters can also be controlled with precision (i.e., impact depth and velocity, impactor shape and size, and number of craniotomies). This model has unique advantages over weight drop and FPI, because it allows for good control over biomechanical parameters. This allows for a relatively consistent and reproducible focal injury with minimized risk for inaccuracy or secondary "rebound" injury. In addition, the CCI device can be scaled for use in rodents or even large animals, such as sheep or non-human primates.

Several laboratories have independently demonstrated the presence of spontaneous seizures in mice or rats after CCI. Early seizures, within 24 h of injury, have been reported in rats (Nilsson et al., 2004; Kochanek et al., 2006) and mice (Hunt et al., 2009). Within weeks after CCI, spontaneous convulsive seizures are present in up to 40% of mice after severe injuries (Hunt et al., 2009, 2010) and 9–20% of mice after more moderate injuries (Hunt et al., 2009, 2010; Bolkvadze and Pitkänen, 2012). CCI injury has also been administered to immature rats, and a percentage of these animals develop spontaneous electrographic seizures (Statler et al., 2009). Seizures after CCI are similar to spontaneous behavioral and electrographic seizures that have been described in rats after lateral FPI (Kharatishvili et al., 2006) and in models of TLE (Racine, 1979; Cronin and Dudek, 1988; Sloviter, 1992; Buckmaster and Dudek, 1997a; Patrylo and Dudek, 1998; Williams et al., 2009; Wuarin and Dudek, 2001; Shibley and Smith, 2002). Although seizure frequency after CCI injury appears considerably lower than in widely utilized chemoconvulsant TLE models, spontaneous seizures after CCI injury occur with roughly similar onset latency as pharmacologically induced TLE (Williams et al., 2009 ; Shibley and Smith, 2002), which appears to be considerably shorter than the seizure onset latency after severe lateral FPI in rats (Kharatishvili et al., 2006). For example, previous studies have suggested that limbic involvement typically does not evolve in rats until several months after FPI (D'Ambrosio et al., 2004, 2005; Kharatishvili et al., 2006), but many mice after CCI injury develop seizures and hippocampal pathology by 8 weeks post-injury.

NEOCORTICAL UNDERCUT

This model of penetrating TBI involves partial isolation of neocortical circuits by surgical undercut to reproduce the deafferentation and white matter damage caused by neocortical TBI (Burns, 1951; Prince and Tseng, 1993). *In vivo* electrographic epileptiform activity develops in intact regions of the neocortex within hours after injury in cats (Topolnik et al., 2003), but clear evidence for spontaneous electrographic seizures has not been demonstrated.

BLAST INJURY

Recently, a new model of penetrating, ballistic-like TBI in rats was developed to more closely recapitulate aspects of war-time TBI (Lu et al., 2011). Injury is delivered to frontal cortex using a computer-controlled hydraulic pressure generator to rapidly inflate and deflate an elastic water balloon on a probe, which creates a cortical cavity in the brain. Injury severity can be managed by amount of inflation. In this model, up to 70% of animals develop seizures 72 h after injury, and the frequency and the duration of epileptic events can be scaled according to injury severity. However, it is unknown whether rats injured by ballistic-like TBI develop long-term spontaneous seizures.

BASIC CELLULAR MECHANISMS OF NEUROTRAUMA

The term "epileptogenesis" refers to a transformation process by which the normal brain develops an increased propensity for generating spontaneous seizures (Lothman et al., 1991). This process typically involves structural alterations in neural circuitry – due to progressive neuronal damage and "self-repair" mechanisms – which develop through a latent period of variable time and culminate with the emergence of spontaneous, recurrent, seizures (Dudek and Spitz, 1997). That this process includes a latent period of variable time suggests a progressive series of cellular changes may be involved. As such, human post-traumatic seizures are often classified according to the time of their presentation after injury: immediate or impact-associated (<24 h after injury), early (<1 week after injury), and late (>1 week after injury; Haltiner et al., 1997; Frey, 2003; Agrawal et al., 2006). This classification scheme is thought to represent different pathophysiological processes (Semah et al., 1998; Agrawal et al., 2006). Understanding the epileptogenic process after TBI should help to elucidate the importance of these cellular mechanisms in PTE and promote new therapeutic targets. Trauma sets into motion a multidimensional cascade of cellular and molecular events that involve three temporally overlapping responses in the brain: primary and secondary injuries and "self-repair" mechanisms (Mendelow and Crawford, 1997; Laurer et al., 2000; Graham et al., 2006; **Figure 1**). An important goal of studies which investigate post-traumatic epileptogenesis is to dissociate injury-induced cellular alterations that promote seizure generation from compensatory and "self-repair" responses.

Primary injury refers to the immediate tissue deformation and compression that occurs within seconds to minutes after mechanical brain insult (Mendelow and Crawford, 1997; Laurer et al., 2000; Graham et al., 2006). There is an immediate release of neurotransmitters, glutamate in particular, which is followed by ion channel activation and calcium influx. This can lead to excitotoxic injury, reflected by mitochondrial damage and energy depletion, neuronal and glial swelling, and cell death. Vascular damage and blood–brain barrier (BBB) disruption can also occur in primary injury. Cortical structures such as the hippocampus are especially vulnerable to neuronal damage after moderate and severe TBI. Moreover, the presence of immediate or early seizures can

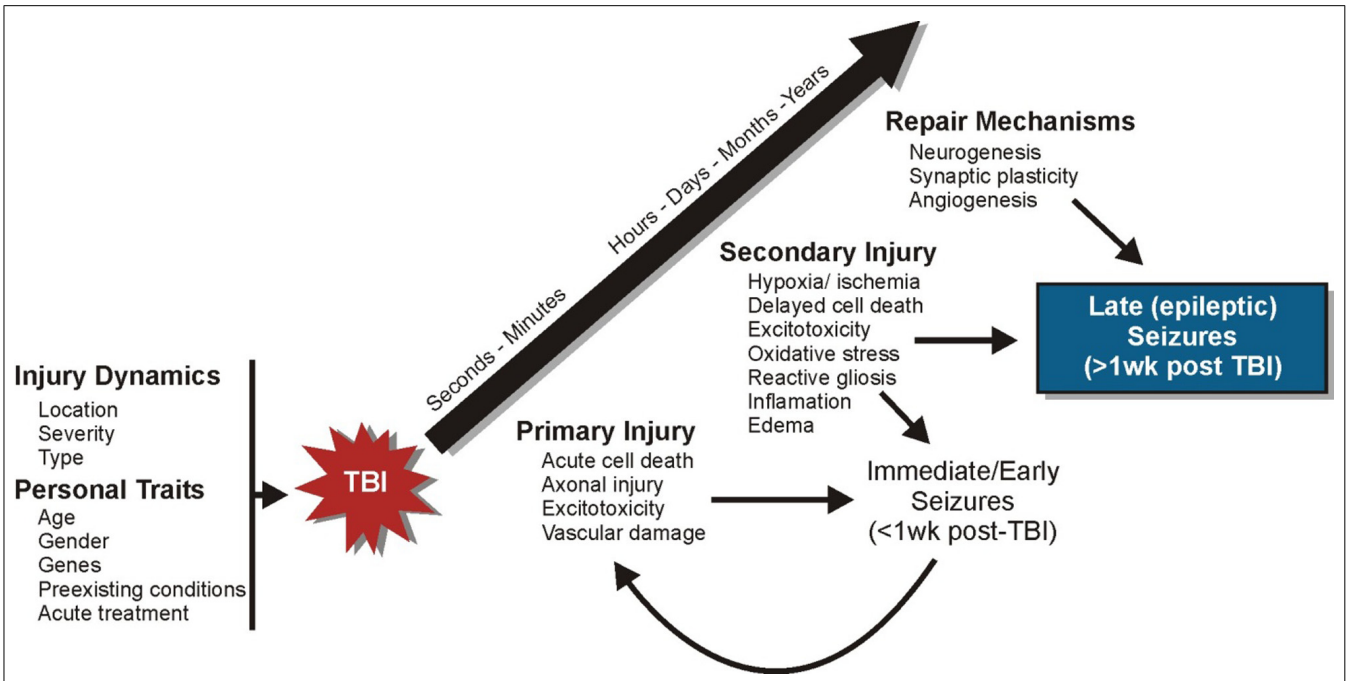

FIGURE 1 | Progression of cellular events following TBI. A series of cellular and molecular events occur following TBI that involve three temporally overlapping responses in the brain: primary and secondary injuries and "self-repair" mechanisms.

further exacerbate initial damage and complicate injury management (Temkin, 2009). These seizures are considered to be injury-induced (i.e., provoked) and are not epileptic, because they occur as a direct result of neurologic and systemic abnormalities of the acute trauma. Strategies to protect against primary injury are typically aimed at taking preventive rather than therapeutic measures (e.g., wearing protective headgear).

Secondary injury involves a myriad of cellular and physiological factors associated with progressive tissue damage (Mendelow and Crawford, 1997; Teasdale and Bannan, 1997; Laurer et al., 2000; Graham et al., 2006). Over time, brain injury triggers inflammatory cascades, growth factor responses, edema, mitochondrial dysfunction, oxidative stress due to the build-up of free radicals and reactive oxygen species, delayed cell death, perturbation of cellular calcium homeostasis, and hypoxia and ischemia. The brain also initiates "self-repair" mechanisms over the course of days to months concurrent with the development of secondary tissue damage. This period is characterized by synaptic circuit remodeling, axon sprouting, synaptic plasticity, gliosis, neurogenesis, and angiogenesis. The time-dependant reorganization of synaptic circuitry can promote increased synchronous neuronal activity that contributes to functional recovery, but may also contribute to spontaneous seizure generation. During this period, seizures that occur spontaneously (i.e., late seizures) are considered to represent PTE because they reflect permanent changes in neuronal structure and function. The primary goal of many neuroprotective measures is to prevent or reduce secondary brain damage and to enhance beneficial "self-repair" mechanisms (Mendelow and Crawford, 1997; Teasdale and Bannan, 1997; Graham et al., 2006). However, repair mechanisms may not be universally beneficial, as some of these same mechanisms (e.g., axon sprouting and synaptic reorganization) are also correlated with the development of spontaneous seizures.

Genetics, gender, age, acute medical treatments, and the agent of injury all likely influence the epileptogenic processes, but the contribution of these personal traits and injury dynamics in epilepsy have not been well established (Pitkänen and McIntosh, 2006). A better understanding of the importance of these factors in post-traumatic epileptogenesis will likely elucidate why some individuals develop PTE after TBI while others do not.

NEURAL CIRCUIT REORGANIZATION

The central dogma in epilepsy research has long been that seizures occur due to some type of imbalance between excitatory and inhibitory neurotransmission (Dudek and Spitz, 1997; McCormick and Contreras, 2001; Nadler, 2003). In humans and animal models of injury-induced epilepsy, the presence of spontaneous seizures is strongly associated with axon sprouting and reorganization of neural circuitry. Within this framework, most experimental injury models show evidence that excitatory connectivity is enhanced and inhibitory influences are decreased. These changes often involve the presence of recurrent excitatory circuits, which form when principal cells are sufficiently interconnected and have long been proposed as a cellular basis for pathologically synchronous neural activity and seizures (Traub and Wong, 1982; Dudek and Spitz, 1997; McCormick and Contreras, 2001).

EXCITATORY CIRCUIT CHANGES

Several laboratories have independently demonstrated reactive plasticity of glutamatergic axons and formation of new, recurrent

excitatory circuits in TBI models (Santhakumar et al., 2000; Kharatishvili et al., 2006, 2007; Hunt et al., 2009, 2012). The dentate gyrus has acted as a model system to study excitatory axon sprouting associated with PTE because it is particularly susceptible to injury and subsequently undergoes structural reorganization (**Figure 2**). Dentate granule cells, which are not normally connected to each other, sprout axon collaterals into the inner molecular layer to form functional recurrent excitatory connections with nearby granule cells during epileptogenesis (Cronin and Dudek, 1988; Cronin et al., 1992; Wuarin and Dudek, 1996, 2001; Lynch and Sutula, 2000; Winokur et al., 2004), a process termed "mossy fiber sprouting." These dramatic changes in local mossy fiber circuits are relatively easy to detect by Timm's histochemistry and are consistently reproduced in human tissue (de Lanerolle et al., 1989; Sutula et al., 1989; Houser et al., 1990; Babb et al., 1991; Zhang and Houser, 1999) and experimental models of TLE (Nadler et al., 1980; Ben-Ari, 1985; Tauck and Nadler, 1985; Cronin and Dudek, 1988; Buckmaster and Dudek, 1997b; Buckmaster et al., 2002; Shibley and Smith, 2002). Mossy fiber sprouting into the inner molecular layer of the dentate gyrus is also a common feature of the epileptic dentate gyrus in TLE patients with a history of head injury (Swartz et al., 2006), and it has been consistently reported in animal models of TBI (Santhakumar et al., 2000; Kharatishvili et al., 2006, 2007; Hunt et al., 2009, 2012). Post-traumatic mossy fiber sprouting is generally more robust after severe versus mild TBI and might be related to whether cortical injury directly impinges upon the hippocampus (Hunt et al., 2012). However, the degree of mossy fiber sprouting after experimental TBI is qualitatively less than the robust, bilateral sprouting observed after experimental status epilepticus, and it is often more regionally localized to areas near the injury.

These anatomical changes in mossy fiber circuits are associated with a functional alteration in neuronal circuitry of dentate granule cells that may contribute to abnormal network synchronization after TBI. Similar to rodent models of TLE (Cronin et al., 1992; Wuarin and Dudek, 1996, 2001; Patrylo and Dudek, 1998; Lynch and Sutula, 2000; Winokur et al., 2004), new functional recurrent excitatory circuits emerge in the presence of mossy fiber sprouting after TBI that are not detected in the normal dentate gyrus (Hunt et al., 2009, 2010). When surgically isolated from afferent input (i.e., entorhinal cortex), spontaneous and evoked reverberating burst discharges, indicative of synchronous network activation, have been observed by single-cell and extracellular field potential recordings from granule cells in slices with mossy fiber sprouting after TBI (Hunt et al., 2009, 2010). Studies using localized glutamate stimulation have suggested monosynaptic granule cell-to-granule cell connections in slices with mossy fiber sprouting, which are absent in the normal dentate gyrus (Hunt et al., 2010). These new excitatory connections among granule cells are only present in brain slices with mossy fiber sprouting and are not detected in slices without sprouting, even in the same injured animal. Although recurrent excitatory circuits are normally masked by recurrent inhibitory circuits and can only be revealed by experimentally altering the extracellular environment to increase excitation, these new excitatory circuits have been

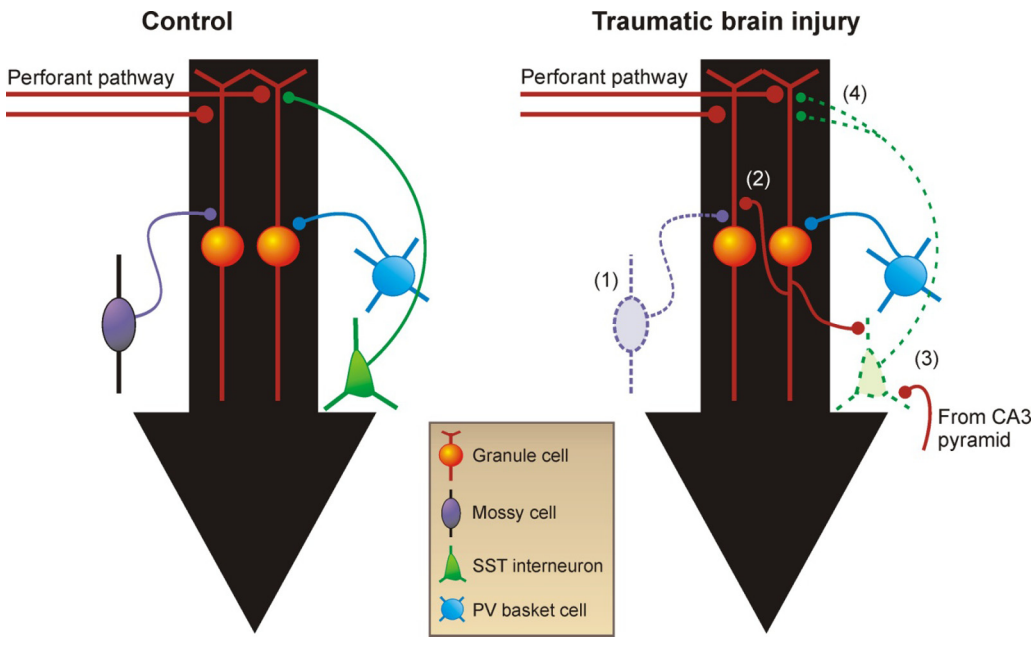

FIGURE 2 | Reorganization of neural circuitry in the dentate gyrus after brain injury. (1) Within days after injury, there are fewer excitatory mossy cells and inhibitory GABA neurons in the hilus. (2) Within weeks after TBI, mossy fibers sprout axon collaterals into the inner molecular layer and form new recurrent excitatory circuits among granule cells. (3) Somatostatin-positive GABA neurons in the hilus that survive TBI receive increased excitatory input from granule cells and CA3 pyramidal cells. (4) These neurons also have increased axon length in the molecular layer in acquired epilepsy models. Black arrows show direction of information flow through the dentate gyrus. PV, parvalbumin; SST, somatostatin.

proposed to form the basis from which synchronous network activity can periodically arise in the dentate gyrus, particularly if inhibition periodically fails. Therefore, mossy fiber sprouting may provide a means for regional granule cell network synchronization after TBI that may be "unmasked" if inhibitory control is impaired (Patrylo and Dudek, 1998).

Other brain regions that are susceptible to injury, such as hippocampus and neocortex also undergo synaptic reorganization after TBI. For example, an increase in spontaneous and evoked burst discharges has been detected in neocortex of rats within 2 weeks after severe CCI injury (Yang et al., 2010). Although it is not known whether these changes are due to the formation of new excitatory circuits in neocortex or enhanced intrinsic excitability of existing circuits after injury, similar findings have been observed at similar time points after neocortical undercut (recently reviewed by Prince et al., 2012), which appear to be associated with an increase in excitatory synapses onto pyramidal neurons. In CA1 of hippocampus, pyramidal neurons show immediate deafferentation following CCI injury, possibly due to loss of CA3 neurons, which is followed by a time-dependent increase in number of synaptic contacts (Scheff et al., 2005) and fiber excitability (Norris and Scheff, 2009). Taken together, these findings suggest that changes in functional excitability of other brain regions might also contribute to seizure generation after TBI, independent of mossy fiber sprouting.

INHIBITORY CIRCUIT CHANGES

GABAergic interneurons form robust local synaptic connections with principal cells and play an important role in controlling cortical activity. Two primary modes of $GABA_A$ receptor-mediated inhibition have been identified: phasic (synaptic) and tonic (extrasynaptic) inhibition (Farrant and Nusser, 2005; Glykys and Mody, 2007; **Figure 3**). Phasic inhibition refers to the transient activation of postsynaptic $GABA_A$ receptors at the synaptic junction following exposure to high concentrations of GABA released from presynaptic vesicles. This form of synaptic communication allows for rapid transmission of information from the presynaptic terminal to the postsynaptic membrane in a spatially and temporally restricted manner. In whole-cell voltage-clamp recordings, synaptic events can be easily identified as inhibitory postsynaptic currents (IPSCs). Small amounts of GABA escape the synaptic cleft and activate high-affinity extrasynaptic $GABA_A$ receptors on the same or adjacent neurons, an event termed "spillover." Tonic inhibition refers to the persistent activation of these extrasynaptic $GABA_A$ receptors by low concentrations of ambient GABA. Phasic and tonic currents can be isolated by high concentrations of $GABA_A$ receptor antagonists. In whole-cell voltage-clamp recordings the tonic current is reflected by a shift in "holding" current.

In the dentate gyrus, the loss of certain populations of hilar interneurons is a common histopathological feature in human PTE (Swartz et al., 2006), and several labs have reported a loss in the number of inhibitory interneurons after experimental TBI (Lowenstein et al., 1992; Toth et al., 1997; Santhakumar et al., 2000; Grady et al., 2003; Hall et al., 2005). Although it is unclear whether subtypes of hilar neurons are preferentially lost after TBI, as is often observed in TLE, inhibitory hilar neuron loss ipsilateral to the site of injury is typically associated with a reduction in synaptic inhibition of granule cells (Hunt et al., 2011; Pavlov et al., 2011; Gupta et al., 2012). In contrast, whether there are changes in tonic inhibition after TBI remain unresolved. One study reported an increase in tonic current amplitude in dentate granule cells

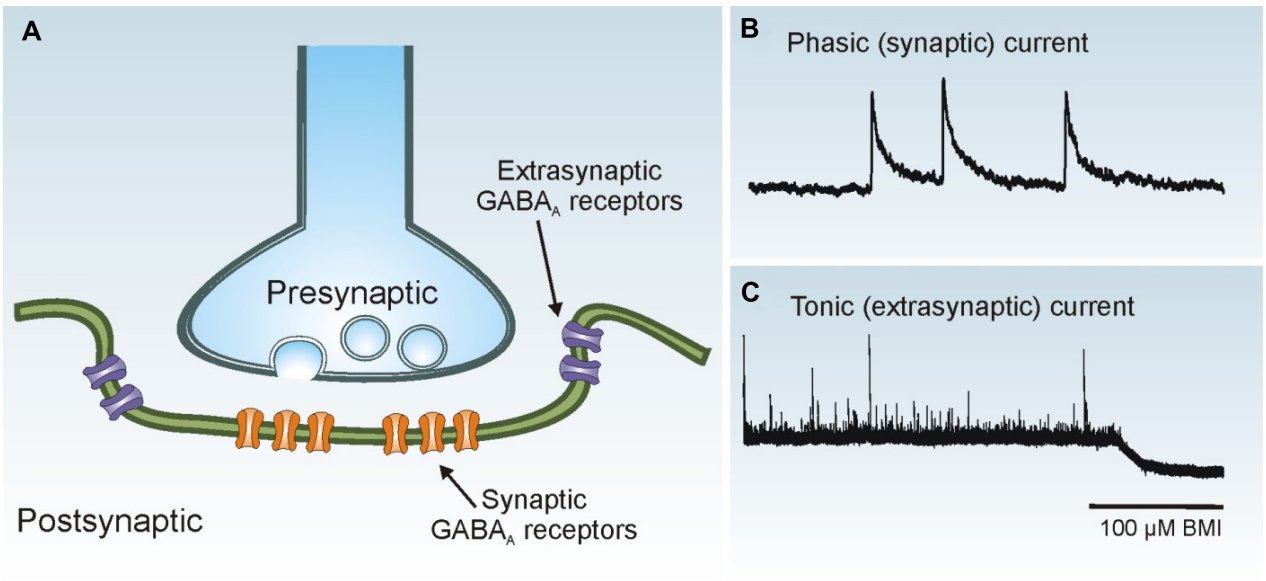

FIGURE 3 | Phasic and tonic $GABA_A$ receptor activation. (A) Synaptic receptors (orange) are located on the postsynaptic membrane just beneath presynaptic release sites, whereas extrasynaptic receptors (purple) are located away from the synaptic junction. **(B)** Phasic (synaptic) IPSCs are rapid events. Three individual IPSCs are shown in a whole-cell voltage-clamp recording obtained from a dentate granule cell. **(C)** The tonic (extrasynaptic) current in this granule cell is revealed as a baseline shift after application of the $GABA_A$ receptor antagonist bicuculline methiodide (BMI; 100 μM).

contralateral to severe CCI injury (Mtchedlishvili et al., 2010), whereas other studies reported no change in tonic current amplitude to granule cells (Pavlov et al., 2011) or a marked decrease in tonic inhibition in semilunar granule cells (Gupta et al., 2012) ipsilateral to moderate FPI. Recent evidence has also shown that there are changes in the expression of $GABA_A$ receptor subunit levels after CCI injury (Mtchedlishvili et al., 2010; Gupta et al., 2012; Raible et al., 2012), which might also affect inhibitory influences after TBI.

Beyond the presence of substantial interneuron loss and a subsequent reduction in synaptic inhibition of granule cells after TBI, relatively little is known about how individual interneuron subpopulations are affected by mechanical trauma. In a recent study, we reported an increase in excitatory input to hilar somatostatin interneurons arising from granule cells and CA3 pyramidal neurons after CCI injury, suggesting that reactive plasticity of glutamatergic axons is not limited to connections among principal neurons (Hunt et al., 2011). These changes were accompanied by an increase in the decay time constant of spontaneous inhibitory synaptic events in granule cells, which is consistent with increased activity or axon sprouting of this dendritically projecting GABA neuron population after injury. Similar changes have also been reported in a model of status epilepticus (Zhang et al., 2009; Halabisky et al., 2010). However, whether this phenomenon represents a compensatory response to boost feedback inhibition in the dentate gyrus, which is compromised by injury, or a pathological response that contributes to seizures (e.g., by a periodic failure of already fragile inhibitory circuits or increased risk for GABA-mediated depolarization in distal dendrites) is unknown. It is also not known how other populations of inhibitory neurons are affected by TBI. Given the relatively large number of ways in which inhibitory circuits can be modified by head injury and the important role of inhibitory neurons in restraining excessive cortical excitability (Chagnac-Amitai and Connors, 1989; Trevelyan et al., 2007; Schevon et al., 2012), future studies aimed at understanding how inhibition is modified after closed-head TBI, including in brain regions outside of the dentate gyrus, should remain an active area of investigation.

BRAIN INFLAMMATION AND BLOOD–BRAIN BARRIER DYSFUNCTION AFTER TBI

While the immune system provides crucial protection against infected or damaged tissue, accumulating evidence points to an involvement of inflammation in the pathophysiology of epilepsy. Heightened or abnormal innate immune responses are noted in individuals with epilepsy, in animal models of this disease, as well as with pro-epileptic insults such as infection, trauma, ischemia/hypoxia, fever, and recurrent seizures (Nelson and Ellenberg, 1976; Pacifici et al., 1995; Carpio et al., 1998; Crespel et al., 2002; Kotila and Waltimo, 2005; Choi and Koh, 2008; Vezzani et al., 2013; Dedeurwaerdere et al., 2012). Importantly, many innate immune processes play significant roles in cell excitability and survival during inflammation, which have the potential to promote network hyper-excitability (Hama et al., 1991; Yamada and Hatanaka, 1994; Schäfers and Sorkin, 2008; Vezzani et al., 2008). Experimental models of epilepsy that do not involve head injury (non-PTE models) have described several inflammatory pathways that contribute to seizure susceptibility (Dedeurwaerdere et al., 2012). Despite these indications, surprisingly very few basic studies have studied the role of the immune system in experimental PTE even though inflammation and BBB breakdown are hallmark features of head injury (Schmidt et al., 2005; Werner and Engelhard, 2007). The causal role of inflammation in PTE development can, at least in part, be assessed by systematic experimental manipulation of individual components of inflammation in animal models. An important consideration in these studies is to discriminate between acute effects of seizure threshold (i.e., anti-convulsant effects) and long-term development of spontaneous seizures (i.e., epileptogenesis), because these processes may have different mechanisms and inflammation may contribute to them in different ways. This section attempts to highlight the need to examine the role of inflammation in epileptogenesis as presently there a very few studies that do so using PTE or non-PTE models.

Work in brain trauma has extensively characterized patterns of immune responses following TBI (detailed review in Morganti-Kossmann et al., 2001; Lucas et al., 2006). Head trauma initially triggers inflammation by a variety of factors including direct damage to the BBB as well as an accumulation of foreign particles, extravasated blood proteins, cellular debris, complement fragments, prostaglandins, and both reactive oxygen and nitrogen species within the brain (Dardiotis et al., 2012). The resulting inflammatory cascade involves the coordinated activity of resident central nervous system (CNS) immune and non-immune cells, circulating immune cells, and transport mechanisms across the BBB. Neurons, astrocytes, and particularly microglia located proximal to the site of injury rapidly respond to trauma with pro-inflammatory signals including chemokines, cytokines, and tissue adhesion molecules (Ransohoff, 2002). The release of these inflammatory mediators supports the further recruitment, migration, and infiltration of leukocytes into the brain parenchyma. The invasion of leukocytes (chiefly neutrophils, monocytes, and lymphocytes) results in further disruption of homeostasis and secretion of pro-inflammatory signals within acutely spared brain regions resulting in a potent inflammatory response to these sites (Morganti-Kossmann et al., 2001). Additional increases in BBB permeability magnify levels of inflammatory units and blood proteins entering brain tissue (Dardiotis et al., 2012).

In the context of epilepsy, an important feature of inflammation is its contribution to ongoing cell death after trauma. In addition to cell loss by excitotoxicity, ischemia, and disruptions in fluid and metabolic homeostasis, inflammatory cytokines enhance signaling components of apoptosis (Morganti-Kossmann et al., 2001). Proteases and free radicals released during inflammation support lipid and protein peroxidation, mitochondrial damage, DNA damage, as well as further induction of apoptotic mechanisms (Tyurin et al., 2000). Conversely, many immune components provide a neuroprotective effect following trauma. The mechanisms underlying these neuroprotective effects are not fully characterized, however inflammatory components can provide neurotrophic support by increasing growth factors, reducing oxidative stress, or via anti-inflammatory signaling (Morganti-Kossmann et al., 2001). Understanding the role that individual inflammatory components serve in post-traumatic cell loss will likely help determine how

they affect PTE development. Components of inflammation also affect cell excitability within the CNS in addition to their effects on cell survival. For example, inflammatory cytokines modulate neurotransmitter levels (Zalcman et al., 1994; Dunn, 2006), communication between neurons and glia (Hanisch, 2002), $GABA_A$ receptor-mediated responses (Miller et al., 1991), and calcium currents (Plata-Salamán and Ffrench-Mullen, 1992; Qiu et al., 1998). Chemokines also affect neuronal excitability by modulation of voltage-dependent channels (sodium, potassium, and calcium), activation of an inward rectifying potassium conductance and increasing the release of neurotransmitters including GABA, glutamate, and dopamine (Fabene et al., 2010). The effects of inflammation on cell loss and cell excitability may serve a role in the development of network hyper-excitability and epileptogenesis following TBI.

There are several challenges to understanding the overall contribution of inflammatory pathways to PTE. Individual components of inflammation typically affect many processes; they are pleiotropic, and are integrative such that one process can affect many others via forward and backward regulatory pathways. For example, treatment with antibodies against interleukin (IL)-6 results in elevated serum levels of tumor necrosis factor alpha (TNF-α) whereas treatment with antibodies against TNF-α results in decreased serum levels of IL-6 (Starnes et al., 1990). Individual inflammatory components also have different temporal expression patterns following head injury (for review, see Korn et al., 2005; Lucas et al., 2006). There is also a complex spatial patterning of post-traumatic inflammation where expression may be specific to cell-type or brain region and may be different between peripheral and central tissues (Morganti-Kossmann et al., 2001; Schmidt et al., 2005; Ravizza and Vezzani, 2006). For example, transgenic mice that over-express IL-6 under a glial fibrillary acidic protein (GFAP) promoter (i.e., to reflect astrocytic production of IL-6) exhibit spontaneous seizures, neuronal loss, neural dysfunction, and breakdown of the BBB (Campbell et al., 1993). In contrast, mice that over-express this same cytokine under a neuron-specific enolase (NSE) promoter display no neuronal damage or seizures (Fattori et al., 1995). All of these characteristics depend on the type of immune component, unique biomechanics of the TBI, as well as the specific traits of the injured individual (Rovegno et al., 2011). As studies continue to document these pathways in TBI it is becoming increasing important to ascertain more specific profiles of individual inflammatory components that include their spatial and temporal patterning as well as their net effect on cell loss and cell excitability.

The PTE field can be guided by reports that characterize or manipulate immune processes in non-PTE models and models of trauma. To date, many links between innate immunity and seizure expression in non-PTE models have been made studying cytokine signaling (Dedeurwaerdere et al., 2012). Cytokines are a diverse family of glycoproteins that are secreted by glia, neurons, leukocytes, endothelial and epithelial cells in response to stress, immune challenge, and injury (Ransohoff, 2002). Cytokine signaling occurs at receptors found on a variety of cells throughout the body via transcription-dependent and transcription-independent mechanisms (Bezbradica and Medzhitov, 2009). Cytokines are often characterized as either pro-inflammatory (e.g., IL-1β, IL-6, and TNF-α) or anti-inflammatory, with the latter exerting their effects by reducing expression of pro-inflammatory cytokines (e.g., IL-4, IL-10, and IL-13; Kadhim et al., 2008). While basal cytokine expression is low, these signals are highly elevated in the acute phase of TBI (Ransohoff, 2002). Transgenic glial over-expression of IL-6 (Campbell et al., 1993) or TNF-α (Akassoglou et al., 1997) results in spontaneous seizures and neurodegeneration including a loss of GABAergic cells in cortical and hippocampal structures. IL-6 delivered to naïve animals intranasally increases their seizure susceptibility to the convulsant PTZ (Kalueff et al., 2004). Brain administration of IL-1β increases the duration of seizures induced acutely by kainic acid, whereas administration of the IL-1 receptor antagonist (IL-1ra) provides anti-convulsant effects (Vezzani et al., 2000). Mice with transgenic glial over-expression of IL-1ra or IL-1 receptor type 1 knock-out mice exhibit reduced susceptibility to convulsant-induced seizures (Vezzani et al., 2000, 2002). The prostaglandin receptor EP2 has recently been targeted using novel inhibitors because of its role in diverse cytokine signaling and neurotoxicity (Jiang et al., 2012). Inhibition of prostaglandin receptor EP2 reduces cytokine expression, markers of astrogliosis and microgliosis, mortality, hippocampal neurodegeneration, and BBB leakage in pilocarpine-treated mice in the absence of acute anticonvulsant effects (Jiang et al., 2012, 2013). These results, particularly the long-term development of spontaneous seizures observed with over-expression of IL-6 and TNF-α indicate a role for cytokines in epileptogenesis that needs to be assessed in models of PTE.

Increases in BBB permeability and tissue adhesion molecules may also reduce seizure threshold or have pro-epileptogenic effects (Quadbeck and Helmchen, 1958; Kasantikul et al., 1983; reviewed in Shlosberg et al., 2010; Kim et al., 2012). The BBB's constituent components: endothelial cells, pericytes, and astrocytic end-feet work in concert to homeostatically regulate the flow of molecules and cells between the vasculature and brain parenchyma (Wolburg and Lippoldt, 2002). The resulting "neurovascular unit" that arises from these structures in combination with local neurons is drastically altered by primary and secondary damage following TBI (Korn et al., 2005). While a positive correlation between BBB permeability and recurrent seizures has been observed following electrically induced status epilepticus (van Vliet et al., 2007), the relationship between seizures and BBB permeability remains complex (Cornford and Oldendorf, 1986; Janigro, 1999; Tomkins et al., 2001; Pavlovsky et al., 2005). Increased BBB permeability allows entry of inflammatory cells/molecules into the brain, while in parallel, inflammatory signaling from sites within the brain can also promote BBB permeability (Abbott, 2000; Huber et al., 2001; Marchi et al., 2007; Zlokovic, 2008). Thus, secondary phases of increased BBB permeability and inflammation can occur in response to immune signaling and these secondary phases may play a role in post-traumatic epileptogenesis (Korn et al., 2005).

Emerging work using animal models has further characterized how BBB permeability is altered in pathological conditions and how individual infiltrates within the extracellular space affect local cell survival and excitability (Seiffert et al., 2004; Ivens et al., 2007; Fabene et al., 2008; Tomkins et al., 2008). Repeated administrations of a granulocyte-specific antibody to reduce populations

of neutrophils produce anti-convulsant and anti-epileptogenic effects in pilocarpine mice (Fabene et al., 2008). Brain slices from animals given experimental disruption of the BBB using bile salts exhibit epileptiform activity (Seiffert et al., 2004). These bile salts result in an extravasation of serum albumin into the brain and this epileptiform activity is similarly observed in slices from animals given direct cortical application of serum albumin (Seiffert et al., 2004). The mechanistic link between extravasation of serum albumin into the brain and epileptiform activity is suggested to be a transforming growth factor beta (TGF-β)-dependent down-regulation of an inwardly rectifying K^+ current in astrocytes that reduces their ability to buffer extracellular K^+ (Ivens et al., 2007; Cacheaux et al., 2009; David et al., 2009). Administration of rapamycin (van Vliet et al., 2012) or an antagonist of the prostaglandin receptor EP2 (Jiang et al., 2013) are both associated with reduced BBB leakage. Administration of IL-1β or transgenic over-expression of TNF-α result in increased BBB permeability (Akassoglou et al., 1997; Ferrari et al., 2004; Ravizza et al., 2008). There is also growing interest in understanding how adhesion molecules that support transport across the BBB may contribute to, as opposed to result from, epileptic pathologies (Dedeurwaerdere et al., 2012). Pilocarpine-induced seizures increase levels of vascular cell adhesion molecules (VCAMs) including intercellular adhesion molecule-1 (ICAM-1), VCAM-1 and P-selectin glycoprotein ligand-1 (PSGL-1) and these increases enhance leukocyte adhesion to CNS vessels *in vivo* (Fabene et al., 2008). Exogenous delivery of antibodies for either α4 integrin or VCAM-1 delivered 1 h after pilocarpine treatment, and repeated (every other day for 20 days), greatly reduces spontaneous seizure expression (Fabene et al., 2008). Interestingly, magnetic resonance imaging of individuals with TBI identifies increased BBB permeability in the majority of cases involving PTE and this enhanced permeability is sustained from days to years post-injury in some individuals (Tomkins et al., 2008). These findings suggest that BBB permeability may be a common feature of inflammation-dependent changes in seizure threshold and/or epileptogenesis. The long-term suppression of spontaneous seizures in pilocarpine-treated mice by *in vivo* treatment with antibodies against α4 integrin, VCAM-1, or granulocytes indicates several important potential targets to treat PTE.

Chemokines (also known as chemoattractant cytokines) may also contribute to the development of epilepsy. Chemokines are chemotactic proteins that are characterized by the relative location of their cysteine (Cys) residues within the NH_2 terminal of each protein. Chemokines are classified as CC, CXC, CX_3C, and XC where CC refers to both Cys residues adjacent to one another, CXC refers to both Cys residues separated by one amino acid, CX_3C refers to both Cys residues separated by three amino acids and XC has only one Cys residue (Moser et al., 2004). There is also a lipopolysaccharide inducible CC chemokine receptor termed L-CCR (Shimada et al., 1998). The central role of chemokines is to direct cell migration including migration of leukocytes during brain inflammation (Ley et al., 2007). Importantly, individual (or classes) of chemokines are known to affect specific types of leukocytes (Moser et al., 2004). Many chemokines are increased after head trauma including CCL3, CCL9, CCL12, CCL10, and CCL2 (also known as monocyte chemoattractant protein MCP-1; Israelsson et al., 2008). Unfortunately few, if any, studies have selectively targeted chemokines to characterize their role in PTE. CCL2 is up-regulated following pilocarpine or kainate induced SE (Fabene et al., 2010). CCL3 and CCL4 are increased in amygdala stimulation induced SE (Guzik-Kornacka et al., 2011). Administration of the small molecule Minozac results in reductions in CCL2, IL-1β, IL-6, and TNF-α; behavioral improvement; and attenuates the increased susceptibility to electroconvulsive shock-induced seizures after experimental closed-head injury (Lloyd et al., 2008; Chraszcz et al., 2010). These results indicate a potential neuroprotective or neurorestorative role for suppressors of chemokines, cytokines, or other pro-inflammatory responses (Lloyd et al., 2008). Targeted approaches are needed to assess the contribution of individual chemokines to epileptogenesis in models of PTE. Identification of a role for individual chemokines can provide clues as to what types of infiltrating leukocytes are pro-epileptogenic based on established relationships between individual chemokines and their target immune cells.

Collectively, a number of studies suggest that inflammation may contribute to the development of PTE. Inflammation or other immune changes are shared features of many seizure disorders and these changes often precede the manifestation of behavioral seizures (i.e., during the latent period; Majores et al., 2004; Ravizza et al., 2008). An emerging concept is that seizures themselves can result in brain inflammation, thereby raising the possibility of a reciprocal relationship between inflammation and seizures that may support or maintain epilepsy (Librizzi et al., 2012). Inflammation affects cell excitability and cell survival and there is some evidence that these effects can promote network hyper-excitability (Miller et al., 1991; Plata-Salamán and Ffrench-Mullen, 1992; Zalcman et al., 1994; Qiu et al., 1998; Ravizza and Vezzani, 2006). There is also a growing need to distinguish immune effects on seizure mechanisms from immune effects on recovery and compensation as there is at least some evidence that individual inflammatory components affect both processes (Morganti-Kossmann et al., 2001). For example, in addition to TNF-α's pro-excitatory and pro-epileptic effects, this inflammatory cytokine serves a neuroprotective role, since mice lacking TNF-α show enhanced lesion size and BBB breakdown following CCI (Sullivan et al., 1999). The pleiotropic and integrative nature of inflammatory components will offer some difficulties in this type of pathway-specific targeting. Nonetheless, this pathway-specific approach needs to be further extended in models of PTE and other forms of epilepsy to characterize how individual components of inflammation affect epileptogenesis. While there are clear challenges in identifying the role of inflammatory processes in the pathophysiology of PTE, the possibility to reduce or block the development of epilepsy after TBI provides great encouragement for work in this area.

SUMMARY

The development of medically intractable epilepsy is one of the most common long-term health problems associated with head injury in humans. Recent work using animal models of TBI has shown substantial evidence for the formation of new, excessive recurrent excitatory synaptic connectivity and alterations to

inhibitory interneurons in this disorder, although these studies have primarily focused on the dentate gyrus and other regions of the injured brain should be investigated in more detail. However, there are a number of important aspects of post-traumatic epileptogenesis that are not fully understood. For example, there is a need for a more complete profile of how individual components of inflammation and BBB permeability contribute to post-traumatic epileptogenesis. Thus, much progress has been made in modeling epilepsy in rodents after mechanical injury and basic mechanisms of neural circuit reorganization associated with epilepsy have been identified in these models, but new developments in this field should continue to identify precise cellular or molecular mechanisms that control the development of epilepsy after head injury.

ACKNOWLEDGMENTS

Supported by Department of Defense (USAMRMC) Grant W81XWH-11-1-0502 (to Bret N. Smith) and a Fellowship from the American Epilepsy Society and Lennox and Lombroso Trust for Epilepsy Research and Training (Jeffery A. Boychuk).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2013; accepted: 22 May 2013; published online: 18 June 2013.
Citation: Hunt RF, Boychuk JA and Smith BN (2013) Neural circuit mechanisms of post-traumatic epilepsy. Front. Cell. Neurosci. 7:89. doi: 10.3389/fncel.2013.00089

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 05 July 2013
doi: 10.3389/fncel.2013.00104

Expressional analysis of the astrocytic Kir4.1 channel in a pilocarpine-induced temporal lobe epilepsy model

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The inwardly rectifying potassium (Kir) channel Kir4.1 in brain astrocytes mediates spatial K^+ buffering and regulates neural activities. Recent studies have shown that loss-of-function mutations in the human gene *KCNJ10* encoding Kir4.1 cause epileptic seizures, suggesting a close relationship between the Kir4.1 channel function and epileptogenesis. Here, we performed expressional analysis of Kir4.1 in a pilocarpine-induced rat model of temporal lobe epilepsy (TLE) to explore the role of Kir4.1 channels in modifying TLE epileptogenesis. Treatment of rats with pilocarpine (350 mg/kg, i.p.) induced acute status epilepticus, which subsequently caused spontaneous seizures 7–8 weeks after the pilocarpine treatment. Western blot analysis revealed that TLE rats (interictal condition) showed significantly higher levels of Kir4.1 than the control animals in the cerebral cortex, striatum, and hypothalamus. However, the expression of other Kir subunits, Kir5.1 and Kir2.1, remained unaltered. Immunohistochemical analysis illustrated that Kir4.1-immunoreactivity-positive astrocytes in the pilocarpine-induced TLE model were markedly increased in most of the brain regions examined, concomitant with an increase in the number of glial fibrillary acidic protein (GFAP)-positive astrocytes. In addition, Kir4.1 expression ratios relative to the number of astrocytes (Kir4.1-positive cells/GFAP-positive cells) were region-specifically elevated in the amygdala (i.e., medial and cortical amygdaloid nuclei) and sensory cortex. The present study demonstrated for the first time that the expression of astrocytic Kir4.1 channels was elevated in a pilocarpine-induced TLE model, especially in the amygdala, suggesting that astrocytic Kir4.1 channels play a role in modifying TLE epileptogenesis, possibly by acting as an inhibitory compensatory mechanism.

Keywords: Kir4.1 channel, astrocytes, temporal lobe epilepsy, status epilepticus, spatial potassium buffering, pilocarpine

INTRODUCTION

The spatial K^+ buffering by astrocytes removes excess extracellular K^+ at synapses and transports them into regions of low K^+ concentration such as blood vessels, regulating neuronal activities (Walz, 2000; Kofuji and Newman, 2004; Simard and Nedergaard, 2004; Butt and Kalsi, 2006). The K^+ buffering currents are mediated by inwardly rectifying potassium (Kir) channels which are expressed in astrocytes (Tanemoto et al., 2000; Hibino et al., 2004; Kofuji and Newman, 2004; Simard and Nedergaard, 2004; Butt and Kalsi, 2006). These comprise Kir4.1 channels, homo-tetramers of Kir4.1 subunits, and Kir4.1/5.1 channels, hetero-tetramers of Kir4.1 and Kir5.1 subunits, which conduct large inward K^+ currents at potentials negative to K^+ equilibrium potential (Tanemoto et al., 2000; Ohno et al., 2007; Su et al., 2007; Furutani et al., 2009). In addition, spatial K^+ buffering is linked to glutamate uptake and/or aquaporin-4-mediated water transport by astrocytes (Nagelhus et al., 1999; Amiry-Moghaddam and Ottersen, 2003; Puwarawuttipanit et al., 2006; Djukic et al., 2007; Kucheryavykh et al., 2007).

Recent clinical studies have shown that mutations in the human gene *KCNJ10* encoding Kir4.1 cause EAST (epilepsy, ataxia, sensorineural deafness, and tubulopathy) or SeSAME (seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance) syndrome consisting of generalized tonic-clonic (GTC) seizures, ataxia, hearing loss, and abnormal renal excretion of electrolytes (Bockenhauer et al., 2009; Scholl et al., 2009). The most frequent mutation of *KCNJ10* was R65P at the cytoplasmic end of transmembrane region (TM)-1 and others include G77R (TM-1), C140R (extracellular loop between TM-1 and TM-2), T164I, A167V (cytoplasmic end of TM-2), R175Q, R199X, and R297C (C-terminal domain; Reichold et al., 2010; Sala-Rabanal et al., 2010; Tang et al., 2010). All these mutations caused drastic decreases in K^+ currents mediated by Kir4.1 and Kir4.1/5.1 channels, suggesting that the impaired functioning of astrocytic Kir4.1 channels causes epileptic seizures by disrupting spatial K^+ buffering. In addition, several SNPs of *KCNJ10* have been shown to be associated with temporal lobe epilepsy (TLE) with febrile seizures (Heuser et al., 2010). Expressional analysis also revealed pathophysiological alterations in Kir4.1 expression in patients with TLE (Das et al., 2012; Heuser et al., 2012; Steinhäuser et al., 2012),

Abbreviations: GFAP, glial fibrillary acidic protein; GTC, generalized tonic-clonic; Kir, inwardly-rectifying potassium; SE, status epilepticus; TLE, temporal lobe epilepsy.

suggesting a potential involvement of Kir4.1 channels in TLE epileptogenesis. However, information on the modulatory role of Kir4.1 in the generation and/or development of TLE is still very limited.

In the present study, we performed expressional analysis of Kir4.1 in a pilocarpine-induced rat model of TLE to explore the pathophysiological role of Kir4.1 channels in TLE epileptogenesis. The expressions of Kir5.1 and Kir2.1, other Kir subunits expressed in astrocytes, were also evaluated for comparison.

RESULTS

PILOCARPINE-INDUCED TLE MODEL

All the TLE rats (N = 11) used herein experienced pilocarpine (350 mg/kg, i.p.)-induced status epilepticus (repeated and sustained clonic seizures) and showed spontaneous seizures (i.e., wild running/jumping and GTC seizures) 7–8 weeks after the pilocarpine treatment. The animals, which were given pilocarpine but did not experience status epileptics and any seizure activity thereafter (7–8 weeks), were used as the control ($N = 11$). Four and seven animals in each group were subjected to Western blot and immunohistochemical analysis, respectively.

WESTERN BLOT ANALYSIS

As reported previously (Connors et al., 2004; Seifert et al., 2009; Harada et al., 2013), Kir4.1 was detected primarily as a tetramer (∼160 kDa) in all brain regions examined in TLE and control rats (**Figure 1A**). Two-way ANOVA revealed no significant interaction [$F(1, 60) = 1.61$, $P = 0.13$], but significant main effects of groups [$F(1, 60) = 23.24$, $P < 0.01$] and regions [$F(9, 60) = 10.80$, $P < 0.01$]. Expression levels of Kir4.1 were relatively high in the striatum (St) and pons/medulla oblongata (P/MO). As compared to control animals, TLE rats showed significantly higher Kir4.1 levels in the frontal cortex (fCx, $P < 0.05$), occipito-temporal cortex (otCx, $P < 0.05$), St ($P < 0.01$), hypothalamus (Ht, $P < 0.05$), and P/MO ($P < 0.01$; **Figures 1A,B**). These changes were region-specific and the Kir4.1 levels in other brain regions [i.e., parieto-temporal cortex (ptCx), hippocampus (Hpc), thalamus (Th), midbrain (Mid), and cerebellum (Cer)] remained unaltered.

In contrast to Kir4.1, Kir5.1 and Kir2.1 subunits were detected mainly as monomers (Kir5.1: 50 kDa, Kir2.1: 45 kDa) in all 10 regions (**Figure 1A**). Levels of Kir5.1 were relatively high in the ptCx and Mid while the Kir2.1 levels were high in the Mid and low in the cerebral cortices and Cer (**Figures 1C,D**). Analysis of Kir5.1 expression showed only a significant main effect of regions [$F(9, 60) = 7.97$, $P < 0.01$] without a significant interaction [$F(9, 60) = 0.32$, $P = 0.96$] or a main effect of groups [$F(9, 60) = 0.77$, $P = 0.38$]. Thus, no significant differences in the expression levels of Kir5.1 were observed between TLE and control rats in all 10 regions (**Figure 1C**). On the other hand, analysis of Kir2.1 expression revealed significant main effects of groups [$F(1, 60) = 7.93$, $P < 0.01$] and regions [$F(9, 60) = 13.9$, $P < 0.01$] without a significant interaction [$F(9, 60) = 1.24$, $P = 0.29$]. Among 10 regions, only the Kir2.1 level in the St was significantly ($P < 0.01$) higher in TLE than in control rats (**Figure 1D**).

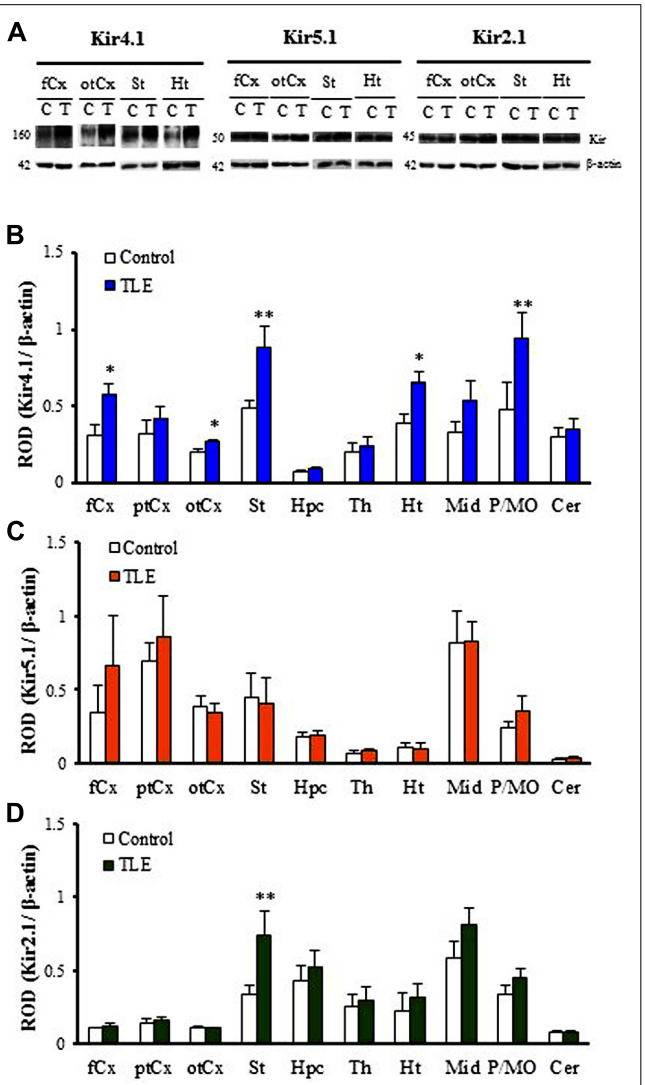

FIGURE 1 | Western blot analysis for Kir4.1, Kir5.1 and Kir2.1 expression in pilocarpine-induced TLE rats. (A) Representative Western blots visualizing Kir4.1, Kir5.1, and Kir2.1 expression in the frontal cortex (fCx), occipito-temporal cortex (otCx), striatum (St), and hypothalamus (Ht). **(B–D)** Regional expression of Kir4.1 **(B)**, Kir5.1 **(C)**, and Kir2.1 **(D)** in pilocarpine-induced TLE rats. Kir expression was expressed as relative optical density (ROD) to β-actin. fCx, frontal cortex; ptCx, parieto-temporal cortex; otCx, occipito-temporal cortex; St, striatum; Hpc, hippocampus; Th, thalamus; Ht, hypothalamus; Mid, midbrain; P/MO, pons/medulla oblongata; Cer, cerebellum. Each column represents the mean ± SEM of four animals. $*P < 0.05$, $**P < 0.01$, significantly different from the control rats.

IMMUNOHISTOCHEMICAL ANALYSIS FOR KIR4.1 EXPRESSION

Since Western blot analysis revealed that pilocarpine-induced TLE rats showed elevated Kir4.1 expression in the fCx and otCx, we further conducted immunohistochemical analysis for Kir4.1 expression using frontal (Bregma +1.68 mm level) and occipito-temporal (Bregma −3.00 mm level) brain slices (**Figure 2A**). With regard to the expression patterns of Kir4.1-immunoreactivity (IR), we have previously shown that Kir4.1 was primarily stained in astrocytes which typically show a stellate-shape and were

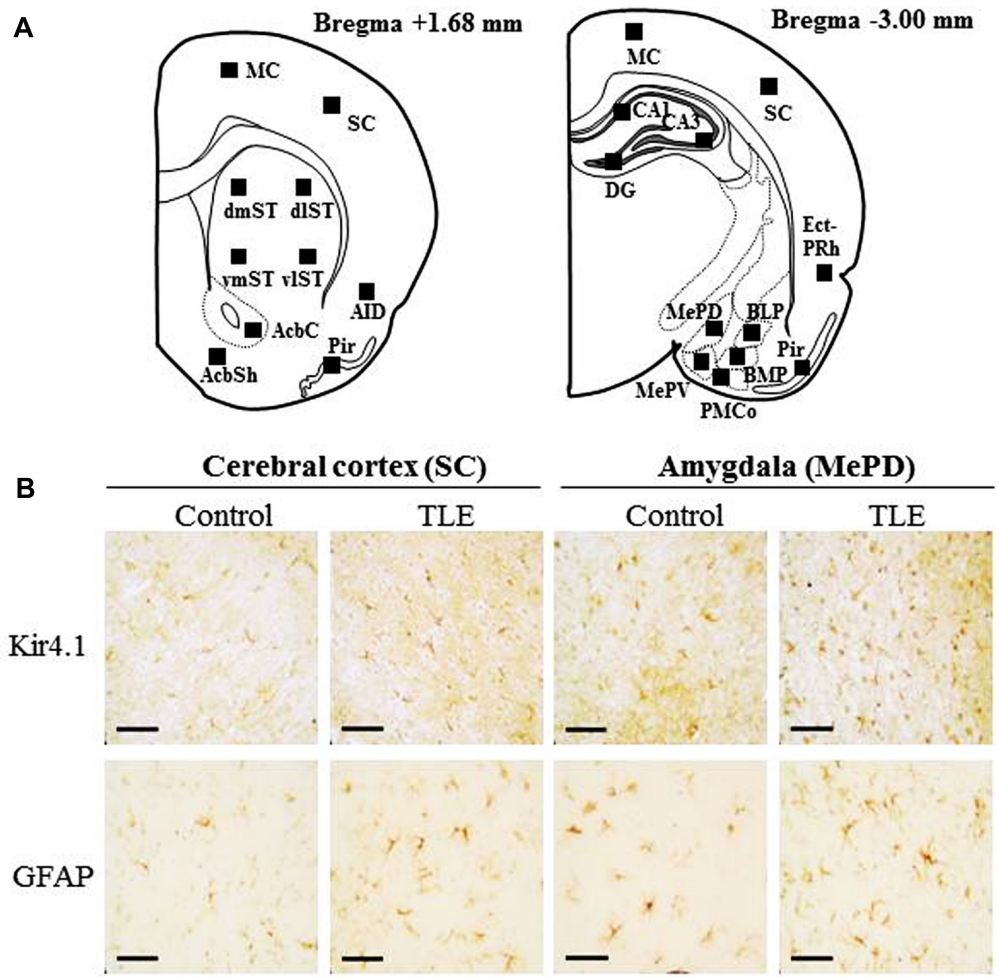

FIGURE 2 | Immunohistochemical analysis of Kir4.1- and GFAP-immunoreactivity (IR)-positive cells in pilocarpine-induced TLE rats. (A) Schematic illustrations of the brain sections selected for quantitative analysis of Kir4.1- and GFAP-IR-positive cells. Squares in each section indicate the area analyzed for counting of Kir4.1- and GFAP-IR-positive cells. The distance from the Bregma is shown on the bottom of each section. MC, motor cortex; SC, sensory cortex; AID, agranular insular cortex; Pir, piriform cortex; dmST, vmST, dlST and vlST, dorsomedial, ventromedial, dorsolateral, and ventrolateral striatum, respectively; AcbC and AcbSh, core and shell regions of nucleus accumbens, respectively; Ect-PRh, ectorhinal–perirhinal cortex; MePV and MePD, medial amygdaloid nucleus, posteroventral and posterodorsal part; BLP, basolateral amygdaloid nucleus, posterior part; BMP, basomedial amygdaloid nucleus, posterior part; PMCo, posteromedial cortical amygdaloid nucleus; CA1, CA3, and DG: CA1, CA3, and dentate gyrus of the hippocampus. **(B)** Representative photographs illustrating the Kir4.1 (upper panels)- and GFAP (lower panels)-positive cells in the sensory cortex (SC) and the medial amygdaloid nucleus, posterodorsal part (MePD). Scale bar: 50 μm.

specifically co-stained with glial fibrillary acidic protein (GFAP; an astrocyte marker; Harada et al., 2013; also see **Figure A1** in Appendix). Although Kir4.1-IR was also found in a small population of round-shaped (small) cells, which might possibly represent oligodendrocyte precursor cells (Maldonado et al., 2013), we omitted them from the analysis and solely counted the stellate-shaped astrocytes probe with anti-Kir4.1 antibody. In addition, to evaluate changes in the total number of astrocytes *per se* and the Kir4.1 expression ratio relative to the total number of astrocytes, we also performed immunohistochemical analysis of GFAP using paired successive slices obtained from the same animal.

In accordance with previous studies (Connors et al., 2004; Seifert et al., 2009; Harada et al., 2013), Kir4.1 was mostly expressed in stellate-shaped cells (**Figure 2B**). Two-way ANOVA revealed significant interaction groups × regions [$F(21, 264) = 1.91$, $P < 0.05$] and significant main effects of groups [$F(1, 264) = 410.45$, $P < 0.01$] and regions [$F(21, 264) = 3.50$, $P < 0.01$]. In pilocarpine-induced TLE rats, Kir4.1 expression was significantly elevated in all brain regions examined [dentate gyrus of the Hpc (DG) and dorsomedial St (dmST): $P < 0.05$, other regions: $P < 0.01$] except for the agranular insular cortex dorsal part (AID; **Figures 2–4**). The number of Kir4.1-IR-positive astrocytes increased two to four times the control levels in TLE animals and these changes were prominent in the sensory cortex (SC), lateral St, and amygdala (**Figures 3A** and **4A**). In addition, the number of GFAP-IR-positive astrocytes *per se* also increased in pilocarpine-induced TLE rats (**Figures 3B** and **4B**). Analysis

of GFAP expression showed significant main effects of groups [$F(1, 264) = 333.16$, $P < 0.01$] and regions [$F(21, 264) = 6.26$, $P < 0.01$] without a significant interaction [$F(1, 264) = 0.86$, $P = 0.65$]. The numbers of GFAP-IR-positive cells in all 22 brain regions examined were significantly [piriform cortex (Pir) B+1.68: $P < 0.05$, other regions: $P < 0.01$] higher in TLE than in control rats. We then compared the Kir4.1 expression ratios relative to the number of astrocytes (Kir4.1-IR-positive cells/GFAP-IR-positive cells). Two-way ANOVA revealed significant interaction [$F(21, 264) = 1.78$, $P < 0.05$] and significant main effects of groups [$F(1, 264) = 12.82$, $P < 0.01$] and regions [$F(21, 264) = 2.36$, $P < 0.01$]. The relative Kir4.1 expression ratios in astrocytes were 0.3–0.8 in most regions of the brain in the control animals (0.557 ± 0.022), but the values were significantly ($P < 0.01$) increased in TLE group (0.652 ± 0.019). These changes were region-specific and significant increases were observed in the posteroventral (MePV, $P < 0.05$) and posterodorsal (MePD, $P < 0.01$) parts of the medial amygdaloid nucleus, the posteromedial cortical amygdaloid nucleus (PMCo, $P < 0.05$), ventrolateal St (vlST, $P < 0.05$), SC ($P < 0.01$), and Pir ($P < 0.05$; **Figures 3C** and **4C**).

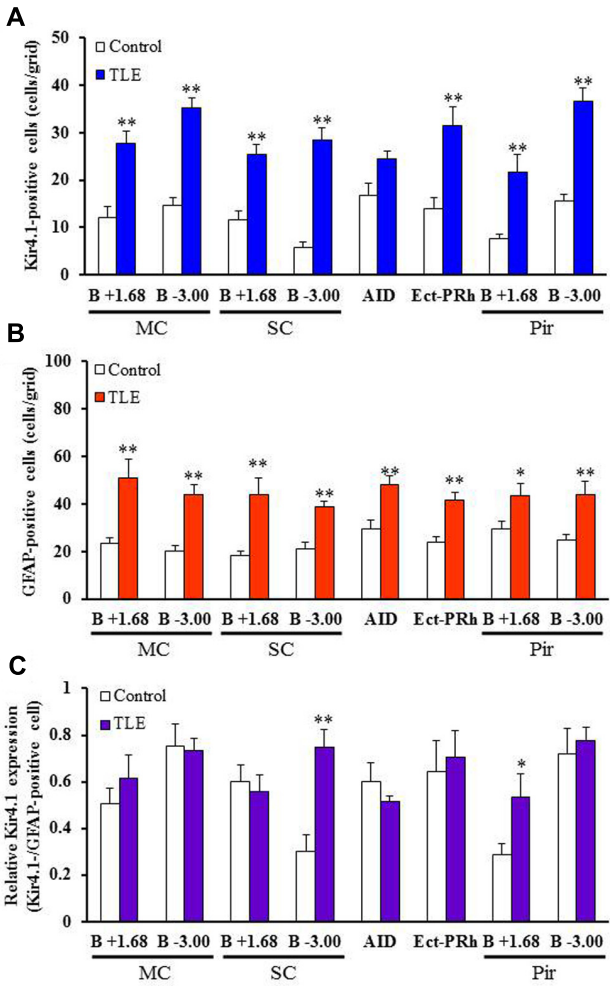

FIGURE 3 | Topographical expression of Kir4.1 and GFAP in the cortical regions of pilocarpine-induced TLE rats. (A,B) Number of Kir4.1 **(A)**- or GFAP **(B)**-immunoreactivity (IR)-positive cells. **(C)** Relative Kir4.1 expression ratios in astrocytes. A pair of successive slices in each region from the same animal was stained with anti-Kir4.1 or anti-GFAP antibody. The Kir4.1 expression ratios were calculated as the ratios of Kir4.1-positive astrocytes relative to the total number of astrocytes (Kir4.1-positive cells/GFAP-positive cells) in each animal. MC, motor cortex; SC, sensory cortex; AID, agranular insular cortex, dorsal part; Ect-PRh, ectorhinal-perirhinal cortex; Pir, piriform cortex. Each column represents the mean ± S.E.M. of seven animals. $*P < 0.05$, $**P < 0.01$, significantly different from control rats.

DISCUSSION

Temporal lobe epilepsy is the most common type of partial complex seizure in adulthood (Hauser et al., 1996; Wieser, 2004). The main features of TLE include (1) localization of seizure foci in the limbic structures (e.g., Hpc and amygdala), (2) existence of a "latent period," a seizure-free time interval following the initial precipitating injury, (3) incidence of mesial sclerosis leading to atrophy (e.g., neuronal loss and gliosis) in the limbic structures (Mathern et al., 1997; Bartolomei et al., 2005; Curia et al., 2008). The pilocarpine-induced TLE model shares important features of human TLE such as (1) presence of a latent period followed by spontaneous recurrent seizures, (2) occurrence of wide spread brain injuries resembling human TLE, (3) similarity of drug responses to human TLE (e.g., relatively resistant to conventional antiepileptics; Leite et al., 1990; Cavalheiro et al., 1991; Glien et al., 2002; Löscher, 2002; Wieser, 2004; Chakir et al., 2006; Curia et al., 2008). The present study demonstrated for the first time that expression of astrocytic Kir4.1 channels mediating spatial K^+ buffering was markedly elevated in a pilocarpine-induced TLE model. The elevation of Kir4.1 expression in the TLE model was characterized by the following points, (1) subunit-specificity for Kir4.1, (2) a partial association with an increase in the number of astrocytes (i.e., astrogliosis) and (3) the most prominent elevation in the amygdala.

In this study, Western blot analysis revealed that the pilocarpine-induced TLE model exhibits a subunit-specific increase in the Kir4.1 expression with negligibly affecting the level of Kir5.1 and Kir2.1 subunits. Kir5.1 subunits, like Kir4.1, are expressed in astrocytes and form heteromeric Kir4.1/5.1 channels with Kir4.1, mediating K^+ buffering (Tanemoto et al., 2000; Hibino et al., 2004; Kofuji and Newman, 2004). In contrast, Kir2.1 subunits are predominantly expressed in neurons to regulate the resting membrane potential while several reports show that astrocytes also express Kir2.1 to some degree in several brain regions (e.g., Pir and olfactory bulb; Howe et al., 2008; Kang et al., 2008). Our results suggest that, among astrocytic Kir channels, Kir4.1 channels play the most important role in modulating TLE epileptogenesis.

Elevation of Kir4.1 expression in the pilocarpine-induced TLE model was widely spread throughout brain regions examined and these changes were generally associated with an increase in the number of astrocytes, which was probably due to astrogliosis following status epilepticus-induced brain injury (Leite et al., 1990; Cavalheiro et al., 1991; Borges et al., 2003; Curia et al., 2008). Although astrogliosis may also contribute to epileptogenesis, it can compensate abnormal discharges and promote tissue repair. Astrocytes can reduce abnormal neural excitation

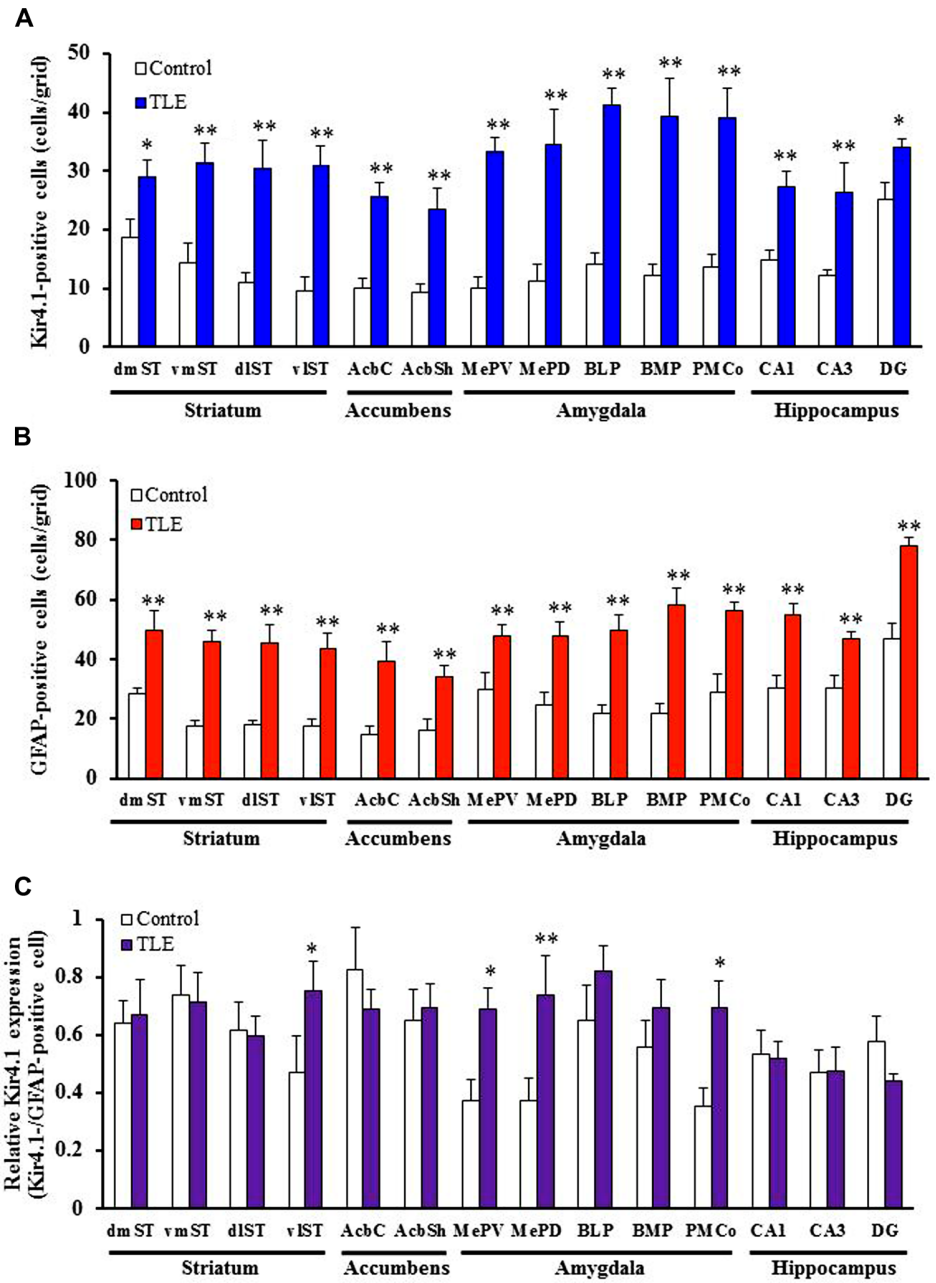

FIGURE 4 | Topographical expression of Kir4.1 and GFAP in the basal ganglia and limbic regions of pilocarpine-induced TLE rats. (A,B) Number of Kir4.1 **(A)**- or GFAP **(B)**-immunoreactivity (IR)-positive cells. **(C)** Relative Kir4.1 expression ratios in astrocytes. A pair of successive slices in each region from the same animal was stained with anti-Kir4.1 or anti-GFAP antibody. The Kir4.1 expression ratios were calculated as the ratios of Kir4.1-positive astrocytes relative to the total number of astrocytes (Kir4.1Kir4.1-positive cells/GFAP-positive cells) in each animal. dmST, vmST, dlST, and vlST, dorsomedial, ventromedial, dorsolateral, and ventrolateral striatum, respectively; AcbC and AcbSh, core and shell regions of the nucleus accumbens, respectively; MePV and MePD, medial amygdaloid nucleus, posteroventral and posterodorsal part; BLP, basolateral amygdaloid nucleus, posterior part; BMP, basomedial amygdaloid nucleus, posterior part; PMCo, posteromedial cortical amygdaloid nucleus; CA1, CA3, and DG, CA1, CA3, and dentate gyrus of the hippocampus. Each column represents the mean ± SEM of seven animals. $*P < 0.05$, $**P$ 0.01, significantly different from control rats.

by spatial buffering of potassium and by taking up synaptically released glutamate. In addition, they can secrete growth factors [e.g., glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF)] and cytokines (e.g., TNF-α) that mediate neuronal survival, axonal/dendritic sprouting, and homeostatic plasticity (Borges et al., 2003; Fellin, 2009). Thus, the up-regulation of Kir4.1 associated with status epilepticus-induced astrogliosis might negatively regulate the TLE epileptogenesis by normalizing extracellular K^+ ($[K^+]_o$) and glutamate ($[glutamate]_o$). Furthermore, significantly higher Kir4.1

expression ratios relative to the number of astrocytes (Kir4.1-IR-positive cells/GFAP-IR-positive cells) were observed region-specifically in the amygdaloid nuclei (i.e., MePV, MePD, and PMCo). These results illustrate the important role of amygdalar Kir4.1 channels in modifying status epileptics-induced epileptogenicity in TLE. Since deficit or knockdown of astrocytic Kir4.1 channels is known to impair K^+- and glutamate-uptake into astrocytes and facilitate seizure generation (Djukic et al., 2007; Kucheryavykh et al., 2007; Bockenhauer et al., 2009; Scholl et al., 2009; Reichold et al., 2010; Sala-Rabanal et al., 2010; Tang et al., 2010), up-regulation of Kir4.1 channels in the pilocarpine TLE model seemed to occur as a compensatory mechanism to the limbic hyperexcitability in TLE epileptogenesis. Indeed, the medial amygdaloid and cortical amygdaloid nuclei are known to be closely linked to kindling epileptogenesis and human epileptic disorders including TLE (Hosford et al., 1995; Morimoto et al., 2004). Although it is known that pilocarpine-induced status epilepticus causes neural damage, sclerosis, and rewiring not only in the amygdala, but also in the Hpc, changes in the relative Kir4.1 expression ratios were not significant in the Hpc (i.e., CA2). This may be due to the relatively low expression level of Kir4.1 in the Hpc as compared to other brain regions (see **Figure 2**).

Evidence is accumulating that dysfunction of astrocytic Kir4.1 channels is causative of seizure activity generation. Specifically, loss-of-function mutations in human Kir4.1 gene (*KCNJ10*) cause the EAST syndrome, including GTC seizures and ataxia (Bockenhauer et al., 2009; Scholl et al., 2009; Reichold et al., 2010; Sala-Rabanal et al., 2010; Tang et al., 2010). It is also suggested that the down-regulation of Kir4.1 expression in the amygdala is related to seizure induction in an animal model of GTC seizures (Harada et al., 2013). Furthermore, recent studies showed the down-regulation and/or impaired functioning of Kir4.1 channels in specimens from patients with TLE (Das et al., 2012; Heuser et al., 2012; Steinhäuser et al., 2012), suggesting a close relationship of Kir4.1 to human TLE. The present results (Kir4.1 up-regulation) in the pilocarpine-induced TLE model, however, were different from the findings of Kir4.1 expression (Kir4.1 down-regulation) in patients with TLE. Although the reasons for this discrepancy are currently uncertain, it may result from the difference in the etiological basis between human TLE and pharmacologically evoked seizure. In fact, we also observed that Kir4.1 expression in the paralimbic cortex was gradually increased during the kindling development induced by pentylentetrazole (Mukai et al., 2013). Alternatively, it may be due to the temporal changes in Kir4.1 expression. Since the present study analyzed the Kir4.1 expression shortly after the occurrence of spontaneous seizures, the down-regulation of Kir4.1 may occur at a more advanced (delayed) stage in the pilocarpine-induced TLE model. Indeed, a recent study showed that Kir4.1 expression was down-regulated by local inflammatory events after TLE-associated brain injury, implying that the down-regulation of Kir4.1 could be a consequence, and not a primary cause, of seizures (Zurolo et al., 2012). Further studies are required to delineate the time course of the Kir4.1 expressional changes and the mechanisms underlying the Kir4.1 up-regulation in the pilocarpine-induced TLE model.

In conclusion, we performed expressional analysis of Kir4.1 in a pilocarpine-induced rat model of TLE to explore the pathophysiological role of Kir4.1 channels in epileptogenesis. Western blot analysis revealed that Kir4.1 levels of TLE rats under an interictal state were significantly increased in the cerebral cortex, St, and Ht while the levels of other Kir subunits, Kir5.1 and Kir2.1, were unaltered. Immunohistochemical analysis demonstrated that TLE rats showed a widespread elevation in Kir4.1 expression which accompanied an increase in the number of astrocytes *per se*. In addition, the Kir4.1 expression ratio relative to the increase in the astrocyte number was also elevated region-specifically in the amygdaloid nuclei in a pilocarpine TLE model. The present findings suggest that astrocytic Kir4.1 channels play a modulatory role in TLE epileptogenesis, possibly by acting as an inhibitory compensatory mechanism. Further studies using patch-clamp and/or microdialysis techniques are necessary to delineate the functional alterations (e.g., changes in Kir4.1-mediated potassium currents, extracellular levels of K^+ and glutamate) of up-regulated Kir4.1 channels in the TLE model.

MATERIALS AND METHODS

ANIMALS

Male SD rats (7 weeks old; Japan SLC, Shizuoka, Japan) were used. Animals were kept in air-conditioned rooms under a 12-h light/dark cycle (light on: 6:00 AM) and allowed *ad libitum* access to food and water. The housing conditions of the rat and animal care methods complied with the NIH guide for the care and use of laboratory animals. The experimental protocols of this study were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences.

PILOCARPINE-INDUCED TLE MODEL

A pilocarpine-induced TLE model was prepared according to methods reported previously (Cavalheiro, 1995; Liu et al., 2008). Briefly, animals were first treated with methyl-scopolamine (1 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA) to reduce peripheral cholinergic side effects and, 30 min later, pilocarpine (350 mg/kg, i.p., Sigma-Aldrich) was injected to induce acute status epilepticus. Pilocarpine-induced status epilepticus was then terminated by the injection of diazepam (10 mg/kg, i.p., CERCINE® INJECTION, Takeda Pharmaceutical Co. Ltd., Osaka, Japan) at 5, 20, 80, 300, and 420 min after the onset of status epilepticus (repeated and sustained clonic seizures). Animals which did not show any seizure activity (status epileptics) within 20 min after the pilocarpine injection were used as the control and treated with diazepam in the same manner as the status epilepticus-experienced rats. All animals were fed for 7–8 weeks after the pilocarpine treatment. Eleven out of the twelve rats which experienced pilocarpine-induced status epilepticus showed spontaneous seizures, (i.e., wild running/jumping and GTC seizures) and were defined as TLE rats. One animal which showed pilocarpine-induced status epilepticus but did not any spontaneous seizure was excluded from the analysis. None of the control animals ($N = 11$) showed any seizures or abnormal behavior during the 7–8 weeks observation period.

WESTERN BLOT ANALYSIS

Temporal lobe epilepsy rats under interictal conditions ($N = 4$) or control rats ($N = 4$) were deeply anesthetized with pentobarbital (80 mg/kg, i.p.). The brain was then removed from the skull, chilled in ice-cold saline and dissected into the following 10 regions (fCx, ptCx, otCx, St, Hpc, Th, Ht, Mid, P/MO, and Cer). Brain samples were then homogenized in an ice-cold lysis buffer (pH 7.5) containing: (in mM) Tris 20, NaCl 150, $MgCl_2$ 10, EDTA 1.0, EGTA 1.0, 1% Triton X-100, and a mixture of protease inhibitors (leupeptin, aprotinin, E-64, pepstatin A, bestatin, and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; Nacalai Tesque, Kyoto, Japan). The homogenate was centrifuged at 15,000*g*, 4°C for 30 min and the supernatant was stored at −80°C for the Western blot analysis.

Western blots were performed as published previously (Ohno et al., 2009; Harada et al., 2013). Briefly, samples were incubated with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 5 min at 95°C. Each sample (40 μg/lane) was then subjected to SDS-PAGE and separated proteins were transferred for 60 min to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was first incubated with a blocking solution containing 0.3–2% skim milk, 25 mM Tris, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5) for 60 min, then with the corresponding primary antibodies overnight (4°C), followed by a 60 min-incubation with the secondary antibody, a goat anti-rabbit IgG-HRP conjugate (1:2000, Santa Cruz Biotechnology, CA, USA) for Kir4.1, a donkey anti-goat IgG-HRP conjugate (1:2000, Santa Cruz Biotechnology) for Kir5.1 or Kir2.1, or a sheep anti-mouse IgG-HRP conjugate (1:2000, GE Healthcare) for β-actin. The primary antibodies used were a rabbit polyclonal antibody against Kir4.1 (1:500, Alomone Labs., Jerusalem, Israel), a goat polyclonal antibody against Kir5.1 (N-12; 1:400, Santa Cruz Biotechnology), a goat polyclonal antibody against Kir2.1 (1:400, Santa Cruz Biotechnology) and mouse monoclonal antibodies against β-actin (1:1000, Sigma-Aldrich). Final detection was performed with the enhanced chemiluminescence methodology (Amersham ECL Western blotting detection reagents and analysis system, GE Healthcare) using a lumino imaging analyzer (LAS-3000, FUJIFILM, Tokyo, Japan). To normalize for protein loading, chemiluminescence of the bands in each lane was standardized to the intensity of the β-actin band in the same lane.

IMMUNOHISTOCHEMICAL ANALYSIS

Brains were obtained from TLE rats (interictal status; $N = 7$) or control rats ($N = 7$) in the same manner as for the Western blot analysis. After fixation in a 4% paraformaldehyde solution for 24 h, brain samples were dehydrated and embedded in paraffin. Formalin-fixed and paraffin-embedded tissue samples were cut into 4-μm thick sections and a pair of successive slices in each brain region was immunohistochemically stained with anti-Kir4.1 or anti-GFAP antibody using the avidin–biotin complex (ABC) method (Ohno et al., 2009, 2012; Harada et al., 2013). Briefly, the fronto- and occipito-temporal brain sections were deparaffinized in xylene and then rehydrated in ethanol. Sections were autoclaved for 10 min to retrieve the antigen. After cooling to room temperature, endogenous peroxidase activity was quenched by 3% H_2O_2 and non-specific binding was blocked using a 5% skim milk solution. Sections were then incubated overnight (4°C) with a rabbit anti-Kir4.1 antibody (1:100, Alomone Labs) and a mouse anti-GFAP antibody (1:100, Progen) in the 5% skim milk solution. Thereafter, they were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (1:400, Vector Laboratories, Burlingame, CA, USA) and a goat anti-mouse IgG secondary antibody (1:400, Sigma-Aldrich) for 60 min and with an avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Kit) for an additional 60 min. Kir4.1- and GFAP-IR was visualized by the diaminobenzidine–nickel staining method.

The number of Kir4.1- or GFAP-IR-positive cells was counted in a 350 × 350 μm^2 grid laid over various regions of the brain (**Figure 4**), which included the following regions: the motor cortex (MC), SC, AID, ectorhinal–perirhinal cortex (Ect-PRh), Pir, dorsolateral St (dlST) and dmST, vlST and ventromedial St (vmST), core (AcbC) and shell (AcbSh) regions of the nucleus accumbens, MePV, MePD, basolateral amygdaloid nucleus posterior part (BLP), basomedial amygdaloid nucleus posterior part (BMP), PMCo, and CA1, CA3, and the DG of the Hpc. Relative expression rate of Kir4.1 was defined as a percentage of the number of Kir4.1-positive cells relative to that of GFAP-positive cells.

STATISTICAL ANALYSIS

All data are expressed as the mean ± SEM. Expressional changes in Kir channel subunits determined by Western blot or immunohistochemical analysis were compared by two-way ANOVA followed by Tukey multiple comparison test. Differences were considered to be statistically significant for values of $P < 0.05$.

ACKNOWLEDGMENTS

This work was partly supported by a Grant in Aid for Scientific Research (22590092) from the Ministry of Education, Science, Sports and Culture of Japan and a research grant from the Japan Epilepsy Research Foundation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2013; accepted: 16 June 2013; published online: 05 July 2013.

*Citation: Nagao Y, Harada Y, Mukai T, Shimizu S, Okuda A, Fujimoto M, Ono A, Sakagami Y and Ohno Y (2013) Expressional analysis of the astrocytic Kir4.1 channel in a pilocarpine-induced temporal lobe epilepsy model. Front. Cell. Neurosci. **7**:104. doi: 10.3389/fncel.2013.00104*

APPENDIX

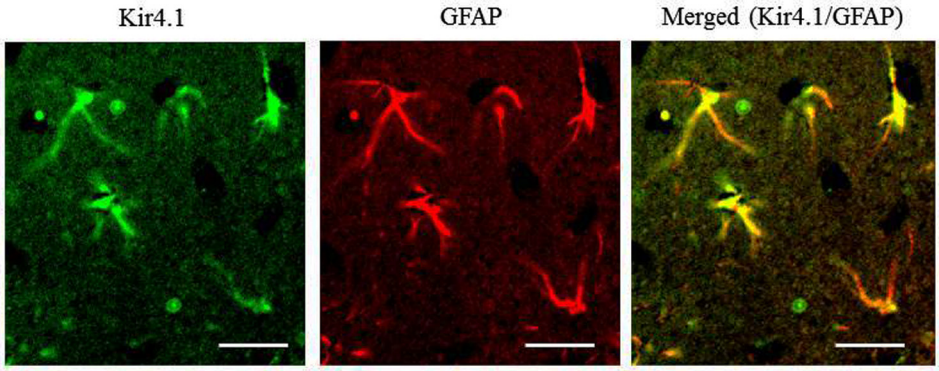

FIGURE A1 | Expressional patterns of Kir4.1 in the rat hippocampus. Typical photograph illustrating a double staining of Kir4.1 with GFAP in the hippocampal CA1 field. The hippocampal section was incubated anti-GFAP antibody (Progen, Heidelberg, Germany) for 24 h at 4°C and then incubated with a FITC (fluorescein isothiocyanate; green fluorescence)-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich) or TRITC (tetramethylrhodamine-5- (and 6)-isothiocyanate; red fluorescence)-conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich) to probe Kir4.1 and GFAP, respectively. Immunofluorescence images were obtained with a confocal laser scanning microscope. Scale bar: 50 μm. All the Kir4.1-immunoreactivity (IR)-positive cells with the stellate-shape were double-stained with anti-GFAP antibody (yellow in a merged picture). Kir4.1-IR was occasionally found in a few round-shaped cells with no GFAP-IR [also negative to a neuronal marker, neuronal nuclear antigen (NeuN)], which were omitted from the analysis.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 31 July 2013
doi: 10.3389/fncel.2013.00115

Role of hormones and neurosteroids in epileptogenesis

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This article describes the emerging evidence of hormonal influence on epileptogenesis, which is a process whereby a brain becomes progressively epileptic due to an initial precipitating event of diverse origin such as brain injury, stroke, infection, or prolonged seizures. The molecular mechanisms underlying the development of epilepsy are poorly understood. Neuroinflammation and neurodegeneration appear to trigger epileptogenesis. There is an intense search for drugs that truly prevent the development of epilepsy in people at risk. Hormones play an important role in children and adults with epilepsy. Corticosteroids, progesterone, estrogens, and neurosteroids have been shown to affect seizure activity in animal models and in clinical studies. However, the impact of hormones on epileptogenesis has not been investigated widely. There is emerging new evidence that progesterone, neurosteroids, and endogenous hormones may play a role in regulating the epileptogenesis. Corticosterone has excitatory effects and triggers epileptogenesis in animal models. Progesterone has disease-modifying activity in epileptogenic models. The antiepileptogenic effect of progesterone has been attributed to its conversion to neurosteroids, which binds to GABA-A receptors and enhances phasic and tonic inhibition in the brain. Neurosteroids are robust anticonvulsants. There is pilot evidence that neurosteroids may have antiepileptogenic properties. Future studies may generate new insight on the disease-modifying potential of hormonal agents and neurosteroids in epileptogenesis.

Keywords: epilepsy, epileptogenesis, neurosteroid, estrogen, progesterone, kindling

INTRODUCTION

Epilepsy, one of the most common serious neurological disorders, is characterized by the unpredictable occurrence of seizures. A seizure is an abnormal electrical discharge in the brain that causes an alteration in consciousness, sensations, and behaviors. The symptoms that occur depend on the parts of the brain affected during the seizure. Common signs of seizures include staring, unusual feelings, twitching, unconsciousness, and jerking in the arms or legs. Epilepsy affects an estimated 3 million Americans and about 65 million people worldwide in a variety of ways (Jacobs et al., 2009; Hesdorffer et al., 2013). About 150,000 new cases of epilepsy are diagnosed in the United States annually (Hesdorffer et al., 2013). Children and adults are the fastest−growing segments of the population with new cases of epilepsy.

Epilepsy is a collective designation for a group of brain disorders consisting of a complex spectrum of different seizure types and syndromes. Epileptic seizures are classified into partial (simple and complex partial seizures) and generalized seizures (absence, tonic–clonic, myoclonic, and atonic seizures). Accurate diagnosis of seizure type and epileptic syndrome is critical for determining appropriate drug therapy and prognosis. The International League Against Epilepsy (ILAE) provided a definition of "seizure" and "epilepsy" (Fisher et al., 2005). A seizure is defined as "a transient occurrence of signs and/or symptoms due to abnormal synchronous neuronal activity in the brain." Epilepsy is defined as "a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures." A single seizure, therefore, does not constitute epilepsy. The diagnosis of epilepsy requires the occurrence of recurrent (two or more) epileptic seizures separated by at least 24 h, unprovoked by any immediate identified cause. Antiepileptic drugs (AEDs) are the mainstay for controlling seizures (**Table 1**). Current drug therapy is symptomatic in that available drugs inhibit seizures, but neither effective prophylaxis nor cure are available. The goal of the therapy is to eliminate seizures without interfering with normal function (Glauser et al., 2006, 2013). Despite many advances in epilepsy research, presently an estimated 30% of people with epilepsy have "intractable seizures" that do not respond to even the best available medication. There is renewed focus on the pathophysiology of epileptogenesis, the process whereby a brain becomes progressively epileptic due to an initial precipitating event.

This article describes the emerging evidence of hormonal influence on epileptogenesis and the potential mechanisms underlying their actions on neuronal excitability and seizure activity. It also describes recent studies on neurosteroid agents that prevent or delay the development of epilepsy. The main focus of the review is on steroid hormones and neurosteroids with seizure-modulating activity. Neuropeptides and other hormones such as oxytocin, neuropeptide-Y, and galanin, which may affect neuronal excitability, are not discussed here because such description is beyond the scope of this article. The seizure-modulating effects of neuropeptides are discussed elsewhere (Robertson et al., 2011).

Table 1 | List of current antiepileptic drugs.

Standard (first generation)	Newer (second generation)
Carbamazepine (Tegretol)	Acetazolamide (Diamox)
Clonazepam (Klonopin)	Clobazam (Onfi)
Chlorazepate (Tranxene)	Ezogabine (Potiga)
Diazepam (Valium)	Felbamate (Felbatol)
Divalproex sodium (Depakote)	Fosphenytoin (Cerebyx)
Ethosuximide (Zarontin)	Lacosamide (Vimpat)
Ethotoin (Peganone)	Lamotrigine (Lamictal)
Lorazepam (Ativan)	Levetiracetam (Keppra)
Mephobarbital (Mebaral)	Oxcarbazepine (Trileptal)
Methsuximide (Celontin)	Parampanel (Fycompa)
Nitrazepam (Mogadon)	Pregabalin (Lyrica)
Phenobarbital (Gardinal)	Progabide (Gabrene)
Phenytoin (Dilantin)	Rufinamide (Banzel)
Primidone (Mysoline)	Tiagabine (Gabitril)
Valproic acid (Depakene)	Topiramate (Topamax)
	Vigabatrin (Sabril)
	Zonisamide (Zonegran)

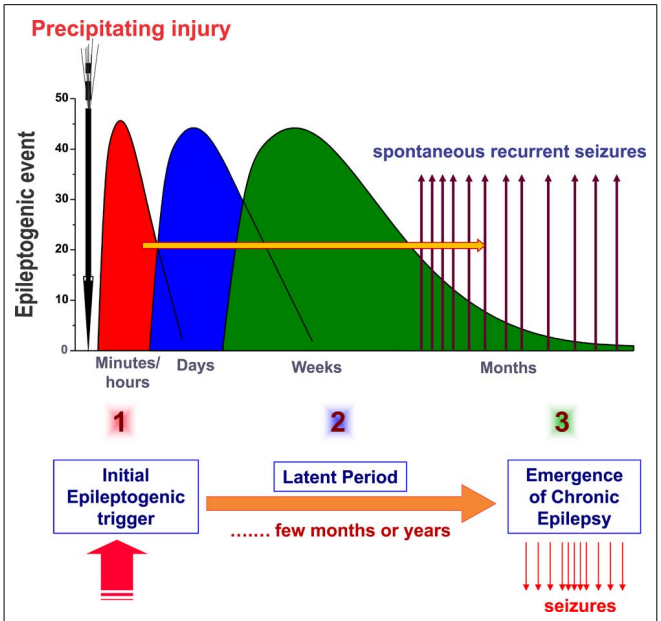

FIGURE 1 | Pathophysiology of epileptogenesis. Epileptogenesis is the process whereby a normal brain becomes progressively epileptic because of precipitating injury or risk factors such as TBI, stroke, brain infections, or prolonged seizures. Epilepsy development can be described in three stages: (1) the initial injury (epileptogenic event); (2) the latent period (silent period with no seizure activity); and (3) chronic period with spontaneous recurrent seizures. Although the precise mechanisms underlying spatial and temporal events remain unclear, epileptogenesis may involve an interaction of acute and delayed anatomic, molecular, and physiological events that are both complex and multifaceted. The initial precipitating factor activates diverse signaling events, such as inflammation, oxidation, apoptosis, neurogenesis, and synaptic plasticity, which eventually lead to structural and functional changes in neurons. These changes are eventually manifested as abnormal hyperexcitability and spontaneous seizures.

OVERVIEW OF EPILEPTOGENESIS AND INTERVENTION STRATEGIES

Epilepsy is a chronic condition with many possible causes. Epilepsy may develop because of an abnormality in neural connectivity, an imbalance in inhibitory and excitatory neurotransmitters, or some combination of these factors. Primary epilepsy (50%) is idiopathic ("unknown cause"). In secondary epilepsy (50%), seizures may result from a variety of conditions including trauma, anoxia, metabolic imbalances, tumors, encephalitis, drug withdrawal, and neurotoxicity (Engel et al., 2007). The molecular mechanisms underlying the development of acquired epilepsy are not very well understood. The term "epileptogenesis" is used to describe the complex plastic changes in the brain that, following a precipitating event, convert a normal brain into a brain debilitated by recurrent seizures (Pitkänen et al., 2009; Pitkänen and Lukasiuk, 2011). Although specific types of epilepsy may have unique pathophysiological mechanisms, a broad hypothesis in this field is that convergent neuronal mechanisms are common in different forms of acquired epilepsy.

The current hypothesis about the pathogenesis of epilepsy (epileptogenesis) involves three stages: (1) the initial precipitating event; (2) the latent period (no seizures); and (3) the chronic period with spontaneous seizures (**Figure 1**). Acquired epilepsy typically develops due to an initial precipitating event such as traumatic brain injury (TBI), stroke, brain infections, or prolonged seizures. The other possible precipitating triggers for epileptogenesis include febrile seizures, metabolic dysfunction, alcohol withdrawal, and status epilepticus, an emergency condition characterized by continuous seizures or repeated seizures without regaining consciousness for 30 min or more (McClelland et al., 2011). Exposure to organophosphorous pesticides and chemical warfare nerve agents, such as soman, can cause epilepsy as a result of cholinergic neurotoxicity and status epilepticus (de Araujo Furtado et al., 2012).

The development of epileptogenesis is thought to be a step-function of time after the brain injury, with a latent period present between the brain injury and the first unprovoked seizure. There are some alternative hypotheses to this notion that view epileptogenesis as a continuous process that extends past the first spontaneous seizure (Dudek and Staley, 2011). Temporal lobe epilepsy (TLE) is one of the common forms of chronic epilepsies (Wieser and ILAE Commission on Neurosurgery of Epilepsy, 2004). TLE is characterized by the progressive expansion of spontaneous seizures originating from the limbic system regions, especially the hippocampus, most often due to neuronal injury. The hippocampal sclerosis, which is characterized by aberrant mossy fiber sprouting and widespread neuronal loss in the dentate hilus and CA1 and CA3 subfields, is the hallmark of epilepsy pathology (Sutula et al., 1989; Buckmaster et al., 2002; Nadler, 2003; Morimoto et al., 2004). However, there is an ongoing debate about whether the hippocampal sclerosis is the basis or the outcome of recurring seizures. There is emerging evidence from refractory models that epilepsy involves "progressive epileptogenesis" much beyond the latent period and the onset of the

first seizure (Williams et al., 2009). Several mechanisms have been described including loss of interneurons (van Vliet et al., 2004; Sloviter and Bumanglag, 2013) and neuroinflammation (Vezzani et al., 2011). There is great variation in the onset of spontaneous seizures following a precipitating factor (Rao et al., 2006; Norwood et al., 2010). Thus, the critical window for effective "antiepileptogenic" interventions remain poorly defined for curing epilepsy in people at risk.

Studies in animal models have provided improved understanding of neurophysiological basis of epileptic seizures (O'Dell et al., 2012; Simonato et al., 2012). Spontaneous seizures arise from hyperexcitable and hypersynchronous neuronal networks and involve both cortical and several key subcortical structures. The general cellular pathway underlying occurrence of epileptic seizures is apparent in three phases: (i) focal epileptogenicity (initiation); (ii) synchronization of the surrounding neurons (sync); and (iii) propagation of the seizure discharge to other areas of the brain (spread). Experimental field and intracellular recordings in isolated brain sections provide a detailed description of neurophysiological abnormalities underlying epileptic regions. Paroxysmal depolarization shift (PDS) is identified as a hallmark of epileptic neurons. It is characteristic of neurons in epileptic cortical zones and consists of an abnormally prolonged depolarization with repetitive spiking reflected as interictal discharges in the electroencephalogram (EEG). High-frequency oscillations, termed ripples (80–200 Hz) and fast ripples (250–600 Hz), are recorded in the EEG of epileptic patients and in animal epilepsy models (Köhling and Staley, 2011; Lévesque et al., 2011). Fast ripples are thought to reflect pathological activity and seizure onset zones.

Gowers (1881) proposed that *seizures beget seizures*. The kindling model has provided a conceptual framework for this idea and for developing new molecular targets for preventing epilepsy (Goddard et al., 1969; McNamara et al., 1992). Post-status epilepticus paradigms are widely used for modeling the epileptogenesis in which a single episode of prolonged seizures (by pilocarpine, kainate, or electrical stimulation) triggers progressive development of seizure activity (Buckmaster and Dudek, 1997; Hellier et al., 1999; Glien et al., 2001; Rao et al., 2006; Löscher, 2012). These chronic models share many features of human limbic epilepsy (Löscher, 2002; Stables et al., 2002). Pilocarpine, kainate, or perforant path stimulation induce acute SE and neuronal injury and follow a pattern of latent period similar to that observed in limbic epilepsy. Like TLE, mossy fiber sprouting, neurodegeneration, and ectopic granule cell proliferation are evident after the latent period (Löscher, 2002; Rao et al., 2006).

Despite decades of research, currently there is no single Food and Drug Administration (FDA)-approved drug that truly prevents the development of epilepsy in people at risk. A variety of intervention approaches have been tested in animal models of epileptogenesis (Acharya et al., 2008; Pitkänen and Lukasiuk, 2011). A number of clinical trials show a lack of antiepileptogenic efficacy of AEDs, including phenytoin and carbamazepine, in patients at high risk for developing epilepsy (Temkin, 2001; Mani et al., 2011). There is a desperate need for drugs that truly prevent the development of epilepsy ("antiepileptogenic agents") or alter its natural course to delay the appearance or severity of epileptic seizures ("disease-modifying agents").

In 2000, National Institute of Neurological Disorders and Stroke (NINDS) and epilepsy research and advocacy groups organized the first "Curing Epilepsy" conference, which marked a turning point for shifting and expanding the focus of epilepsy research toward cures for epilepsy and the prevention of epilepsy in those at risk (Jacobs et al., 2001). During the past decade, there has been increasing research emphasis on the prevention of epileptogenesis and translation of lead discoveries in this field into therapies for curing epilepsy (Jacobs et al., 2009; Simonato et al., 2012). The Institute of Medicine (IOM) released a consensus report in 2012 on public health dimensions of the epilepsies focusing on promoting health and understanding epilepsy (Austin et al., 2012; Hesdorffer et al., 2013). The IOM report, *Epilepsy Across the Spectrum: Promoting Health and Understanding*, provided 13 recommendations for future work in the field of epilepsy. The report contains research priorities which include one key recommendation on prevention of epilepsy.

ROLE OF STEROID HORMONES IN EPILEPTOGENESIS

Steroid hormones play a key role in the neuroendocrine control of neuronal excitability and seizure susceptibility (**Table 2**; Herzog, 2002; Reddy, 2003a, 2010; Verrotti et al., 2007). Steroid hormones are synthesized and secreted from ovarian, gonadal, and adrenal sources. In men, the main circulating steroids are androgenic steroids (testosterone and dihydrotestosterone) and adrenal corticosteroids (cortisol and aldosterone). Deoxycorticosterone (DOC) is also released from adrenal cortex in response to stress. In women, the primary reproductive steroid hormones are estrogens and progesterone, which are released during the menstrual cycle. The early follicular phase is associated with low levels of estrogens and progesterone. The synthesis and secretion of estrogens and progesterone from the ovaries are controlled primarily by hypothalamic gonadotropin releasing hormone (GnRH) and the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). As ovulation approaches, the level of estrogen rises and triggers a large surge of LH leading to ovulation.

Table 2 | List of steroid hormones and neurosteroids that affect seizure susceptibility.

Anticonvulsant steroids	Proconvulsant steroids
Progesterone	Estradiol
Allopregnanolone	Pregnenolone sulfate
Pregnanolone	DHEA sulfate
Dihydroprogesterone	Cortisol
Androstanediol	11-Deoxycortisol
Etiocholanone	
Dihydrotestosterone	
Deoxycorticosterone	
Dihydrodeoxycorticosterone	
Allotetrahydrodeoxycorticosterone	

Following ovulation, the ruptured follicle luteinizes and forms a corpus luteum that secretes progesterone and estrogen. Estradiol is secreted in the second half of the follicular phase and increases to a peak at midcycle, while progesterone is elevated during the luteal phase and declines before menstruation begins.

The cyclical changes of estrogens and progesterone are now widely believed to be important in the pathogenesis of catamenial epilepsy, a menstrual cycle-related seizure disorder in women with epilepsy (Reddy, 2009a, 2013). Catamenial epilepsy is a multifaceted neuroendocrine condition in which seizures are clustered around specific points in the menstrual cycle, most often around perimenstrual or periovulatory period. Generally, estrogens are found to be excitatory or proconvulsant, while progesterone has powerful antiseizure effect and reduces seizures, and thus they play a central role in the pathophysiology of epilepsy in women (Reddy, 2009a, 2013). Progesterone is an intermediate precursor for the synthesis of neurosteroids, which are increased in parallel during the ovarian cycle. There is emerging evidence that endogenous neurosteroids influence seizure susceptibility and epileptogenesis (Reddy, 2011; Reddy and Rogawski, 2012).

PROGESTERONE

Progesterone is an endogenous anticonvulsant hormone with substantial impact on seizure susceptibility. The potential molecular pathways for the progesterone modulation of seizure activity are illustrated in **Figure 2**. Progesterone is an appealing hormone for prophylactic interventions on epilepsy development, due to its multifunctional modulatory actions in the brain. Progesterone has long been known to have antiseizure activity in animal models (Selye, 1942; Craig, 1966; Kokate et al., 1999a; Frye and Scalise, 2000; Reddy et al., 2004), and in clinical studies (Bäckström et al., 1984; Herzog, 1995, 1999). Women with epilepsy are prone to

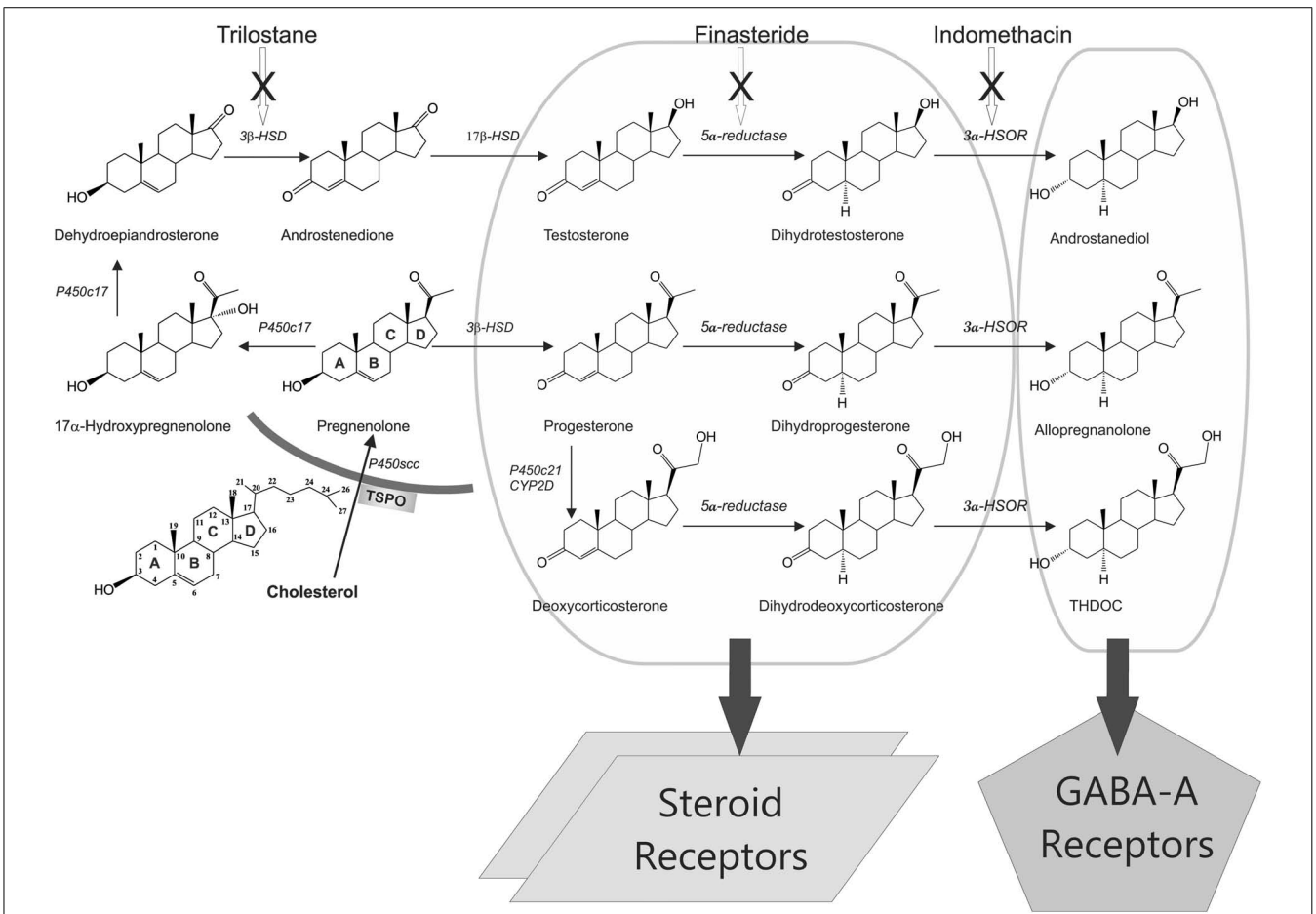

FIGURE 2 | Biosynthesis and targets of steroid hormones and neurosteroids in the brain. Enzymatic pathways for the production of three prototype neurosteroids allopregnanolone, THDOC, and androstanediol are illustrated from cholesterol. Steroid hormones progesterone, deoxycorticosterone, and testosterone undergo two sequential A-ring reduction steps catalyzed by 5α-reductase and 3α-HSOR to form the 5α,3α-reduced neurosteroids. The conversion of intermediate precursor steroids into neurosteroids occurs in the hippocampus and several other regions within the brain, where they can affect neuronal function. As evident from the pathways, many modifications are made by the same enzymes, which can be blocked by specific inhibitors (trilostane, finasteride, and indomethacin). There are two mechanisms by which steroid hormones affects neuronal function: (i) binding to steroid receptors (PRs, ARs, or MRs; left panel) and (ii) conversion to GABA-A receptor-modulating neurosteroids (right panel). Progesterone, deoxycorticosterone, or testosterone binding to their cognate steroid receptors could lead to activation of gene expression in the brain. Neurosteroids rapidly modulate neuronal excitability by direct interaction with inhibitory GABA-A receptors in the brain.

seizures in response to decreased levels of progesterone during perimenstrual periods (Herzog et al., 1997; Reddy, 2009a). Indeed, the incidence of epilepsy is generally lower in women than in men (Hauser et al., 1993; Christensen et al., 2005; McHugh and Delanty, 2008). This gender difference could be caused by ovarian hormones such as progesterone. Although progesterone is known to inhibit stimulation-evoked seizures in kindling models (Holmes and Weber, 1984; Mohammad et al., 1998; Lonsdale and Burnham, 2003), it has not been investigated widely for potential disease-modifying effect in epileptogenic models. In the kindling model, progesterone has been shown to impair or retard epileptogenesis (Holmes and Weber, 1984; Edwards et al., 2001; Reddy et al., 2010).

Recently progesterone has been evaluated in an National Institutes of Health (NIH)-sponsored, multicenter clinical trial as a treatment for epilepsy in women (Herzog et al., 2012). This randomized, double-blind, placebo-controlled, phase III, multicenter, clinical trial compared the efficacy and safety of adjunctive cyclic natural progesterone therapy versus placebo treatment of intractable seizures in 294 subjects randomized 2:1 to progesterone or placebo, stratified by catamenial and non-catamenial status. The results indicate lack of significant difference in proportions of responders between progesterone and placebo groups. However, a more restricted analysis on a subset of the data found a significantly higher responder rate in women with perimenstrual seizure exacerbation. These findings suggest that progesterone may provide a clinically important benefit for a subset of women with perimenstrual catamenial epilepsy. The dramatic response to progesterone, which is a neurosteroid precursor, in women with perimenstrual catamenial epilepsy is attributable to the unique neurosteroid sensitivity of perimenstrual catamenial seizures (Reddy, 2009a).

Previous studies have shown that progesterone supports the normal development of neurons, and that it reduces the extent of brain damage after TBI (Roof et al., 1994; Cutler et al., 2005, 2007; Stein, 2013). It has been observed in animal models that females have reduced susceptibility to TBI and this protective effect has been hypothesized to be caused by increased circulating levels of progesterone in females (Roof and Hall, 2000; Meffre et al., 2007). A number of additional studies have confirmed that progesterone has neuroprotective effects (Gibson et al., 2008; Singh and Su, 2013). Promising results have also been reported in human clinical trials. Recently, two clinical studies have evaluated progesterone as a treatment for moderate to severe TBI (Wright et al., 2007; Xiao et al., 2008). These studies demonstrated the efficacy of progesterone as a neuroprotective agent in TBI. Progesterone is highly efficacious in reducing disability and death in TBI. Progesterone has neuroprotective properties in acute models of ischemic injury, stroke, and astroglial dysfunction (Koenig et al., 1995; Jiang et al., 1996; He et al., 2004), suggesting its beneficial effects in brain injury.

Progesterone targets multiple molecular and cellular mechanisms relevant to epileptogenesis, and may therefore be a natural disease-modifying agent. Progesterone's cellular actions are mediated by the progesterone receptors (PRs), which are expressed in the hypothalamus, neocortex, hippocampus, and limbic areas (Brinton et al., 2008). Progesterone is an intermediate precursor for the synthesis of neurosteroids (see Role of Neurosteroids in Epileptogenesis). Progesterone's antiseizure activity is mediated mainly by its conversion to allopregnanolone, a neurosteroid and positive modulator of GABA-A receptors with broad-spectrum antiseizure properties (Belelli et al., 1989; Kokate et al., 1999a; Frye et al., 2002; Kaminski et al., 2004; Reddy et al., 2004). Moreover, neurosteroids can modulate PRs via intracellular metabolism to analogs that bind to PRs (Rupprecht et al., 1993). Progesterone may modulate signaling cascades of inflammation, apoptosis, neurogenesis, and synaptic plasticity (Patel, 2004; Vezzani, 2005; Stein and Sayeed, 2010), and therefore, progesterone may directly exert disease-modifying effects on epileptogenesis.

Recently, progesterone has been tested in rodent models of hippocampus epileptogenesis (Reddy et al., 2010; Reddy and Mohan, 2011; Reddy and Ramanathan, 2012). At low, non-sedative doses, progesterone treatment for 2 weeks significantly suppressed the rate of development of kindled seizure activity evoked by daily hippocampus stimulation in mice, indicating a disease-modifying effect of progesterone on limbic epileptogenesis. There was a significant increase in the rate of "rebound or withdrawal" kindling during drug-free stimulation sessions following abrupt discontinuation of progesterone treatment. A washout period after termination of progesterone treatment prevented such acceleration in kindling. The molecular mechanisms underlying progesterone's attenuating effect on kindling development remains unknown. There are several potential mechanisms by which progesterone could inhibit epileptogenesis, including activation of PRs, synthesis of neurosteroids, modulation of oxidative cascades, and promoting neuroprotection. The effect of progesterone on the early kindling progression is reduced in mice lacking PRs, which provide evidence that PRs may be partly involved in progesterone's disease-modifying effects (Reddy et al., 2010). These findings are consistent with the role of PRs in progesterone inhibition of epileptiform activity in the hippocampus (Edwards et al., 2000). Despite the early attenuation, late stage kindling progressed normally in the PRKO mice, suggesting that PRs are not involved in the later part of the kindling progression. However, the extent to which PRs mediate the progesterone's disease-modifying effect remains unclear. Collectively, the disease-modifying effect of progesterone may occur through a complex mechanism partly involving PR-dependent and PR-independent pathways.

Progesterone is rapidly metabolized into neurosteroids pregnanolone and allopregnanolone, which could mediate progesterone's attenuating effects on kindling epileptogenesis. This possibility is supported by emerging evidence that neurosteroids can retard the development of spontaneous seizures in post-SE models of epileptogenesis (Biagini et al., 2006, 2009). It is suggested that 5α-reductase converts progesterone to allopregnanolone and related neurosteroids that retard epileptogenesis. To further test this hypothesis, we utilized the mouse hippocampus kindling model of epileptogenesis and investigated the effect of finasteride, a 5α-reductase and neurosteroid synthesis inhibitor (Reddy and Ramanathan, 2012). In a kindling model in adult mice, pre-treatment with finasteride significantly blocked progesterone's inhibition of epileptogenesis (Reddy and Mohan, 2011), and led to complete inhibition of the progesterone-induced retardation of limbic epileptogenesis in mice (Reddy and Ramanathan, 2012).

Therefore, neurosteroids such as allopregnanolone may mediate the disease-modifying effect of progesterone in the kindling model (see Role of Neurosteroids in Epileptogenesis).

In addition, progesterone's modulation of inflammation is suggested as an appealing mechanism because pro-inflammatory molecules and oxidative signaling has been found to be activated in animal models of epilepsy (Patel, 2004; Vezzani, 2005). Progesterone has pleiotropic effects on inflammation and cell growth/survival (He et al., 2004; Stein and Sayeed, 2010) that may contribute to its attenuating effects on epileptogenesis. Progesterone inhibits secreted phospholipase A2 enzyme, a very high level target in the inflammatory cascade that has been shown to induce neurodegeneration through glutamate release (DeCoster et al., 2002; Yagami et al., 2002). Progesterone has been shown in numerous preclinical models to be neuroprotective after injury (Roof et al., 1994; Koenig et al., 1995; Jiang et al., 1996; Cutler et al., 2007). Recently, two clinical studies have evaluated progesterone as a treatment for moderate to severe TBI (Wright et al., 2007; Xiao et al., 2008). The progesterone was administered over a period of 3 or 5 days beginning within 8 or 11 h of the injury. In both studies, the groups receiving progesterone had significantly fewer deaths than those receiving placebo. In addition, there was evidence of improved functional outcomes in the progesterone-treated groups, suggesting that progesterone is highly efficacious in reducing morbidity and mortality in TBI, which is a leading cause of epilepsy in adults and military persons.

TESTOSTERONE

Testosterone has marked impact on seizure susceptibility. The potential biosynthetic pathways of testosterone metabolism are illustrated in **Figure 2**. Testosterone is known to produce both proconvulsant and anticonvulsant effects depending on the animal model and the seizure type (Reddy, 2008). Both animal and clinical studies show that testosterone enhances seizure activity by metabolism to estrogens (Isojarvi et al., 1988; Thomas and Yang, 1991; Herzog et al., 1998; Edwards et al., 1999; El-Khayat et al., 2003). Epidemiological data indicate that the occurrence of focal and tonic–clonic epileptic seizures is ∼50% higher in intact than in castrated dogs (VMDB Report, 2003). On the contrary, testosterone and related androgens have protective effects against seizures induced by pentylenetetrazol and kainic acid (Schwartz-Giblin et al., 1989; Frye and Reed, 1998; Frye et al., 2001a,b; Reddy, 2004b). Moreover, studies in orchidectomized or castrated animals have shown that decreased testosterone is associated with higher incidence of seizures and replacement with testosterone attenuates seizures (Grigorian and Khudaverkian, 1970; Thomas and McLean, 1991; Pericić et al., 1996; Pesce et al., 2000). It is demonstrated that testosterone modulation of seizure susceptibility occurs through its conversion to neurosteroids with "anticonvulsant" and "proconvulsant" actions, and hence the net effect of testosterone on neural excitability and seizure activity depends on the levels of distinct testosterone metabolites within the brain (Reddy, 2004a,b). Unlike estradiol, which generally facilitates seizures (Bäckström, 1976; Hom and Buterbaugh, 1986; Buterbaugh, 1989; Woolley, 2000), androstanediol has been shown to produce powerful antiseizure effects (Reddy, 2004b; Kaminski et al., 2005). Testosterone might have a biphasic effect on seizures: proconvulsant at higher doses, anticonvulsant at lower doses. However, testosterone itself has not been reported to improve seizures clinically (Herzog et al., 1998). Reductions of seizures were observed only when testosterone was given together with an estrogen synthesis inhibitor (Herzog et al., 1998), suggesting the estradiol modulation of seizure activity.

In many men with epilepsy, testosterone deficiency is an unusually common clinical observation (Macphee et al., 1988; Herzog, 1991; El-Khayat et al., 2003). TLE surgery has been shown to reduce seizure occurrence and normalize serum androgen concentrations in men with epilepsy (Bauer et al., 2000). Alterations in testosterone levels, therefore, may possibly contribute to exacerbation of seizures. The introduction of finasteride (*Propecia*), which inhibits dihydrotestosterone and androstanediol synthesis, for the treatment of male pattern baldness led to recurrent seizures, which then subsided once the drug was discontinued. Finasteride-induced seizure exacerbation has also been reported recently (Herzog and Frye, 2003). There is a new case report implicating endogenous neurosteroids in TLE (Pugnaghi et al., 2013). Two-week phenytoin treatment has been shown to affect the hippocampal levels of testosterone, cytochrome P450 (CYP) isoforms, and androgen receptor (AR) expression (Meyer et al., 2006). The increased metabolism of testosterone leading to augmented androgen metabolite formation most likely led to enhanced expression of CYP19 and AR in hippocampus, which is a critical area for limbic epileptogenesis.

Aromatase is the key enzyme for the conversion of testosterone to estradiol, a neuroactive steroid that promotes seizures. Aromatase is expressed in discrete areas in the brain such as hippocampus and neocortex that are involved in epileptogenesis. Aromatase inhibitors could decrease brain excitability by decreasing local estradiol levels and therefore, could be beneficial for the treatment of epilepsy (MacLusky et al., 1994). Consequently, aromatase inhibitors have been proposed as a suitable approach to seizure therapy in some men with epilepsy. Some aromatase inhibitors have been tested in men with epilepsy: testolactone, letrozole, and anastrozole. Herzog et al. (1998) tested the efficacy of testosterone and testolactone in men with intractable complex partial seizures. Improvement in seizure control was reportedly achieved with testosterone therapy when testosterone was used along with testolactone. In a case report, letrozole has been shown to improve seizure control in a 61-year-old man with epilepsy (Harden and MacLusky, 2004, 2005). In a pilot study, the safety and efficacy of add-on anastrozole therapy was tested in men with intractable epilepsy. Men with the greatest seizure reduction showed unexpectedly elevated levels in FSH, a pituitary-derived gonadotropin. Hence, the outcome of trials with three distinct aromatase inhibitors – testolactone, letrozole, and anastrozole – suggests a beneficial treatment modality for men with epilepsy (Harden and MacLusky, 2005). Therefore, it is likely that aromatase inhibitors could be potential agents for interruption of proepileptogenic estrogens in the brain.

ESTROGENS

Although estrogens can affect seizure susceptibility, the role of various estrogens in epileptogenesis is poorly understood. In general, estrogens have proconvulsant and epileptogenic properties

in animals and humans (Scharfman and MacLusky, 2006). There are limited studies that support protective effects of estrogens, but it may act as a anticonvulsant under some conditions (Velíšková, 2006). Estradiol has been widely investigated in animal epilepsy models. The effect of estrogens on seizure susceptibility is highly variable and depends on factors such as treatment duration, dosage, hormonal status, and the seizure model (Velíšková and Velísek, 2007). Early studies of estradiol administration to ovariectomized rats revealed proconvulsant effects (Reddy, 2009a). The effect of estrogens on hippocampus seizure susceptibility is controversial (Scharfman and MacLusky, 2006). While estradiol has been shown to be proconvulsant in several studies, there is also evidence that support lack of effect or protective effect of estrogens (Reibel et al., 2000; Velíšková et al., 2000; Velíšková and Velísek, 2007). The effect of circulating estrogens has been studied in female rats with epilepsy (Scharfman et al., 2008, 2009). Epileptic female rats show cyclic increases in epileptiform activity in EEG recordings that coincide with their ovarian cycle, mostly attributable to estrogens.

Estradiol has been known to play a role in the exacerbation of seizures in women with epilepsy (Logothetis et al., 1959; Bäckström, 1976; Jacono and Robinson, 1987). Plasma estradiol levels are found to increase during both the follicular and luteal phase of the normal menstrual cycle. Bäckström (1976) was the first investigator to characterize the relationship between seizures and steroid hormones. In women with epilepsy, a positive correlation between seizure susceptibility and the estrogen-to-progesterone ratio was observed, peaking in the premenstrual and preovulatory periods and declining during the midluteal phase. Logothetis et al. (1959) have demonstrated that intravenous infusions of estrogen were associated with rapid interictal epileptiform activity in women with epilepsy, and seizures were exacerbated when estrogen was given premenstrually. Therefore, it is hypothesized that estrogens may facilitate some forms of catamenial seizures observed during these phases. The periovulatory catamenial exacerbation has been attributed to the midcycle surge of estrogen that is relatively unopposed by progesterone until early luteal phase (Logothetis et al., 1959). An increase in the ratio of estrogen-to-progesterone levels during perimenstrual period might at least partly contribute to the development of perimenstrual catamenial epilepsy (Bonuccelli et al., 1989; Herzog et al., 1997). A recent report from the Nurses' health study in 114,847 nurses identified key factors associated with seizures in women with epilepsy (Dworetzky et al., 2012). Menstrual irregularity at ages 18–22 years was specifically associated with an increased risk of epilepsy. Menstrual irregularity during follow-up and early age at menarche increased the risk of isolated seizures. Oral contraceptive uses are not associated with isolated seizure or epilepsy.

GLUCOCORTICOIDS

Pituitary–adrenal hormones have long been known to affect epileptogenesis (Aird and Gordan, 1951; Rose et al., 1979; Weiss et al., 1993; Joëls, 2009; Borekci et al., 2010). Acute stress raises seizure threshold in animals, but chronic stress is known to be a clear risk factor for precipitating seizures in patients with epilepsies. Stress increases plasma and brain concentrations of corticosteroids and neurosteroids. Acute physical or psychological stress causes increased production of hypothalamic corticotrophin releasing hormone (CRH), which is transported via hypophyseal portal system to the pituitary, where it increases both adrenocorticotropic hormone (ACTH) synthesis and secretion. Major physiological effects result from ACTH's action on adrenal cortex to increase the circulating levels of corticosteroids, principally the glucocorticoid cortisol, and the mineralocorticoid DOC. Cortisol is a major corticosteroid secreted from the adrenal cortex. Cortisol is an excitatory steroid. It elicits proconvulsant and epileptogenic effects (Roberts and Keith, 1995; Joëls, 1997). DOC elicits inhibitory effects and protects against seizures (Reddy, 2003b). An imbalance in cortisol and DOC and other corticosteroids may contribute to susceptibility or resistance to epileptogenesis.

Stress enhances epileptogenesis. In general, chronic or repeated stress has been shown to enhance vulnerability to epileptogenesis in animal models (Joëls, 2009). Corticosterone, the major adrenal steroid in rodents, has been tested extensively in animal models (Weiss et al., 1993; Kumar et al., 2007, 2011; Joëls, 2009; Borekci et al., 2010; Desgent et al., 2012). Prolonged exposure to elevated corticosterone, used as a model of chronic stress, accelerates limbic epileptogenesis (Kumar et al., 2007). Exposure to repeated experimental stress accelerates the development of limbic epileptogenesis, an effect which may be related to elevated corticosterone levels (Jones et al., 2013). Chronic low-dose corticosterone supplementation is shown to enhance epileptogenesis in the rat amygdala kindling model (Kumar et al., 2007). Episodic corticosterone treatment elicits a striking acceleration in kindling epileptogenesis and triggers long-term changes in hippocampal CA1 neurons (Karst et al., 1999). Overall, corticosterone – with other stress hormones – rapidly enhances CA1/CA3 hippocampal activity shortly after stress and could imposes a risk for neuronal injury, such as during epileptic activity. In the hippocampus, stress-induced elevations in neurosteroids promote inhibitory tone mediated through GABA-A receptors. Under conditions of repetitive stress, hormonal influences on the inhibitory tone might diminish and instead, increased excitation become more apparent. In agreement, perinatal stress and elevated corticosteroid levels accelerate epileptogenesis and lower seizure threshold in rodent epilepsy models (Salzberg et al., 2007; Lai et al., 2009; Desgent et al., 2012). Therefore, exposure to stressful events during a critical phase in epileptogenesis could impose lasting deleterious effects on the course of epilepsy.

Deoxycorticosterone, a mineralocorticoid precursor with anesthetic and antiseizure properties, is also produced in the adrenal zona fasciculate. Although the antiseizure properties of DOC in human were first described in 1944 (Aird, 1944; Aird and Gordan, 1951), the mechanisms underlying the brain actions of DOC were only recently identified. The antiseizure activity of DOC requires its enzymatic conversion to 3α,21-dihydroxy-5α-pregnan-20-one (THDOC), a neurosteroid that is a powerful positive allosteric modulator of GABA-A receptors (Reddy, 2003b; **Figure 2**). THDOC is released during physiological stress nearly exclusively from adrenal sources (Purdy et al., 1990; Reddy, 2003b). Plasma and brain levels of THDOC rise rapidly following acute stress (Purdy et al., 1991; Concas et al., 1998; Reddy and Rogawski, 2002). Acute stressors such as swimming, foot shock, or carbon dioxide exposure elicit an increase in allopregnanolone and

THDOC concentrations in plasma and in brain (Barbaccia et al., 1996, 1997; Vallée et al., 2000). Stress-induced THDOC and neurosteroids have been demonstrated to elevate seizure threshold (Reddy and Rogawski, 2002) and contributes to neuroprotection (Reddy, 2003b, 2006).

11-Deoxycortisol (pregn-4-ene-17,21-diol-3,20-dione; DC) is an immediate precursor of cortisol. DC acts as a competitive antagonist of glucocorticoid receptor *in vitro*, but is ineffective as a glucocorticoid antagonist *in vivo* due to adrenal 11-hydroxylation (Cutler et al., 1979). Nearly 50 years ago, Heuser and Eidelberg (1961) observed that systemic administration of large doses of DC succinate induces long-lasting seizure activity in rats and cats. The mechanism underlying pro-epileptic properties of DC has been studied recently (Kaminski et al., 2011). DC is capable of inducing long-lasting status epilepticus in rodents that is refractory to several anticonvulsant drugs. In electrophysiological studies, DC is shown to accelerate the decay time of the inhibitory post-synaptic currents mediated by GABA-A receptors in brain slices, indicating that it significantly impedes GABAergic inhibition which may lead to paroxysmal epileptiform network activity and convulsive seizures. Because DC is an endogenous substance, it is suggested to contribute to an increased seizure propensity in some clinical situations. However, the specific role of DC to the pathophysiology of epileptogenesis is remains unclear.

Physical activity has been suggested as a positive disease-modifying factor for preventing or delaying the development of epilepsy. Exercise has beneficial effects on epileptogenesis (Arida et al., 1998, 2007, 2010; Silva de Lacerda et al., 2007). Exercise treatment reduced brain susceptibility in the kindling or the pilocarpine model of epilepsy. Behavioral analysis showed a reduced frequency of seizures during physical exercise program. Metabolic, electrophysiological, and immunohistochemical studies have confirmed the positive influence of exercise on epilepsy (Arida et al., 1999, 2007). Although a variety of factors can contribute to such favorable responses, the mechanisms remain poorly understood. Dendritic plasticity, increased neurogenesis, induction of trophic factors and release of neurosteroids are some factors underlying the inhibitory effects of exercise on epileptogenesis. Enrichment of environment has been shown to delay kindling epileptogenesis in rats (Auvergne et al., 2002). It is likely that endogenous neurosteroids may be involved in the neuroprotective effects of exercise and enriched environment. Thus, prevention of loss of interneurons, reduced GABA-A receptor plasticity, and decrease in axonal sprouting could contribute to the disease-modifying effect of exercise and enriched environment.

Despite acute stress-induced seizure protection in animals (Perićić et al., 2000; Reddy and Rogawski, 2002), patients, and clinicians are not likely to recognize a reduction in seizure frequency associated with stress. It is well known that emotional factors can affect seizure control (Temkin and Davis, 1984). In general, stressful events are associated with more frequent epileptiform spikes and seizures (Frucht et al., 2000). Indeed, stress has been reported to trigger seizure activity in persons with epilepsy (Temkin and Davis, 1984; Frucht et al., 2000). During stressful episodes adrenal hormone levels are expected to fluctuate, possibly affecting epileptogenic events. In agreement with this hypothesis, perinatal stress and elevated steroid levels have been shown accelerate epileptogenesis and lower seizure threshold in various animal models for epilepsy (Joëls, 2009).

Acute stress has anticonvulsant-like effects, while chronic stress is known to induce epileptic seizures. How can such contradictory observations be reconciled? Although the exact pathophysiology of possible seizure facilitation by stress is unknown, there are certainly many neural and endocrine pathways through which stress can alter neuronal excitability and thereby affect seizure susceptibility. The extent of seizure susceptibility during stress might therefore represent a balance between anticonvulsant (e.g., neurosteroids) and proconvulsant factors (e.g., glucocorticoids and CRH). Stress-induced seizures would thus occur when the balance is shifted to favor the proconvulsant factors, surpassing the anticonvulsant action of endogenous neurosteroids (Reddy, 2006). Although little is known regarding proconvulsant factors, stress can increase brain levels of "proconvulsant" sulfated neurosteroids such as pregnenolone sulfate (PS) and dehydroepiandrosterone sulfate (DHEAS; see Role of Neurosteroids in Epileptogenesis). Additionally, repeated episodes of stress and neurosteroid release might lead to a sort of neurosteroid withdrawal-induced hyperexcitable state (Reddy et al., 2001, 2012) and could predispose patients to stress-induced seizures. Nevertheless, alleviating the effects of stress by pharmacological interventions may help reduce the epileptogenicity in people with risk factors for epilepsy.

ROLE OF NEUROSTEROIDS IN EPILEPTOGENESIS

Neurosteroids are steroids synthesized within the brain with unconventional rapid effects on neuronal excitability. It is well known that steroid hormones such as progesterone and DOC can exert anticonvulsant actions (Selye, 1941; Clarke et al., 1973). The anticonvulsant properties of progesterone and DOC are predominantly due to their conversion in the brain to neurosteroids allopregnanolone (3α-hydroxy-5α-pregnane-20-one, AP) and allotetrahydrodeoxycorticosterone (THDOC), respectively (Reddy, 2003a; Reddy et al., 2004; **Figure 2**). A variety of neurosteroids are known to be synthesized in the brain (Baulieu, 1981; Kulkarni and Reddy, 1995). The most widely studied are AP, THDOC, and androstanediol. These neurosteroids are produced via sequential A-ring reduction of the steroid hormones by 5α-reductase and 3α-hydroxysteroid-oxidoreductase (3α-HSOR) isoenzymes (Reddy, 2009a). The androgenic neurosteroid androstanediol (5α-androstan-3α,17β-diol; **Figure 2**) is synthesized from testosterone (Reddy, 2004a,b).

In the periphery, the steroid precursors are mainly synthesized in the gonads, adrenal gland, and feto-placental unit, but synthesis of these neurosteroids likely occurs in the brain from cholesterol or from peripherally derived intermediates. Since neurosteroids are highly lipophilic and can readily cross the blood–brain barrier, neurosteroids synthesized in peripheral tissues accumulate in the brain (Reddy and Rogawski, 2010). Recent evidence indicates that neurosteroids are present mainly in principal neurons in many brain regions that are relevant to focal epilepsies, including the hippocampus and neocortex (Agís-Balboa et al., 2006; Saalmann et al., 2007; Do Rego et al., 2009). The biosynthesis of neurosteroids is controlled by the translocator protein (18 kDa; TSPO), formerly called peripheral or mitochondrial benzodiazepine receptor (Rupprecht et al., 2009, 2010). Activation of TSPO by endogenous

signals and ligands facilitates the intramitochondrial flux of cholesterol and thereby promotes neurosteroid synthesis. It is suggested that TSPO ligands might be an alternative approach for neurosteroid therapeutics (Nothdurfter et al., 2011). Currently, synthetic analogs of endogenous neurosteroids are under clinical trial for treatment of epilepsy (Reddy and Rogawski, 2010, 2012).

POTENTIATION OF PHASIC AND TONIC INHIBITION

Neurosteroids rapidly alter neuronal excitability through direct interaction with GABA-A receptors (Harrison and Simmonds, 1984; Majewska et al., 1986; Harrison et al., 1987; Gee et al., 1988; Purdy et al., 1990; Hosie et al., 2007, 2009), which are the major receptors for the inhibitory neurotransmitter GABA. Activation of the GABA-A receptor by various ligands leads to an influx of chloride ions and to a hyperpolarization of the membrane that dampens the excitability. Allopregnanolone and other structurally related neurosteroids act as positive allosteric modulators and direct activators of GABA-A receptors (**Figure 3**). At low concentrations, neurosteroids potentiate GABA-A receptor currents, whereas at higher concentrations, they directly activate the receptor (Harrison et al., 1987; Reddy and Rogawski, 2002). Like barbiturates, neurosteroid enhancement of GABA-A receptors occurs through increases in both the channel open frequency and channel open duration (Twyman and Macdonald,

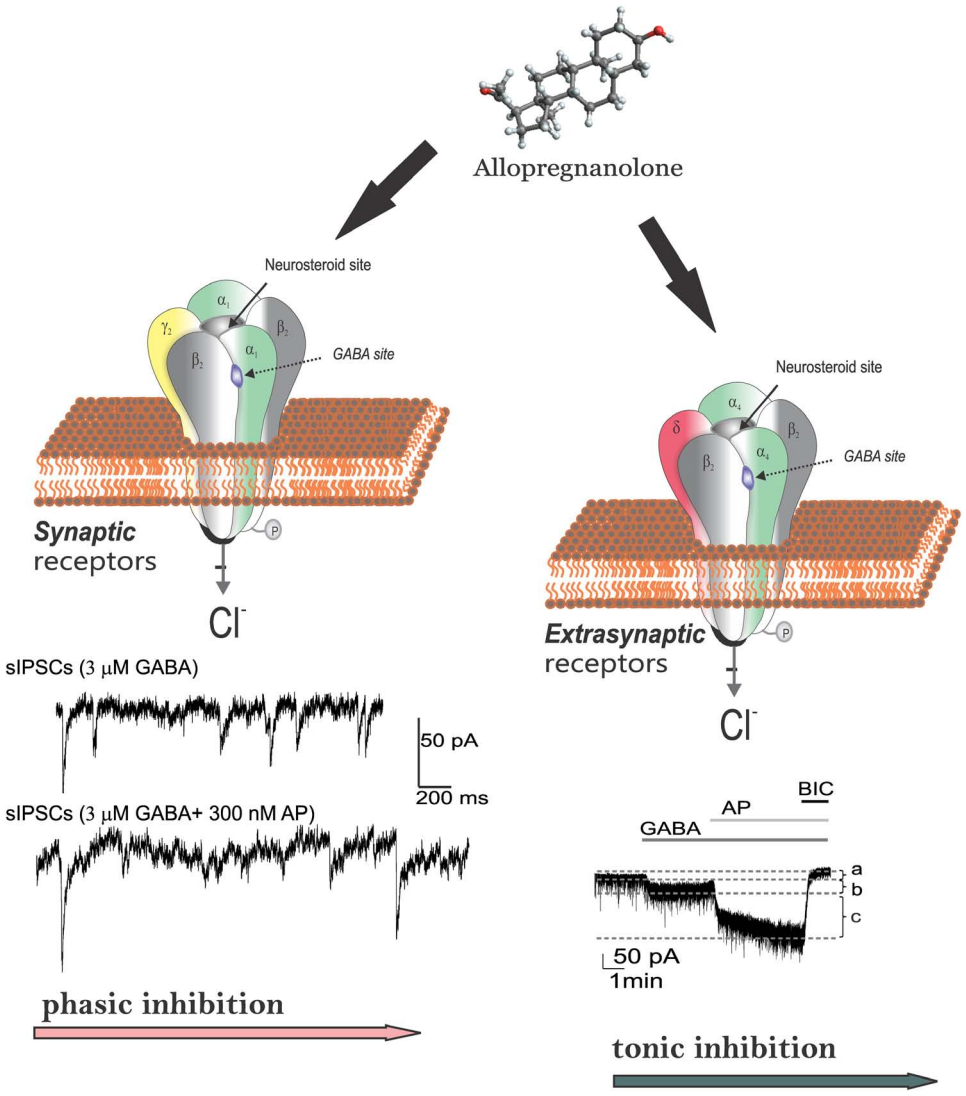

FIGURE 3 | Neurosteroid modulation of GABA-A receptors in the brain. Allopregnanolone and related neurosteroids binds and enhances GABA-A receptor-mediated inhibition in the brain. GABA-A receptors are pentameric with five protein subunits that form the chloride ion channel pore. Neurosteroids directly binds to the "neurosteroid binding sites" and potentiate the GABA-gated chloride currents. The neurosteroid binding sites are distinct from sites for GABA, benzodiazepines, and barbiturates. There are two types of GABA-A receptors with different functions. Post-synaptic GABA-A receptors, which are pentameric chloride channels composed of 2α2βγ subunits, mediate the phasic portion of GABAergic inhibition, while extrasynaptic GABA-A receptors, pentamers composed of 2α2βδ subunits, primarily contribute to tonic inhibition in the hippocampus. Neurosteroids activate both synaptic and extrasynaptic receptors and enhance the phasic and tonic inhibition, and thereby promote maximal network inhibition in the brain.

1992; Lambert et al., 2009; Ramakrishnan and Hess, 2010). The GABA-A receptor is a pentamer consisting of five subunits that form a chloride channel. Sixteen subunits (α1-6, β1-3, γ1-3, δ, ε, θ, and π subunits) have been identified so far. The GABA site is located at the interface between α and β subunits. Benzodiazepines bind at the interface between α and γ subunits and they interact with subunit combinations α1,2,3,5β2γ2.

Activation of GABA-A receptors produces two forms of inhibition: phasic inhibition generated by the rapid, transient activation of synaptic GABA-A receptors by presynaptic GABA release, and tonic inhibition generated by the persistent activation of extrasynaptic GABA-A receptors, which can detect extracellular GABA (**Figure 3**). There are major differences between synaptic and extrasynaptic GABA-A receptors (**Table 3**). The extrasynaptic GABA-A receptors are distributed within the hippocampus (α4βδ, α5βδ, or α1βδ), neocortex (α4βδ, α5βδ), thalamus (α4βδ), striatum (α4βδ), hypothalamus (α4βδ), and cerebellum (α6βδ). Although GABA activates synaptic (γ2-containing) GABA-A receptors with high-efficacy, GABA activation of the extrasynaptic (δ-containing) GABA-A receptors are limited to low-efficacy activity characterized by minimal desensitization and brief openings. Such tonic currents are particularly evident in dentate granule cells, which play a major role in hippocampus excitability. The high sensitivity of δ-containing receptor channels to neurosteroid modulation may be dependent on the δ-subunit or the low-efficacy channel function that it confers. There is evidence that neurosteroids preferentially enhance low-efficacy GABA-A receptor activity independent of subunit composition (Bianchi and Macdonald, 2003).

The effect of neurosteroids on GABA-A receptors occurs by binding to discrete sites on the receptor–channel complex that are located within the transmembrane domains of the α- and β-subunits (Hosie et al., 2006, 2007), which they access by lateral membrane diffusion (Chisari et al., 2009, 2010; **Figure 3**). The binding sites for neurosteroids are distinct from the recognition sites for GABA, benzodiazepines, and barbiturates (Hosie et al., 2009). Androgenic neurosteroids such as androstanediol may interact with these sites, and a recent study indicates that this agent is a positive allosteric modulator of GABA-A receptors (Reddy and Jian, 2010). Although neurosteroids act on all GABA-A-receptor isoforms, they have large effects on extrasynaptic δ-subunit containing GABA-A receptors that mediate tonic currents (Belelli et al., 2002; Wohlfarth et al., 2002). The potentiation of δ-subunit-containing receptors by THDOC and other neurosteroids is selective for channels with low-efficacy gating characteristics marked by brief bursts and channel openings in conditions of both low and high GABA concentrations, and neurosteroids can thereby preferentially increase the efficacy of these receptors based on pharmacokinetics which are not yet fully understood (Bianchi and Macdonald, 2003). Neurosteroids therefore markedly enhance the current generated by δ-subunit-containing receptors even in the presence of saturating GABA concentrations. Consequently, GABA-A receptors that contain the δ-subunit are highly sensitive to neurosteroid potentiation and mice lacking δ-subunits show drastically reduced sensitivity to neurosteroids (Mihalek et al., 1999; Spigelman et al., 2002). Tonic current causes a steady inhibition of neurons and reduces their excitability. Neurosteroids therefore could play a role in setting the level of excitability by potentiation of tonic inhibition during seizures when ambient GABA rises (Stell et al., 2003).

The pharmacological profile of major neurosteroids is outlined in **Table 4**. Allopregnanolone-like neurosteroids are powerful antiseizure agents. Exogenously administered neurosteroids exhibit broad-spectrum anticonvulsant effects in diverse rodent seizure models (Reddy, 2010). Neurosteroids protect against seizures induced by GABA-A receptor antagonists, including pentylenetetrazol and bicuculline, and are effective against pilocarpine-induced limbic seizures and seizures in kindled animals (Belelli et al., 1989; Kokate et al., 1994; Frye, 1995; Wieland et al., 1995; Reddy and Rogawski, 2001, 2010; Kaminski et al., 2004, 2005; Reddy et al., 2004). Like other GABAergic agents, they may exacerbate generalized absence seizures (Snead, 1998; Citraro et al., 2006). The potencies of neurosteroids in models where they confer seizure protection vary largely in accordance with their activities as positive allosteric modulators of GABA-A receptors (Reddy, 2004a,b; Kaminski et al., 2005). Like other GABAergic agents,

Table 3 | An overview of synaptic (αβγ2-containing) and extrasynaptic (αβδ-containing) GABA-A receptors in the brain.

Synaptic GABA-A receptors	Extrasynaptic GABA-A receptors
Pentameric chloride ion channels	Pentameric chloride ion channels
Contributes to phasic inhibition	Contributes to tonic inhibitiion
Low GABA affinity	High GABA affinity
High GABA efficacy	Low GABA efficacy
Pronounced desensitization	Moderate or low desensitization
Mainly synaptic localization	Perisynaptic and extrasynaptic sites
Benzodiazepine sensitive	Benzodiazepine insensitive
Potentiated by neurosteroids	Highly potentiated by neurosteroids
Not blocked by low [Zn^{2+}]	Blocked by low [Zn^{2+}]
Distributed widely within the brain: cortex, hippocampus, amygdala, limbic structures, thalamus, hypothalamus, cerebellum	Selective distribution in few brain regions: hippocampus, neocortex, thalamus, hypothalamus, cerebellum

Table 4 | Pharmacological profile of major neurosteroids in animal models.

Seizure model	Allopregnanolone	THDOC	Androstanediol
Kindling models			
Hippocampus kindling	3.5	ND	50 (36-64)
Amygdala kindling	14 (8–23)	15 (10–30)	ND
Chemoconvulsant models			
Pentylenetetrazol	12 (10–15)	19 (77–122)	40 (27–60)
Bicuculline	12 (10–15)	12 (10–15)	44 (24–81)
Picrotoxin	10 (5–19)	10 (5–19)	39 (21–74)
N-Methyl-D-aspartate	>40	>40	>200
Kainic acid	>40	>40	>200
4-Aminopyridine	>40	>40	>200
Electroshock models			
Maximal electroshock	29 (19–44)	48 (35–66)	ND
6-Hz stimulation	14 (10–19)	ND	ND
Status epilepticus models			
Pilocarpine	7 (4–13)	7 (4–13)	81 (45–133)

The profile of neurosteroids is expressed in terms of ED_{50}, which is the dose in mg/kg producing seizure protection in 50% of animals. Values in parentheses are 95% confidence limits. ND, not determined.

neurosteroids are inactive or only weakly active against seizures elicited by maximal electroshock. Neurosteroids are highly active in the 6-Hz model, a better paradigm in which limbic-like seizures are induced by electrical stimulation of lower frequency and longer duration than in the maximal electroshock test (Kaminski et al., 2004). Androstanediol, but not its 3β-epimer, produced a dose-dependent suppression of behavioral and electrographic seizures in the mouse hippocampus kindling (Reddy and Jian, 2010). In addition, neurosteroids are also highly effective in suppressing seizures due to withdrawal of GABA-A receptor modulators including neurosteroids and benzodiazepines, as well as other types of agents such as ethanol and cocaine (Devaud et al., 1996; Tsuda et al., 1997; Reddy and Rogawski, 2001; Gangisetty and Reddy, 2010). In contrast to benzodiazepines, where utility in the chronic treatment of epilepsy is limited by tolerance, anticonvulsant tolerance is not evident with neurosteroids (Kokate et al., 1998; Reddy and Rogawski, 2000), which indicate that neurosteroids are more effective than benzodiazepines for long-term treatment. Novel therapeutic approaches are being developed based on the emerging information on neurosteroid interaction with GABA-A receptors (Reddy and Rogawski, 2009; Murashima and Yoshii, 2010).

ANTIEPILEPTOGENIC ACTIVITY

Neurosteroids may play a role in chronic epilepsy. Neurosteroid modulation of tonic activation of extrasynaptic GABA-A receptors can regulate excitability during epileptogenicity. Given the complex plasticity in GABA-A receptors in epilepsy, it is difficult to predict the functional outcome of altered subunit compositions. A consistent finding from studies that have used various models of chronic epilepsy is that tonic conductances are largely preserved in epileptic brain around the time when synaptic inhibition is reduced (Mtchedlishvili et al., 2001; Sun et al., 2007; Zhang et al., 2007). Studies in a status epilepticus model of TLE have shown a striking reduction in δ-subunit containing GABA-A receptors in the dentate gyrus (Peng et al., 2004; Zhang et al., 2007), suggesting that neurosteroid effects on non-synaptic GABA-A receptors may be reduced. There was a compensatory increase in γ2-subunit, so that tonic inhibition is preserved though the efficacy of THDOC in modulating tonic current is decreased. In addition, neurosteroid modulation of synaptic currents is diminished in dentate gyrus granule cells and α4-subunit-containing receptors are expressed at synaptic sites (Sun et al., 2007). All of these changes may exacerbate seizures in epileptic animals and reduce the potency but not efficacy of endogenous neurosteroids. The expression of neurosteroidogenic enzymes such as P450scc and 3α-HSOR appears to be elevated in the hippocampus in animals and human subjects affected by TLE (Stoffel-Wagner et al., 2000; VMDB Report, 2003; Biagini et al., 2009). If local neurosteroidogenesis is enhanced, this may in part counteract the epileptogenesis-induced changes.

There is emerging evidence that endogenous neurosteroids play a role in regulating epileptogenesis (Edwards et al., 2001; Biagini et al., 2006, 2009, 2010; Reddy et al., 2010; **Figure 4**). Using the kindling model, we demonstrated that the development and persistence of limbic epileptogenesis are impaired in mice lacking PRs (Reddy and Mohan, 2011). To explore mechanisms underlying the observed seizure resistance, we investigated the role of neurosteroids using finasteride, a 5α-reductase inhibitor that blocks the synthesis of progesterone-derived neurosteroids. We determined the rate of rapid kindling in both control animals and those which had received injections of progesterone with or without concurrent finasteride treatment (Reddy and Mohan, 2011). Progesterone produced a significant delay in the rate of kindling and pretreatment with finasteride blocked progesterone's inhibition of kindling epileptogenesis (Reddy and Ramanathan, 2012). These findings are consistent with a contributory role of neurosteroids in limbic epileptogenesis. Thus, it is possible that inhibition of neurosteroids could incite mechanisms that may promote epileptogenesis.

The P450sc is a critical enzyme for the biosynthesis of neurosteroids (**Figure 2**). It is present in neurons, oligodendrocytes, astrocytes, and activated microglia. Following pilocarpine-induced status epilepticus in the rat, the neurosteroidogenic enzyme P450scc is upregulated for several weeks, suggesting that it may be associated with promotion of neurosteroidogenesis (Biagini et al., 2009). Ordinarily, rats develop spontaneous recurrent seizures following a latent period of similar duration to the period during which P450scc is elevated. The role of neurosteroids in delaying seizure onset in the pilocarpine model is confirmed using finasteride, which can exacerbate seizures by inhibition of neurosteroid synthesis. Inhibiting neurosteroid synthesis with finasteride accelerated the onset of spontaneous recurrent seizures (Biagini et al., 2006), suggesting that endogenous neurosteroids play a role in restraining epileptogenesis, or at least act to inhibit the expression of seizures.

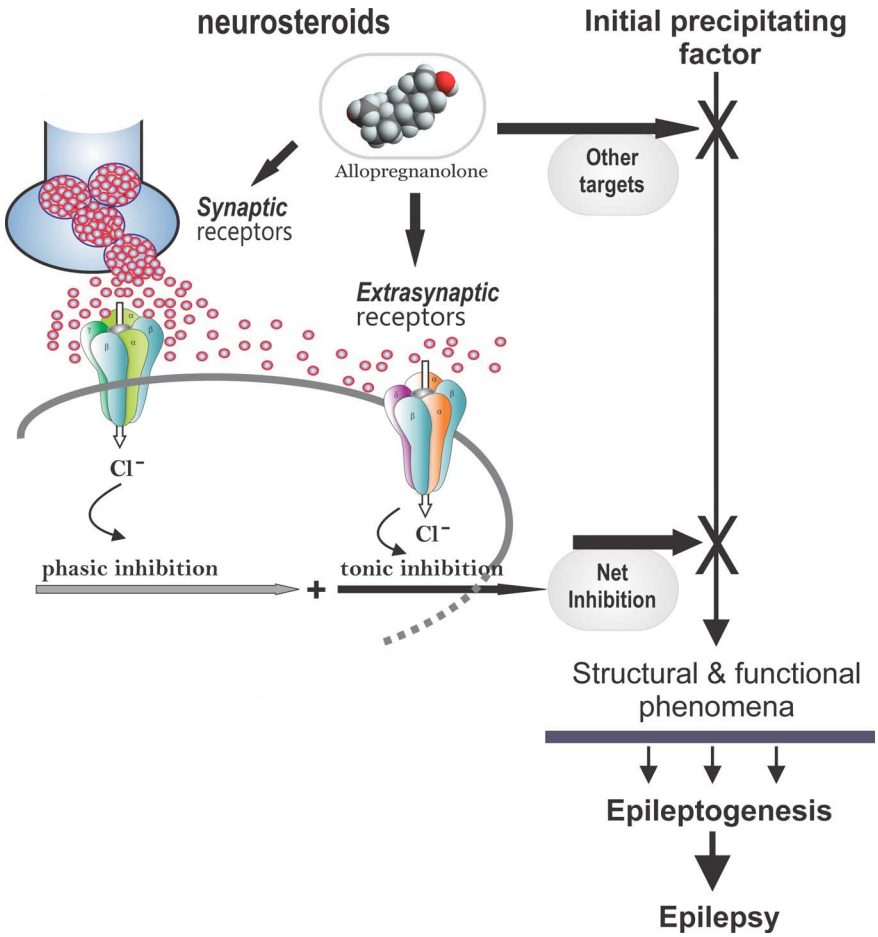

FIGURE 4 | Potential molecular mechanisms of neurosteroid interruption of epileptogenesis. In the brain, allopregnanolone and related neurosteroids may retard epileptogenesis by the interruption of one or more of the pathways leading to development of epilepsy, which generally occurs following an initial precipitating event. Neurosteroids such as allopregnanolone binds to synaptic and extrasynaptic GABA-A receptors and enhances phasic and tonic inhibition within the brain, and thereby may affect epileptogenesis. Other potential mechanisms include modulation of neuroinflammation and neurogenesis in the brain.

The development of epilepsy is linked to complex alterations in neuroplastic mechanisms. Dysregulation of neurosteroid synthesis may also play a role. This premise is being tested in various epileptogenic models (Reddy and Mohan, 2011). We investigated the role of the prototype endogenous neurosteroid allopregnanolone in controlling limbic epileptogenesis. Treatment with finasteride, a neurosteroid synthesis inhibitor, resulted in a significant increase in epileptogenesis in the hippocampus kindling model (Ramanathan and Reddy, 2011). Exogenous administration of allopregnanolone, at doses that produce levels similar to gonadotropins, markedly inhibited epileptogenesis. In female epilepsy rats, finasteride treatment exacerbates seizure frequency (Lawrence et al., 2010). Neurosteroid-mediated increase in tonic inhibition in the hippocampus could inhibit the spread of the seizure discharge from the hippocampal focus and thereby suppress the rate of development of behavioral kindled seizure activity without affecting the focal electrographic discharges (**Figure 4**). The exact mechanisms are unclear. Increased tonic inhibition by allopregnanolone is shown to impair the NMDA (*N*-methyl-D-aspartate) receptor-mediated excitability in the hippocampus (Shen et al., 2010). It is likely that such a mechanism may underlie the progesterone's disease-modifying effects in the kindling model. Based on these pilot studies, it is suggested that augmentation of neurosteroid synthesis may represent a unique strategy for preventing or retarding epileptogenesis.

PROCONVULSANT AND EPILEPTOGENIC ACTIVITY

Neurosteroids that are sulfated at C3 have inhibitory actions on GABA-A receptors (Park-Chung et al., 1999). PS and DHEAS block GABA-A receptors at low micromolar concentrations (Majewska, 1992). These "sulfated steroids" act as non-competitive antagonists of the GABA-A receptor by interacting with a site that is distinct from that of neurosteroids such as allopregnanolone and THDOC (Majewska and Schwartz, 1987; Majewska et al., 1990; Park-Chung et al., 1999). The steroid negative modulatory action on GABA-A receptors occurs through a reduction in channel opening frequency, although the precise mechanism of block is not well understood (Mienville and Vicini,

1989; Akk et al., 2001). PS has been shown to possess agonistic actions on NMDA receptors (Wu et al., 1991) and presynaptic sites (Mtchedlishvili and Kapur, 2003). Given their abundance in brain, it seems reasonable that PS and DHEAS could function as endogenous neuromodulators. In contrast to allopregnanolone, sulfated neurosteroids PS and DHEAS exert proconvulsant or convulsant actions (Reddy and Kulkarni, 1998; Kokate et al., 1999b). Direct injection of PS to rodent brain elicits seizures which increase in severity and frequency with time and eventually progress to status epilepticus, tonic hindlimb extension, and death of the animals (Kokate et al., 1999b; Williamson et al., 2004). However, systemic administration of PS does not induce overt seizure activity (Kokate et al., 1999b). The pharmacology of PS seizures is poorly understood, but both clonazepam and allopregnanolone can effectively protect against PS-induced seizure activity (Kokate et al., 1999b). The proconvulsant effect of DHEAS is reduced by progesterone, an intermediate precursor of neurosteroid synthesis. Moreover, exogenous application or endogenous stimulation of DHEAS modulates hippocampal GABA inhibition possibly by entraining hippocampal neurons to theta rhythm (Steffensen, 1995), suggesting a potential physiological relevance of the proconvulsant effects of DHEAS in animals.

GENDER DIFFERENCES IN EPILEPSY AND NEUROSTEROIDS

Epilepsy shows sex differences in incidence, progression, and severity, as well as in responsiveness to therapy. The incidence of epilepsy is generally higher in males than in females. More women than men are diagnosed with idiopathic generalized epilepsy, but localization-related symptomatic epilepsies are more frequent in men, and cryptogenic localization-related epilepsies are more frequent in women (Hauser, 1997; Christensen et al., 2005). Sex differences have been described in patients with TLE, with respect to distinct regional distribution of brain dysfunction during interictal periods as well as to the extent of neuronal damage. Women tend to have less structural atrophy than men, regardless of the seizure rate. During brain development, sex hormones have organizational effects leading to permanent differences between males and females in distinct brain regions (Velíšková, 2009). However, the precise mechanisms underlying the sex-dependent differentiation of the specific neuronal circuits, particularly brain regions involved in seizure control, are not clear. Many factors are involved in determining sex differences in seizure susceptibility, including the presence of sexual dimorphism in brain structures involved in seizure generation and control, in regional connectivity, sensitivity of neurotransmitter systems, receptor distribution, and dependence on hormonal milieu and on changes in sex hormone levels during the life span.

Neurosteroids exhibit a strong gender differences in their pharmacological effects with more potency in females than males (Reddy, 2009b). Steroid hormones such as progesterone and testosterone play a key role in the gender-related differences in susceptibility to seizures. However, the precise mechanism underlying such sexual dimorphism is obscure. Many of the biological actions of steroid hormones are mediated through intracellular receptors. Studies have suggested that these sex differences in seizure sensitivity are due to gender-specific distribution of steroid hormones or other sexually dimorphic characteristics in specific brain areas relevant to epilepsy. For example, estradiol reduces seizure-induced hippocampal injury in ovariectomized female but not in male rats, suggesting that the effects of estradiol on seizure threshold and damage may be determined by sex-related differences in the hormonal environment. Neurosteroids may play a key role in gender-related differences in seizure susceptibility (Reddy, 2009b). Both progesterone and allopregnanolone protect against experimental seizures in both male and female mice lacking PRs (Reddy et al., 2004). However, female mice exhibit significantly enhanced sensitivity to the protective activity of allopregnanolone as compared to males. In the pilocarpine seizure test, androstanediol has similar increased potency in female mice, which is not related to differences in pharmacokinetics of this neurosteroid. These results underscore the possible role of endogenous neurosteroids in gender-related differences in seizure susceptibility and protection.

POTENTIAL SIDE EFFECTS OF NEUROSTEROIDS

Steroid hormones such as progesterone and DOC have long been known to have sedative, anesthetic, and antiseizure properties (Aird, 1944; Gyermek et al., 1967; Green et al., 1978). Studies during the past three decades have uncovered that neurosteroids mediate such rapid effects of steroid hormones in the brain (Reddy et al., 2004, 2005). Unlike steroid hormones, the acute effects of neurosteroids are not related to interactions with classical steroid hormone receptors that regulate gene transcription. Moreover, neurosteroids are not themselves active at intracellular steroid receptors. They modulate brain excitability primarily by interaction with neuronal membrane receptors and ion channels, principally GABA-A receptors. Therefore, like other GABAergic agents, neurosteroids have sedative and anxiolytic properties (Reddy, 2003a). At high doses, neurosteroids cause anesthetic effects. This feature is exemplified with alphaxolone, which was introduced as intravenous anesthetic in 1970s. It was withdrawn later from the market due to toxicity of the solvent used for formulation of this synthetic neurosteroid. Although natural neurosteroids can be used for therapeutic purpose in patients with epilepsy, certain obstacles prevent the clinical use of endogenous neurosteroids. First, natural neurosteroids such as allopregnanolone have low bioavailability because they are rapidly inactivated and eliminated by glucuronide or sulfate conjugation at the 3α-hydroxyl group. Secondly, the 3α-hydroxyl group of allopregnanolone may undergo oxidation to the ketone, restoring activity at steroid hormone receptors (Rupprecht et al., 1993). Synthetic neurosteroids, which are devoid of such hormonal actions, could provide a rational alternative approach to therapy (Reddy and Kulkarni, 2000). Recently, a number of synthetic analogs of allopregnanolone and other neurosteroids are tested in animals and human trials. Ganaxolone, the 3β-methyl analog of allopregnanolone, is a synthetic neurosteroid analog that overcomes these limitations (Carter et al., 1997). Results from the clinical trials of ganaxolone demonstrated a benign side effect profile (Reddy and Woodward, 2004). More than 900 subjects (adults and children) have received this neurosteroid in Phase 1 and 2 trials. Overall, the drug is safe and well tolerated. The

Table 5 | Pipeline of new drugs for epilepsy under development.

Agent	Pharmacological profile
BGG492 (Novartis)	A competitive AMPA/kainate receptor antagonist
Brivaracetam (UCB)	A novel high-affinity synaptic vesicle protein 2A (SV2A) ligand
CPP-115 (Catalyst)	A GABA transaminase inhibitor (vigabatrin derivative)
ICA-105665 (Pfizer)	A highly selective opener of neuronal Kv7 (KCNQ) potassium channels
T2000 (Taro)	A non-sedating barbiturate (prodrug of diphenylbarbituric acid)
Tonabersat (Upsher-Smith)	A novel mechanism of uncoupling of neuronal gap junctions
UCB-0942 (UCB)	A new pre-and post-synaptic inhibitor
VX765 (Vertex)	A selective inhibitor of interleukin converting enzyme
YKP3089 (SK Life)	Novel mechanism of action
2-Deoxy-D-glucose (NeuroGenomeX)	A glucose analog and glycolytic inhibitor
Ganaxolone (Marinus)	A synthetic neurosteroid and GABA-A receptor modulator
Imepitoin (BI)	A low-affinity partial agonist at the benzodiazepine site of the GABA-A receptor
NAX 810-2 (NeuroAdjuvants)	Galanin receptor GalR1 and GalR2 agonist
Valnoctamide (Hebrew Univ)	Valproic acid second generation derivative

most common side effect is reversible dose-related sedation. Other adverse events reported by few subjects include dizziness, fatigue, and somnolence, which indicate its GABAergic effects.

CONCLUSION

The major part of the pathophysiology of epilepsy is epileptogenesis, whereby a normal brain becomes progressively epileptic because of injury factors. Despite increased scientific awareness, there is a large gap in our understanding of epileptogenesis and many questions remain unanswered regarding the cellular and molecular mechanisms underlying the "irreversible conversion" of a normal brain into epileptic brain predisposed to recurrent seizures. Such gaps in our knowledge about epilepsy's risk factors, comorbidities, and outcomes limit the ability of clinical programs to prevent epilepsy and its consequences. Since the Curing Epilepsy conference in 2000, there has been more focused research on optimizing approaches in preventing epilepsy and there is emphasis on several translation models. Reduction in neuroinflammation and neurodegeneration is a widely targeted approach for curing epilepsy. Despite intense search for drugs that interrupt epileptogenesis, presently there is no FDA-approved drug available for prevention of epilepsy development in patients at risk. It is essential to find a lead target, such as a receptor or signaling pathway that is crucial for the progression of epileptogenesis that can be disrupted by pharmacological agents to prevent or retard epilepsy.

A variety of pharmacological agents have been tested in animal models of epileptogenesis and in clinical trials in patients with a risk factor for epilepsy. However, the outcomes are not highly promising. The pipeline of new drugs for clinical development is very limited. A summary of agents that are currently in development for epilepsy is listed in **Table 5**. Steroid hormones, especially corticosteroids, estrogen, and progesterone play an important role in epilepsy. Repetitive stress and corticosteroids increases the risk of epilepsy. There is emerging evidence that neurosteroids may play a role in limbic epileptogenesis. Neurosteroids that enhance the GABAergic inhibition within the brain are potent anticonvulsants and they regulate neural excitability networks by enhancing the phasic and tonic inhibition in the hippocampus, a critical region involved in the TLE. Tonic inhibition is therefore an attractive target for antiepileptogenic drugs (**Figure 4**). The effects of stress and hormonal changes on neuronal excitability are most likely mediated by neurosteroids. Therefore, menstrual, gonadal, and stress-related fluctuations in neurosteroids or dysfunction in their synthesis can alter epileptogenesis in people at risk for epilepsy. As pleotropic agents, steroid hormones can modify, interrupt or reverse the epileptogenic process, such as cell loss, neuroinflammation, neurogenesis, astrogliosis, and axonal sprouting.

ACKNOWLEDGMENTS

The original research described in this article was supported in part by the NIH grants NS051398, NS052158, NS076426, and NS071597 (to Doodipala Samba Reddy) and the seed grant of TAMHSC Women's Health in Neuroscience (WHIN) program.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 April 2013; accepted: 01 July 2013; published online: 31 July 2013.

Citation: Reddy DS (2013) Role of hormones and neurosteroids in epileptogenesis. Front. Cell. Neurosci. 7:115. doi: 10.3389/fncel.2013.00115

frontiers in
CELLULAR NEUROSCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 18 September 2013
doi: 10.3389/fncel.2013.00154

Age dependency of trauma-induced neocortical epileptogenesis

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Trauma and brain infection are the primary sources of acquired epilepsy, which can occur at any age and may account for a high incidence of epilepsy in developing countries. We have explored the hypothesis that penetrating cortical wounds cause deafferentation of the neocortex, which triggers homeostatic plasticity and lead to epileptogenesis (Houweling et al., 2005). In partial deafferentation experiments of adult cats, acute seizures occurred in most preparations and chronic seizures occurred weeks to months after the operation in 65% of the animals (Nita et al., 2006, 2007; Nita and Timofeev, 2007). Similar deafferentation of young cats (age 8–12 months) led to some acute seizures, but we never observed chronic seizure activity even though there was enhanced slow-wave activity in the partially deafferented hemisphere during quiet wakefulness. This suggests that despite a major trauma, the homeostatic plasticity in young animals was able to restore normal levels of cortical excitability, but in fully adult cats the mechanisms underlying homeostatic plasticity may lead to an unstable cortical state. To test this hypothesis we made an undercut in the cortex of an elderly cat. After several weeks this animal developed seizure activity. These observations may lead to an intervention after brain trauma that prevents epileptogenesis from occurring in adults.

Keywords: sleep, wake, trauma, excitability, epileptogenesis, seizure, epilepsy

INTRODUCTION

Epilepsy is used to describe over 40 different types of neurological pathologies resulting from different etiologies. The main common features of epilepsy are the presence of unprovoked seizures and the abnormal local neuronal synchronization (Timofeev et al., 2012). Traumatic brain injury in particular is a major risk factor for epileptogenesis (Feeney and Walker, 1979; Temkin et al., 1995; Annegers et al., 1998). Cortical trauma leads to paroxysmal activity within 24 h in up to 80% of patients with penetrating wounds and stops within a 48 h period (Kollevold, 1976; Dinner, 1993). In Vietnam and Croatia, post-war epidemiological studies reported that about 50% of patients with penetrating cranial wounds develop recurring seizures 10–15 years after the trauma (Salazar et al., 1985; Marcikic et al., 1998).

Trauma-induced epilepsy is poorly controlled by the currently available medication. Early administration of anticonvulsant medication decreases the percentage of early posttraumatic seizures but does not prevent chronic epilepsy (Temkin et al., 1990, 1999; Chang and Lowenstein, 2003). Thus, understanding the mechanisms of trauma-induced epileptogenesis (TIE) – the set of latent processes caused by the initial insult that lead to the development of epilepsy – may lead to the development of new preventive approaches (**Figure 1**).

Both the cortex and the underlying white matter are damaged in the vast majority of brain-penetrating wounds. We review what is known about the changes that occur in the cortex following brain trauma. Experiments with direct damage of the cortex and the underlying white matter in young and adult cats will be described in detail below. The evidence points toward homeostatic mechanisms that may account for the differences between the effects of brain trauma in young and adult cats.

CORTICAL ACTIVITY DURING STATES OF VIGILANCE

There are three major states of vigilance: waking (W), slow-wave sleep (SWS), and rapid eye movement (REM) sleep. During normal levels of cortical activity, excitation and inhibition are balanced. At the level of neocortex, persistent synaptic activity and neuronal firing characterize both waking state and REM sleep, but in contrast, during SWS the membrane potential oscillates between depolarized and hyperpolarized states. Changes in the state of vigilance are controlled by shifts in the levels of neuromodulation.

During waking state, electroencephalography (EEG) activities are characterized by low-amplitude, high-frequency oscillations

Abbreviations: cAMP, cyclic adenosine monophosphate; EEG, electroencephalography; EMG, electromyography; EOG, electrooculography; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; HCN, hyperpolarization-activated cyclic nucleotide-gated; IPSC, inhibitory postsynaptic current; IPSP, inhibitory postsynaptic potential; KCNQ, delayed rectifier voltage-gated potassium channel; LFP, local field potential; PDS, paroxysmal depolarizing shift; REM, rapid eye movement; SWS, slow-wave sleep; TIE, trauma-induced epileptogenesis; W, wake

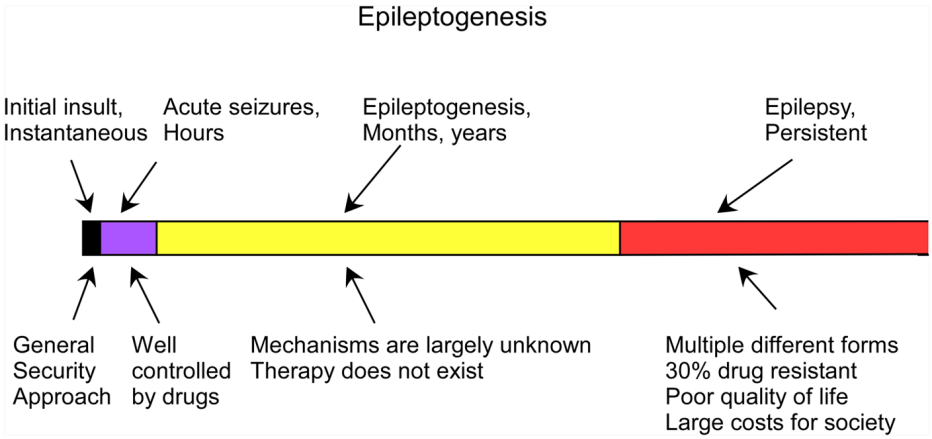

FIGURE 1 | Time course of the development of epilepsy from brain trauma (adapted from Timofeev, 2011).

in the beta and gamma frequency ranges in a majority of cortical regions (Niedermeyer, 2005). In this state, the membrane potential of cortical neurons is relatively depolarized (around −62 mV), display continuous excitatory and inhibitory synaptic activity, and spontaneously fire action potentials (Timofeev et al., 2000, 2001; Steriade et al., 2001; Mukovski et al., 2007; Rudolph et al., 2007). During quiet wakefulness, an 8–12 Hz alpha rhythm is generated over the visual cortex in awake human subjects with closed eyes (Compston, 2010) and an 8–12 Hz mu rhythm is recorded over the somatosensory cortex during immobility (Rougeul et al., 1972; Rougeul-Buser et al., 1975; Bouyer et al., 1983). Because of their relatively high amplitude, neurons contributing to these rhythms are likely to have synchronous membrane potential fluctuations in the same frequency bands. Indeed, whole-cell patch recordings from neurons in the barrel cortex of mice have large, coordinated amplitude fluctuations of the membrane potential prior to and following whisking, but these fluctuations were dramatically reduced during whisking (Crochet and Petersen, 2006; Poulet and Petersen, 2008; Gentet et al., 2010). These fluctuations appear to be slower than the mu rhythm recorded in humans and cats, but faster than sleep slow oscillation recorded in mice (Fellin et al., 2009). Recordings from cell bodies demonstrated that coordinated inhibitory activity dominated during quiet wakefulness, in responses to visual stimuli, as well as in other states of vigilance (Rudolph et al., 2007; Haider et al., 2013).

The main electrographic characteristic of SWS is the presence of slow rhythmic activity (Blake and Gerard, 1937), which includes not only slow waves composing the core of the slow oscillation (Steriade et al., 1993), but also spindles and faster activity including beta oscillations, gamma oscillations, and ripples grouped by the slow oscillation (Steriade, 2006). Intracellular recordings during SWS demonstrate that depth-positive (surface-negative) waves of local field potential (LFP) are accompanied with periods of disfacilitation, which results in significant hyperpolarization and silence of cortical neurons with dramatically reduced or abolished synaptic activity. During depth-negative (surface-positive) waves, cortical neurons display intense synaptic activity, neuronal depolarization, and mean membrane potentials similar to those recorded in quiet wakefulness states (Timofeev et al., 2000, 2001; Steriade et al., 2001). In intracellular recordings from non-anesthetized rats, when slow/delta frequencies ranges were present in the LFP, nearby neurons also displayed synchronous alternation of de- and hyperpolarizing states. When the LFP was more activated, neurons displayed relatively stable values of membrane potential and the LFP–intracellular synchrony was decreased (Okun et al., 2010). The amplitude of slow waves recorded intracellularly is quite large (around 10 mV) and similar in different cortical regions in anesthetized animals. During sleep there are area-dependent differences between cortical areas and the intracellular slow-wave amplitude in motor and somatosensory areas is lower but higher in visual and associative areas (Chauvette et al., 2011). Interestingly, in sleep-deprived rats isolated slow waves accompanied with silencing of neuronal firing can be found in otherwise awake behaving animals (Vyazovskiy et al., 2011). In adult human brain, slow-wave activity originate in frontal (Massimini et al., 2004) or medial prefrontal cortex (Nir et al., 2011). However, in young children the slow-wave activity is more intense over occipital cortex, shifting to parietal areas in adolescents and becoming stronger in frontal cortex in adults (Kurth et al., 2010). During aging, slow-wave activity decreases in all cortical regions and this decrease is more pronounced in males than in females (Carrier et al., 2011). Neuronal recordings from cortical slices maintained *in vitro* (Sanchez-Vives and McCormick, 2000), as well as from multisite intracellular recordings *in vivo* (Chauvette et al., 2010) in response to optogenetic stimulation (Beltramo et al., 2013; Stroh et al., 2013) demonstrated that the activity starts preferentially in cortical layer V. However, experiments on epileptic patients suggest that superficial cortical layers play a leading role in the generation of spontaneous cortical active states (Cash et al., 2009; Csercsa et al., 2010).

Rapid eye movement sleep is characterized by activated EEG, complete disappearance of muscle tone, and rapid ocular movements (Luppi et al., 2013). Similar to waking state the membrane potential of cortical neurons is relatively stable, depolarized, and neurons fire spontaneous action potentials (Steriade et al., 2001; Timofeev et al., 2001).

CORTICAL ACTIVITY DURING STATES OF EPILEPSY

Multiple brain structures are involved in seizure generation. In neocortical epilepsy the neocortex is a primary source of epileptic activity (Timofeev, 2010). Neocortical seizures that are primarily focal often become secondarily generalized tonic–clonic seizures (Crunelli and Leresche, 2002). Electrographically, these seizures are commonly composed of spike-wave/polyspike-wave EEG discharges at 1.0–2.5 Hz and runs of fast spikes at 7–16 Hz (Timofeev and Steriade, 2004). LFP recordings revealed the presence of ripple activity during spike components of spike-wave complexes, in particular at the onset of electrographic seizures (Grenier et al., 2003). During the spike component of spike-wave complexes, both excitatory and inhibitory cortical neurons are implicated in the generation of paroxysmal depolarizing shift (PDS). Within PDS, a majority of regular-spiking (mainly pyramidal) neurons generates only one or a few spikes, while fast-spiking (mainly inhibitory) interneurons fire throughout PDS with very high frequencies (Timofeev et al., 2002). Therefore, inhibitory activity dominates synaptic components of spikes of spike-wave complexes. Given that during seizure activity the reversal potential for $GABA_A$ inhibitory postsynaptic potential (IPSP) is shifted toward more depolarized values (Cohen et al., 2002; Timofeev et al., 2002), the reversed IPSPs contribute to the generation of PDS.

Chemical synaptic interactions might not be the most important mechanism that generates a PDS. During seizures the extracellular concentrations of Ca^{2+} decreases and K^+ increases (Heinemann et al., 1977; Pumain et al., 1983; Somjen, 2002). A reduction in the extracellular Ca^{2+} by itself impairs the presynaptic release of neurotransmitter (Katz, 1969). However, simultaneous reduction in Ca^{2+} and increase in K^+ in the extracellular milieu to the levels attained during seizure activity also prevents action potential propagation dramatically impairing chemical synaptic interactions (Seigneur and Timofeev, 2010). Therefore mechanisms other than chemical synaptic interactions may be responsible for short-range synchronization, such as electrical coupling via gap junctions, ephaptic interactions, and extracellular communication via activity-dependent ionic changes (Jefferys, 1995; Timofeev et al., 2012). Several research groups have demonstrated on several models of neocortical epilepsy that thalamocortical neurons are not a major contributor to the generation of these cortical seizures (Steriade and Contreras, 1995; Pinault et al., 1998; Timofeev et al., 1998; Steriade and Timofeev, 2001; Meeren et al., 2002; Polack and Charpier, 2006; Polack et al., 2007; Nita et al., 2008b). The runs of paroxysmal fast EEG spikes also have a purely cortical origin since thalamocortical neurons do not show significant oscillatory activity when fast runs occur (Timofeev et al., 1998). At the level of neocortex, fast runs start and terminate almost simultaneously over large distances, suggesting the presence of a common input responsible for turning on and off these fast runs. However, within fast runs, the synchrony is loose; neighboring sites of neocortex (<1 mm inter-electrode distance) can oscillate with different frequencies (Boucetta et al., 2008). The fast-spiking neurons usually oscillate at double the frequency of nearby recorded LFP (Timofeev et al., 1998). These observations suggest that during seizure, long-range (mainly chemical) synaptic interactions do not have a leading role in the synchronization of neuronal activity (Timofeev et al., 2012).

Neocortical seizures are nocturnal, occurring more often during SWS (Timofeev, 2011), and when they occur during SWS, the secondary generalized seizures last much longer than during wake (Bazil and Walczak, 1997). Why should SWS be a factor in the onset of cortical seizures? One of the major differences between SWS and other states of vigilance is the low activity of neuromodulatory systems and, as a result, the network cannot maintain permanent active states. Therefore, the main difference between SWS and other states of vigilance in the cortex is the presence of hyperpolarized silent states.

Several types of anesthesia also create alternating silent and active states. If the anesthetic used does not increase GABAergic processes and does not decrease gap-junction communications, it is often a seizure-triggering factor. In particular, ketamine–xylazine anesthesia in cats induces slow oscillation (alternation of active and silent states). The duration of silent states in anesthetized animals was 150–200% longer than during sleep, depending on the cortical area (Chauvette et al., 2011). As a result, 75% of cats maintained under ketamine–xylazine anesthesia for several hours exhibited electrographic seizures (Boucetta et al., 2008). An increase in network silence may be a factor contributing to seizure onsets because prolonged network silence increases neuronal excitability (reviewed in Timofeev, 2011).

PARTIAL CORTICAL DEAFFERENTATION IS A MODEL FOR TRAUMA-INDUCED EPILEPTOGENESIS

Epilepsy induced by a penetrating wound progresses through the same stages of epileptogenesis as other forms of acquired epilepsy. Multiple forms of TIE has been described, but much less attention was paid to epileptogenesis triggered by penetrating wounds (Hunt et al., 2013). In this experimental model a large part of axons connecting a given cortical area with other brain regions is severed (as example see Figure 2 in Timofeev et al., 2010). Our previous experiments on cats demonstrated that immediate reaction to brain penetration, in which only slight cortical but large white matter damage was produced, resulted in a dramatic reduction of LFP amplitudes in areas above the damaged white matter. About 3–4 h after the cortical undercut was produced, there were two major outcomes. In 30% of anesthetized cats slow-wave activity was fully or partially recovered. However, in the remaining 70% of animals, slow oscillatory activity was periodically transformed into paroxysmal discharges (Topolnik et al., 2003a,b). An example of electrographic seizure in ketamine–xylazine-anesthetized cat with an undercut cortex is shown in **Figure 2**. The slow oscillation in the undercut cortex is different from the normal slow oscillation: in acute conditions, above the undercut area, silent states last longer than usual (**Figure 2**, left, see also Figure 4 in Topolnik et al., 2003b). The electrographic seizure evolves continuously from the slow oscillation. Seizure onset is characterized by a shortening of both active and silence states and a slight increase in the amplitude of depolarization during active states (**Figure 2**, middle). The body of seizure was associated with a slight, steady hyperpolarization and a dramatic increase in the amplitude during PDS (**Figure 2**, right). Under anesthesia, the seizures usually terminated with postictal depression characterized by EEG flattening and neuronal hyperpolarization (**Figure 2**). This paroxysmal activity usually lasted for 8–10 h and then spontaneously stopped.

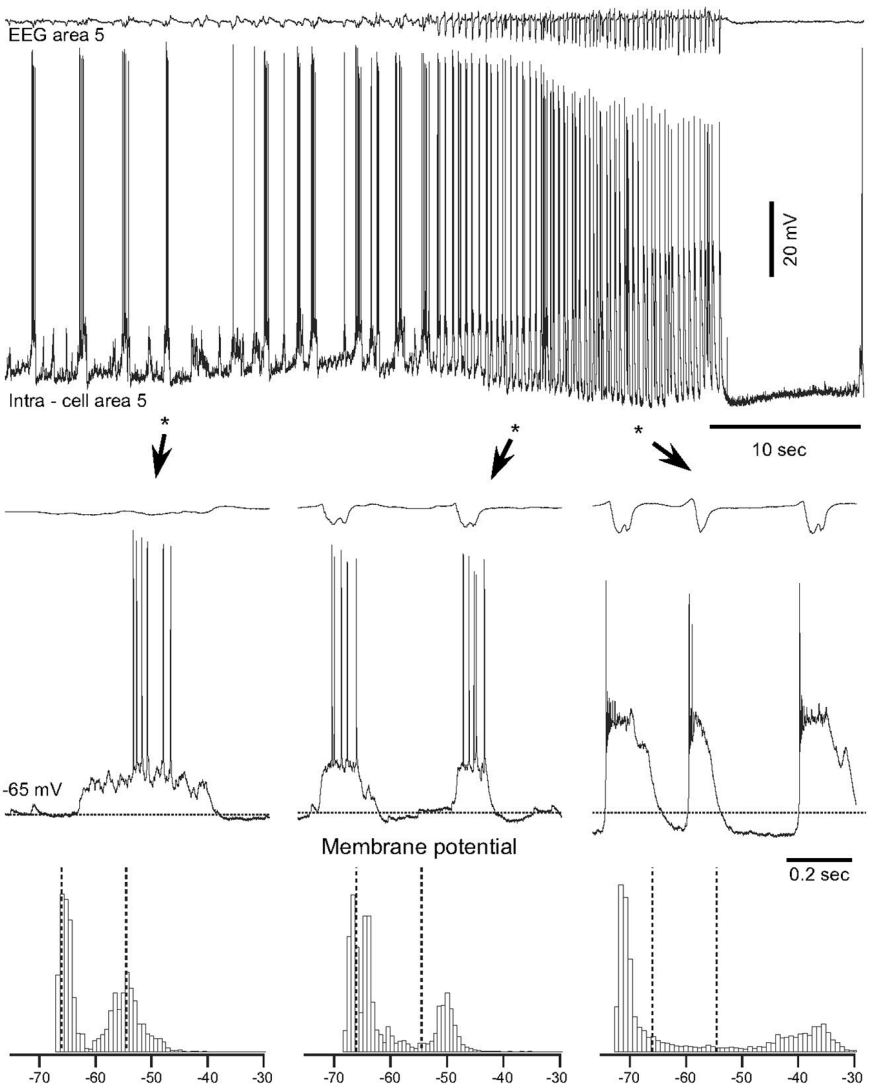

FIGURE 2 | Spontaneous electrographic seizures in partially deafferented cortex associated with increases in the maximal neural depolarization and hyperpolarization. Upper panel shows EEG and intracellular recordings from a neuron in area 5, 3 h after the undercut. Three fragments depicted by stars and arrowheads are expanded below. Horizontal dotted lines in middle panels indicate the level of membrane potential (−65 mV). Bottom panels show histograms of the membrane potential (V_m) distribution during corresponding periods of seizure. Dotted lines indicate the initial level of V_m during the slow oscillation. Note a shift of V_m during seizure in both depolarizing and hyperpolarizing directions (Topolnik et al., 2003b).

The early stages of seizure activity (0–4 days after undercut) without anesthesia were not investigated previously in the undercut model of epileptogenesis in cats (but see below). At 4–5 days following partial cortical deafferentation, *paroxysmal oscillatory activity was observed in a subset of electrodes surrounding the traumatized area*, but not within undercut (see **Figure 3A** for electrode location) (Nita et al., 2006, 2007). With time, electrographic paroxysmal activity spreads to other cortical regions. In experiment shown in **Figure 3B** during early phases of epileptogenesis (5 days after undercut) the paroxysmal activities occurred in marginal gyrus (electrodes 5, 7, and 9), but not in suprasylvian gyrus (electrodes 12–14) in which the undercut was made. Often paroxysmal discharges occurred in contralateral foci (electrodes 9 and 10), likely due to callosal transmission of synchronous neuronal firing from paroxysmal focus. These paroxysmal discharges were primarily composed of spike-and-wave complexes (**Figure 3C**). Similar pattern was seen in 10 days from the undercut. However, in 30 days from partial deafferentation of suprasylvian gyrus the paroxysmal activities could be detected on multiple electrodes including the undercut cortex. In 1.5–4 months from the deafferentation, when most of the investigated areas revealed periodic paroxysmal discharges (**Figure 3B**), behavioral seizures began in 65% of cats (Nita et al., 2007). Our data indicate that seizures usually invaded the undercut cortex within a month or more, suggesting that axonal sprouting between intact and undercut cortex might play a role in the propagation of seizures. Electrographic seizures were present during waking state, were dramatically enhanced during SWS, but were absent during REM sleep. Neuronal activity during

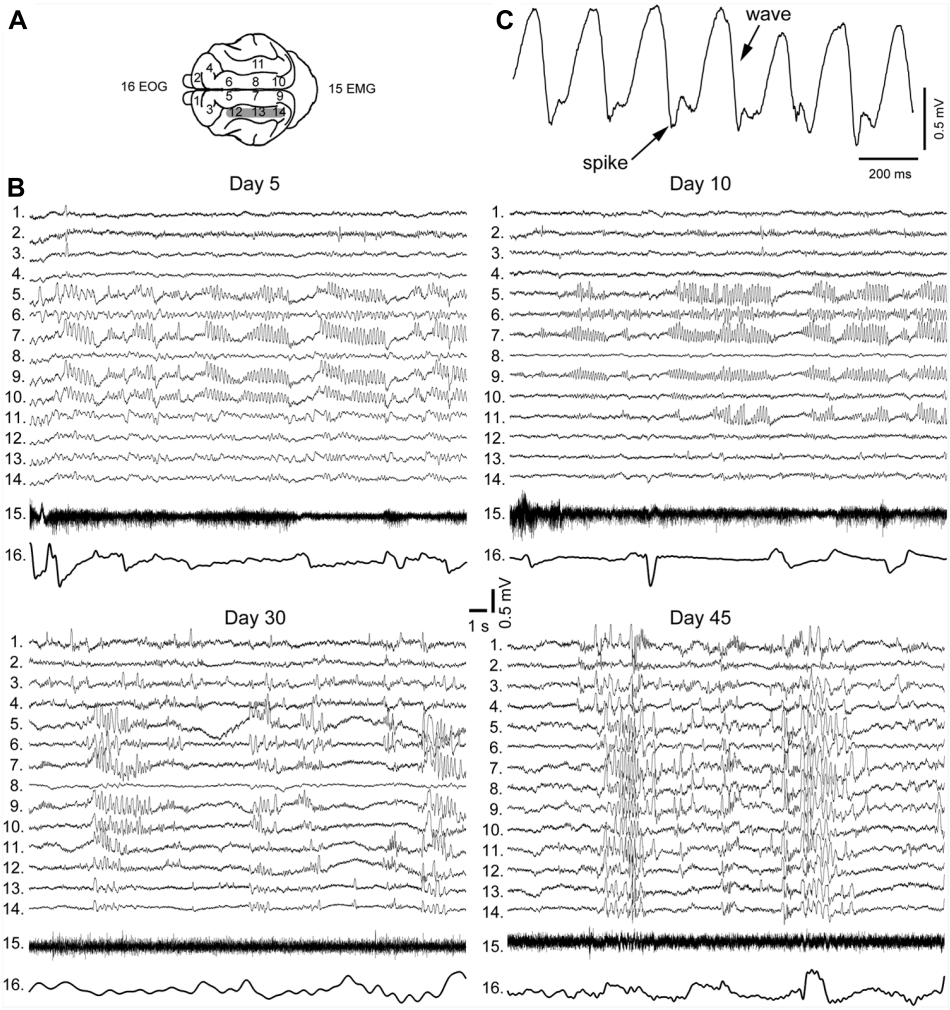

FIGURE 3 | Electrographic signatures of partial deafferentation-induced epileptogenesis in a cat. (A) Location of recording electrodes in cats brain. **(B)** Paroxysmal activities recorded at 5, 10, 30, and 45 days post-surgery. Note an increasing with time number of electrodes revealing paroxysmal discharges. **(C)** An expanded segment of paroxysmal discharge showing spike and wave pattern (modified from Nita et al., 2007).

brain activated states in partially deafferented cortex, even outside seizures was different than activity in intact cortex. Similar to intact cortex, during SWS, neurons oscillated between depolarizing and hyperpolarizing states (see above). However, silent (hyperpolarizing) states in partially deafferented cortex were also found during both quiet wakefulness and even REM sleep (Timofeev et al., 2010). These and related data suggest that *prolonged network silence during seizure-free periods is a major contributing factor in TIE* (Timofeev, 2011).

OTHER MODELS OF EPILEPTOGENESIS

Repetitive brain stimulation induces kindling that eventually leads to the development of spontaneous seizure activity (Goddard, 1967). We recently investigated electrographic activity in cats undergoing neocortical kindling. Repetitive neocortical stimulation was not efficient in generating kindling or seizures if applied during stable states of vigilance. However, when the kindling stimulation was delivered in the first minute of a transition from SWS to wake, it was highly efficient in triggering seizure activity (Nita et al., 2008a,b). After the end of these seizures, cats displayed continuing oscillatory activity with a frequency around 1.5 Hz lasting from tens of minutes and progressively increasing to hours (Nita et al., 2008a). During the continuing seizure activity intracellular recordings from cortical neurons revealed the alternation of active and silent states, although silent states were not accompanied by large amplitude hyperpolarization. Therefore, the overall presence of silence in this cortical network was increased.

Another model for epileptogenesis is chronic GABA infusion, which suppresses activity in the region of infusion. Paroxysmal activity emerges upon withdrawal from chronic GABA infusion (Brailowsky et al., 1988, 1989; Silva-Barrat et al., 1992). The hyperpolarization induced by GABA infusion is consistent with the hypothesis that reduced activity or periodic silence in some cortical territories for days, weeks, or months favor the occurrence of seizures.

STRUCTURAL CHANGES ACCOMPANYING PARTIAL CORTICAL DEAFFERENTATION

Cutting the majority of fibers arriving to a given region triggers a set of morphological and functional changes. Axonal transection causes some neurons to degenerate and others that survive may exhibit axonal sprouting.

Neuronal degeneration

Partial cortical deafferentation produced a dramatic reduction (≈25%) in cortical thickness (Avramescu et al., 2009) suggesting a reduction in the number of cortical cells in the affected region. Detailed examination of the cortex above the white matter transection has demonstrated the presence of delamination, reduction in the number of neurons, shrinkage as well as change in neuronal orientation in cortical depth (Avramescu et al., 2009). These features are similar to those described in the developmental condition called shaken infant syndrome (Marin-Padilla et al., 2002) although mechanisms leading to these changes might be different. The neuronal loss was not equally distributed over the cortical depth profile. There was a major loss of excitatory neurons in deep cortical layers (below 1.5 mm) with a moderate loss in superficial layers. By contrast, there was a highly significant loss of GABAergic neurons in both superficial and deep, but not in middle cortical layers. Why GABAergic neurons were reduced in number is not clear. These cells in the neocortex possess short axons that normally do not reach white matter. It is generally agreed that recurrent seizures may induce neuronal loss in affected brain regions (Cendes, 2005). However, in the cortical undercut model, seizures always started and generally dominated in areas surrounding the undercut cortex (Topolnik et al., 2003b; Nita et al., 2006, 2007) but not within the undercut cortex. In the region surrounding the undercut cortex, there was no significant alteration in the distribution of neuron types as observed within the undercut region (Avramescu et al., 2009). The cause of interneuronal loss remains to be investigated. One of the causes might be a higher dependency of interneurons upon aerobic metabolism than other types of cortical neurons (Sloper et al., 1980; Ribak et al., 1982). Another cause would be a high kainate neurotoxicity of interneurons due to the presence of AMPA/kainate receptors with high calcium permeability (Iino et al., 1990; Weiss et al., 1990). These are possibly the main two factors responsible for preferential loss of GABAergic cells in the undercut model. In layer V pyramidal neurons from the undercut cortex of rats, there was a tendency toward reduction of basal and apical dendritic branches, but these differences did not reach the level of significance (Salin et al., 1995; Avramescu et al., 2009). The preservation of layer V neurons in the undercut model might look surprising, because the axons of these neurons were severed. However, layer V neurons possess very extensive local intracortical connectivity (Markram et al., 1997), which helped them to survive cutting of extracortically going axon. In corticospinal neurons axotomy induced a reduction in the size of cell bodies (Tseng and Prince, 1996).

Axonal sprouting

Sometimes severed axons grow to re-innervate the targeted tissue. Axonal damage of corticospinal neurons at the level of the cervical spinal cord, however, did not induce noticeable sprouting within neocortex (Tseng and Prince, 1996). In contrast, a cortical undercut produced local sprouting characterized by increased total axonal length, increase in the number of axonal collaterals and number of axonal swellings (Salin et al., 1995) suggesting that local factors and not the damage of axon per se may trigger the axonal sprouting process. Glutamate uncaging experiments revealed that cortical undercut increased the number of "hot spots" on layer V pyramidal neurons accompanied with a decrease in the amplitude of individual excitatory postsynaptic current (EPSCs; Jin et al., 2006); the increased number of "hot spots" was also found in fast-spiking interneurons (Jin et al., 2011). The level of "inhibitory hot spots" was decreased for both pyramidal cells and interneurons (Jin et al., 2011). Direct investigation of connectivity patterns performed *in vivo* revealed that in partially deafferented cortex the connection probability between neurons was increased starting at 2 weeks after the undercut (Avramescu and Timofeev, 2008). The amplitude of EPSPs in chronic stages was significantly increased compared to controls at 2 and 6 weeks from the undercut, but it was significantly lower at 4 weeks. The coefficient of variation of responses was decreased with time suggesting a more reliable functioning of implicated synapses. It should be noted that synchronous network activity controls axonal sprouting after cortical trauma (Carmichael and Chesselet, 2002). Therefore, cortical trauma induces a pathological loop: axonal sprouting contributes to increased network synchronization leading to seizure and synchronous cortical paroxysmal activity in the partially deafferented cortex directly contributes to the reinforcement of sprouting. Altogether, a partial cortical isolation increases the number and the duration of silent states in the cortical network, which boosts neuronal connectivity and synaptic (network) excitability.

CHANGES IN THE INTRINSIC EXCITABILITY IN THE PARTIAL CORTICAL DEAFFERENTATION MODEL

Following cortical trauma, intrinsic currents also undergo changes that increase neuronal excitability. In acute conditions, the relative number of intrinsically bursting neurons doubles both in the undercut cortex and in surrounding areas (Topolnik et al., 2003a), which was likely induced by local changes of K^+ concentration due to direct neuronal damage (Jensen et al., 1994; Jensen and Yaari, 1997). The membrane potential of neurons is more hyperpolarized within the undercut cortex compared to surrounding areas (Topolnik et al., 2003a). The neuronal excitability and the overall firing, particularly in deep layers, is decreased within the undercut cortex (Topolnik et al., 2003a). Overall this results in a misbalance in excitability in the undercut and surrounding areas, creating conditions for the generation of acute seizures. Indeed, we found, using detailed conductance based models of the thalamocortical network including Na^+ and K^+ ion concentration dynamics, that change in the normal balance of ionic concentrations (particularly an increase in extracellular K^+ concentrations) may promote epileptiform discharges and lead to PDS (Frohlich et al., 2010; Krishnan and Bazhenov, 2011).

In chronic conditions the signs of changes of intrinsic excitability are opposite. Starting from 2 weeks after isolation in *in vivo* conditions the input resistance of neurons as well as their intrinsic excitability, which is measured as the number of spikes elicited by

a given current pulse or as instantaneous firing rate, is increased (Avramescu and Timofeev, 2008). Similar finding was obtained *in vitro* (Prince and Tseng, 1993), suggesting that it is the network excitability and not necessarily the network properties of the traumatized tissue that is affected. Altogether the changes in intrinsic and synaptic excitability produce an increase in the duration of the silent state and a compensatory increase in the instantaneous spontaneous firing rates ($R = 0.87$, $p < 0.01$), suggesting that a homeostatic regulation of the neuronal excitability took place (Avramescu and Timofeev, 2008).

HOMEOSTATIC PLASTICITY IN BRAIN TRAUMA RECOVERY AND EPILEPTOGENESIS

Brain excitability is maintained at a level via homeostatic mechanisms that is neither too low nor too high. Silencing a cortical culture network for 2 days upregulates synaptic excitability and an increase in network activity down-regulates excitatory synaptic efficacy (Turrigiano et al., 1998; Watt et al., 2000; Murthy et al., 2001), but not all connections (Kim and Tsien, 2008). Conversely, prolonged levels of enhanced activity induced by the blockade of synaptic inhibition or elevated $[K^+]_o$, reduces the size of mEPSCs (Lissin et al., 1998; Turrigiano et al., 1998; Leslie et al., 2001). Similar activity-dependent changes in mEPSC size have been observed in spinal cell cultures (O'Brien et al., 1998). Synaptic scaling occurs post-synaptically in part by changes in the number of open channels (Turrigiano et al., 1998; Watt et al., 2000), although all synaptic components may increase (Murthy et al., 2001) including numbers of postsynaptic glutamate receptors (Rao and Craig, 1997; Lissin et al., 1998; O'Brien et al., 1998; Liao et al., 1999). There is a similar regulation of NMDA currents by activity (Watt et al., 2000; see however Lissin et al., 1998). Interestingly, mIPSCs are scaled down with activity blockade, opposite in direction to changes in excitatory currents. This effect is reversible (Rutherford et al., 1997) and is accompanied by a reduction in the number of opened $GABA_A$ channels and $GABA_A$ receptors clustered at synaptic sites (Kilman et al., 2002). In addition, intrinsic excitability is regulated by activity. After chronic blockade of activity, Na^+ currents increase and K^+ currents decrease in size, resulting in an enhanced responsiveness of pyramidal cells to current injection (Desai et al., 1999). Some of these processes may also occur *in vivo* (Desai et al., 2002). Thus, homeostatic plasticity also controls the levels of neuronal activity through intrinsic mechanisms (Turrigiano et al., 1998; Murthy et al., 2001). In recent studies, we have demonstrated that (a) during TIE, cortical neurons undergo long-lasting silent periods during all states of vigilance (Nita et al., 2007), (b) in a neocortical kindling model of epilepsy, seizures are followed by continuing outlasting activity (Nita et al., 2008a,b). This outlasting activity can last for up to 2 h and consist of silent and active states. Therefore, silent periods are increased in both models of epileptogenesis.

Based on the experimental data, we developed network computational models in which partial cortical deafferentation led to up-regulation of the neuronal excitability and the development of seizure-like activity (Houweling et al., 2005; Frohlich et al., 2006, 2008; Volman et al., 2011a,b, 2012, 2013). First, we found that only sufficiently strong deafferentation leads to the pathological network synchronization; after a weak deafferentation homeostatic plasticity was able to recover the normal asynchronous network activity (Houweling et al., 2005). Therefore, we predicted the existence of a critical degree of deafferentation (a threshold) for pathological network reorganization. Second, we found that both spatially defined (Houweling et al., 2005) and randomly deafferented group of neurons may lead to pathological bursting (Frohlich et al., 2008). Third, we found that the network, to be prone to paroxysmal bursting should include a population of cells with relatively high density of intact neurons and a population of cells with high levels of deafferentation and low spontaneous activity (Volman et al., 2011a,b). This suggests that, in the heterogeneous networks, epileptic activity should arise near the boundary of intact and deafferented areas and propagate to the deafferented population as observed experimentally (Topolnik et al., 2003b; Nita et al., 2006, 2007). Fourth, our studies predicted a critical role of interaction between neurons and glial cells in TIE (Volman et al., 2012, 2013). More recently we developed a sophisticated network model implementing both homeostatic plasticity and ion concentration dynamics (Gonzalez et al., 2013). This study revealed that the threshold between normal and pathological network activity (Frohlich et al., 2010) is reduced after deafferentation followed by homeostatic scaling. Therefore, after deafferentation even physiological level fluctuations of the input to the network may trigger transition to recurrent epileptiform activity that would be impossible in the normal (healthy) network.

Importantly, our modeling studies suggest an existence of bistability between normal and pathological (paroxysmal) activity in the same network depending on its initial connectivity structure. Homeostatic scaling can lead to the different dynamical network states depending on the initial connectivity: either to recovery of normal activity (when the damage was small) or pathological paroxysmal activity (when the damage was large).

WHY DO NOT ALL ANIMALS DISPLAY DEAFFERENTATION-INDUCED EPILEPTOGENESIS?

We have conducted many experiments in which cortical undercut was used to trigger epileptogenesis. Most of the anesthetized cats presented acute seizure activity that stopped after several hours (Topolnik et al., 2003a,b), but only 65% of cats developed seizures in chronic conditions (Nita et al., 2006, 2007; Nita and Timofeev, 2007). These animals weighted more than 2.5 kg and their age was unknown. Since 2008, new regulations of the Canadian Council on Animal Care recommended that all experiments be performed on animals bred for research. Breeders sell only young cats (8–14 month), weighing only 2.0–2.5 kg. Since 2008 experiments on the partial deafferentation model of epileptogenesis were performed only on young cats. Given recent technological advances we now record electrographic activity immediately after the end of surgery, using a wireless system (Grand et al., 2013).

The first major finding was that, as in previous experiments, cats developed acute seizures (**Figure 4**). These seizures started at the border between the undercut and intact cortex [anterior part of the left suprasylvian gyrus (L. Supra) in **Figure 4**] and then other cortical areas became involved. Behavioral seizures started tens of seconds later. In the example shown in **Figure 4**, the behavioral seizure started with eye deflection, which happened 25 s after the onset of electrographic seizure. About 5 s later, the motor cortex got involved in paroxysmal activity and at the same time

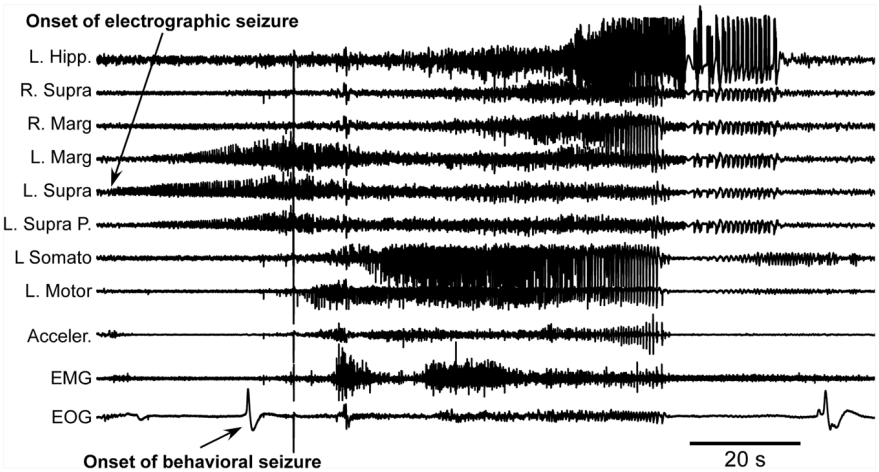

FIGURE 4 | Acute seizure recorded 8 h after the undercut and 6 h after the end of anesthesia. Electrographic seizure starts in the anterior part of the suprasylvian gyrus and then propagates to other cortical areas and hippocampus. Behavioral seizure starts with eye movement and later with muscle contractions. Muscle activity occurs when motor cortex displays electrographic seizure. L, left; R, right; P, posterior; Hipp, hippocampus; Supra, suprasylvian gyrus; Marg, marginal gyrus; Somato, somatosensory cortex; Motor, motor cortex; Acceler, accelerometer; EMG, electromyogram; EOG, electrooculogram.

the behavioral seizure was also detected with electromyography (EMG) electrodes and accelerometer. Ipsilateral hippocampus and contralateral cortical electrodes showed involvement into seizure activity somewhere in the middle part of the entire seizure. Motor seizure detected with EMG electrodes and accelerometer stopped simultaneously with LFP paroxysmal activity in the motor cortex. The electrographic seizure remained ongoing in the hippocampus and several other cortical areas for another 20 s. The end of the seizure was characterized by a major eye movement. The seizure depicted in the **Figure 4** occurred 12 h after the undercut and 6 h after the end of anesthesia. Because these recordings were made after the end of anesthesia, and our previous investigations of acute seizures were done in cats anesthetized with ketamine–xylazine (Topolnik et al., 2003a,b; Avramescu and Timofeev, 2008), we concluded that anesthesia was not a factor leading to seizure development. These types of seizures stopped within 48 h from the undercut, suggesting that they were elicited by acute conditions created by the tissue damage (increased extracellular concentration of K^+, glutamate, and other immediate actors of brain damage) but probably not by tissue reorganization.

The second major finding was that in semi-chronic and chronic conditions the young cats showed an increase in slow-wave activity, mainly in the undercut cortex, but also in neighboring locations of the same hemisphere (**Figure 5**). The potential epileptogenesis was investigated in nine young cats (age 10–14 months at the time of surgery). Experiments on three of them were done in head-restrained conditions similar to previous experiments on adult cats (Nita et al., 2007) and the remaining six animals were freely moving. In freely moving cats, we used 24 h per day wireless recordings accompanied with continuous video recordings (Grand et al., 2013). The recordings lasted between 2 and 4 months. In chronic conditions, none of these animals showed any sign of epileptic activity: neither electrographic nor behavioral. Given our previous database, the absence of seizures after four initial experiments was surprising. Because these experiments were done on bred cats, we thought that their genetic background might prevent these animals to develop epileptogenesis. Therefore the other five cats for these experiments were bought from a different breeder to ensure a different genetic background. None of these animals developed epilepsy either. All rhythmic movements recorded in these animals were physiological: scratching, chowing, walking, etc. These movements on occasion were synchronized with a maximum of one LFP channel, normally located within motor or somatosensory cortical areas. In all these animals, the slow-wave activity was increased above undercut area, and slow waves appeared at all states of vigilance, although more rarely in a wake state and more often in a SWS state. Often, but not always, slow waves were enhanced in the whole undercut hemisphere during quiet wakefulness (**Figure 5**).

We need to explain a puzzling set of observations: (i) partial cortical deafferentation induces acute seizures in almost all investigated cats both under anesthesia and without anesthesia; (ii) partial cortical deafferentation results in chronic seizures in 65% of cats of unknown age, and (iii) partial cortical deafferentation does not trigger epileptogenesis and as a result, chronic seizures in young animals. Clearly the presence of acute seizures was not a sufficient factor to trigger epileptogenesis.

Reduced network activity (undercut, tetrodotoxin, or other) upregulates neuronal excitability via both intrinsic and synaptic mechanisms. The exact mechanisms are multiple, including increased density of sodium channels, axonal sprouting, increased neurotransmitter release, increased number of postsynaptic receptors, etc. *However, we hypothesized that in young animals there is a second-order mechanism that prevents the compensatory upregulation of excitability to go to a pathological level. This conclusion is supported by multiple studies of homeostatic plasticity on either cultures or young animals (Turrigiano, 2011). In adult animals, at least partial deafferentation also leads to an increase in both intrinsic*

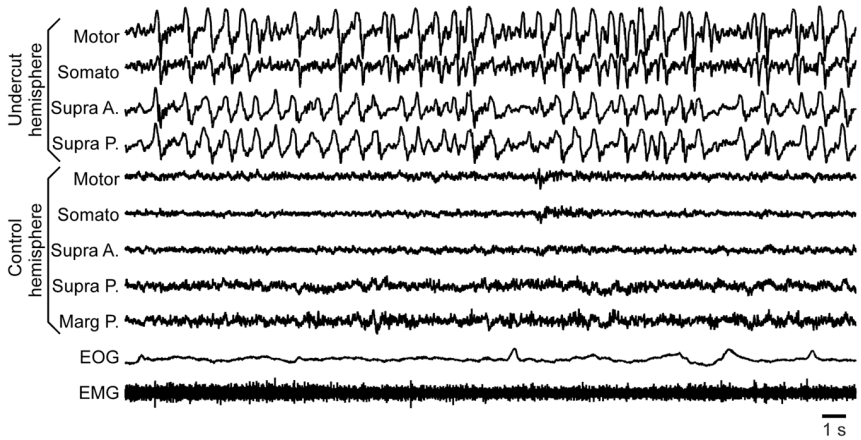

FIGURE 5 | Increased slow-wave activity in a partially deafferented hemisphere. All abbreviations are as in **Figure 4**.

and synaptic excitability via multiple mechanisms (Avramescu and Timofeev, 2008; Avramescu et al., 2009). We propose that in adult animals the upper sensor, the one that evaluates at which level the increase in excitability needs to be stopped is not present or malfunctions, which leads to overt hyperexcitation and the onset of seizures.

Our first step to confirm this hypothesis was to obtain an adult cat from the first breeder described above. The breeder received the cat 7 years ago as an adult and used it for reproduction. We performed undercut underneath the suprasylvian gyrus on this animal. Similar to other cats it had some acute seizures that stopped after 3 days from the undercut. Mild electrographic paroxysmal discharges reappeared 20 days after the surgery and their intensity progressively increased over several weeks. At 1.5 month from the partial cortical deafferentation the first behavioral seizures started. **Figure 6** shows an example full seizure that occurred 3.5 months from the undercut. These seizures usually started during quiet wakefulness. In the example shown in **Figure 6** the cat had a period of long REM sleep (**Figure 6A**), followed by a brief (40 s) period of SWS (**Figure 6B**) and followed by a brief (40 s) period of waking state. Awakening was characterized by postural adjustment (**Figure 6B**), but thereafter the animal did not move, suggesting that this was a period of quiet wakefulness. The onset of seizure was characterized by several major deflections on all recorded channels (**Figure 6D**), but thereafter the motor components of seizure recorded with accelerometers, EMG, and electrooculography (EOG) electrodes were accompanied with low amplitude phase locked LFP deflections in motor and visual cortices (**Figure 6E**). The seizure lasted for about 8 min. After the end of the seizure, the cat again went into a quiet state of wakefulness characterized by intense mu-rhythm activity recorded in the somatosensory cortices of both hemispheres.

Our studies suggest that various network properties, including the rate of homeostatic scaling and specifics of the neuronal glial interaction may affect the network susceptibility to epileptiform activity after homeostatic scaling triggered by deafferentation. We propose that some of these properties can change during development making adult animals more prone to seizures. The exact origin of these differences is still an open question that needs to be investigated.

CONCLUSION

The proposed dynamics of neuronal excitability produced by penetrating wounds are shown in the **Figure 7**. Acute trauma often elicits acute seizures that last for several hours. These seizures are not formally epileptic because they are produced as normal brain responses to the immediate sequelae of trauma: increased levels of extracellular K^+, glutamate and other immediate factors of brain damage. Penetrating brain wounds reaching white matter also deafferent cortical regions. During normal brain operations, the excitation and inhibition are balanced (Shu et al., 2003; Haider et al., 2006) with a bias toward inhibition (Rudolph et al., 2007). Deafferentation removes a number of excitatory inputs to the affected cortical area, which can no longer maintain prolonged active states characterizing waking state and REM sleep.

Several physiological changes occur following deafferentation. During SWS the silent, hyperpolarized states become longer. A large number of neurons degenerate, particularly in deep cortical layers cortex (Avramescu et al., 2009), and in humans the onset of spontaneously active states in epileptic patients shifts toward more superficial layers (Cash et al., 2009). Increased network silence triggers a set of processes that upregulate cellular and network excitability that bring the deafferented neocortex to a normal level of excitability.

Here we propose that in young animals there is another set of processes that controls the extent of up-regulation and excitability: when physiological levels of excitability are attained, the up-regulation processes are stopped. However, in adult animals the sensor(s) for the upper limit of excitability is reduced or absent. Thus the up-regulation of network excitability becomes uncontrollable and the cortex becomes epileptic. The exact nature of this upper sensor is unknown, but it can share some known mechanisms of age-related cognitive impairment (Samson and Barnes, 2013). It can be an age-dependent altering of cyclic adenosine monophosphate (cAMP) signaling that controls neuronal excitability via hyperpolarization-activated

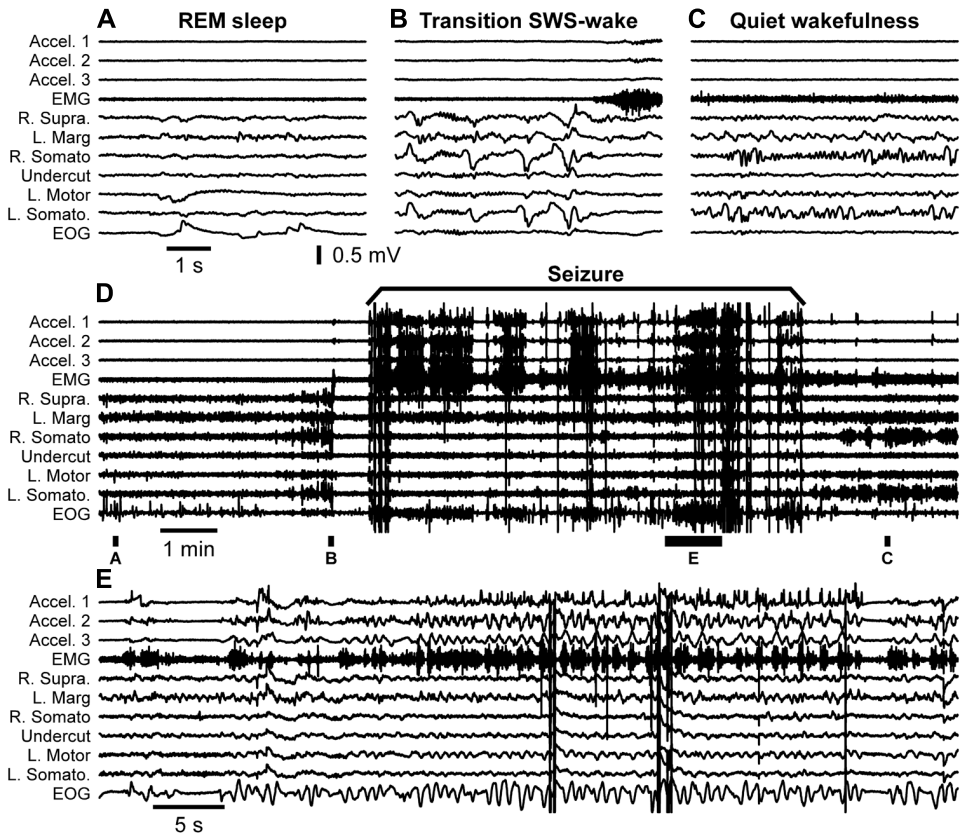

FIGURE 6 | Spontaneous seizure recorded in a cat 3.5 months after partial deafferentation of the suprasylvian gyrus. The seizure occurred during quiet wakefulness. The full extent of seizure as well as pre and post seizure states of the brain is shown in the panel **(D)**. Panels **(A–C,E)** are segments expended in time as indicated in panel **(D)**. All abbreviations are as in **Figure 4**.

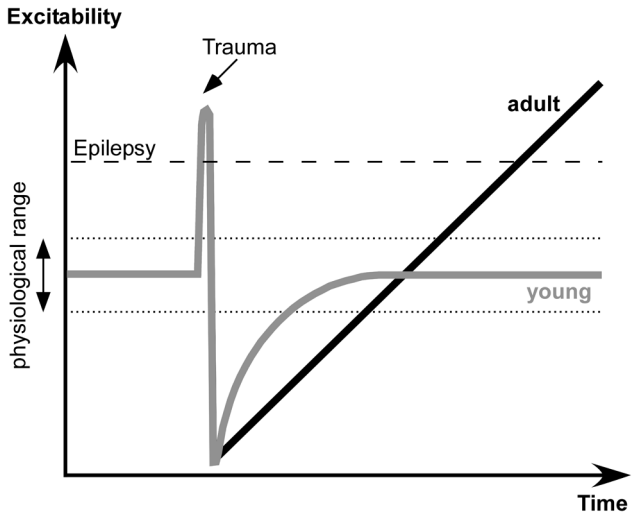

FIGURE 7 | Proposed dynamics of network excitability induced by brain trauma in young and adult subjects.

cyclic nucleotide (HCN) or KCNQ channels (Wang et al., 2011), age-dependent changes in a control of inhibitory activities (Bories et al., 2013), changes in action potential properties (Luebke and Chang, 2007), change in a control of synaptic excitability in upper layer neurons (Luebke et al., 2004), or other changes.

Current therapies try to control seizures by increasing GABA inhibition or reducing neuronal excitability by blocking Na^+ channels. In view of the evidence presented here that silencing cortical activity may trigger epileptogenesis, these treatments may be counterproductive. Epileptogenesis is a latent period that has not yet been the target for antiepileptic therapy. The first step is to identify the mechanisms for the deafferentation-induced up-regulation of neuronal excitability. We have proposed that the controller in young animals that limits normal excitability is absent in adults. If it is possible to upregulate this controller and partially recover the normal network function in the deafferented region in animal models, the same intervention may also mitigate the development of trauma-induced epilepsy in adult humans.

ACKNOWLEDGMENTS

This study was supported by Canadian Institutes of Health Research (MOP-37862, MOP-67175), the National Science and Engineering Research Council of Canada (grant 298475), the National Institute of Neurological Disorders and Stroke (1R01-NS060870 and 1R01-NS059740), and the Howard Hughes Medical Institute.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 June 2013; accepted: 26 August 2013; published online: 18 September 2013.

*Citation: Timofeev I, Sejnowski TJ, Bazhenov M, Chauvette S and Grand LB (2013) Age dependency of trauma-induced neocortical epileptogenesis. Front. Cell. Neurosci. **7**:154. doi: 10.3389/fncel.2013.00154*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 30 August 2013
doi: 10.3389/fncel.2013.00138

Dentate gyrus network dysfunctions precede the symptomatic phase in a genetic mouse model of seizures

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Neuronal circuit disturbances that lead to hyperexcitability in the cortico-hippocampal network are one of the landmarks of temporal lobe epilepsy. The dentate gyrus (DG) network plays an important role in regulating the excitability of the entire hippocampus by filtering and integrating information received via the perforant path. Here, we investigated possible epileptogenic abnormalities in the function of the DG neuronal network in the Synapsin II (Syn II) knockout mouse (Syn II$^{-/-}$), a genetic mouse model of epilepsy. Syn II is a presynaptic protein whose deletion in mice reproducibly leads to generalized seizures starting at the age of 2 months. We made use of a high-resolution microelectrode array (4096 electrodes) and patch-clamp recordings, and found that in acute hippocampal slices of young pre-symptomatic (3–6 week-old) Syn II$^{-/-}$ mice excitatory synaptic output of the mossy fibers is reduced. Moreover, we showed that the main excitatory neurons present in the polymorphic layer of the DG, hilar mossy cells, display a reduced excitability. We also provide evidence of a predominantly inhibitory regulatory output from mossy cells to granule cells, through feed-forward inhibition, and show that the excitatory-inhibitory ratio is increased in both pre-symptomatic and symptomatic Syn II$^{-/-}$ mice. These results support the key role of the hilar mossy neurons in maintaining the normal excitability of the hippocampal network and show that the late epileptic phenotype of the Syn II$^{-/-}$ mice is preceded by neuronal circuitry dysfunctions. Our data provide new insights into the mechanisms of epileptogenesis in the Syn II$^{-/-}$ mice and open the possibility for early diagnosis and therapeutic interventions.

Keywords: epilepsy, synapsins, dentate gyrus, mossy cells, excitatory inhibitory balance

INTRODUCTION

Epilepsy is a debilitating nervous system disorder mainly characterized by abnormal synchronization of neuronal activity. Current treatments are often unsatisfactory and have undesirable side effects and a number of patients present intractable seizures, for which surgical treatment is the only option (Wilson et al., 1977). This clearly indicates that mechanistically epileptic seizures are far from being understood.

The hippocampal formation is part of the limbic system and represents an important integration site, receiving inputs from most brain areas mainly via the perforant path. Intractable medial temporal lobe epilepsies (MTLE) are often successfully treated by excision of the hippocampus and nearby regions of the brain (Schwartzkroin, 1994). Therefore, this brain area appears to be highly seizure-prone and provides a convenient model to study epileptiform events *in vitro*. The major subdivisions of the hippocampus are the dentate gyrus (DG) and the *cornu ammonis* (CA), further divided in the CA3-CA1 regions. The DG has stemmed a lot of interest due to its densely packed, hyperpolarized granule cells thought to have a gate role, filtering synaptic input mainly received from the perforant path (Amaral et al., 2007). Granule cells are locally regulated by excitatory hilar mossy cells and by several types of inhibitory interneurons (Amaral et al., 2007; Hsu, 2007). There are several theories that propose mossy cells to be key players in hippocampal seizure-like events (Scharfman and Myers, 2012). These are based on several mossy cell characteristics: (i) they are highly excitable and receive input from thousands of granule cells; (ii) they are very sensitive to excitotoxicity; (iii) their axonal projections extend up to millimeters along the septo-temporal axis of the hippocampus, making them suitable candidates for seizure spread; (iv) temporal-lobe epilepsy is often associated with hilar cell loss in patients and in some laboratory models (Santhakumar et al., 2000; Ratzliff et al., 2002; Jinde et al., 2012, 2013; Scharfman and Myers, 2012).

There are several animal models widely used in the study of seizures and epilepsy, both *in vitro* and *in vivo*. Acute pharmacological models are obtained mostly by increasing excitation or lowering inhibition (blocking K^+-channels with 4-aminopyridine, using a low Mg^{2+} concentrations in the recording solution to relieve the NMDA receptor Mg^{2+} block, treatment with bicuculline or penicillin to block $GABA_A$ receptors) (Gutnick et al., 1982; Voskuyl and Albus, 1985; Witte, 1994;

Westerhoff et al., 1995). Chronic models are obtained with kainic acid injection, leading to loss of CA3 neurons in the hippocampus (Nadler, 1987), or with pilocarpine injection, causing excessive stimulation of M1 muscarinic receptors in the hippocampus and subsequent neuronal loss (Turski et al., 1983). Another murine epilepsy model, the so-called "kindling" model is based on the finding that repeated seizures can lead to development of epilepsy. Experimental kindling can be triggered, either electrically or chemically, by inducing repeated, short, focal seizures that eventually lead to the appearance of more severe, chronic seizures (Goldberg and Coulter, 2013). The molecular mechanism of kindling is unclear, but there are data supporting the role of BDNF upregulation in the progression of kindling (Garriga-Canut et al., 2006). In all these models of chemically or electrically induced epilepsy, as well as in other models (e.g., stroke or traumatic brain injury), the process of epileptogenesis starts within minutes to months and requires neuroanatomical changes, neural network alterations, activation of inflammatory cascades, post-translational modifications of existing proteins, activation of immediate early genes and several transcriptional changes (Rakhade and Jensen, 2009; Hunt et al., 2013). One comprehensive study evaluated the change in RNA expression across all annotated rat genes after pilocarpine treatment (Okamoto et al., 2010). Overall, there were about 1400 genes that changed their expression throughout the progression of epileptogenesis, with a group of 128 genes that were found to be consistently overexpressed at all stages. The proteins encoded by these genes were involved in the immune response, cell motility, apoptosis, and intracellular signaling cascades (including the mTOR signaling pathways) (Okamoto et al., 2010; Goldberg and Coulter, 2013). This study suggests that epileptogenesis is a highly complex process, involving very diverse groups of genes.

Epilepsy is an inheritable condition and mutations in several genes are associated with seizure disorders in human patients (Poduri and Lowenstein, 2011). Genetic manipulations of these target genes in mice have provided mouse models of epilepsy that are of invaluable help for the understanding of the neuropathophysiological changes in epileptogenesis (Mantegazza et al., 2010). One of the family of genes mutated in several epileptic patients, synapsins (Syns), are a family of abundant neuronal phosphoproteins playing important roles in synaptogenesis, synaptic transmission and plasticity. In mammals, they are encoded by three related genes, *SYN1-3* (Kao et al., 1999), translated into ten protein isoforms through alternative splicing (Syn Ia/Ib, Syn IIa/IIb, and IIIa/IIIf) (Cesca et al., 2010; Fassio et al., 2011). The best described function of Syns is the control of neurotransmitter release by clustering synaptic vesicles (SVs) and reversibly tethering them to the actin cytoskeleton, thus maintaining the integrity of the recycling pool (Benfenati et al., 1992). Upon activity-dependent phosphorylation, Syns detach from SVs and render them free to fuse with the plasma membrane (Giovedi et al., 2004; Menegon et al., 2006; Messa et al., 2010). This is an oversimplified view, since experiments have shown that the regulation of neurotransmitter release by Syns is much more subtle and finely tuned (Cesca et al., 2010; Fassio et al., 2011). In both excitatory and inhibitory synapses Syns appear to control downstream events of SV exocytosis, such as docking and fusion and, implicitly, the size of the readily releasable pool (RRP) (Baldelli et al., 2007; Gitler et al., 2008; Valente et al., 2012; Medrihan et al., 2013).

All mutant mice lacking one or more Syn isoforms are prone to epileptic seizures, except for Syn III$^{-/-}$ mice (Cesca et al., 2010) with Syn II$^{-/-}$ mice showing the strongest epileptic phenotype (Etholm et al., 2012). Genetic mapping analysis identified SYN2 among a restricted number of genes significantly contributing to epilepsy predisposition (Cavalleri et al., 2007; Lakhan et al., 2010). Synapsin II appears to have a specific role in preventing synaptic depression and maintaining the SV recycling pool at central excitatory synapses and at the neuromuscular junction (Rosahl et al., 1995; Coleman et al., 2008). Moreover, in DG inhibitory synapses, Syn II controls the dynamics of neurotransmitter release leading to an increase in the RRP responsible for the synchronous release in the detriment of the asynchronous GABA release (Medrihan et al., 2013). Syn II, but also Syn I knockout mice (Syn I$^{-/-}$ and Syn II$^{-/-}$) present mild emotional memory deterioration as they age (Greco et al., 2013), and are highly seizure prone. After 2–3 months of age, both Syn II$^{-/-}$ mice and Syn I$^{-/-}$ mice become epileptic and exhibit partial, secondarily generalized epileptic seizures triggered by novelty stimuli such as handling, loud noise or mating (Etholm et al., 2012). One of the most important questions regarding the Syn II$^{-/-}$ mouse epileptic model concerns the absence of seizures before the age of 2 months. Our aim in this study was to investigate the neuronal function in the pre-symptomatic phase of this mouse model. Given the large body of experimental evidence pointing toward the importance of the DG in epileptogenesis, here we have searched for pathological impairments of its circuitry in pre-symptomatic Syn II$^{-/-}$ mouse brain slices, using a combination of electrophysiological and morphological techniques.

MATERIALS AND METHODS

ANIMALS

Experiments were performed on 3–6 weeks (pre-symptomatic animals) or 4–6 months-old (after seizure onset) homozygous Syn II knockout (Syn II$^{-/-}$) mice generated by homologous recombination and age-matched C57BL/6J wild-type (WT) animals. All experiments were carried out in accordance with the guidelines established by the European Community Council (Directive 2010/63/EU of September 22nd, 2010) and were approved by the Italian Ministry of Health. Adequate measures were always taken to minimize animal pain or discomfort.

PREPARATION OF SLICES

Mice were anesthetized with isofluran by inhalation, decapitated and the brain dissected out in ice cold cutting solution containing (mM): 87 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 25 glucose, and 75 sucrose saturated with 95% O_2 and 5% CO_2. Horizontal, 400 μm thick, cortico-hippocampal slices were cut using a Microm HM 650 V vibratome equipped with a Microm CU 65 cooling unit (*Thermo Fisher Scientific, Waltham MA, USA*). Slices were cut at 2°C in the bath solution. After cutting, slices were left to recover for 45–60 min at 35°C and for another hour at room temperature in artificial cerebrospinal fluid

(aCSF) containing in mM: 125 NaCl, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$ (bubbled with 95% O_2–5% CO_2). The same solution was used for the perfusion of slices during recordings.

PATCH-CLAMP RECORDINGS

Whole-cell recordings were performed with a Multiclamp 700B/Digidata1440A system (*Molecular Devices, Sunnyvale, CA*) using an upright BX51WI microscope (*Olympus, Tokyo, Japan*). For all experiments we used a high-K gluconate intracellular solution containing (in mM): 126 K gluconate, 4 NaCl, 1 $MgSO_4$, 0.02 $CaCl_2$, 0.1 BAPTA, 15 Glucose, 5 HEPES, 3 ATP, and 0.1 GTP. The pH was adjusted to 7.3 with KOH and osmolarity was adjusted to 290 mosmol/l using sucrose. Patch-pipette resistance was 3–6 MΩ when filled with intracellular solution. Somatic access resistance (R_a) was continuously monitored, and cells with unstable R_a (20% changes) or with values larger than 15 MΩ were excluded from the analysis.

Mossy cells were identified by their shape, which has a triangular appearance in an infrared differential interference contrast image, size (mossy cells are clearly larger that surrounding interneurons) and location (deep hilus). Mossy cells were also filled with AlexaFluor 568 (40 μM in the pipette solution) to make the specific complex spines (named thorny excrescences) on the proximal dendrites visible in epifluorescence imaging (see **Figure 4C**). For cells that could not be clearly visualized, electrophysiological features, such as firing frequency adaptation during positive current injection, reduced afterhyperpolarization, broad action potentials, and high frequency/large amplitude spontaneous excitatory postsynaptic currents (sEPSPs) in the presence of bicuculline/CGP 55845, were used to identify them. Intrinsic cell properties were calculated from recordings performed in the presence of 50 μM D-APV, 10 μM CNQX, 5 μM CGP 55845, and 30 μM bicuculline. For the recording of miniature excitatory postsynaptic currents (mEPSCs), aCSF cointaining 5 μM CGP 55845, 30 μM bicuculline, and 0.3 μM TTX was used. All experiments were performed at a holding potential (V_h) of -70 mV in the presence of 30 μM bicuculine and 5 μM CGP 55845 (all from *Tocris Bioscience, Ellisville, MO*).

Granule neurons were selected based on their oval shape and their middle position in the granule layer. Since dentate granule neurons can be in different stages of maturation we recorded only mature neurons in which $R_m < 300$ MΩ (Liu et al., 1998).

In experiments where extracellular stimulation was performed, a monopolar stimulation electrode (a glass pipette filled with aCSF) was placed in the hilus, close to the granule cell layer and connected to an external stimulator (*A-M Systems, Sequim WA*).

PATCH-CLAMP DATA ANALYSIS

All data were acquired with Clampex and analyzed offline with Clampfit 10.2 (*Molecular Devices, Sunnyvale CA, USA*), MiniAnalysis (*Synaptosoft, Decatur GA, USA*), Excel and GraphPad.

In the current-clamp mode, 30 current steps lasting 1 s, starting from −100 pA in 10 pA increments, were applied to both granule and mossy cells. To precisely determine the resting membrane potential (V_m) value, this was continuously recorded for 1 min, plotted in a histogram, and fitted with a Gaussian curve. V_m was taken as the mean of the distribution. To determine the *start* (V_s) *of an action potential (AP)*, phase plane plots were constructed and V_s was considered the voltage point where dV/dt exceeded 10 mV/ms. The AP amplitude was measured as the difference between the maximum voltage reached during the overshoot minus V_s. The afterhyperpolarization (AHP) value was considered as the point where *dV/dt* was equal to zero, after the repolarization phase. The *rheobase* was estimated as the smallest current step that elicited an AP. For calculating the *input resistance* (R_{in}), the steady state voltage values from the first step above and below zero current (−10 and +10 pA), plus the zero current trace were plotted against the injected current and fitted with a linear regression. R_{in} was taken as the slope of the fitting line. The coefficient of determination R^2 was always higher than 0.95.

For the analysis of mEPSCs, 30 s of each recording were imported into MiniAnalysis (*Synaptosoft Inc., New Jersey*). Events' peaks were identified manually because of the presence of multipeak events due to the high frequency of spontaneous events. Subsequently, the software automatically calculated amplitude, rise, and decay. Minimum amplitude threshold was set to 8 pA. For the analysis of mEPSC kinetics, a set of individual 40–60 events was chosen in which the rise and the decay phase were very clear. The events were all aligned at the point of 50% rise and averaged for further calculations. The decay phase was best fitted with a monoexponential equation on the 90–10% decay phase. The cumulative distributions of amplitudes and frequencies were constructed by pooling all values, and analyzed with a Kolmogorov–Smirnov test for distributions.

HIGH-DENSITY ACTIVE-PIXEL-SENSOR MICROELECTRODE ARRAY (MEA) RECORDINGS

The APS-MEA, extensively described elsewhere (Ferrea et al., 2012), consists of a microelectrode array chip and an amplification system designed to provide simultaneous extracellular recordings from 4096 electrodes at a sampling rate of 7.7 kHz. Each square pixel measures 21 × 21 μm, and the array is integrated with an electrode pitch (center-to-center) of 42 μm. Pixels are arranged in a 64 × 64 array configuration, yielding an active area of 7.22 mm^2, with a pixel density of 567 pixels/mm^2. The three on-chip amplification stages provide a global gain of 60 dB, with a 0.1–5 kHz band-pass filter. This bandwidth is suitable for recording both slow LFP signals and fast APs. The acquisition is controlled by the BrainWave software (*3Brain Gmbh, Switzerland*). For stimulation experiments of the DG, a monopolar stimulation electrode placed on the medial perforant path and connected to an external stimulator (*A-M Systems, Sequim WA*) was used. Clear evoked responses separated from the stimulation artifact were obtained using stimulation intensities between 100 and 500 μA (for 30 μs). Large-scale field recordings were acquired using BrainWave and analyzed offline with MatlabR2010a, Clampfit 10.2 and ImageJ. For the analysis of the peak amplitude, 3 representative channels (named "pixels") from the molecular layer and 3 from the hilus were exported in Matlab (MathWorks, http://www.mathworks.it/) and then imported in Clampfit for analysis. The

maximum amplitude of the evoked events on each channel was measured, averaged (10 events/experiment) and normalized to the control amplitude. For the analysis of the mean amplitude for the entire area, an image at the point of highest response for the respective area was imported in ImageJ and the mean intensity was calculated after normalization of the maximum intensity to the maximum voltage of the same response.

IMMUNOFLUORESCENCE

WT and Syn II$^{-/-}$ mice were deeply anesthetized with 20% urethane (0.1 ml/10 gm) and perfused transcardially with 0.1 M phosphate buffer containing 4% paraformaldehyde (pH 7.4). Brains were subsequently postfixed overnight in paraformaldehyde solution, and then washed in PBS, infiltrated with a 30% sucrose PBS solution for cryoprotection and frozen in OCT. Ten μm thick horizontal sections were cut using a Leica CM3050 S Cryostat and collected on SuperFrost slides. Sections were incubated 1 h in blocking buffer (2% NGS, 1% BSA, 0.1% CFG, 0.1% Triton X-100, 0.05% Tween in PBS), then for 2 h in blocking buffer containing primary antibodies. After several washes in PBS, sections were incubated with blocking buffer containing fluorochrome-conjugated secondary antibodies (*Invitrogen*, 1:500), washed and mounted using Prolong Gold antifade reagent with DAPI staining (*Invitrogen*) for fluorescence microscopy observation on a Leica SP5 confocal laser scanning fluorescence microscope. The mean fluorescence intensity ratio between Syn I or Syn II and calretinin were calculated with the software ImageJ. Antibodies used were: Anti-Synapsin 1 (Mouse, 1:500, SYSY #106 001; Rabbit, 1:200 G-177), Anti-Synapsin 2 (Mouse, 1:200, clone 19.21), Anti-panSynapsins (Rabbit, 1:500 G143), Anti-Calretinin (Guinea Pig, 1:1000, SYSY# 214 104).

ELECTRON MICROSCOPY

Acute slices were fixed by immersion in 1.3% glutaraldehyde in 66 mM sodium cacodylate buffer. Subsequently, slices were post-fixed with 1% OsO4 in 1.5% $K_4Fe(CN)_6$ in 0.1 M sodium cacodylate, en bloc stained with 0.5% uranyl acetate, dehydrated through a series of graded ethanol solutions, washed in propylene oxide and flat embedded in Embed 812 between two Aclar sheets. After 48 h of polymerization at 60°C, a small region corresponding to the DG was excised, glued with cyanoacrylate glue on blocks of resin and cut with a Leica EM UC6 ultramicrotome. Ultrathin sections (thickness: 70–90 nm) were collected on Formvar carbon coated copper grids. Grids were observed in a JEOL JEM-1011 microscope operating at 100 kV using an ORIUS SC1000 CCD camera (*Gatan*). Total and docked SV densities were calculated using the software ImageJ.

STATISTICAL ANALYSIS

All data are expressed as means ± s.e.m. All statistics were performed with GraphPad Prism. For comparison between WT vs. Syn II$^{-/-}$ experiments, two-tailed unpaired Student's *t*-test was used. For multiple comparisons, one or two-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test or Kruskal-Wallis test followed by Dunn's *post-hoc* test was used, depending on the type of data. The level of significance was set at $p < 0.05$.

RESULTS

NETWORK IMPAIRMENTS OF DG IN PRE-SYMPTOMATIC SYN II$^{-/-}$ MICE

Our first step was to investigate the response of different areas of the DG to the stimulation of the perforant path in horizontal brain slices containing the hippocampus and rhinal cortices from pre-symptomatic (3–6 weeks) Syn II$^{-/-}$ mice. To this aim, we employed a high-resolution Active Pixel Sensor microelectrode array system (APS-MEA, 4096 electrodes: see Materials and Methods) (Ferrea et al., 2012). Field postsynaptic potentials (fPSPs), evoked by the stimulation of the perforant path with an extracellular electrode, were detected in the granule layer of the DG and they further propagated to the hilus (**Figure 1A**). The mean amplitude of the response, calculated over the entire activated region in the granule cell layer, was similar in WT and Syn II$^{-/-}$ slices (153.7 ± 15.2 μV for WT vs. 135.6 ± 10.6 μV for Syn II$^{-/-}$, $n = 13$ slices for each genotype; two-tailed unpaired Student's *t*-test, $p = 0.342$) (**Figures 1B**,**D**). On the other hand, the mean amplitude of the response in the hilar region was significantly reduced in Syn II$^{-/-}$ slices (143.0 ± 19.4 μV for WT vs. 87.13 ± 10.7 μV for Syn II$^{-/-}$, $n = 12$ slices for each genotype; two-tailed unpaired Student's *t*-test, $p = 0.013$) (**Figures 1C**,**D**). The stimulus amplitude was set to 400–500 μA based on previous input-output curves performed for 3 selected electrodes in each of the two regions. At all stimulation intensities, a reduced amplitude of the responses in the Syn II$^{-/-}$ hilar region with respect to the WT was observed (**Figures 1E**,**F**). The recorded signal on several neighboring electrodes on the APS-MEA correlated with the fine anatomy of the DG and its polarity corresponded to current sinks in the dendritic-granule layer (negative) and to current sources in the hilus (positive) (**Figure 1D**). Moreover, the propagation time of the evoked fPSP, measured between the peaks of the response from one representative electrode in the granule and hilar regions, was significantly longer in Syn II$^{-/-}$ than in WT slices (2.8 ± 0.78 ms for WT vs. 5.6 ± 0.59 ms for Syn II$^{-/-}$, $n = 6/5$ slices; two-tailed unpaired Student's *t*-test, $p = 0.032$) (**Figure 1G**), suggesting the presence of functional impairments of the hilar region.

The hilus of the DG contains hilar mossy cells and inhibitory interneurons, whose activity modulate the excitability of granule neurons (Scharfman and Myers, 2012), by forming a regulatory loop. We have previously shown that the plasticity of the young Syn II$^{-/-}$ excitatory and inhibitory synapses upon train stimulation of the perforant path does not significantly differ from that of WT synapses (Medrihan et al., 2013). Since we observed a reduced signal in the hippocampal hilus, we reasoned that a sustained train of stimuli would reveal possible dysfunctions of the granule layer-hilus network function. Thus, we stimulated the perforant path with a train of 20 Hz for 5 s and measured the ratio between the first and the last evoked fPSP response of the train over the entire granule cell layer area (**Figures 2A**,**B**). To our surprise, the depression induced by a train of stimuli was significantly higher in WT than in Syn II$^{-/-}$ slices (0.45 ± 0.03 for WT vs. 0.73 ± 0.08 for Syn II$^{-/-}$, $n = 4/5$ slices; two-tailed unpaired Student's *t*-test, $p = 0.033$) (**Figure 2C**). Based on previous results showing that

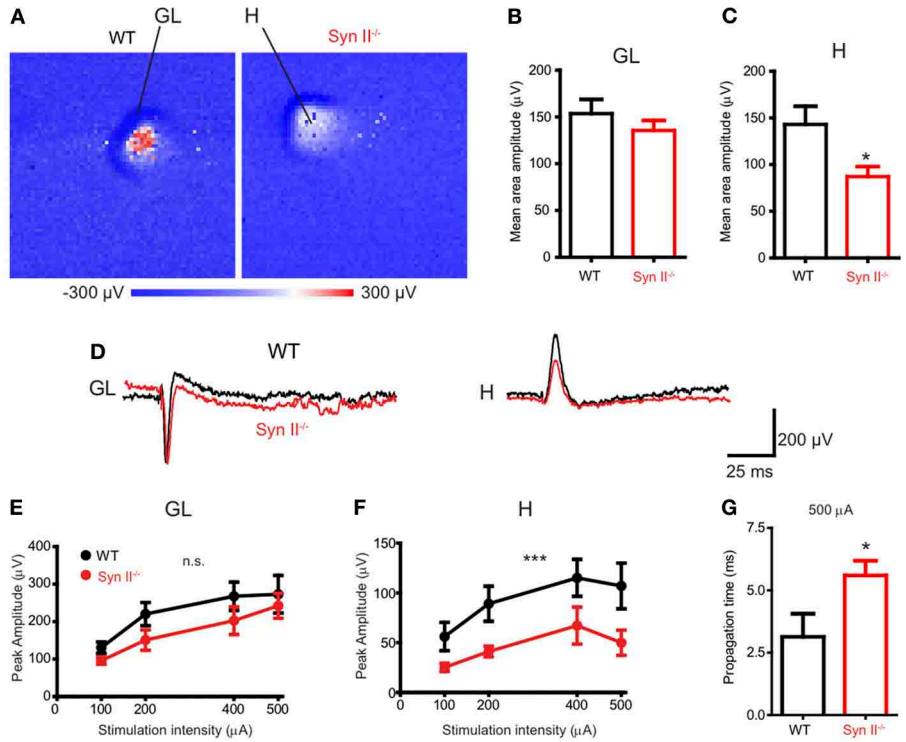

FIGURE 1 | APS-MEA extracellular field recordings show decreased activity in the hilus upon stimulation of the perforant path. (A) Color-coded fPSP activity in the entire APS-MEA chip (each pixel represents one electrode) in WT and pre-symptomatic Syn II$^{-/-}$ slices with arrows showing the activated areas (GL, granule layer; H, hilus) upon perforant path stimulation. **(B,C)** Mean (±s.e.m.) amplitude of the entire response area from WT and pre-symptomatic Syn II$^{-/-}$ in the granule layer **(B)** and hilus **(C)**; $^{*}p < 0.05$, two-tailed unpaired Student's *t*-test. **(D)** Representative traces from one APS-MEA electrode from both genotypes located in the granule layer and hilus, respectively. **(E,F)** Stimulation-response curves representing the mean (±s.e.m.) peak amplitudes of three randomly selected electrodes from the granule layer **(E)** or hilus **(F)** at increasing stimulation intensities; $^{***}p < 0.001$, Two-Way ANOVA. **(G)** Mean (±s.e.m.) stimulus propagation time from GL to H at a stimulation intensity of 500 µA; $^{*}p < 0.05$, two-tailed unpaired Student's *t*-test.

perforant path plasticity is unaltered in Syn II$^{-/-}$, these results point toward a reduced feedback inhibition of the granule cell layer.

SYNAPSIN II IS ABUNDANTLY EXPRESSED IN TERMINALS OF BOTH GRANULE AND MOSSY NEURONS

Next, we confirmed the presence of Syn II in the DG. Acute brain slices from 6 weeks old WT mice were immunolabeled for endogenous Syns I and II and for the mossy cell marker calretinin (Blasco-Ibanez and Freund, 1997) (**Figure 3A**). Since in some species calretinin is also a marker for GABAergic interneurons (Scharfman and Myers, 2012), we co-immunolabeled for GABA and noticed that calretinin immunoreactive hilar cells are negative for GABA (data not shown), suggesting that they are indeed hilar mossy cells. Both Syn I and Syn II puncta were abundantly observed in the hilar region of the DG, where mossy fiber terminals are localized. Remarkably, Syn II, but not Syn I, positive puncta were present in high density in the inner molecular layer, colocalizing with the hilar cell terminals. We therefore measured the mean intensity ratio between either Syn isoform and calretinin in the inner molecular layer of the DG (0.09 ± 0.007 for Syn I/calretinin and 0.61 ± 0.11 for Syn II/calretinin, $n = 3$ mice; two-tailed unpaired Student's *t*-test, $p = 0.034$) and in the hilar region (0.195 ± 0.0994 for Syn I/calretinin and 0.229 ± 0.1157 for Syn II/calretinin, $n = 3$ mice; two-tailed unpaired Student's *t*-test, $p = 0.837$) (**Figures 3B–D**). To further prove the specific presence of Syn II at mossy cell terminals, we stained brain slices from Syn I$^{-/-}$ and Syn II$^{-/-}$ mice respectively for calretinin (red) and all Syn isoforms (pan-synapsin antibody, green). While in the Syn I$^{-/-}$ slices the pan-synapsin staining is visible in the IML, it was completely absent in the same region of the Syn II$^{-/-}$ slices (**Figure 3E**, bottom panels).

REDUCED SYNAPTIC ACTIVITY IN MOSSY CELLS FROM PRE-SYMPTOMATIC SYN II$^{-/-}$ MICE

Since we noticed an impaired function of the hilar region of the DG (**Figure 1**) and Syn II is highly expressed at the mossy fiber terminals (**Figure 3**), we next investigated the synaptic input received by hilar mossy cells from granule neurons. First, we used electron microscopy to morphologically evaluate the number and spatial distribution of SVs within the mossy fiber terminals in the hilar region of the DG of pre-symptomatic Syn II$^{-/-}$ and WT mice (**Figure 4A**). The total density of SVs in Syn II$^{-/-}$ synapses

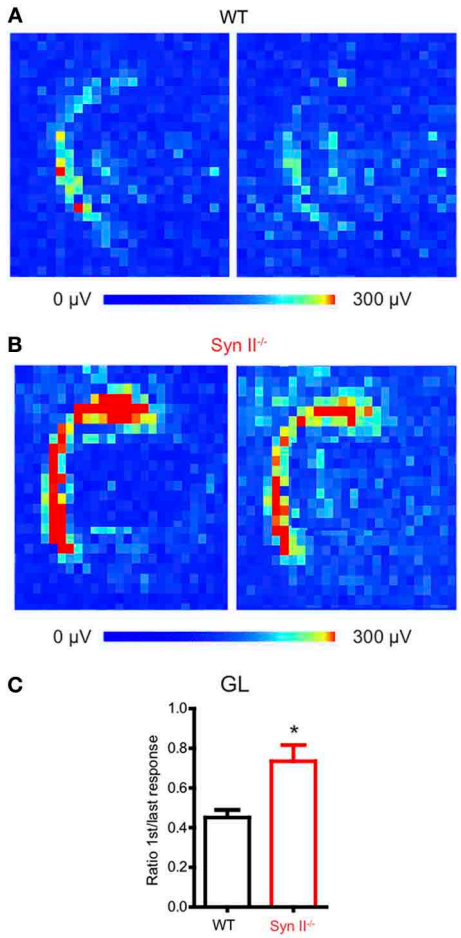

FIGURE 2 | Perforant path depression is reduced in slices from young Syn II$^{-/-}$ mice. (A,B) Color-coded fPSP activity on a selected area on the APS-MEA chip representing the response of the DG granule layer from WT **(A)** and pre-symptomatic Syn II$^{-/-}$ **(B)** slices to the first (left) and the last (right) perforant path extracellular stimulation in a train of 5 s at 20 Hz. **(C)** Mean (±s.e.m.) ratio between the mean granule layer amplitude of the first and the last response in the train for both genotypes; $^*p < 0.05$, two-tailed unpaired Student's *t*-test.

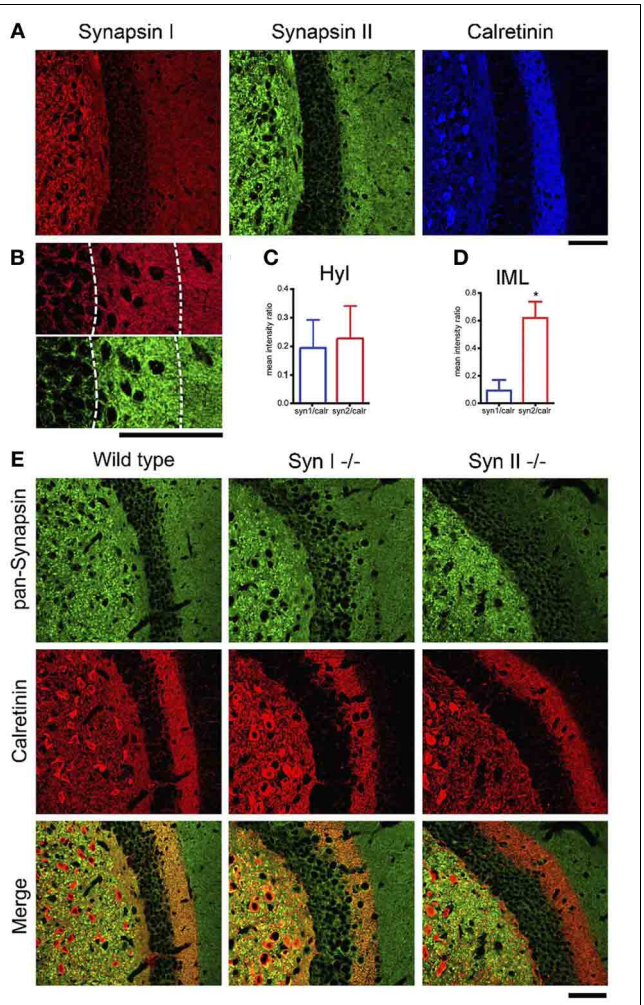

FIGURE 3 | SynII is highly colocalized with the mossy cell marker calretinin. (A) Representative images of the DG region in brain slices from 6 weeks old WT mice immunostained for Syn I, Syn II, and calretinin (scale bar 50 μm); **(B)** Representative images of the inner molecular layer area (IML) of the DG immunostained for Syn I (upper panel) or Syn II (lower panel) (scale bar, 50 μm); **(C,D)** Mean (±s.e.m.) ratios between Syn I/calretinin and Syn II/calretinin signals in the hilus (Hyl, **C**) and inner molecular layer (IML, **D**) of the DG. $^*p < 0.05$, two-tailed unpaired Student's *t*-test. **(E)** Representative images of the DG region in brain slices from 6 weeks old WT (left column), Syn I$^{-/-}$ (middle column) or Syn II$^{-/-}$ (right column) mice immunostained for all synapsin isoforms (pan-Syn antibody) and calretinin. Right panels display the merge of the two signals (scale bar, 50 μm).

was significantly lower than that of WT synapses (198.7 ± 13.7, $n = 44$ terminals/3 mice and 89.27 ± 10.8 SVs/μm^2, $n = 51$ terminals/3 mice, for WT and Syn II$^{-/-}$ respectively; two-tailed unpaired Student's *t*-test, $p = 0.0003$; **Figures 4A,B**). However, the number of docked SVs did not differ between genotypes (9.42 ± 0.6, $n = 19$ terminals/3 mice and 9.70 ± 0.7 SVs/μm, $n = 19$ terminals/3 mice, for WT and Syn II$^{-/-}$ respectively; two-tailed unpaired Student's *t*-test, $p = 0.782$) (**Figures 4A,B**), as previously reported for Syn deletions at various synapses (Gitler et al., 2004; Medrihan et al., 2013).

To functionally analyze these synapses, we recorded mEPSCs from pre-symptomatic Syn II$^{-/-}$ hilar mossy cells after blocking GABA receptors and Na^+ channels with bicuculline (30 μM), CGP55845 (5 μM) and TTX (0.3 μM). To distinguish mossy cells from the surrounding hilar neurons, we filled the patch pipette with AlexaFluor568-containing intracellular solution. This enabled the detection of moss-resembling spines (called "thorny excrescences") present on the proximal dendrites of these cells (**Figure 4C**). As reviewed before (Henze and Buzsaki, 2007; Scharfman and Myers, 2012), hilar mossy cells are highly excitable, receiving massive input from the mossy fibers, and their mEPSCs have an unusually large amplitude and frequency in comparison with other central synapses (**Figure 4D**). Both the amplitude and the frequency distributions of mEPSCs were significantly shifted toward smaller values in Syn II$^{-/-}$ hilar mossy neurons ($n = 11$ neurons/7 mice for WT and 6 neurons/3 mice for Syn II$^{-/-}$; Kolmogorov–Smirnov test,

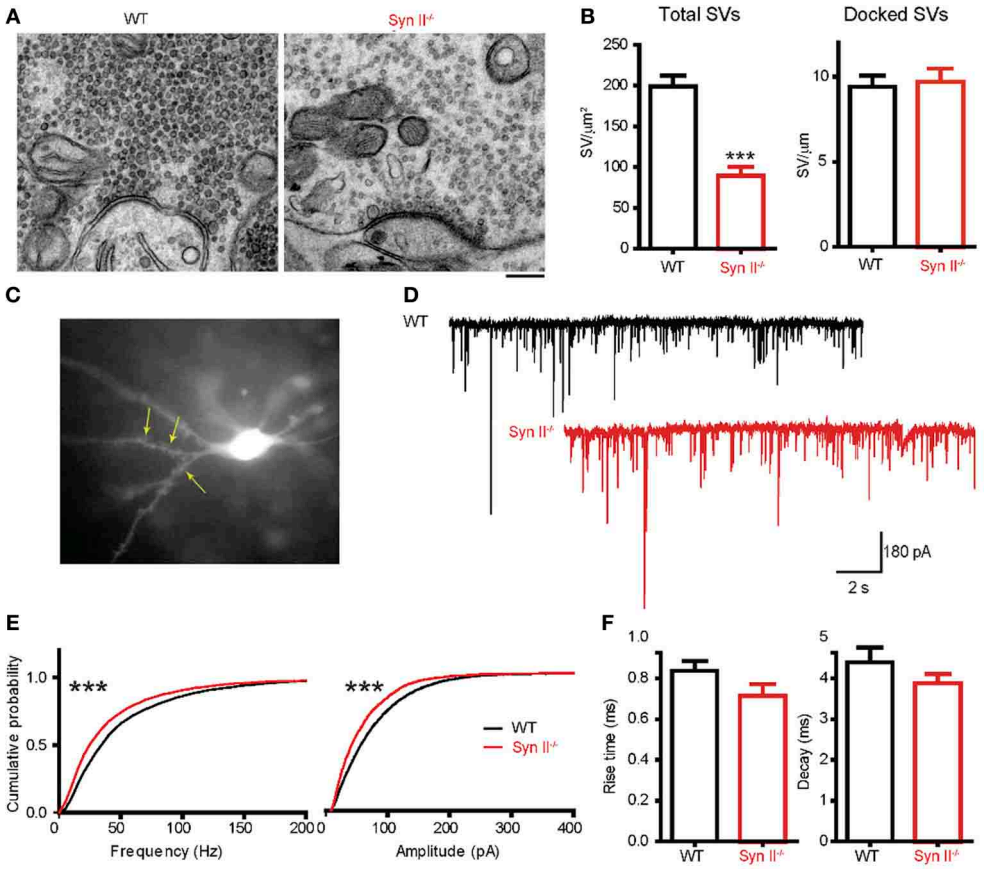

FIGURE 4 | A decreased number of mossy fiber synaptic vesicles is associated with reduced mEPSC frequency and amplitude in mossy cells from pre-symptomatic Syn II$^{-/-}$ mice. (A) Transmission electron microscopy images of mossy fiber terminals in the DG hilus of brain slices from WT (black bars) and pre-symptomatic Syn II$^{-/-}$ (red bars) mice (scale bar 200 nm). **(B)** Mean (±s.e.m.) density of total SVs and number of docked SVs in presynaptic terminals of WT and Syn II$^{-/-}$ neurons; $^{***}p < 0.001$, two-tailed unpaired Student's *t*-test. **(C)** Representative Syn II$^{-/-}$ hilar mossy cell patch-clamped in an acute brain slice and filled with AlexaFluor568. Yellow arrows point toward thorny excrescences that are visible in the focal plane. **(D)** Representative mEPSC traces recorded in mossy cells from WT (black lines) and pre-symptomatic Syn II$^{-/-}$ (red lines) mice in the presence of GABA receptor and Na^+ channel blockers. **(E)** Cumulative distributions of the amplitudes and frequencies of mEPSCs in WT and Syn II$^{-/-}$ neurons; $^{***}p < 0.001$, Kolmogorov–Smirnov test. **(F)** Mean (±s.e.m.) rise-time (10–90%) and mono-exponential τ of decay of mEPSCs from WT (black bars) and Syn II$^{-/-}$ (red bars) neurons.

$p < 0.001$) (**Figure 4E**). Such a leftward shift of frequencies indicates the presence of a presynaptic involvement. Although the distribution of mEPSC amplitudes in Syn II$^{-/-}$ was also left-shifted, it was not associated with a change in the kinetic parameters of the response (rise-time 10–90%: 0.83 ± 0.04 vs. 0.71 ± 0.05 ms, $p = 0.168$; decay τ: 4.39 ± 0.35 vs. 3.87 ± 0.23 ms, $p = 0.374$; two-tailed unpaired Student's *t*-test) (**Figure 4F**), suggesting an overall decreased excitatory presynaptic input on Syn II$^{-/-}$ hilar mossy cells from young mice.

HILAR MOSSY CELLS OF PRE-SYMPTOMATIC SYN II$^{-/-}$ MICE DISPLAY DECREASED EXCITABILITY

Extracellular fPSPs are the summation of a series of events, notably synaptic activity and synchronous firing of APs by groups of neurons (Buzsaki et al., 2012). Thus, in the next experiment, we evaluated the firing rate of hilar mossy neurons from pre-symptomatic Syn II$^{-/-}$ in the current clamp configuration. In the presence of specific antagonists that fully block synaptic activity, mossy cells were injected with 30 current steps, lasting 1 s and ranging from −100 to +200 pA, in 10 pA increments (**Figures 5A**,**B**). The firing rate of mossy neurons was lower in Syn II$^{-/-}$ slices with respect to WT recordings (**Figure 5B**) and was accompanied by a significant increase in the rheobase (45.0 ± 6.7, $n = 11$ neurons/5 mice for WT vs. 83.3 ± 8.8 for Syn II$^{-/-}$, $n = 6$ neurons/4 mice; two-tailed unpaired Student's *t*-test, $p = 0.003$) (**Figure 5C**, left). Input resistance, a parameter correlated with the firing rate, was also significantly reduced in Syn II$^{-/-}$ (396.0 ± 45.2 MΩ, $n = 11$ neurons/5 mice for WT vs. 248.7 ± 23.2 for Syn II$^{-/-}$, $n = 6$ neurons/4 mice; two-tailed unpaired Student's *t*-test, $p = 0.031$) (**Figure 5C**, right). On the contrary, recording from granule cells revealed no differences in the firing rates of WT and Syn II$^{-/-}$ neurons (**Figures 5D**,**E**), with no genotype-dependent difference in either input resistance or rheobase (data not shown). Other intrinsic membrane properties

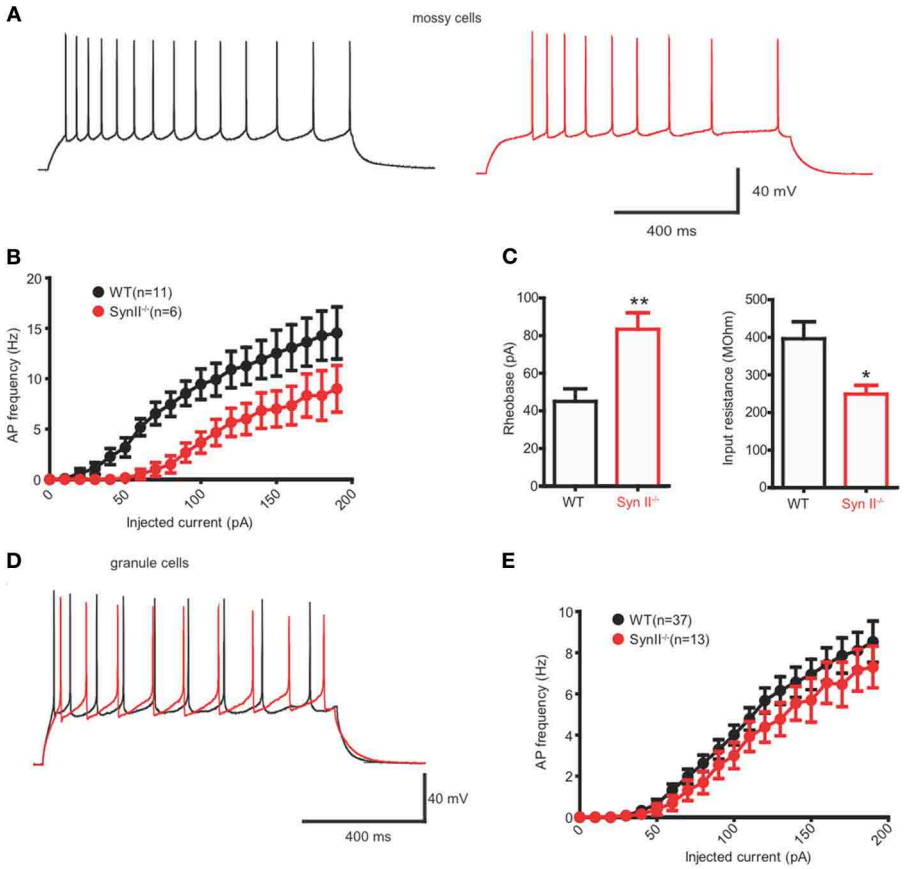

FIGURE 5 | Hilar mossy cells of pre-symptomatic Syn II$^{-/-}$ mice display decreased excitability. (A) Representative traces of whole cell current-clamp recordings from hilar mossy neurons in acute slices of WT (black) and pre-symptomatic Syn II$^{-/-}$ (red) mice. **(B)** Action potential (AP) frequency plotted as a function of the injected current for both genotypes. **(C)** Mean (±s.e.m.) rheobase and input resistance; $^{*}p < 0.05$, $^{**}p < 0.01$, two-tailed unpaired Student's *t*-test. **(D,E)** Representative traces of whole cell current-clamp recordings from granule neurons **(D)** in acute slices of WT (black) and pre-symptomatic Syn II$^{-/-}$ (red) mice and AP frequency **(E)** plotted as a function of the injected current for both genotypes.

(resting and threshold potential, AP amplitude, half-amplitude width, after-hyperpolarization current) were similar for the two genotypes in both mossy and granule neurons (data not shown). These results indicate that the reduced fPSPs in the hilar region of pre-symptomatic Syn II$^{-/-}$ mice (**Figure 1**) are associated with reduced excitability of Syn II$^{-/-}$ mossy cells, but not granule cells.

ADULT SYN II$^{-/-}$ HILAR MOSSY CELLS RECAPITULATE THE PHENOTYPE OF PRE-SYMPTOMATIC SYN II$^{-/-}$ MICE

To verify if the reduced excitability of hilar mossy cells in pre-symptomatic Syn II$^{-/-}$ slices persists after the initiation of epileptic seizures in these mice, we repeated the experiments from **Figures 4**, **5** on adult (4–6 months old) Syn II$^{-/-}$ mouse slices. As in pre-symptomatic mice, both the amplitude and the frequency distributions of mEPSCs were significantly shifted toward lower values in Syn II$^{-/-}$ hilar mossy neurons ($n = 4$ neurons/3 mice for WT and 6 neurons/3 mice for Syn II$^{-/-}$; Kolmogorov–Smirnov test, $p < 0.001$) (**Figures 6A**,**B**). The smaller amplitude distribution of Syn II$^{-/-}$ cells was not accompanied by any change in the kinetic parameters of the response with respect to the WT (rise-time 10–90%: 1.36 ± 0.2 vs. 1.08 ± 0.1 ms, $p = 0.296$; decay τ: 5.58 ± 1.1 vs. 5.63 ± 0.6 ms, $p = 0.967$; two-tailed unpaired Student's *t*-test) (**Figure 6C**). Moreover, the firing rate of mossy cells was lower in adult Syn II$^{-/-}$ (**Figures 6D**,**E**), with a significant increase in rheobase (60.0 ± 5.7, $n = 3$ neurons/3 mice for WT vs. 85.0 ± 8.6 pA for Syn II$^{-/-}$, $n = 8$ neurons/3 mice; two-tailed unpaired Student's *t*-test, $p = 0.043$) (**Figure 6F**, left) and a decrease in input resistance (422.0 ± 12.5 MΩ, $n = 3$ neurons/3 mice for WT vs. 269.0 ± 38.0 for Syn II$^{-/-}$, $n = 8$ neurons/3 mice; two-tailed unpaired Student's *t*-test, $p = 0.042$) (**Figure 6F**, right). These results show that the cellular phenotype of Syn II$^{-/-}$ mice appears long before the appearance of an overt epileptic phenotype.

THE INHIBITORY OUTPUT OF HILAR MOSSY CELLS TO GRANULE CELLS IS REDUCED IN BOTH PRE-SYMPTOMATIC AND SYMPTOMATIC SYN II$^{-/-}$ MICE

The axons of hilar mossy cells project into the inner molecular layer of the DG, where they make excitatory synapses directly with granule cells, or with GABA interneurons, leading to disynaptic inhibition of granule cells (Scharfman and Myers, 2012; Jinde

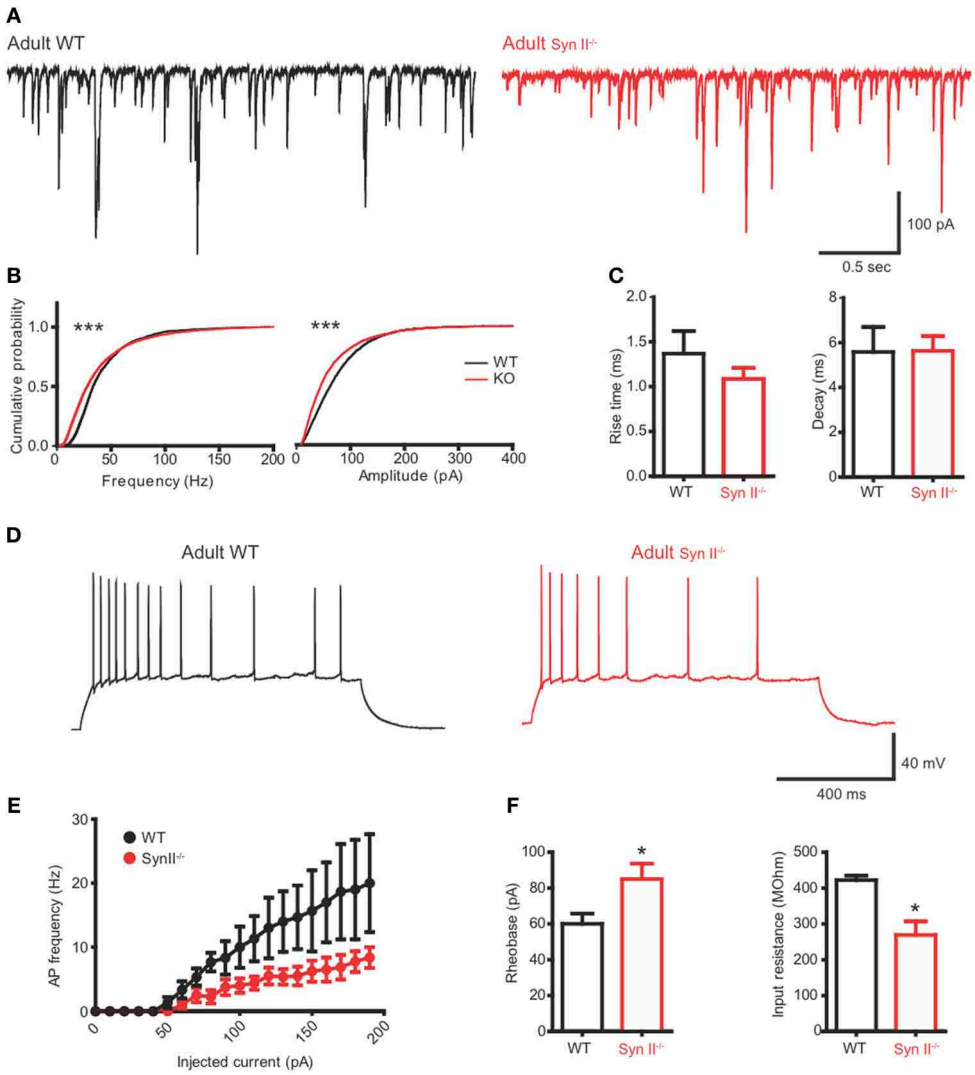

FIGURE 6 | The hilar mossy cell phenotype of pre-symptomatic Syn II$^{-/-}$ mice is maintained in adult symptomatic Syn II$^{-/-}$ mice. (A) Representative mEPSC traces **(A)** and cumulative distributions **(B)** of their amplitude and frequency from 4 to 6 months old WT (black) and Syn II$^{-/-}$ (red) mossy cells; ***$p < 0.001$, Kolmogorov–Smirnov test. **(C)** Mean (±s.e.m.) rise-time (10–90%) and mono-exponential τ of decay of mEPSCs from 4–6 months old WT (black bars) and Syn II$^{-/-}$ (red bars) neurons. **(D)** Representative traces of current-clamp recordings from hilar mossy neurons in acute slices of WT (black) and symptomatic Syn II$^{-/-}$ (red) mice. **(E)** Frequency of APs plotted as a function of the injected current for both genotypes. **(F)** Mean (±s.e.m.) rheobase and input resistance; *$p < 0.05$, two-tailed unpaired Student's *t*-test.

et al., 2013). Since Syn II is abundantly and specifically expressed in the mossy cell terminals of the inner molecular layer (**Figure 3**), the next step was to evaluate the net effect of the mossy cell output on granule cell activity. To this aim, we patched granule neurons from the granule cell layer and stimulated the axons of mossy cells in the region below the granule layer (**Figure 7A**). When granule neurons are voltage-clamped at -80 mV, close to the reversal potential of Cl^-, the stimulation should result in an evoked excitatory inward response non-contaminated by inhibition. On the contrary, when the clamped voltage is shifted to 0 mV, the Cl^- drive will be predominant, and the stimulation should elicit a net inhibitory, outward response representing the fast-forward inhibition resulting from the intermediate activation of GABA interneurons (**Figure 7B**). Single stimulation did not reveal any difference between the amplitude of both eEPSC and eIPSCs in WT and pre-symptomatic and adult Syn II$^{-/-}$ slices ($n = 16$ neurons/6 mice for WT, 10 neurons/4 mice for young Syn II$^{-/-}$ and 13 neurons/3 mice for adult Syn II$^{-/-}$; One-Way ANOVA followed by the Bonferroni's multiple comparison test, $p = 0.344$ and 0.751 for eEPSCs and eIPSC, respectively) (**Figures 7C,D**). Instead, the application of a 40 Hz tetanic stimulation revealed that depression was significantly increased at inhibitory synapses in both young and adult Syn II$^{-/-}$ granule neurons (**Figure 7F**), while it was similar between genotypes at excitatory synapses (**Figure 7E**). We quantified this effect by measuring the ratio between the evoked excitatory and inhibitory responses (E/I

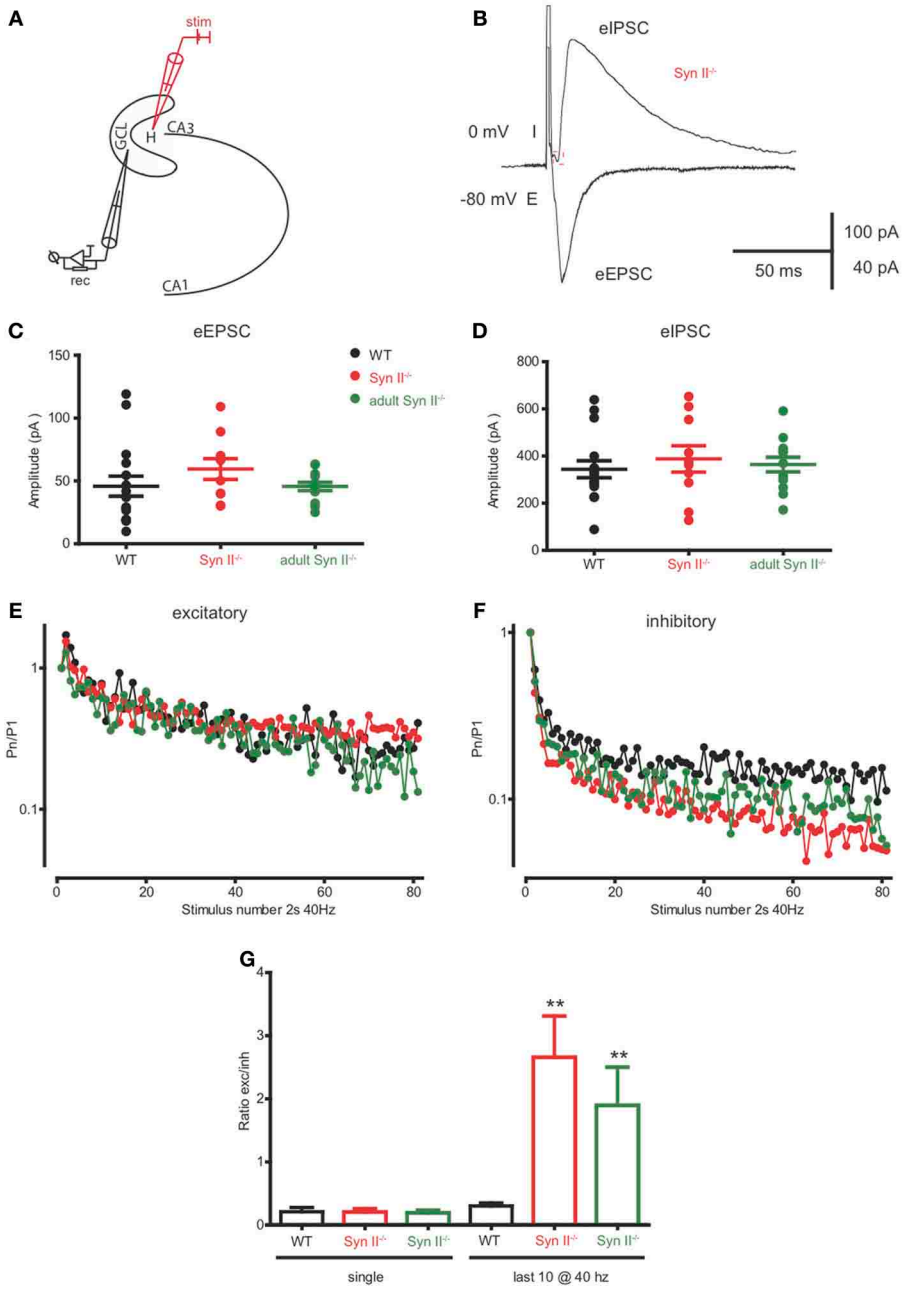

FIGURE 7 | The inhibitory output of hilar mossy cells to granule cells is reduced in both pre-symptomatic and symptomatic Syn II$^{-/-}$ mice. (A) Scheme of the experimental setup. **(B)** Representative traces of an eEPSC (−80 mV, inward) and an eIPSC (0 mV, outward) recorded in the voltage-clamp configuration from the same pre-symptomatic Syn II$^{-/-}$ granule cell after the stimulation of the perforant path. **(C,D)** Aligned dot-plots representing the amplitude of the eEPSCs **(C)** or eIPSCs **(D)** from young WT (black), pre-symptomatic (red) and symptomatic (green) Syn II$^{-/-}$ mice. **(E,F)** Plots of the normalized mean amplitude of excitatory **(E)** or inhibitory **(F)** responses vs. time showing the multiple-pulse depression during a 2-s train at 40 Hz in young WT (black), pre-symptomatic (red) and symptomatic (green) Syn II$^{-/-}$ mice. **(G)** Mean (±s.e.m.) ratio between the amplitudes of eEPSCs and eIPSCs from the same granule neurons (young WT, black; pre-symptomatic Syn II$^{-/-}$, red; symptomatic Syn II$^{-/-}$, green) in the case of a single stimulus (left) or for the last 10 stimuli in a 40 Hz train (right); $^{**}p < 0.001$, Kruskal–Wallis test followed by Dunn's multiple comparison test.

ratio) on the same granule cell. Single pulse stimulation of the mossy cell axon produced postsynaptic inhibitory and excitatory currents whose ratio was similar between genotypes ($p = 0.938$; One-Way ANOVA followed by the Bonferroni's multiple comparison test) (**Figure 7G**, left). However, when the E/I ratio was measured for the last 10 responses in the train, it was significantly increased in both pre-symptomatic and symptomatic Syn II$^{-/-}$ slices, with a decrease of inhibitory responses (E/I

ratio = 0.3 ± 0.04 for WT, $n = 6$ neurons/3 mice; 2.6 ± 0.6 for young Syn II$^{-/-}$, $n = 7$ neurons/4 mice; 1.8 ± 0.6 for adult Syn II$^{-/-}$, $n = 6$ neurons/3 mice; $p = 0.0011$; Kruskal–Wallis test followed by the Dunn's multiple comparison test) (**Figure 7G**, right). This change in the ratio between excitation and inhibition may be responsible for the hyperexcitability of the DG under conditions of sustained high frequency synaptic input, as seen in **Figure 2**.

DISCUSSION

Epilepsy affects 1% of the general population, and 0.5% of children (Cowan, 2002). In the last 15 years, with the advent of genetic screening of epileptic families, many causative mutations in epileptogenic genes have been identified. The vast majority of these genes code for ion channels, or ion channel auxiliary subunits, with Na^+ channels as the main "actors" (Gardiner, 1999; Poduri and Lowenstein, 2011). Some mutations have been also found in genes that encode proteins involved in the presynaptic SV release machinery or cell metabolism (Cavalleri et al., 2007; Striano et al., 2008; Suls et al., 2009; Pearl et al., 2011). As a result of these findings, more than 15 transgenic and knockout mouse models have been generated to aid in the study of epilepsy (Mantegazza et al., 2010). These models are superior to pharmacologically induced models. They often closely resemble the human pathology and offer the possibility to study the evolution of the disease and test new therapeutic strategies. These organisms can be studied in the pre-symptomatic phase, to investigate the mechanisms that lead to epileptogenesis, and distinguish them from subsequent secondary mechanisms that lead to the aggravation of symptoms.

As mentioned above, *SYN2* alterations in humans seem to confer predisposition for epilepsy. Previous studies have suggested an association of *SYN2* rs3773364 A>G polymorphism with febrile seizures in the UK, Irish, and Finnish cohorts (EPIGEN Epilepsy Genetic Consortium; Cavalleri et al., 2007) and in Indian patients with idiopathic epilepsy (Lakhan et al., 2010), but not in the Australian cohort (Cavalleri et al., 2007) or in Malaysian epileptic patients (Haerian et al., 2011). Remarkably, mice lacking one or more Syn isoforms are all prone to epileptic seizures, with the exception of Syn III$^{-/-}$ mice (Rosahl et al., 1995; Cesca et al., 2010; Etholm et al., 2011, 2012; Ketzef et al., 2011; Farisello et al., 2013). The model used in the present study is the Syn II$^{-/-}$ mouse developing an overt epileptic phenotype around the age of 2 months (Bogen et al., 2011; Etholm et al., 2011, 2012). It remains unclear why absence of Syn I or II leads to hyperexcitability. An early study (Rosahl et al., 1995) investigated mice lacking Syn I, Syn II, or both isoforms. By using extracellular recordings from CA1 pyramidal neurons, it was shown that paired-pulse facilitation was increased in the Syn I$^{-/-}$, but not in the Syn II$^{-/-}$ or in the double Syn I/II$^{-/-}$ mouse. On the contrary, post-tetanic potentiation was decreased in the latter two genotypes, but not in the Syn I$^{-/-}$ mouse. The Syn II$^{-/-}$ and Syn I/II$^{-/-}$ mice also underwent severe synaptic depression upon repetitive stimulation (Rosahl et al., 1995).

We have recently shown that pre-symptomatic Syn I/II/III$^{-/-}$ mice display an impaired tonic current, due to defects in GABA release and spillover and leading to diffuse hyperexcitability of hippocampal pyramidal neurons (Farisello et al., 2013). A similar study (Ketzef et al., 2011) showed that, increased field responses were elicited in these mice during the pre-symptomatic phase. They further suggest that in pre-symptomatic animals a number of compensatory mechanisms take place that enhance both inhibition and excitation, and eventually culminate with the onset of seizures. In a parallel study (Boido et al., 2010), the action of the antiepileptic drug levetiracetam was investigated in Syn I/II/III$^{-/-}$ mouse slices. They used 4-aminopyridine to evoke epileptic-like events and showed that levetiracetam ameliorated abnormal activity more efficiently in WT than in Syn I/II/III$^{-/-}$ mice. This finding can be explained by the decreased levels of the levetiracetam receptor SV2A due to the marked decrease in SV density observed in these animals (Gitler et al., 2004).

The basic question is why this phenotype appears so late in post-natal development, if other phenotypic traits, such as the sharp loss of the reserve pool of SVs in nerve terminals (Baldelli et al., 2007; Cesca et al., 2010; Lignani et al., 2013), are altered much earlier. This view has been extended by more recent studies showing that Syns are actively involved in post-docking steps of exocytosis, delivering SVs to the active zones and affecting the kinetics and synchronization of release (Hilfiker et al., 1998; Bykhovskaia, 2011; Medrihan et al., 2013). In the case of Syn II, it is interesting that the protein expression profile increases from birth to reach a plateau around postnatal day 60, coinciding with the onset of seizures (Bogen et al., 2011). Thus, it is possible that the overt epileptic phenotype appears only when the synapses require the full expression of Syns to maintain a proper balance between excitatory and inhibitory transmission. Moreover, epileptogenesis is a multifactorial disorder that involves a cascade of molecular, cellular and network alterations occurring over a long time interval after the initial insult (Rakhade and Jensen, 2009; Hunt et al., 2013). The latent phase of epileptogenesis lasts months to years in humans, and between 2 and 12 weeks in rodent models (Rakhade and Jensen, 2009). In the case of a genetic model as Syn II$^{-/-}$ mice, the synaptic dysfunctions during the first 2 months of life hinder the development of a proper balance between excitation and inhibition, eventually leading to seizures. Another important developmental aspect is the expression of GABA receptors and GABA synthetic enzymes that do not reach full expression until before the fourth week of life in rats. Furthermore, it is well known that in the first weeks of life GABA acts as an excitatory neurotransmitter, due to the increased intracellular Cl^- concentration (Rakhade and Jensen, 2009). These aspects could explain the late seizure onset in our mouse model. Although mossy cells have a decreased output and a lower excitatory drive on inhibitory interneurons, this does not significantly alter the interneuron output, since GABA-mediated inhibition is initially weak. This is probably a simplistic explanation, since a number of ion channels, ion pumps and neurotransmitter synthases reach full expression a few weeks after birth in rodents, making it a difficult task to pinpoint one single event that is crucial in seizure onset.

A specific characteristic of temporal lobe epilepsy is the loss of hilar neurons in the hippocampus. It was assumed for a long

time that mossy cells are the main population to decrease in number, despite the fact that, until very recently, no specific marker to distinguish them from inhibitory interneurons was available. Recent results have shown that hilar mossy cells and GABA-positive interneurons are equally decreased in number in a head trauma epilepsy model (Santhakumar et al., 2000), suggesting that mossy cell survival might be as important as their loss in increasing seizure propensity. Currently, there are several theories regarding the reason why mossy cell loss could lead to hippocampal hyperexcitability. The first one is the "*cell loss-induced axonal sprouting*" hypothesis (Wenzel et al., 2000). It is based on the fact that granule cell axons massively innervate mossy cells. A partial loss of mossy cells, by triggering mossy fiber sprouting, would abnormally innervate other granule cells and thus create recurrent excitation loops. The second theory is the "*dormant basket cell*" hypothesis (Sloviter, 1991). This hypothesis proposes a role for mossy cells in exciting the inhibitory basket cells, present in the granule cell layer. A decrease in the number of mossy cells would then lead to a hypoactivation of inhibitory interneurons and to dentate granule cells hyperexcitability. The third theory, the "*irritable mossy cell*" hypothesis, emphasizes the role of the surviving mossy cells that would undergo various alterations, leading to the amplified activity of the granule cells (Scharfman and Myers, 2012). A more recent theory integrates the latter two (Scharfman and Myers, 2012), basing itself on anatomical data and paired recordings showing that mossy cells synapses preferentially target interneurons locally (Scharfman, 1995) and have projections that extend along the septo-temporal hippocampal axis which mainly synapse on granule cells (Buckmaster et al., 1996). Furthermore, it seems that mossy cells do not connect only with basket cells, but with a range of different inhibitory interneurons also in the hilus. Thus, the role of mossy cells in regulating hippocampal activity is rather complex and difficult to predict.

It is not known which, if any, of these theories represents the true mechanism by which the hippocampus becomes hyperexcitable. An essential aspect is the fact that mossy cells convey feedback from the CA3 pyramidal neurons to the granule cells. This circuit configuration might provide the frame for reverberant activity and the transition from brief, interictal events, to seizure-like activity (Scharfman et al., 2001). To test any of the above-described theories, one would need a tool to specifically eliminate mossy cells from the hippocampus and study the subsequent changes. This experiment was performed by Jinde et al. (2012), who generated a conditional transgenic mouse selectively expressing the diphtheria toxin receptor in the mossy cells. After a few weeks of diphtheria toxin injection in the DG, massive mossy cell degeneration was observed, accompanied by a transient increase in theta power during exploration, deficits in contextual discrimination, and increased anxiety. Patch-clamp recordings of granule cells showed that the frequency, but not the amplitude, of both sEPSCs and sIPSCs was decreased (Jinde et al., 2012). These results strengthen the view that mossy cells convey both direct excitatory and indirect/feed-forward inhibitory input to granule cells, and that the balance between these two inputs plays a key role for the spatial and temporal control of granule cell excitability in the DG. Nonetheless, the animals did not exhibit any epileptiform activity (Jinde et al., 2012), confirming that, as it is in our case, the hypoexcitability of mossy cells is not the sole culprit for epileptic hippocampal activity.

In the present study, we dissected the local expression of Syn II in the mouse hippocampus and showed that it is enriched in regions were granule cell axons project, as well as in the inner molecular layer, where inhibitory synapses from the hilus and granule cell interneurons, and excitatory projections from hilar mossy cells are found. Notably, in this area, the, expression of the mossy cell marker calretinin overlapped with the expression of Syn II (**Figure 3**). Upon single stimulation of the perforant path in pre-symptomatic Syn II$^{-/-}$ mice we found a decreased field response and a longer latency in the hilus, suggesting impairments in the synaptic release mechanism at mossy fiber terminals and/or a decreased responsiveness of hilar cells. Moreover, synaptic depression in response to tetanic stimulation was significantly decreased in the granule cell layer of the Syn II$^{-/-}$ mice. Based on previous work showing that perforant path activation is similar in Syn II$^{-/-}$ and WT age matched mice (Medrihan et al., 2013) and considering that somatic granule cell properties were unchanged, we suggest that these changes arise from an altered feedback received from the hilar region. Indeed, patch-clamp recordings of mossy cells demonstrated that the dysfunction lies on both sides: on one hand, vesicular release from mossy fibers is reduced, and on the other hand, mossy cell excitability is reduced. These changes appeared before the overt epileptic phenotype and persisted in the adulthood. Finally, the dissection of the excitatory and inhibitory inputs to granule cells from mossy cells revealed that, under conditions of sustained high frequency synaptic activity, the excitation/inhibition ratio is significantly increased in both pre-symptomatic and symptomatic Syn II$^{-/-}$ mice.

It is difficult to explain why the loss of a presynaptic protein leads to decreased excitability of mossy cells. The latter could be the expression of a homeostatic mechanism operating at the level of the hippocampal network that fights against hyperexcitability and absolutely requires the full expression of Syn II when plasticity in the adult brain decreases. This hypothesis suggests that one of the functional roles of Syns I and II, whose expression strongly increases after the first month of postnatal life and remains high throughout adulthood (Lohmann et al., 1978; Bogen et al., 2009), is to provide a high degree of plasticity to the adult synapses.

The functional changes in the DG of the Syn II$^{-/-}$ may not be all due to the sole absence of synapsin and high-throughput profiling studies will prove useful to investigate the relative expression of other genes/proteins involved in epileptogenesis. However, these studies would be difficult to design in our case, since the absence of synapsin II may have developmental long term effects on many aspects of the maturation of the hippocampal network. The profiling studies are usually investigating the changes induced by the application of a drug or trauma or a mutation in a transcription/repressor factor that can directly lead to changes in the transcription machinery (Bando et al., 2011; Pernot et al., 2011; Goldberg and Coulter, 2013). In our case any change in DNA/RNA/proteins might reflect adaptive responses to the primary changes in synaptic transmission

and network excitability induced by the synapsin II mutation. Indeed, the Syn II$^{-/-}$ mouse is characterized, like all other synapsin mutants, by a severe reduction in the density of the SVs, leading to a general decrease in the expression of SV proteins, such as synaptophysin I, SV2 and Rab5 (Rosahl et al., 1995).

Overall, our results reinforce the idea that mossy cells have a predominantly inhibitory effect within the hippocampus, through feedback inhibition on granule cells and that impairments in their function could trigger, or participate in an excitation/inhibition imbalance at the level of the DG of the hippocampus that represents a key factor for epilepsy predisposition. However, epileptogenesis is a multifactorial process that follows a precise temporal sequence of events in most animal models of epilepsy (pilocarpine, kindling, trauma) leading to progressive neural circuitry alterations (Rakhade and Jensen, 2009; Hunt et al., 2013). Thus, our findings suggest that the hypoexcitability of mossy cells is just one factor among others that weakens the DG circuitry and renders it more prone to hyperexcitability in the animal adult. Studies on experimental models of epilepsy, such as the Syn II$^{-/-}$ mouse, could shed light on the complex synaptic mechanisms orchestrating network excitability and contribute to ameliorate diagnosis and prognosis of idiopathic epilepsy linked to synaptic abnormalities.

ACKNOWLEDGMENTS

We thank Drs. Hung-Teh Kao (Brown University, Providence, RI) and Paul Greengard (The Rockefeller University, New York, NY) for providing us with the Syn II mutant mouse strain. We also thank Dr. Gabriele Lignani and Federico Pecoraro for help with the experiments and useful discussions. This study was supported by research grants from the Italian Ministry of University and Research (PRIN to Fabio Benfenati and Pietro Baldelli), the Italian Ministry of Health Progetto Giovani (to Pietro Baldelli) and the Compagnia di San Paolo, Torino (to Fabio Benfenati and Pietro Baldelli). The support of Telethon-Italy (Grant GGP09134 to Fabio Benfenati and GGP09066 to Pietro Baldelli) is also acknowledged.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2013; accepted: 08 August 2013; published online: 30 August 2013.

Citation: Toader O, Forte N, Orlando M, Ferrea E, Raimondi A, Baldelli P, Benfenati F and Medrihan L (2013) Dentate gyrus network dysfunctions precede the symptomatic phase in a genetic mouse model of seizures. Front. Cell. Neurosci. 7:138. doi: 10.3389/fncel.2013.00138

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 30 August 2013
doi: 10.3389/fncel.2013.00139

Are vesicular neurotransmitter transporters potential treatment targets for temporal lobe epilepsy?

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The vesicular neurotransmitter transporters (VNTs) are small proteins responsible for packing synaptic vesicles with neurotransmitters thereby determining the amount of neurotransmitter released per vesicle through fusion in both neurons and glial cells. Each transporter subtype was classically seen as a specific neuronal marker of the respective nerve cells containing that particular neurotransmitter or structurally related neurotransmitters. More recently, however, it has become apparent that common neurotransmitters can also act as co-transmitters, adding complexity to neurotransmitter release and suggesting intriguing roles for VNTs therein. We will first describe the current knowledge on vesicular glutamate transporters (VGLUT1/2/3), the vesicular excitatory amino acid transporter (VEAT), the vesicular nucleotide transporter (VNUT), vesicular monoamine transporters (VMAT1/2), the vesicular acetylcholine transporter (VAChT) and the vesicular γ-aminobutyric acid (GABA) transporter (VGAT) in the brain. We will focus on evidence regarding transgenic mice with disruptions in VNTs in different models of seizures and epilepsy. We will also describe the known alterations and reorganizations in the expression levels of these VNTs in rodent models for temporal lobe epilepsy (TLE) and in human tissue resected for epilepsy surgery. Finally, we will discuss perspectives on opportunities and challenges for VNTs as targets for possible future epilepsy therapies.

Keywords: vesicular neurotransmitter transporters, temporal lobe epilepsy, epileptogenesis, antiepileptic drugs, *SLC17*, *SLC18*, *SLC32*

INTRODUCTION

Epilepsy is one of the most common acquired chronic neurologic disorders, affecting approximately 1% of the human population and displaying an annual incidence of 50,000–100,000 persons (Pitkänen and Sutula, 2002; Pitkänen and Lukasiuk, 2009; Fridley et al., 2012). The disorder has disastrous implications for the quality of life of the patients, concerning independent living, education and employment, mobility and personal relationships. In Europe alone, health costs increased considerably, reaching more than €15.5 billion (Baulac and Pitkanen, 2008). Despite the availability of a large number of antiepileptic drugs (AEDs), clinically proven to suppress or prevent seizures, in 30–40% of patients symptoms cannot be controlled (Baulac and Pitkanen, 2008; Abou-Khalil and Schmidt, 2012).

Epilepsy is characterized by spontaneous, recurrent seizures (SRS), caused by abnormal synchronized, high frequency neuronal discharges (Pitkänen and Sutula, 2002; Baulac and Pitkanen, 2008; O'dell et al., 2012). Especially in patients with temporal lobe epilepsy (TLE), marked by partial complex seizures of temporal-lobe origin, a progressive development of the disorder is observed (Pitkänen and Sutula, 2002; Baulac and Pitkanen, 2008). Generally, TLE is initiated by an initial precipitating injury, such as status epilepticus (SE), head trauma, brain infection, stroke, or febrile seizures. This insult triggers a cascade of devastating neurobiological events and histological and biochemical changes during a latency period of 5–10 years. In this time range the patient remains free from symptoms or complications, though an epileptic state is being established in the brain, called epileptogenesis, leading to the occurrence of SRS and the diagnosis of epilepsy (Pitkänen and Sutula, 2002; Sharma et al., 2007; Pitkänen and Lukasiuk, 2009; O'dell et al., 2012).

The main mechanisms of action of currently available AEDs can be classified into four broad categories: (1) modulation of voltage-dependent sodium, calcium (Ca^{2+}) or potassium (K^{+}) channels, (2) alterations in γ-aminobutyric acid (GABA)ergic inhibition via actions on $GABA_A$ receptors or on GABA synthesis, reuptake or degradation, (3) decreased synaptic excitation via actions on ionotropic glutamate receptors and (4) modulation of neurotransmitter release via presynaptic mechanisms, with most relevant action on glutamate release (Porter et al., 2012) (**Figure 1**). Despite the fact that some AEDs have proven neuroprotective properties and act by multiple mechanisms of action, they are often only transiently effective and anticonvulsant. Consequently, treatment might be successful at the onset of SRS (silent period), however, as the disease progresses, many patients develop tolerance and become pharmacoresistant (Abou-Khalil and Schmidt, 2012; Kobow et al., 2012; O'dell et al., 2012). Alternative therapies are limited and invasive, including seizure foci resection, vagus nerve stimulation and brain stimulation (Fridley et al., 2012). Obviously, there is an urgent need

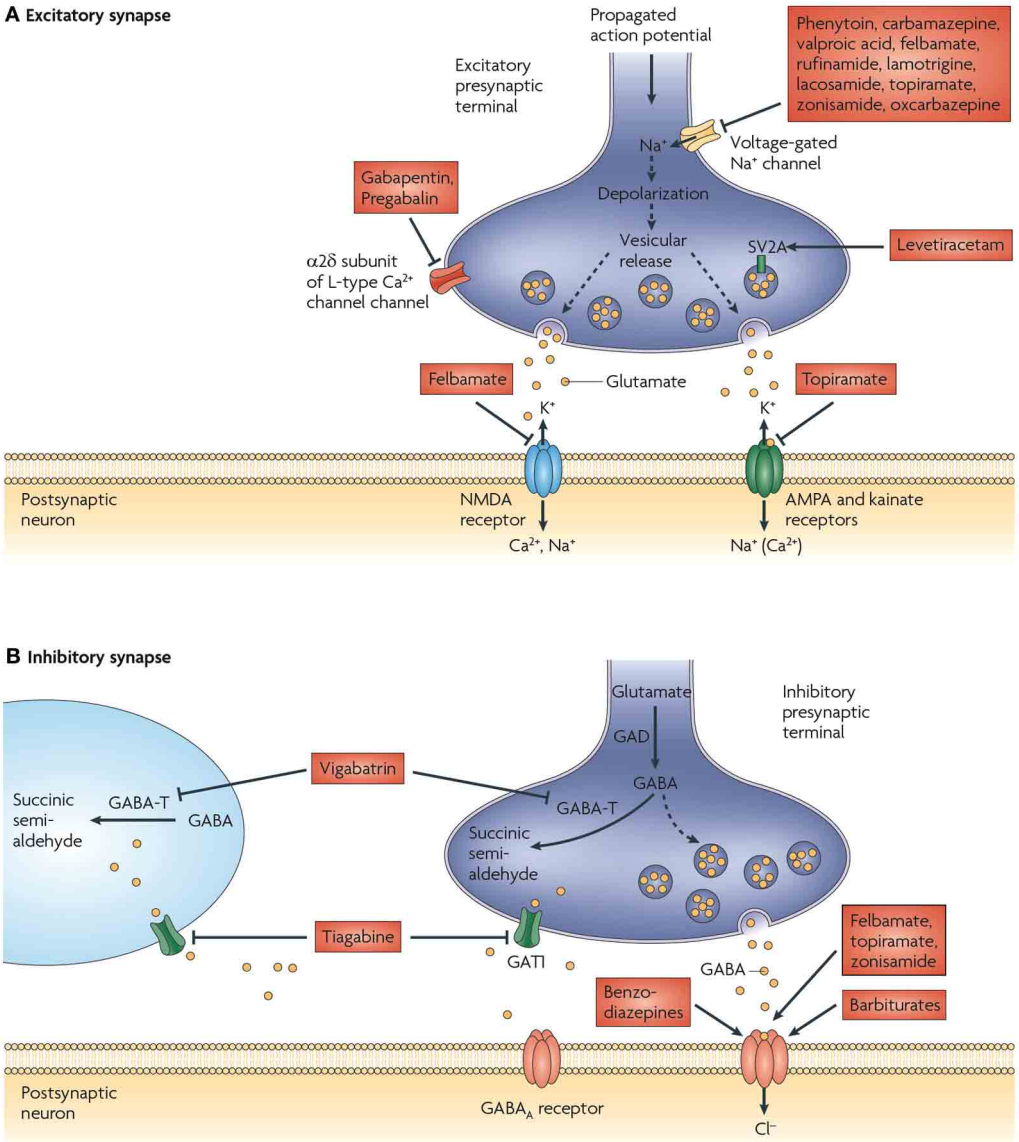

FIGURE 1 | Proposed mechanisms of action of currently available antiepileptic drugs (AEDs) at excitatory and inhibitory synapses. (A) Currently available AEDs are thought to target several molecules at the excitatory synapse. These include voltage-gated Na^+ channels, synaptic vesicle glycoprotein 2A (SV2A), the α2δ subunit of the voltage-gated Ca^{2+} channel, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, and NMDA (N-methyl-d-aspartate) receptors. Many of the AEDs can modulate voltage-gated Na^+ channels. This would be expected to decrease depolarization-induced Ca^{2+} influx and vesicular release of neurotransmitters. Levetiracetam is the only available drug that binds to SV2A, which might have a role in neurotransmitter release. Gabapentin and pregabalin bind to the α2δ subunit of voltage-gated Ca^{2+} channels, which is thought to be associated with a decrease in neurotransmitter release. Excitatory neurotransmission at the postsynaptic membrane can be limited by topiramate (acting on AMPA and kainate receptors) and felbamate (acting on NMDA receptors). **(B)** AED targets at inhibitory synapses have also been proposed. These include the γ-aminobutyric acid (GABA) transporter GAT1 (also known as *SLC6A1*), which is inhibited by tiagabine, leading to a decrease in GABA uptake into presynaptic terminals and surrounding glia, and GABA transaminase (GABA-T), which is irreversibly inhibited by vigabatrin. This decreases the metabolism of GABA in presynaptic terminals and glial cells. The benzodiazepines, barbiturates, topiramate, and felbamate have been found to enhance inhibitory neurotransmission by allosterically modulating GABAA receptor-mediated Cl^- currents. However, the action of each of these drugs is different and is dependent on the subunit conformation of the GABAA receptor complex. GAD, glutamic acid decarboxylase. Figure is modified, with permission, from Bialer and White (2010).

of non-invasive treatments that prevent or modify the development of epilepsy, in other words, antiepileptogenic or disease-modifying drugs, respectively (Baulac and Pitkanen, 2008). In future validation, new targets should be able to achieve at least one of these two aims (Kobow et al., 2012).

In order to identify new targets, the molecular and cellular mechanisms behind the genesis of epilepsy need to be unveiled. Although countless studies have tried to understand the pathogenesis and progression of TLE, the order of deleterious events during epileptogenesis still remains unknown (O'dell et al., 2012).

Moreover, the outcome after the initial insult is considerably variable and at present there are no reliable biomarkers or surrogate disease markers available (Kobow et al., 2012). Current innovative strategies for future AED targets include ion channels and other potential molecular targets (see Meldrum and Rogawski, 2007 and Bialer and White, 2010 for recent reviews) such as neuronal gap junctions (Belousov, 2012; Belousov and Fontes, 2013), neuropeptides (Casillas-Espinosa et al., 2012; Portelli et al., 2012a,b), astrocytic gap junctions and Kir channels (Kovacs et al., 2012; Steinhauser et al., 2012), the cystine/glutamate antiporter or system x_{c-} (De Bundel et al., 2011; Lewerenz et al., 2013), mTOR (McDaniel and Wong, 2011), distinct inflammatory pathways (Vezzani et al., 2013), adenosine kinase (Boison, 2010, 2012), aquaporin channels (Binder et al., 2012; Kovacs et al., 2012) and Ca^{2+}-dependent gliotransmission (Carmignoto and Haydon, 2012). It should be taken into consideration that all targets are chosen based on animal models, of which many are not clinically meaningful considering the precipitating cause. In addition, the use of human disease-affected brain tissue is of utmost importance in the search for new drug targets, still there are some disadvantages when human brain tissue is used for investigation. First of all, most patients included in these studies suffered from seizures for many years and secondly, these patients have been treated with specific AEDs. Moreover, it is very unlikely that only one cellular or molecular pathway is responsible for the variety of syndromes and degrees of epilepsy (Kobow et al., 2012).

An attractive new molecular target for AEDs might be represented by vesicular neurotransmitter transporters (VNTs) (**Figure 2**). The rationale of their potential use is related on the nature of epilepsy that is characterized by spontaneous, recurrent seizures, caused by abnormal synchronized, high frequency neuronal discharges. Neuronal discharges correspond to chemical transmission, which is essential for normal communication and functioning of the brain. This involves accumulation of neurotransmitters into secretory vesicles, achieved by various types of VNTs, followed by their exocytotic release into the extracellular space (Chaudhry et al., 2008a; Omote et al., 2011). VNTs are small proteins responsible for packing synaptic vesicles with neurotransmitters, thereby determining the amount of neurotransmitter released per vesicle through fusion in both neurons and glial cells. Based on their substrate specificity and amino acid sequence similarity, to date, nine VNTs have been divided into three subclasses; *SLC17*, *SLC18* and *SLC32* gene families. The *SLC17* gene family consists of the three vesicular glutamate transporters (VGLUT1, VGLUT2, and VGLUT3), the vesicular excitatory amino acid transporter (VEAT), and the vesicular nucleotide transporter (VNUT) (Reimer, 2013). The *SLC18* gene family comprises the vesicular monoamine transporters (VMAT1 and VMAT2) for serotonin (5-HT), dopamine (DA), noradrenaline

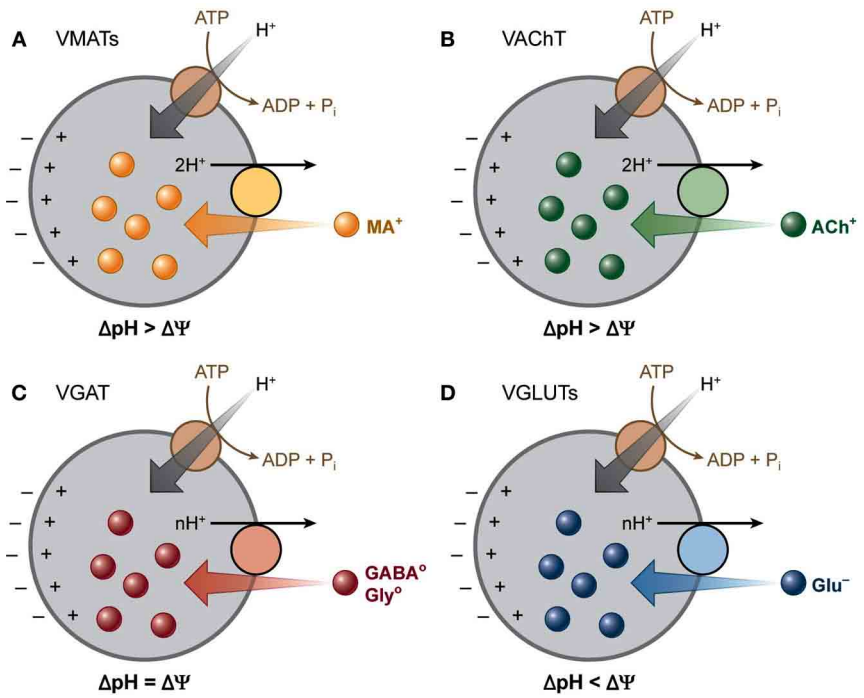

FIGURE 2 | Vesicular neurotransmitter transporters depend differentially on the two components of the electrochemical gradient of H^+ ($\Delta\mu_{H+}$). A V-ATPase generates a $\Delta\mu_{H+}$ across the vesicle membranes. The vesicular transporters use this gradient to drive the transport of transmitters into secretory vesicles by coupling the translocation of transmitter to H^+ running down $\Delta\mu_{H+}$. The different vesicular transporters rely to different extents on the two components (ΔpH and $\Delta\psi$) of this gradient. **(A)** VMATs and **(B)** VAChT transport their positively charged substrates coupled to the exchange of two H^+, and hence rely primarily on ΔpH. **(C)** GABA and glycine are transported as neutral zwitterions by VGAT, which depends equally on both the chemical and the electrical component of $\Delta\mu_{H+}$. **(D)** VGLUTs transport the negatively charged glutamate and thus rely more on $\Delta\psi$ than ΔpH. [Modified from Chaudhry et al. (2008b) with permission].

(NE) and histamine and the vesicular acetylcholine transporter (VAChT) (Eiden et al., 2004). Finally, the *SLC32* gene family consists of the vesicular GABA transporter (VGAT) (Gasnier, 2004).

The import of neurotransmitters depends on a proton electrochemical driving force ($\Delta\mu_{H+}$) generated by the vacuolar H^+-ATPase. Despite the utmost important function of VNTs, the main regulation of this vesicular transport remains unknown. Two mechanisms have been suggested to modulate vesicular transport: (1) influencing the membrane potential ($\Delta\psi$) by cation and anion fluxes and (2) a direct interaction between the heterotrimeric G-protein, Gαo2 and VNTs (Omote et al., 2011; Blakely and Edwards, 2012; Hnasko and Edwards, 2012) (**Figure 2**).

Each VNT has always been considered as a specific marker of the respective nerve cells containing that particular neurotransmitter or structurally related neurotransmitters. Recently, it has been observed that several neuronal populations co-release classical neurotransmitters (see for a review Hnasko and Edwards, 2012). The co-transmitters might influence each other's uptake, by influencing the $\Delta\mu_{H+}$, or they can be gathered in distinct vesicles (Hnasko and Edwards, 2012). Glutamate co-release by cholinergic neurons and monoaminergic neurons is most studied and introduced the term of "vesicular synergy," since vesicular co-accumulation of glutamate by vesicular glutamate transporter 3 (VGLUT3) in cholinergic and serotoninergic neurons, results in higher vesicular import of acetylcholine (ACh) and 5-HT, respectively. The anionic influx of one of the substrates of VGLUT3 (glutamate, Cl^- or P_i) probably creates a lumen-positive $\Delta\psi$ and consequently increases the $\Delta\mu_{H+}$ for ACh and 5-HT vesicular accumulation (El Mestikawy et al., 2011). Due to this co-release neurotransmission might become more complex and expose unraveled roles for VNTs therein. Here we have reviewed the limited literature available on VNTs and epilepsy and their potential role as treatment targets for TLE.

Yet, the only example of a vesicular protein as a target for the treatment of epilepsy is the synaptic vesicle protein 2A (SV2A), the binding site of levetiracetam (LEV). *In vitro* and *ex vivo* binding studies, using SV2A knock-out ($^{-/-}$), heterozygous ($^{+/-}$) and wild-type ($^{+/+}$) mice, identified SV2A as the binding target for LEV. In addition, these transgenic mice were phenotyped in kindling and distinct acute seizure models, unveiling a decreased seizure threshold and accelerated kindling development of the $SV2A^{+/-}$ mice compared to the $SV2A^{+/+}$ mice. $SV2A^{-/-}$ mice, on the other hand, exhibit early severe seizures and die within 2–3 weeks after birth (Kaminski et al., 2012). Other data from these transgenic mice demonstrated the role of SV2A in modulation of vesicular exocytosis (Xu and Bajjalieh, 2001; Budzinski et al., 2009; Chang and Sudhof, 2009; Wan et al., 2010; Yao et al., 2010; Joshi et al., 2013). Although LEV failed in classical seizure screening tests, this drug significantly inhibited the development of seizure kindling and displayed a potential antiepileptogenic effect in the chronic pilocarpine post-SE rat model (Kaminski et al., 2012). A comparative study showed a significant decrease of SV2A protein expression in hippocampus of pharmacoresistant human TLE patients and hippocampus of rats during the latent and chronic phase of the pilocarpine post-SE rat model (van Vliet et al., 2009). These data are consistent with the previously mentioned decreased seizure threshold and accelerated epileptogenesis in $SV2A^{+/-}$ mice (Kaminski et al., 2012). Moreover, these results might explain the loss of the initial efficacy of LEV in refractory TLE patients (Kaminski et al., 2012; Lee et al., 2013). At present, LEV is a worldwide commonly used second generation AED, approved as adjunctive and monotherapy treatment of partial-onset seizures with or without secondary generalization, and adjunctive treatment of myoclonic seizures associated with juvenile myoclonic epilepsy and primary generalized tonic-clonic seizures associated with idiopathic generalized epilepsy (Lyseng-Williamson, 2011). Brivaracetam, a rationally designed LEV derivative, which showed an increased affinity to the LEV-binding site and more potent and complete seizure suppression in animal models of partial and generalized seizures, is currently tested in clinical trial studies (Kaminski et al., 2012).

These data prove that vesicular proteins are accessible and propose a promising role for proteins, involved in vesicle exocytosis, as novel targets for the development of AEDs. Unfortunately, only few studies have followed this concept and brivarecetam is the only new AED in the pipeline targeting a vesicular protein. As outlined for SV2A, results from transgenic mice, distinct acute and chronic rodent epilepsy models and resected human epileptic tissue are crucial to constitute a clear and complete conception of new, potential AED targets. Consequently, in this review we will use the same outline structure to describe the known literature on the different VNTs in epilepsy. Unveiling and understanding the structure of VNTs is of utmost importance to deduce their functional domains and their physicochemical properties in order to develop compounds that can interact with these transporters. Therefore, we will also shortly describe and show the known VNT structure models and the most active and used modulators of their activity. In conclusion, we will point out the remaining challenges for research on VNTs as possible targets for future epilepsy therapies.

SLC17 FAMILY

The *SLC17* gene family consists of nine members (Reimer, 2013). Three VGLUTs (VGLUT1, VGLUT2, and VGLUT3), the VEAT, and the VNUT are responsible for the vesicular transport of nucleotides and anionic neurotransmitters, glutamate and aspartate. In addition, four sodium-dependent phosphate transporters (NPT1, NPT3, NPT4, and NPT homologue) are part of this family as well. These NPTs are not involved in neurotransmission, but exert voltage driven organic anion elimination of toxic xenobiotics in the kidney and are therefore not further discussed in this review (Omote et al., 2011).

The human SLC17 protein family is categorized as a subgroup of anion transporters within the major facilitator superfamily (MFS). The MFS is the largest group of secondary active transporters, regulating the transport of a wide variety of substrates, such as inorganic ions, sugars, amino acids, and xenobiotics, across cellular and intracellular membranes (Pao et al., 1998; Law et al., 2008). MFS proteins contain 12 transmembrane (TM) helices, divided in two six-TM halves surrounding a central aqueous cavity, in which the substrate-binding site is located. Alternating opening of the binding site on one of both sides of

the membrane is achieved by rigid body rotation of the N- and C-terminal halves, called "rocker switch" mechanism (Abramson et al., 2003; Law et al., 2008; Dang et al., 2010). This mechanism enables "uphill" transport of substrate, coupled to "downhill" transport of driving ions such as Na^+ or protons (Pietrancosta et al., 2012).

VESICULAR GLUTAMATE TRANSPORTERS

Glutamate is the most abundant and major excitatory neurotransmitter of the brain, mediating fast synaptic transmission (Casillas-Espinosa et al., 2012; Mehta et al., 2013). Glutamate is crucial for synaptic plasticity (e.g., long-term potentiation, LTP), learning, memory and other cognitive functions. In addition, this neurotransmitter is an important substitute source of energy for neuronal cells in case of glucose deficiency (Mehta et al., 2013). On the other hand, excessive glutamatergic neurotransmission and subsequent glutamate excitotoxicity has been observed in various neurological diseases, such as epilepsy, Alzheimer's disease, Parkinson's disease, multiple sclerosis and stroke. This emphasizes both the crucial and highly toxic role glutamate can play in the brain and the necessity of accurate controlled extracellular levels of glutamate (Casillas-Espinosa et al., 2012; Mehta et al., 2013). VGLUTs are crucial for the storage of glutamate in synaptic vesicles and the subsequent exocytotic release into the synaptic cleft. Synaptic glutamate activates pre- and post-synaptic metabotropic (mGluR) and ionotropic glutamate receptors. The mGluRs are modulators of the synaptic glutamate signal transmission. Activation of group I (mGluR 1, 5) can enhance neuronal excitability, while activation of group II (mGluR 2, 3) and III (mGluR 4, 6, 7, 8) decreases presynaptic glutamate release (see for a review Ure et al., 2006). The ionotropic glutamate receptors include the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainic acid (KA) receptors. These glutamatergic receptors have been intensively studied in both rodent epilepsy models and human epileptic tissue, since excessive activation of these receptors induces glutamate excitotoxicity (see for a review Ghasemi and Schachter, 2011; Matute, 2011; Rogawski, 2013). KA receptors are for example excessively stimulated by local or systemic administered KA, a well-known proconvulsant, used in both acute and chronic rodent epilepsy models to induce convulsions and SE (initial precipitating injury) respectively. Finally, the excitatory amino acid reuptake transporters (EAATs) are of utmost importance for the termination of glutamatergic neurotransmission as well as for the prevention of neurotoxicity. In addition, system x_c^- has been proposed as the most important source of extracellular glutamate in mouse hippocampus and the loss of system x_c^- was shown to decrease the convulsion threshold in distinct acute rodent epilepsy models (De Bundel et al., 2011). Although the involvement of glutamate transporters in epilepsy has been proven in several animal models and human tissue, they are rarely proposed as target for new AED development strategies (Hinoi et al., 2005; De Bundel et al., 2011; Lewerenz et al., 2013).

VGLUTs mediate the import of glutamate into synaptic vesicles. Controlling the activity of these transporters could thus potentially modulate the efficacy of glutamatergic neurotransmission (Takamori, 2006). Indeed, overexpression of VGLUT will increase the amount of glutamate released per vesicle and as such changes in VGLUT expression may affect quantal size and glutamate release under not only physiologic, but also pathologic conditions (Fremeau et al., 2004; Wojcik et al., 2004; Wilson et al., 2005).

In **Figures 3A,B**, the 2D and 3D molecular structure of VGLUT1 are shown, based on transmembrane segment prediction and topology of bacterial MFS proteins. Filled black circles represent residues facing the center of the pore (gray volume) that is open to the cytoplasmic side. Three arginine residues (R80 from helix 1, R176 from helix 4, and R314 from helix 7) that are exposed to the pore are shown in sticks. The first and last highly variable 60 residues of the N- and C-terminal are not shown in the 3D model (Almqvist et al., 2007).

VGLUT1 and VGLUT2 are complementarily expressed in distinct subsets of glutamatergic neurons in the central nervous system (CNS) (Fremeau et al., 2001; Herzog et al., 2001; Varoqui et al., 2002; Boulland et al., 2004). VGLUT1 is found in astrocytes of the dentate-molecular layers, the stratum radiatum of CA1 hippocampus, the frontal cortex, and the striatum (Bezzi et al., 2004; Zhang et al., 2004; Potokar et al., 2009; Ormel et al., 2012a), while VGLUT2 expression is more restricted and only observed in hippocampal astrocytes (Bezzi et al., 2004). VGLUT3 is expressed in the hippocampus, frontal cortex and in Bergmann glia of the cerebellum. VGLUT3 is relatively more abundant and expressed on distinct synaptic-like microvesicles (SMLVs) than VGLUT1 (Ormel et al., 2012b). Since astrocytes express VGLUTs on SLMVs and since only overexpression of VGLUT3 *in vitro* resulted in increased Ca^{2+} dependent astroglial glutamate exocytosis, it has been postulated that this glutamate transporter is crucial for astroglial glutamate release (Ni and Parpura, 2009; Ormel et al., 2012b). As already mentioned in the introduction, VGLUT3 protein expression is observed in serotoninergic, cholinergic and GABAergic neuronal populations (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002; Gras et al., 2008; Seal et al., 2008).

VGLUT1-immunoreactivity (IR) was reduced in punctate structures of one or several layers of the peritumoral neocortex of patients with epilepsy that was secondary to low-grade tumors, a common cause of epilepsy in which the epileptogenic region presents a loss of neurons and excitatory synapses. This decrease was correlated with gliosis, neuronal loss, and a decrease in the number of asymmetrical synapses (Alonso-Nanclares and De Felipe, 2005). In TLE patients without hippocampal sclerosis, VGLUT1 mRNA levels were decreased, whereas VGLUT1-IR was increased. The authors postulated that this increase could represent a higher vesicular glutamate storage capacity, which may increase glutamatergic transmission, and can contribute to higher extracellular glutamate levels and excitability. In patients with hippocampal sclerosis, on the other hand, both VGLUT1 protein and mRNA levels were decreased in subfields with severe neuron loss, in accordance with the previous study in peritumoral neocortex. Furthermore, upregulated VGLUT1 reactivity could be detected in the dentate gyrus of these patients, indicating that new glutamatergic synapses are formed in the layer with mossy fiber sprouting (van der Hel et al., 2009). As we already mentioned in the introduction, patients are treated with specific

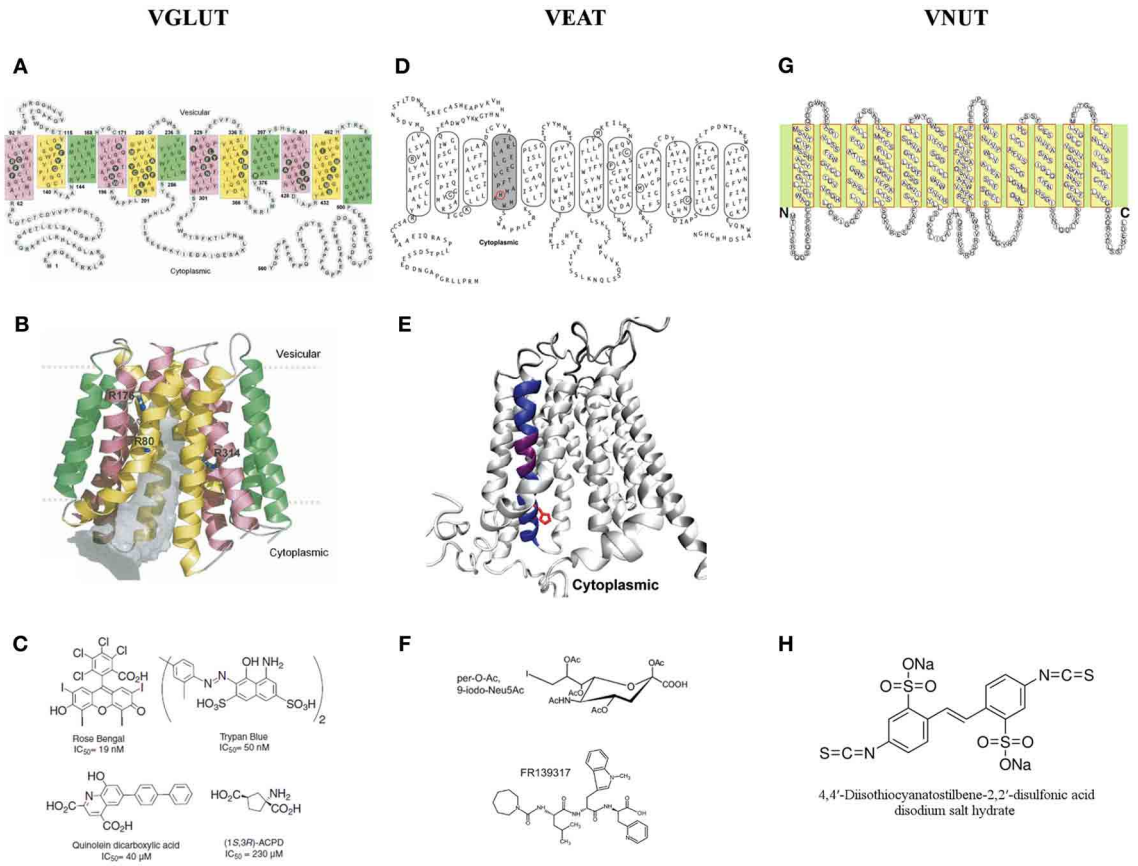

FIGURE 3 | A two and three dimensional molecular structure of the members of the SLC17 family. (A,B) The vesicular glutamate transporter 1 (VGLUT1) (Almqvist et al., 2007), **(D,E)** the vesicular excitatory amino acid transporter (VEAT) (Courville et al., 2010) and **(G)** the vesicular nucleotide transporter (VNUT) (Sawada et al., 2008). **(C)** The different families of VGLUT modulators (Pietrancosta et al., 2010). **(F)** The most bioactive analog of sialic acid: per-O-Ac,9-iodo-Neu5Ac and the novel VEAT ligand identified by virtual high-throughput screening: FR139317 (Pietrancosta et al., 2012). **(H)** 4,4′-diisothiocyanatostilbene-2,2′-disulfonate, the only known inhibitor of ATP transport *in vitro*.

AEDs. Consequently, there is a possibility that these drugs influence VGLUT-IR. Indeed, treatment of seizure sensitive Mongolian gerbils with high doses of valproic acid, a Na^+ channel blocker, but not vigabatrin, a GABA transaminase inhibitor, reduced the VGLUT1/2-IR in the dentate gyrus of these animals. Moreover, the enhanced VGLUT1/2-IR in the seizure sensitive gerbils, compared with the seizure resistant animals, could be closely related to the hyperexcitability of granule cells or the low threshold for seizures in these gerbils (Kang et al., 2005).

Several studies investigated both VGLUT1/2 expression and/or mRNA levels in different animal models of epilepsy. Although models for different types of epilepsy were used, almost all studies showed increased expression of VGLUT1 or VGLUT2 in particular brain regions. Kim et al. showed enhanced VGLUT1-IR in both hippocampi following hypoxic ischemia, although in this model VGLUT2 immunodensity remained unaltered (Kim et al., 2005). On the contrary, VGLUT2 expression was increased in regions of hippocampal heterotopia in methylazoxymethanol exposed animals (Harrington et al., 2007) as well as in the cortex of the genetic absence epilepsy rat from Strasbourg (GAERS) (Touret et al., 2007). In the pilocarpine model of TLE, where a striking loss of mossy cells occurs during the latent period, VGLUT1 mRNA-containing hilar neurons were decreased, together with an associated loss of VGLUT1-containing terminals in the dentate gyrus inner molecular layer. Furthermore, axonal sprouting of granule and pyramidal cells induced aberrant VGLUT1-containing terminals at the chronic stage. VGLUT1-IR was recovered in the inner molecular layer and enhanced in the CA1-CA3 dendritic layers (Boulland et al., 2007). Decreased VGLUT1 levels, detected in the human studies and in the latent period after pilocarpine-seizure induction, are possibly a reflection of the occurring neuronal loss. The enhanced VGLUT1/2-IR in symptomatic animals displaying chronic seizures, on the other hand, may reflect the reorganization of the glutamatergic neurons characterized by sprouting of glutamatergic fibers and associated neosynaptogenesis, together with increased glutamate release in brain regions affected by recurrent seizures (Boulland et al., 2007; Touret et al., 2007).

Therefore, in order to find out whether a severe reduction in VGLUT2 protein can affect seizure generation and thus whether the VGLUT2 protein is crucially involved in the origin of seizure activity, we investigated seizure susceptibility in VGLUT2$^{+/-}$

mice. We showed that, compared to their wild-type littermates, VGLUT2$^{+/-}$ mice are more susceptible to pentylenetetrazole, a chemoconvulsant known to induce generalized seizures (Schallier et al., 2009). These results are suggestive of an important role for VGLUT2 in generalized seizures. However, more studies are needed to fully understand the mechanism behind the altered seizure susceptibility of the VGLUT2$^{+/-}$ mice. It would not be surprising if compensatory up- or downregulation of proteins related to glutamatergic neurotransmission occur. Indeed, although there are no studies that investigated compensations in the VGLUT2$^{+/-}$ mice, increased neuronal synthesis of glutamate, decreased cortical and hippocampal GABA and decreased GLAST (glutamate reuptake transporter) levels have been shown in the VGLUT1$^{+/-}$ mice (Garcia-Garcia et al., 2009).

Recently, mice lacking VGLUT3 were produced to determine the physiological role of this glutamate transporter. These VGLUT3$^{-/-}$ mice exhibit primary, generalized non-convulsive epilepsy (Seal et al., 2008). Although more research should be performed exploring the role of VGLUT3 in seizure generation and epilepsy, this observation strengthens the idea that this transporter is possibly involved in the protection against absence seizures.

Up to date, three types of VGLUT inhibitors (**Figure 3C**) have been characterized: dyes (e.g., Rose Bengal and Trypan Blue) (Naito and Ueda, 1985; Ozkan and Ueda, 1998; Bole and Ueda, 2005; Almqvist et al., 2007), substituted quinolones (e.g., quinolein dicarboxylic acid) (Bartlett et al., 1998; Carrigan et al., 1999, 2002), and glutamate analogs [e.g., (1S,3R)-ACPD] (Pietrancosta et al., 2010). Of these three types, dyes have the highest affinity toward VGLUTs, especially Rose Bengal, the only competitive inhibitor with the highest affinity of all. The quinolones, on the other hand, display the highest selectivity, while the glutamate analogs have only very low activity on glutamate uptake, which can be explained by the low affinity of VGLUTs toward glutamate itself (Pietrancosta et al., 2010).

VESICULAR EXCITATORY AMINO ACID TRANSPORTER

The co-localization of glutamate and aspartate was already demonstrated in synaptic vesicles in hippocampal CA1 neurons and SLMVs of pinealocytes (Fleck et al., 1993; Yatsushiro et al., 1997; Gundersen et al., 1998). Moreover, aspartate might be involved in both excitatory and inhibitory synapses in the hippocampus (Gundersen et al., 1991, 1998, 2004). Only recently, Morland and coworkers observed ATP-dependent vesicular uptake of L-aspartate in the brain. Moreover, vesicular L-aspartate uptake, relative to the L-glutamate uptake, was twice as high in the hippocampus as in the whole brain, indicating a crucial role of aspartate signaling in this brain region (Morland et al., 2013). *In vitro* data suggest that NMDA receptor-mediated excitotoxicity is likely caused by extrasynaptic NR1-NR2B NMDA receptor activation by aspartate, which implicates an important role of aspartate signaling in pathologies marked by excitotoxicity. However, the necessity of further studies under physiologic conditions and the development of tools to distinguish aspartate and glutamate postsynaptic responses was emphasized, in order to determine the contribution of aspartate to synaptic transmission (Nadler, 2011). In addition, 30 min after administration of valproate, a broad spectrum AED, aspartate levels are significantly decreased in both excitatory and inhibitory presynaptic nerve terminals in rat hippocampus, whereas glutamate and GABA levels remained unchanged. Consequently, the anticonvulsant effect of valproate might be at least partly achieved by reduced extrasynaptic NMDA receptor-mediated excitatory signaling in the brain (Morland et al., 2012).

Miyaji et al. unveiled the role of sialin as a vesicular aspartate transporter in hippocampal synaptic vesicles and pineal SLMVs (Miyaji et al., 2008). Through *in vitro* reconstitution of mouse and human sialin in liposomes, it was shown that this transporter, which is predominantly expressed in hippocampal neurons (Aula et al., 2004; Yarovaya et al., 2005), is responsible for membrane potential ($\Delta\psi$)-driven aspartate and glutamate transport into synaptic vesicles in addition to H^+/sialic acid co-transport in lysosomes. Since aspartate and glutamate are excitatory amino acids, the sialin transporter should be called the VEAT (Miyaji et al., 2008, 2010, 2011).

Mutations in the *SLC17A5* gene, encoding sialin, can elicit two autosomal recessive lysosomal storage disorders: Salla disease and infantile sialic acid storage disease (ISSD). Both disorders are marked by accumulation of sialic acid in lysosomes (Verheijen et al., 1999). Several studies implied a direct correlation between the activity of sialin transport and the severity of the disease phenotype. Mutant forms of sialins in persons suffering from ISSD show complete absence of H^+/sialic co-transport activity, whereas the mutant forms of sialins found in Salla disease patients still exhibit 20–60% of normal H^+/sialic co-transport (Morin et al., 2004; Wreden et al., 2005; Myall et al., 2007; Ruivo et al., 2008). Salla disease and ISSD disorders predominantly affect the CNS, eliciting varying degrees of developmental delay in motor and cognitive skills, epilepsy, and premature death and are marked by cytoplasmatic vacuoles and hypomyelination (Prolo et al., 2009).

Incorporation of forms of mouse and human *SLC17A5* protein, associated with Salla disease, in proteoliposomes completely abolished the aspartate and glutamate import, whereas H^+/sialic acid co-transport was significantly decreased. These results suggest that loss of aspartatergic (and combined glutamatergic) neurotransmission could contribute to the severe neurological defects of Salla disease (Miyaji et al., 2008, 2010, 2011).

Figure 3D represents a 2D topology model for rat and human sialin with manual adjustments. TM4 (shaded) traces out a large aqueous cavity that forms a part of the substrate permeation pathway and displays substrate-induced alterations in accessibility of substituted cysteine residues in TM4. Mutated residues in the lysosomal free sialic acid storage disorders are circled. The 3D model of sialin (**Figure 3E**), with TM4 colored in blue and purple, is based on the crystal structure of the glycerol 3-phosphate transporter. His-183, a TM4 residue affected by a disease-associated mutation (H183R), is depicted in red in (circle in panel D; side chain in panel E) (Courville et al., 2010).

To further investigate potential mechanisms underlying the pathology of the free sialic acid storage disorders, Prolo et al. characterized sialin-deficient mice. These sialin$^{-/-}$ mice display poor coordination, seizures, failure to thrive, and premature death. Histological characterization of these knock-out mice also revealed prominent vacuolar lesions and a marked decrease in

myelin throughout the CNS with the exception of the parasympathetic nervous system. In conclusion, these mice can be used as an appropriate model for free sialic acid storage disorders, since their phenotype appears to be consistent with these disorders (Prolo et al., 2009).

Surprisingly, Morland and coworkers could not observe a difference in ATP-dependent L-aspartate uptake in synaptic vesicles from sialin$^{-/-}$ and sialin$^{+/+}$ mice. Moreover, overexpression of sialin in PC12 cells did not result in significant vesicular uptake of L-aspartate and depolarization-induced depletion of L-aspartate from hippocampal nerve terminals was similar in hippocampal slices from both phenotypes. These *in vitro* results suggest that either sialin is present in insufficient amounts in the vesicular membrane, or sialin does not transport L-aspartate into synaptic vesicles under physiological conditions, contrary to the findings of Miyaji et al. (2008). This discrepancy might be explained by the fact that Miyaji and coworkers used hippocampal P2 fraction for their studies, which is likely to contain non-vesicular membranes and is called the crude synaptosomal fraction. Moreover, a proteomic study (Takamori et al., 2006) did not detect sialin among the other synaptic vesicle proteins, purified from rat brain. On the other hand, no evidence was found of non-vesicular release of L-aspartate, which confirms the exocytotic release in hippocampus, though, after vesicular accumulation by another transporter than sialin (Morland et al., 2013).

Recently, using structure-activity, homology modeling, molecular docking, and mutagenesis studies, the substrate-binding site of sialin has been successfully predicted and a 3D cytosol-open model of sialin has been built and validated. This model predicts small molecule binding to sialin and allows screening of new potential ligands targeting the VEAT. A first pilot virtual high-throughput screening appointed pseudopeptide FR139317 (**Figure 3F**) to be a competitive inhibitor, showing >100-fold and ∼5-fold higher affinity, than the natural substrate, N-acetylneuraminic acid and the most bioactive analog, per-O-Ac,9-iodo-Neu5Ac (**Figure 3F**) respectively. This increased activity of FR139317 may be due to novel polar interactions, in particular with TM VIII (Tyr-335) (for more details see Pietrancosta et al., 2012).

VESICULAR NUCLEOTIDE TRANSPORTER

Burnstock was the first to indicate ATP as a neurotransmitter and eventually its release and co-release was demonstrated in both peripheral and CNS (Burnstock et al., 1972; Burnstock, 1976, 2007). ATP is highly concentrated (up to 100 mM) in neuronal, synaptic vesicles together with other nucleotides, though at lower concentrations, and in granules in adrenal chromaffin cells (Burnstock, 2007). On the other hand, Ca^{2+}-triggered ATP release by astrocytes has been repeatedly observed *in vitro* and could be blocked by inhibition of the vacuolar H^+-ATPase (Newman, 2001; Coco et al., 2003; Pascual et al., 2005; Pangrsic et al., 2007; Zhang et al., 2007; Pryazhnikov and Khiroug, 2008).

Vesicular astrocytic ATP is postulated to be the major, if not the sole, determinant of astrocytic Ca^{2+} wave propagation in hippocampal astrocyte cultures, mediated by G-protein coupled P2Y1 receptors, although further studies should elucidate if astrocytic ATP contributes to Ca^{2+} waves *in vivo* and is involved in neuron-glia interactions (Bowser and Khakh, 2007). In addition, the involvement of another ATP receptor, P2X7, located on neurons and glia, has been observed in the pathophysiology of epilepsy in distinct chronic rodent epilepsy models. However, due to paradoxical results of P2X7 ligands in the pilocarpine and kainate models, additional experiments in distinct rodent epilepsy models, are needed to unveil the role of P2X7 in TLE (Engel et al., 2012).

Astrocytic release of ATP is proposed to play a role in hippocampal heterosynaptic depression. Extracellularly ATP is degraded into adenosine by EctoATPases. Adenosine in turn activates presynaptic adenosine A1 receptors and suppresses glutamate release from other afferents (Pascual et al., 2005; Serrano et al., 2006). Moreover, adenosine has been demonstrated to be an endogenous anticonvulsant and neuroprotectant. Extracellular adenosine levels are controlled by metabolic reuptake through nucleoside transporters and phosphorylation by adenosine kinase (ADK). ADK overexpression and adenosine deficiency have been observed in different rodent models as well as in human tissue resected from patients with hippocampal sclerosis and TLE (Boison, 2012; Masino et al., 2012). Recently Masino et al. have demonstrated that anticonvulsant effects of the ketogenic diet are due to reduction of the expression of ADK in mice and the enhancement of A1 receptor signaling (Masino et al., 2012).

On the other hand it has been shown that adenosine can potentiate hippocampal neuronal activity via binding to adenosine A2a receptors, without affecting presynaptic glutamate release or postsynaptic glutamatergic conductance. Adenosine deficiency in epilepsy can lead to decreased A2a receptor signaling and might be an explanation for comorbidities such as disturbed psychomotor control, sleep disorder and depression (Boison, 2012). Moreover, microdialysis experiments suggest a crucial role of post-synaptic A2a receptors in the anticonvulsant effect of 2-chloroadenosine and the attenuation of evoked glutamate release by 2-chloro-N^6-cyclopentyladenosine, both well-known adenosine A1 receptor agonists, in the acute local pilocarpine rat model for limbic seizures (Khan et al., 2000, 2001).

VNUT is encoded by the human and mouse *SLC17A9* gene (Reimer and Edwards, 2004; Fredriksson et al., 2008; Sreedharan et al., 2010). Proteoliposomes reconstituted with purified, recombinant *SLC17A9* transporter exhibit $\Delta\psi$-driven, Cl^- dependent ATP transport, similar to the ATP transporter endogenously expressed in synaptic vesicles and chromaffin granules. Suppression of endogenous *SLC17A9* expression in PC12 cells using small interfering RNA (siRNA) decreased KCl-triggered release of ATP, confirming the involvement of this transporter in vesicular storage and subsequent exocytosis of ATP (Sawada et al., 2008).

VNUT is 430 amino acid residues long with 12 putative TM helices with ∼23–29% identity and 41–48% similarity to that of other SLC17 members (**Figure 3G**) (Sawada et al., 2008). To the best of our knowledge we are unaware of the excistence of a VNUT 3D model.

VNUT is predominantly expressed in the brain and adrenal gland. Immunohistochemical studies revealed *SLC17A9* protein expression in astrocytes (Sawada et al., 2008). Whether astrocytic ATP is released by lysosomes (Zhang et al., 2007) or

smaller vesicles ∼300 nm (Coco et al., 2003; Pangrsic et al., 2007; Pryazhnikov and Khiroug, 2008) is still subject for discussion.

By contrast, ATP release from central neurons is not well-studied. It has only recently been demonstrated that ATP release from cultured rat hippocampal neurons is attenuated by RNAi-mediated knockdown of VNUT. Strong VNUT-IR is observed in the cerebellar cortex and the olfactory bulb. In the hippocampus VNUT has been observed in both excitatory and inhibitory presynaptic neurons (Larsson et al., 2012).

Nowadays, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (**Figure 3H**), also known to inhibit VGLUT, is the only inhibitor proven to block ATP transport *in vitro* (Thompson et al., 2005; Sawada et al., 2008).

SLC18 FAMILY

SLC18 transporters transport cationic neurotransmitters, such as ACh, NE, 5-HT, DA, and histamine, into synaptic vesicles. The *SLC18* gene family consists of three members: the VMAT1, the VMAT2, and the VAChT (Omote et al., 2011). The three members of the SLC18 family display significant sequence homology (Parsons, 2000; Bravo and Parsons, 2002). Similar to the SCL17 family these transporters consists of 12 TM domains (Wimalasena, 2010).

VESICULAR MONOAMINE TRANSPORTERS

Several studies investigated the role of monoaminergic neurotransmission in the pathophysiology of epileptogenesis and epilepsy and its co-morbidities: anxiety and depression. Direct enhancement of hippocampal extracellular DA or 5-HT levels has been shown to exert both anticonvulsant and antidepressant activities (Smolders et al., 2008), although SRS in pilocarpine-induced epileptic rats are associated with increased mesolimbic dopaminergic activity (Cifelli and Grace, 2012). Rocha et al. observed alterations of the dopaminergic system in the neocortex of patients with TLE (Rocha et al., 2012). Moreover, reduction of brain 5-HT levels facilitates the induction of SE by pilocarpine administration and increases the frequency of SRS. Activation of postsynaptic 5-HT_{1A} receptors shows antiepileptic activity in different TLE models, such as maximal dentate activation (Orban et al., 2013). Furthermore, depletion of 5-HT levels, after administration of 5,7-dihydroxytryptamine into the median raphe nucleus of rats, significantly increased the incidence of pilocarpine-induced SE and the frequency of seizures during the chronic phase of this epilepsy model (Trindade-Filho et al., 2008). NE has been proposed as a potential biomarker for the efficacy of vagus nerve stimulation, an effective adjunctive treatment for medically refractory epilepsy. Vagus nerve stimulation enhances extracellular hippocampal NE levels, which could be at least partly responsible for its seizure-suppressing effect in the intrahippocampal pilocarpine rat model (Raedt et al., 2011). Monoaminergic neurons and their projection fibers are not only found in the cortex, striatum and thalamus, but also in the hippocampus (Joels and Baram, 2009; Sukumar et al., 2012). In addition, monoaminergic control of neurogenesis in the adult midbrain (salamander) and hippocampus (rodents) have been suggested (Park and Enikolopov, 2010; Berg et al., 2011). In conclusion, most studies imply that upregulation of the monoaminergic system could have anticonvulsant activity.

VMAT is responsible for the transport of monoamine neurotransmitters: DA, 5-HT, NE, epinephrine, and histamine, from the cytoplasm into synaptic vesicles via a electrochemical gradient generated by vacuolar type H^+-ATPase (Schuldiner et al., 1995). Two VMAT isoforms, VMAT1 and VMAT2, have been molecularly cloned (Erickson et al., 1992; Liu et al., 1992). Human VMAT1 is mainly expressed in peripheral endocrine cell populations, such as the adrenal gland, whereas the expression of human VMAT2 is largely confined to neuronal, histaminergic cells. VMAT2 has a consistently higher affinity for most monoamines, particularly histamine (Erickson et al., 1996).

Sequence analysis of human VMAT2 unveilled that most variable regions are located near the N- and C-terminal and in the large glycosylated loop between TMD I and II (**Figure 4A**) (Wimalasena, 2010). Biochemical studies (Thiriot and Ruoho, 2001; Thiriot et al., 2002) demonstrated that Cys 430 in TMD XI is essential for the recognition and binding of VMAT inhibitors. Moreover, using a recombinant VMAT2 construct with a thrombin cleavage site between TMD VI and VII demonstrated that Cys 117 in the loop between TMD I and II and Cys 324 in the loop between TMD VII and VIII form a disulfide bond in human VMAT2, which contributes to the structural integrity and efficient monoamine transport (Wimalasena, 2010). The structure is predicted by usingTMbase-A database of membrane spanning protein segments. Distinct conserved amino acids in human VMAT1, human VMAT2, rat VMAT1, rat VMAT2, and bovine VMAT2 are colored. The shown amino acid numbering is based on the sequence of human VMAT2 (Wimalasena, 2010).

Three independent groups almost simultaneously published the generation of $VMAT2^{-/-}$ mice. These knock-out mice show lack of feeding behavior and die shortly after birth, which can be partially rescued by amphetamine administration. No differences in monoaminergic cell populations and their projections were observed in $VMAT2^{-/-}$ mice, however, monoamine storage and vesicular release are severely disrupted and brain monoamine levels are dramatically reduced, compared to their wild-type littermates (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997). This critical role of VMAT2 in monoamine storage in the CNS and their subsequent exocytotic release is confirmed by *in vitro* and *in vivo* data from VMAT2 heterozygous mice (Fon et al., 1997) and *in vitro* overexpression of VMAT2 in ventral midbrain neurons (Pothos et al., 2000).

A first minor link between epilepsy and VMAT2 was observed in the perinatal asphyxia rat model. Perinatal asphyxia is suggested to induce a variety of brain disorders, including spasticity, epilepsy, mental retardation, attention deficit disorders, and minimal brain disorder syndromes, and could be the initial trigger for subsequent psychiatric and neurodegenerative diseases. Three months after asphyxia was induced in rat pups, tyrosine hydroxylase (TH)-IR was decreased in the striatum, hippocampus, thalamus, frontal cortex, and cerebellum, while VMAT-IR was increased in the striatum. This significant increase of VMAT2 is suggested to be a compensation for deficient dopaminergic/noradrenergic innervation, as a consequence of decreased TH (Kohlhauser et al., 1999).

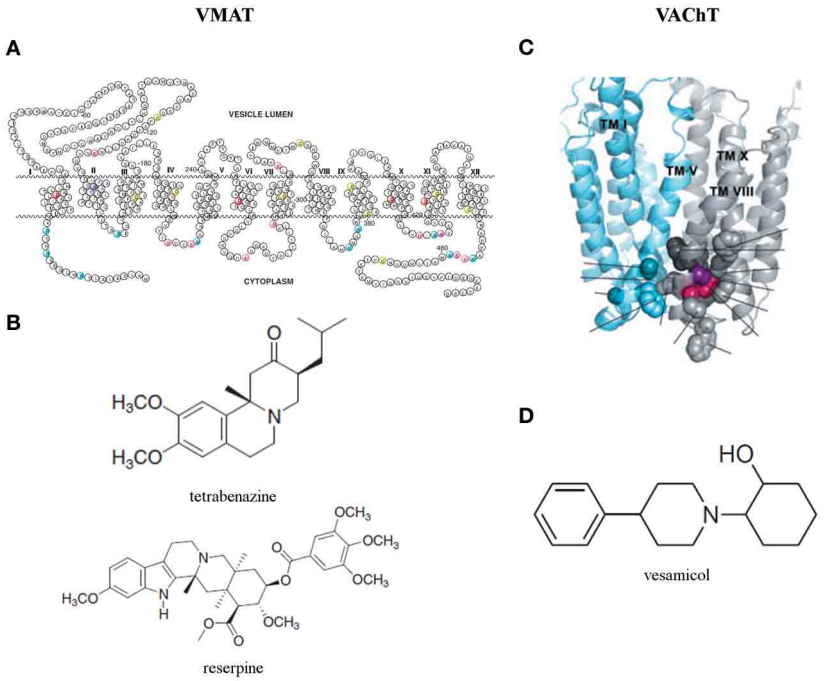

FIGURE 4 | (A) Predicted secondary structure of the human vesicular monoamine transporter 2 (VMAT2) and **(B)** two classical and now commonly used VMAT inhibitors: tetrabenazine and reserpine (Wimalasena, 2010). **(C)** Three dimensional homology model of the vesicular acetylcholine transporter (VAChT) (Khare et al., 2010) and **(D)** its most studied inhibitor: vesamicol (Kozaka et al., 2012).

Following KA-induced SE both TH and norepinephrine transporter (NET), but not VMAT2 mRNA levels are transiently elevated in locus coeruleus (LC) neurons. Noradrenergic LC efferents innervate most forebrain areas and are activated postictally in epilepsy kindling models. Moreover, LC stimulation has been demonstrated to exert seizure-suppressing effects in several epilepsy animal models. Consequently, it is suggested that the increase of TH and NET, responsible for synthesis and reuptake of NE, respectively, could be an adaptive mechanism to restore intracellular NE levels and enable LC neurons to counteract hyperexcitability (Bengzon et al., 1999).

Only recently, it has been shown that VMAT2 protein is mainly expressed in the cytoplasm and axons of neurons of the hippocampus and temporal lobe cortex of rats and humans. Moreover, the expression level of VMAT2 mRNA and protein has been determined in resected neocortices of TLE patients (Jiang et al., 2013) and in the hippocampus and adjacent cortices of rats in different stages of the post-SE pilocarpine model (Jiang et al., 2013). VMAT2 is transiently increased in the acute stage after pilocarpine-induced epileptic seizures, but its expression is clearly decreased after SRS in the hippocampus and temporal lobe cortices of TLE rats. In line with this last observation, the expression level of VMAT2 mRNA and protein in TLE patients is significantly reduced compared to non-epileptic control subjects. Taking previously mentioned studies into account, it is suggested that the transiently increased VMAT2 might be a compensatory response to enhance monoaminergic neurotransmission to counteract hyperexcitability and epileptic seizures. The decrease in VMAT2 expression after SRS is consistent with the known involvement of monoaminergic alterations in epileptic comorbidities, such as anxiety and depression, and might contribute to the epilepsy disease progression (Jiang et al., 2013).

The potency of two classical and now commonly used VMAT inhibitors, tetrabenazine for VMAT2 and resperine for both isoforms, was confirmed on both human VMAT isoforms for almost two decades (Erickson et al., 1996). Comparison with other VMAT inhibitors point to the optimal positioning of the nitrogen and the carbonyl oxygen of tetrabenazine in the binding site to explain the high affinity of this specific VMAT2 inhibitor. Consequently, since resperine does not contain carbonyl oxygen and nitrogens, its extended hydrophobic tail must provide significant non-specific contribution toward its high binding affinity. Indeed, the binding site of the transporter is known to be particularly hydrophobic. Other inhibitors lacking these important constituents are substantially weaker inhibitors for VMAT in comparison with tetrabenazine and resperine (**Figure 4B**) (Wimalasena, 2010).

VESICULAR ACETYLCHOLINE TRANSPORTER

ACh is secreted both centrally and peripherally, and is known to regulate a plethora of physiological functions. Preclinical and clinical data indicate that cholinergic activity in the entorhinal cortex (EC), which is highly innervated by cholinergic terminals from the basal forebrain (Lewis et al., 1967; Lysakowski et al., 1989; Gaykema et al., 1990), may endorse and maintain epileptiform discharges and kindling (Turski et al., 1989; Saucier and Cain, 1996; Gloveli et al., 1999). The EC propagates cholinergic activity to the stratum oriens of cornu ammonis 1 (CA1), the

dentate hilus and many, if not all, interneurons of the hippocampus. Cholinergic functions are altered in the epileptic temporal lobe, however, the exact role and nature of these changes in the pathogenesis of the disease are still as yet to be understood (Friedman et al., 2007). Cholinergic dysfunction (for review see Friedman et al., 2007) and acetylcholine-esterase (AChE) upregulation (Zimmerman et al., 2008) have been observed in the epileptic temporal lobe, however, the exact role of these changes in the pathogenesis of the disease are only recently demonstrated by Gnatek et al. (2012). AChE mRNA and protein levels are fast and strongly increased after SE in mice hippocampus (Gnatek et al., 2012), probably as an initial attempt to maintain homeostasis by reducing ACh levels and subsequently neuronal excitability (Meshorer et al., 2005). Upregulated AChE mRNA was found in principle and inhibitory interneurons, endothelial cells and activated microglia (Gnatek et al., 2012). These observations suggest a possible role in increased blood-brain barrier (BBB) permeability (van Vliet et al., 2007; Weissberg et al., 2011) and in microglial-mediated innate immune response (Vezzani et al., 2011). The upregulation of AChE after SE loosens the brain's immune and anti-inflammatory response and facilitates epileptogenesis. Indeed, transgenic mice overexpressing AChE displayed a robust increase in IL-1β mRNA levels and accelerated epileptogenesis compared to wild-type FVB/N mice (Gnatek et al., 2012).

ACh acts on two different classes of receptors: muscarinic ACh receptors (mAChRs) and nicotinergic ACh receptors (nAChRs). Pilocarpine, a non-selective mAChR agonist, is a widely used chemoconvulsant in both actue and chronic animal epilepsy models (Meurs et al., 2008; Portelli et al., 2009; Bankstahl et al., 2012; Portelli et al., 2012b). The M1 mAChR is crucial for the initiation of pilocarpine-induced seizures, and subsequent maintenance of these seizures necessitates NMDA receptor activation (Maslanski et al., 1994; Smolders et al., 1997). Moreover, M1 receptor knockout mice were found to be highly resistant to pilocarpine-induced seizures (Hamilton et al., 1997). M1 receptor activation highly affects K^+ conductances, such as K^+ current blockade thereby leading to a slow depolarization, blockade of the Ca^{2+}-dependent slow K^+ current that is responsible for the afterhyperpolarization that pursues a burst discharge, and blockade of a transient outward K^+ that regulates excitability in hippocampal neurons (Millan et al., 1993; Friedman et al., 2007). M1 receptor downregulation has been reported following seizure induction in several models, whilst kindled animals show significantly increased expression of both M1 and M3 receptors 28 days following kindling (Friedman et al., 2007). The preponderance of highly sensitive nAChRs is found presynaptically where they stimulate neurotransmitter release, which accordingly influences synaptic efficacy and plasticity, spike-timing-dependent plasticity, frequency-dependent filtering as well as overall signal-to-noise ratio in the cortex (Miwa et al., 2011). Overactivation of nAChRs is thought to be linked to epilepsy (Picard et al., 2006; Miwa et al., 2011). Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is the first human epilepsy for which a mutation has been described and is caused by mutations in the α_4 or β_2 subunits of nAChR (Steinlein and Bertrand, 2010). ACh was found to induce seizure-like events in both control and epileptic hippocampal-entorhinal slices, which were completely blocked by the non-specific muscarinic antagonist atropine, partially blocked by the M1 receptor antagonist pirenzepine, and unaffected by the non-specific nicotinic antagonist mecamylamine (Zimmerman et al., 2008).

VAChT, predominantly found in synaptic vesicles, is responsible for loading ACh from the cytoplasm to synaptic vesicles (Prado et al., 2013). Like VGLUTs, VAChT appears to be a very slow transporter and thus is prone to highly influence ACh release (Varoqui and Erickson, 1996; Hori and Takahashi, 2012). VAChT expression levels are known to be affected in Alzheimer's and Huntington's disease (Efange et al., 1997; Smith et al., 2006; Chen et al., 2011), however, not much is known on changes that may occur in an epileptic brain. A 3D model of VAChT has been designed by Schuldiner et al. based on the crystal structure of the MFS protein glycerol-3-phosphate phosphate antiporter (**Figure 4C**) (Khare et al., 2010).

Only one study is available where VAChT binding-site density was investigated in the human hippocampal formation from epileptic patients that underwent anterior temporal lobectomy. No reduction but rather a slight trend toward increased VAChT binding sites was observed when compared to autopsy controls, as opposed to a significant reduction in mAChRs. This indicates the relative preservation of the cholinergic projecting terminals that is consistent with an axon sparing lesion as opposed to neuronal cell loss in the process of hippocampal sclerosis (Pennell et al., 1999). An explanation for the slight increase in VAChT binding sites may be due to a relative concentration of septohippocampal presynaptic terminals due to synaptic reorganization in the setting of hippocampal atrophy. However, the authors do report an overall reduction in total VAChT per hippocampal formation when compared to autopsy controls due to significant hippocampal atrophy.

In the preclinical setting, there is currently only one study where the role of VAChT in epileptic mechanisms was directly investigated using the pilocarpine animal model of epilepsy (Guidine et al., 2008). Homozygous VAChT knock-down mice were used, resulting in 70% less VAChT expression and a similar deficit in ACh release, since complete VAChT knock-out mice do not survive as a result of compromised respiratory activity (De Castro et al., 2009). These VAChT homozygous knock-down mice were found to show hyperactivity and deficits in spatial memory acquisition as well as lack of behavioral flexibility (Martyn et al., 2012). The authors hypothesized that innate cholinergic hypofunction would lead to receptor upregulation, and hence an increased susceptibility to the convulsive effects of pilocarpine. Their hypothesis held up since the VAChT-deficient mice showed a reduced seizure threshold following pilocarpine administration, which they propose is due to M1 receptor upregulation or overactivation.

Finally, AChE mRNA is increased and mRNA levels of the ACh-synthesizing enzyme choline acetyltransferase (ChAT) and VAChT are decreased following acute stress (Kaufer et al., 1998). Stress is still the most frequently self-reported trigger of seizures. Moreover, enhanced severity of this stress or anxiety increases the risk of subsequent seizures (for review see Friedman et al., 2011).

Acute stress transiently enhances acetylcholine transmission and neuronal excitability. Consequently, mRNA levels of the early immediate transcription factor c-Fos, a marker of neuronal hyperexcitation, are robustly elevated. c-Fos has binding sites in the promotors of the previously mentioned cholinergic key genes (AChE, ChAT and VAChT) and will consequently cause long-lasting changes in their expression in neocortex and hippocampus, resulting in a reduction in ACh levels. This delayed secondary phase of suppressed neuronal excitability correlates with the delayed neuropsychiatric pathologies that characterize post traumatic stress disorder, including depression, irritability, and impaired cognitive performance (Kaufer et al., 1998).

The location of the binding sites of Ach and vesamicol, a well-studied inhibitor (**Figure 4D**), on VAChT are recently investigated by inducing mutations, in and around W331 (**Figure 4C**) and nearby the luminal end of the transporter. Hitherto, the existence of a spatial cluster of residues close to vesicular lumen, strongly correlated with the affinitiy for ACh and vesamicol. The cluster consists of invariant W331, highly conserved A334 and invariant F335 in TM VIII and invariant C391 in TM X (Khare et al., 2010). However, the mechanistic model for VAChT (Varoqui and Erickson, 1996) displaying two binding sites for ACh, one close to cytoplasm and the other close to the vesicular lumen with transfer of bound ACh between them during transport, cannot be excluded (Khare et al., 2010). Recently, two vesamicol analogues, o-iodo-trans-decalinvesamicol (OIDV) or o-bromo-trans-decalinvesamicol (OBDV), were synthesized and their affinities to VAChT were assessed by *in vitro* binding assays. Both displayed greater binding affinity to VAChT than vesamicol. Moreover, OIDV was able to penetrate the BBB and might be a VAChT imaging probe with high affinity and selectivity (Kozaka et al., 2012).

SLC32 FAMILY

The *SLC32* gene family consists of only one member, the vesicular GABA transporter (VGAT), responsible for the vesicular accumulation of electrically neutral substrates, GABA, and glycine (McIntire et al., 1997; Gasnier, 2004). Since GABA and glycine are known to be inhibitory neurotransmitters, the VGAT transporter is also called the VIAAT (Sagne et al., 1997).

Although the VNTs are divided into different families, they were all thought to share a common overall topology with an even number of transmembrane domains and cytosolic localized N- and C-terminals. However, using epitope-specific antibodies and mass spectrometry, Martens and coworkers showed that the VGAT possesses an uneven number of transmembrane domains, with the N-terminus located in the cytoplasm (**Figure 5**) (Martens et al., 2008).

VESICULAR GABA TRANSPORTER

As the major inhibitory neurotransmitter in the brain, GABA is an important modulator of hyperexcitability in epilepsy patients and experimental animal models. Some years ago GABA's involvement in epilepsy was suggested (Snodgrass, 1992; Roberts, 2000) and it soon became clear that impairment of GABA functions produced seizures, whereas enhancement resulted in an anticonvulsant effect (Olsen and Avoli, 1997). Accordingly, several old (Holland et al., 1992) and more modern AEDs (Rogawski and Loscher, 2004) act by enhancing the efficacy of GABA-mediated mechanisms proving the GABA theory of epilepsy (Meldrum, 1989; Treiman, 2001). The exception of absence epilepsy proves the role. Indeed, new evidence indicates that increased, rather than decreased, GABAergic inhibition characterizes this generalized form of epilepsy. In particular, enhanced tonic $GABA_A$ inhibition, resulting from a malfunction of the astrocytic GABA transporter GAT-1, occurs in thalamocortical neurons of all

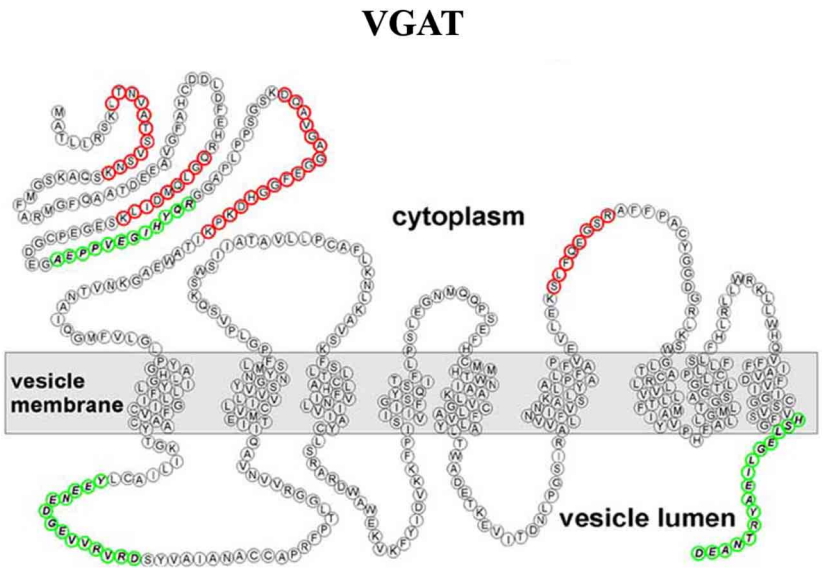

FIGURE 5 | Refined transmembrane topology of the vesicular GABA transporter (VGAT), the only member of the SLC32 gene family (Martens et al., 2008).

well-established pharmacologic and genetic models of the disease (Cope et al., 2009; Di Giovanni et al., 2011). Aberrant tonic current has also been shown to have a pathological role in TLE. GABA receptor-mediated inhibition is reduced in the hippocampi of animals during SE (Joshi and Kapur, 2012). SE also causes long-term changes in plasticity of hippocampal GABA receptors consisting of down-regulation of the subunits in the dentate gyrus granule cells, and up-regulation in interneurons. Surprisingly, the level of tonic inhibition in granule cells remains unchanged, consistent with the idea that it is mediated by $GABA_A$ receptors containing different subunits (Ferando and Mody, 2012). These findings have important pharmacological implications and extrasynaptic $GABA_A$ receptors might become an important AED target.

Of the new AEDs recently launched, tiagabine and vigabatrin (γ-vinyl GABA) act selectively through the GABA-ergic system. The former inhibits neuronal and glial uptake of GABA (including GAT-1) whilst the latter increases the synaptic concentration of GABA by inhibition of GABA-aminotransferase. Other new anticonvulsants are gabapentin and its structure-related pregabalin that are GABA analogues. They easily cross the blood-brain barrier and increase brain synaptic GABA, but their effects are complex and have not been fully characterized (Rogawski and Loscher, 2004; Czapinski et al., 2005). The older benzodiazepine-like AEDs and barbiturates work with different modes of action, through positive allosteric modulation of $GABA_A$ receptors. The use of benzodiazepines is currently limited to acute treatment in SE since their use in chronic therapy is characterized by negative side effects, such as sedation, muscle relaxation, and especially the development of tolerance and dependence (Rogawski and Loscher, 2004).

Interestingly, VGAT activity depends equally on both pH and $\Delta\psi$, differently from the other VNTs (McIntire et al., 1997). *In vitro* studies have shown that VGAT in GABAergic synaptic terminals acts as a co-transporter or symporter for Cl^- with GABA (Juge et al., 2009). During refilling, two Cl^- ions accompany GABA as it enters the synaptic vesicle. High rates of exchange strongly depend on the size of the pH gradient in the vesicle lumen vs. cytoplasm. A recent study showed that the voltage-gated Cl^- ion channel called CLC-3 co-localizes with VGAT in perisomatic synaptic endings on CA1 pyramidal neurons and plays a pivotal role in regulating inhibitory synaptic function by optimizing GABA loading into presynaptic vesicles (Riazanski et al., 2011). CLC-3 might help GABAergic interneurons to maintain rapid firing by ensuring optimal pH differences between the vesicle lumen and cytoplasm, and the availability of protons for rapid GABA loading into vesicles or alternatively act as a Cl^- transporter, providing a pathway to clear the vesicle of high Cl^- concentrations (Riazanski et al., 2011). These results are noteworthy, particularly that CLC-$3^{-/-}$ mice develop spontaneous seizures that resembled TLE with hippocampal sclerosis and nearly complete bilateral destruction of the hippocampus (Stobrawa et al., 2001). In CLC-$3^{-/-}$ mice, VGAT-dependent GABA release is reduced and feedback inhibition is eliminated leading to limbic seizures. With prolonged seizures, principal neurons become excitotoxic and widespread degeneration occurs (Naegele, 2012).

VGAT plays a fundamental role in embryonic development. Indeed, $VGAT^{-/-}$ mice are not viable and die between embryonic day (E)18.5 and birth. They exhibit a hunched posture and are completely immobile and stiff, most likely symptoms of overexcitation (Wojcik et al., 2006; Saito et al., 2010). $VGAT^{-/-}$ foetuses showed significant increases in both GABA and glycine contents in the forebrain that were not derived from elevated amounts of GABA-synthesizing enzymes. GABA and glycine accumulate in the GABAergic and glycinergic neurons, respectively, but they are not degraded in the glial cells of $VGAT^{-/-}$ mice. These observations bear important consequences for understanding the functional roles of VGAT from the cellular to the whole-body level (Saito et al., 2010). It has been reported that glutamic acid decarboxylase (GAD)65 and not GAD67 forms a complex with heat shock cognate 70, cysteine string protein and VGAT in the nerve terminal. This complex appears to be the necessary machinery required for efficient GABA synthesis and packaging into synaptic vesicles through VGAT (Jin et al., 2003; Buddhala et al., 2009).

VGAT expression increases during development, however, in several brain regions expression levels of VGAT are already high at birth and in some cases they decrease in the course of brain development (Boulland and Chaudhry, 2012). In the hippocampal formation, a shift in the localization of the VGAT nerve terminals has been observed. At early postnatal stages, they are present in dendritic layers while later in the development they target the perikaryon of the principal cells. This redistribution of GABAergic nerve terminals occurs at around P7 which is also the stage at which the postsynaptic response to GABA stimulation switches from membrane depolarization to membrane hyperpolarization. Thus, redistribution of GABAergic nerve terminals together with changes in the expression of Cl^- transporters is responsible for the conversion of the GABA action from excitation toward inhibition (Boulland and Chaudhry, 2012). In the normal adult rat hippocampus, VGAT immunoreactive puncta is strongly detected in all pyramidal cell layers of the CA1-3 regions and the granule cell layer of the dentate gyrus (Chaudhry et al., 1998; Kang et al., 2003; Boulland et al., 2007; Choi et al., 2010).

There is only sparse data on VGAT in human epilepsy available. In a case report of Unverricht-Lundborg disease, the most common progressive myoclonic epilepsy, VGAT-IR was decreased in the cortex of the examined patient (Buzzi et al., 2012). In the resected cortex from pediatric epilepsy surgery patients with type II cortical dysplasia GAD-labeled neurons displayed larger somata and VGAT and GABA transporter 1 staining showed a dense plexus surrounding cytomegalic neurons (Andre et al., 2010). Finally, cortical tubers of tuberous sclerosis complex patients showed decreased levels of GAD65, VGAT, GluR subunit 1 and $GABA_A$ receptor subunits α1 and α2 (White et al., 2001). However, until now nothing is known on VGAT in TLE patients.

As far as the experimental models of epilepsy are concerned, the density of VGAT terminals is reduced in an animal model of cortical dysplasia (Zhou and Roper, 2010), in pre-seizure seizure-sensitive gerbil (Kang et al., 2003) and picrotoxin-induced kindling rat hippocampi (Jiang et al., 2004), although VGAT-IR is unaltered in KA-induced seizures (Sperk et al., 2003). Contrasting evidence exists for changes in VGAT in epileptogenesis induced by pilocarpine. One study reported that at 1 day after

pilocarpine-induced SE VGAT-IR is not changed (Kwak et al., 2006), instead it is significantly reduced in the dentate gyrus at 1–2 weeks after SE and recovered to the control level at 5 weeks after SE (Kwak et al., 2006). Conversely, other studies support an increased rather than a decreased synthesis of VGAT that occurs after SE and persists until the chronic stage (Boulland et al., 2007; Choi et al., 2010). Indeed, a marked increase in VGAT-IR in all pyramidal cell layers and the granule cell layer of dentate gyrus due to an up-regulation of VGAT mRNA synthesis at 1–2 weeks reaching a maximum level of labeling at 12 weeks after SE has been reported (Boulland et al., 2007; Choi et al., 2010). Therefore, the increased synthesis of VGAT is likely to lead to an increased GABA release from all remaining GABAergic interneurons. Such increased expression of proteins is involved in presynaptic GABAergic transmission and could be associated with increased activity of remaining interneurons (Boulland et al., 2007). This hypothesis is supported by electrophysiological studies showing that, in chronic pilocarpine-treated animals, interneurons are hyperactive (Cossart et al., 2001).

A marked selective decrease in the number of VGAT mRNA-containing neurons in the hilus of the dentate gyrus has been observed (Boulland et al., 2007). This finding is consistent with the cell death of GAD-containing neurons, previously demonstrated in this model (Obenaus et al., 1993). The reduction of VGAT-IR reported after excitotoxic insult might be also explained by VGAT cleavage. Recently, it has been shown that VGAT is cleaved under KA-induced conditions *in vitro* and *in vivo* giving rise to a truncated product that lacks a punctate synaptic distribution. VGAT cleavage was mediated by calpains, and the stability of the cleavage product suggests that the protease plays a modulatory role rather than a destructive effect in excitotoxic neuronal damage (Gomes et al., 2011). VGAT cleavage by calpain leads to the formation of a new and stable truncated VGAT form (tVGAT), which is not targeted to the synapse. This is expected to decrease the release of GABA by exocytosis, thereby changing the equilibrium between excitatory and inhibitory synaptic activity in the early phases after the excitotoxic insult (Gomes et al., 2011). VGATs are not limited to hippocampal GABA interneurons. Indeed, mRNA is also expressed in the dentate gyrus and in mossy fiber synaptosomes and increase after kindling in rats (Lamas et al., 2001). Therefore, granule cells and their mossy fibers, besides being glutamatergic, contain the machinery for the synthesis and vesiculation of GABA. This further supports the notion that local synaptic molecular changes enable mossy fibers to release GABA in response to enhanced excitability as a protective mechanism in response to seizures (Gomez-Lira et al., 2002).

CONCLUDING REMARKS

Epilepsy is one of the most common acquired chronic neurologic disorders, with disastrous implications for the quality of life of the patients. Despite the availability of a large number of AEDs, still 30–40% of patients remain pharmacoresistant (Pitkänen and Sutula, 2002; Baulac and Pitkanen, 2008; Pitkänen and Lukasiuk, 2009; Abou-Khalil and Schmidt, 2012; Fridley et al., 2012). Therefore, there is an urgent need of new non-invasive anti-epileptogenic or disease-modifying treatments (Baulac and Pitkanen, 2008). Several innovative targets for future AED are currently intensively investigated. In this review we focused on the role of VNTs in epilepsy and summarized the available evidence in **Table 1**.

The future perspectives and challenges to fully elucidate the involvement of VNTs and their subsequent possible use as targets for the treatment of TLE are discussed below.

VGLUTs

Taken together, all the data here reviewed indicate that VGLUTs might be involved in different types of seizures and epilepsy. As expected VGLUTs are the most studied VNTs, although to date, there are no data available on the expression levels of VGLUT2/3 in human epileptic tissue. Furthermore, activity studies need to be performed. Unfortunately, although several competitive and non competitive VGLUT inhibitors have been identified with affinities into the nanomolar range (Thompson et al., 2005; Chaudhry et al., 2008a; Pietrancosta et al., 2010), there is still a lack of specific *in vivo* VGLUT-inhibitors to further investigate and confirm present consistent results.

In addition, it is worthwhile mentioning that a recent study by Juge et al. linked fasting and excitatory neurotransmission through Cl^--dependent regulation of VGLUT activity (Juge et al., 2010). The ketogenic diet is often used to control epilepsy (Joshi et al., 2013). Although fasting does not affect the activity of glutamate reuptake transporters (Bough et al., 2007), the vesicular transporters are possibly targeted by the ketone bodies, in particular acetoacetate and β-hydroxybutyrate, produced by this diet. These metabolites can enter the brain, serving as substrates for energy production in neurons. Ketone bodies can compete with Cl^-, an anion which is absolutely necessary for optimal VGLUT functioning, by shifting the Cl^- dependence to a higher concentration. Furthermore, Juge et al. showed that acetoacetate can reversibly suppress glutamate release and seizures evoked by 4-aminopyridine in the brain. Thus, the competitive interaction between Cl^- and ketone bodies can turn VGLUT activity off upon binding of the latter, causing a reduction in glutamatergic neurotransmission *in vivo*. The identification of ketone bodies as physiological modulators of VGLUTs advances the search for new approaches in the development of drugs to treat neurological disorders caused by excessive glutamatergic neurotransmission, such as epilepsy (Juge et al., 2010).

VEAT

The role of sialin in epilepsy and epileptogenesis is far from known. Moreover, the involvement of sialin in the vesicular loading of aspartate has recently been questioned, based on *in vitro* data from sialin$^{(-/-)}$ mice (Morland et al., 2013). Of course, it cannot be excluded that these transgenic mice develop compensatory mechanisms to counteract the loss of sialin. Consequently, there is a need for the development of specific inhibitors of sialin to confirm the role of sialin as VEAT *in vivo*.

Currently commercial antibodies cannot be used for immunolocalization of sialin and from previous immunohistochemical studies (Aula et al., 2004; Yarovaya et al., 2005), using "home-made" anti-sialin antibodies, presence in synaptic vesicles could not be confirmed. Therefore, production of new distinct

Table 1 | Summary of literature regarding transgenic mice with disruptions in vesicular nucleotide transporters (VNTs) in different models of seizures and epilepsy, as well as the known alterations and reorganizations in the expression levels of these VNTs in rodent models for temporal lobe epilepsy (TLE) and in human tissue resected for epilepsy surgery.

	Human epileptic tissue	mRNA/protein alterations	References	Animal epilepsy models/transgenic animals	Alterations mRNA/protein/seizure threshold	References
VGLUT1	Punctate structures of the peritumoral neocortex	Protein level ↓	Alonso-Nanclares and De Felipe, 2005	Seizure sensitive gerbils (Snodgrass)	Protein level ↑	Kang et al., 2005
	TLE without hippocampal sclerosis	mRNA level ↓	van der Hel et al., 2009	Hypoxic ischemia (both hippocampi)	Protein level ↑	Kim et al., 2005
		Protein level ↑	van der Hel et al., 2009	Post-SE pilocarpine model [latent period, hilar neurons, dentate gyrus (DG)]	mRNA level ↓	Boulland et al., 2007
	TLE with hippocampal sclerosis (subfields with neuronal loss)	mRNA level ↓	van der Hel et al., 2009	Post-SE pilocarpine model (chronic period, CA1-CA3)	Protein level ↑	Boulland et al., 2007
		Protein level ↓	van der Hel et al., 2009			
	TLE with hippocampal sclerosis (DG)	Protein level ↑	van der Hel et al., 2009			
VGLUT2	No studies			Seizure sensitive gerbils (DG)	Protein level ↑	Kang et al., 2005
				Hypoxic ischemia (both hippocampi)	Protein level unaltered	Kim et al., 2005
				Methylazoxymethanol explosion (hippocampus)	Protein level ↑	Harrington et al., 2007
				Symptomatic GAERS (cortex)	Protein level ↑	Touret et al., 2007
				VGLUT2$^{(+/-)}$	Seizure threshold ↓ (pentylenetetrazole)	Schallier et al., 2009
VGLUT3	No studies			VGLUT3$^{(-/-)}$	Spontaneous absence seizures	Seal et al., 2008
VEAT	No studies			Sialin$^{(-/-)}$	Spontaneous seizures	Prolo et al., 2009
VNUT	No studies			No studies		
VMAT2	Medically intractable TLE (neocortices)	mRNA level ↓	Jiang et al., 2013	Perinatal asphyxia rat model (striatum)	Protein level ↑	Kohlhauser et al., 1999
		Protein level ↓	Jiang et al., 2013	Following KA-induced status epilepticus (locus coeruleus)	mRNA level unaltered	Bengzon et al., 1999
				Acute stage post-SE pilocarpine model (hippocampus and cortex)	Protein level ↑	Jiang et al., 2013
				After SRS post-SE pilocarpine model (hippocampus and cortex)	Protein level ↓	Jiang et al., 2013

(Continued)

Table 1 | Continued

	Human epileptic tissue	mRNA/protein alterations	References	Animal epilepsy models/transgenic animals	Alterations mRNA/protein/seizure threshold	References
VAChT	TLE with hippocampal sclerosis	Protein level ↑ trend	Pennell et al., 1999	homozygous VAChT knock-down mice	Seizure threshold ↓ (pilocarpine)	De Castro et al., 2009
VGAT	Unverricht-Lundborg disease (cortex)	Protein level ↓	Buzzi et al., 2012	Cortical dysplasia (CD)	Protein level ↓	Zhou and Roper, 2010
	Pediatric epilepsy type II (CD) (cortex)	Dense plexus surrounding cytomegalic neurons	Andre et al., 2010	Seizure sensitive gerbils	Protein level ↓	Kang et al., 2003
	Tuberous sclerosis complex (cortical tubers)	mRNA level ↑	White et al., 2001	Picrotoxin-induced kindling	Protein level ↓	Jiang et al., 2004
				KA-induced seizures	Protein level unaltered	Sperk et al., 2003
				Post-SE pilocarpine model (hippocampus, 1 day)	Protein level unaltered	Kwak et al., 2006
				Post-SE pilocarpine model (1–2 weeks only DG)	Protein level ↓	Kwak et al., 2006
				Post-SE pilocarpine model (5 weeks only DG)	Protein level unaltered	Kwak et al., 2006
				Post-SE pilocarpine model (hilus of the DG 1–12 weeks)	Number of VGAT mRNA-containing neurons ↓	Boulland et al., 2007
				Post-SE pilocarpine model (hippocampus 1–12 weeks)	mRNA level ↑ (time dependent)	Boulland et al., 2007
				Post-SE pilocarpine model (hippocampus 1–12 weeks)	Protein level ↑ (time dependent)	Boulland et al., 2007

anti-sialin antibodies, which can be tested for specificity in both Western Blotting and immunohistochemistry in sialin$^{(-/-)}$ vs. wiltype brain tissue, is necessary.

However, since sialin$^{(-/-)}$ mice clearly exhibit seizures, this transporter, whether or not crucial for vesicular import of aspartate, still might be a novel AED target. Subsequently, the currently available tools should be used to further investigate this role. The seizure threshold of sialin$^{(+/-)}$ can be compared to their wildtype littermates in different animal models for epilepsy and moreover, the expression levels of VEAT in human epileptic tissue should be determined.

VNUT

The lack of tools is a major issue in order to unravel the possible involvement of VNUT in epilepsy and epileptogenesis. Although ADK overexpression and adenosine deficiency are considered to be pathological hallmarks in distinct rodent models and in human tissue resected from TLE patients (Boison et al., 2012), still nothing is known about mRNA or protein expression levels of VNUT in those tissues. Surprisingly, only one home-made antibody against VNUT has been described until now (Larsson et al., 2012). Moreover, the development of specific inhibitors might provide more insights in the role of VNUT in epilepsy as well.

VMAT

A few studies, including both animal models for epilepsy and human epileptic tissue, indicate the possible involvement of VMAT in acute seizure generation and epileptogenesis.

Recently, Narboux-Neme et al. generated a conditional deletion of VMAT2 in raphe 5-HT neurons by Cre-recombinase expressed under the control of the 5-HT transporter gene (SERT, slc6a4) promoter. These VMAT2$^{sert-cre}$ mice show an almost complete depletion of 5-HT in the brain. Nonetheless, raphe neurons are normally developed and exert normal innervations of target regions. The conditional VMAT2$^{-/-}$ results in a depressive-like phenotype and an anxiety-like response (Narboux-Neme et al., 2011). To further investigate whether VMAT inhibition would indeed negatively affect the course of

disease progression in epilepsy, these inhibitors could be administrated at different stages of the chronic post-SE pilocarpine model (Jiang et al., 2013). Similarly, it would be most interesting to subject the conditional VMAT2$^{-/-}$ mice to chronic models of epilepsy.

VAChT

The precise role VAChT plays in epilepsy still needs to be unraveled. The sparse data from human epileptic tissue and homozygous VAChT knock-down mice, suggest that inhibition of this VNT might lead to protection against seizures. However, cholinergic neurotransmission in the hippocampus is linked to cognitive functions, such as learning and memory. Hippocampal LTP is negatively affected in homozygous VAChT knock-down mice, which is of great importance in learning and memory processes (Martyn et al., 2012; Bliss and Collingridge, 2013). This indicates that a substantial attenuation of VAChT, consequently leading to hippocampal cholinergic deficits, may not be ideal in the clinical situation. Incidentally, cognitive and behavioral impairments are co-morbidities associated with epilepsy (Berg, 2011). Neuropharmacologists aim to find AED treatments that not only suppress seizures and hinder the epileptogenic process, but also prohibit any cognitive dysfunctions in patients with TLE. In an attempt to prevent or reduce worsening of epilepsy-related co-morbidities together with attenuating epileptic seizures, it would be of interest to investigate the actions of VAChT blockers, in animal models of epilepsy to confirm whether similar effects on seizures, epilepsy progression and cognitive functioning will be obtained as in the VAChT knock-down mouse model.

VGAT

Despite the fact that no drugs acting specifically on VGAT have been identified to date, it has been shown that vigabatrin inhibits VGAT with an affinity similar to GABA (McIntire et al., 1997), an effect that is present only acutely and is lost after chronic treatment with vigabatrin (Engel et al., 2001). This could explain the proconvulsant effect of vigabatrin during the first hours following its administration (Löscher et al., 1989). Selective high-affinity VGAT inhibitors should broadly impair inhibition and would thus be of limited therapeutic interest. Still, they could have provided probes for *in vivo* imaging of inhibitory nerve terminals in the human brain, with potential applications for monitoring the progression or treatment of neurodegenerative diseases characterized by a loss of GABAergic neurons such as epilepsy, Huntington's disease, and brain ischemia. These transporters are particularly interesting as specific markers for GABAergic neurons whose expression levels could reflect the demand for synaptic transmission and their average activity. This could be used in addition to the earlier described intrahippocampal injection of fluorochromated anti-VGAT-C in mice for specific GABAergic synapses labeling *in vivo* (Martens et al., 2008).

Moreover, an interesting strategy for new AEDs targeting VGAT might be related to its expression. For instance, vigabatrin has been shown to be capable of increasing VGAT-IR in the gerbil hippocampus following spontaneous seizures (Kang et al., 2003). Similarly, the opening of ATP-sensitive potassium (KATP) channels by diazoxide prevented seizures and resulted in an up-regulation of VGAT mRNAs and VGAT protein production in hippocampus, and a down-regulation of GAT-1 and GAT-3 gene and protein expressions in picrotoxin-induced kindling in rats (Jiang et al., 2004).

Consequently, epigenetics and microRNA (miRNA) controllers should be considered as hot topic therapeutic interventions to regulate VNTs expression levels.

Epigenetics is defined as information that is heritable during cell division other than the DNA sequence itself, including DNA methylation or histone tail modifications, which can produce lasting alterations in chromatin structure and gene expression (Kobow and Blumcke, 2011). The methylation hypothesis of Kobow and Blümcke postulates that seizures are able to induce epigenetic chromatin modifications and consequently deteriorate epileptogenesis and contribute to structural brain lesions and cognitive dysfunction (Kobow and Blumcke, 2011). Moreover, parallel as in cancer, epigenetic modifications can affect multidrug transporters and induce pharmacoresistance in epilepsy (Kobow et al., 2013). Distinct histone deacetylase and DNA methyltransferase inhibitors are currently under clinical investigation as possible novel epigenetic treatment strategies (Kobow and Blumcke, 2011). More research on epigenetic chromatin modifications will certainly lead to a better understanding of the pathomechanisms involved in epileptogenesis and to novel possible antiepileptogenic compounds or biomarkers (for review see Lubin, 2012; Roopra et al., 2012). For example in prostate cancer, *SCL18A2* hypermethylation was observed in ∼90% of all cases, subsequently epigenetic silencing of VMAT2 was defined as a novel adverse predictor of biochemical recurrence after radical prostatectomy (Sorensen et al., 2009).

miRNA on the other hand is a group of small non-coding RNA able to control post-transcriptional gene expression by fine-tuning protein production, through sequence-specific binding within the 3′ untranslated region of mRNA transcripts (Jimenez-Mateos and Henshall, 2013). This translational control of miRNA is observed in neurons (Kosik, 2006), astroytes (Tao et al., 2011) and microglia (Ponomarev et al., 2011) and seems to be involved in the process of epileptogenesis as well as the maintenance and progression of the epileptic state (for review see Jimenez-Mateos and Henshall, 2013). By use of genome-wide miRNA profiling in human hippocampus of autopsy control and mesial TLE patients with and without hippocampal sclerosis, three distinct miRNA signatures were observed. Moreover, deregulated miRNA targets components of key pathways in TLE, including VGLUT1 (Kan et al., 2012). Although several approaches have been examined to modulate individual miRNA expression, such as viral vectors, miRNA decoys and miRNA "sponges," a better understanding of miRNA biology and function is necessary before an eventually clinical translation of these possible new therapeutic interventions (for review see Brown and Naldini, 2009).

CONCLUSION

Epilepsy remains a hard-to-treat disorder for millions of patients. There is an urgent need of non-invasive antiepileptogenic or disease-modifying treatments. VNTs are appealing new intracellular targets for future AEDs. Nevertheless, LEV and brivaracetam are, respectively, hitherto the only approved AED and the

only AED currently in the pipeline targeting a vesicular protein (Kaminski et al., 2012). Much more research is needed to improve our understanding of the role of VNTs in normal and pathological conditions, as this will certainly lead to new therapeutic strategies for TLE and other CNS disorders.

ACKNOWLEDGMENTS

Joeri Van Liefferinge is supported by the agency for Innovation by Science and Technology (IWT/SB/101344). Joeri Van Liefferinge, Ilse Smolders, and Ann Massie acknowledge the Fund for Scientific Research Flanders (FWO, Belgium, grant G038412N) and the Queen Elisabeth Medical Foundation (G.S.K.E.) for financial support Ilse Smolders and Giuseppe Di Giovanni are indebted to the EU COST Action CM1103 "Structure-based drug design for diagnosis and treatment of neurological diseases: dissecting and modulating complex function in the monoaminergic systems of the brain" for supporting their international collaboration.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 June 2013; accepted: 11 August 2013; published online: 30 August 2013.

Citation: Van Liefferinge J, Massie A, Portelli J, Di Giovanni G and Smolders I (2013) Are vesicular neurotransmitter transporters potential treatment targets for temporal lobe epilepsy? Front. Cell. Neurosci. 7:139. doi: 10.3389/fncel.2013.00139

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 12 September 2013
doi: 10.3389/fncel.2013.00149

Seizure-like activity in hyaluronidase-treated dissociated hippocampal cultures

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The extracellular matrix (ECM) plays an important role in use-dependent synaptic plasticity. Hyaluronic acid (HA) is the backbone of the neural ECM, which has been shown to modulate α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptor mobility, paired-pulse depression, L-type voltage-dependent Ca^{2+} channel (L-VDCC) activity, long-term potentiation and contextual fear conditioning. To investigate the role of HA in the development of spontaneous neuronal network activity, we used microelectrode array recording and Ca^{2+} imaging in hippocampal cultures enzymatically treated with hyaluronidase. Our findings revealed an appearance of epileptiform activity 9 days after hyaluronidase treatment. The treatment transformed the normal network firing bursts and Ca^{2+} oscillations into long-lasting "superbursts" and "superoscillations" with durations of 11–100 s. The changes in Ca^{2+} transients in hyaluronidase-treated neurons were more prominent then in astrocytes and preceded changes in electrical activity. The Ca^{2+} superoscillations could be suppressed by applying the L-VDCC blocker diltiazem, whereas the neuronal firing superbursts could be additionally suppressed by 6-cyano-7-nitroquinoxaline-2,3-dione as an antagonist of AMPA/kainate receptors. These results suggest that changes in the expression of HA can be epileptogenic and that hyaluronidase treatment *in vitro* provides a robust model for the dissection of the underlying mechanisms.

Keywords: extracellular matrix, hyaluronic acid, AMPA receptors, L-type Ca^{2+} channels, neuronal network, microelectrode array, Ca^{2+} imaging, seizure

INTRODUCTION

The extracellular matrix (ECM) plays an important role in regulating use-dependent synaptic plasticity. Distinct aggregates of ECM molecules surround cell bodies and proximal dendrites of some central neurons, forming so-called perineuronal nets (PNNs; Dityatev et al., 2010). These nets are heterogeneous in their structure and composition and are composed of molecules produced by both neurons and astrocytes (Dityatev et al., 2010), such as hyaluronic acid (HA), chondroitin sulfate proteoglycans of the aggrecan family, tenascin-R and link proteins (Bruckner et al., 2000; Galtrey and Fawcett, 2007; Frischknecht and Seidenbecher, 2008). HA is a large, negatively charged, non-branched polymer composed of repeated disaccharides of glucuronic acid and *N*-acetylglucosamine. Recently, HA has been shown to affect both α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptor mobility and paired-pulse modulation in hippocampal cultures (Frischknecht et al., 2009). Another study established a mechanistic link between HA and long-term potentiation (LTP) by showing that removing HA by hyaluronidase (Hyase) suppresses L-type voltage-dependent Ca^{2+} channel (L-VDCC)-mediated currents in hippocampal slices, reduces Ca^{2+} transients in postsynaptic dendrites and spines, and specifically abolishes an L-VDCC-dependent component of LTP (Kochlamazashvili et al., 2010). Furthermore, the injection of Hyase before contextual fear conditioning impairs the formation/retention of fear memories. A mathematical modeling study highlighted that the ECM may function as a memory substrate by showing that remodeling of ECM may lead to a bistability in which two different stable levels of average firing rates can coexist in a spiking network (Kazantsev et al., 2012).The multiple roles played by the ECM in healthy brain neuroplasticity suggest that it could be an important factor for pathogenic plasticity associated with epileptogenesis (Dityatev, 2010), which results in the transformation of normal neuronal network activity into spontaneous recurrent epileptiform discharges (seizures; Robert et al., 1998). Although accepted to be important, the exact contribution of the multiple structural and functional pathological forms of plasticity in hippocampal circuit alteration during epileptogenesis remains to be elucidated. For most forms of epilepsy, it is unknown how the hippocampus becomes hyperexcitable and how hyperexcitable cells integrate into a pathologically functioning circuit, resulting in the generation of spontaneous recurrent seizures. In recent years, microelectrode array (MEA) and optical imaging technologies have developed rapidly, providing new options to perform long-term, detailed analysis of neural circuitry dynamics.

In this study, we used these technologies to describe the development of epileptiform activity following Hyase treatment *in vitro*. Our findings revealed that Hyase destructed the ECM of

PNNs, surrounding cell bodies and the proximal dendrites of parvalbumin (PV)-expressing interneurons, and induced a slow development of seizure-like activity. The treatment transformed normal network spiking bursts into long-lasting "superbursts" and caused the appearance of neuronal and astrocytic Ca^{2+} "superoscillations." Seizure-like activity in Hyase-treated cultures persisted for at least 9 days and could be suppressed by an L-VDCC blocker but not by an NMDA receptor antagonist. These results suggest that changes in the expression of HA can be epileptogenic and the underlying mechanisms may involve changes in Ca^{2+} oscillations.

MATERIALS AND METHODS

CELL CULTURES

Hippocampal cells were dissociated from embryonic mice (on embryonic day 18) and plated with a high initial density of approximately 9000 cells/mm^2 on MEAs (Alpha MED Science, Japan) pretreated with the adhesion promoting molecule polyethyleneimine (Sigma P3143). We used so high-density to mimic tissue conditions and have long-lasting recordings of network activity. The cultures develop a complex pattern of activity as previously described (Pasquale et al., 2008). C57BL6J mice were killed by cervical vertebra dislocation, according to the protocols approved by the National Ministry of Public Health for the care and use of laboratory animals and by the Bioethics Committee of the Nizhny Novgorod State Medical Academy. Embryos were removed and decapitated. The entire hippocampi were dissected under sterile conditions. Hippocampi were cut in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS-minus). After enzymatic digestion for 25 min by 0.25% trypsin (Invitrogen 25200-056) at 37°C, cells were separated by trituration (10 passes) using a 1 ml pipette tip. After being passed, the solution was centrifuged at 1500 × *g* for 2 min, and the cell pellet was immediately re-suspended in Neurobasal medium (Invitrogen 21103-049) with 2% B27 (Invitrogen 17504-044), 0.5 mM L-glutamine (Invitrogen 25030-024), and 5% fetal calf serum (PanEco K055) (NBM1). The dissociated cells were seeded in a 40 µl droplet covering the center of the culture dish on a 1 mm^2 electrode region of the MEA, forming a dense monolayer (Potter and DeMarse, 2001). After the cells had adhered (usually within 2 h), the dishes were filled with 0.8 ml of NBM1. After 24 h, the plating medium was replaced by a medium containing Neurobasal medium with 2% B27, 1 mM L-glutamine, and 0.4% fetal calf serum (NBM2) without any antibiotics or antimycotics. Glial growth was not suppressed because glial cells are essential for long-term culture maintenance. One half of the medium was changed every 2 days. The cells were cultured under constant conditions of 37°C, 5% CO_2, and 95% air at saturating humidity in a cell culture incubator (MCO-18AIC, Sanyo).

Phase-contrast images of the cultures were taken weekly to record the culture status using a DMIL HC (Leica, Germany) inverted microscope with a 10×/0.2Ph1 objective. Experiments were performed when the cultures reached the 17th day *in vitro* (DIV).

ELECTROPHYSIOLOGICAL METHODS

Extracellular potentials were recorded simultaneously through 64 planar indium tin-oxide (ITO) platinum black electrodes with the integrated MED64 system (Alpha MED Science, Japan). MEAs were 8 × 8 (64) with a 50 µm × 50 µm electrode size and a 150 µm spacing, and the sampling rate was 20 kHz/channel.

All of the signal analyses and statistics were performed using custom-made software (Matlab®).

SPIKE DETECTION

The detection of recorded extracellular spikes was based on threshold calculation using the signal median:

$$T = N_S\sigma, \sigma = median\left(\frac{|x|}{0.6745}\right), \quad (1)$$

where x is the band-pass-filtered (0.3–8 kHz) data signal, σ is an estimate of the standard deviation of the signal without spikes (Quiroga et al., 2004), and N_S is a spike detection coefficient determining the detection threshold (Pimashkin et al., 2011). In signal processing the threshold estimation based on the median of the signal in a form of Eq. 1 is less dependent on the frequency of the spikes than the estimation based on standard deviation. Coefficient 0.6745 in Eq. 1 is used for normalization of the median of the absolute signal to standard deviation. $N_S = 4$ was used for all data, resulting in a reliable detection of spikes with amplitudes greater than 20 µV. The minimal interspike interval was set to 1 ms. Detected spikes were plotted in raster diagrams.

SPONTANEOUS ACTIVITY ANALYSIS

Small burst detection

To analyze the effect of Hyase on neural network activity, we recorded spontaneous bursting activity for 10 min. To detect small bursts, we calculated the total spiking rate (TSR) accounting for the total number of spikes from all electrodes within 50 ms time bins. A fast appearance of a large number of spikes over multiple electrodes in a small (50 ms) time bin was used as a criterion for small burst appearance (for more details, see Pimashkin et al., 2011). Spontaneous activity in the culture consists of a basal stochastic activity observed in a fraction of cells and short bursting episodes. The basal activity was consisted of a spike trains (∼1 spike per 10–100 ms). To detect bursts we used threshold detection based on the statistical characteristics of the spontaneous activity TSR(t). Burst threshold was set to be $T_{Burst} = 0.1 \times \sigma_{TSR}$, where σ_{TSR} is standard deviation of TSR(t). To exclude the basal activity, the burst detection threshold coefficient was empirically set to 0.1, giving the best estimate for the burst initiation and ending points recognized in the raster diagram. Simulation of bursts with the frequency of occurrence up to 5 Hz revealed that the estimated duration of bursts was biased less than 10% of real values. Statistical analysis of the bursting activity characteristics was performed by analysis of variance (ANOVA) tests ($p < 0.05$).

Superburst detection

Superbursts in the electrical activity recorded from the multielectrode arrays were detected as follows. First, we defined a Gaussian function with an effective width equal to 50 s. Next, we iteratively moved that function from the beginning of the recording to the end in 10 ms time steps and calculated a cross-correlation of the function with the TSR. The resulting cross-correlation indicated how much of the synchronized activity (bursts) was recorded in

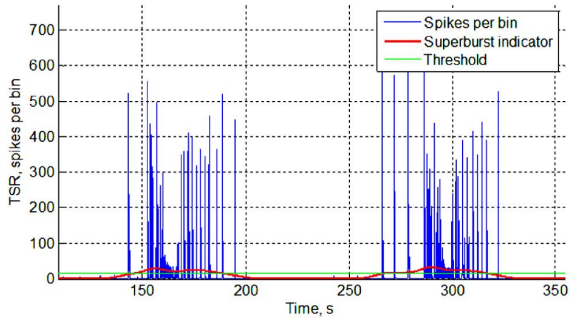

FIGURE 1 | Superburst detection. TSR (blue), total spiking rate over all electrodes in every consecutive 50 ms bin. The superburst indicator (red) reflects the cross-correlation of the TSR with a 50-s long Gaussian window. The estimated threshold for superburst detection is shown in green (for details, see Spontaneous Activity Analysis).

the 10 s window (**Figure 1**). To detect superbursts in the spiking activity, we applied threshold detection, in which the threshold was estimated as the spiking superburst detection accuracy coefficient multiplied by the standard deviation of the calculated cross-correlation. The superburst detection accuracy coefficient was found empirically and was equal to 0.4. All time points that crossed the threshold were defined as the beginnings and endings of the superbursts.

Ca^{2+} IMAGING

Dye loading

Oregon Green 488 BAPTA-1 AM (OGB-1) (0.4 μM; Invitrogen) was dissolved in dimethylsulfoxide (DMSO) with 4% pluronic acid and NBM2 (pH 7.4) and gassed with 95% O_2 and 5% CO_2 at 35.5°C. After 40-min incubation for near-full absorption of the OGB-1 molecules by the cultured cells, the exposed culture was washed for 15 min with NBM2 in the absence of dye. Additionally, the exposed dissociated cultures were also loaded with the astrocyte-specific indicator sulphorodamine SR101 (10 μM; Invitrogen S359; Nimmerjahn et al., 2004).

Optical techniques

A confocal laser scanning microscope, Zeiss LSM 510 (Germany), with a W Plan-Apochromat 20×/1.0 objective was used to investigate the spontaneous activity of the neuronal and astrocytic network. Cytosolic Ca^{2+} was visualized via OGB-1 excitation with the 488 nm line of Argon laser radiation and emission detection with a 500–530 nm filter, while astrocytes were visualized with SR101, which was excited by 543 nm radiation from a He–Ne laser and detected with the use of a 650–710 nm filter for emission. Time series of 256 × 256-pixel images with a 420 μm × 420 μm field of view were recorded at a rate of 4 Hz. A confocal pinhole of 1 Airy unit ensured an axial optical slice resolution of 1.6 μm.

Image analysis

Quantitative evaluation of Ca^{2+} transients was performed off-line using custom-made software in C++ Builder. Cell regions from fluorescent images were manually selected. The Ca^{2+} fluorescence for each cell in each frame was calculated as the average fluorescence intensity (F, relative units from 0 to 255) of the pixels within the defined cell region. Single Ca^{2+} signals were found using the following algorithm. First, each trace from all of the cells was filtered by averaging two neighboring points in the sample set. Next, we calcuated a simple derivative of the signal by calculating a difference between each pair of consequent points. The pulses were found from the derivative of the trace using a threshold detection algorithm. The threshold was estimated as the detection accuracy coefficient multiplied by the standard deviation of the derivative of the trace. Suprathreshold points on the derivative of the trace were taken as the beginnings and endings of the pulses. The detection accuracy coefficient was empirically set to 0.45.

To detect superoscillations, we filtered the signal with a low-pass elliptic filter (0.2 Hz) that removed any regular short calcium pulses. Then, we calculated the derivative of the filtered signal. Each point of the derivative was estimated as an average of the differences of the 20 subsequent pairs of points. This definition allowed us to clearly visualize the superoscillation beginnings and endings. Next, we applied a threshold detection algorithm to detect these superoscillations. The threshold was estimated as the superoscillation detection accuracy coefficient multiplied by the standard deviation of the derivative of the trace. All of the time points that crossed the threshold were defined as the beginnings and endings of the superoscillation. The superoscillation detection accuracy coefficient was empirically set to 0.8.

IMMUNOCYTOCHEMISTRY

Staining

The cultured cells were fixed for 15 min in 4% formaldehyde containing phosphate-buffered saline (PBS; pH 7.4), washed in PBS and permeabilized for 30 min with 0.1% Triton X-100 (Sigma 93443-100ML) and 2% bovine serum albumin (BSA). Subsequently, the cells were incubated for 2 h at room temperature in PBS containing 1% BSA and the appropriate mixture of the primary antibodies: rabbit polyclonal anti-aggrecan (AB1031, Millipore) to stain PNNs and chicken anti-microtubule-associated protein 2 (MAP2) (AB15452, Millipore) to stain neurons. After washing in PBS, the cell cultures were incubated for 2 h at room temperature with the following secondary antibodies: goat anti-rabbit conjugated Alexa Fluor 555 (A21429, Invitrogen) and goat anti-chicken conjugated Alexa Fluor 647 (A 21245, Invitrogen). The immunostained cultures were examined under a confocal laser scanning microscope (Zeiss LSM510, Germany), with a W Plan-Apochromat 20×/1.0 objective. The laser intensity, gain and offset were held constant for each analysis. Quantitative evaluation was performed using Image J (Research Service Branch, NIH).

Quantification of PNNs

The number of MAP2-positive neurons bearing a PNN was determined on cultures that were double-labeled with anti-MAP2 and anti-aggrecan antibodies. In five 420 μm × 420 μm fields of view, we sampled all MAP2-immunopositive hippocampal neurons and assessed the presence of PNNs surrounding each individual neuron ($N = 5$ cultures/DIV).

PHARMACOLOGICAL AGENTS

Drugs were applied to cultures using a pipette. One group received 100 μl of hyaluronidase (Hyase, from *Streptomyces hyalurolyticus*,

Sigma H1136; 75 U/ml), and the second group received Hyase that had been boiled for 30 min (control group). The Hyase was dissolved in PBS, added on the 17th DIV to the cell cultures and incubated at 35.5°C for 1 day, i.e., the culture medium was changed on the 18th DIV.

Pharmacological analysis was performed on DIV 20. AMPA and NMDA glutamate receptors were blocked by 10 μM 6-Cyano-7-nitroquinoxaline-2.3-dione (CNQX; Sigma, C127) and 10 μM 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP; Sigma, C104), respectively. L-VDCCs were blocked by 10 μM diltiazem.

STATISTICAL ANALYSIS

All data quantification is presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a two-way ANOVA implemented in the SigmaPlot 11.0 program (Systat Software Inc.). Student–Newman–Keuls (SNK) was used as a post hoc ANOVA test. The difference between groups was considered significant if the p value was less than 0.05.

RESULTS

Hyaluronic acid is the backbone of neural ECM, which is enriched in PNNs. To remove HA and study its role in neural network activity, we treated hippocampal cultures with Hyase. Because aggrecan has been shown to be a key component of the ECM of PNNs (Giamanco et al., 2010), we used aggrecan immunostaining to characterize the efficacy of the treatment. **Figure 2** shows hippocampal neurons labeled by anti-MAP2 antibodies and PNNs labeled by anti-aggrecan antibody in the control treatment (**Figure 2A**) and after Hyase treatment (**Figures 2B,C**). The treatment was done on DIV 17 when the number of PNNs in the control group reached a steady-state level (**Figure 2D**).

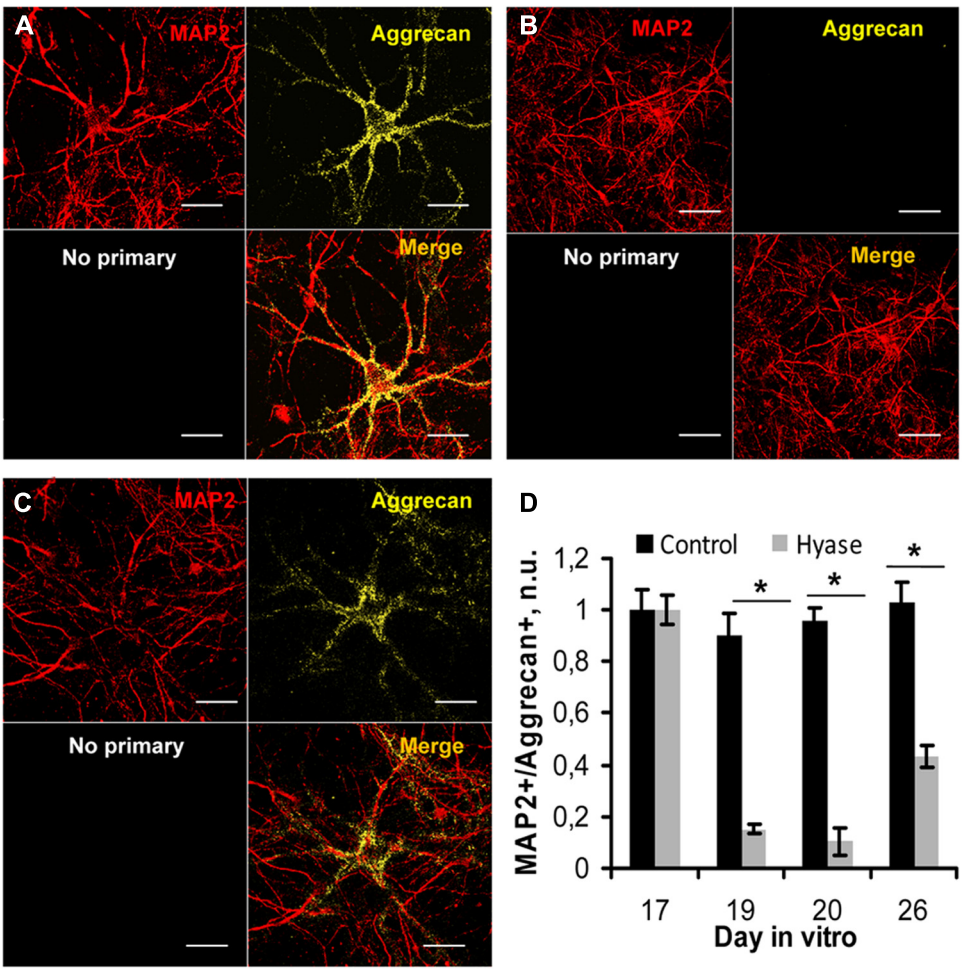

FIGURE 2 | The effect of hyaluronidase on PNNs in the primary hippocampal cultures (A–C). PNNs, stained by aggrecan antibodies (yellow), envelop hippocampal cultured neurons, which were labeled by MAP2 antibodies (red). Hippocampal neurons in the control **(A)** and Hyase-treated primary hippocampal cultures **(B,C)** 2 days after Hyase treatment, i.e., on DIV 19 **(B)**, and 9 days after Hyase treatment, i.e., on DIV 26 **(C)**. "No primary" panels (black) represent immunostainings done without primary antibodies **(A–C)**. Scale bar: 20 μm. **(D)** The fraction of MAP2-positive neurons bearing aggrecan-positive PNNs at different time intervals after Hyase treatment (ANOVA; *$p < 0.05$; $N = 5$ cultures/DIV). The data are from five independent culture preparations. The values shown were normalized by the mean fractions of PNNs in the control and Hyase groups on the 17th DIV. n.u., normalized units; MAP2, microtubule-associated protein 2; Hyase, hyaluronidase.

Counting the PNNs in the control and Hyase-treated primary hippocampal cultures revealed a strong reduction in the fraction of aggrecan-immunopositive neurons 2 (ANOVA $p = 0.001$), 3 (ANOVA $p < 0.001$), and 9 ($p = 0.019$) days after Hyase treatment (**Figure 2D**). Between days 3 and 9, however, there was an increase in the fraction of PNNs (ANOVA $p = 0.037$), suggesting PNN re-formation during this time interval.

Previous studies demonstrated that spontaneous spiking activity appears in high-density dissociated hippocampal cultures after 8–10 days of development on MEAs (Wagenaar et al., 2006). On DIV 16, dissociated culture activity became stabilized. To establish whether the Hyase treatment changes the spontaneous network activity, we analyzed raster plots of electrical spiking activity and spike rate diagrams (**Figures 3A1–A4,B1–B4**). We revealed significant changes in spontaneous spiking activity 9 days after Hyase treatment. Short network bursts with duration of less than 10 s (**Figures 3D1,D2**) were transformed into seizure-like superbursts of activity with durations of 15–35s (**Figures 3C2,E1,E2**). These changes in the spontaneous spiking activity of Hyase-treated cultures appeared on days 5–7 following the treatment and were observed during the next 4 days.

Statistical analysis revealed that the superbursts (**Figures 3C1–C4**) consist of a large number of network bursts, which are characterized by a low number of spikes per burst and a short interburst interval. Moreover, the general number of network bursts per 10 min increased significantly from day to day after Hyase treatment (19 DIV: 513.3 ± 36; 20 DIV: 712 ± 54; 26 DIV: 827 ± 62, ANOVA; $p < 0.05$ compared to DIV 17; $N = 5$), and this parameter was significantly different between the Hyase and control groups on DIV 26 (Control: 257 ± 21, ANOVA $p < 0.05$; $N = 5$).

In the control group on the 26th DIV, we recorded short superbursts with a frequency of 1.5 ± 0.3 per 10 min and a mean duration of 10.5 ± 0.5 s. After Hyase treatment, we observed long superbursts with a frequency of 3.3 ± 0.5 per 10 min and a mean duration of 36.5 ± 1.4 s (ANOVA; $p < 0.05$; $N = 5$).

Spontaneous Ca^{2+} oscillations appear in dissociated hippocampal cultures beginning on DIV 7 (unpublished observation). On the DIV 17, a large number of neurons have similar patterns of Ca^{2+}oscillations, which are quite distinct from the patterns of Ca^{2+} oscillations in astrocytes (**Figures 4A1,B1**). Both neuronal and astrocytic patterns of Ca^{2+} activity were fairly stable and did not change significantly up to the 26th DIV (**Figures 4A2–A4**). The duration of the neuronal oscillations was approximately 6 s (**Figures 4C,D**) and the duration of the astrocytic Ca^{2+} oscillations was approximately 10 s on DIV 17–26. The Hyase treatment significantly changed the spontaneous Ca^{2+} activity (**Figures 4B2–B4,4C–F**), and already on the second day after Hyase application, we observed the appearance of neuronal Ca^{2+} superoscillations with a duration of 94.78 ± 12.33 s (**Figure 4C**). The superoscillation duration then showed a significant decrease (20 DIV: 50.95 ± 1.25 s; 26 DIV: 28.54 ± 2.71 s; ANOVA $p < 0.001$ for both days compared to the 19th DIV; $N = 5$). Additionally, an increase in the duration of the astrocytic Ca^{2+} oscillations was observed after Hyase application (19 DIV: 16.78 ± 1.21 s; 20 DIV: 18.03 ± 2.48 s; 26 DIV: 17.75 ± 2.53 s; ANOVA; $p < 0.05$ for all days compared to DIV 17; $N = 5$). We also observed that some astrocytes synchronized their Ca^{2+} activity with that of neurons starting from the 2nd day after the Hyase treatment (**Figures 4B2–B4**).

Next, we attempted to block the Hyase-induced superbursts of spiking activity and Ca^{2+} superoscillations in the neuronal and glial networks (**Figures 5** and **6**). Seizure-like elevations in the burst activity of Hyase-treated cultures could be suppressed within 5 min after treatment by the L-VDCC blocker diltiazem (**Figures 5B1,C,D**) but not by the NMDA receptor antagonist CPP (**Figures 5B2,C,D**). An AMPA/kainate receptor antagonist, CNQX, also suppressed superbursts. Neuronal Ca^{2+} superoscillations could also be blocked by diltiazem (**Figure 6B1**, upper curve; **Figure 6C**) but not by CPP (**Figure 6**). Diltiazem also significantly reduced the number of astrocytic Ca^{2+} oscillations after the Hyase treatment (**Figure 6B1**, lower curve; **Figure 6D**).

DISCUSSION

Here, we demonstrated that a Hyase treatment, which results in the removal of HA as the backbone of the neural ECM, leads to a slow development of epileptiform activity in cultured hippocampal neurons. These data provide a new *in vitro* model of epileptogenesis that can be easily induced and studied using MEA technology and optical imaging to characterize the processes underlying epileptogenesis.

Many animal models of epilepsy already exist. The majority involve experimental induction of a brain injury through the administration of a chemoconvulsant or electrical stimulation, both of which induce an episode of seizures. Subsequent injury-induced plasticity includes the appearance of mossy fiber sprouting (Parent and Lowenstein, 1997), an increased rate of neurogenesis in the dentate gyrus (Parent et al., 1997), increased activity of microglia, and an induction of reactivity in astrocytes (Wetherington et al., 2008). In addition to cell death and anatomical changes in neurons and glia, seizures induce significant functional plasticity in surviving hippocampal neurons, including alterations in the function of Ca^{2+}- and hyperpolarization-activated mixed cationic channels (Shah et al., 2004; Jung et al., 2010) and K^+ channels (Bernard et al., 2004), changes in neurotransmitter receptors, including $GABA_A$ receptors (Pathak et al., 2007), glutamate receptors (Doherty and Dingledine, 2001), chloride transporters (Pathak et al., 2007) and excitatory amino acid transporters (Crino et al., 2002; Eid et al., 2008).

Thus, multiple mechanisms could underlie epileptogenesis. Because Hyase treatment leads to a disruption of PNNs around PV-expressing GABAergic interneurons, it is plausible to suggest that one of the causes of Hyase-induced epileptiform activity is an imbalance in excitation and inhibition due to impaired PV interneuron function. These cells represent the most frequent group of inhibitory cells, and they are widely distributed across the brain (Karetko and Skangiel-Kramska, 2009). However, PNN removal with another enzyme, chondroitinase ABC, has been found to reduce the firing threshold of these cells *in vitro* (Dityatev et al., 2007), which would promote rather than deter GABAergic inhibition.

Other possible mechanisms may be related to alterations of the perisynaptic ECM by Hyase. An elegant study by Frischknecht et al. (2009) used Hyase or chondroitinase ABC to remove PNNs from

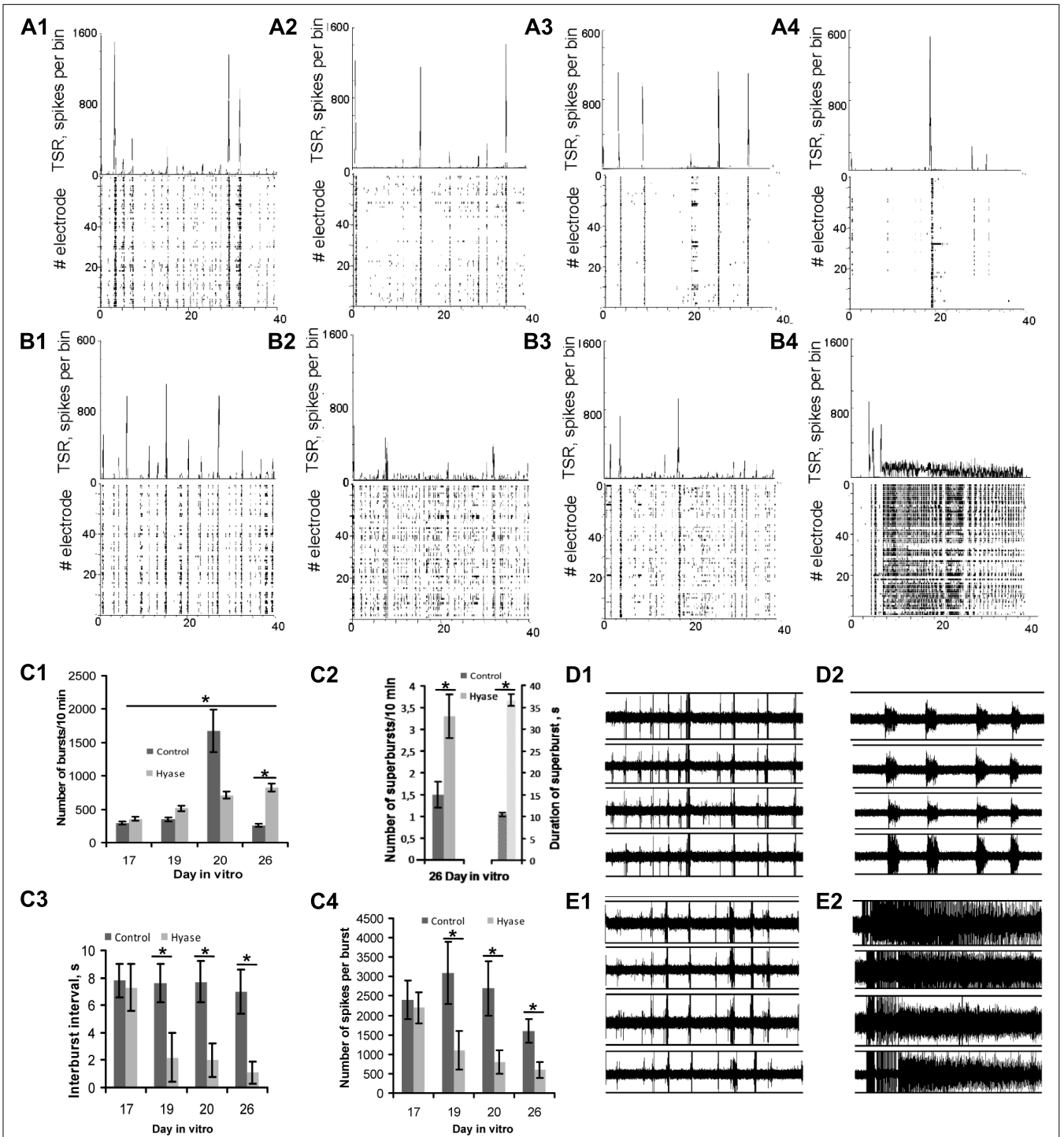

FIGURE 3 | Raster plots of electrical spiking activity over 64 electrodes (lower rows) and total spike rate diagram (upper row) in primary hippocampal cultures (A1–A4,B1–B4). Hippocampal neuronal network activity in intact cultures on the 17th DIV **(A1,B1)**. **(A2,A3)** Neuronal network activity in control cultures on DIV 19 **(A2)**, DIV 20 **(A3)**, and DIV 26 **(A4)**. **(B2–B4)** Development of activity in Hyase-treated cultures 2 days after Hyase treatment on DIV 19 **(B2)**, 3 days after Hyase treatment on DIV 20 **(B3)**, and 9 days after Hyase treatment on DIV 26 **(B4)**. In the latter panel, a seizure-like superburst is shown. **(C1)** Number of network bursts per 10 min after Hyase treatment (ANOVA; $*p < 0.05$; $N = 5$). **(C2)** Number and duration of network superbursts per 10 min on day 9 after Hyase treatment. **(C3)** Interburst interval (s) after Hyase treatment (ANOVA; $*p < 0.05$; $N = 5$). **(C4)** Number of spikes per burst after Hyase treatment (ANOVA; $*p < 0.05$; $N = 5$). **(D1,D2,E1,E2)** Spike recording from 4 electrodes of MED64 probes: **(D1)** intact culture on the 17th DIV; **(D2)** the same culture on the 26th DIV, 9 days after boiled Hyase application; **(E1)** intact culture on DIV 17; **(E2)** the same culture on DIV 26, 9 days after Hyase application. TSR, total number of spikes over all electrodes per 50 ms bin; Hyase, hyaluronidase.

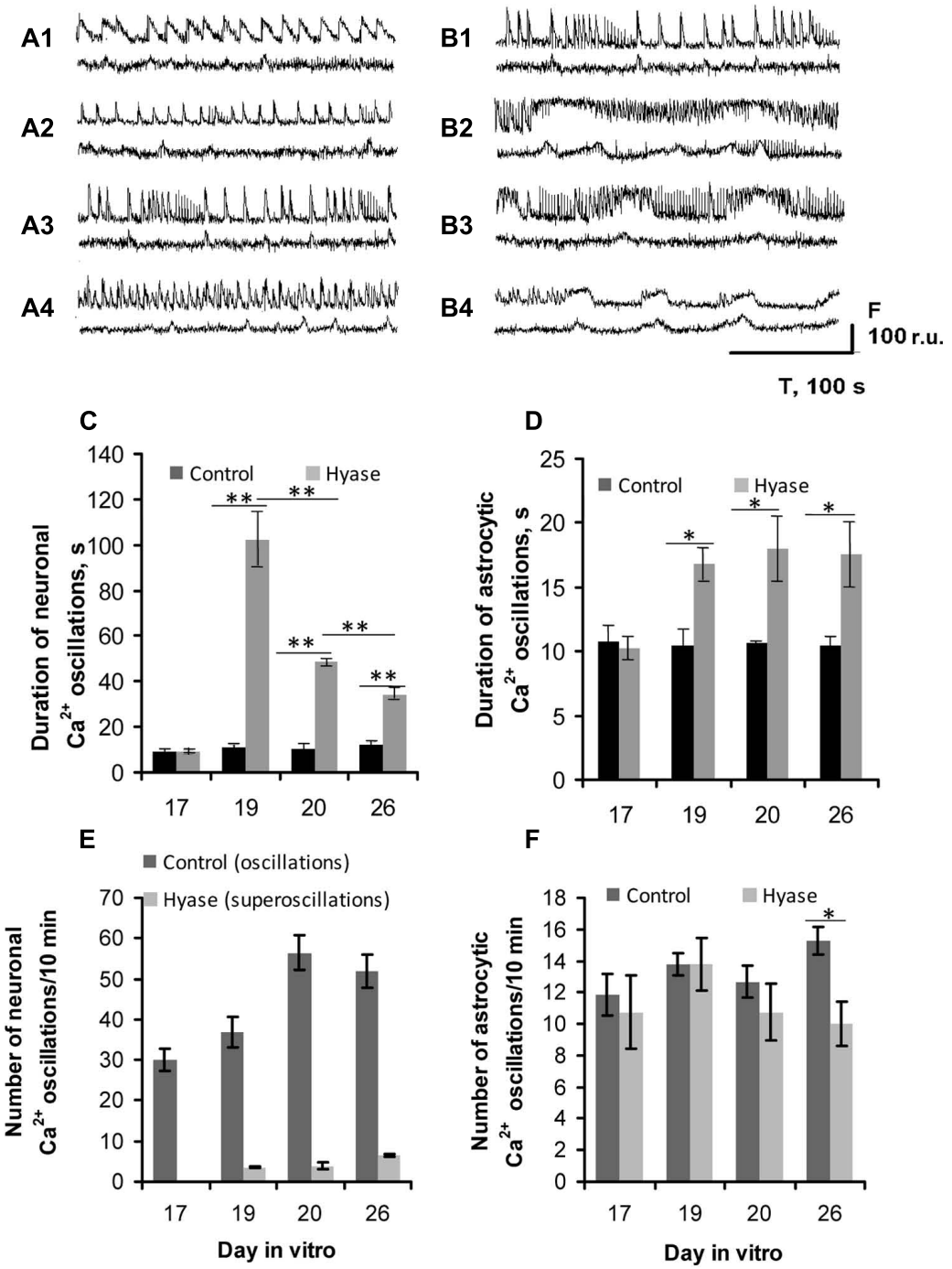

FIGURE 4 | Hyaluronidase-induced Ca^{2+} superoscillations in the neuronal (upper curves) and glial (lower curves) networks in dissociated hippocampal cultures. (A,B) Spontaneous Ca^{2+} oscillation recordings from: **(A1)** DIV 17, intact; **(A2)** DIV 19, control; **(A3)** DIV 20, control; **(A4)** DIV 26, control; **(B1)** DIV 17, intact; **(B2)** DIV 19, 2nd day after Hyase application, showing the appearance of neuronal Ca^{2+} superoscillations with maximal duration; **(B3)** DIV 20, 3rd day after Hyase application, some astrocytic Ca^{2+} transients demonstrate synchronization with the neuronal superoscillations; **(B4)** DIV 26, 9th day after Hyase application, the neuronal Ca^{2+} superoscillations are shorter but the astrocytic oscillations are longer compared to DIV 19. **(C)** Time-course of the changes in the neuronal Ca^{2+} oscillation duration after Hyase application. **(D)** Time-course of the changes in the astrocytic Ca^{2+} oscillation duration after Hyase application. **(E)** Number of neuronal and **(F)** astrocytic Ca^{2+} oscillations after Hyase application. Neuronal Ca^{2+} superoscillations appear 2 days after the Hyase treatment on DIV 19, and there is no such activity in the control cultures. There is no difference between the Control and Hyase groups in the frequency of astrocytic Ca^{2+} oscillations before DIV 26, but they differ on DIV 26 (Control 15.75 ± 0.89, Hyase 10.3 ± 0.9, ANOVA; $p < 0.05$; $N = 5$). The astrocytic Ca^{2+} oscillations after Hyase treatment become less frequent but longer. $^{*}p < 0.05$; $^{**}p < 0.001$ indicate significant differences by ANOVA, $N = 5$.

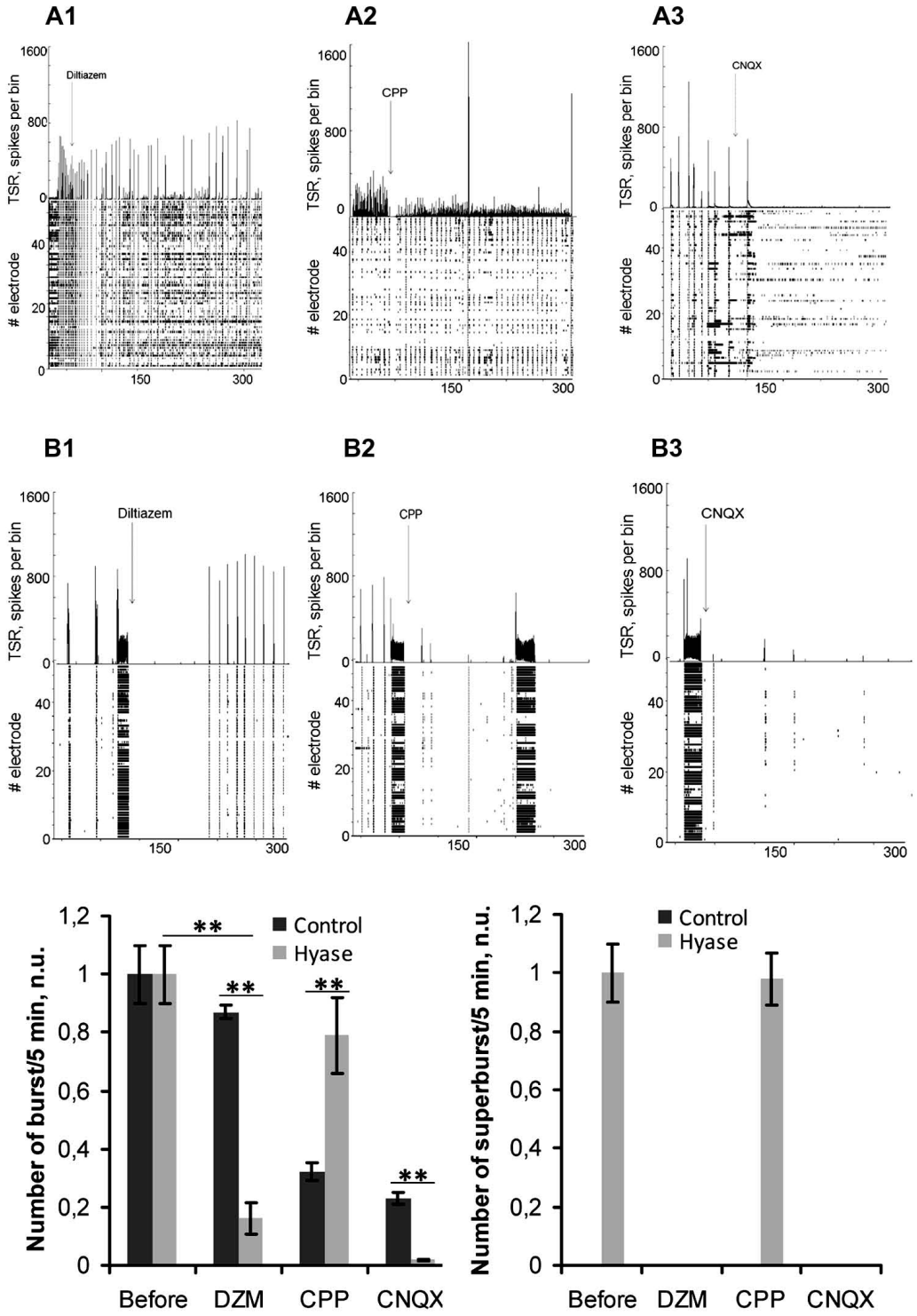

FIGURE 5 | Pharmacological suppression of Hyase-induced superbursts of neuronal spiking activity. (A,B) Spontaneous burst recordings on the 26th DIV, 5 min after blocker application: **(A1)** Control group treated with diltiazem; **(A2)** Control group treated with CPP; **(A3)** Control group treated with CNQX; **(B1)** Hyase group treated with diltiazem; **(B2)** Hyase group treated with CPP; **(B3)** Hyase group treated with CNQX. **(C,D)** Number of bursts and superbursts after blocker application. CPP: (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, 10 μl; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione, 10 μl; Hyase, hyaluronidase; DZM, diltiazem, 10 μM. Mean ± SEM are shown, $^{**}p < 0.001$ indicate significant differences by ANOVA, $N = 5$.

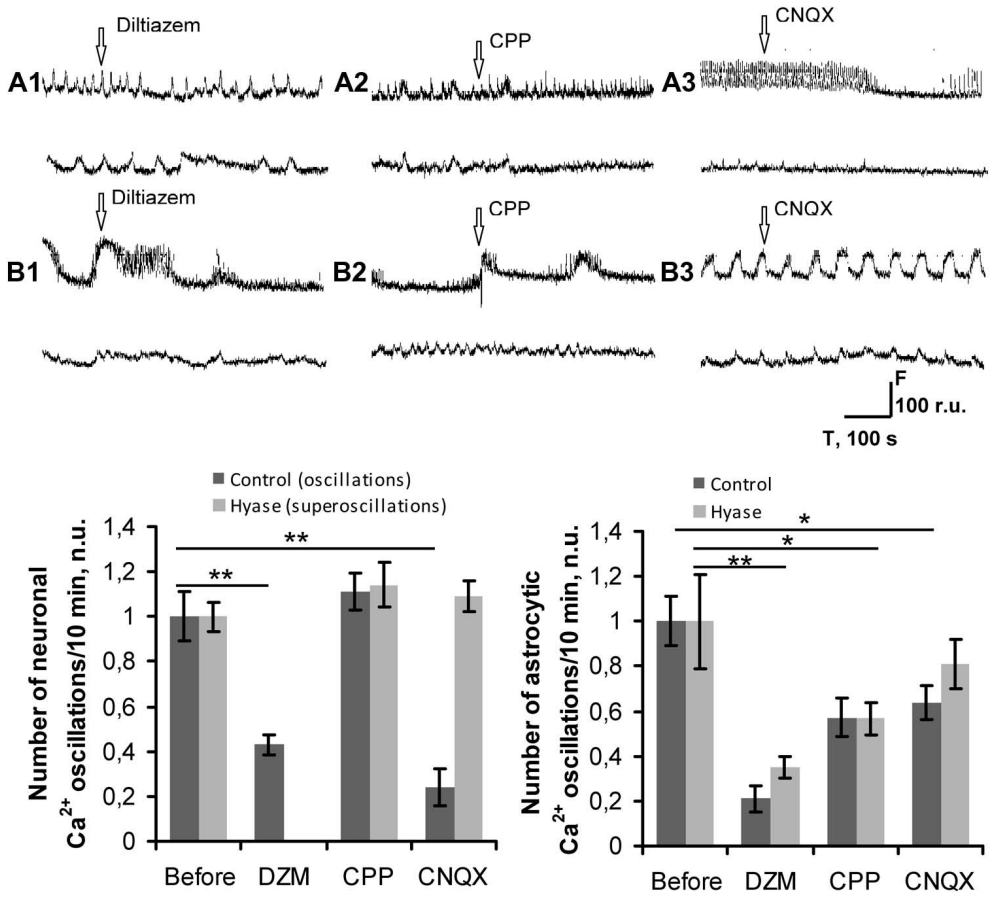

FIGURE 6 | Pharmacological suppression of Hyase-induced long-lasting Ca^{2+} oscillations in neuronal networks. (A,B) Spontaneous Ca^{2+} oscillations recordings on DIV 26, 5 min after blocker application. Upper curves show neuronal transients, lower curves represent astrocytic transients. **(A1)** Control group treated with diltiazem; **(A2)** Control group treated with CPP; **(A3)** Control group treated with CNQX; **(B1)** Hyase group treated with diltiazem; **(B2)** Hyase group treated with CPP; **(B3)** Hyase group treated with CNQX. **(C)** Blockage of L-VDCCs decreases the neuronal Ca^{2+} oscillations in control neurons and decreases both Ca^{2+} oscillations in astrocytes and Ca^{2+} superoscillations in Hyase-treated neurons. **(D)** Number of astrocytic Ca^{2+} oscillations. There is no difference between the control and Hyase-treated groups. CPP, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, 10 μl; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione, 10 μl; Hyase, hyaluronidase; DZM, diltiazem, 10 μM. Mean ± SEM values are shown, $*p < 0.05$; $**p < 0.001$ indicate significant differences by ANOVA, $N = 5$.

cultured rat hippocampal neurons and revealed that a perisynaptic net-like structure in the ECM influences the mobility of AMPA receptors, creating a barrier for their movement into and out of excitatory synapses. ECM removal impairs paired-pulse depression of excitatory postsynaptic currents (EPSCs) in cultures and thus might promote hyperexcitation in cultured pyramidal cells (Frischknecht et al., 2009).

Our MEA recordings revealed that the spontaneous electrical activity of mature dissociated hippocampal neurons grown on MEAs occurs in the form of synchronized burst discharges, but the pattern of these burst discharges is different from the long-lasting "superburst" discharges induced by Hyase treatment. These "superbursts" became most prominent 9 days after the Hyase treatment. Our Ca^{2+} imaging data demonstrates that the Hyase treatment also transformed the normal neuronal Ca^{2+} oscillations into long-lasting "superoscillations" with mean durations of up to 100 s already on the 2nd day after the Hyase treatment. Thus, dramatic changes in Ca^{2+} oscillations precede changes in spiking activity, suggesting that superoscillations may represent a key event leading to changes in the expression of genes that determine neuronal excitability. In addition, changes in Ca^{2+} signaling in astrocytes were detected after the Hyase treatment, although at a smaller scale than in neurons.

Elevations in burst activity and neuronal Ca^{2+} transients in the Hyase-treated neurons and astrocytes was suppressed by an application of the L-VDCC blocker diltiazem, although not by a NMDA receptor antagonist, whereas the superbursts of spiking activity were additionally suppressed by CNQX, an antagonist of AMPA receptors. The latter is not surprising because CNQX mostly blocks excitatory transmission. The elimination of superbursts and superoscillations by diltiazem is at first glance surprising because HA was reported to support L-VDCC activity and the Hyase treatment leads to an acute deficit in neuronal L-VDCC-mediated currents (Kochlamazashvili et al., 2010). It is plausible to assume that this acute deficit in L-VDCC activity might be later overcompensated by overexpression of L-VDCCs after the Hyase

treatment, which might lead to the generation of Ca^{2+} superoscillations and downstream effects on neuronal excitability/excitatory transmission. This hypothesis remains to be verified in follow-up studies. The presented experimental data support the view that the remodeling of ECM may lead to generation of epileptiform activity *in vitro* in the form of superbursts. Such epileptogenic degradation of HA and ECM remodeling may be due to genetic factors or triggered by insults such as stroke or a brain injury. Thus the described experimental model could be potentially valuable for development of new class of broadly applicable anti-epileptogenic drugs, which would target the HA-based neural ECM and/or ECM-mediated signaling.

ACKNOWLEDGMENTS

The research was supported by the Government of Russian Federation grant for Leading Scientists No11.G34.31.0012 and the COST Action BM1001 "Brain Extracellular Matrix in Health and Disease."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 June 2013; accepted: 22 August 2013; published online: 12 September 2013.

Citation: Vedunova M, Sakharnova T, Mitroshina E, Perminova M, Pimashkin A, Zakharov Y, Dityatev A and Mukhina I (2013) Seizure-like activity in hyaluronidase-treated dissociated hippocampal cultures. Front. Cell. Neurosci. 7:149. doi: 10.3389/fncel.2013.00149

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 08 November 2013
doi: 10.3389/fncel.2013.00195

Immune mechanisms in epileptogenesis

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Epilepsy is a chronic brain disorder that affects 1% of the human population worldwide. Immune responses are implicated in seizure induction and the development of epilepsy. Pre-clinical and clinical evidence have accumulated to suggest a positive feedback cycle between brain inflammation and epileptogenesis. Prolonged or recurrent seizures and brain injuries lead to upregulation of proinflammatory cytokines and activated immune responses to further increase seizure susceptibility, promote neuronal excitability, and induce blood–brain barrier breakdown. This review focuses on the potential role of innate and adaptive immune responses in the pathogenesis of epilepsy. Both human studies and animal models that help delineate the contributions of brain inflammation in epileptogenesis will be discussed. We highlight the critical role of brain-resident immune mediators and emphasize the contribution of brain-infiltrating peripheral leukocytes. Additionally, we propose possible immune mechanisms that underlie epileptogenesis. Several proinflammatory pathways are discussed, including the interleukin-1 receptor/toll-like receptor signaling cascade, the pathways activated by damage-associated molecular patterns, and the cyclooxygenase-2/prostaglandin pathway. Finally, development of better therapies that target the key constituents and processes identified in these mechanisms are considered, for instance, engineering antagonizing agents that effectively block these pathways in an antigen-specific manner.

Keywords: seizure, epilepsy, epileptogenesis, immune response, inflammation, microglia, astrocytes, T lymphocytes

INTRODUCTION

Epilepsy is a chronic neurological condition characterized by recurring seizures, and is often accompanied by cognitive deficits and mood disorder (Devinsky, 2004; Pellock, 2004; Jones et al., 2008). It affects approximately 1% of the world population, thus represents one of the most common brain disorders. Epilepsy arises from diverse etiologies including genetic, structural, metabolic, or in other instances, the cause is unknown. There is currently no medication available to effectively prevent epilepsy by targeting the mechanisms underlying the enduring predisposition to recurrent seizures, and nearly half of the patients with epilepsy fail to respond to anticonvulsants that only alleviate symptoms. Thus, there is a pressing need for the development of effective disease-modifying therapies that treat the underlying pathology. Such development can best be accomplished through an in depth understanding of the disease mechanisms.

Until a decade ago, epilepsy research focused on alterations of neuronal activities. Such neurocentric emphasis failed to address questions that arose in more complex models of epileptogenesis. A cumulative body of knowledge has suggested that the pathogenesis of epilepsy is associated with non-neuronal components, such as the glial cells that exceedingly outnumber neurons, brain vascular cells, and more importantly leukocytes from the periphery. Despite a long-held belief that the brain is an immunoprivileged site due to the vascular blood–brain barrier (BBB) that tightly regulates infiltration of blood constituents and the lack of a lymphatic drainage, mounting evidence has supported the critical role of immune responses in the initiation and maintenance of epilepsy (Vezzani and Granata, 2005; Vezzani and Baram, 2007; Choi et al., 2009; Riazi et al., 2010). Ongoing brain inflammation has the potential to lower seizure threshold, which in turn may promote neuronal excitability through modifications of neuronal channels, alterations of neurotransmitter uptake or release, and regulation of BBB permeability (Viviani et al., 2007; Wetherington et al., 2008; Friedman et al., 2009; Vezzani et al., 2011a).

Both innate and adaptive immune responses can be primed in the brain with the contribution of resident immune cells

Abbreviations: ACTH, adrenocorticotrophic hormone; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BBB, blood–brain barrier; CNS, central nervous system; COX-2, cyclooxygenase-2; CSF, cerebrospinal fluid; DAMPs, damage-associated molecular patterns; EEG, electroencephalography; FDA, U.S. food and drug administration; GABA, gamma-aminobutyric acid; GABA-BR, gamma-aminobutyric acid beta receptor; GLuR3, glutamate receptor 3; HMGB1, high mobility group box 1; ICAM-1, intracellular adhesion molecule-1; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL-1β, interleukin-1β; IL-1R/TLR, interleukin-1 receptor/toll-like receptor; IL-6, interleukin-6; IVIG, intravenous gammaglobulin; LPS, lipopolysaccharide; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl D-aspartate receptor; NR2B, NMDA receptor subtype 2B; PAMPs, pathogen-associated molecular patterns; Smad, small mothers against decapentaplegic; TGF-β, transforming growth factor-β; TLE, temporal lobe epilepsy; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VGCC, N-type voltage-gated calcium channel; VGKC LGI1, glycine receptor, voltage gated potassium channel leucine-rich glioma-inactivated 1.

and mediators, as well as leukocytes infiltrating from the periphery (Ransohoff et al., 2003; Banks and Erickson, 2010). The innate arm of the response involves the activation of the IL-1 receptor/toll-like receptor (IL-1R/TLR) signaling pathways through ligation of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), activation of the cyclooxygenase-2 (COX-2) pathway, and initiation of the transforming growth factor-β/small mothers against decapentaplegic (TGF-β/Smad) signaling cascade. Inflammatory mediators produced by the innate immune system remodel the BBB by enhancing its permeability and upregulating leukocyte adhesion molecules on the endothelium, which acts to attract lymphocytes of the adaptive immune system leading to their infiltration into the CNS.

This review focuses on the roles of immune responses in the pathogenesis of epilepsy by summarizing the most recent findings generated from human studies and animal models that help delineate the contributions of brain inflammation in epileptogenesis. We will evaluate the causal relationship between inflammation and seizure activities and the positive feedback loop they establish by revisiting the experimental evidence from *in vivo* and *in vitro* models. Furthermore, we will provide mechanistic insights into the immunological cascade that precedes the establishment of epilepsy and assess the influences of immune mediators apart from the neurological aspect of seizure induction. Finally, we will propose potential mechanisms that underlie epileptogenesis and discuss development of therapies targeting the key constituents and processes identified in these mechanisms.

PRE-CLINICAL DATA UNDERSCORING THE RELEVANCE OF IMMUNE RESPONSES IN EPILEPSY

Rodent models of epilepsy have provided ample evidence supporting the role of immune responses in the precipitation of epilepsy, modulation of seizure threshold, orchestration of seizure recurrence, regulation of brain cell survival or attrition, and rewiring of neuronal circuits that may lead to establishment of hyperexcitable neuronal networks (Dube et al., 2005; Kulkarni and Dhir, 2009; Riazi et al., 2010; Vezzani et al., 2011a,b). Adult and immature rats and mice are frequently used to elucidate the role of various immunological pathways that are potentially involved in seizure generation. Administration of proinflammatory or anti-inflammatory agents to rats and mice has been used to assess the influence of these immune mediators on latency to onset, frequency, duration, and phenotype of provoked seizures. Furthermore, the inflammatory pathways can be blocked pharmacologically in wildtype animals or manipulated in transgenic mice to evaluate their role in seizure severity (Kulkarni and Dhir, 2009; Maroso et al., 2010; Vezzani et al., 2011a). Additionally, the availability of genetically modified mice with impaired or constitutively hyperactive immunoregulatory pathways enables more detailed mechanistic studies of inflammation-related epileptogenesis (Campbell et al., 1993; Probert et al., 1997). Guinea pigs models, though less common, have also been used to elucidate the contribution of peripheral immune cells to seizure induction (Librizzi et al., 2010).

RESIDENT IMMUNE MEDIATORS OF THE BRAIN

It has long been appreciated that chemically induced or electrically stimulated prolonged seizures in rats and mice lead to induction of robust immune responses in the seizure-laden brain (Minami et al., 1991; Eriksson et al., 2000; De Simoni et al., 2000; Vezzani et al., 2000; Turrin and Rivest, 2004; Voutsinos-Porche et al., 2004; Gorter et al., 2006; Jung et al., 2006; Yoshikawa et al., 2006; Aronica et al., 2007; Dhote et al., 2007; Lee et al., 2007; Kulkarni and Dhir, 2009; Holtman et al., 2010; Polascheck et al., 2010). Immunohistochemical analyses performed on sections of affected brains have revealed that various cell type, including microglia, astrocytes, neurons, ependymal cells in the ventricles, and endothelial cells of the BBB, were involved in the waves of inflammation associated with seizure induction (Takao et al., 1990; Ban et al., 1991; Turrin and Rivest, 2004; Chakravarty and Herkenham, 2005; Ravizza and Vezzani, 2006). Activation of the IL-1R/TLR inflammatory pathway was among the first to be identified in these brain resident immune cells. Constitutive expression of IL-1R and TLR has been detected in the brain, though at a suboptimal level. Upon stimulation, such as viral or bacterial infections, cellular injuries, ischemia, and seizures, upregulation of these receptors is readily detectable (Ericsson et al., 1995; Allan et al., 2005; Peltier et al., 2010; Zurolo et al., 2011). Activation of TLRs, particularly TLR2 and TLR4, through systemic or cortical delivery of lipopolysaccharide (LPS) to rats, results in rapid changes in neuronal excitability, e.g., alteration in synaptic transmission and regulation of long-term potentiation (Bellinger et al., 1993; Plata-Salaman and Ffrench-Mullen, 1994; Schneider et al., 1998; Rodgers et al., 2009). The excitatory effect of IL-1β has also been reported to be associated with the reduction of gamma-aminobutyric acid (GABA) inhibition and reduced outward current of voltage-gated Ca^{2+} channels in the hippocampus (Zeise et al., 1997; Wang et al., 2000; Viviani et al., 2007; Schafers and Sorkin, 2008). The role of IL-1β in seizure sensitivity was further supported by an *in vitro* system that demonstrated that *N*-methyl-D-aspartate (NMDA)-mediated Ca^{2+} influx is enhanced by activation of Src-dependent NMDA receptor subtype 2B (NR2B) phosphorylation (Viviani et al., 2003). In addition to PAMPs, DAMPs have been recently shown to stimulate TLRs, especially TLR4, to exacerbate seizures. The endogenous signals of DAMPs, such as high mobility group box 1 (HMGB1), can be released by stressed neurons or activated microglia and astrocytes, suggesting that LPS may have mimicked the endogenous DAMP pathway to cause seizure (Maroso et al., 2010). Astrocytes can also enhance seizure induction through increased release of glutamate when the IL-1R/TLR pathway is activated (Wetherington et al., 2008; Friedman et al., 2009).

After the activation of the IL-1R/TLR pathway in the glial population, upregulation of COX-2 and prostaglandins in neurons often ensues (Yamagata et al., 1993; Rozovsky et al., 1994; Yoshikawa et al., 2006; Kulkarni and Dhir, 2009). Inhibition of COX-2 activation prior to seizure induction results in increased mortality and exacerbated seizures behaviors in mice (Baik et al., 1999; Toscano et al., 2008). In contrast, ablation of COX-2 production after seizure induction has been shown to be neuroprotective and resulted in decreased production of inflammatory cytokines by glia and prevented leakage of BBB in a conditional

COX-2 knockout mouse strain (Serrano et al., 2011). However, constitutive inhibition of COX-2 failed to prevent the recurrent of unprovoked seizures (Holtman et al., 2010). Thus, it remains unclear whether induction of COX-2 in neurons leads to enhanced epileptogenesis.

After the induction of status epilepticus, serum albumin is detected in the brain suggesting that in BBB failure may contribute to the development of epilepsy (van Vliet et al., 2007). Extravasation of albumin into the cerebral cortex as a result of compromised BBB leads to activation of the TGF-β signaling pathway in astrocytes, and hence increases local inflammation. Such inflammatory responses in the brain parenchyma would most likely induce another wave of neuronal hyperactivation and attrition, which leads to excretion of danger signals, such as DAMPs that would further activate glia to boost inflammation. Thus, a positive feedback loop involves seizure, glia, neurons, and immune responses in the brain.

INFILTRATING IMMUNE MEDIATORS OF THE BRAIN

It has long been appreciated that prolonged seizures lead to upregulation of adhesion molecules on brain endothelial cells to facilitate extravasation of circulating leukocytes. Expression of E-selectin, P-selectin, intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) are increased on the endothelial cells of the brain (Bell and Perry, 1995; Librizzi et al., 2007). The ligands of these molecules, integrins and mucins, are expressed by circulating leukocytes after seizure and facilitate rolling and tethering of granulocytes and lymphocytes (Fabene et al., 2008). Blockade of α4β1 integrins on leukocytes inhibits infiltration of this cell population into the brain, and therapeutic inhibition of α4 integrin activation prevents induction of seizure, and even development of epilepsy (Fabene et al., 2008).

CLINICAL EVIDENCE OF THE INVOLVEMENT OF IMMUNE RESPONSES IN EPILEPSY

ANTI-INFLAMMATORY THERAPIES THAT ARE EFFECTIVE AT TREATING EPILEPSY

The efficacy of anti-inflammatory medications, such as corticosteroids and adrenocorticotrophic hormone (ACTH), in the treatment of some pediatric epilepsies that do not respond to conventional anticonvulsants was one of the first lines of clinical evidence that epilepsy has an immune inflammatory component (Hrachovy et al., 1983; Mackay et al., 2004). It has been shown that ACTH had superior efficacy in the cessation of spasms, improved developmental prognosis, and normalization of electroencephalography (EEG; Snead et al., 1983; Baram et al., 1996; Kivity et al., 2004). In the treatment of refractory epileptic encephalopathies, such as West syndrome, Ohtahara syndrome, Dravet syndrome, Lennox-Gastaut syndrome, Landau-Kleffner syndrome, epilepsy with continuous spike waves during slow-wave sleep and drug-resistant myoclonic atonic epilepsy, a significant proportion of ACTH- and steroid-treated pediatric patients were reported to be seizure-free for an extended period of time, albeit relapsed over time (Yamatogi et al., 1979; Snead et al., 1983; Donat, 1992; Engel, 2001).

In addition to, the corticosteroid therapies, intravenous gammaglobulin (IVIG) has been considered as another potential treatment for refractory epilepsy (Eibl and Wedgwood, 1989). The mechanisms of IVIG induced immunomodulation include suppression of proinflammatory cytokines, interference with antibody-dependent cytotoxicity through Fc receptor blockade, dampening innate immune responses by inhibition of phagocytosis by antigen presenting cells and complement uptake, as well as neutralization of autoantibodies. IgG has been reported to be readily detected in the cerebrospinal fluid (CSF) after a single dose of IVIG in neuromuscular disorders, suggesting that IVIG is capable of crossing the BBB (Cutler et al., 1970; Sekul et al., 1994; Dalakas, 1998). Furthermore, the compromised BBB in many types of epilepsy might further facilitate delivery of IVIG into the brain to exert local immuno- and neuro-modulating effects for seizure alleviation. In a double-blind clinical trial, seven doses of IVIG was administered over a time period of 6 weeks, and more than 50% of the patients in the treatment group had a significant reduction of seizures (van Rijckevorsel-Harmant et al., 1994). Similar effects of seizure reduction and temporary EEG normalization were observed in another trial using pediatric patients (Hart et al., 1994). The use of IVIG in intractable epilepsy and status epilepticus merits further investigation as consistent efficacious outcome has not been achieved and the dosing regimen remains to be optimized.

Blockade of cell-adhesion molecules involved in lymphocyte trafficking has also shown promise in ameliorating seizure severity in epilepsy. Natalizumab, an FDA approved humanized antibody specific to a homing molecule (α4β1 integrin) that directs lymphocyte migration to inflamed tissues, including the brain, has been shown to significantly reduce generalized seizures and status epilepticus in adult patients who also suffered from a autoimmune demyelinating disease, multiple sclerosis (Ley et al., 2007; Sotgiu et al., 2010; Fabene et al., 2013)

EPILEPTOGENIC TRIGGER WITH AN INTRINSIC INFLAMMATORY NATURE

Febrile status epilepticus is intrinsically associated with immune responses. Genetic susceptibility to inflammation, though not an obligatory factor, has been suggested to lower the seizure threshold, as nearly 30% of febrile seizure patients have such a family history. In addition, mutations in the IL-1β gene segment predispose patients to prolonged febrile convulsions (Millichap, 1959; Virta et al., 2002; Kanemoto et al., 2003). An elevation in a number of proinflammatory cytokines caused by neurotropic viral infections, for instance, human herpesvirus-6 and influenza viruses, is also commonly associated with febrile seizures in infants and young children (Hall et al., 1994; Chiu et al., 2001). Detection of viral DNA is more frequent in the CSF of patients with repetitive febrile seizures than in those patients with a single seizure (Kondo et al., 1993). Increased levels of Th1 and Th2 cytokines, such as interferon-γ (IFN-γ) and interleukin-6 (IL-6), have been reported in influenza-infected patients who later developed febrile seizures, when compared with the virally infected control subjects without seizures (Chiu et al., 2001; Masuyama et al., 2002; Kawada et al., 2003).

Rasmussen's encephalitis, a prototypic childhood inflammatory epilepsy, is a progressive immune-mediated brain disorder characterized by focal recurrent seizures (epilepsia partialis

continua), unilateral hemispheric atrophy, progressive neurological dysfunction and intractable epilepsy. Rasmussen encephalitis is associated with T-cell activation and production of proinflammatory cytokines by activated glia. Effector $CD8^+$ cytotoxic T lymphocytes are proposed to induce astrocytic and neuronal apoptosis and degeneration, one of the hallmarks of Rasmussen's encephalitis (Bauer et al., 2007). An autoantigen, a glutamate receptor, GluR3, has been detected in this disease. Removal of the GluR3-specific antibody from the circulation has been shown to ameliorate seizure severity, promote neurological functions, and improve the disease prognosis. However, anti-GluR3 antibody is not present in most cases and immune modulation including IVIG, steroid and plasmapheresis has limited efficacy (Rogers et al., 1994; He et al., 1998; Mantegazza et al., 2002; Watson et al., 2004). Hemispherectomy remains the only "cure" of disease progression.

In recent years, an increasing number of autoantibodies have been detected in serum and CSF of patients with new onset drug-resistant focal epilepsy. A definitive diagnosis and therapy have thus been possible for cases that would have been previously categorized as viral or idiopathic encephalitis/epilepsies. The California Encephalitis Project found that the frequency of autoimmune encephalitis was greater than any single viral etiology. Anti-*N*-methyl D-aspartate receptor (NMDAR) encephalitis was, in fact, the most frequent cause of immune-mediated encephalitis (Armangue et al., 2013). Autoimmune/inflammatory epilepsy is defined as immunologically mediated disorder where recurrent seizures are prominent feature and immune etiology is suggested by detection of neuronal antibodies, presence of inflammatory changes in CSF or upon MRI, or immunotherapy-responsive symptoms and exclusion of other etiologies. Autoimmune/inflammatory epilepsies include limbic encephalitis (both paraneoplastic and non-paraneoplastic), non-limbic encephalitis complicated by seizures, seizures in the context of autoimmune disease, and neural antibody-mediated CNS disorders where seizures are a significant feature. Disease causing autoantibodies have been detected against the following specific neuronal surface proteins: NMDAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), γ-aminobutyric acid beta receptor (GABA-BR), glycine receptor, voltage gated potassium channel leucine-rich glioma-inactivated 1 (VGKC LGI1), VGKC CASPR2 and P/Q or N-type voltage-gated calcium channel (VGCC; Armangue et al., 2012).

The paraneoplastic type is thought to be caused by self-reactive T lymphocytes, although antibodies specific for intracellular components are often detectable (Dalmau and Rosenfeld, 2008). The non-paraneoplastic category is consistently associated with seizure-inducing autoantibodies that target extracellular membrane components, including voltage-gated channels, NMDAR, and glutamic acid decarboxylase (Bien et al., 2007; Dalmau et al., 2008; Vincent and Bien, 2008; Dalmau, 2009; Vincent et al., 2010). These antibodies cause seizures by modifying neuronal excitability, which has been recapitulated in an *in vitro* model by measuring hippocampal neuronal firing frequency following autoantibody treatment (Vianello et al., 2008). Due to its autoimmune nature, limbic encephalitis responds to immunotherapies, including steroids and IVIG through the mechanisms of dampening T-cell immunity and neutralizing autoantibodies (Dalmau, 2009). Another type of autoimmune epilepsy that also responds to corticosteroid treatments is Hashimoto's encephalopathy. Self-reactive antibodies in this syndrome target thyroid peroxidase or thyroglobulin, and are very potent in seizure induction (Castillo et al., 2006; Watemberg et al., 2006).

DETECTION OF IMMUNE MEDIATORS IN THE BRAIN OF PATIENTS WITH EPILEPSY

Leukocyte accumulation in the perivascular space, and occasionally the parenchyma, of epileptic brain in pediatric as well as adult patients provide another line of evidence that immune cell invasion of the central nervous system (CNS) may be critical in orchestrating epileptogenesis. $CD3^+$ lymphocytes and myeloid-derived macrophages have been detected in resected brain samples of patients diagnosed with temporal lobe epilepsy (TLE; Ravizza et al., 2008). A subset of $CD3^+$ cells, cytotoxic $CD8^+$ T lymphocytes, has also been found in the gray and white matter of epileptic patients with tuberous sclerosis complex (Boer et al., 2008). Brain-infiltrating granulocytes and T cells have been additionally demonstrated in epilepsy of diverse etiologies (Fabene et al., 2008). In line with the above findings, our laboratory and others have detected a myriad of immune-related genes that are upregulated in surgical specimens from patients with intractable TLE using a global survey of changes in gene expression. These immune-related genes include chemokines, complement components, metalloproteinases and their inhibitors, adhesion molecules, immune receptors such as MHC molecules and Fc receptors, and heat shock proteins (Aronica and Gorter, 2007; van Gassen et al., 2008).

PROPOSED IMMUNE MECHANISMS OF EPILEPTOGENESIS

Understanding the immune mechanisms underlying epileptogenesis provide insights into the development of more effective target-specific immunotherapies rather than general treatments that non-specifically suppress or regulate the immune responses. Ample experimental and clinical evidence has suggested that inflammation in the brain is likely to predispose, precipitate, and perpetuate epileptogenesis, however, protective immune responses that promote neuronal repair and restores homeostasis also exist in epilepsy as well as other immune-mediated neurological disorders, such as multiple sclerosis, Parkinson's disease, and Alzheimer's neurodegeneration. Thus, whether the immune response detected during the initiation and development of epilepsy is always deleterious to the survival of brain cells or perhaps may also mediate neuroprotective functions merits further in-depth investigation.

The current dominant view of immune-mediated epileptogenesis entails contributions from both brain-resident cells capable of innate immune responses as well as peripherally derived infiltrating innate and adaptive immune effector cells. The pathological triggering events that are initiated in the brain or the periphery for a variety of reasons, such as simple febrile seizures, trauma, stroke or infection, may lead to an inflammatory cascade. Activation of glia, neurons, and endothelial cells that constitute the BBB most likely result in the release proinflammatory cytokines, such as IL-1β and TNF-α, and danger signals, such as HMGB1.

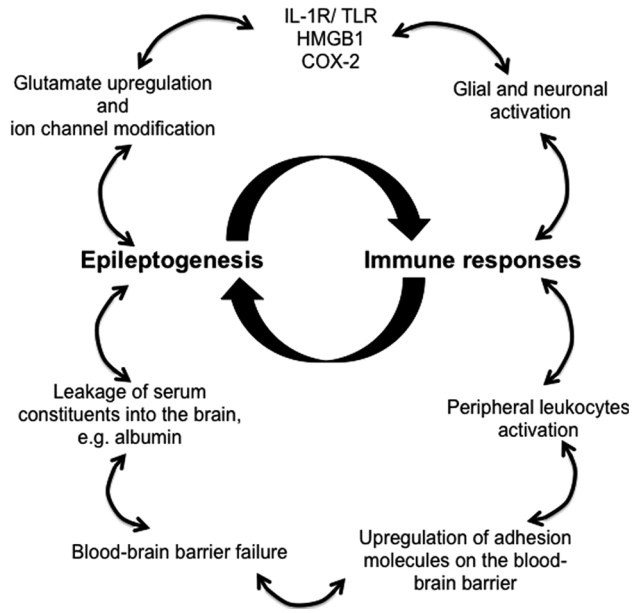

FIGURE 1 | Proposed immune mechanisms in epileptogenesis. The relationship between the immune system and the development of epilepsy is non-linear, but rather better represented as an amplifying feedback loop. The immune response predisposes, precipitates and perpetuates epileptogenesis through activation of resident brain cells (glia and neurons) and facilitation of peripheral leukocyte infiltration. These immune mediators release proinflammatory cytokines and chemokines into the brain parenchyma and the blood, thereby activating downstream signaling cascades and compromising blood–brain barrier, which leads to pathophysiological outcomes, reoccurrence of seizures, and ultimately the development of epilepsy.

These factors activate cognate pathways in neurons to cause an intracellular calcium ion surge, which results in modification of voltage-dependent ion channels. Dysregulated ion channels directly enhance the neuronal hyperexcitability and reduce seizure threshold. In addition, proinflammatory cytokines also stimulate chronic release of neuroexcitatory transmitters, inhibit uptake of these neurotransmitters by the glial population, and restrict the recycling of GABA receptors (Hu et al., 2000; Bezzi et al., 2001; Stellwagen et al., 2005; Ferguson et al., 2008). COX-2 and prostaglandin can also be involved in such a process that remodels the neuronal network by mobilizing intracellular calcium storage and an increase in cAMP production.

The inflammatory milieu and the neuronal hypersynchronization in the CNS are often accompanied by BBB leakage, which introduces tightly regulated blood components, such as albumin and potassium ion, into the brain (Seiffert et al., 2004; Oby and Janigro, 2006; Aronica et al., 2007; Ivens et al., 2007; Shlosberg et al., 2010). Increased leukocyte adhesion to the endothelial cells further modifies the BBB through cytoskeletal organization, which results in enhanced leukocyte infiltration into the brain (Greenwood et al., 2002). Upon entering the brain, activated peripheral immune cells are capable of generating free radicals, releasing additional chemokines, cytokines, nitric oxide, and cytotoxic enzymes to establish a self-amplifying cascade to further precipitate epileptogenesis.

CONCLUSION

Animal models and clinical evidence highlight the involvement of CNS resident and peripherally derived infiltrating immune mediators in seizure induction and epilepsy development (Vezzani et al., 2011a). Robust immune responses in the brain decrease seizure threshold, enhance neuronal excitability, induce BBB failure, promote synaptic reorganization, and regulate epileptogenesis (**Figure 1**). Despite the appreciation of the critical role of immunity in epileptogenesis and the advancements made in the recent years in understanding the immunological mechanisms underlying epilepsy, novel diagnostic measures and effective therapeutic treatments that targets immunological pathways are still lacking.

In addition to, the glial populations and neurons, which are the major brain-resident immune mediators in epilepsy, peripheral leukocytes that infiltrate the brain are also being investigated for their contribution to epileptogenesis as a result of a compromised BBB, for instance, macrophages, monocytes, dendritic cells, αβ T lymphocytes, γδ T lymphocytes, and regulatory cells. Several inflammatory signaling pathways have been identified which initiate immune responses involving the aforementioned immune mediators. Activation of the IL-1R/TLR pathway may be due to brain injury or infection, but could also be caused by DAMPs, such as HMGB1. Furthermore, COX-2 induced production of prostaglandins is capable of triggering brain inflammation (Fabene et al., 2008; Serrano et al., 2011). Therefore, pharmacological blockade of these signaling pathways and inhibitors that antagonizing the main immune mediators have the potential of becoming the next generation of effective anti-epileptic treatment.

ACKNOWLEDGMENTS

The work was supported by NIH/NINDS R01NS073768 to Sookyong Koh and Stephen D. Miller, as well as a post-doctoral fellowship 262243 from the Epilepsy Foundation of America to Dan Xu.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 June 2013; accepted: 08 October 2013; published online: 08 November 2013.

Citation: Xu D, Miller SD and Koh S (2013) Immune mechanisms in epileptogenesis. Front. Cell. Neurosci. 7:195. doi: 10.3389/fncel.2013.00195

This article was submitted to the journal Frontiers in Cellular Neuroscience.