GLIAL DYSFUNCTION IN EPILEPTOGENESIS

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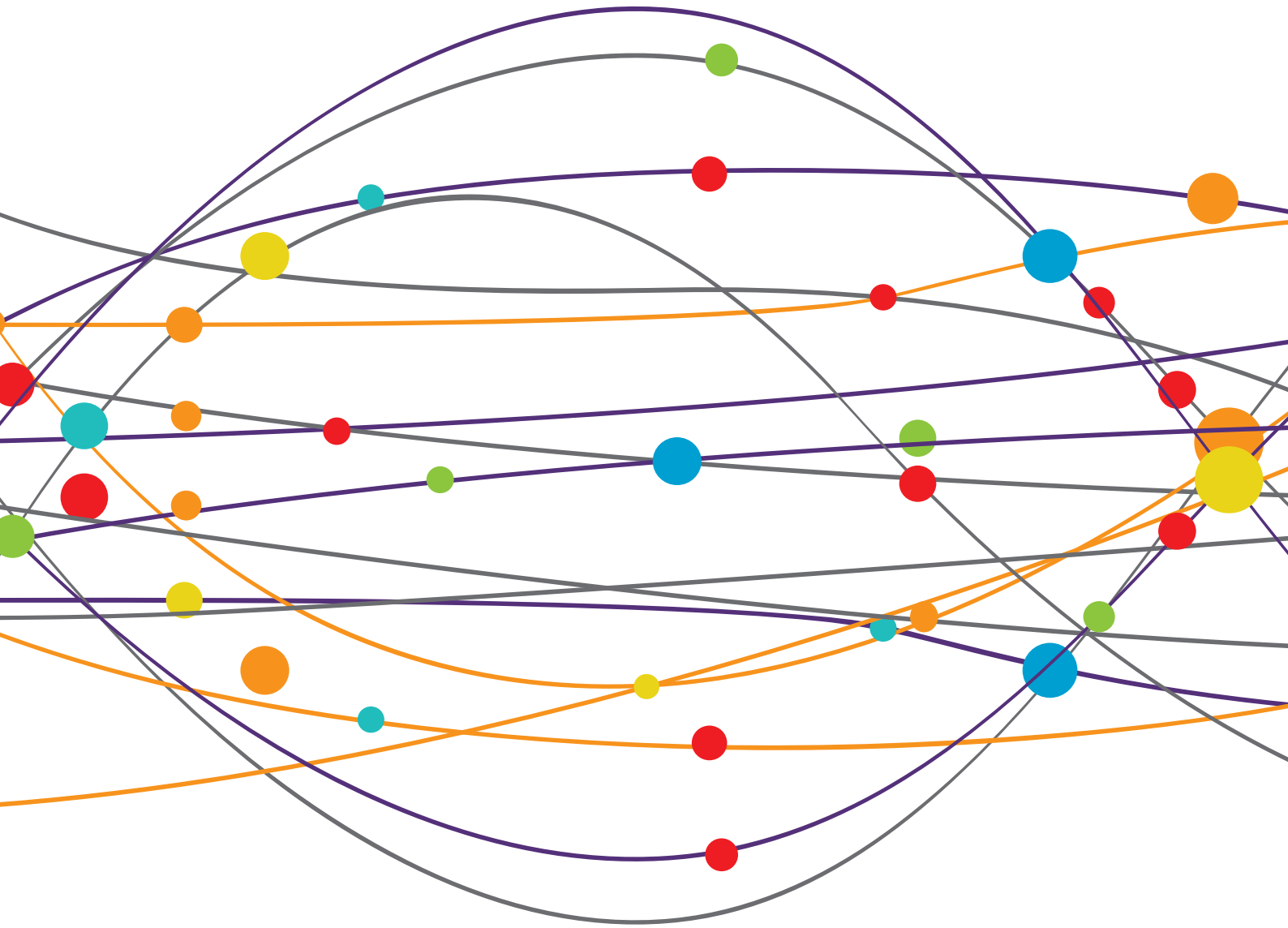

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GLIAL DYSFUNCTION IN EPILEPTOGENESIS

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EDITORIAL
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Editorial: Glial Dysfunction in Epileptogenesis

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Editorial on the Research Topic

Glial Dysfunction in Epileptogenesis

Epilepsy is one of the most common neurological disorders affecting around 1% of the world's population (1). Epilepsy research has so far mainly focused on how to inhibit epileptic discharges resulting in different symptomatic treatment options targeted at neurons.

Despite the introduction of more than 20 novel anti-seizure medications since the early 1990s, the proportion of people with drug-refractory epilepsy has remained notably stable at ∼30% (2).

Given the expanding spectrum of important functions ascribed to the non-neuronal constituents of the brain, we can now observe a paradigm shift where glial cells are included in the equation of epilepsy pathogenesis. With our novel understanding of glial cells as central organizers of homeostatic functions and as major contributors to inflammation and brain excitability, we are approaching novel curative treatment strategies for epilepsy. In the future, targeting dysfunctional and/or reactive glia or glia-mediated inflammatory processes may thus prevent initiation and progression of epilepsy, and yield true anti-epileptogenic medications.

In this special issue (SI) of Frontiers in Neurology, we brought together experts in this new area of epilepsy research, and provide a balanced collection of seven original studies and eight review articles.

The eBook both starts and ends with reviews of one of the core homeostatic functions of astrocytes, which is glutamate handling in the central nervous system. Glutamate clearance is highly relevant for epilepsy pathogenesis as excess glutamate directly could trigger neuronal discharges and epileptic activity. The first article, presented by Peterson and Binder (USA), provides a systematic overview of the functional components of astrocyte glutamate control. Astrocytes express both glutamate transporters (GLT-1 and GLAST in rodents/EAAT 1 and 2 in humans), as well as metabotropic glutamate receptors (mGluR)3 and mGluR5. Peterson and Binder demonstrate evidence for dysregulation of these channels across patients with epilepsy and preclinical seizure models.

After uptake, astrocytes conduct the intracellular metabolization of glutamate and ammonia to glutamine. Sandhu et al. from the group of Tore Eid (USA) highlight the importance of the enzyme glutamine synthetase (GS), and provide evidence for the association of astrocytic GS deficiency or dysfunction in discrete brain regions in several types of epilepsy, including mesial temporal lobe epilepsy, neocortical epilepsies, and glioblastoma-associated epilepsy. These findings are reinforced by several studies using experimental inhibition or deletion of GS in specific brain regions, and by this mimic different human epilepsy forms including their comorbidities.

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Alcoreza et al. from the group of Harald Sontheimer (USA) provide information on astrocytic glutamate receptor dysregulation in epilepsy, presenting yet another view of the delicate regulatory processes of glutamate homeostasis. They also highlight the importance of extracellular space volume alterations and dysregulation of the water channel aquaporin-4 as integral parts of epilepsy pathophysiology and discuss evidence for upregulation of system x–c, a cystine/glutamate antiporter expressed by astrocytes in epileptic tissue.

Kinboshi et al. (Japan) focuses on another important homeostatic function of astrocytes, which is K^+ spatial buffering. This process mainly depends on inwardly rectifying potassium (Kir) 4.1 channels and gap junction coupling. Astrocytes rapidly transport K^+ from areas of high neuronal activity, where $[K^+]_{EC}$ increases, to regions with lower K^+ levels via the astrocyte network through gap junctions. This K^+ clearance mechanism is not only critical for maintaining K^+ homeostasis and preventing neural hyperexcitability but is also linked to glutamate uptake during normal brain function. There is ample evidence for Kir4.1 dysfunction in epilepsy and recent studies indicate that inhibition of Kir4.1 channels facilitates the expression of brain-derived neurotrophic factor (BDNF), an important modulator of epileptogenesis in astrocytes.

Verhoog et al. from the group of Erwin van Vliet (The Netherlands) enriches this eBook with a substantial review of state-of-the-art literature on dysfunctional astrocytes in epilepsy, also including the role astrocyte Ca^{2+} signaling, altered blood brain barrier (BBB) function and blood flow regulation.

The series of contributions to this eBook on glia-derived inflammation in epilepsy could not have a better opening than with an original study on febrile seizures, provided by Brennan et al. from the group of Tallie Baram (USA). The potential role of aberrant microglia and astrocyte function during epileptogenesis is important because the involved mediators provide targets for intervention and prevention of epilepsy. By performing experimental febrile status epilepticus in rat pups, the authors elicited a strong inflammatory response leading to a rapid and sustained upregulation of pro-inflammatory cytokines. In the attempt to curb epileptogenesis, several pathways involving cytokines, microRNAs, high mobility group B-1 (HMGB1) and prostaglandin E2 signaling were targeted by using network-specific interventions as well as global anti-inflammatory approaches. The failure of selectively decreasing the expression of downstream inflammatory cascades, and the emergence of intolerable side effects, illustrates that the intricate, cell-specific and homeostatic interplays among these networks constitute a serious challenge to tailored interventions that aim to prevent epileptogenesis.

Another approach to curb epileptogenesis is presented in the study of Wyatt-Johnson et al. (USA). Microglial survival and proliferation are regulated by the colony-stimulating factor 1 receptor (CSF1R). The authors used the CSF1R inhibitor PLX3397 in a rat pilocarpine model of status epilepticus (SE). This led to suppression of microgliosis and hippocampal astrogliosis but did not improve or worsen the memory deficits in these animals.

Neuroinflammation is not only an integral part of TLE pathogenesis, but also regarded as a hallmark of traumatic brain injury (TBI) and subsequent post-traumatic epilepsy (PTE). Sun et al. (China) presents an organized review focusing particularly on glial cell activation, peripheral leukocyte infiltration, inflammatory cytokine release and BBB disruption in PTE.

BBB disruption is a hallmark of many pathological brain insults and has been associated with the development and progression of focal epilepsy, although the underlying molecular mechanisms are not fully understood. A proposed mechanism is the activation of transforming growth factor beta (TGFβ) signaling in astrocytes by extravasated albumin, impairing the ability of astrocytes to properly interact with neurons and eventually leading to epileptiform activity. Henning et al. (Germany) used the unilateral intracortical kainate mouse model of TLE with hippocampal sclerosis (HS) and revealed pronounced albumin extravasation already 4 h after SE induction. Inhibition of the TGFβ pathway by the specific TGFβ receptor 1 (TGFβR1) kinase inhibitor IPW-5371 slightly attenuated acute and chronic epileptiform activity but did not reduce the extent of HS or affect astrocytic gap junction coupling, which is thought to play a role in TLE-HS epileptogenesis (3). The same group, with Müller et al. (Germany) as first author, presents another original study employing the same mouse model. Accompanied by loss of GABAergic interneurons and/or synaptic inhibition, as shown in various epilepsy models and in human epilepsy, they found a pronounced GABA accumulation in reactive astrocytes of the sclerotic mouse hippocampus.

Together, their data provide evidence that the preserved tonic inhibitory currents in the epileptic brain are mediated by GABA overproduction and release from astrocytes, adding another potential target for antiepileptogenic drug therapy.

Vila Verde et al. (Italy) contribute with an elegant approach using the guinea pig epilepsy model (4) and, for the first time, provide evidence that seizures *per se* induce IL-1β biosynthesis in astrocytes, increased BBB permeability, and morphological changes typically observed in activated glial cells, in the absence of blood borne inflammatory molecules and leukocytes. They further found that serum albumin extravasation into the brain parenchyma exacerbates neuronal hyperexcitability by inducing astrocytic and microglial activation.

Ahmed et al. from the group of Brooks-Kayal (USA) provide one out of 2 "omics"-contributions investigating upstream effects of epileptogenesis. Using a mouse pilocarpine TLE model they utilized the moderate throughput technique of Reverse Phase Protein Arrays (RPPA) and measured levels of proteins comprising components of major signaling pathways and cellular complexes and found time- and region-specific changes in correlations among levels of functionally related proteins affecting both neurons and glia. Among these they identified changes of levels of the MTOR pathway component pS6, and detailed responses of multiple components of the MTOR, MAPK, JAK/STAT and apoptosis pathways, NMDA receptors, and additional cellular complexes.

In the other "omics"- approach, also relatable to the Vila Verde et al. study presented earlier exploring the effects of seizure activity *per se*, Berger et al. (Norway) investigated

upstream-effects of early epileptogenesis in the contralateral hippocampus (CLH) of mice treated with the intracortical kainate model of TLE with HS. They found that the CLH, despite the absence of morphological changes, shows substantial changes in gene expression and DNA methylation in both glia and neurons, but displays a significantly lower number of glial genes up- and downregulated compared to earlier results from the ipsilateral hippocampus (5). Furthermore, several genes and pathways potentially involved in "anti-epileptogenic effects" were upregulated in the CLH, suggesting compensatory mechanisms to prevent morphological alterations like neuronal death and reactive gliosis.

While most contributions to this eBook have focused on glial dysfunction during epileptogenesis in TLE with HS, two contributions broach the issue of other epilepsy conditions. The first one is a review on Tuberous sclerosis complex (TSC) by Zimmer et al. from the group of Elenora Aronica (The Netherlands). TSC represents the prototypic monogenic disorder of the mammalian target of rapamycin (mTOR) pathway dysregulation and is associated with structural and functional brain abnormalities and intellectual disability. So far, research conducted in TSC has been largely neuron-centered. This review highlights recent achievements in TSC research focusing on glial cells, which now are believed to be integral parts of the pathological features of this condition. These cells and their inter-glial crosstalk may offer new insights into the common neurobiological mechanisms underlying epilepsy and the complex cognitive and behavioral comorbidities that are characteristic of the spectrum of mTOR-associated neurodevelopmental disorders.

The last contribution is a review by Gobbo et al. (Germany). The authors provide a substantial overview of the physiology and pathology of cortico-thalamo-cortical oscillations. The electrographic hallmark of childhood absence epilepsy and other idiopathic forms of epilepsy are 2.5–4 Hz spike and wave discharges (SWDs) originating from abnormal electrical oscillations of the cortico-thalamo-cortical network. SWDs are generally associated with sudden and brief non-convulsive epileptic events mostly generating impairment of consciousness and correlating with attention and learning as well as cognitive deficits. The authors deep-dig into this topic and provide substantial information on the role of astroglia (including interstitial fluid homeostasis, K^+ clearance, neurotransmitter uptake, gap junction function, gliotransmission, astroglial Ca^{2+} signaling, and finally reactive astrogliosis and cytokine release) in the modulation of excitation and inhibition in the brain as well as on the development of aberrant synchronous network activity, also bridging over to sleep disturbances.

CONCLUSION

In conclusion, this eBook embraces the complex and multifaceted contributions of glia function and dysfunction to epileptogenesis and illustrates in various ways the intricate interplay between glia and neurons in the etiology and pathogenesis of the epilepsies. Today, research on glia in epilepsy is still in its infancy. We allow us to postulate that increased research focus on glia in combination with novel technology represents an opportunity to develop therapeutic niches, including disease-modifying treatments and true anti-epileptogenic drugs (6).

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KH, MdC, and CS wrote the eBook. All authors contributed and validated the Editorial.

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REVIEW
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Astrocyte Glutamate Uptake and Signaling as Novel Targets for Antiepileptogenic Therapy

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Astrocytes regulate and respond to extracellular glutamate levels in the central nervous system (CNS) *via* the Na^+-dependent glutamate transporters glutamate transporter-1 (GLT-1) and glutamate aspartate transporter (GLAST) and the metabotropic glutamate receptors (mGluR) 3 and mGluR5. Both impaired astrocytic glutamate clearance and changes in metabotropic glutamate signaling could contribute to the development of epilepsy. Dysregulation of astrocytic glutamate transporters, GLT-1 and GLAST, is a common finding across patients and preclinical seizure models. Astrocytic metabotropic glutamate receptors, particularly mGluR5, have been shown to be dysregulated in both humans and animal models of temporal lobe epilepsy (TLE). In this review, we synthesize the available evidence regarding astrocytic glutamate homeostasis and astrocytic mGluRs in the development of epilepsy. Modulation of astrocyte glutamate uptake and/or mGluR activation could lead to novel glial therapeutics for epilepsy.

Keywords: epilepsy, astrocytes, glutamate transporters, metabotropic glutamate receptors, GLT-1, GLAST, mGluR3, mGluR5

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INTRODUCTION

Epilepsy is a common neurological disorder and is characterized by the occurrence of unprovoked seizures. Epilepsy is a major public health problem, affecting more than 65 million people worldwide (1). Healthcare cost estimates associated with epilepsy in the United States range from $9.6 to $12 billion per year (2). TLE is the most common form of epilepsy with focal seizures. TLE is also frequently associated with refractory epilepsy. Approximately 1/4 of patients with TLE develop refractory epilepsies that are pharmaco-resistant to currently available antiepileptic drugs (AEDs) (3).

AEDs work primarily by targeting neurons through modulation of ion channels, enhancement of inhibitory neurotransmission or attenuation of excitatory neurotransmission (4). Most AEDs target channels on neurons to exert their antiepileptic effects. Newer generation AEDs still primarily target neurons but through novel mechanisms and unique binding sites [e.g., AMPA-R, CMRP2, SV2A, or inhibition of carbonic anhydrase activity (5)]. Modulation of neurotransmission can consequently lead to dose-dependent "neurotoxic" adverse effects which are common undesired effects associated with AED usage. Adverse cognitive and behavioral effects of AEDs have been shown to lead to AED discontinuation in up to one-third of patients (6). Therefore, new non-neuronal targets that could potentially have fewer side effects should be considered and further investigated.

Neuronal hyperexcitability is a major contributor to epilepsy but increased evidence suggests that changes in astrocytes can contribute to the development of epilepsy (7–13). Astrocytes are involved in ionic homeostasis, regulation of extracellular space volume and clearance of neurotransmitters. Astrocytes are a critical component of the tripartite synapse, where they are involved in the active control of neuronal activity and synaptic neurotransmission. Astrocytes regulate extracellular glutamate levels *via* Na^+-dependent glutamate transporters, glutamate transporter-1 (GLT-1) and glutamate aspartate transporter (GLAST). GLT-1 is responsible for ∼90% of glutamate uptake in the adult dorsal forebrain and is crucial for the maintenance of low extracellular glutamate to permit efficient synaptic transmission (14). The human homologs of GLAST and GLT-1 are EAAT1 and EAAT2, respectively. In this review we will be referring to these transporters in pre-clinical and clinical studies by GLAST and GLT-1. Aside from perisynaptic glutamate uptake, astrocytes can also sense extracellular glutamate to more readily adapt to their microenvironments through metabotropic glutamate receptors mGluR3 and mGluR5. These G-protein coupled receptors can differentially modulate the expression of glutamate transporters and glutamate release therefore indirectly regulating synaptic activity. This review will provide an overview of what we currently understand regarding the regulation of astrocytic glutamate transporters and receptors in the development of epilepsy. Targeting glutamate uptake and/or glutamate receptor activation through astrocytes could lead to novel treatment options for patients with refractory epilepsies.

DYSREGULATION OF GLUTAMATE UPTAKE IN EPILEPTOGENESIS

GLT-1 and GLAST are the primary transporters responsible for glutamate clearance in the central nervous system (CNS) following excitatory neuronal transmission. It is crucial to maintain low levels of basal extracellular glutamate in the brain to permit efficient and localized synaptic transmission. Evidence of increased glutamate levels have been observed in patients suffering from TLE and in preclinical seizure models (15–17). The vast majority of GLT-1 is astrocytic with synaptic localization, with ∼5–10% of expression in neurons (18, 19). Mice that globally lack GLT-1 develop lethal spontaneous seizures, while transgenic mice that overexpress GLT-1 have a higher seizure threshold than wild-type mice, suggesting that GLT-1 plays an important role in preventing seizures and protection against glutamate toxicity (20, 21). In multiple preclinical studies, GLT-1 protein levels have been shown to be downregulated during the development of epilepsy (**Figure 1**). Perisynaptic GLT-1 at the plasma membrane in astrocytes is significantly reduced around CA3-CA1 synapses during the latent period following systemic kainate-induced status epilepticus (SE) (22). Hippocampal GLT-1 total protein levels have been found to be downregulated following intrahippocampal kainate-induced SE (11). Crude synaptosomal GLT-1 levels, which include components of the tripartite synapse, are also reduced nearly 80% 1 week following intrahippocampal kainate induced-SE in the hippocampus early in the epileptogenic process (13). These data suggest that the pool of transporters available for glutamate uptake at excitatory synapses is substantially reduced in epileptogenesis. The kainic acid (KA) model of TLE is characterized by a period of SE, that serves as the initial insult, followed by a latency period where the mice are seizure-free followed by the occurrence of spontaneous recurrent seizures (23–25). Downregulation in GLT-1 protein levels observed in these studies interestingly coincides with the approximate onset of spontaneous seizures, demonstrating that glutamate transporter dysregulation could contribute to the development of epilepsy (13, 22, 26–28). Interestingly, GLT-1 protein levels were found to be upregulated in in a spontaneously epileptic rat, a double mutant (zi/zi, tm, tm), compared to control Wistar rats (29). GLT-1 protein levels have also been shown to be disrupted in patients with TLE (8, 9). GLT-1 levels have been found to be decreased in the hippocampus of TLE patients with hippocampal sclerosis (HS) in most (8, 9) but not all (30) studies. In patients with decreased GLT-1, severe neuronal cell loss was observed suggesting that loss of glutamate transporters could exacerbate neurotoxicity in epilepsy (8, 9).

GLAST has also shown to be dysregulated in the epileptic brain. GLAST is found in the dorsal forebrain postnatally and homogenously distributed among astrocytic soma and endfeet compared to its counterpart GLT-1 (18, 31). GLAST-deficient mice have significantly longer seizure duration compared to wild-type mice suggesting that GLAST also plays a role in seizure susceptibility (32). In a preclinical model of TLE, synaptosomal GLAST protein levels were elevated in the epileptic hippocampus while overall total protein levels were unchanged at chronic time points (13). GLAST protein levels were found to be significantly lower in a spontaneously epileptic rat, a double mutant (zi/zi, tm, tm), compared to control Wistar rats (29). In TLE patients with HS, GLAST protein levels have been shown to be downregulated while GLAST protein levels are unchanged in TLE patients without HS (8). Astrocytic glutamate synthetase is responsible for the rapid conversion of intracellular glutamate to glutamine and is a prerequisite for efficient glutamate clearance from the extracellular space. Loss of glutamine synthetase has also been observed in patients with TLE which could have an impact on glutamate transporter clearance (30). These findings suggest that glutamate transporter dysregulation could contribute to increased extracellular glutamate and ictogenesis in the epileptic brain.

REGULATION OF ASTROCYTIC GLUTAMATE RECEPTORS IN EPILEPTOGENESIS

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors (GPCRs) important in synaptic neuromodulation. These receptors can be divided into three separate families: Group I, Group II, and Group III, based on their structure and downstream function (33). Metabotropic glutamate receptors found on astrocytes can influence astrocytic functions in physiology and disease. Astrocytes dominantly

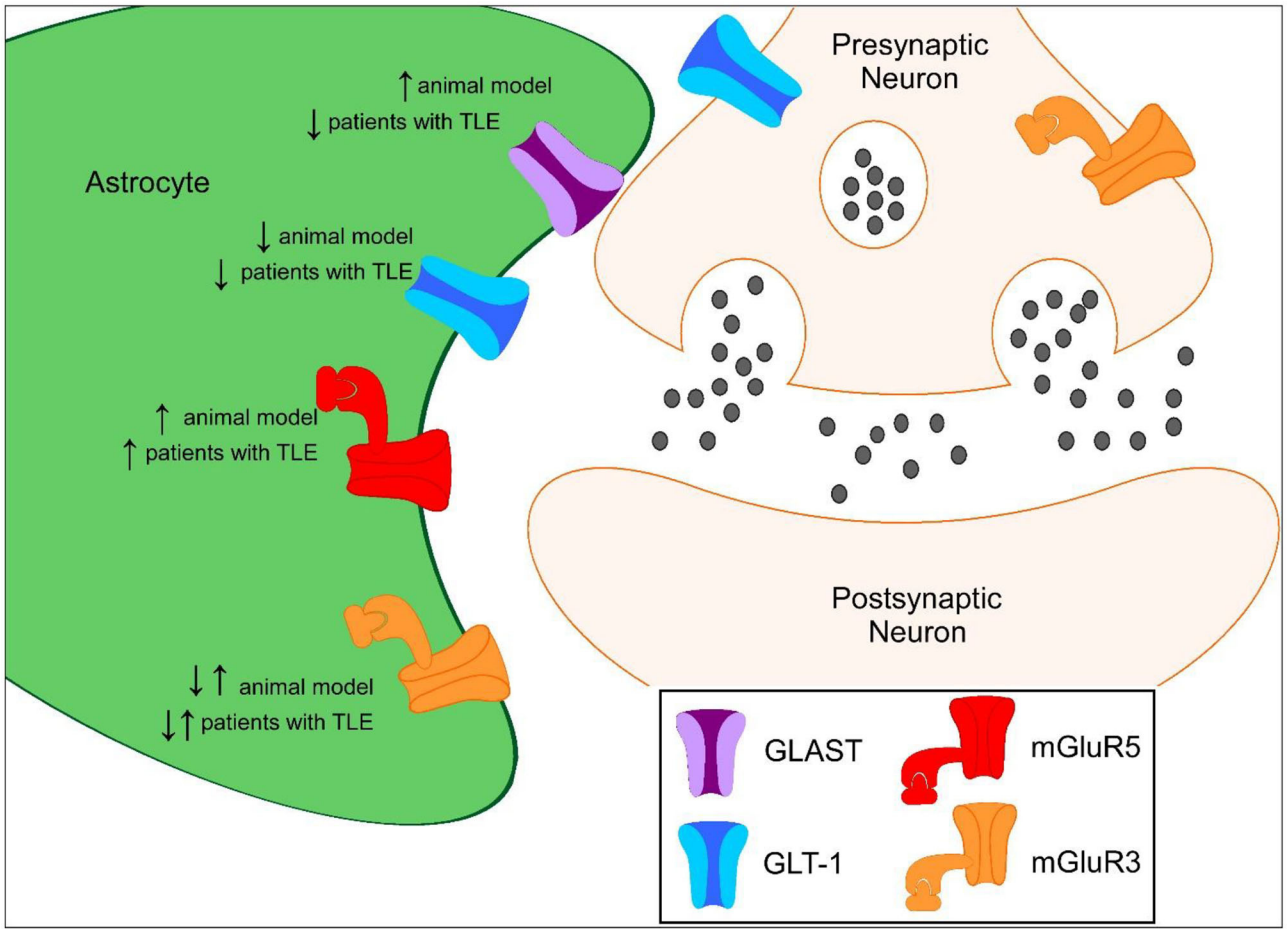

FIGURE 1 | Proposed protein expression of glutamate transporters and mGluRs in the hippocampus during epileptogenesis compared to controls. ↑ represents increased protein expression observed compared to control. ↓ represents decreased protein expression observed compared to control. ↓↑ represents decreased and increased in protein expression observed compared to control.

express mGluR3 and mGluR5 receptors and differential regulation of these receptors has been observed in epilepsy (10, 34, 35). Astrocytic mGluR5 signaling plays an important role in astrocytic motility, ensheathment and glutamate transport in the developing brain (36, 37). Expression of astrocytic mGluR5 is typically limited to the first few weeks of brain development (37–39). Activation of Group I mGluR5 receptors, coupled to $G\alpha_q$ proteins, has been shown to acutely alter GLT-1 activity by increasing glutamate clearance in astrocytes (40) but chronic stimulation can lead to a reduction in astrocytic GLT-1 and GLAST levels resulting in reduced glutamate transport (35).

Differential expression of astrocytic mGluRs have been reported in patients and preclinical models of epilepsy. mGluR5 levels have been shown to be overexpressed in murine seizure models. mGluR5 expression in reactive astrocytes is persistently upregulated following electrically induced SE in a kindling model and in TLE (34). Selective positive modulation of mGluR5 in the Theiler's murine encephalomyelitis virus (TMEV)-induced model of epilepsy attenuates seizures (41). Additionally, selectively knocking out astrocytic mGluR5 signaling during epilepsy slows glutamate clearance through glutamate transporters, suggesting that mGluR5 plays an important function in regulating these transporters in epileptogenesis (42).

Multiple studies have shown that mGluR5 levels are also increased in patients with TLE (10, 43). mGluR5 expression levels in patients have been associated with seizure frequency. Lower mGluR5 expression was found to be negatively correlated with seizure frequency and epilepsy duration in patients with TLE (non-HS) (10). Conditional knockout of mGluR5 signaling from astrocytes slowed glutamate clearance in epileptogenesis (42). These data support the hypothesis that mGluR5 upregulation could act as a compensatory mechanism to counterbalance the hyperexcitability observed in epilepsy.

It is important to note that activation of mGluR5 has also been shown to lead to increased excitability. For example, stimulation of group I mGluRs, including mGluR5, elicits ictal-like events in hippocampal slices (44). Following SE, mGluR5 activation has also been shown to enhance astrocytic calcium signals during the latency period of epileptogenesis in the pilocarpine model of TLE (45). Moreover, increases in astrocytic

calcium transients can lead to release of gliotransmitters, including glutamate, and activation of NMDA receptors (46). In one study, intrahippocampal perfusion of the mGluR group 1 agonist, DHPG (R,S-3,5-dihydroxyphenylglycine), induced seizures while infusion of the mGluR5 receptor antagonist, MPEP (2-methyl-6-(phenylethynyl)-pyridin), attenuated pilocarpine-induced seizures (**Table 1**) (52).

Hyperexcitability associated with mGluR5 activation has also been observed in other neurological diseases. For example, preclinical data suggest that in Fragile X Syndrome, a genetic form of autism, the absence of fragile X mental retardation protein (FMRP) leads to overstimulation of the mGluR5 pathway enhancing glutamatergic signaling contributing to phenotypes observed in this disease (53–55). Interestingly, treatment of *Fmr1* knockout mice with negative modulators of mGluR5 ameliorates phenotypes (56, 57). These studies indicate that although acute activation of mGluR5 can decrease excitability, chronic stimulation, which could occur in a diseased state, can be detrimental.

Activation of Group II mGluR3 receptors, which are coupled to $G\alpha_i$ proteins in astrocytes, may have neuroprotective functions including increasing the capacity for glutamate clearance in the CNS through upregulation of glutamate transporters (35, 58). mGluR3 receptor activation has been shown to upregulate GLT-1 and GLAST protein levels promoting increased glutamate uptake in astrocytes (35, 58). mGluR3 receptors are also found in the presynaptic terminals of glutamatergic neurons (59). mGluR3 receptor agonists have also been shown to protect neurons from excitotoxicity and astrocytes from nitric oxide-induced death (60). Astrocyte-specific mGluR3 expression is markedly increased at early and chronic time points following SE in CA3 and hilar region following electrically induced SE in a kindling model and in TLE (34). A reduction in astrocyte-specific mGluR3 was observed in the molecular layer and stratum lacunosum moleculare of the hippocampus at chronic time points (34). mGluR2/3 expression was also found to be markedly decreased both acute and chronic time points following pilocarpine-induced SE (50).

Whether mGluR3 expression levels are upregulated or downregulated in patients with TLE is controversial. One study found mGluR2/3 expression is downregulated (50) while a separate study showed that mGluR2/3 is upregulated in the hippocampi of TLE patients (61). Whether this discrepancy is due to study design, severity or stage of epilepsy, region-specific effects, or technical differences remains to be determined. Future studies could further examine the use of selective negative modulators of mGluR5 or positive modulators of mGluR3 as an alternative therapeutic approach to treat epilepsy.

ASTROCYTIC GLUTAMATE UPTAKE AND TARGETING OF GLUTAMATE RECEPTORS AS THERAPIES FOR REFRACTORY EPILEPSIES

Non-neuronal targets, including glial cells, are an attractive alternative approach to treat patients whose seizures are not well-controlled with currently available AEDs. Increasing astrocytic glutamate uptake capacity by upregulation of glutamate transporters has been shown to have neuroprotective

TABLE 1 | Positive and negative outcomes of glutamate transporter modulation and mGluR agonists/antagonist in preclinical seizure models.

Drug candidate	Selectivity	Dose	Model	Antiepileptic effect	Other effects	References
17AAG	HSP90β inhibitor	50 μl, 200; mg/kg, i.p.	KA model of TLE	↓ seizures	↑ GLT-1 ↓ astrogliosis	(47)
Ceftriaxone	GLT-1 transcriptional activator	200 mg/kg; i.p.	Knock out mouse model of TSC	↓ seizures	↑ GLT-1 ↓ glutamate ↓ neuronal death	(48)
APDC	Group II mGluR agonist	0.6 nmol; i.c.v. infusion	DL-HCA model of seizure	↓ seizures		(49)
APDC	Group II mGluR agonist	12.5, 50, 200, 400, and 600 mg/kg; i.v.	Pilocarpine model of TLE	No effect		(50)
DCG-IV	Group II mGluR agonist	0.5 μl, 1 nm; intra-amygdaloid	Kindling of the basolateral amygdala	↓ seizures		(51)
DCG-IV	Group II mGluR agonist	0.6 nmol/side; i.c.v. infusion	DL-HCA model of seizure	Partial effect		(49)
DCG-IV	Group II mGluR agonist	5–100 nmol/side; i.c.v. infusion	DL-HCA model of seizure	↑ seizures		(49)
DCG-IV	Group II mGluR agonist	1 μM; intrahippocampal	Pilocarpine model of TLE	Partial effect	↓ extracellular glutamate	(52)
DCG-IV	Group II mGluR agonist	10 μ; intrahippocampal	Pilocarpine model of TLE	Partial effect	↑ glutamate and GABA	(52)
DHPG	Group I mGluR agonist	1 mM; intrahippocampal	Pilocarpine model of TLE	↑ seizures	↑ glutamate and GABA	(52)
Cyclobutylene AP5	Group II mGluR agonist	4, 8, and 16 nmol/side; i.c.v. infusion	DL-HCA model of seizure	↓ seizures		(49)
MPEP	mGluR5 antagonist	50 mg/kg; i.p.	Pilocarpine model of TLE	↓ seizures	↓ glutamate and GABA	(52)
MPEP	mGluR5 antagonist	1 μg/g; I.V.	Pilocarpine model of TLE	No effect	↓ neuronal death	(45)

Up-arrow notation represents an increase and down-arrow notation represents a decrease in the table.

and anti-epileptic effects. Seizures were significantly reduced and astrogliosis was attenuated when mice were administered an HSP90β inhibitor to increase GLT-1 expression in a mouse model of TLE (47). Ceftriaxone, a β-lactam antibiotic, has also been shown to upregulate GLT-1 protein expression and reduce seizures in multiple preclinical studies (48, 62, 63). Treatment with ceftriaxone has shown negative adverse side effects including impairment in synaptic plasticity and memory recognition (64, 65). Ceftriaxone affects many pathways in the CNS, therefore, it is currently not well-understood if these adverse effects are a result of GLT-1 activation. Nevertheless, selectively targeting aberrant astrocytes could reduce adverse side effects. Intraspinal delivery of AAV8-Gfa2-GLT1 has been used to selectively increase GLT-1 protein expression under the truncated glial fibrillary acidic protein promotor in a model of spinal cord injury showing promising results (66). Gene therapy could potentially be used to target subpopulations of astrocytes by selecting genes known to be overexpressed in the epileptic brain. For example, adenosine kinase is strikingly upregulated in reactive astrocytes after kainic acid-induced SE and its promotor could be used to selectively target this cell population (67).

The mGluR5 receptor antagonist MPEP reduced seizures when administered i.p. in the pilocarpine seizure model (52). In contrast, another study also using the pilocarpine seizure model found that MPEP suppressed neuronal death but did not result in a change in synaptic activity, suggesting that astrocytes could have neurotoxic roles in epilepsy through increased gliotransmission (45). Future studies should further examine mGluR5 antagonists as potential adjunctive therapies to decrease the severe neuronal loss observed in TLE patients with HS. The mGluR2/3 agonist, APDC, was shown to reduce seizure in the DL-homocysteic acid (DL-HCA) seizure model (49). In the pilocarpine seizure model, APDC did not reduce seizures nor neuronal death (50). These studies indicate that selection of agonist/antagonists can have differential outcomes. Two Group II mGluR agonists targeting mGluR2/3, cyclobutylene AP5 and DCG-IV, have both demonstrated positive effects on seizure control in the DL-homocysteic acid (DL-HCA) seizure model and kindling model of TLE (49, 51). Interestingly, at higher doses DL-HCA has been shown to have pro-epileptic effects (49, 52). Thus, activation of Group II mGluRs may be another promising avenue for alternative therapies for treating epilepsy.

CONCLUSION

Astrocytes play a critical role in the development and progression of epilepsy (7, 8, 30, 68–76). Astrocytic glutamate uptake is dysregulated in both preclinical models and in patients with TLE leading to increases in basal glutamate levels, and activation and signaling of astrocytic metabotropic glutamate receptors, mGluR3 and mGluR5, is also altered in animal models and patients with TLE. It is not clear yet whether targeting glutamate transporters and receptors would be more effective as a novel antiepileptic (controlling seizures in pharmacoresistant epilepsies) or antiepileptogenic (disease-modifying prevention of development of epilepsy after epileptogenic insults) strategy. Future studies should distinguish antiepileptic vs. antiepileptogenic effects, for example of GLT1 upregulation in appropriate animal models. Targeting of altered "epileptic" glutamate metabolism and signaling in astrocytes has the potential of efficacy with fewer side effects compared to traditional suppression of glutamatergic neurotransmission in neurons. This could lead to novel approaches to antiepileptic, antiepileptogenic, and/or neuroprotective therapies.

AUTHOR CONTRIBUTIONS

AP did an exhaustive literature search, generated a complete draft of the review, and prepared the table and figure. DB also reviewed the literature, provided detailed comments, and edits to the review and the table and figure. All authors contributed to the article and approved the submitted version.

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REVIEW
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Tuberous Sclerosis Complex as Disease Model for Investigating mTOR-Related Gliopathy During Epileptogenesis

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Tuberous sclerosis complex (TSC) represents the prototypic monogenic disorder of the mammalian target of rapamycin (mTOR) pathway dysregulation. It provides the rational mechanistic basis of a direct link between gene mutation and brain pathology (structural and functional abnormalities) associated with a complex clinical phenotype including epilepsy, autism, and intellectual disability. So far, research conducted in TSC has been largely neuron-oriented. However, the neuropathological hallmarks of TSC and other malformations of cortical development also include major morphological and functional changes in glial cells involving astrocytes, oligodendrocytes, NG2 glia, and microglia. These cells and their interglial crosstalk may offer new insights into the common neurobiological mechanisms underlying epilepsy and the complex cognitive and behavioral comorbidities that are characteristic of the spectrum of mTOR-associated neurodevelopmental disorders. This review will focus on the role of glial dysfunction, the interaction between glia related to mTOR hyperactivity, and its contribution to epileptogenesis in TSC. Moreover, we will discuss how understanding glial abnormalities in TSC might give valuable insight into the pathophysiological mechanisms that could help to develop novel therapeutic approaches for TSC or other pathologies characterized by glial dysfunction and acquired mTOR hyperactivation.

Keywords: tuberous sclerosis (TSC), mammalian target of rapamycin (mTOR), epilepsy, astrocyte, microglia, oligodendrocyte, glia, epileptogenesis

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INTRODUCTION

Tuberous sclerosis complex (TSC) is a rare, genetic multisystem disorder with a prevalence of ∼1:6,000 newborns. Common symptoms in TSC include benign tumor growth in kidney, heart, lung, eyes, skin, and brain (1). Characteristic lesions in the brain are cortical tubers and ventricular subependymal nodules, which may progress

into subependymal giant cell astrocytomas (SEGAs) (2–4). Neurological manifestations include epilepsy, neurodevelopmental delay, and TSC-associated neuropsychiatric disorders (TANDs), such as intellectual disability and autism spectrum disorder (ASD) (5–7). Moreover, as one of the most debilitating symptoms, TSC represents the most common genetic cause for pediatric epilepsy, with roughly 85% of cases developing seizures, predominantly within the first year of life, and 60% eventually presenting with refractory epilepsy (8, 9). Because uncontrolled seizure activity aggravates cognitive comorbidities, immediate seizure management after or ideally before epilepsy onset is crucial for normal cognitive development of patients (10–12). Currently, the most effective long-term treatment for epilepsy in TSC is vigabatrin, a highly effective drug against infantile spasms in TSC patients (13–15), whereas a subgroup of eligible patients benefits from adjunctive everolimus [mammalian target of rapamycin (mTOR) inhibitor] treatment or surgical resection of the suspected epileptogenic lesion (14, 16–19).

TSC is caused by loss-of-function mutations in the tumor suppressors *TSC1* or *TSC2*, both of which are negative regulators of the mTOR (20, 21). Purely heterozygous germline mutations, as well as mosaic mutations, have been detected in TSC patients (21, 22). mTOR is a serine/threonine protein kinase and the catalytic subunit of mTOR complex 1 (mTORC1) and mTORC2. Under normal conditions, mTOR activity is tightly controlled by upstream regulators and acts as important sensor of cellular energy status and homeostasis. Environmental stimuli, such as cytokines or growth factors can stimulate mTOR, enabling cells to dynamically respond to various extracellular cues via adaptation in metabolism or cellular growth (23, 24). Mutations in either *TSC1* or *TSC2* lead to uncoupling from upstream regulators and abnormal hyperactivation of mTORC1, causing growth of the characteristic lesions during brain development. While TSC represents the prototypic monogenic disorder of mTOR hyperactivation, other malformations of cortical development, such as megalencephaly, hemimegalencephaly, and focal cortical dysplasia (FCD) are also characterized by aberrant mTOR activation due to acquired mutations in various mTOR regulators (25). Importantly, all share histopathological and clinical characteristics with TSC; hence, this spectrum of diseases is collectively referred to as mTORopathies [reviewed in (26, 27)].

Importantly, mTOR hyperactivity seems to be directly linked to epileptogenesis as mTOR inhibitors can suppress seizures in preclinical TSC models (28, 29), as well as in clinical studies aimed at treating TSC and SEGAs (16–19, 30, 31). Current consensus is that mTOR inhibitors induce a temporary anticonvulsant effect as do currently available antiepileptic drugs, but may also possess disease-modifying potential (15, 32). The clear causative role of mTOR as epileptogenic driver, as well as implications of mTOR activation in acquired epilepsies (33–36), makes TSC an attractive disease model to utilize as translational prototype for epilepsy in general. Despite the progress in understanding the role of the mTOR signaling pathway, there is still a lack in pinpointing the precise cellular substrates responsible for producing seizures. Interestingly, although the neuropathological hallmarks of TSC are primarily found in tubers, some studies showed that the seizure focus in TSC brains could also originate from the surrounding normal-appearing cortex, based on seizure freedom after resection of the perituberal zone, tissue analysis, and electrocorticographic recordings (37–40). However, further progress in the careful examination and advances in the identification of novel histopathological markers may make a discrimination between tuber and perituber obsolete, eventually. Nevertheless, surgical resection of the tuber leads to seizure relief in 50% to 60% of cases, suggesting an important role in epileptogenesis in at least a subset of patients with a clear-cut epileptogenic "driver" lesion (41–44).

In the brain of TSC patients, mTOR hyperactivity promotes development of often multifocal brain lesions characterized by aberrant glioneuronal proliferation, cortical dyslamination, and hypomyelination, along with the presence of dysplastic neurons and improperly developed giant cells (4, 27, 45–47). TSC tissue obtained from surgery due to refractory epilepsy usually presents with a heterogeneous frequency of the aforementioned histopathological hallmarks between patients (27, 46). TSC lesions are thought to arise by the Knudson hypothesis, also known as the "two-hit" hypothesis (48). Accordingly, somatic mutations in either *TSC1* or *TSC2*, resulting in the loss of wild-type alleles, have been detected in different types of TSC neoplastic lesions and to a lesser extent in cortical tubers (21, 49, 50). Thus, it is still an ongoing matter of discussion whether monoallelic inactivation of TSC1/TSC2 is sufficient for tuber development or if the second hit occurs in a specific cellular component complicating its identification (21, 49, 50). Cell specificity, mutation load, and mutation timing during brain development likely give rise to the diverse neuropathological presentations. Recent evidence from *in vitro* cell cultures and organoid models of TSC revealed that mTORC1 activity during cortical development is tightly controlled, and mTORC1 suppression is required for proper neurogenesis (51). Of note, mTORC1 hyperactivity promotes gliogenesis, likely explaining the increased number of glia in tubers (52–56). More specifically, mTORC1 was shown to activate STAT3 signaling, which represents a major driver of gliogenesis during development (53, 57–60) Furthermore, gliosis and activation of inflammatory signaling pathways are histopathological hallmarks of TSC (46, 52, 61–64). Accordingly, although dysfunctional neuronal circuitry is ultimately required for the development of epilepsy and mTOR can directly regulate neuronal structure, function, and plasticity (65–68), accumulating evidence shows that glial cells represent a crucial element in the pathogenesis of TSC and might pose novel therapeutic strategies (64). This review will focus on the role of glial dysfunction related to mTOR hyperactivity and its contribution to comorbidities, such as epilepsy and TANDs in TSC. In this context, while many studies primarily focused on neuroglial crosstalk, we will emphasize aberrant interglial communication as an essential aspect of TSC. Finally, studying glial abnormalities in TSC might give valuable insight into pathophysiological mechanisms, which could help to develop novel therapeutic approaches for TSC or other pathologies characterized by gliopathic changes and acquired mTOR hyperactivation (summarized in **Figure 1**).

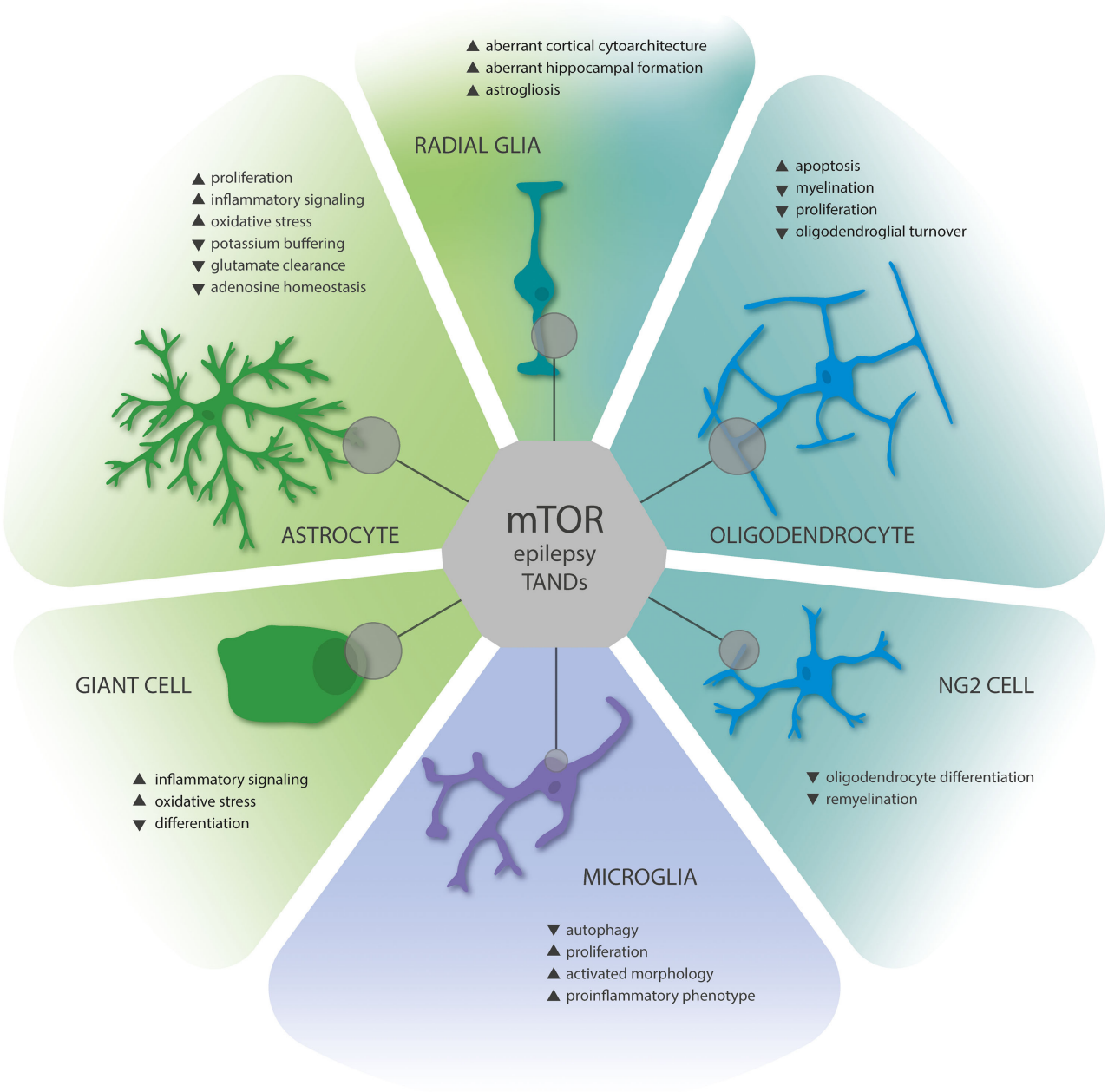

FIGURE 1 | Summary of gliopathic changes due to mTOR hyperactivation in TSC brain lesions. Astrocytes display increased proliferation, activation, and enhanced expression of proinflammatory mediators. Moreover, astrocytes are characterized by decreased homeostatic functions related to ion homeostasis and neurotransmitter metabolism. Radial glia, the neuroglial precursors of astrocytes, oligodendrocytes, and neurons, contribute to malformations of cortical development and aberrant gliogenesis, as well as the formation of giant cells, which display characteristics of proinflammatory glia. Oligodendrocyte dysfunction leads to hypomyelination and disturbed remyelination, and their proliferation is reduced. While dysfunction of NG2 glia in TSC deserves further investigation, they are crucially involved in myelination and crosstalk with neurons, thus representing an essential component of TSC gliopathology. Finally, as for astrocytes, microglia are characterized by enhanced proliferation, activation, and expression of proinflammatory mediators; however, these changes are likely secondary to mTOR activation in the TSC brain. Collectively, these changes contribute to epilepsy and neuropsychiatric comorbidities in TSC. The influence of mTOR signaling on the individual cell types is indicated by the size of gray circles.

ASTROCYTES

Astrocytes display distinct functional changes in a variety of epilepsies with different etiologies, and it becomes increasingly clear that they play crucial roles in the process of epileptogenesis, including TSC (69, 70). Neuropathological hallmarks in resected cortical tubers of TSC patients include increased expression of glial fibrillary acidic protein (GFAP), vimentin, and S100β, as

well as higher numbers of astrocytes. Moreover, these astrocytes often present dysplastic and reactive phenotypes compared to the perituberal area and control brain tissue (71, 72). While most studies characterize the total population of astrocytes, some report different subpopulations of astrocytes in TSC (52, 72). One study characterized two subpopulations of astrocytes: "reactive" cells, which are large and vimentin positive and reveal mTOR activation, and "gliotic" astrocytes, which are smaller, do not show mTOR activation, and resemble gliotic astrocytes found in hippocampal sclerosis (HS) (52). Gliotic astrocytes, as in HS, present with decreased expression of inwardly rectifying potassium (Kir) channel subunit Kir4.1, a decrease in the glutamate transporters excitatory amino acid transporter 1 (EAAT1) and EAAT2, and a decrease in glutamine synthetase, all of which represent proepileptogenic changes (70). The authors of this study concluded that the gradual transformation from reactive to gliotic astrocytes might represent a major driving force for the morphological dynamics of tubers over time (52). Another study discriminated between normal astrocytes (no mTOR activation, vimentin-negative, and GFAP-positive), reactive astrocytes (no mTOR activation, vimentin-positive, and GFAP-positive), and dysplastic astroglia (mTOR activation, vimentin-positive, and GFAP-negative), the latter representing an expression pattern common to immature astrocytes and radial glia (72). Taken together, both studies support the notion that populations of improperly differentiated astrocytes with mTOR activation, as well as properly developed, reactive astrocytes without mTOR activation, contribute to TSC pathology. Here, the aforementioned continuum of pathological changes in astrocytes and the precise cellular composition of the tissue might reflect the intrinsic epileptogenicity of the tuber. Importantly, the functional changes in TSC astrocytes are likely caused by a combination of the reactive state in response to seizures known from other diseases, such as mesial temporal lobe epilepsy (TLE), which could induce secondary mTOR activation (33), but also general disturbance in protein translation caused by sustained mTOR activation in mutation-carrying cells. Ultimately, both astrocyte subpopulations could end up having different pathogenic origins, but similar functional outcomes in terms of expression of Kirs, EAATs, or glutamine synthetase, further increasing the epileptogenic potential of the tuber. Whether the different degrees of mTOR activation underlie the wide diversity of astrocyte functions and phenotypes in TSC deserves further investigation. However, for neurons, it has already been shown that extent of mTOR hyperactivity correlates with seizure severity and associated neuropathology (73). Finally, in addition to intrinsic astrocytic properties, maintenance of a non-reactive state in astrocytes was also shown to depend on neuronal mTORC1 signaling, adding yet another level to altered astrocyte function in TSC (74).

The most striking evidence for astrocytic contribution to epileptogenesis in TSC comes from a conditional *Tsc1* knockout mouse model (referred to as *Tsc1*^{GFAP} mice), in which *Tsc1* is specifically deleted in GFAP-expressing cells during embryonic development, leading to mTOR hyperactivity in these cells (75, 76). Notably, *Tsc1* deletion is also induced in GFAP-positive neural progenitor cells and can be found in neurons, thereby blurring the specific contribution of astrocytes to some extent (77). While this model does not recapitulate all pathological hallmarks of human TSC (most notably lacking tuber formation and giant cells), development of spontaneous recurrent seizures arises in all animals at 1 month of age. This occurs likely via diffuse astrocyte proliferation and dispersion of neurons, causing altered neuronal circuitry. Interestingly, even post-natal deletion of *Tsc1* at 2 weeks of age leads to development of epilepsy in half of the animals, although in a less severe form (77). Consequently, *TSC1* deletion appears to be the initial insult followed by a latent stage of epileptogenesis, which in TSC patients might be even prenatally. Notably, treatment of *Tsc1*^{GFAP} mice with the mTOR inhibitor rapamycin suppressed seizures, whereas vigabatrin reduced seizures and partially inhibited mTOR activity, astrogliosis, and neuronal disorganization (29, 78). Interestingly, TSC patients present with differences in disease severity, depending on the underlying mutation, with *TSC2* mutations causing a more severe neurological and cognitive phenotype (22, 79–81). In conjunction with this, *Tsc2*^{GFAP} mice present with more severe epilepsy than *Tsc1*^{GFAP} mice (82).

While the growth advantage of astrocytes plays an apparent role in disruption of neuronal circuits, astrocytes in this model also display functional changes. A pathological hallmark of acquired epilepsy is impaired potassium buffering by astrocytes (83). Its implication in epileptogenesis is based on increased extracellular potassium upon neuronal depolarization, reduced astrocytic clearance of excess potassium, and consequently neuronal hyperexcitability and seizures. Key players in astrocytic potassium buffering represent aquaporins, Kirs, and connexins, which all display dysregulation in TSC-null astrocytes, *Tsc1*^{GFAP} mice, and surgically resected TSC tissue (84–87). Another well-established player in neuronal hyperexcitability is impaired astrocyte-mediated clearance of glutamate, which can predispose neurons to sustained excitability, excitotoxicity, and epileptiform activity. Astrocytes in human TSC display altered glutamate receptor expression, whereas *Tsc1*^{GFAP} mice present with decreased expression of glutamate transporters, implying altered extracellular glutamate metabolism (72, 88, 89). Pharmacological upregulation of glutamate transporters in astrocytes of *Tsc1*^{GFAP} could reduce seizure frequency and some of the pathological changes, exemplifying the likely importance of extracellular glutamate clearance in TSC (88). Lastly, increased astrogliosis and consequent enhanced astrocytic adenosine kinase activity in epilepsy models and various epileptogenic pathologies, including TSC, result in a deficient homeostatic adenosine tone at the synapse and reveal a direct link between astrocyte activation and network excitability (90, 91).

Besides the reported changes in potassium buffering, glutamate clearance, and adenosine homeostasis, TSC is also characterized by inflammatory changes, and astrocytes are supposed to be both source and target of inflammatory signaling therein (92–95). Indeed, human tuber and SEGA tissue also display activation of inflammation in astrocytes, in particular, the toll-like receptor 4 (TLR-4), interleukin 1β (IL-1β), and complement pathways, as well as increased expression of IL-17, intercellular adhesion molecule 1, tumor necrosis factor α (TNF-α), and nuclear factor κB (NF-κB) (61–63, 96–98). In

particular, several large-scale RNA-sequencing studies confirmed that neuroinflammation is a hallmark of TSC-associated lesions, and the retrieved data were enriched for both astrocyte and microglial specific genes (21, 63, 99, 100). Additionally, microRNAs (miRNAs) involved in the regulation of astrocytic inflammatory responses are upregulated in TSC (101). In comparison, astrocytes in *Tsc1*^{GFAP} mice also present with increased IL-1β and C-X-C motif chemokine 10 expression, most notably, preceding the development of seizures, and are also characterized by increased microglial proliferation (74). Collectively, proinflammatory changes represent an important pathogenic mechanism by further activating astrocytes and could also reinforce mTOR-related dysfunctional processes, e.g., the immunoproteasome pathway, which might represent a direct molecular link between inflammation, mTOR activation, and epilepsy in TSC and other mTORopathies (102).

An additional pathogenic mechanism frequently encountered and closely linked to inflammation in epilepsy is oxidative stress (OS) (103–105). OS is defined as disturbance in the cell's redox state and was shown to be highly correlated with inflammation in dysmorphic neurons, giant cells, and glia of TSC and other mTORopathies (98). Glial cells in TSC displayed higher expression of the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) (98). Both enzymes produce mediators that contribute to OS and inflammation, thereby supporting the notion that glia are mediators of these pathogenic processes in TSC. In addition, giant cells revealed strong expression of OS (iNOS, cysteine/glutamate antiporter) and inflammation (COX-2, TLR-4) markers, as well as accumulation of NF-κB in the nucleus, supporting the strong correlation between these two processes (98). Another article pointed at the critical role of OS promoting an environment that favors the positive selection of cells with higher antioxidant capacity due to aberrant mTOR activation (106). Further research into OS in TSC revealed that the proinflammatory miRNA-155 might contribute to this sustained activation of antioxidant pathways in giant cells and astrocytes, exemplifying the link between OS and brain inflammation (107). Furthermore, the induction of sustained, miR155-mediated antioxidant signaling in astrocytes led to dysregulation of iron metabolism, which could result in the potentiation of OS in TSC (107).

A final pathological hallmark of TSC is the disruption of the blood–brain barrier (BBB) (108), with implications for a modulatory role of matrix metalloproteinases in BBB remodeling (62, 109–113). In this context, chronic BBB dysfunction and epileptogenesis after status epilepticus (SE)–induced epilepsy could be reduced via treatment with rapamycin, pointing toward a more general role of mTOR-dependent BBB remodeling during epileptogenesis in epilepsy (34, 35, 114).

GIANT CELLS AND RADIAL GLIA

In the context of gliopathy in TSC, it is noteworthy that giant cells represent a cell type with features of immaturity, highlighting the absence of differentiation to macroglial or neuronal lineage cells prior to migration into the developing cortex (72, 115–117). While the exact precursor of giant cells is unclear, the differential expression of glial (GFAP and S100 protein), neuronal (neurofilament, synaptophysin, MAP2), and neuroglial progenitor (SOX2, nestin, vimentin, CD133, β_1-integrin) markers suggests that these cells reflect intermediary, undifferentiated stages of cellular development (45, 62, 115, 116, 118, 119). Many of the expression changes in astrocytes mentioned before are recapitulated in giant cells in tubers; however, on average, they display high heterogeneity, likely due to variation in the frequency of mutations based on the "two-hit" hypothesis (21, 49). Accordingly, balloon cells in FCD, a cell type histologically resembling giant cells in TSC, have been shown to also carry pathogenic somatic, second-hit variants of mTOR regulatory genes, and their density correlates with genetic findings (120). Moreover, non–cell-autonomous effects of the mutation influencing both the interglial and neuroglial crosstalk during cortical development may also contribute to the histological phenotype of malformed cells. Thus, giant cells and balloon cells might represent an important glioneuronal cell type in the generation of disturbed cellular architecture in developmental malformations related to mTOR dysregulation. Functionally, giant cells might contribute to brain inflammation by expressing complement factors and attracting immune cells already very early in development (62, 121). Moreover, they might be actively involved in the aberrant neuronal circuitry leading to the neurological manifestations of TSC by expressing glutamate and γ-aminobutyric acid (GABA) receptors and transporters (72, 122, 123).

One proposed precursor for giant cells are radial glia, progenitor cells of astrocytes and neocortical neurons, and oligodendrocyte progenitors cells (OPCs) (124, 125). Radial glia are localized in the subventricular zone of the developing brain, giving rise to the proliferative, astrogliogenic/neurogenic niche in the developing brain, as well as providing the physical substrate for neurons to migrate along toward their cortical destination (126). In light of this, radial glia perform vital functions in the formation of the cortex, and their malfunction is hypothesized to give rise to improperly differentiated cells, i.e., giant cells and dysmorphic neurons, and malformed cortical cytoarchitecture. Studies on radial glia-specific *Tsc1* or *Tsc2* knockout mice displayed characteristic features of human TSC, such as aberrant cortical architecture, hippocampal disturbances, astrogliosis, and spontaneous seizures (127–129). Importantly, these alterations displayed specific phenotypic differences between *Tsc1* and *Tsc2* knockout mice. Moreover, organoid model systems revealed that higher mTOR baseline activation in outer radial glia, a feature linked to the stemness of progenitor cells (130), is specific to primate corticogenesis, suggesting that this cell niche is highly susceptible to perturbations due to germline or somatic mutations in the mTOR pathway and thereby could induce aberrant formation of giant cells in the TSC brain (131, 132). This primate-specific feature could also explain the limitations of most TSC model organisms to reflect the histopathological features of TSC, such as tubers and giant cells. The aforementioned studies imply strong phenotypic effects, depending on the timing of the mutation, as well as the cell type affected, potentially explaining the phenotypic heterogeneity of dysmorphic cells

and in particular astrocytes in human TSC (27). Another highly intriguing finding from these studies on brain organoid development revealed that cellular subtype differentiation of progenitor cells might be perturbed *in vitro* due to enhanced mTOR-dependent glycolysis and endoplasmic reticulum (ER) stress (132, 133), features also implicated in TSC (134, 135).

OLIGODENDROCYTES

The central nervous system (CNS) pathology of TSC comprises a range of white matter abnormalities, detectable in presurgical magnetic resonance imaging (MRI) (136, 137), as well as in resected lesional tissue (138). While cortical tubers have classically been the neuropathological hallmark feature observed in these patients, the widespread hypomyelination/dysmyelination has emerged as a synonymous and prominent indication for clinical phenotypes in TSC patients. The cells responsible for the development and maintenance of an intact white matter of the brain are specialized cells called oligodendrocytes. They undergo a complex and precisely timed cycle of proliferation, migration, and differentiation to finally generate myelin by concentrically wrapping axons with multilamellar sheets of plasma membrane consisting of specific proteins and lipids (139). Two distinct terms in regard to white matter pathologies have been established, namely, demyelination and hypomyelination. The term *demyelination* is generally used if there is loss of myelin, occurring after a normal myelin development. This pathology has been studied accurately in patients suffering from multiple sclerosis (27, 140). Hypomyelination, on the other hand, may emerge if myelin production is disturbed or was never initiated, as seen in TSC patients (27).

Recent technological advances in MRI including diffusion tensor imaging (DTI) and fractional anisotropy (FA) have further emphasized hypomyelination in TSC (141, 142). Data revealed that regions involved in the processing of visual auditory and social stimuli contain more dysmyelinated axons in patients, hence supporting behavioral and cognitive characteristics (142). In addition, a major neuropathological aspect is the limited myelination in resected lesions of TSC patients. A recent study has reported a possible involvement of oligodendroglial turnover, indicating that the inhibition of oligodendroglial cell maturation, supposedly due to constitutive activation of mTOR, may lead to insufficient myelination in TSC patients (138). A principal feature of diseases with abnormal white matter is an oligodendroglial pathology that is frequently associated with cognitive impairments (64). The hypothesis that a dysfunctional white matter and hence abnormal neural circuitry account for the neurological manifestations in TSC has been further investigated by a plethora of studies. Interestingly, TSC patients with ASD have more crucial white matter abnormalities compared to patients without ASD (143, 144).

Oligodendroglial development, from an OPC (also called NG2 glia) to the maintenance of an intact myelin sheath, is tightly controlled by a myriad of both extracellular and intracellular factors, with two regulatory pathways in focus: the mitogen-activated protein kinase kinase/extracellular signal-regulated kinases 1 and 2/mitogen-activated protein kinase (Mek/ERK1/2-MAPK), and the mTOR signaling pathway (145, 146). Specifically, during oligodendrocyte lineage progression and initiation of myelination, the mTOR pathway via mTORC1 has emerged as a key player involved in this process (146). In a recent study, the involvement of mTOR signaling in cytoskeletal reorganization during oligodendrocyte development, as well as in initiation of myelination, was demonstrated. Moreover, the importance of the mTOR pathway on oligodendroglial branching complexity was observed (147). One study demonstrated a decrease in both myelin content and oligodendrocytes in and around cortical lesions of mTORopathy specimens (138). This decrease was linked to elevated mTOR expression and a possible impairment of oligodendroglial turnover, suggesting that mTOR pathway mutations cause a defect in oligodendrocytes (138). Thus, high lesional-specific mTOR activation combined with a decreased number of oligodendrocytes may further strengthen the hypothesis of mTOR pathway-dependent modulation of oligodendroglial differentiation and myelination properties. A plethora of studies have proven the essential role of mTOR signaling on the complex differentiation of oligodendrocytes to the maintenance of an intact myelin sheath (148–150).

Animal models have delved further into the causal relationship between mTOR pathway signaling and proper CNS myelin formation and maintenance. However, there is still considerable uncertainty with regard to the cell autonomous effects of TSC ablation in oligodendrocytes or aberrant signaling from TSC-deficient neurons or astrocytes that may indirectly influence myelination processes in the brain. It has now been suggested that CNS myelination, specifically myelin-associated lipogenesis, and protein gene regulation are mainly dependent on mTORC1 function (151). The same authors demonstrated that oligodendrocyte-specific enhancement of mTORC1, via loss of TSC1, results in abnormal myelination in mice (151). Remarkably, brains of $Tsc2^{Olig2}$ KO mice reveal distinct hypomyelination, further supporting a cell-autonomous effect of TSC2 inactivation on oligodendrocytes (152). Grier et al. (153) drew attention to the important but more transient contribution of mTORC2 signaling in myelinogenesis by utilizing a mouse model lacking the rapamycin-insensitive companion of mTOR (Rictor), a functional component of the mTORC2, in NG2 glia. They were able to observe that loss of Rictor in these cells decreases and delays the expression of myelin related proteins and causes a developmental hypomyelination (153).

Besides cell-autonomous effects, a recent study supports the role of abnormal neuron–oligodendroglia communication causing hypomyelination employing induced pluripotent stem cell–derived neuronal and oligodendroglial cultures from TSC patients. Interestingly, neuron–oligodendrocyte cocultures from these patients revealed increased oligodendrocyte proliferation but a decrease in maturation (154). In terms of neuron–glia interaction, it was shown that *Tsc1* mutant mice display a striking delay in myelination supporting the hypothesis of an underlying abnormal neuron–oligodendrocyte communication that causes white matter pathologies (155). Further, neuron-specific ablation of *Tsc1* in a mouse model results in an increase in connective

tissue growth factor secretion that leads to a decrease in the number of oligodendrocytes (156).

In conclusion, there is evidence that mTOR signaling is indeed fundamental to oligodendrocyte differentiation and myelination, as well as critical indications that both cell-autonomous effects and interactions between neurons and oligodendrocytes cause hypomyelination in mTORopathies. Because the outcome of the mTOR pathway hyperactivation observed in TSC patients as well as in animal models is hypomyelination and not the expected hypermyelination, at least five mechanisms were hypothesized to be responsible for this paradoxical impact on myelinogenesis. The constitutive mTORC1 signaling might account for (1) a delayed onset of myelination, (2) triggering non-physiological toxic effects, such as ER stress or apoptosis of oligodendrocytes, (3) TSC subunits exerting non-canonical functions that are independent of mTORC1, (4) suppressing mTORC2 functions, and (5) a negative feedback on mTORC1-independent targets, such as Mek-Erk 1/2 and/or PI3K-Akt pathways [for a detailed review, see (157)].

Because of the emerging evidence for a link between decreased myelin content and the development of neurological deficits, achieving remyelination of axons takes center stage in multiple sclerosis research, implying that it might be beneficial for mTORopathy patients as well (158, 159). As far as disease control is concerned, an important question is whether the observed hypomyelination in TSC patients may be reversible by reducing the constitutive activation of the mTOR signaling pathway. Latest research emphasizes the use of rapamycin or the rapamycin analog, everolimus. Only few researchers have addressed the question if and how the white matter is altered after treatment with an mTOR inhibitor. A pharmacological therapy administering everolimus was able to decrease mean diffusivity and increase FA during DTI measurements in TSC patients (160). In terms of everolimus treatment period, recent data support the hypothesis that longer periods improve the white matter microstructural integrity even more (161). In summary, evidence from experimental and human studies indicates that hypomyelination could be reversed by treatment with everolimus; however, the mechanism of action needs to be studied in more detail.

NG2 GLIA

Apart from astrocytes and oligodendrocytes, NG2 glial cells represent a third macroglial subtype in the CNS, which has received much attention in the past decade [for a detailed review, see (162)]. In the literature, these cells are primarily referred to as OPCs, but also as complex cells, synantocytes, polydendrocytes, and GluR cells, as they depict glial and neuronal functions (163, 164). NG2 glial cells are substantially spread in both gray and white matter of the developing as well as the adult brain (165, 166). A remarkable feature found in cells expressing the proteoglycan NG2 is their proliferative and differentiation potential throughout life (166, 167). Interestingly, in post-natal white matter, these cells mainly differentiate into myelinating oligodendrocytes (168–170), and especially following demyelination, this process is amplified (171). In contrast, NG2 glia in the gray matter retain their neuronal–glial properties post-natally (11). These cell populations receive direct neuronal glutamatergic and GABAergic synaptic input and express voltage-gated ion channels for K^+, Na^+, and Ca^{2+} that can trigger long-term potentiation; however, they do not generate action potentials themselves (172–175). The precise functional changes of these cells in response to synaptic input remain largely unknown except some evidence on modulation of inward rectifying potassium channels (176) and proliferation (177, 178). Interestingly, cleaved NG2 was shown to rescue diminished neuronal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) currents in NG2 knockout mice, establishing a reciprocal signaling loop between NG2 and neurons (179). Apart from these neuronal properties, NG2 glia in the human hippocampus do not couple to other glia via gap junctions, such as astrocytes and lack glutamate transporters, while expressing Kirs (180). Moreover, NG2 ablation was shown to induce microglia-mediated neuroinflammation and neuronal death in the hippocampus. The authors concluded that reduced NG2-derived trophic support via hepatocyte growth factor was responsible for this loss of neurons (181). Furthermore, NG2-derived transforming growth factor β2 (TGF-β2) signaling to TGF-β receptor 2 on microglia was shown to be a key regulator of microglial CX3CR1-mediated immune responses, and deficiency of this signaling axis via NG2 ablation led to neuronal loss and inflammation (182). Hence, the ability of NG2 glia to respond to neuronal inputs, as well as retaining a high proliferative potential in the human brain, makes this cell type another interesting glial cell in the context of TSC.

So far, only one study directly characterized NG2 cells in TSC tubers (52). Although these authors concluded that there were no detectable morphological alterations in oligodendrocytes and NG2 cells, they also acknowledged the lack of knowledge concerning specific activation markers for these cell types. Moreover, this study mainly evaluated morphological changes from an astrocyte perspective and did not convincingly rule out functional changes in NG2 glia (52). Because mTOR is an essential regulator of oligodendrocyte differentiation during development (157), its therapeutic potential was investigated by several studies. Moreover, the OPC-specific NG2 proteoglycan appears be directly linked to mTOR and to positively regulate its activity (183). Deletion of either *TSC1* or phosphatase and tensin homolog (*PTEN*) in NG2 cells, both negatively regulating mTOR, induced an increase in mTORC1 activity. Interestingly, whereas *TSC1* deletion in these cells led to the expected hypomyelination and impaired oligodendrocyte development, ablation of *PTEN* resulted in enhanced NG2 glia proliferation and oligodendrocyte lineage progression. This suggests the involvement of an mTORC1-independent PTEN-downstream signaling process. Further, also deletion of the PTEN-AKT downstream target glycogen synthase kinase 3β (*GSK3β*) resulted in a comparable increase in differentiation of oligodendrocytes (184). These results may indicate a possible remyelination mechanism via inhibition of the PTEN-AKT-GSK3β pathway. McLane et al. (185) further revealed that an ablation of *TSC1* affects oligodendroglia differently, depending

on the olig odendroglial lineage stage. A deletion of *TSC1* from NG2 glia speeds up the remyelination process, although *TSC1*-deficient proteolipid protein–positive oligodendrocytes decelerate remyelination.

Although most research on NG2 glia focused on their function as OPCs, there are also emerging lines of evidence that link them to neuronal function and microglia-mediated neuroinflammation. In the context of TSC, it would be interesting to study models of mTOR activation specifically in gray matter NG2 cells, where they reportedly serve more diverse functions.

MICROGLIA

Opposed to other neuroglia that are brain-borne, microglia arise from yolk sac–primitive macrophages and invade the brain during development before the BBB is fully formed (186–190). This early migration is a well-preserved mechanism among species, thereby emphasizing the important role of microglia during brain development (188, 191–193). Indeed, the phagocytic function of microglia is most prominent during development as they are capable of phagocytosing newly formed neurons and synapses in the developing brain (194–196). In the adult brain, ramified microglia surveil the brain environment with their processes and migrate to areas of need in response to activation cues, such as chemokine signaling (197). In response to distress signals, microglia can become activated, which is accompanied by a variety of morphological and molecular changes (198, 199). In general, two states of activation can be distinguished: a proinflammatory state (classically M1) that allows immune responses against pathogens and dysfunctional cells, which at the same time might exert damage on surrounding healthy tissue; and an anti-inflammatory state (classically M2) that is thought to be central in repair processes (200–202). However, thanks to sequencing data, microglia activation was identified to be a continuum in which many subtypes can be distinguished (203–206). In addition to their classical role as resident immune-competent cells and noteworthy in the context of TSC, microglia were also shown to modulate neuronal activity directly (207) and can be activated in response to excessive neuronal activity in epilepsy (208).

Several studies have shown increased density and activation of microglia cells in the brains of TSC patients (45, 62, 110, 121, 209). In cortical tubers, microglia with an activated morphology were found throughout the lesional tissue, mostly localized in close proximity to dysmorphic neurons and giant cells with mTOR activation, indicated by phosphorylation of the mTOR target ribosomal protein S6 kinase (pS6K), as well as around blood vessels (62). Similarly, in other epilepsies characterized by mTOR activation, such as FCD, TLE, and Rasmussen encephalitis, increased expression of microglial markers has been found in the respective brain lesions (33, 209–213). Moreover, in TLE patients with glial scarring due to drug-resistant epilepsy, mTOR activation was mostly found in microglia and to a lesser extent in astrocytes (33). Functionally, these microglia have been suggested to have a damaging role as they were shown to colocalize with several proinflammatory markers and surrounded cells expressing caspase 3, indicating that they might be involved in apoptosis (62). Moreover, in FCD lesions and glioneuronal tumors, the number of microglia has been correlated with seizure frequency of the patients (210, 214). Although it remains difficult to pinpoint whether microglia activation is causative or consequential of neurological deficits in these pathologies, the colocalization with pS6-positive cells indicates that microglia respond to mTOR hyperactivation in TSC. Sun et al. (215) showed that microglia activation in FCD and TSC might be partially caused by reductions in the immune modulatory molecules CD47 and CD200 on neurons and their respective receptors on microglia. When exogenously introduced, these molecules could potentially exert anti-inflammatory effects on microglia by suppressing proinflammatory cytokines, such as IL-6 (216).

Several attempts have been made to study aberrant mTOR activation in microglia and its impact on their function. Zhao et al. (217) showed that deletion of the *Tsc1* gene in CX3CR1-expressing cells (referred to as $Tsc1^{CX3CR1}$), either congenitally or post-natally, increased microglial mTOR activity and their overall number in the cortex and hippocampus. All of the $Tsc1^{CX3CR1}$ mice developed spontaneous recurrent seizures around 5 weeks of age, as well as two-thirds of the post-natally induced conditional knockout mice. However, that same year, Zhang et al. (218) reported that CX3CR1-Cre driver lines in $Tsc1^{CX3CR1}$ animals target not solely the alleged microglia cells, but also NeuN- and rarely GFAP-positive cells. Therefore, they concluded that the effects seen in $Tsc1^{CX3CR1}$ mice were not exclusively driven by reactive microglia but could also be elicited by affected neurons and astrocytes. Furthermore, post-natal induction of the knockout, which had a higher specificity for microglia showing only 5% of non-microglial cells affected, did not result in spontaneous ictal activity (218), in contrast to the previous study (217). These studies emphasize that it is essential to precisely target and characterize cell types in TSC KO models, as only small percentages of affected neurons can lead to increased neuronal excitability (219). Nevertheless, isolated microglia from $Tsc1^{CX3CR1}$ animals displayed clear cellular alterations. Thus, these two studies support the role of mTOR-dependent microglial abnormalities, and its role in epileptogenesis, especially in the context of inflammation, cannot be excluded.

In another model, direct manipulation of the mTOR pathway was induced by *in utero* electroporation of constitutively active Rheb, an mTORC1 activator. With this method, Nguyen et al. (73) observed that mTOR hyperactivity resulted in a global increase in Iba1-positive microglia that were both larger and had a more activated morphology. Furthermore, these Iba1-positive microglia were positively correlated with seizure frequency. However, with this technique, not only microglial cells were targeted. Indeed, mTOR activation also induced hypertrophy and cytoarchitectural misplacement of neurons in these animals, which together with the activated microglia were concluded to be responsible for seizure generation. Finally, in the BV2 microglial cell line direct activation of mTOR by MHY1485 treatment *in vitro* induced expression of proinflammatory cytokines, such as TNF-α, IL-6, and HMGB1, and decreased anti-inflammatory cytokines, such as TGF-β and IL-10. Furthermore,

microglia displayed a shift from an anti-inflammatory toward a proinflammatory subtype, and markers of autophagy were reduced due to mTOR activation (220).

Besides direct genetic or chemical activation of the mTOR signaling pathway, the majority of research on the interaction of microglia and mTOR is supported by experiments that evaluated microglia in disease states characterized by increased mTOR activity through various brain injuries and/or by means of chemical mTOR inhibition. For example, pilocarpine-induced SE resulted in mTOR activation in neurons and microglia, and subsequent rapamycin treatment could alleviate microgliosis and had beneficial effects on cognitive performance of animals (221). Moreover, kainic acid–injected rats treated with rapamycin displayed reduced microglial activation (35). In contrast, rapamycin treatment in an electrically induced post-SE model did not change expression of inflammation markers or number of CD11b/c and CD68-positive cells, indicating that rapamycin did not affect brain inflammation in this model. Other studies have used brain injuries, such as stroke or vascular dementia in combination with mTOR blockage to evaluate microglial changes. Treatment with rapamycin or its derivatives everolimus or sirolimus could revert medial cerebral artery occlusion (MCAO)–induced increases of cytokines and/or chemokines, as well as promote anti-inflammatory microglial polarization (222, 223). In this same study, Raptor^{CX3CR1} mice, characterized by lacking regulatory-associated protein of mTOR (Raptor) specifically in microglia leading to mTORC1 activation, undergoing MCAO were found to have similar beneficial responses to chemical mTOR inhibition in terms of microglia activation and cytokine induction. Treatment with everolimus in mice with bilateral common carotid artery stenosis, a vascular dementia model that induces mTOR activation, also caused a shift toward anti-inflammatory microglia due to a loss of inhibitory feedback of mTORC1 on PI3K, alternatively activating the prosurvival kinase Akt (224). Likewise, spinal cord injury induced increases in OX42-positive microglia, which could be attenuated by treatment with wortmannin, an inhibitor of the PI3K/Akt/mTOR pathway (225). Interestingly, according to Yang et al. (226), because of its ability to also interact with mTORC2, everolimus is more effective than rapamycin in counteracting lipopolysaccharide (LPS)/kainic acid–induced microglial responses. Of note, some authors argued that the anti-inflammatory effect of mTOR inhibitors might be mediated primarily by other cell types than microglia (222). Despite these claims, in pure microglia cell cultures, such as the BV2 and N9 cell line, inhibition of mTOR activity after oxygen glucose deprivation, LPS, or a cytokine challenge reduced both microglia activation and inflammation (224, 227–229). Furthermore, LPS-stimulated N9 microglia even exerted neuroprotective effects after rapamycin treatment, as conditioned medium could suppress neurotoxicity in a neuronal cell line (229). Lastly, in a kainic acid–induced SE model, the long-term epileptogenic effects of early life seizures could be reduced via treatment with an inhibitor of microglia activation, minocycline, directly linking microglial activation and epileptogenesis (230).

Finally, assuming microglial activation secondary to mTOR-driven malformations of cortical development in TSC, depletion of resident microglia, and repopulation of the brain with fresh microglia might offer a drastic, yet promising therapeutic option to resolve chronic neuroinflammation (231). Importantly, this approach could relieve the neuroinflammatory burden in the post-natal TSC brain even after aberrant brain development. Adjunctive with mTOR inhibitors, this approach could target source (mTOR hyperactivation) and symptom (microglia activation) of TSC brain malformations simultaneously and offer a valuable disease-modifying therapy.

INTERGLIAL CROSSTALK

While neuron–glia interactions are the focus in many of the studies discussed here, interactions between glia may offer new therapeutic and diagnostic opportunities (**Figure 2**). In the context of neuroinflammation, bilateral signaling between microglia and astrocytes likely plays an essential role in brains of TSC patients. For example, *Tsc1*^{GFAP} mice do not only display alterations in astrocytes, but microglia number and size were abnormally increased in cortex and hippocampus, pointing toward an indirect effect of mTOR hyperactivation in astrocytes on microglia (232). However, the importance of microglia in the induction of a reactive phenotype in astrocytes has been shown (233), and *Tsc1*^{CX3CR1} mice also display increased proliferation and reactive changes of astrocytes (217). Together, this reinforcing crosstalk might be crucial for the maintenance of a proinflammatory environment in TSC with contributions from functionally normal glia, as well as glia with cell-autonomous mTOR-related alterations. The effect of microglia is likely contributing to the proinflammatory environment of TSC tubers as their functions involve inflammation initiation and propagation in conjunction with astrocytes (233, 234). Moreover, microglia activation may exert proinflammatory/damaging effects on oligodendrocytes and neurons, contributing to neuronal dysfunction and resulting neurological comorbidities and hyperexcitability (235, 236).

Next to microglia, particularly interesting in the context of interglial crosstalk in TSC is the role of astrocytes to directly influence the production and survival of cells of the oligodendrocyte lineage (237, 238). Accordingly, reactive and enlarged dysplastic astrocytes with enhanced activation of mTOR and gain of aberrant functions in cortical tubers, including a proinflammatory phenotype, may pose detrimental in the function of other glia in TSC. In support of this, there is a growing body of evidence that supports the concept of astrocytopathies within the field of childhood white matter disorders in which dysfunctional astrocytes have been suggested to drive degeneration of the white matter (239, 240). As mentioned previously, astrocytic gap-junction coupling in TSC models is disturbed (85), and heterotypic gap-junction coupling between astrocytes and oligodendrocytes was shown to be essential for (re-)myelination in animal models (241–243). Moreover, dysregulation of glutamate metabolism by astrocytes in TSC (72, 88, 89) could promote excitotoxic cell death in oligodendrocytes as they express functional *N*-methyl-D-aspartic acid, AMPA, and kainate-type receptors that mediate toxic effects

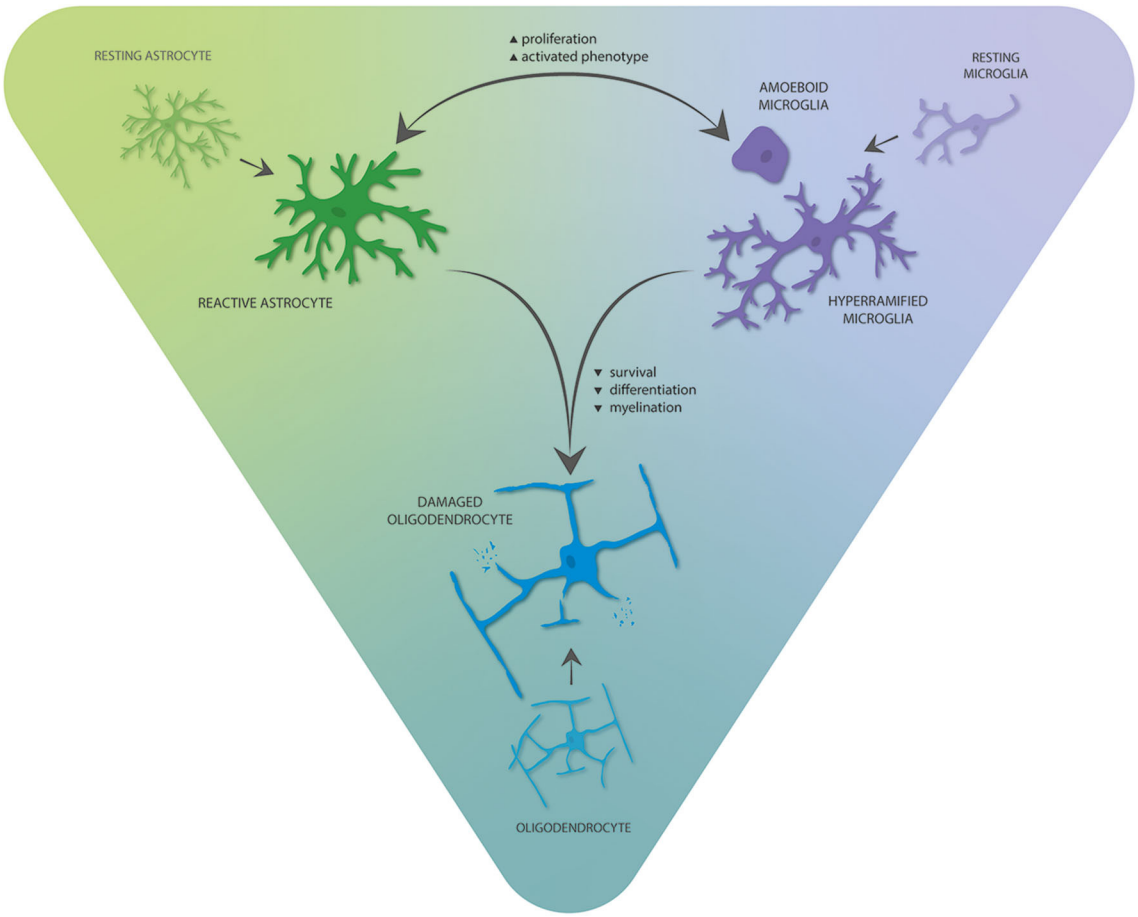

FIGURE 2 | Interglial crosstalk of the three main glial cell types in the TSC brain. Astrocytes and microglia can stimulate and reinforce proliferation and phenotypic activation of each other, thereby promoting proinflammatory gene expression. These alterations mediate negative consequences on oligodendrocyte survival, differentiation, and myelination.

of excess glutamate (244–247). Moreover, astrocyte-specific NF-κB activation in TSC might also play a role in suppressing myelination (248). Lastly, evidence from the "twitcher" mouse model supports the role of microglial COX-2 in demyelination. Here, secreted microglial prostaglandins (PGDs) could stimulate PGD receptors on astrocytes, inducing astrogliosis as indicated by hypertrophy and a rise in intracellular calcium, and blocking this pathway increased oligodendrocyte survival (249). Because increased COX-2 expression is observed not only in glia, but also giant cells/balloon cells and dysmorphic neurons in TSC and FCD (98), this specific crosstalk might link hypomyelination to COX-2 expression.

As for mature oligodendrocytes, NG2 function in TSC likely depends on other glia. *In vitro*, it was shown that astrocyte- and microglia-conditioned medium exerts important effects in the development of oligodendrocytes, with astrocytic factors promoting oligodendrocyte survival and microglial factors supporting differentiation and myelination (250). Considering aberrant number and function of both cell types already early in development, this interglial crosstalk might contribute to the hypomyelination observed in TSC. Moreover, NG2 glia survival and differentiation can be impaired by OS and TNF produced by activated microglia (251–253). In essence, astrocytes and microglia could participate in the pathological link between OS, inflammation, and the dysregulated iron metabolism in TSC by inducing aberrant oligodendrocyte maturation and myelination in TSC (252, 254). Importantly, OS-dependent dysregulation of histone–deacetylase activity could promote astrogenesis/neurogenesis over oligodendrogenesis potentially contributing to the disturbed cell ratio observed in TSC brain tissue (252).

CONCLUSIONS

While the most debilitating CNS symptoms of TSC, epilepsy, and neurodevelopmental comorbidities ultimately result from neuronal dysfunction, it is also clear that glial alterations contribute and shape the complex mechanisms generating these symptoms. Moreover, glia provide the proliferative precursor

of pre-natal and post-natal neurons in the form of radial glia and astrocytes, respectively. It is important to stress that in TSC there is likely a mixture of cells with cell-autonomous mTOR activation because of intrinsic TSC mutations and cells with regular mTOR activity that respond to changes due to this intrinsically dysfunctional cellular substrate. Nevertheless, the major triad of glial cells displays conserved features in response to mTOR activation in TSC, TSC models, and conditions of mTOR hyperactivation.

Although it is likely that increased proliferation of astrocytes and resulting physical disruption of neuronal circuits can impact epileptogenesis in TSC, studies on surgically resected tubers and TSC models suggest that astrocytes also present with epileptogenic functional changes. More importantly, these changes are likely caused by a mixture of primary astrocytic changes during brain development due to mTOR activation and secondary effects that promote reactive states of astrocytes, such as brain inflammation later on. Finally, astrocyte dysfunction in TSC recapitulates findings from other epileptogenic pathologies, thus potentially representing common astrocytopathic mechanisms of epilepsy that could be targeted by novel astrocyte-centered therapies.

For oligodendrocytes, it is of utmost interest to find targets by which the endogenous remyelination in TSC patients might be enhanced. The mTOR signaling pathway has been proposed to be an attractive target to promote remyelination; however, recent results emphasize the importance of correctly applied therapeutics, because what may be beneficial to OPC development might be noxious to myelinating oligodendrocytes.

Lastly, alterations in microglial functions in TSC might be caused by cell-autonomous mTOR activation or secondary to the altered brain environment in TSC. Whether their activation depends on either or both is not clearly defined yet; however, the presence of proinflammatory microglia upon mTOR activation likely contributes to pathology, while a shift toward an anti-inflammatory phenotype via mTOR inhibition might have beneficial effects.

Although challenging, a better understanding of the complexity of the glial pathology in TSC may provide opportunities for novel therapeutic approaches targeting glial-mediated mechanisms. In particular, a combinatorial therapy targeting different glial cell types and their crosstalk might be translated into disease-modifying treatments for various epilepsies associated with a deregulation of mTOR. Considering the evidence for mTOR inhibition not only rescuing neuronal, but also glial dysfunction, in preclinical TSC models (29, 255, 256), mTOR inhibitors, which were recently approved by the US Food and Drug Administration and European Medicines Agency for the treatment of refractory seizures associated to TSC starting from the age of 2 years (257), represent promising candidates to target TSC gliopathy. Finally and most importantly, mTOR inhibition as therapy of TSC could potentially be extrapolated to other genetic and acquired epilepsies (258, 259).

AUTHOR CONTRIBUTIONS

TZ and EA drafted the content of this review. TZ, DB, VG, EV, and AM contributed the specific topics and assisted in the final editing and revision of the manuscript. EA conceived the idea and was invited to participate in the editorial issue. All authors contributed to the article and approved the submitted version.

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Differential Glial Activation in Early Epileptogenesis—Insights From Cell-Specific Analysis of DNA Methylation and Gene Expression in the Contralateral Hippocampus

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Background and Aims: Morphological changes in mesial temporal lobe epilepsy with hippocampal sclerosis (mTLE-HS) are well-characterized. Yet, it remains elusive whether these are a consequence of seizures or originate from a hitherto unknown underlying pathology. We recently published data on changes in gene expression and DNA methylation in the ipsilateral hippocampus (ILH) using the intracortical kainate mouse model of mTLE-HS. In order to explore the effects of epileptic activity alone and also to further disentangle what triggers morphological alterations, we investigated glial and neuronal changes in gene expression and DNA methylation in the contralateral hippocampus (CLH).

Methods: The intracortical kainic acid mouse model of mTLE-HS was used to elicit status epilepticus. Hippocampi contralateral to the injection site from eight kainate-injected and eight sham mice were extracted and shock frozen at 24 h post-injection. Glial and neuronal nuclei were sorted by flow cytometry. Alterations in gene expression and DNA methylation were assessed using reduced representation bisulfite sequencing and RNA sequencing. The R package edgeR was used for statistical analysis.

Results: The CLH featured substantial, mostly cell-specific changes in both gene expression and DNA methylation in glia and neurons. While changes in gene expression overlapped to a great degree between CLH and ILH, alterations in DNA methylation did not. In the CLH, we found a significantly lower number of glial genes up- and downregulated compared to previous results from the ILH. Furthermore, several genes and pathways potentially involved in anti-epileptogenic effects were upregulated in the CLH. By comparing gene expression data from the CLH to previous results from the ILH (featuring hippocampal sclerosis), we derive potential upstream targets for epileptogenesis, including glial *Cox2* and *Cxcl10*.

Conclusion: Despite the absence of morphological changes, the CLH displays substantial changes in gene expression and DNA methylation. We find that gene expression changes related to potential anti-epileptogenic effects seem to dominate compared to the pro-epileptogenic effects in the CLH and speculate whether this imbalance contributes to prevent morphological alterations like neuronal death and reactive gliosis.

Keywords: epilepsy, NeuN, TLE, glia, neuron, gene expression, DNA methylation, epigenetics

INTRODUCTION

Epileptogenesis describes the transformation of a normally functioning brain into an epileptic brain (1, 2). For mesial temporal lobe epilepsy with hippocampal sclerosis (mTLE-HS), this process often involves an initial incident (i.e., prolonged febrile seizure, inflammation, or cerebral trauma), followed by a clinically silent latent phase, and, ultimately, seizures of increasing frequency and severity (3). Pathological hallmarks of mTLE-HS are well-characterized in both humans and in animal models and predominantly consist of progressive neuronal cell death and reactive gliosis (4–14). The underlying mechanisms of these features remain elusive, and their further disentanglement is of paramount importance for the development of truly anti-epileptogenic drugs (15, 16).

In this paper, we use a combined analysis of cell-specific gene expression and DNA methylation to investigate epileptogenesis in a mouse model for mTLE-HS. Gene expression by means of RNA sequencing is a well-established approach for investigating biological function (17, 18). A cell-specific approach, i.e., the separation of neurons and glia prior to downstream analysis, has been used in various previous studies (19–21) and facilitates the detection of more subtle effects and the determination of the cellular origin of the observed DNA methylation and gene expression alterations (22).

DNA methylation contributes to cell-specific gene expression (23–26) and is altered in both epileptic human tissue (27) and animal models of epilepsy (22, 28–30). Amendable by, among other things, neuronal activity (31), nutrition (32), and newer epigenetic tools (33), it represents a modifiable potential upstream mechanism in epileptogenesis.

We recently published a study on neuronal and glial DNA methylation and gene expression changes at 24 h post-kainate-induced status epilepticus, a time point relevant to early epileptogenesis (11, 34). These findings from the ipsilateral hippocampus (ILH) revealed a number of significant gene expression alterations in both neurons and glia. We further found a number of epilepsy-relevant genomic loci with a significant association of differential gene expression and differential DNA methylation (22). These observations originated from the intracortical kainate mouse model, where both hippocampi are exposed to epileptic activity but only the ILH gradually develops morphological changes (e.g., neuronal death and reactive gliosis) similar to human mTLE-HS (11) (**Figure 1**). In contrast, the contralateral hippocampus (CLH) is only exposed to epileptic activity and regarded as "free from morphological alterations" (11, 35). As such, it is often used as an internal control for the ILH (11).

The aims of this study were to on glia- and neuron-mediated downstream effects of epileptic activity based on gene expression changes in the CLH. We further elaborated on potential upstream targets for hippocampal sclerosis and epileptogenesis within genes exclusively differentially expressed in the ILH (and not CLH). Lastly, we explored the potential role of DNA methylation on cell-specific gene expression in early epileptogenesis.

Abbreviations: *CLH*, contralateral hippocampus comparison (specifically, comparison of the contralateral hippocampi of the kainic acid group *vs.* the sham group); *GO*, Gene Ontology; *ILH*, ipsilateral hippocampus comparison (specifically, comparison of the ipsilateral hippocampi of the kainic acid group *vs.* the sham group); *KEGG*, Kyoto Encyclopedia of Genes and Genomes; *mRNAseq*, mRNA sequencing; *mTLE-HS*, mesial temporal lobe epilepsy with hippocampal sclerosis; *RRBS*, reduced representation bisulfite sequencing.

METHODS

The methods applied in this study follow the same protocol as previously described in Berger et al. (22). Raw data are available under GEO accession GSE153976.

Animals

Adult male C57/BL6N mice (Janvier Labs, France), acquired at an age of 8 weeks, were acclimatized for 4 weeks in a controlled environment (21–23°C, 12-h dark/light cycles). One to four animals were housed per cage, with water and food available *ad libitum*. All animal procedures were approved by the Norwegian Food Safety Authority (project number FOTS: 14198), the Center for Comparative Medicine, Oslo University Hospital and the University of Oslo.

Intracortical Kainic Acid Mouse Model of Mesial Temporal Lobe Epilepsy With Hippocampal Sclerosis

We used the intracortical mouse model for mTLE-HS, described in detail by Bedner et al. (11), to elicit status epilepticus. In brief, the mice were anesthetized with a mixture of medetomidine (0.3 mg/kg, i.p.) and ketamine (40 mg/kg, i.p.) and kept on a heating blanket. For mice in the kainic acid group, kainate (70 nl, 20 mM, Tocris) was injected above the right

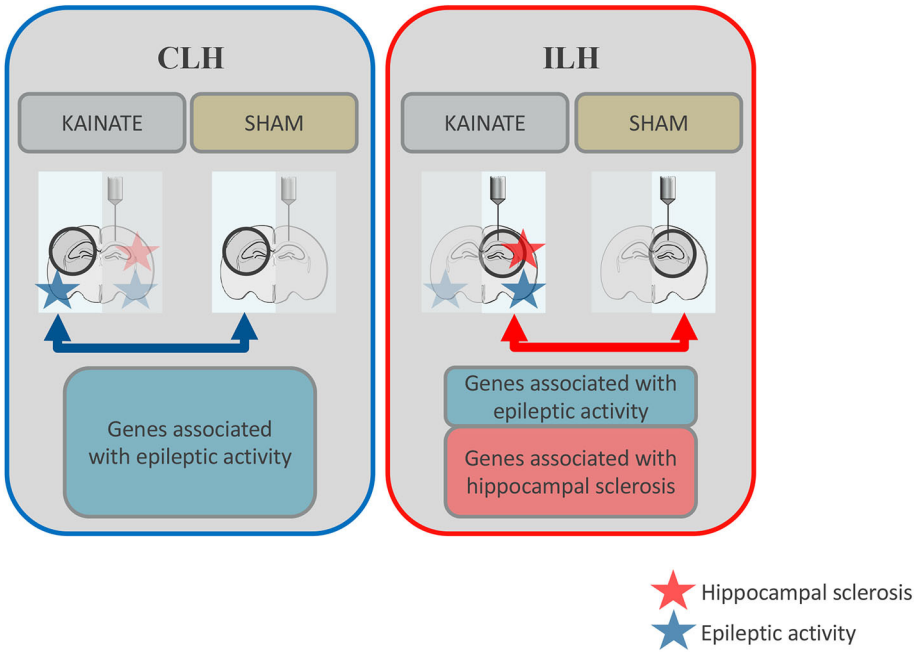


FIGURE 1 | Schematic overview: Differential gene expression in the contralateral and ipsilateral hippocampi at 24 h after status epilepticus induction. Both the ipsilateral and contralateral hippocampi were exposed to epileptic activity (upon a status epilepticus lasting several hours), but only the ipsilateral hippocampus gradually develops morphological alterations such as reactive gliosis and neuronal death (hippocampal sclerosis). In this study, gene expression was compared between epileptic mice (kainate injected over the right, ipsilateral, hippocampus) and sham mice (saline injected at the same location) for the contralateral hippocampus (CLH, *blue arrow*). This data was compared to previous data on gene expression from the ipsilateral hippocampus (ILH, *red arrow*). Differentially expressed genes associated with epileptic activity are represented by the *blue boxes*. Differentially expressed genes potentially associated with morphological alterations typical of mesial temporal lobe epilepsy with hippocampal sclerosis (mTLE-HS) are represented by the *red box*.

hippocampus (= ipsilateral) by a Hamilton pipette (Hamilton Company, NV) at a depth of 1.7 mm at anteroposterior −2 mm, lateral +1.5 mm in relation to bregma. After the procedure, anesthesia was stopped with atipamezole (300 mg/kg, i.p.). Buprenorphine (0.1 mg/kg, s.c.) was applied at 4 and 12 h after the intervention. In order to ensure successful execution of technical procedures, only animals displaying convulsive seizures (Racine grade 5) within the first 4 h after termination of the procedures were included in further analysis. For sham animals, 0.9% NaCl was used instead of kainate for the intracortical injection.

Tissue Collection and Pooling

Cervical dislocation was performed 24 h after status epilepticus, and hippocampi were extracted. Thereafter, each hemisphere was placed in a 2-ml polypropylene tube, instantly shock frozen in liquid nitrogen, and stored at −80°C. Left hippocampi (= contralateral) were pooled in 2-ml tubes from four (kainic acid group, $n = 4$; sham group, $n = 4$) or two (kainic acid group, $n = 4$; sham group, $n = 4$) mice prior to further processing. The number of mice amounted to eight per group (eight kainic acid and eight sham) and the number of biological samples to three per group (three samples in the kainic acid and three samples in the sham group). Tissue was kept on dry ice during pooling.

Fluorescence-Activated Nuclear Sorting

Cell nuclei were sorted into NeuN+ nuclei (referred to as neurons) and NeuN– nuclei (referred to as glia) by a modified version of Jiang et al. (36) (for technical limitations and restrictions in interpretability, see *Limitations*). Hippocampi were placed on ice immediately after pooling, and 1 ml homogenization buffer was added. GentleMACS dissociator (Miltenyi) was used to homogenize the tissue. The homogenate was subsequently filtered through a 70-μm filter and debris removed by density gradient centrifugation using Debris Removal Solution (Miltenyi). Nuclear pellets were resuspended in 100 μl incubation buffer per 1×10^6 nuclei and Anti-NeuN Alexa Fluor 488 (Merck Millipore) added (0.1 μg/ml per sample). The samples were incubated for 1 h on ice, protected from light. Adult mouse liver was used as a NeuN-negative control sample and processed in parallel with hippocampal tissue. The nuclei were sorted into NeuN+ and NeuN– fractions using a FACSAria (BD Biosciences), followed by centrifugation, and pellets were resuspended in lysis buffer for DNA and RNA isolation. For further details, see **Supplementary Document**.

Isolation of DNA and Total RNA From Sorted Nuclei

MasterPure Complete DNA and RNA Purification Kit (Epicenter) was used to extract DNA from sorted nuclei.

DNA purity was evaluated on NanoDrop and the DNA concentration assessed on Qubit (DNA HS assay). Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) and RNA was up-concentrated with the RNA Clean & Concentrator-5 kit (Zymo Research). RNA integrity and concentration were analyzed on Bioanalyzer with the RNA Pico Kit (Agilent Technologies). For further details, see **Supplementary Document**.

Reduced Representation Bisulfite Sequencing

A modified version of the gel-free protocol provided by Boyle et al. (37) was used for reduced representation bisulfite sequencing (RRBS) library preparation. Main changes comprised the inclusion of a two-sided size selection before bisulfite conversion and sample pooling after completion of single libraries. Libraries representing the contralateral and ipsilateral hemispheres were prepared and sequenced in parallel, and sequencing pools contained either 14 libraries run twice on NextSeq500 (50% PhiX spike-in, 75-bp single reads) or15 libraries sequenced over two lanes on HiSeq2500 (10% PhiX spike-in, 50-bp single reads). The library preparation procedure is described in detail in **Supplementary Document**.

High-Throughput mRNA Sequencing

SMART-Seqv4 Ultra Low InputRNA Kit for Sequencing (Takara Bio) was used to amplify messenger RNA (mRNA) from total RNA, and the resulting complementary DNA (cDNA) was used as the input in library preparation with the ThruPlex DNAseq Kit (Rubicon Genomics). Libraries representing the contralateral and ipsilateral hemispheres were prepared and sequenced in parallel, and sequencing pools contained either 12 libraries sequenced on NextSeq500 (75-bp single reads) or 27 libraries sequenced over three lanes on HiSeq3000 (150-bp paired-end reads). Details regarding mRNA sequencing (mRNAseq) library preparation are given in **Supplementary Document**.

Computational Methods

Bioinformatic Handling and Quality Control of mRNAseq Data

The mRNAseq reads were trimmed with Trim Galore! v0.4.3 and aligned by the Rsubread (the R interface of the Subread software) (38). Quality control of the BAM files was done with Picard/CollectRnaSeqMetrics. The featureCounts function of Rsubread was used for counting the number of reads mapping uniquely to each gene, based on the comprehensive gene annotation for mm10 in the GENCODE release M16 (www.gencodegenes.org/mouse/release_M16.html). Only reads aligning to mRNA regions were used in further analysis.

The expression levels (normalized counts) of a neuronal gene (*Rbfox3*), glial genes (*Aldh1l1*, *Cx3cr1*, and *Mbp*), as well as pericyte (*Pdgfrb*) and endothelial (*Pecam1*) genes were visualized to verify the enrichment of neurons and glia in the NeuN+ and NeuN− fractions. In order to validate our cell sorting procedures and discover outliers, a multidimensional scaling plot of the mRNAseq data was produced. For this, we used the edgeR function plotMDS to compute point coordinates, using the top 100 most variable genes, and ggplot2 (39) to produce the final plots.

Bioinformatic Handling and Quality Control of RRBS Data

The RRBS raw data underwent trimming with Trim Galore! v0.4.3, with parameters "–rrbs–illumina," and quality control with FastQC. Alignment was done with Bismark v0.20 (powered by Bowtie2) using the mouse genome mm 10 as reference. The Picard tool CollectRrbsMetrics v2.18.15 was used for quality control of the BAM files.

An MDS plot of the RRBS data set was produced in a similar fashion to the mRNAseq, using the 100 most variable loci.

The bisulfite conversion rates were estimated in two ways. Firstly, by Picard/CollectRrbsMetrics, which measures the conversion of non-CpG cytosines. This statistic may be unreliable in neurons, where the methylation of non-CpG cytosines occurs with non-negligible frequency. To account for this, we also performed an alternative estimate of the conversion rates directly from the untrimmed fastq files, targeting the (unmethylated) cytosines added in the end-repair step of the RRBS preparation (private bash script). Samples whose conversion rate estimates were below 98% in both methods were excluded.

Annotation

Coordinates of the genes, exons, and introns were obtained from the M16 release of GENCODE's comprehensive annotation, restricted to autosomal genes. Annotation of CpG sites was performed with the R package annotatr (40), supplying details of the gene regions overlapping each CpG. In particular, promoter regions were defined as the 1-kb segments upstream of the transcription start sites, and upstream regions were defined as ranging from −5 to −1 kb relative to the transcription start sites.

Analysis of Differential Gene Expression

Analysis of differential gene expression between the kainic acid group and the sham group samples was performed with the R package edgeR (41). Preparatory steps included removal of genes without the official HGNC symbol, removal of genes with a low read count (determined by the edgeR function filterByExpr with default parameters), and normalization adjusting for different library sizes (done with calcNormFactors). The differential gene expression analysis followed a standard edgeR workflow based on a quasi-likelihood negative binomial generalized log-linear model fitted to the count data. Data from glial and neuronal cells were analyzed separately. The significance threshold was set to a false discovery rate (FDR) of 25%.

Analysis of Differential DNA Methylation

Loci exhibiting differential DNA methylation between the kainic acid group and the sham group samples were identified with edgeR, following a workflow for RRBS data recently published by the edgeR authors (42). In brief, this treats the methylated and unmethylated counts at each locus as independent variables following a negative binomial distribution. As for differential gene expression, the differential DNA methylation analysis was carried out separately for neuronal and glial cells, with a

FDR of 25% as the significance threshold. Preparatory steps included removing all CpG sites where more than 10% of the samples had either very low coverage (< 8 reads) or very high coverage (>99.5 quantile across all sites and samples). In addition to the a standard differential DNA methylation analysis of individual CpG sites, aggregated analyses were performed for various genomic regions defined by the gene annotation, including upstream segments, promoters, UTR5's, exons, introns, gene bodies (i.e., the union of all exons and introns of a specific gene), and UTR3's. For the aggregated analysis, the input was the mean counts across all the covered CpGs within the region.

Combined Differential Gene Expression and Differential DNA Methylation Analysis

In order to identify genes for which both gene expression and DNA methylation differed significantly between the kainic acid group and sham group, a combined analysis of differential gene expression and (aggregated = differentially methylated regions) differential DNA methylation was performed for each genomic feature. For each feature type (upstream, promoter, UTR5, exon, intron, gene body, and UTR3), only the genes surviving filters in the corresponding aggregated differential DNA methylation data set were kept and used as inputs in a new differential gene expression analysis. Co-incidence of differential gene expression and differential DNA methylation was declared for features surviving a FDR cutoff of 25% in both analyses.

Functional Enrichment Analysis

Enrichment analyses of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed with the goana and kegga functions of edgeR, with the parameter species = "Mm".

Selection of Relevant Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Terms

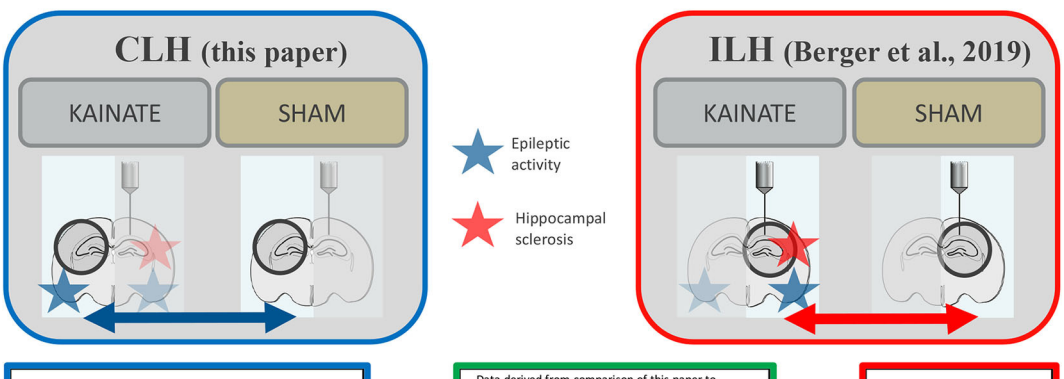
Epileptogenesis-relevant GO and KEGG terms in neurons and glia were selected manually among the complete lists of respective terms in **Supplementary Table** (sheets 2, 3, 5, 6, 22, 23, 25, and 26) based on reviews on the subject (4, 15) and personal knowledge.

RESULTS

A systematic overview of all data is given in **Figure 2**.

Quality Control

The bisulfite conversion rates of the included samples were above 98% (**Supplementary Figure 1**). The multidimensional scaling



Data from this paper

CLH			
	mRNAseq / DGE		
cell type	list	GO	KEGG
neuron	Table 1 / sheet 1	sheet 2	sheet 3
glia	Table 2 / sheet 4	sheet 5	sheet 6
	RRBS DMCpGs		
cell type	list	GO	KEGG
neuron	sheet 7	sheet 8	sheet 9
glia	sheet 10	sheet 11	sheet 12
	RRBS DMR		
cell type	list	GO	KEGG
neuron	sheet 13	sheet 14	sheet 15
glia	sheet 16	sheet 17	sheet 18
cell type	DMR+DGE		
neuron	sheet 19		
glia	sheet 20		

Data derived from comparison of this paper to Berger *et al.* 2019

exclusively ILH (and not CLH)			
	mRNAseq / DGE		
cell type	list	GO	KEGG
neuron	Table 5 / sheet 21	sheet 22	sheet 23
glia	Table 6 / sheet 24	sheet 25	sheet 26

Data from Berger *et al*., 2019

ILH	
cell type	mRNAseq / DGE
neuron	sheet 27
glia	sheet 28

FIGURE 2 | Data set overview. Overview of provided data in paper (**Tables 1**, **2**, etc.) and **Supplementary Table** (sheets 1, 2, etc.) from the CLH (comparison of the contralateral hippocampi of the kainic acid group *vs*. sham group) and the ILH (comparison of the ipsilateral hippocampi of the kainic acid group *vs*. sham group as in Berger et al. (22).

plots for RRBS and mRNAseq (**Supplementary Figure 2**) clearly distinguished NeuN+ (referred to as neurons) and NeuN– (referred to as glia) fractions. As shown in the normalized mRNAseq plots (**Supplementary Figure 3**), the NeuN+ fraction was enriched for neuronal mRNA and the NeuN– fraction for glial mRNA. For further details regarding quality control of the differential gene expression and differential DNA methylation data, see **Supplementary Document**.

Differential Gene Expression in the Hippocampus Contralateral to Kainate Injection

For the analysis of differentially expressed genes, mouse hippocampi contralateral to the kainate injection site were compared to the corresponding hippocampi of sham (saline)-treated animals 24 h post-injection. Differential gene expression was measured in neurons and glia separately. In neurons, 115 genes were upregulated and 16 downregulated (ratio of upregulated to downregulated genes, 7.19) (**Table 1** and **Supplementary Table**, sheet 1). In glia, 74 genes were upregulated and 22 downregulated (ratio of upregulated to downregulated genes, 3.36) (**Table 2** and **Supplementary Table**, sheet 4). We found an overlap of 27 genes differentially expressed in both neurons and glia. All of these were upregulated in both cell types. Comprehensive results from the GO term analysis and KEGG pathway analysis can be found in **Supplementary Table** (sheets 2, 3, 5, and 6). Selected results considered relevant for epileptogenesis are listed in **Table 3** for neurons and **Table 4** for glia cells.

Comparison of Differential Gene Expression Between the Contralateral and Ipsilateral Hippocampus

Comparison of differential gene expression in CLH to ILH (22) revealed similar numbers of differentially expressed genes for neurons, while there was a marked difference in the glia. In the neuronal fraction, 115 genes were upregulated in the CLH (**Table 1** and **Supplementary Table**, sheet 1) while 132 genes were upregulated in the ILH (**Supplementary Table**, sheet 27). Sixteen genes were downregulated in the CLH (**Table 1** and **Supplementary Table**, sheet 1) and 15 genes downregulated in the ILH (**Supplementary Table**, Sheet 27). In glia, only half of the number of genes were upregulated in the CLH (74 genes; **Table 2** and **Supplementary Table**, sheet 4) compared with the ILH (147 genes; **Supplementary Table**, sheet 28). The difference was even more pronounced for the downregulated genes (22 in the CLH *vs*. 85 in the ILH).

Overlap of Differentially Expressed Genes in the Contralateral and Ipsilateral Hippocampus

A comparison of genes differentially expressed in the CLH to those differentially expressed in the ILH (**Supplementary Table**, sheets 27 and 28) (22) revealed that a large number of differentially expressed genes coincided between CLH and ILH. This was the case both in the neurons and glia (**Figure 3**).

TABLE 1 | Differentially expressed genes in neurons in the contralateral hippocampus (CLH) at 24 h after kainate-induced status epilepticus.

Gene symbol	logFC	FDR	Gene description
UPREGULATED GENES (*N* = 115)			
Sdc1	**4.79**	**0.00**	**Syndecan 1**
Socs3	4.34	0.00	Suppressor of cytokine signaling 3
Cd1d1	3.99	0.00	CD1d1 antigen
Col27a1	**3.87**	**0.00**	**Collagen, type XXVII, alpha 1**
Gal	**6.07**	**0.00**	**Galanin**
Inhba	3.64	0.00	Inhibin beta-A
Lhfp	1.96	0.01	Lipoma HMGIC fusion partner
Ccn4	2.51	0.01	Cellular communication network factor 4
Tnc	2.27	0.01	Tenascin C
Megf11	2.17	0.01	Multiple EGF-like-domains 11
Nptx2	3.47	0.01	Neuronal pentraxin 2
Gipr	3.98	0.01	Gastric inhibitory polypeptide receptor
Pmepa1	2.54	0.01	Prostate transmembrane protein, androgen induced 1
Parp3	**3.47**	**0.01**	**Poly (ADP-ribose) polymerase family, member 3**
Nedd9	**1.72**	**0.02**	**Neural precursor cell expressed, developmentally downregulated gene 9**
Egr2	2.62	0.02	Early growth response 2
Fosb	3.36	0.02	FBJ osteosarcoma oncogene B
Crispld2	2.96	0.02	Cysteine-rich secretory protein LCCL domain containing 2
Pros1	2.71	0.03	Protein S (alpha)
Vim	3.14	0.03	Vimentin
Rgs4	2.58	0.03	Regulator of G-protein signaling 4
Prss23	2.39	0.03	Protease, serine 23
Ptgs2	2.89	0.04	Prostaglandin-endoperoxide synthase 2
Trh	6.51	0.04	Thyrotropin-releasing hormone
Sik1	2.08	0.04	Salt inducible kinase 1
Tll1	3.71	0.04	Tolloid-like
Fgl2	2.53	0.04	Fibrinogen-like protein 2
Fos	**2.99**	**0.04**	**FBJ osteosarcoma oncogene**
Adgrf4	2.41	0.05	Adhesion G protein-coupled receptor F4
Bag3	2.04	0.05	BCL2-associated athanogene 3
Arc	**2.12**	**0.06**	**Activity regulated cytoskeletal-associated protein**
Csrnp1	2.45	0.06	Cysteine–serine-rich nuclear protein 1
Angptl4	2.41	0.06	Angiopoietin-like 4
Ccl12	3.71	0.07	Chemokine (C-C motif) ligand 12
1700071M16Rik	1.92	0.07	1700071M16Rik
Fam129b	1.48	0.07	Family with sequence similarity 129, member B
Cemip2	1.68	0.07	Cell migration inducing hyaluronidase 2
Bmp3	2.11	0.07	Bone morphogenetic protein 3
Trib1	2.01	0.09	Tribbles pseudokinase 1
Rara	1.84	0.09	Retinoic acid receptor, alpha
Syndig1l	1.95	0.09	Synapse differentiation inducing 1 like
Dmp1	2.01	0.09	Dentin matrix protein 1
Cdk18	2.34	0.09	Cyclin-dependent kinase 18
Trib2	1.81	0.09	Tribbles pseudokinase 2 (Source: MGI symbol)

(Continued)

TABLE 1 | Continued

Gene symbol	logFC	FDR	Gene description
Gadd45g	2.14	0.09	Growth arrest and DNA-damage-inducible 45 gamma
Serinc2	1.99	0.09	Serine incorporator 2
Vgf	2.20	0.09	VGF nerve growth factor inducible
Tpbg	1.70	0.09	Trophoblast glycoprotein
Sulf1	1.39	0.10	Sulfatase 1
Srxn1	1.89	0.10	Sulfiredoxin 1 homolog
Acvr1c	2.22	0.10	Activin A receptor, type IC
Timp1	3.26	0.11	Tissue inhibitor of metalloproteinase 1
Ptx3	3.28	0.11	Pentraxin-related gene
Gpr3	1.93	0.11	G-protein-coupled receptor 3
Homer1	1.66	0.12	Homer scaffolding protein 1
Clcf1	2.67	0.12	Cardiotrophin-like cytokine factor 1
Cd1d2	3.31	0.12	CD1d2 antigen
Pappa	2.35	0.13	Pregnancy-associated plasma protein A
C2cd4b	2.39	0.14	C2 calcium-dependent domain containing 4B
Atf3	3.58	0.14	Activating transcription factor 3
Fndc9	2.91	0.14	Fibronectin type III domain containing 9
Acan	1.68	0.14	Aggrecan
Sbno2	1.99	0.16	Strawberry notch 2
Stk40	1.60	0.16	Serine/threonine kinase 40
Trip10	1.61	0.16	Thyroid hormone receptor interactor 10
Nfkbie	2.08	0.16	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, epsilon
Anxa2	2.29	0.16	Annexin A2
Sphk1	2.41	0.16	Sphingosine kinase 1
Serpina3n	1.99	0.16	Serine (or cysteine) peptidase inhibitor, clade A, member 3N
Gfra1	1.63	0.16	Glial cell line derived neurotrophic factor family receptor alpha 1
Rasl11a	1.57	0.16	RAS-like, family 11, member A
Ier5	1.84	0.16	Immediate early response 5
Dgat2l6	3.38	0.16	Diacylglycerol *O*-acyltransferase 2-like 6
Hpgd	1.88	0.16	Hydroxyprostaglandin dehydrogenase 15 (NAD)
Pear1	3.10	0.16	Platelet endothelial aggregation receptor 1
Kif18a	1.63	0.16	Kinesin family member 18A
Prex1	1.98	0.16	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1
Plpp4	1.97	0.16	Phospholipid phosphatase 4
Adamts6	1.66	0.16	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif 6
Dusp5	**1.74**	**0.16**	**Dual specificity phosphatase 5**
Col5a3	1.99	0.18	Collagen, type V, alpha 3
Tnfrsf12a	2.04	0.18	Tumor necrosis factor receptor superfamily, member 12a
Ier2	2.09	0.18	Immediate early response 2
Tgfbr2	1.52	0.18	Transforming growth factor, beta receptor II
Ptgs1	**2.04**	**0.18**	**Prostaglandin-endoperoxide synthase 1**
Cdkn1a	2.41	0.18	Cyclin-dependent kinase inhibitor 1A
Cgref1	1.67	0.19	Cell growth regulator with EF hand domain 1
Arl4d	2.01	0.19	ADP-ribosylation factor-like 4D
Pipox	2.07	0.19	Pipecolic acid oxidase
Fosl2	1.62	0.19	Fos-like antigen 2
Pik3r6	1.76	0.20	Phosphoinositide-3-kinase regulatory subunit 5
Ccn1	2.26	0.20	Cellular communication network factor 1
Ltbp1	1.67	0.20	Latent transforming growth factor beta binding protein 1
Btg2	1.74	0.20	BTG anti-proliferation factor 2
Prlr	2.08	0.20	Prolactin receptor
Zfp36	2.22	0.20	Zinc finger protein 36
Efemp2	1.49	0.20	Epidermal growth factor-containing fibulin-like extracellular matrix protein 2
Rasa4	1.91	0.21	RAS p21 protein activator 4
Cd300lb	6.27	0.21	CD300 molecule like family member B
Sv2c	2.18	0.21	Synaptic vesicle glycoprotein 2c
Bdnf	1.78	0.21	Brain derived neurotrophic factor
Medag	2.15	0.21	Mesenteric estrogen-dependent adipogenesis
Mt1	2.12	0.21	Metallothionein 1
S100a10	1.93	0.21	S100 calcium binding protein A10
Npy	**2.23**	**0.23**	**Neuropeptide Y**
Notch1	1.80	0.24	Notch 1
Sstr2	2.15	0.24	Somatostatin receptor 2
Rbms1	1.40	0.24	RNA binding motif, single-stranded interacting protein 1
F2r	1.45	0.24	Coagulation factor II (thrombin) receptor
Chst5	1.77	0.24	Carbohydrate (*N*-acetylglucosamine 6-*O*) sulfotransferase 5
Msn	2.05	0.24	Moesin
Mfap4	**1.70**	**0.24**	**Microfibrillar-associated protein 4**
Adra1a	1.54	0.25	Adrenergic receptor, alpha 1a
Emp1	2.12	0.25	Epithelial membrane protein 1
Gadd45b	2.45	0.25	Growth arrest and DNA-damage-inducible 45 beta
DOWNREGULATED GENES (*N* = 16)			
Cxcl12	−1.88	0.04	Chemokine (C-X-C motif) ligand 12
Fbxl7	−1.43	0.06	F-box and leucine-rich repeat protein 7
Ogn	−2.54	0.06	Osteoglycin
Plk5	−2.54	0.10	Polo like kinase 5
Capn3	−2.00	0.11	Calpain 3
Atad2	−1.19	0.13	ATPase family, AAA domain containing 2
Pde7b	−1.51	0.16	Phosphodiesterase 7B
Prtg	−1.49	0.18	Protogenin
Per3	−1.45	0.19	Period circadian clock 3
Cys1	−1.52	0.19	Cystin 1
Smo	−1.76	0.20	Smoothened, frizzled class receptor
Gm12216	−1.50	0.21	Gm12216
Stxbp6	−1.47	0.24	Syntaxin binding protein 6
Cd34	−1.54	0.24	CD34 antigen
Plcg2	−1.54	0.24	Phospholipase C, gamma 2
Aqp11	−1.39	0.25	Aquaporin 11

Differentially expressed genes in neurons in the contralateral hippocampus (FDR < 0.25). logFC, log fold change; FDR, false discovery rate.

TABLE 2 | Differentially expressed genes in glia in the contralateral hippocampus (CLH) at 24 h after kainate-induced status epilepticus.

Gene symbol	logFC	FDR	Gene description
UPREGULATED GENES (*N* = 74)			
Socs3	**5.55**	**0.00**	**Suppressor of cytokine signaling 3**
Fos	**5.82**	**0.00**	**FBJ osteosarcoma oncogene**
Serpina3n	4.19	0.00	Serine (or cysteine) peptidase inhibitor, clade A, member 3N
Cebpd	2.82	0.01	CCAAT/enhancer binding protein delta
Tnfrsf12a	3.85	0.01	Tumor necrosis factor receptor superfamily, member 12a
Gadd45b	**5.36**	**0.01**	**Growth arrest and DNA-damage-inducible 45 beta**
Emp1	4.30	0.01	Epithelial membrane protein 1
Ier5l	2.81	0.01	Immediate early response 5-like
S1pr3	3.68	0.01	Sphingosine-1-phosphate receptor 3
Egr1	4.17	0.01	Early growth response 1
Timp1	5.61	0.01	Tissue inhibitor of metalloproteinase 1
Egr2	3.11	0.01	Early growth response 2
Tubb6	**3.24**	**0.01**	**Tubulin, beta 6 class V**
Cd44	3.89	0.01	CD44 antigen
Gadd45g	**3.13**	**0.02**	**Growth arrest and DNA-damage-inducible 45 gamma**
Ccl2	3.28	0.02	Chemokine (C-C motif) ligand 2
Junb	3.68	0.02	Jun B proto-oncogene
Ptx3	5.09	0.02	Pentraxin related gene
Fosb	3.92	0.02	FBJ osteosarcoma oncogene B
Tm4sf1	4.35	0.02	Transmembrane 4 superfamily member 1
Sphk1	3.78	0.02	Sphingosine kinase 1
Gm3448	2.79	0.02	Predicted gene 3448
Ccl12	4.33	0.02	Chemokine (C-C motif) ligand 12
Vgf	3.00	0.02	VGF nerve growth factor inducible
Fstl4	2.60	0.03	Follistatin-like 4
Myc	2.84	0.03	Myelocytomatosis oncogene
Itga5	3.38	0.03	Integrin alpha 5 (fibronectin receptor alpha)
Cebpb	2.35	0.03	CCAAT/enhancer binding protein (C/EBP), beta
Arid5a	2.05	0.03	AT rich interactive domain 5A (MRF1-like)
Ier3	2.61	0.04	Immediate early response 3
Thbd	2.01	0.04	Thrombomodulin
Adamts1	2.86	0.05	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 1
Ecm1	**2.09**	**0.05**	**Extracellular matrix protein 1**
Sbno2	2.62	0.05	Strawberry notch 2
Slc39a14	2.19	0.05	Solute carrier family 39 member 14
Hmga1b	2.22	0.05	High mobility group AT-hook 1B
Il1r1	1.90	0.06	Interleukin 1 receptor, type I
Klk9	3.35	0.06	Kallikrein related-peptidase 9
Dusp5	**2.25**	**0.07**	**Dual specificity phosphatase 5**
Ucn2	5.70	0.08	Urocortin 2
Gpr151	3.15	0.08	G protein-coupled receptor 151
Ier2	2.71	0.08	Immediate early response 2
Mafk	2.96	0.08	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein K
Csrnp1	2.60	0.08	Cysteine–serine-rich nuclear protein 1
Itga7	1.74	0.09	Integrin alpha 7
Fosl2	2.05	0.10	Fos-like antigen 2
Bcl3	5.25	0.10	B cell leukemia/lymphoma 3
Atf3	4.24	0.12	Activating transcription factor 3
Fosl1	2.36	0.13	Fos-like antigen 1
Hmga1	2.49	0.13	High mobility group AT-hook 1
Loxl1	2.05	0.13	Lysyl oxidase-like 1
Mchr1	2.23	0.13	Melanin-concentrating hormone receptor 1
Kdm6b	1.71	0.13	KDM1 lysine (K)-specific demethylase 6B
Odc1	1.90	0.13	Ornithine decarboxylase, structural 1
Cd244a	2.96	0.13	CD244 molecule A
Msr1	3.59	0.15	Macrophage scavenger receptor 1
Sv2c	2.70	0.15	Synaptic vesicle glycoprotein 2c
Tma16	1.82	0.16	Translation machinery associated 16
C2cd4b	2.69	0.16	C2 calcium-dependent domain containing 4B
Egr4	2.41	0.16	Early growth response 4
Hspb1	3.46	0.17	Heat shock protein 1
Tnc	1.67	0.17	Tenascin C
Srxn1	1.92	0.17	Sulfiredoxin 1 homolog
Ahnak2	2.75	0.17	AHNAK nucleoprotein 2
Wwtr1	1.64	0.17	WW domain containing transcription regulator 1
Gpr3	2.00	0.18	G-protein coupled receptor 3
Zfp36	2.57	0.18	Zinc finger protein 36
Trib1	1.95	0.18	Tribbles pseudokinase 1
Il11	2.88	0.21	Interleukin 11
Rhoj	2.62	0.21	Ras homolog family member J
Hcar2	3.17	0.21	Hydroxycarboxylic acid receptor 2
Cdh22	2.02	0.24	Cadherin 22
Pvr	1.49	0.25	Poliovirus receptor
Slc7a1	1.23	0.25	Solute carrier family 7 member 1
DOWNREGULATED GENES (*N* = 22)			
Nat8f4	−2.30	0.01	*N*-acetyltransferase 8 (GCN5-related) family member 4
Hapln1	−2.80	0.03	Hyaluronan and proteoglycan link protein 1
Aifm3	−2.17	0.03	Apoptosis-inducing factor, mitochondrion-associated 3
Btbd17	−2.31	0.03	BTB (POZ) domain containing 17
Nwd1	−2.35	0.04	NACHT and WD repeat domain containing 1
Gdpd2	−2.40	0.06	Glycerophosphodiester phosphodiesterase domain containing 2
Slc2a5	−2.28	0.08	Solute carrier family 2 member 5
P2ry12	−2.63	0.09	Purinergic receptor P2Y, G-protein coupled 12
Gpr165	−2.10	0.10	G protein-coupled receptor 165
Tet1	−1.41	0.13	Tet methylcytosine dioxygenase 1
2900052N01Rik	−2.38	0.15	2900052N01Rik
Susd5	−2.04	0.15	Sushi domain containing 5

(Continued)

TABLE 2 | Continued

Gene symbol	logFC	FDR	Gene description
Maf	−1.49	0.17	Avian musculoaponeurotic fibrosarcoma oncogene homolog
Fn3k	−1.87	0.17	Fructosamine 3 kinase
Tmem255b	−2.02	0.17	Transmembrane protein 255B
Traf4	−1.75	0.17	TNF receptor associated factor 4
Fam228a	−1.73	0.17	Family with sequence similarity 228, member A
Paqr7	−1.94	0.20	Progestin and adipoQ receptor family member VII
Gpr34	−2.49	0.21	G protein-coupled receptor 34
Sowaha	−1.46	0.21	Sosondowah ankyrin repeat domain family member A
Phkg1	−1.77	0.25	Phosphorylase kinase gamma 1
Folh1	−2.08	0.25	Folate hydrolase 1

Differentially expressed genes in glia in the contralateral hippocampus (FDR < 0.25). logFC, log fold change; FDR, false discovery rate.

TABLE 3 | Selection of relevant Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms of the differentially expressed genes in neurons in the contralateral hippocampus (CLH) at 24 h after kainate-induced status epilepticus.

	Upregulated in neurons (115 genes)	Downregulated in neurons (16 genes)
GO	Cell death	Positive regulation of calcium ion transport
	Regulation of cell proliferation	Axon guidance
	Cell communication	Vesicle organization
	MAPK cascade	Regulation of DNA-binding transcription factor activity
	Cell surface receptor signaling pathway	Axonogenesis
	Regulation of transcription, DNA-templated	Growth factor activity
	Vasculature development	Programmed cell death
	Inflammatory response	Blood vessel morphogenesis
KEGG	Cytokine-cytokine receptor interaction	Axon guidance
	cAMP signaling pathway	NF-kappa B signaling pathway
	ECM–receptor interaction	Leukocyte transendothelial migration
	IL-17 signaling pathway	–
	TGF-beta signaling pathway	–
	TNF signaling pathway	–
	MAPK signaling pathway	–
	p53 signaling pathway	–
	VEGF signaling pathway	–
	JAK-STAT signaling pathway	–

Relevant GO and KEGG terms among the significantly differentially expressed genes (FDR < 0.25).

TABLE 4 | Selection of relevant Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms of the differentially expressed genes in glia in the contralateral hippocampus (CLH) at 24 h after kainate-induced status epilepticus.

	Upregulated in glia (74 genes)	Downregulated in glia (22 genes)
GO	Regulation of angiogenesis	G protein-coupled nucleotide receptor activity
	Regulation of cell motility	Purinergic receptor activity
	Positive regulation of cell migration	Oxidation-reduction process
	Positive regulation of cell death	–
	Angiogenesis	–
	Regulation of interleukin-1 beta production	–
	Tumor necrosis factor production	–
	Neurogenesis	–
KEGG	TNF signaling pathway	–
	IL-17 signaling pathway	–
	ECM-receptor interaction	–
	MAPK signaling pathway	–
	Cytokine-cytokine receptor interaction	–
	Growth hormone synthesis, secretion and action	–
	JAK-STAT signaling pathway	–
	VEGF signaling pathway	–
	p53 signaling pathway	–
	PI3K-Akt signaling pathway	–
	NF-kappa B signaling pathway	–

Relevant GO and KEGG terms amongst significantly differentially expressed genes (FDR < 0.25).

For neurons, 77 upregulated genes and eight downregulated genes were differentially expressed in both the CLH and ILH (**Figure 3** and **Supplementary Table**, sheets 1 and 27). This constitutes a proportion of 65.22% (up) and 50.00% (down) of all the differentially regulated genes in the CLH. For glia, we found an overlap of 55 upregulated genes and an overlap of 17 downregulated genes between the CLH and ILH (**Figure 3** and **Supplementary Table**, sheets 4 and 28). This translates to a proportion of 74.32% (up) and 77.27% (down) of all the differentially regulated genes in the CLH. All overlapping genes between the CLH and ILH showed concordant expression.

Genes Exclusively Differentially Expressed in the Ipsilateral Hippocampus

In neurons, we found 55 genes to be upregulated and seven downregulated exclusively in the ILH (**Table 5** and **Supplementary Table**, sheet 21). In glia, 92 genes were upregulated and 68 genes were downregulated exclusively in the ILH (**Table 6** and **Supplementary Table**, sheet 24). The relevant GO and KEGG terms of these differentially expressed genes are listed in **Tables 7**, **8**, and a detailed list of the GO and KEGG terms is to be found in **Supplementary Table** (sheets 22, 23, 25, and 26).

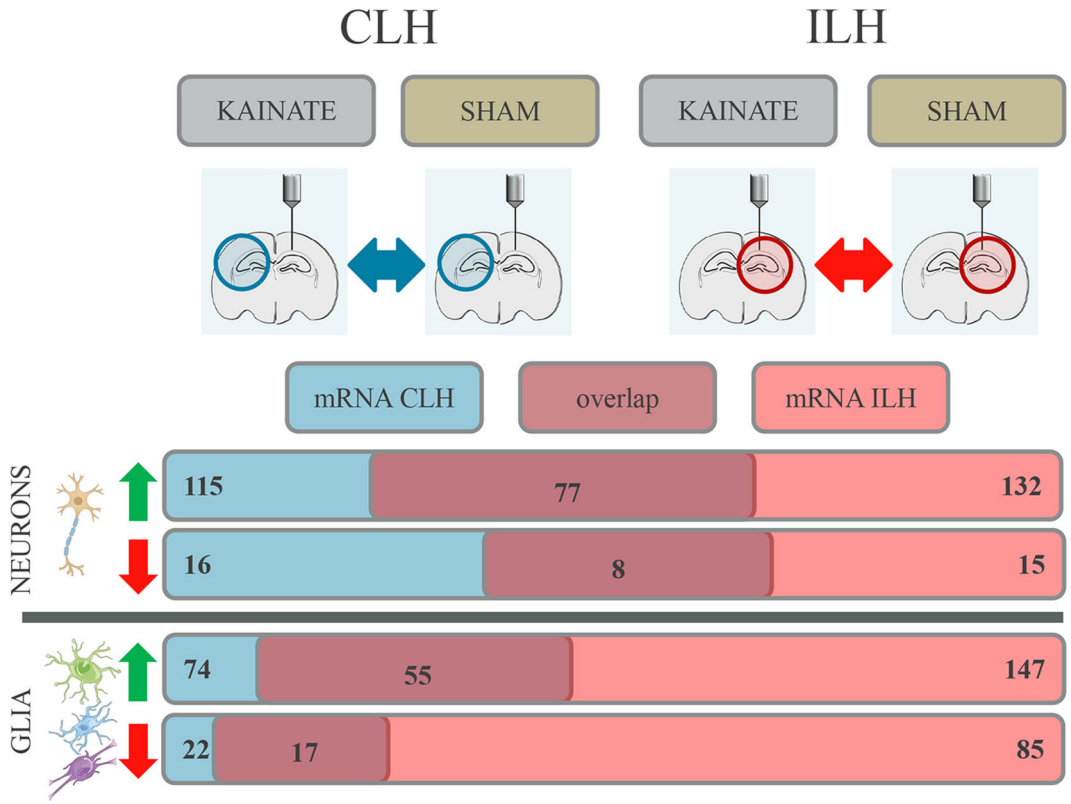


FIGURE 3 | Differential gene expression with overlap in the contralateral (CLH) and ipsilateral hippocampus (ILH) in both neurons and glia at 24 h after kainate-induced status epilepticus. Number of genes in the neurons and glia showing differential expression in the CLH (numbers given in *blue box*), in the ILH (numbers given in *red box*), and in both hemispheres (numbers given in *dark red box*).

Differential Methylation in the Hippocampus Contralateral to Kainate Injection

Differentially methylated CpGs were analyzed comparing left (contralateral) hippocampi of the kainic acid group to the sham group at 24 h after status epilepticus induction. For an overview of the number and distribution of the differentially methylated sites and the differentially methylated regions, see **Figure 4**. For a detailed list of the differentially methylated CpGs, differentially methylated regions, and the associated GO and KEGG terms, see **Supplementary Table** (differentially methylated CpGs: sheets 7 and 10; differentially methylated regions: sheets 13 and 16; differentially methylated CpGs GO: sheets 8 and 11; differentially methylated CpGs KEGG: sheets 9 and 12; differentially methylated regions GO: sheets 14 and 17; differentially methylated regions KEGG: sheets 15 and 18).

Overlap of Differentially Methylated CpGs Between Neurons and Glia

Ten CpG sites (0.12% of all differentially methylated CpGs in the CLH) were hypermethylated and six CpG sites (0.09%) hypomethylated in both neurons and glia. Twenty-one CpGs (0.18%) were hypermethylated in neurons and hypomethylated in glia and 13 hypomethylated in neurons and hypermethylated in glia (0.18%).

Association Between Differential Methylation and Differential Gene Expression in the Contralateral Hippocampus

In order to investigate a possible statistical association between differential DNA methylation and differential gene expression in the CLH, significantly differentially methylated regions and differentially expressed genes were aligned. No general trend in the association between the differentially methylated regions (upstream, promoter, UTR5, exon, intron, gene body, and UTR3) and differential gene expression was found (see figures in **Supplementary Table**, sheets 19 and 20), but significant alterations in DNA methylation and gene expression coincided at 11 genomic loci for neurons and four genomic loci for glia (**Supplementary Table**, sheets 19 and 20).

TABLE 5 | Differentially expressed genes in neurons exclusively in the ILH (and not the CLH) at 24 h after kainate-induced status epilepticus.

Gene symbol	logFC.ILH	FDR.ILH	Gene description
UPREGULATED GENES (*N* = 55; ILH: 132)			
Hspa1a	5.46	0.01	Heat shock protein 1A
Mapk4	2.28	0.01	Mitogen-activated protein kinase 4
Hspa1b	4.82	0.02	Heat shock protein 1B
Pcdh11x	2.26	0.03	Protocadherin 11 X-linked
Pde6b	2.91	0.03	Phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide
Wisp1	2.09	0.03	WNT1-inducible-signaling pathway protein 1
9330188P03Rik	3.35	0.03	RIKEN cDNA 9330188P03 gene
Hspb1	**4.00**	**0.03**	**Heat shock protein 1**
Lbh	3.01	0.03	Limb-bud and heart
Rrad	4.89	0.04	Ras-related associated with diabetes
4931440P22Rik	1.70	0.04	RIKEN cDNA 4931440P22 gene
Cyr61	2.90	0.04	Cysteine-rich angiogenic inducer 61
Zbtb46	1.58	0.05	Zinc finger and BTB domain containing 46
Bach1	1.72	0.05	BTB and CNC homology 1, basic leucine zipper transcription factor 1
Samd4	1.90	0.05	Sterile alpha motif domain containing 4
Npas4	3.24	0.06	Neuronal PAS domain protein 4
Adam19	1.62	0.08	A disintegrin and metallopeptidase domain 19 (meltrin beta)
Pim1	2.43	0.08	Proviral integration site 1
Mapkapk3	1.97	0.09	Mitogen-activated protein kinase-activated protein kinase 3
Cdh4	1.45	0.09	Cadherin 4
Kdm6b	1.57	0.09	KDM1 lysine (K)-specific demethylase 6B
Spp1	**3.14**	**0.09**	**Secreted phosphoprotein 1**
Sorcs3	2.28	0.09	Sortilin-related VPS10 domain containing receptor 3
Uck2	1.35	0.10	Uridine–cytidine kinase 2
Plce1	1.40	0.10	Phospholipase C, epsilon 1
Tgfb1i1	1.66	0.10	Transforming growth factor beta-1-induced transcript 1
Frrs1	1.87	0.12	Ferric-chelate reductase 1
Blnk	2.81	0.12	B cell linker
Rgs20	1.74	0.12	Regulator of G-protein signaling 20
Itprip	1.88	0.13	Inositol 1,4,5-triphosphate receptor interacting protein
Smad7	1.83	0.13	SMAD family member 7
Svil	1.52	0.13	Supervillin
Mir132	**3.39**	**0.15**	**MicroRNA 132**
Zdhhc22	1.85	0.17	Zinc finger, DHHC-type containing 22
Amotl1	1.71	0.18	Angiomotin-like 1
Serpina3i	2.75	0.18	Serine (or cysteine) peptidase inhibitor, clade A, member 3I
Ifit1	2.33	0.18	Interferon-induced protein with tetratricopeptide repeats 1
Kcnip3	1.67	0.18	Kv channel interacting protein 3, calsenilin
Odc1	1.57	0.18	Ornithine decarboxylase, structural 1
Igsf9b	2.27	0.18	Immunoglobulin superfamily, member 9B
Spred1	1.62	0.18	Sprouty protein with EVH-1 domain 1, related sequence
Samd11	2.19	0.19	Sterile alpha motif domain containing 11
Scd4	2.01	0.19	Stearoyl-coenzyme A desaturase 4
Dusp4	1.88	0.19	Dual specificity phosphatase 4
Tspan9	1.68	0.19	Tetraspanin 9
Eva1b	2.00	0.19	Eva-1 homolog B (*C. elegans*)
Btc	2.40	0.19	Betacellulin, epidermal growth factor family member
St8sia2	2.11	0.20	ST8 alpha-*N*-acetyl-neuraminide alpha-2,8-sialyltransferase 2
Tm4sf1	2.49	0.20	Transmembrane 4 superfamily member 1
Cdh22	1.77	0.20	Cadherin 22
Itga5	2.11	0.21	Integrin alpha 5 (fibronectin receptor alpha)
Mapk6	1.51	0.22	Mitogen-activated protein kinase 6
Egr4	1.91	0.23	Early growth response 4
Itpkc	1.84	0.23	Inositol 1,4,5-trisphosphate 3-kinase C
Drd1	**1.82**	**0.25**	**Dopamine receptor D1**
DOWNREGULATED GENES (*N* = 7; ILH: 15)			
Echdc2	−1.77	0.08	Enoyl coenzyme A hydratase domain containing 2
Cyp7b1	−2.28	0.10	Cytochrome P450, family 7, subfamily b, polypeptide 1
Gstm6	−1.57	0.16	Glutathione S-transferase, mu 6
Crlf1	−1.97	0.19	Cytokine receptor-like factor 1
Macrod1	−1.52	0.20	MACRO domain containing 1
Gm35339	−1.43	0.20	Predicted gene, 35339
6330420H09Rik	−2.15	0.22	RIKEN cDNA 6330420H09 gene

Differentially expressed genes in neurons exclusively in the ipsilateral hippocampus (FDR < 0.25).
logFC, log fold change; FDR, false discovery rate; ILH, ipsilateral hippocampus; CLH, contralateral hippocampus.

Differential DNA Methylation, and Association of Differential Methylation With Differential Gene Expression, in the Contralateral Hippocampus Compared With the Ipsilateral Hippocampus

Only a fragment of the differentially methylated CpGs and the differentially methylated regions overlapped between the CLH and ILH (22). Of all the differentially methylated CpGs in neurons in the CLH, 48 (0.44% of all the differentially methylated

TABLE 6 | Differentially expressed genes in glia exclusively in the ILH (and not CLH) at 24 h after kainate-induced status epilepticus.

Gene symbol	logFC.ILH	FDR.ILH	Gene description
UPREGULATED GENES (*N* = 92; ILH: 147)			
Ch25h	5.14	0.00	Cholesterol 25-hydroxylase
Lilr4b	4.88	0.00	Leukocyte immunoglobulin-like receptor, subfamily B, member 4B
Calca	4.68	0.01	Calcitonin/calcitonin-related polypeptide, alpha
Spp1	**4.71**	**0.01**	**Secreted phosphoprotein 1**
Fn1	2.64	0.01	Fibronectin 1
Fgl2	2.97	0.01	Fibrinogen-like protein 2
Rasgef1c	3.04	0.01	RasGEF domain family, member 1C
Ifit3	**2.69**	**0.01**	**Interferon-induced protein with tetratricopeptide repeats 3**
Iigp1	**3.09**	**0.02**	**Interferon inducible GTPase 1**
Rasl11a	1.99	0.02	RAS-like, family 11, member A
Btc	3.29	0.02	Betacellulin, epidermal growth factor family member
Nptx2	2.91	0.03	Neuronal pentraxin 2
Adam8	3.04	0.03	A disintegrin and metallopeptidase domain 8
Inhba	2.61	0.03	Inhibin beta-A
Lilrb4a	4.02	0.03	Leukocyte immunoglobulin-like receptor, subfamily B, member 4A
Cd300lf	3.60	0.03	CD300 molecule like family member F
Cacng5	2.10	0.03	Calcium channel, voltage-dependent, gamma subunit 5
Ifi204	**4.09**	**0.03**	**Interferon activated gene 204**
Dab2	2.15	0.04	Disabled 2, mitogen-responsive phosphoprotein
Ifi207	3.11	0.04	Interferon activated gene 207
Parp3	2.74	0.04	Poly(ADP-ribose) polymerase family, member 3
Rasip1	2.30	0.05	Ras interacting protein 1
Lpl	1.88	0.06	Lipoprotein lipase
Tpbg	1.72	0.06	Trophoblast glycoprotein
Gcnt2	2.42	0.06	Glucosaminyl (*N*-acetyl) transferase 2, I-branching enzyme
Serpine1	3.97	0.06	Serine (or cysteine) peptidase inhibitor, clade E, member 1
Oasl2	2.47	0.06	2'-5' Oligoadenylate synthetase-like 2
Ptgs2	**2.43**	**0.07**	**Prostaglandin-endoperoxide synthase 2**
Slc10a6	3.88	0.07	Solute carrier family 10 (sodium/bile acid cotransporter family), member 6
Ahnak	1.95	0.07	AHNAK nucleoprotein (desmoyokin)
Nedd9	1.33	0.07	Neural precursor cell expressed, developmentally downregulated gene 9
Rai14	1.61	0.07	Retinoic acid induced 14
Layn	1.95	0.08	Layilin
Col16a1	2.51	0.08	Collagen, type XVI, alpha 1
Atp10a	2.07	0.08	ATPase, class V, type 10A
Gal	3.44	0.08	Galanin and GMAP prepropeptide
Mx1	3.40	0.08	MX dynamin-like GTPase 1
Irgm1	1.56	0.09	Immunity-related GTPase family M member 1
Gldn	3.04	0.09	Gliomedin
Cchcr1	1.58	0.09	Coiled-coil alpha-helical rod protein 1
Slc5a3	1.82	0.10	Solute carrier family 5 (inositol transporters), member 3
Socs2	1.76	0.10	Suppressor of cytokine signaling 2
Il4ra	**1.81**	**0.10**	**Interleukin 4 receptor, alpha**
Irf7	2.39	0.10	Interferon regulatory factor 7
Nlrc5	2.21	0.10	NLR family, CARD domain containing 5
Fgf18	2.32	0.11	Fibroblast growth factor 18
Ifit3b	2.41	0.11	Interferon-induced protein with tetratricopeptide repeats 3B
Strip2	1.74	0.12	Striatin interacting protein 2
Has2	3.19	0.12	Hyaluronan synthase 2
Mir212	4.52	0.12	MicroRNA 212
Flnc	3.71	0.12	Filamin C, gamma
Map3k6	2.39	0.12	Mitogen-activated protein kinase kinase kinase 6
Timeless	1.39	0.12	Timeless circadian clock 1
Snhg15	1.56	0.13	Small nucleolar RNA host gene 15
Mamstr	2.09	0.14	MEF2 activating motif and SAP domain containing transcriptional regulator
Clcf1	2.36	0.14	Cardiotrophin-like cytokine factor 1
Bdnf	**1.81**	**0.14**	**Brain-derived neurotrophic factor**
Rnf138rt1	5.32	0.15	Ring finger protein 138, retrogene 1
Slfn10-ps	2.78	0.16	Schlafen 10, pseudogene
Amotl1	1.65	0.16	Angiomotin-like 1
Mir132	**3.30**	**0.17**	**MicroRNA 132**
Serpina3i	2.64	0.17	Serine (or cysteine) peptidase inhibitor, clade A, member 3I
Hmox1	1.87	0.17	Heme oxygenase 1
Lrtm2	1.62	0.18	Leucine-rich repeats and transmembrane domains 2
Spred3	1.72	0.18	Sprouty-related EVH1 domain containing 3
Vmn1r15	6.73	0.18	Vomeronasal 1 receptor 15
Rtp4	1.91	0.18	Receptor transporter protein 4
Rnf125	2.28	0.18	Ring finger protein 125
Slfn2	2.93	0.18	Schlafen 2
Piezo2	1.68	0.19	Piezo-type mechanosensitive ion channel component 2
Anxa2	2.01	0.19	Annexin A2
Gpd1	1.68	0.19	Glycerol-3-phosphate dehydrogenase 1 (soluble)
Cyr61	2.08	0.19	Cysteine-rich angiogenic inducer 61
Plaur	2.39	0.19	Plasminogen activator, urokinase receptor
Ifit1	2.11	0.20	Interferon-induced protein with tetratricopeptide repeats 1

(Continued)

TABLE 6 | Continued

Gene symbol	logFC.ILH	FDR.ILH	Gene description
Itga2b	1.93	0.20	Integrin alpha 2b
Fgfr4	2.25	0.20	Fibroblast growth factor receptor 4
Bst2	2.06	0.20	Bone marrow stromal cell antigen 2
Gm6225	2.35	0.21	Predicted gene 6225
Cbln4	1.60	0.21	Cerebellin 4 precursor protein
Serpina3m	2.79	0.22	Serine (or cysteine) peptidase inhibitor, clade A, member 3M
Akap12	1.34	0.22	A kinase (PRKA) anchor protein (gravin) 12
Sdc1	1.59	0.22	Syndecan 1
Ndst1	1.59	0.22	*N*-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 1
Npas4	2.45	0.22	Neuronal PAS domain protein 4
Tspan4	1.89	0.23	Tetraspanin 4
Klk6	2.76	0.23	Kallikrein related-peptidase 6
Cxcl10	**2.90**	**0.23**	**Chemokine (C-X-C motif) ligand 10**
Col7a1	1.75	0.23	Collagen, type VII, alpha 1
Plce1	1.17	0.24	Phospholipase C, epsilon 1
Peak1	1.41	0.24	Pseudopodium-enriched atypical kinase 1
Itga1	1.36	0.25	Integrin alpha 1
DOWNREGULATED GENES (*N* = 68; ILH: 85)			
Pcx	−2.11	0.02	Pyruvate carboxylase
Shroom2	−2.28	0.03	Shroom family member 2
Gpr12	−2.22	0.04	G-protein-coupled receptor 12
Ccdc13	−1.80	0.05	Coiled-coil domain containing 13
Cygb	−1.88	0.05	Cytoglobin
Ankub1	−2.23	0.06	Ankrin repeat and ubiquitin domain containing 1
Siglech	−2.16	0.06	Sialic acid binding Ig-like lectin H
Itpka	−1.70	0.06	Inositol 1,4,5-trisphosphate 3-kinase A
Hpca	−1.84	0.07	Hippocalcin
Ppp1r1b	−1.75	0.08	Protein phosphatase 1, regulatory inhibitor subunit 1B
Nkain4	−2.55	0.08	Na^+/K^+ transporting ATPase interacting 4
Kctd4	−2.09	0.08	Potassium channel tetramerization domain containing 4
Gstm6	−1.67	0.08	Glutathione S-transferase, mu 6
Shisa8	−2.21	0.09	Shisa family member 8
2810468N07Rik	−2.22	0.09	RIKEN cDNA 2810468N07 gene
Abca9	−1.97	0.09	ATP-binding cassette, sub-family A (ABC1), member 9
Chn1	−1.71	0.10	Chimerin 1
Ntsr2	−2.14	0.11	Neurotensin receptor 2
Myh14	−1.76	0.11	Myosin, heavy polypeptide 14
Fam234a	−1.77	0.11	Family with sequence similarity 234, member A
Faah	−1.50	0.12	Fatty acid amide hydrolase
Tppp3	−1.76	0.12	Tubulin polymerization-promoting protein family member 3
Abca6	−1.46	0.12	ATP-binding cassette, sub-family A (ABC1), member 6
Gnai1	−1.90	0.13	Guanine nucleotide binding protein (G protein), alpha inhibiting 1
Cfap100	−1.48	0.14	Cilia and flagella associated protein 100
Grm3	**−2.01**	**0.14**	**Glutamate receptor, metabotropic 3**
Phgdh	−1.66	0.15	3-Phosphoglycerate dehydrogenase
Selplg	−2.14	0.15	Selectin, platelet (p-selectin) ligand
Epn2	−1.61	0.17	Epsin 2
Rlbp1	−1.78	0.18	Retinaldehyde binding protein 1
Pantr1	−1.72	0.18	POU domain, class 3, transcription factor 3 adjacent non-coding transcript 1
Plk5	−2.14	0.18	Polo-like kinase 5
Nat8f1	−1.91	0.18	*N*-acetyltransferase 8 (GCN5-related) family member 1
1700066M21Rik	−1.65	0.18	RIKEN cDNA 1700066M21 gene
Adi1	−1.61	0.18	Acireductone dioxygenase 1
Tmem191c	−1.45	0.18	Transmembrane protein 191C
Gmnc	−2.55	0.18	Geminin coiled-coil domain containing
Zfp763	−1.51	0.18	Zinc finger protein 763
Slc25a18	−1.79	0.19	Solute carrier family 25 (mitochondrial carrier), member 18
Hhip	−2.01	0.19	Hedgehog-interacting protein
Calb1	−1.51	0.19	Calbindin 1
Chst5	−1.74	0.19	Carbohydrate (*N*-acetylglucosamine 6-*O*) sulfotransferase 5
Trim59	−2.18	0.19	Tripartite motif-containing 59
Olfml1	−2.24	0.19	Olfactomedin-like 1
Mturn	−1.41	0.19	Maturin, neural progenitor differentiation regulator homolog (*Xenopus*)
Gstm1	−1.80	0.19	Glutathione S-transferase, mu 1
Enho	−1.63	0.19	Energy homeostasis associated
Prodh	−1.86	0.19	Proline dehydrogenase
Slc27a1	−1.71	0.19	Solute carrier family 27 (fatty acid transporter), member 1
Pacsin3	−1.44	0.19	Protein kinase C and casein kinase substrate in neurons 3
Htr1a	−1.95	0.19	5-Hydroxytryptamine (serotonin) receptor 1A
Dll3	−1.72	0.19	Delta-like canonical Notch ligand 3
Map6d1	−1.60	0.19	MAP6 domain containing 1
Prrg1	−1.61	0.19	Proline-rich Gla (G-carboxyglutamic acid) 1
Carns1	−1.88	0.20	Carnosine synthase 1
Tle2	−1.48	0.20	Transducin-like enhancer of split 2
Macrod1	−1.45	0.20	MACRO domain containing 1
Nrgn	−1.51	0.20	Neurogranin
Plin3	−2.18	0.21	Perilipin 3
Grhpr	−1.38	0.21	Glyoxylate reductase/hydroxypyruvate reductase

(Continued)

TABLE 6 | Continued

Gene symbol	logFC.ILH	FDR.ILH	Gene description
Sult1a1	−2.19	0.21	Sulfotransferase family 1A, phenol-preferring, member 1
Pls1	−1.58	0.22	Plastin 1 (I-isoform)
Lin7b	−1.69	0.22	Lin-7 homolog B (*C. elegans*)
Armh4	−1.53	0.22	Armadillo-like helical domain containing 4
Panx2	−1.33	0.23	Pannexin 2
Appl2	−1.76	0.23	Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2
Grhl1	−1.01	0.23	Grainyhead-like transcription factor 1
Pigz	−1.71	0.24	Phosphatidylinositol glycan anchor biosynthesis, class Z

Differentially expressed genes in glia exclusively in the ipsilateral hippocampus (FDR < 0.25).
logFC, log fold change; FDR, false discovery rate; ILH, ipsilateral hippocampus; CLH, contralateral hippocampus.

TABLE 7 | Selection of relevant Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms of the differentially expressed genes in neurons in the ipsilateral hippocampus (but not in the contralateral hippocampus) at 24 h after kainate-induced status epilepticus.

	Upregulated in neurons (55 genes)	Downregulated in neurons (7 genes)
GO	Ion binding	Monocarboxylic acid metabolic process
	MAP kinase activity	Oxidation-reduction process
	Protein phosphorylation	Carboxylic acid metabolic process
	Cell communication	Positive regulation of cell proliferation
	Regulation of spindle assembly	Lipid metabolic process
	Protein serine/threonine kinase inhibitor activity	–
	Regulation of synaptic plasticity	–
	Cell–cell adhesion	–
KEGG	MAPK signaling pathway	–
	VEGF signaling pathway	–
	Calcium signaling pathway	–
	Inositol phosphate metabolism	–
	Antigen processing and presentation	–
	ECM–receptor interaction	–
	IL-17 signaling pathway	–
	Phosphatidylinositol signaling system	–

Relevant GO and KEGG terms among the significantly differentially expressed genes (FDR < 0.25).

CpGs in neurons) were also differentially methylated in neurons in the ILH (22 hypermethylated and 26 hypomethylated). In glia, seven differentially methylated CpGs (0.11%) were differentially methylated in both the CLH and ILH (four hypermethylated and three hypomethylated). Regarding differentially methylated regions, 17 were overlapping between the ILH and CLH in neurons (16 hypermethylated and one hypomethylated) and two (both hypermethylated) in glia. Some of these overlapping differentially methylated CpGs and differentially methylated regions were linked to genes with epilepsy- and DNA methylation-relevant functions like TGF-beta signaling, DNA methyltransferase activity, or angiogenesis, but none of these overlapped with the differentially expressed genes in the ipsilateral or contralateral hippocampus (**Supplementary Table**).

Only one gene, *Spp1*, had an association between differential DNA methylation and differential gene expression in both the CLH and ILH (neurons). *Spp1* was upregulated in both CLH and ILH. This coincided with upstream and promoter hypermethylation in the CLH and upstream and promoter hypomethylation in the ILH. No overlaps for differential DNA methylation and differential gene expression associations were found for glia when comparing CLH to ILH (**Supplementary Table**) (22).

TABLE 8 | Selection of relevant Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms of the differentially expressed genes in glia in the ipsilateral hippocampus (but not in the contralateral hippocampus) at 24 h after kainate-induced status epilepticus.

	Upregulated in glia (92 genes)	Downregulated in glia (68 genes)
GO	Cell differentiation	Dendrite
	Immune system process	Modulation of chemical synaptic transmission
	Positive regulation of cell differentiation	Calcium channel regulator activity
	Positive regulation of cell motility	Regulation of synaptic plasticity
	Extracellular space	Glutamine family amino acid metabolic process
	Vasculature development	Myelin sheath
	Regulation of programmed cell death	Glutamate receptor signaling pathway
	–	GABA-ergic synapse
KEGG	ECM–receptor interaction	Glutathione metabolism
	PI3K–Akt signaling pathway	cAMP signaling pathway
	MAPK signaling pathway	Pyruvate metabolism
	Toll-like receptor signaling pathway	Glycine, serine and threonine metabolism
	Cytokine–cytokine receptor interaction	ABC transporters
	IL-17 signaling pathway	Glutamatergic synapse
	Complement and coagulation cascades	–

Relevant GO and KEGG terms among the significantly differentially expressed genes (FDR < 0.25).

DISCUSSION

In this study, we investigate alterations in gene expression and DNA methylation in glia and neurons in mouse hippocampi contralateral to intracortical kainic acid application. We found

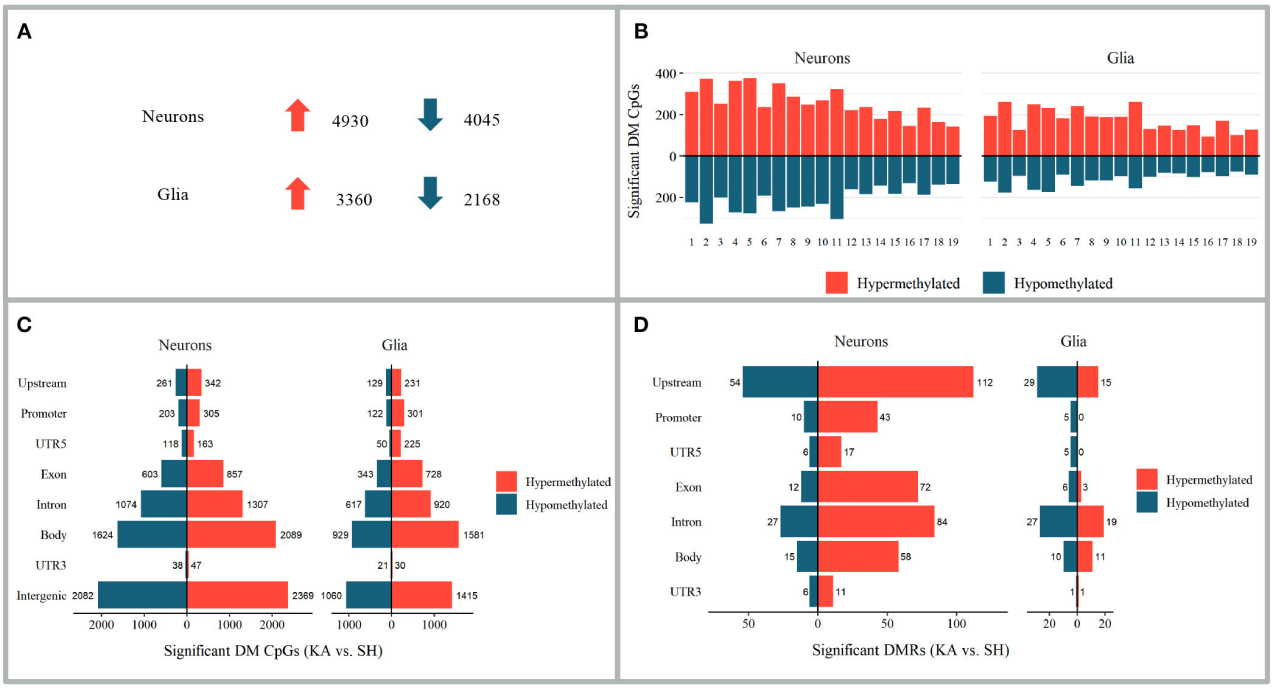


FIGURE 4 | Alterations in DNA methylation 24 h after kainate-induced status epilepticus in hippocampi contralateral to injection site. **(A–C)** Differentially methylated CpGs of the kainic acid group *vs.* the sham group. **(A)** Number of differentially methylated CpGs in neurons and glia; *upward arrow* implies hypermethylation and *downward arrow* hypomethylation. **(B)** Chromosomal distribution of differentially methylated CpGs. **(C)** Distribution of differentially methylated CpGs among genomic features. **(D)** Distribution of differentially methylated regions among genomic features.

fulminant changes of both the gene expression and DNA methylation in glia and neurons in the CLH at 24 h after kainate-induced status epilepticus.

Based on our findings, we will discuss possible beneficial and detrimental responses to epileptic activity in the CLH. We will further illuminate potential genetic targets relevant to hippocampal sclerosis by comparing alterations in gene expression in the CLH to gene expression in the ILH. Lastly, we will discuss DNA methylation and its role for gene expression regulation in early epileptogenesis.

Differential Gene Expression in the Contralateral Hippocampus Only Exposed to Epileptic Activity

We found that differential gene expression in the contralateral hippocampus at 24 h after kainate-induced status epilepticus mainly occurs cell-specific, with only a minor overlap of genes differentially expressed in the neurons and glia. This may reflect the complementary characteristic of neuron–glia interactions in epilepsy (4) and is comparable to our previous findings from cell-specific gene expression in the ipsilateral hippocampus (22).

In the CLH, the primary factor affecting differential gene expression is related to epileptic activity upon status epilepticus (**Figure 1**) (11). Differentially expressed genes comprise diverse inflammatory responses, synaptic signaling, and DNA methylation machinery in both neurons and glia (**Tables 3**, **4**). Many of the gene expression changes seen in the CLH (lacking hippocampal sclerosis) overlap with our previous findings from the ILH [comprising hippocampal sclerosis at chronic time points: **Supplementary Table** 27 and 28 (22)]. This may appear unexpected since the CLH does not show morphological changes as seen in the ILH like reactive gliosis and neuronal death. A previous study on the ipsilateral and contralateral gene expression changes in a unilateral kainic acid epilepsy model also found a large overlap of the differentially expressed genes in the ipsilateral and contralateral hippocampus (43). The authors created different subsets of genes in order to distinguish between the effect of the kainic acid-induced lesion and epileptic seizures. A comparison of our data obtained at an early time point of epileptogenesis from the CLH to these results at a chronic stage of epileptogenesis reveals several genes overlapping with the "seizure" gene set (neurons: *Gal*, *Fos*, *Parp3*, *Nedd9*, *Mfap4*, *Dusp5*, *Col27a1*, *Sdc1*, *Ptgs1*, and *Arc*; glia: *Tubb6*, *Fos*, *Ecm1*, and *Dusp5*).

We further find a great degree of overlap between the gene expression changes seen in the CLH with other studies of various animal models for epilepsy (29, 44), gene expression material from the resected hippocampi of temporal lobe epilepsy (TLE) patients (45), and even with genomic data from animal models of reactive gliosis (46).

If the gene expression response in the CLH is so similar to both the ILH (with morphological alterations such as neuronal cell death and reactive gliosis 3 months after status epilepticus initiation or earlier) (11) and diverse models of epilepsy and reactive gliosis, why does the CLH not develop comparable morphological changes? The question whether epileptic activity can lead to morphological changes has long been a matter of controversial debate and is, to date, unanswered. While several previous studies claim that seizures mediate epileptogenic effects (47, 48), others postulate that seizures *per se* do not promote epileptogenesis (35, 49).

A hypothesis as to why the CLH remains free of hippocampal sclerosis may be that it is exposed to fewer detrimental or a larger number of beneficial effects, or both. As for fewer detrimental effects, one apparent characteristic in the CLH is the significantly lower number of glial genes up- and downregulated compared to the ILH. Only half the number of genes are up- and only a quarter of the number of genes are downregulated compared to the ILH. With several glial genes coding for pro-inflammatory pathways (**Supplementary Table**, sheets 4–6), this less pronounced glial activation in the contralateral hippocampus may be related to the lack of morphological changes characteristically observed at later time points. With regard to the gene expression changes with possible beneficial effects in the CLH, we find several seizure-alleviating and even potential anti-epileptic genes and pathways upregulated. Within the glial genes in the CLH, more genes overlapped with a gene set previously related to a "beneficial" type of astrocyte (A2) than with the gene set of "detrimental" astrocytes (A1) (46), possibly representing a glia-mediated endogenic anti-epileptogenic process in early epileptogenesis. Other epileptic activity-induced genes with seizure-alleviating or potentially even anti-epileptogenic effects include *Gal*, *Socs3*, and *NPY*. GAL (galanin) has previously been shown to exhibit anti-seizure effects and comprises potential anti-epileptogenic qualities (50). The gene expression levels of *Galanin* are elevated in neurons in the CLH, possibly revealing epileptic activity-related homeostatic effects. Further, we find the gene expression levels of NPY (neuropeptide Y), a neuropeptide recently successfully shown to attenuate seizures in slices of medication-refractory TLE (51), elevated in neurons in the CLH. Lastly, we find elevated levels of SOCS3 in glia in the CLH. *Socs3* codes for the suppressor of cytokine signaling 3 protein. This protein reduces the pro-inflammatory responses of, among others, IL-6, IFN, IL2, Il12, and NfkB signaling pathways and reduces astrocytic chemokine production (52). Thus, *Socs3* expression potentially represents another example of an endogenic reaction aiming at reducing the detrimental effects of seizures.

In sum, we speculate that anti-epileptogenic effects may outweigh pro-epileptogenic effects and thus prevent morphological alterations like neuronal death and reactive gliosis in the CLH. In fact, we find a higher fraction of GO terms anticipating anti-epileptogenic effects like "neurogenesis" (glia) and a lower number of GO terms indicating pro-epileptogenic qualities like "negative regulation of neuronal death" (neurons) in the CLH (**Supplementary Table**, sheets 2 and 5) compared to the ILH (22).

Potential Upstream Targets of Hippocampal Sclerosis and Epileptogenesis

If one were to speculate which genes in our ipsilateral and contralateral findings in early epileptogenesis were most likely potential candidate genes driving hippocampal sclerosis and epileptogenesis, one could hypothesize that these would have to be exclusively found on the list of differentially expressed genes in the ILH. Featuring morphological changes like reactive gliosis and neuronal death, the ILH is associated with epileptogenesis (**Figure 1**).

For neurons, genes only differentially expressed in the ILH comprise pathways within various inflammatory responses and epilepsy-relevant genes like *Mir132* (53) and *Drd1* (54) (**Table 5** and **Supplementary Table**, sheet 21). In glia, genes upregulated in the ILH but not in the CLH include several interferon- and interleukin-associated genes like *Ifit3*, *Iigp1*, *Ifi204*, and *Il4ra*, other inflammatory genes previously associated with epilepsy like *Ptgs2* (Cox2) (55), and epilepsy-related genes like *Bdnf* (56) and *Mir132* (53) (**Table 6** and **Supplementary Table**, sheet 24). Downregulated genes in glia involve, among others, *Grm3*, a gene encoding for the metabotropic glutamate receptor 3, previously shown to be downregulated in experimental and human mTLE (57).

Within these genes exclusively differentially expressed in the ILH (and not CLH), one could check for overlaps with the top target genes in the reactive gliosis gene set mentioned earlier. Glial *CxCl10* and *Ptgs2* (*Cox2*) are exclusively differentially expressed in both the ILH (22) and in a previous genomic analysis of reactive gliosis (46). CXCL10, a chemokine elevated in various central nervous system (CNS) pathologies like Alzheimer's disease (58), multiple sclerosis (59), and Rasmussen encephalitis (60), has been shown to elicit elevated neuronal excitability after acute (61) and chronic exposure (62). Produced in astrocytes (63), it mediates neuronal death *via* Ca^{2+}-dependent apoptosis (64). *Ptgs2*, coding for COX2, a cyclooxygenase exerting pro-epileptogenic effects in epileptogenesis (55), represents another potential glial upstream target for anti-epileptogenic intervention. These findings are in line with previous studies on the importance of glia-driven inflammatory pathways in epileptogenesis (4, 65).

As mentioned, the number of genes differentially expressed by the glia in the ILH are significantly higher than those in the CLH. This possibly indicates a more pronounced glial reaction triggered by the combination of epileptic activity and kainate in the ILH. This is supported by the notion of previously reported glial responses to kainic acid injection (66). In the intracortical model of mTLE-HS, the effects of epileptic activity and kainate are difficult to disentangle. Both kainate (67–69) and epileptic activity (47, 48, 70) can exert cytotoxic effects that, in combination, might be potentiated (71, 72). A previous genomic analysis of the ipsilateral and contralateral hippocampi of kainate-injected rats in chronic epilepsy (43) created a "kainic acid" gene set—a list of genes presumably induced by kainic acid. We find a surprisingly small overlap of these "kainic acid genes" with our data (exclusively ILH: *Spp1* and *Hspb1* in neurons and

Spp1 in the glia), possibly indicating that the singular effect of kainate may not be of primordial importance for downstream effects like hippocampal sclerosis and epileptogenesis after all (for restrictions in interpretability, see *Limitations*). Further, our goal was to identify upstream gene expression alterations possibly leading to hippocampal sclerosis, and as such, the exact cause of these alterations may be of secondary importance as long as they lead to epileptogenesis-relevant hallmarks.

DNA Methylation and Its Role for Gene Expression in Early Epileptogenesis

In line with previous studies (22, 73, 74), DNA methylation occurs mainly in a cell-specific manner in the CLH. Regarding the methylation of singular CpG sites, hypermethylation slightly outweighs hypomethylation in both neurons and glia, both with regards to differentially methylated CpGs in total and differentially methylated CpGs within genomic regions. This trend is similar to the DNA methylation dynamics observed at 24 h in the ILH (22) and to previous data from DNA methylation alterations in a rat model of chronic epilepsy (75). Differentially methylated regions were mostly hypermethylated in neurons and hypomethylated in glia. This represents a near inversion of the methylation pattern of the differentially methylated regions in the ILH, where most differentially methylated regions in neurons were hypomethylated and most differentially methylated regions in the glia were hypermethylated (22). Previous studies of epilepsy-related DNA methylation in acute phases of epilepsy in animal models found no general trend toward hyper- or hypomethylation (30) or a tendency toward hypomethylation (76).

One possible reason for the higher ratio of hypomethylated differentially methylated regions in glia in the CLH is the higher levels of gene expression of *Gadd 45b* and *Gadd 45g*, which both are capable of DNA demethylation (77). In the CLH, significant alterations of differential DNA methylation and differential gene expression coincided at several genomic loci (**Supplementary Table**, sheets 19 and 20), e.g., at epilepsy-relevant genes like *Spp1* (78) in neurons and *Atf3* (79) in glia. Differential gene expression and differential DNA methylation coincide at epilepsy-related loci in both the CLH and ILH, yet the overlap of differential methylation between the CLH and ILH is marginal. There are no genomic loci in both the CLH and ILH at which differential DNA methylation and differential gene expression coincide in both hippocampi. While several previous studies revealed various associations between DNA methylation and gene expression in epilepsy (28, 29, 75), more recent studies have claimed a more restricted importance of DNA methylation for gene expression in epilepsy (80). The general role of DNA methylation for the regulation of gene expression appears to be highly tissue- and context-specific (81) and may not be the primary factor determining gene expression in early epileptogenesis. Thus, how changes in DNA methylation are related to differential gene expression in early epileptogenesis remains unclear.

Limitations

Considered a solid marker of mature neurons (82, 83), NeuN (Rbfox3) may not stain all CNS neurons (84). As such, the NeuN– fraction (referred to as glia) may, apart from astrocytes, oligodendrocytes, and microglia, contain a minor fraction of non-glial cells (e.g., endothelial cells, pericytes, and neurons) (84–86).

At steady state, RNA sequencing (RNAseq) is a solid approach for the estimation of protein abundance, and as such, biological function, yet in transition states, distortions in this correlation may occur (17, 18). Hence, we may under- or overestimate biological effects based on our interpretation of the differential gene expression results 24 h after injection. Also, posttranscriptional (87) and posttranslational mechanisms (88) may account, among other things, for a non-linear correlation between mRNA and protein abundance. These shortcomings may also contribute to an explanation as to why the CLH, which features many of the same differentially regulated gene transcripts as the ILH, does not feature morphological alterations.

A previous study on gene expression revealed a mainly stage-specific (acute, latent, or chronic) gene expression profile in epileptogenesis (89). As such, the comparison of our gene set, representing relatively acute changes of kainic acid-induced status epilepticus, to previous data from a chronic time point of epileptogenesis (43) should be interpreted with caution.

Regarding the only marginal overlap of differential DNA methylation between the CLH and ILH and the non-existent overlap of genomic loci with the association between differential gene expression and differential DNA methylation, one possible cause is that the method for detecting differential DNA methylation, RRBS, does not include all CpGs (37). RRBS covers most CpGs in promoters and CpG islands (but not all) and has a low coverage at, for example, CpG shores and enhancers (37). We might *ergo* have missed specific genomic loci at which differential DNA methylation and differential gene expression coincide.

CONCLUSION

In this study we found substantial changes in gene expression and DNA methylation 24 h after status epilepticus in the mouse hippocampus contralateral to the site of kainate injection. This begs the question why the CLH, in contrast to the ILH, does not develop hippocampal sclerosis? In the CLH we found an overweight of upregulated genes with potential anti-epileptogenic properties. Further, we detected a significantly lower number of differentially regulated genes in glia. We therefore hypothesize that both an overweight of upregulated genes and pathways with potential downstream anti-epileptogenic effects and a lower number of genes and pathways with pro-epileptogenic qualities in glia contribute to prevent epileptogenesis in the CLH. Gene expression changes in terms of nuclear mRNA may, however, only be one among many factors when it comes to finally determining cellular responses upon external stimuli. Also the role of DNA methylation for gene expression remains still uncertain in this model as we only found

a marginal overlap of differentially methylated sites between the CLH and ILH. In order to further disentangle the cell- and stage-specific orchestration of epileptogenesis, it is essential to perform longitudinal animal studies including the investigation of acute and chronic time points of epileptogenesis. Finally, studies exploring neuronal and glial gene expression in human tissue are required in order to evaluate the clinical relevance of these findings.

DATA AVAILABILITY STATEMENT

Raw data is available under GEO accession code GSE153976.

ETHICS STATEMENT

The animal study was reviewed and approved by Norwegian Food Safety Authority, the Center for Comparatice Medicine, Oslo University Hospital and the University of Oslo.

AUTHOR CONTRIBUTIONS

TB: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing–original draft, and writing–review & editing. KH: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing–original draft, and writing–review & editing. KS: conceptualization, funding acquisition, methodology, project administration, resources, supervision, and writing–review and editing. ET: conceptualization, methodology, project administration, resources, supervision, and writing–review & editing. CN: investigation and writing–review & editing. HH: conceptualization, investigation, methodology, project administration, writing–original draft, and writing–review & editing. MV: conceptualization, data curation, formal analysis, methodology, project administration, software, supervision, validation, visualization, writing–original draft, and writing–review & editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.573575/full#supplementary-material

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REVIEW
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Astrocytes as Guardians of Neuronal Excitability: Mechanisms Underlying Epileptogenesis

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Astrocytes are key homeostatic regulators in the central nervous system and play important roles in physiology. After brain damage caused by e.g., status epilepticus, traumatic brain injury, or stroke, astrocytes may adopt a reactive phenotype. This process of reactive astrogliosis is important to restore brain homeostasis. However, persistent reactive astrogliosis can be detrimental for the brain and contributes to the development of epilepsy. In this review, we will focus on physiological functions of astrocytes in the normal brain as well as pathophysiological functions in the epileptogenic brain, with a focus on acquired epilepsy. We will discuss the role of astrocyte-related processes in epileptogenesis, including reactive astrogliosis, disturbances in energy supply and metabolism, gliotransmission, and extracellular ion concentrations, as well as blood-brain barrier dysfunction and dysregulation of blood flow. Since dysfunction of astrocytes can contribute to epilepsy, we will also discuss their role as potential targets for new therapeutic strategies.

Keywords: glia, astrogliosis, seizures, epilepsy, treatment, gliotransmission, blood-brain barrier, neuroinflammation

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INTRODUCTION

Epilepsy is a common neurological disease that is estimated to affect roughly 1–2% of the population (1). Despite the fact that quite some anti-epileptic drugs (AEDs) have been developed in the last decades, a large number of patients still fail to respond to these AEDs. This is associated with increased morbidity and mortality and since these patients need life-long care this is also an economic burden for society. Furthermore, patients feel stigmatized and report a reduced quality of life (2). Therefore, it is of crucial importance to find novel drug targets in order to develop novel therapeutic strategies. Moreover, disease-modifying therapies are currently not available and require a better understanding of the underlying disease processes. In the past two decades, astrocytes have been increasingly acknowledged as key players in the etiology and pathogenesis of epilepsy. Therefore, astrocytes should be considered as promising targets for new therapeutic strategies.

The human brain is comprised of ∼100 billion cells, classically divided into neurons and glial cells, although new types of brain cells are still being discovered up to date (3, 4). Glia cells in the central nervous system are typically classified into four cell types: (1) astrocytes, (2) microglia, (3) oligodendrocytes, and (4) their progenitors, neuron-glial antigen 2(NG2)-glia (5). For almost a century it was believed that glial cells outnumbered neurons 10:1 (6). However, it has been shown that the actual ratio of glial cells compared to neurons is closer to 1:1 and may in fact be lower than

1 (6, 7). Nevertheless, the remarkably conserved numerical relationship between glia and neurons over 90 million years of evolution supports the notion that glial cells are crucial for normal brain functioning (8). These numbers suggest a far more prominent role for astrocytes in the brain than long considered. In addition to its vast number, it is estimated that a single astrocyte touches and interacts with up to 2 million synapses with its processes (9).

Although all four glial cell types play a pivotal role in normal brain function, in this review we will focus on astrocytes which are key homeostatic regulators in the central nervous system and play important roles in the pathophysiology of epilepsy (10).

For many years, astrocytes were regarded as "glue" that bound neuronal elements together, providing mere structural support for the brain. In fact, astrocytes are playing a pivotal role in brain homeostasis. From recent transcriptome studies it became clear that different subtypes of astrocytes exist that are not only anatomically and spatiotemporally restricted, but also show varying degrees of heterogeneity of morphology and physiology in distinct brain regions (11, 12). The relevance of astrocytic heterogeneity is also evident in its distinct subpopulations and cortical layer-specific gene signatures that underline the comprehensive involvement of astrocytes in physiology (13–15). More sophisticated research strategies paired with a systemic evaluation and comparison of different glial markers will lead to a better understanding of the role of astrocytes in the central nervous system under physiological and pathophysiological conditions.

Astrocytes have been shown to be involved in important processes such as brain inflammation (16, 17) and oxidative stress (18), energy supply and metabolism (19–21), support of synaptic function and plasticity (22, 23), extracellular balance of neurotransmitters (24, 25), extracellular water and ion homeostasis (26, 27), blood-brain barrier (BBB) maintenance (28, 29), and regulation of blood flow [(30, 31); **Figure 1**].

Although astrocytes employ many processes that protect the brain from hyperexcitability, dysregulation of glial functions may cause hyperexcitability or promote the development of epilepsy by a multitude of mechanisms. In the following paragraphs, we will focus on the underlying processes that can promote epileptogenesis, including astrogliosis, disturbed energy metabolism and gliotransmission, alterations in extracellular ion concentrations, as well as dysfunction of the BBB and dysregulation of blood flow (**Figure 2**).

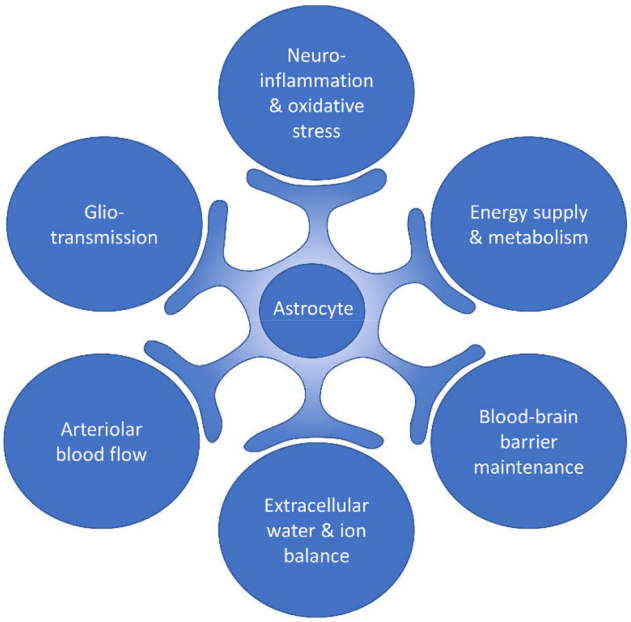

FIGURE 1 | Processes within the brain in which astrocytes are involved. Astrocytes have been shown to be involved in important processes such as neuroinflammation and oxidative stress, energy supply and metabolism, blood-brain barrier maintenance, extracellular water and ion balance, arteriolar blood flow, and gliotransmission.

ASTROGLIOSIS

Due to brain injury induced by status epilepticus, stroke or traumatic brain injury, astrocytes receive "instructions" from their environment (**Figure 3A**) and in response to these molecular signals, the number of astrocytes increases and the astrocyte expression profile as well as its morphology, biochemistry and functionality changes, a process called reactive astrogliosis (32, 33). In turn, reactive astrocytes can send "instructions" to their environment (**Figure 3B**). The term reactive astrogliosis has been introduced in the nineteenth century to characterize morphological and behavioral changes within astrocytes upon pathophysiological conditions caused by various central nervous system diseases. In the beginning, efforts were focused on the morphological changes astrocytes experience during reactive astrogliosis, but over the past three decades a body of evidence has been collected that support astrogliosis heterogeneity and acknowledges a spectrum of molecular, cellular and functional changes within astrocytes upon reactive astrogliosis (32, 34).

The existence of spatiotemporal and anatomically localized subtypes of astrocytes needs to be taken into account when evaluating astrogliosis in the context of experimental epilepsy models, including the consequential effects on epileptogenesis and related neurobehavioral comorbidities, by employing genetic targeting studies and pharmacological therapies.

Cell-specific transcriptomics have revealed that astrocytes undergo massive changes in gene expression when they switch to a reactive phenotype (33). One of the most prominent changes during reactive astrogliosis is characterized by cell hypertrophy and upregulation of glial fibrillary acidic protein (GFAP), vimentin, nestin, and/or inducible nitric oxide synthase (iNOS) (35, 36). In addition, reactive astrocytes may produce and release a variety of factors, including pro-inflammatory cytokines (37), complement factors (38), gliotransmitters (39–41), reactive oxygen species (ROS) (42), trophic factors (43), and vascular endothelial growth factor (VEGF) [(44); **Figure 3**].

In particular, pro-inflammatory cytokines may affect astrocytes profoundly and cause changes that perpetuate

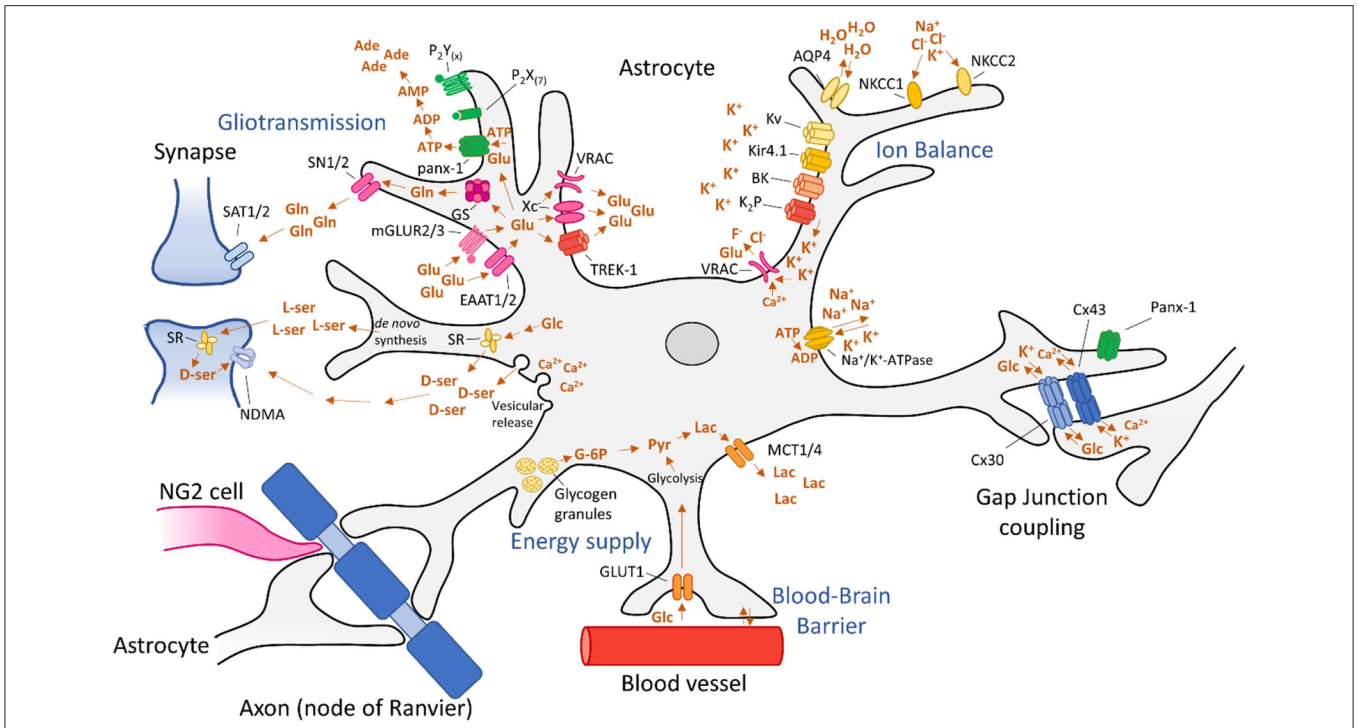

FIGURE 2 | Astrocytic processes involved in epileptogenesis. Dysregulation of astrocyte functions can lead to epileptogenesis via disturbed energy metabolism and gliotransmission, alterations in extracellular ion concentrations, as well as dysfunction of the blood-brain barrier and dysregulation of blood flow. These mechanisms are discussed in detail in the main text.

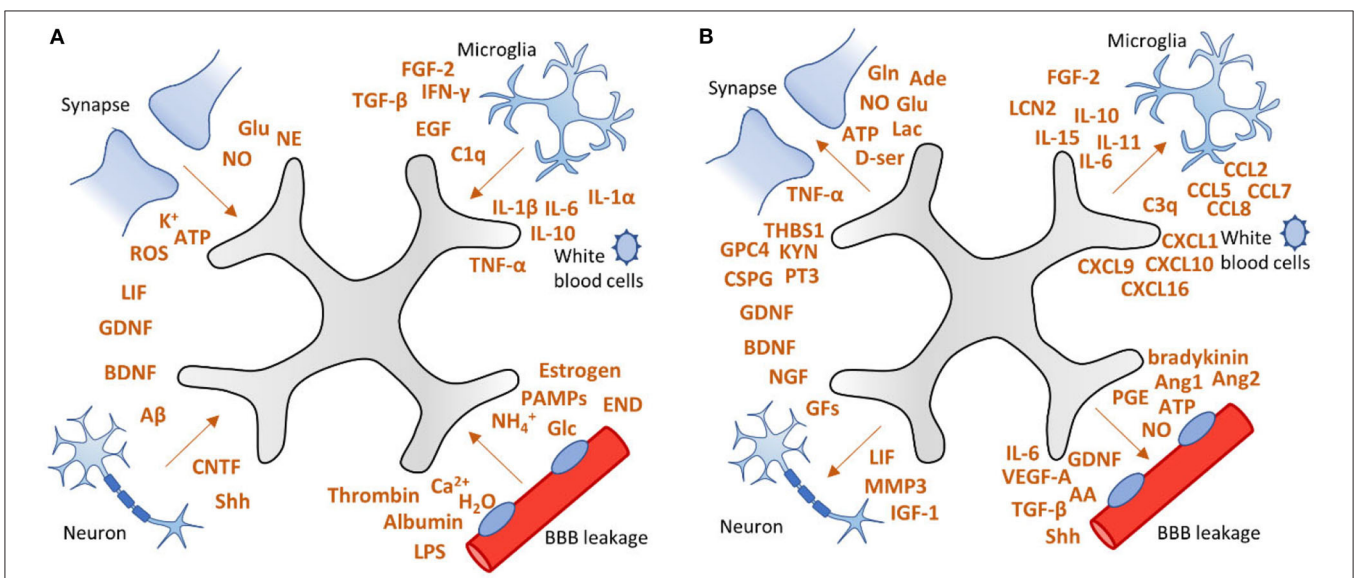

FIGURE 3 | Factors involved in astrogliosis. **(A)** After brain injury, astrocytes can receive "instructions" from their environment and respond to a plethora of signaling molecules. **(B)** In turn, astrocytes send "instructions" to their environment by releasing a variety of factors, including pro-inflammatory cytokines, growth factors, neurotransmitters, as well as vascular mediators. This vicious cycle may lead to persistent activation of astrocytes which can contribute to epileptogenesis. Adapted from Sofroniew (32).

astrogliosis and promote epileptogenesis (45, 46). Cytokines are widely studied in the context of reactive astrogliosis (47) and epilepsy (48). In this paragraph, we will focus on cytokines that exacerbate epilepsy progression and may therefore be interesting for therapeutic intervention. The most studied cytokines regarding astrogliosis and epilepsy are interleukin-1 beta

(IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α); pro-inflammatory cytokines that can be released by reactive astrocytes (49, 50) and activated microglia (51). In a complex pathology such as epilepsy, more cytokines are playing roles in the alleviation and exacerbation of the disease. **Figure 3** shows a fraction of cytokines involved in astrocytosis. For further reading into cytokine involvement in epilepsy the reader is directed to the following reviews (45, 46, 52).

Numerous studies have shown upregulation of IL-1β, IL-6, and TNF-α in animals with (recurrent) seizures (53–59) and patients with epilepsy (60–63). IL-1β can affect neurotransmitter receptors (64, 65), induce calcium influx by N-methyl-D-aspartate (NMDA) and 3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-mediated mechanisms in neurons (66, 67), lead to alterations in expression of microRNAs in astrocytes (68–70), as well as potassium channels (71), metalloproteinases (72), altered astrocytic glutamate uptake (73, 74) and calcium uptake (75), and induces astrocytic release of other pro-inflammatory cytokines (50). IL-6 activates the Grp130/JAK/STAT pathway and thereby induces the release of additional pro-inflammatory cytokines, further endorsing inflammation (76). In addition, high levels of IL-6 lead to decreased astrocytic glutamate uptake via excitatory amino acid transporter 2 (EAAT2; formerly glutamate transporter 1) and even promote the release of glutamate by improving the activity of the astrocytic cystine/glutamate antiporter (Xc^-) (76). Finally, IL-6 increases BBB permeability (77).

Similar to IL-6, TNF-α decreases astrocytic uptake of glutamate (78, 79) via purinergic signaling, thereby activating presynaptic NDMA receptors (80). Furthermore, TNF-α increases excitatory strength of AMPA receptors and at the same time decreases the amount of γ-aminobutyric acid $(GABA)_A$ receptors in neurons, impairing inhibitory signaling (81). Finally, release of pro-inflammatory cytokines often stimulate additional release of cytokines (50), and it is suggested that this perpetual exacerbation of inflammatory signaling contributes to epilepsy (16, 82, 83). VX-765, a small molecule inhibitor of interleukin-converting enzyme (ICE)/caspase-1, thereby inhibiting biosynthesis of IL-1β, has been shown to reduce the number and duration of seizures in rats (84) and mice (85) and has even been tested in Phase II clinical trials (ClinicalTrials.gov Identifier: NCT01501383). In a Phase IIa randomized double blind placebo-controlled study in drug-resistant focal onset epilepsy, VX-765 had delayed beneficial effects (subjects with ≥50% reduction in seizure frequency) that persisted after drug discontinuation (86). Furthermore, the IL-1 receptor antagonist Kineret (anakinra) showed a drastic improvement of seizure control in patients with super-refractory status epilepticus secondary to febrile infection-related epilepsy syndrome (FIRES) (87–90), as well as in patients with drug-resistant epilepsy (91, 92).

To our knowledge, there is no therapy that directly targets IL-6 or its receptor IL-6R, but it has been shown that the antiepileptic drug valproate affects IL-6 serum levels, hinting at a possible mechanistic involvement of IL-6 (93).

Another example is Adalimumab, a TNF-α monoclonal antibody that has been tested in Rasmussen's encephalitis, leading to seizure improvement in a small cohort of patients (94). Furthermore, n-3 docosapentaenoic acid-derived protectin D1 is a pro-resolving mediator that was administered to epileptic mice, showing subsequent downregulation of IL-1β and TNF-α mRNA and consequently a 50% decrease of seizure activity and a 40% decrease in seizure duration (95). Finally, 1400 W, an inhibitor of inducible nitric oxide synthase (iNOS/NOS-II) showed promising results in a rat model of kainic acid-induced epilepsy, since it suppressed astrogliosis, microgliosis, neurodegeneration, mossy fiber sprouting, and had disease modifying effects (96).

Reactive astrogliosis is implicated in acquired and genetic types of epilepsy, including neurodevelopmental diseases [i.e., tuberous sclerosis complex; (17, 97–99)]. Changes of activity and gene expression of key proteins that are involved in epilepsy pathology such as glutamine synthetase (GS) (100), adenosine kinase (ADK) (101, 102), Aquaporins (AQPs) including AQP4 (103, 104), inward rectifying potassium (K_{ir}) channels including $K_{ir}4.1$ (105, 106), and monocarboxylate transporters (MCTs) (107, 108) have been observed in resected brain tissue of patients with temporal lobe epilepsy (TLE). Initially, the astrocytic response can be beneficial for the brain, promoting restoration of brain homeostasis. However, a vicious cycle may lead to persistent astrogliosis which can affect metabolic activity (109–111), ion buffering (112), gap junction (GJ) connectivity (113, 114), neurotransmitter uptake (115, 116), and promotes neuronal death, BBB dysfunction (44), and onset of seizures (117, 118). In the following chapters we will elaborate how these changes can affect neuronal excitability and contribute to epileptogenesis.

Besides affecting molecular pathways, astrocytes participate in bilateral signaling with microglia (119, 120). This interglial crosstalk has implications on both physiological and pathological processes (121, 122). Astrocytes influence microglial behavior by releasing molecules that regulate microglial functions. In turn, microglia are able to drive astrocytes from a neuroprotective to a neurotoxic phenotype (123), thereby potentially affecting the ability of astrocytes to protect against neuronal excitability. This bidirectional crosstalk may induce a persistent inflammatory environment under pathological conditions and may therefore exacerbate disease severity. Recent studies have shown that activated microglia induce neurotoxic phenotypes in astrocytes by secretion of pro-inflammatory mediators such as IL-1α, TNF-α, and complement component subunit 1q (C1q) (119, 124). Crosstalk between astrocytes and microglia may also be involved in epileptogenesis and should be taken into account when conducting studies into the mechanisms that drive epilepsy. Although attention has been primarily focused on astrocyte interactions with other central nervous system cell types, there is recent evidence that astrocyte functionality is influenced by the gut microbiome, and that this cross-talk between gut microbiota and brain, involving astrocytes, may have crucial implications in the development and progression of central nervous system disorders (125, 126). For instance, different types of gut bacteria may positively or negatively modulate the astrocytic inflammatory response (126–128).

ENERGY SUPPLY AND METABOLISM

Under physiological conditions, glucose is the primary metabolic substrate of the brain and is required to maintain the transmembrane potential of neurons (21). Glucose is transferred from the blood into the brain by glucose-transporters (GLUTs). Then, glucose is metabolized into glucose-6-phosphate (G-6P) by hexokinase [(129); **Figure 2**]. Subsequently, it can undergo two types of metabolization: glycolysis or metabolization by the pentose phosphate pathway. During glycolysis, pyruvate is formed, producing ATP. Pyruvate can then be oxidized in mitochondria by the tricarboxylic acid (TCA) cycle or by oxidative phosphorylation, producing 30–34 molecules of ATP at the cost of oxygen (129). In addition to neuronal energy supply, astrocytes are also equipped with a glucose-metabolism to meet the local energy demand. In fact, in times of hypoglycaemia and during periods of high neuronal activation, astrocytes take over the energy supply completely (130, 131). Astrocytes are able to process glucose by a mechanism similar to that of neurons. Astrocytes express glucose transporter 1 (GLUT1) allowing for glucose uptake (**Figure 2**). Glucose is then metabolized into G-6P by hexokinase (HK) and further into lactate, via pyruvate by an isoenzyme of lactate dehydrogenase (LDH) 5 (20). Subsequently, lactate is exported from astrocytes into the extracellular space by monocarboxylate transporters (MCTs) 1 and 4 (132) and taken up into neurons, which convert lactate into pyruvate. This alternative pathway constitutes the astrocyte-neuron lactate shuttle (21). Alternatively, G-6P is converted into G-1P by phosphoglucomutase (PGM) and then into uridine triphosphate (UDP)-glucose by UDP-glucose pyrophosphate (UDPGPP) (130). Finally, UDP-Glucose is converted into glycogen by glycogen synthase (GYS). Glycogen can then be stored in glycogen granules, usually clustered in places of great synaptic density (133). When required, glycogen can be metabolized back to G-6P via the same route in reverse or mediated by glycogen phosphatase (GP) (134).

Glucose Sustains Synaptic Activity During Seizures

During seizures, excessive synaptic activity causes a rapid drop of glucose and a corresponding rise in lactate. Surrounding tissue responds to this by increasing glucose-administration to neurons by increasing blood perfusion and volume (135). At the same time, glucose can be distributed by astrocytes via by gap junctions (GJs) to reach distal neurons [(136); **Figure 2**]. During the excessive energy demands of seizures, astrocyte-derived lactate becomes an essential energy source for neurons (21). Furthermore, astrocytes can store glycogen which can supply energy to neurons via the lactate shuttle to sustain neuronal activity during seizures. Therefore, reducing brain glucose levels is considered anticonvulsive (137).

One way of achieving this is by the ketogenic diet, which is a low-carbohydrate and high-fat and adequate protein diet (138, 139). Thereby, the brain switches from a glucose-sustained metabolism to ketosis during which ketones, such as β-hydroxybutyrate, acetoacetate, and acetone are formed, which are thought to be important mediators for the suppression of seizures during the diet (140). In addition to the ketogenic diet, other antiepileptic diets have been proposed such as the modified Atkins diet, the medium-chain triglyceride ketogenic diet, and the low glycaemic index treatment (141, 142). Ketogenic diets are quite efficient in the alleviation of seizures in children, but also in adults with refractory epilepsy (138, 143, 144). However, it is difficult to adhere to the diet since it is not palatable. Furthermore, weight loss, constipation, high level of low-density lipoprotein, and elevated total cholesterol are most frequently reported as adverse effects (143, 145). Therefore, alternative approaches to inhibit glycolysis or interfere with lactate formation are studied. For instance, the use of the glycolysis inhibitor such as 2-deoxy-2-glucose has been proposed as a direct mechanism of lowering brain glucose, which has acute anticonvulsant and chronic antiepileptic actions in various epilepsy models (146–148). Furthermore, inhibition of LDH suppresses pilocarpine and kainic acid-induced seizures. Interestingly, LDH is also inhibited by the AED stiripentol (149). Another approach is to utilize GJ blockers that impair astrocytic intercellular glucose trafficking, thereby partially reducing the required energy for epileptiform activity (150). Taken together, these data imply that targeting specific brain glucose-pathways is an ambitious and challenging, but also a promising approach to interfere with epileptogenesis. Reducing glucose levels may be achieved by specific diets, local glycolysis-inhibition or by inhibition of GJs.

Gap Junctions

Astrocytic GJs are comprised of two "hemichannels" which are made up of 6 subunits or connexins (Cx) (151). Astrocytes predominantly express connexins Cx43, but also Cx30, Cx26, Cx40, Cx45, and Cx46 (152, 153). One of the functional properties of GJs is to facilitate inter-astrocyte transportation of glucose and glucose-metabolites (150). In addition, GJs are able to propagate intercellular Ca^{2+} signaling through release of ATP (153, 154). Furthermore, GJs permit potassium transport between astrocytes, allowing K^+ influx to redistribute to sites of lower concentration, supporting spatial K^+ buffering (discussed in detail in a following paragraph). GJs reduce the threshold for seizures by facilitating spatial K^+ buffering and glutamate transport. The involvement of GJs in spatial K^+ buffering is reflected in the $AQP4^{-/-}$ mouse model in which increased GJ coupling compensates for the loss of K^+ uptake assisted by AQP4 (155). In line with this, mice with GJ-coupling deficiencies were shown to develop seizures and have problems with glutamate and K^+ clearance (156).

Neuroprotective properties of GJs have been reported and therapeutically interfering with GJ functionality may introduce side effects (157). Moreover, uncoupling (loss of connectivity through loss of GJs) of astrocytic endfeet has been found to precede neurodegeneration and spontaneous seizure generation in a mouse model of TLE (158). Different expression patterns have been reported in studies on animal models and human tissue (159, 160). In astrocytes of sclerotic human hippocampal tissue, expression of connexins appears unchanged (161). It has been proposed that instead subcellular reorganization or post-translational modification of connexins accounts for the loss of

GJ functionality in TLE. This could explain the variability in connexin expression in TLE found in earlier studies (159).

On the other hand, GJs may fuel synaptic and epileptiform activity by intracellular trafficking of metabolites to sustain neuronal activity at sites of high demand. GJs facilitate the spread of Ca^{2+} waves contributing to epileptogenesis by introducing a feedback loop from neurons to astrocytes (162). Furthermore, neuronal GJs are thought to be involved in the synchronous discharges during seizure activity (163, 164). These data suggest that inhibiting GJs has anticonvulsive effects.

Indeed, it has been shown that GJ blockers such as carbenoxolone (165–167), mefloquine (168), quinine (166, 167, 169), and quinidine (170) alleviate seizure severity in various animal models of epilepsy, although a general consensus on the exact mode of action and the efficacy of these compounds is still lacking. Anandamide and oleamide are fatty acids of the endocannabinoid family that have been demonstrated to inhibit intercellular GJs from glial cells (171, 172). Both anandamide (173, 174) and oleamide (175, 176) have been shown to have anticonvulsant effects *in vivo*, hinting at the involvement of glial networks in seizures.

GLIOTRANSMISSION

The concept of "gliotransmission" remains one of the most controversial topics in astrocyte biology. The term gliotransmitter is loosely defined as chemically active transmitters that origin from glial cells which may participate in or affect the excitatory or inhibitory network of neurons. Numerous studies have been performed showing a plethora of astrocytic released gliotransmitters: (1) amino acids including glutamate (177–179), D-serine (180, 181), GABA (182–184) and glycine (185–187), (2) nucleotides, such as adenosine 5′-triphosphate (ATP) (188–190), (3) organic acids including lactate (191–193), taurine (194, 195), and homocysteic acid (196, 197), and (4) peptides such as atrial natriuretic peptide (ANP) (198) and brain-derived neurotrophic factor (BDNF) (199, 200). Some argue that cytokines are in a way also gliotransmitters as they are chemically active too and may affect neuronal excitability, albeit mainly via indirect mechanisms. However, in this review we will further focus on the most studied gliotransmitters: glutamate, D-serine, and ATP and give an insight on how these gliotransmitters affect neuronal excitability.

Ca^{2+}-Dependent Gliotransmitter Release

In the early 1990s the first Ca^{2+} imaging studies were performed, showing increased astrocytic intracellular Ca^{2+} concentrations after local synaptic activity (201–203). A general consensus developed stating that astrocytes are in fact "excitable" cells and may respond to a wide range of neuronal factors and synaptically released spill-over neurotransmitters, and at the same time release so-called gliotransmitters that can communicate to neurons (24). In addition, newer imaging techniques showed that astrocytes appear to facilitate spontaneous focal Ca^{2+} oscillations or transients (204–206) and may even propagate Ca^{2+} signals to adjacent astrocytes (207–209). Unfortunately, due to limitations in experimental approaches required to understand the complexity of gliotransmission, it proved difficult to replicate findings in different models, or translate data from *in vitro* to *in vivo*. A heated debate followed in which contrasting evidence from various studies raised the question whether or not astrocytes contribute to information processing within the neural circuitry under physiological conditions (210). In addition, the dependence on Ca^{2+} signaling has been challenged time and again and is under critical review (211). To go further into this debate is beyond the scope of this review and the reader is directed to excellent literature on the topic (210, 212, 213).

Nowadays, a strong foundation of evidence that supports the bidirectional communication between neurons and astrocytes established the concept of a tripartite system that was originally proposed in the late 1990s (214). Progress on research neuron-glia crosstalk showed that the central role of astrocytes, besides regulation of brain homeostasis, is information processing. A body of evidence supports the existence of coordinated neuron-astrocyte network signaling, in which astrocytes are able to modulate neuronal excitability and synaptic transmission (206, 215–217). In turn, neuronal communication to astrocytes influences astrocytic signaling which may have implications in epilepsy (215, 218).

Two types of astrocyte "excitation" are well documented: neuron-dependent excitation and spontaneous excitation (24). There is evidence of Ca^{2+}-dependent astrocytic release of different types of gliotransmitters including glutamate (204, 219–221), D-serine (222–224), and ATP (225, 226). To what extent these mechanisms are in fact dependent on Ca^{2+} or how they may or may not play a role in synaptic transmission under physiological conditions is discussed elsewhere (211, 227, 228).

Ca^{2+}-Independent Mechanisms of Gliotransmitter Release

In addition to Ca^{2+}-dependent mechanisms of gliotransmitters, several Ca^{2+}-independent mechanisms have been identified for some, but not all gliotransmitters. Astrocytes facilitate glutamate release by targeting the two-pore domain K^+ channel (TREK-1) (220), through the pannexin-1 (panx-1) (229), and Cx43 hemichannels (230, 231), by volume-regulated anion channels (VRACs) (194), reversible glutamate transporters (232–234), and *in vitro* via the (Xc^-) (235, 236) and the ionotropic P2X purinoceptor 7 (P_2X7R) [(237); **Figure 2**]. Astrocytic ATP is released through GJ channels such as panx-1 (229, 238) and Cx43 hemichannels (231, 239), and in culture via mechanically-induced release of ATP by P_2X7R (240, 241).

The relevance of these mechanisms is demonstrated by the changes that occur under pathophysiological circumstances such as in the epileptogenic brain (242–244), during astrogliosis (245), or upon swelling of astrocytes (246). Reactive astrocytes display increased expression and activation of hemichannels such as Cx43 (247) and panx-1 (248, 249), which is generally believed to result in increased gliotransmitter release (234). Moreover, during epilepsy, the opening probability of both astrocytic and neuronal hemichannels is increased, augmenting local excitotoxicity (250).

Using transgenic mouse models, ATP release through panx-1 channels has been shown to enhance neuronal excitability (251, 252). Furthermore, panx-1 in conjunction with P_2X7R potentiates seizure activity in an animal model of epilepsy as well as in brain slices of patients with TLE (252). Panx-1 channels are exciting new targets as global panx-1 inhibition has anticonvulsive effects in animal models of epilepsy (168, 251).

Similar to panx-1, global inhibition of P_2X7R reduces epilepsy severity after kainic acid-induced epilepsy (253), although additional *in vivo* data indicates that this is mainly due to neuronal effects (244, 254). Inhibiting P_2X7R presumably affects astrocytes and other glial cells indirectly as well, by blocking P_2X7R-mediated excitotoxic IL-1β release (255). P_2X7R inhibitors such as Brilliant Blue G, A438079, AFC-5128, and JNJ-47965567 could attenuate chemically-induced kindling but did not possess remarkable effects in acute screening tests when administrated alone (256, 257).

Taken together, these data indicate that modulating astrocytic gliotransmitter release pathways may affect neuronal excitability. Interestingly, in a recent review several experimental pharmacological agents were highlighted as tools to control astrocyte signaling (258). These agents were tested in preclinical models, but some antiepileptic drugs may exert similar effects. This needs to be studied in more detail, as well as the use of these agents as novel therapeutic approaches. In the following paragraphs we will further elaborate on ways that gliotransmitters influence the pathophysiology of epilepsy.

Glutamate

Astrocytes are able to influence extracellular concentrations of glutamate and an excess of extracellular glutamate is one of the mechanisms driving hyperexcitability (259, 260). Under physiological conditions, astrocytes restrict the diffusion of glutamate in the synaptic cleft and take up and recycle glutamate in a process called the glutamate/GABA-glutamine cycle [(25, 261); **Figure 2**]. In this cycle, glutamate is taken up by astrocytic glutamate-uptake channels such as the excitatory amino acid transporter 1 (EAAT1; formerly Na^+-glutamate cotransporter) and EAAT2. Glutamate is then converted into glutamine by glutamine synthetase (GS) at the cost of ammonia and ATP. Interestingly, astrocyte subpopulations that express GS also co-express EAAT1 and EAAT2, emphasizing the link between the two mechanisms (262). After the conversion, glutamine is shuttled back to neurons through release by N system transporters (SN) 1 and 2 on the astrocytic membrane followed by neuronal uptake through system A transporters (SAT) 1 and 2 (263). It is then converted back into glutamate by neuronal glutaminase. In this cycle there are two steps by which astrocytes regulate glutamatergic excitability: (1) by removing excess glutamate from the extracellular space, and (2) by regulating the glutamine release from the astrocytic cytoplasm. In addition to glutamate uptake and conversion to glutamine, astrocytes are also able to synthesize glutamine *de novo*, by employing glycolytic enzymes and the TCA cycle, which produces glutamate from α-ketoglutarate and can then be converted to glutamine by GS (264).

Under pathophysiological circumstances, the regulation of the glutamate/GABA-glutamine cycle is perturbed, which can contribute to epileptogenesis. *In vivo* microdialysis experiments in the human brain showed that extracellular glutamate concentrations were chronically increased in the epileptogenic hippocampus compared to non-epileptic hippocampus (265, 266). This is likely the result of a failing glutamate uptake system from astrocytes in concert with a decreased ability to convert glutamate to glutamine.

Glutamate-Uptake Channels

Downregulation of glutamate-uptake channels such as the EAAT1 (267–269) and EAAT2 (267, 269, 270) has been frequently reported in animal models of epilepsy. Furthermore, EAAT1 deletion causes prolonged seizure activity (271) and EAAT2 knockout mice exhibit spontaneous and recurrent seizures (272). In patients with TLE, EAAT1, and EAAT2 are also downregulated and this is colocalized with GFAP and the proliferation marker Ki-67, suggesting that this is dependent on astrogliosis (273, 274). Transcriptional reactivation of EAAT2 by a small molecule reduced the frequency of spontaneous seizures by 50% in a mouse model of tuberous sclerosis complex, postulating that restoring glutamate-uptake channels is seizure ameliorating (275). The loss of EAAT2 is not only evident on mRNA expression level, but the protein itself is also internalized and subsequently degraded (276). Therefore, preventing the degradation of EAAT2 may pose as an effective treatment for epilepsy as was recently shown in a mice model of kainic acid-induced epilepsy (277).

Glutamine Synthetase

A growing body of evidence supports the notion that pathophysiological events such as epileptic seizures (278, 279) or astrogliosis (280, 281) result in a downregulation and corresponding decrease in immunoreactivity of GS (282). In accordance, chronic treatment with a GS-inhibitor caused spontaneous seizures in rats and increased local extracellular glutamate concentrations by 47%, showing that GS-deficiency alone is enough to evoke ictal events (260).

The exact mechanism of the lowered extracellular glutamate concentration due to GS-deficiency is still unknown, although several hypotheses have been proposed: (1) loss of GS leads to impaired clearance of glutamate because of a reduced conversion to glutamine, and (2) accumulating glutamate in astrocytes constitutes a concentration-dependent gradient that results in astrocytic glutamate release (282).

Indirectly, GS-deficiency may also contribute to hyperexcitability (282). Because glutamine is the precursor for the inhibitory neurotransmitter GABA, a reduction in astrocytic glutamine production evokes a local shortage of GABA. As the main inhibitory neurotransmitter of the brain, a local GABA shortage increases neuronal excitability and neuronal network synchronization (283). A second way that GS-deficiency affects local excitability is that a reduction in the glutamine metabolism consequently consumes less ammonia. Previously, it has been shown that high concentrations of local ammonia is neurotoxic and may even cause excitotoxicity by affecting chloride transport

(284, 285). It is presently unclear to what extent these indirect mechanisms contribute to ictogenesis and research into this would certainly contribute to our understanding of how a GS-deficiency could cause epileptic seizures.

Evidence suggests that GS expression is dependent on neuronal survival as downregulation of GS in patients with TLE coincides with neuronal loss (100, 285). It has been proposed that neuronal loss precedes GS downregulation, and in turn, GS downregulation increases excitability (285). In addition, the resulting increase in extracellular glutamate may result in neuronal cell death (286), feeding a disease exacerbating cycle (285).

Other pathological changes within astrocytes such as swelling (194) and ischemia (233) may also affect glutamate release and extracellular concentrations. From all this data it becomes evident that the glutamatergic mechanisms that underlie excitability are exceedingly intricate and complex. Perturbations in any of the aforementioned glutamatergic mechanisms may lead to an increase of excitatory network activity, and eventually epilepsy (287).

D-Serine

Presently, all mechanisms regarding D-serine release from astrocytes appear to be either directly linked to intracellular Ca^{2+} concentration (i.e., vesicular release), or are receptor activation-dependent, which is indirectly linked to local Ca^{2+} concentration (288). Amongst these are the adenosine type 2A receptor ($A_{2A}R$) (289), bradykinin-type2 (B_2) receptor (290), ephrinB3 receptor (291), ionotropic (292), and metabotropic (223) glutamate receptors, transforming growth factor (TGF)-β receptor (293), as well as muscarinic (294) and nicotinic (295) acetylcholine receptors. Despite its extensive regulation, it was recently proposed that astrocytic D-serine is not available in sufficient amounts to modulate synaptic activity *in vivo*, under physiological conditions (296). Instead, astrocytic *de novo* synthesized L-serine that is required for the production of D-serine in neurons may affect synaptic activity after conversion to D-serine by neuronal serine racemase (SR) (297, 298). It is noteworthy however, that the profound effects of pathophysiological conditions such as epileptic seizures or astrogliosis dramatically change the behavior and expression profiles of astrocytes, which may in turn affect the dynamics of D-serine production. In culture (224) and in brain slices (222), astrocytes are able to express SR, and most notably, in an animal model of traumatic brain injury, it was shown that the switch from neuronal SR to astrocytic SR was in part responsible for traumatic brain injury-induced synaptic damage (299). Furthermore, increased release of D-serine may contribute directly to neuronal excitotoxicity by acting upon the NMDA receptor as a co-agonist together with glutamate (**Figure 2**). Indeed, it has been shown that lowering D-serine in epileptic rats by administering a competitive SR-inhibitor resulted in reduced seizure duration and severity, dependent on ERK signaling (300). These data suggest that modulating D-serine production could pose as a strategy for epilepsy treatment.

ATP

The actions of ATP and its metabolite adenosine arguably extend even further than that of glutamate or D-serine by acting upon purinergic receptors, influencing astrocytes, neurons, microglia, oligodendrocytes, and blood vessels (301). The complexity of ATP-mediated effects is demonstrated by studies that report both excitatory and inhibitory consequences from astrocytic ATP release. For instance, it has been shown that ATP activates the astrocytic ionotropic P_2X and metabotropic P_2Y receptors resulting in increased GABA release (302). Furthermore, ATP released by astrocytes may induce action potentials on inhibitory interneurons, thereby decreasing the excitatory network output (303). In contrast, astrocytic ATP negatively regulates GABAergic inhibitory transmission on post-synaptic neurons (226), suggesting that astrocytic ATP release may augment ictogenesis. Moreover, it has been shown that astrocytic ATP activates neuronal P_2X receptors leading to pro-epileptic effects (304), including enhanced pre-synaptic release of glutamate (305). As argued earlier, purinergic signaling through P_2X receptors is mediated by ATP release through panx-1 channels. However, it appears there is a clear distinction between astrocytic and neuronal panx-1, and surprisingly, astrocytic panx-1 may even be seizure alleviating (306) [for review see (234, 307)]. It is hypothesized that worsening of seizure activity in mice deficient of astrocytic panx-1 is likely connected to increased ADK levels in astrocytes.

Adenosine Kinase

ADK is a key metabolic enzyme of astrocytes that catalyses the conversion of adenosine into adenosine monophosphate. Therefore, modulation of ADK expression is of interest in the context of epilepsy. Adenosine is a potent anticonvulsant and is released during seizures (17). It is a substrate for the adenosine receptor family of which the $A_{1A}R$ and $A_{2A}R$ are the most studied. Anti-epileptic effects are mainly mediated by $A_{1A}R$ signaling which activates K_{ir} channels and inhibit Ca^{2+} channels, but also exert astrocyte-function modulating effects by stimulatory coupling to K^+ and Cl^- ion channels (308–310). Since neuronal excitability is modulated by activation of A_{1A}, A_{2A}, A_{2B}, and A_3 receptors, the equilibrium of intra- and extracellular adenosine critically affects epilepsy severity (311).

Synaptic adenosine is mainly regulated by ADK, because uptake of adenosine into astrocytes is quickly equilibrated by nucleoside transporters (ENTs) (310). Upon brain injury, ADK is transiently downregulated for ca. 2 h, recovering to baseline levels over the course of 24 h (101). This acute response to stress results in increased adenosine levels, enhancing protective effects against brain injury, including status epilepticus and traumatic brain injury, through increased activation of $A_{1A}R$ (310). However, elevated synaptic adenosine levels also activate the $A_{2A}R$, which signaling may in turn desensitize and downregulate the $A_{1A}R$ (312, 313). Indeed, it has been shown that in epileptogenic circuits, stimulation of $A_{2A}R$ downregulates $A_{1A}R$ (314, 315). Recently, it has been shown that a 3-fold induction of $A_{2A}R$ is present in astrocytes within the hippocampus of patients with TLE (316). Increased $A_{2A}R$ signaling promotes astrogliosis by various mechanisms including by increased stimulation of

glutamate release, synaptic actions of BDNF in the hippocampus and through the Akt/NF-κB pathway (317–320). The shift in $A_{1A}R/A_{2A}R$ signaling also causes a change from inhibition to promotion of cell proliferation and may contribute to the development of proliferative scar-forming astrocytes (310). Moreover, after the initial downregulation of ADK, its expression increased in reactive astrocytes (101). This is also confirmed in experimental animal models of epilepsy and human TLE brain slices (102). ADK inhibitors have since been developed (321–323) and tested in animal models of epilepsy (324, 325). Unfortunately, the first line of ADK inhibitors showed liver toxicity side effects, but recently efforts have been made to develop novel ADK inhibitors which may present a viable therapeutic strategy for epilepsy in the future (326).

WATER AND ION TRANSPORT

One of the functions of astrocytes is to maintain homeostatic extracellular water and ion balance in the brain. Changes in ion or water balance affect local synaptic activity by modifying the concentration gradient upon which the electrochemical potential is based. Ultimately, this may result in hyperexcitability by mechanisms discussed below. To ensure homeostatic ion balance is preserved, astrocytes express a plethora of passive, ATP-, voltage-, and volume-gated ion channels (**Figure 2** and **Table 1**).

Intra- to extracellular water balance is of significance for epilepsy because it directly correlates to local osmolarity that plays a role in excitability. Astrocytes are particularly sensitive to changes in extracellular osmolarity (351). For instance, as a result of traumatic brain injury, up to ∼30% of patients develop hyponatremia. This causes a decrease in osmolarity, after which astrocytes swell considerably (352, 353). As astrocytes swell up by water uptake, the extracellular space volume decreases. In turn, astrocytes respond by activating VRACs that work to restore the concentration equilibrium by expelling osmolytes and anions such as F^- and Cl^- [(351, 354, 355); **Figure 2**]. These mechanisms are of interest, because the volume of the extracellular space affects synaptic activity (26). In addition, opening of VRACs is accompanied with substantial amounts of glutamate (356, 357). These VRACs open primarily in astrocytes with high concentrations of K^+ or during hypo-osmolar conditions that often occur during ictal events, although it has been proposed that Ca^{2+} signaling may induce swelling and thereby open VRACs as well (39, 354). Due to lack of selectivity and inability to differentiate between astrocytic and neuronal channels, modulation of VRACs has not been tested in animal models of epilepsy, but may pose an interesting avenue for seizure treatment by potentially lowering extracellular glutamate levels. Care should be taken when following this approach as a tight regulation of osmolarity and the volume of the extracellular space is required for homeostatic brain function.

Spatial Potassium Buffering

The most critical ion flux governed by astrocytes in relation to epilepsy is that of potassium. In a process called spatial potassium buffering, astrocytes clear the extracellular space of excess K^+ during neuronal repolarization. To ensure rapid uptake of K^+ ions, astrocytes express different types of K^+-channels, including K_{ir} channels, Ca^{2+}-sensitive potassium (BK) channels, voltage-gated potassium (K_v) channels, two-pore domain (K_2P) channels and several co-transporters [(358); **Figure 2** and **Table 1**]. Upon entering astrocytes, K^+ is dispersed to areas of lower potassium concentration and travels intercellularly to adjacent astrocytes by GJs. The spatial buffering model is based on the fact that the low resting potential of astrocytes provide a driving force for K^+ uptake in regions of high neuronal activity.

Perturbations in the astrocytic K^+ buffering is therefore directly responsible for increased neuronal activity and excitability. In addition, high extracellular K^+ concentrations may affect the activity of ion and water transporters such as AQP4, EAAT2, Na^+/Ca^{2+}-exchanger (NCX), sodium-potassium pump Na^+/K^+-ATPase, and $Na^+/K^+/Cl^-$-cotransporter (NKCC) (355). Furthermore, increases in the extracellular K^+ concentration induce opening of panx-1 channels, may cause seizure activity by release of ATP and glutamate (252, 307, 355).

TABLE 1 | Selection of ion and water transporters associated with homeostatic astrocyte function and epileptiform activity in disease.

Ion	Transporter	Alteration	Expression in temporal lobe epilepsy
H_2O	AQP1	Causing astrogliosis (327)	↑ (329)
	AQP4	Mislocalization (328)	↑ Overall (330, 331) ↓ Perivascular (331, 332)
	AQP9		↓ (329)
	EAAT1		No change (100) ↓ Hippocampus (274)
K^+	BK		↓ Mossy fibers (335)
	K_2P		↑ CA1 ↓ Dentate gyrus (336)
	$K_{ir}4.1$		↓ Hippocampus, Perivascular (106)
	$K_{ir}5.1$		No change (337)
	$K_{ir}2.1$		↑ CA1, CA3, Dentate gyrus (338)
	K_v		↑ Hippocampus (339)
	Na^+/K^+-ATPase		↓ (Suggested) (340)
	NKCC1	Transient upregulation (333, 334)	↑ Subiculum, hippocampus (341)
Na^+	EAAT1		No change (100), ↓ Hippocampus (274)
	EAAT2		No change (100), ↓ Hippocampus (274)
	Na^+/K^+-ATPase		↓ (Suggested) (340)
	NCX		↓ Dentate gyrus (342)
	NKCC1	Transient upregulation (333, 334)	↑ Subiculum, hippocampus (341)
	TRPA1		↑ (343)
	TRPCs		↑ (344–346)
	TRPV1		↑ Cortex, Hippocampus (347)
Ca^{2+}	AMPA	Different splice variant (348)	↑ Hippocampus (349)
	NMDA	Subcellular relocation	↑ Dentate gyrus (350)
	NCX		↓ Dentate gyrus (342)
	P_2X7		No change (253)
	PMCA	Transient upregulation	↑ Dentate gyrus (342)
	TRPA1		↑ (343)
	TRPCs		↑ (344–346)
	TRPV1		↑ Cortex, Hippocampus (347)
Cl^-	NKCC1	Transient upregulation (333, 334)	↑ Subiculum, hippocampus (341)

↑: *upregulation*, ↓: *downregulation*.

Aquaporins

The integral membrane protein AQP4 is responsible for most of the water uptake by astrocytes (**Figure 2**), but a total of 13 human AQPs (0–12) have been characterized (26, 359). Of those, expression and protein levels of AQP1, 3, 4, 5, 8, 9, and 11 have been shown in rodent brains (329). Aside from aquaporins, there are additional mechanisms to transport water, for instance via co-transporters such as EAAT1 (360).

It is hypothesized that concomitant water uptake by AQP4 during spatial K^+ buffering decreases the volume of the extracellular space, thereby inducing an increase of $[K^+]_0$, which in turn stimulates astrocytic K^+ uptake (358, 361). In epileptic foci, elevated extracellular potassium concentrations due to impaired K^+ uptake by astrocytes may cause hyperexcitability (following paragraphs) (362). Surprisingly, AQP4 expression is increased in the hippocampus of patients with TLE (329, 330). However, local expression of the protein at perivascular astrocytic endfeet is lost. This is due to downregulation of the dystrophin gene that encodes for the protein responsible for anchoring AQP4, which causes the AQP4 channel to be mislocalized (363, 364). Decrease of perivascular AQP4 channels has been shown to perturbate spatial potassium buffering (103, 365). *In vivo* models of acute epilepsy with $AQP4^{-/-}$ mice showed elevated seizure thresholds, which can be explained by the increase in extracellular space volume from impaired water uptake (366). In addition, prolonged seizure activity was measured, likely due to impaired K^+ uptake (159, 331). Recently, it was shown that loss of perivascular AQP4 precedes seizure onset after kainic acid-induced epilepsy in rats, suggesting an involvement in epilepsy etiology (367). Taken together, these data suggest a that dysregulation of AQP4 plays an important role in epilepsy pathology.

Expression of other members of the AQP family such as AQP1 and AQP9 is also frequently reported to be altered in animal models of seizures (368), epilepsy (369), and traumatic brain injury (370, 371). In addition, expression changes in resected brain tissue of patients with TLE have been reported. Transcriptome and ELISA analysis showed that AQP1 expression is increased and AQP9 is decreased in human hippocampal sclerotic tissue compared to adjacent neocortex tissue (329). Moreover, AQP1 and AQP4 have been shown to play a role in cell growth and migration, and may be involved in glial scar formation (327, 372). Overexpression of AQP1 may therefore exacerbate disease progression by worsening astrogliosis. AQP9 is an aquaglyceroporin, meaning it is also able to shuttle glycerol, urea, and monocarboxylates such as lactate, suggesting that loss of AQP9 may disrupt local lactate levels which could affect neuronal excitability (327). Evidently, the functionality of aquaporins extends far beyond their primary function of channeling water molecules and it is important that the mechanisms behind these proteins are elucidated, to help understand their impact on epilepsy pathophysiology.

Inward Rectifying Potassium Channels

Under physiological conditions, the main inward rectifying potassium channel $K_{ir}4.1$ is abundantly expressed in cortical astrocytes, as well as in the hippocampus and thalamus (373, 374). In addition, heteromeric channels of $K_{ir}4.1/5.1$ are expressed in astrocytes of the forebrain. Furthermore, expression of several members of the $K_{ir}2$ and $K_{ir}6$ families have been reported in astrocytes (375, 376).

$K_{ir}4.1$ has been shown to colocalize with AQP4, suggesting a functional role for water transport in relation to K^+ buffering (104). *In vitro* experiments have shown that $K_{ir}4.1$ is able to directly bind to α-syntrophin, a member of the dystrophin-complex that has been shown to assist in AQP4 localization (377, 378). However, expression and immunoreactivity of $K_{ir}4.1$ is not altered in $AQP4^{-/-}$ mice, nor is AQP4 immunoreactivity altered in $K_{ir}4.1^{-/-}$ mice, suggesting that functionality of neither transporter is fully dependent on the other (332, 379). Nevertheless, clearance of extracellular K^+ by $K_{ir}4.1$ is partially dependent on simultaneous water flux by AQP4, to enable proper osmolarity for K^+ distribution and uptake. Recently, the synergy between AQP4 and $K_{ir}4.1$ channel mediated K^+ uptake has been validated by a mathematical model of neuroexcitation (380). Furthermore, in an experiment where heterologous AQP4 and $K_{ir}4.1/5.1$ were co-expressed in *Xenopus* oocytes, cell shrinkage produced K^+ currents, indicating another, more direct functional coupling between AQP4 and K_{ir} channels (381).

During astrogliosis, proliferative astrocytes are shifted toward an immature phenotype in which they lose $K_{ir}4.1$ and EAAT1 functionality, reducing spatial K^+ buffering and impairing glutamate uptake (382, 383). Accordingly, K_{ir} is often reported to be downregulated in animal models of epilepsy (71, 384). More specifically, loss of K_{ir} immunoreactivity is located on astrocytic processes within epileptic foci, but not on astrocytes of the surrounding tissue (385). Furthermore, a significant loss of $K_{ir}4.1$ immunoreactivity has been reported in resected hippocampal tissue of TLE patients (106). Interestingly, the loss of $K_{ir}4.1$ was associated with loss of AQP4-associated proteins α-syntrophin and dystrophin, further emphasizing the link between $K_{ir}4.1$, dystrophin-complex, and AQP4 localization. Decrease or loss of $K_{ir}4.1$ or $K_{ir}4.1/5.1$ channels undoubtedly cause perturbations in spatial K^+ buffering, but functional mechanisms modulating epileptogenesis remain unidentified. Recently, it was shown that antagonism of $K_{ir}4.1$ or suppression of $K_{ir}4.1$ expression by siRNAs induces synthesis of BDNF (386). Expression of BDNF is upregulated in several animal models of epilepsy and in human epileptic disorders (387, 388). One way BDNF is proposed to promote seizures is by reduction of inhibitory synaptic transmission of $GABA_A$ receptor signaling (389). In addition, release of BDNF has been shown to downregulate expression of K^+/Cl^--cotransporter (KCC2) (390, 391). Knock-out of KCC2 has been shown to induce hyperexcitability in mice (392). This shows that impaired $K_{ir}4.1$ signaling may result in hyperexcitability by a multitude of mechanisms.

Other Potassium Channels

BK channels are expressed in astrocytic endfeet and they regulate vasodilation and vasoconstriction (**Figure 2**). BK channels are sensitive to calcium levels, membrane potential, and certain types of arachidonic acid (AA) metabolites which can lead to vasodilation or vasoconstriction (discussed in one of the following paragraphs) (358, 393). This is an important astrocytic

property that supports the dynamic neuronal energy demand. In addition, calcium-dependent (BK_{Ca}) channels regulate K^+ export from astrocytes, directly affecting local excitability (394).

Under physiological conditions, BK channels participate in the spatial K^+ buffering that is required for normal brain function. A specific subunit of the protein (β_4) prevents the channel to contribute to neuronal membrane repolarization, which allows the channel to protect the brain from hyperexcitability (395). A gain-of-function mutation or a loss of β_4 subunit activity removes the protective function and is associated with epileptic seizures (396, 397). In animal models of epilepsy, the β_4 subunit is downregulated and a switch is made in subtypes of the BK channels, resulting in faster gating (335, 398). BK blockers may reverse the adverse effects of β_4 subunit loss and have been able to reduce action potential firing in brain slices of epileptic rats (399) and reduce seizure activity *in vivo* in mice (400). Recently, efforts were made to develop BK blockers, that selectively target BK channels with a gain-of-function mutation in the β_4 subunit, posing as a new strategy for therapy aimed at patients with retractable epilepsy (397).

Specific roles for other K^+ channels such as K_v or K_2P channels (**Figure 2**) remain elusive. Downregulation of K_v channels in astrocytes upon seizure activity has been reported and agonists of K_v have been suggested as anticonvulsant therapies, but additional research is required to understand how K_v channels are involved in excitability (339, 401). K_2P channels are open at rest and thereby aid K_{ir} channels in driving the membrane potential of astrocytes to the K^+ equilibrium, a feature thought to promote glutamate uptake (358, 402).

Sodium Channels

Although astrocytes are considered non-excitable cells (in the classical sense that they are unable to produce action potentials), they dynamically express all 9 isoforms of Na_v sodium channels, with $Na_v1.5$ as the main voltage-gated sodium channel (VGSC) (403). Expression of VGSCs is increased upon brain insult and during astrogliosis and appear to assist via a mechanism involving NCX (403–405). Little is known about the exact function of sodium channels in astrocytes, but it is believed that continuous Na^+ influx is required to maintain $[Na^+]_i$ for activity of Na^+/K^+-ATPase [(406); **Figure 2**]. Na^+/K^+-ATPase assists in extracellular K^+ buffering by uptake of K^+ while simultaneously releasing Na^+ at the cost of ATP. In fact, Na^+/K^+-ATPase, rather than $K_{ir}4.1$, is responsible for most of the net uptake of K^+. Changes in the activity of Na^+/K^+-ATPase have been proposed as an underlying mechanism for epilepsy (340, 407). Mutations in the gene encoding Na^+/K^+-ATPase were shown to cause seizure activity in animals (408) and patients with epilepsy (409).

Another mechanism supporting spatial K^+ buffering is through the $Na^+/K^+/Cl^-$ co-transporter (NKCC1; **Figure 2**), which has been shown to play a role in astrocytic swelling under conditions of high extracellular K^+ (410–412). This is another example stressing the importance of the volume regulation of the extracellular space. Na^+-transport can also be facilitated by members of the transient receptor potential (TRP) family, including "ankyrin" TRPA1, "canonical" TRPC1, TRPC4, TRPC5, and "vanilloid" TRPV4 receptors (413, 414). In addition to VGSCs and ion cotransporters, Na^+ is transported over the membrane in conjunction with various other mechanisms such as glutamate uptake by EAAT1 (415), glutamine export by Na^+/H^+-coupled neutral amino acid transporters (SN1) and SN2 (416), and lactate shuttling by Na^+/K^+-ATPase (417).

Calcium Transporters

Many astrocytes functions occur in response to focal or global Ca^{2+} transients. Therefore, a tight regulation of intra- and extracellular levels of Ca^{2+} is vital for homeostatic astrocytic functionality. Ca^{2+} can permeate the membrane through a variety of channels, including plasmalemmal Ca^{2+}-ATPase (PMCA), TRPA1, TRPC1, TRPC4, TRPC5, TRPV1, ionotropic glutamate receptors AMPA and NMDA, purinergic receptors (i.e., P_2X7) and by several ion exchangers of which the NCX is the most relevant [(153); **Table 1**]. Of note, astrocytes express all 3 isoforms of NCX and it has been shown that NCX colocalizes with Na^+/K^+-ATPase and glutamate receptors (418).

One mechanism in which focal Ca^{2+} transients in astrocytes regulate brain homeostasis is mediated by TRPA1. Influx of Ca^{2+} by TRPA1 regulates GABAergic transmission via the astrocytic GABA3 transporter (419) and D-serine release (420).

TRPCs are involved in store-operated Ca^{2+} entry and have been shown to contribute to Ca^{2+}-mediated glutamate release in astrocytes (413). On the other hand, glutamate can activate astrocytic NMDA receptors and thereby induce Ca^{2+} influx, although they are ∼2 times less permeable than their neuronal counterparts (421, 422).

Chloride Transporters

Anions are also transported across astrocytic membranes. Astrocytes express different isoforms of potassium-chloride and cation-chloride cotransporters of the solute carrier 12 (SLC12) gene family, which include NKCC1, Na^+/Cl^--cotransporter (NCC) and KCC1, KCC3, KCC4 (423–425). Mounting evidence suggests KCC2 is neuron-specific, but some experimental data shows that KCC2 may be present in astrocytes (424, 425). The main role of KCCs in astrocytes is volume regulation, whereas in neurons they regulate membrane potential by keeping intracellular Cl^- levels low, to enable GABAergic transmission (425, 426).

Astrogliosis causes a downregulation of KCC2 and NKCC1 in cortical pyramidal neurons, thereby preventing the Cl^- gradient required for GABAergic transmission (287). In contrast, increased expression of NKCC1 has been found in hippocampal sclerotic tissue of patients with TLE (427). During the development of neurons, the ratio between KCC2 and NKCC1 changes, as KCC2 is upregulated and NKCC1 is downregulated in mature neurons (428). Considering that astrocytes may express both KCC2 and NKCC1, and at the same time appear to differentiate to an immature state during astrogliosis, it is plausible that this change in expression is also reversed in astrocytes in the sclerotic hippocampus. The shift in expression of KCC2 and NKCC1 has been shown in the subiculum of TLE patients, but is yet to be confirmed in astrocytes specifically (341).

Antagonism of NKCC1 reduces seizure frequency in patients with TLE (429). Interestingly, inhibition of NKCC1 with the diuretic bumetanide does not influence K^+ buffering post-stimulation (430). In this study it was found that neither $K_{ir}4.1$

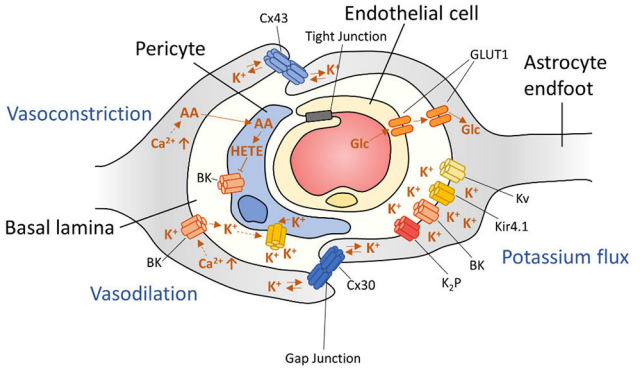

FIGURE 4 | Schematic representation of arteriolar regulation at the neurovascular unit. Astrocyte signaling is able to influence neuronal vascularity by inducing both vasoconstriction and vasodilation through mechanisms that involve BK channels.

nor NKCC1 inhibition changed K^+ buffering after neuronal activation, but that Na^+/K^+-ATPase was mostly responsible for the post-stimulation K^+ uptake. Nevertheless, the development of selective NKCC1 inhibitors may prove rewarding in the clinic (431).

BLOOD-BRAIN BARRIER DYSFUNCTION

The BBB functions as a physical barrier to protect the brain from toxins, undesirable metabolites and ions that could permeate the brain from the blood stream. The BBB is comprised of endothelial cells that are connected via tight junctions [(28); **Figure 4**]. This physical barrier is considered the "first line of defence" for the brain. Astrocytes ensheath with their endfeet the endothelial cells (**Figure 2**) and serve as a "second line of defence." Together with neurons, other glia cells and mural cells, they form the neurovascular unit. The main function of astrocytes at the BBB is the control of nutrient exchange with the bloodstream and maintaining BBB integrity (432).

Endothelial cells at the BBB express several transporter proteins and channels such as GLUT1, several amino acid carriers including EAAT1, EAAT2, EAAT3, and L-system for large neutral amino acids (LAT1), specific transporters (i.e., for nucleosides, nucleobases), non-specific transporters, such as multidrug transporters (MDTs), and organic ion transporters (28, 433). Importantly, astrocytes are able to alter the expression or activity of endothelial transporters including GLUT1 (434, 435) and MDTs such as P-glycoprotein (P-gp) (436, 437). Moreover, astrocytes may affect BBB permeability directly by changing the density of tight junctions (438, 439), for instance through release of angiopoietin 1 and 2, ATP, endothelin-1, fibroblast growth factor, glial cell line-derived neurotrophic factor (GDNF), glutamate, retinoic acid, nitric oxide or VEGF (440, 441).

Astrogliosis and neuroinflammation can lead to BBB dysfunction. Under inflammatory conditions, bradykinin is released in the blood, increasing BBB permeability by acting on endothelial (B_2) receptors (442). In addition, bradykinin induces IL-6 release from astrocytes through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (443) resulting in modulation of endothelial tight junctions (77). Moreover, following brain injury astrocyte-mediated inflammation causes transient opening of the BBB (444). BBB dysfunction is common in epilepsy and can contribute to the development and progression of epilepsy (365, 445–448). In the following paragraphs we will discuss several mechanisms by which BBB dysfunction contributes to epilepsy pathophysiology.

VEGF Signaling

Downregulation or loss of the proteins that make up tight junctions, such as zonula occludens (ZO-1), occludin, and claudins results in opening of the BBB. Loss of tight junctions is shown to be caused by increased expression of the VEGF receptor 1 and 2 in a rat model of pilocarpine-induced epilepsy (449). In addition, in an animal model of kainic acid-induced epilepsy it was shown that upregulation of VEGF-R1 and VEGF-R2 caused downregulation of ZO-1 (450). Furthermore, astrocyte-released VEGF has been shown to downregulate tight junction proteins claudin-5 and occludin (44). Several studies report increased VEGF release and receptor expression in patients suffering from refractory TLE (449, 451). The primary role of VEGF is to induce angiogenesis, which is correlated with seizure frequency (449). Angiogenesis and down-regulation of ZO-1 could be reversed by neutralization of VEGF, suggesting that VEGF signaling is involved in BBB dysfunction. This was demonstrated by oral administration of the VEGF pathway inhibitor sunitinib, which prevented seizures and epilepsy development in pilocarpine-induced seizures in rats, showing the potential of anti-angiogenesis therapies.

Albumin Leakage

In epilepsy, angiogenesis is spatially correlated to leakage of serum proteins into the brain parenchyma (452). Under pathophysiological conditions, BBB leakage exposes the brain to plasma proteins such as IgG and albumin (445, 449, 453). Subsequently, astrocytes are able to internalize serum albumin by binding to the TGF-β receptors, inducing epileptogenesis via a mechanism similar to TGF-β_1 induced TGF-β signaling (453). Importantly, TGF-β_1 was upregulated during gliosis in periods after SE (454). Furthermore, TGF-β_1 has been shown to downregulate $K_{ir}2.3$ in reactive astrocytes (455). In turn, albumin-induced TGF-β signaling causes impaired GJ coupling and down-regulates $K_{ir}4.1$ (453). This shows that TGF-β signaling interferes with potassium buffering in at least two distinct mechanisms involving inward rectifying channels. In addition, albumin extravasation into the brain has been shown to (1) transiently affect GJ coupling (456), (2) induce GFAP expression (457), (3) upregulate pro-inflammatory cytokine IL-6 (458), (4) reduce astrocyte potassium and glutamate clearance (459), and (5) induce excitatory, but not inhibitory synaptogenesis, contributing to potential hyperexcitability (460). Together, these data show that BBB dysfunction can promote epileptogenesis.

Multidrug Transporters

BBB opening is associated with increased expression of MDTs (448, 461). Several studies have reported upregulation of genes encoding for MDTs, including P-gp (MDR1 gene), multidrug resistance protein (MRP) 1, MRP2, MRP5, and breast cancer resistance protein (BRCP) in the epileptogenic brain (448, 462–465). Overexpression of MDTs is shown in endothelial cells, but also in astrocytic endfeet and neurons. These transporters have been shown to affect drug transport in the brain and it has been proposed that drug resistance in patients with refractory epilepsy may be due to changes in MDTs (466–468). For instance, increased expression of P-gp causes enhanced efflux of AEDs, impairing AED entry into the brain. Indeed, P-gp blockers can increase AED levels in the brain and overcome pharmacoresistance in animal models of epilepsy (469–471), suggesting co-administration of antiepileptic drugs and a P-gp blocker may prove useful in patients as well. In contrast, recent experiments based on measurements of extracellular fluid unbound drug concentrations and mathematical models predicting drug target site concentrations, suggest that P-gp expression does not translate to BBB permeability for all AEDs, as other factors may affect target-site concentration more profoundly, such as brain tissue binding (472). Moreover, it is unclear whether changes in MDT expression are different between various animal models or correlate to different types of disease progression. Patient data confirms these speculations as it appears that therapeutic success appears to be influenced by the heterogeneity of the etiology of the seizures (473, 474). On the other hand, mounting evidence from patients that were co-administered with AEDs and P-gp blockers (i.e., Verapamil) show improved clinical outcome compared to AED only treatment (475–478).

Arteriolar Blood Flow Regulation

Astrocytes also aid in the local regulation of vasoconstriction and vasodilation. Changes in intracellular Ca^{2+} at astrocytic endfeet can induce two major arteriolar regulating pathways: (1) The cytoplasmic phospholipase A2 (PLA_2) pathway and (2) BK channel mediated vascular control [(393, 479); **Figure 4**]. Increased PLA_2 activity results in production of AA, which is metabolized into various vasoactive compounds and is also able to pass the cell membrane to pericytes. Inside pericytes, AA is then metabolized into the vasoconstrictive 20-hydroxyeicosatetraenoic acid (20-HETE).

Different concentrations of Ca^{2+} in astrocytic endfeet are also able to regulate arteriolar dilation or vasoconstriction by induction of BK-channels that release K^+ in the perivascular space—a space formed by the envelopment of astrocytic processes around arterioles (31, 393). In addition, BK channels respond to components of the PLA_2 pathway, such as 20-hydroxyeicosatetraenoic acid (20-HETE), epoxy-eicosatetraenoic acids (EET)s, and prostaglandin E2 (PGE_2). Importantly, these mechanisms are not mutually exclusive, and even overlap. Efforts of blocking either pathway individually did not result in total impairment of vascular control, emphasizing the extent of vascular control for homeostatic brain function (393). Together, these mechanisms can regulate cerebral blood-flow in the brain.

During epileptic seizures cerebral blood-flow and also cerebral blood volume are transiently increased as a response to the high energy demand of neurons (135). However, these increases are not sufficient to meet metabolic demands of synchronously activated neurons during ictal events. Several studies have found impaired neurovascular coupling in epilepsy (135, 480, 481). In some studies, this is correlated to hypoxia-induced tissue damage. Others suggest the possibility to predict ictal events based on increased cerebral perfusion preceding seizure onset in the clinic (482). Vast Ca^{2+} waves at astrocytic endfeet recorded during ictal events have been shown to regulate local arteriole responses, and these effects could be blocked by pharmacological inhibition of the Ca^{2+} signals in astrocytic processes (483). Although the underlying mechanisms behind the regulation of cerebral microcirculation in epilepsy are poorly defined, these data emphasize how astrocytes may control the neuronal micro environment during seizures.

CONCLUDING REMARKS

Under physiological conditions, astrocytes protect neurons from becoming hyperexcitable. However, under pathophysiological conditions found before and during epilepsy, the evident and complex involvement of astrocytes in the neuronal network is perturbed. In this review we showed how aberrant astrocytic signaling and changes in astrocyte function contribute to the development and aggravation of epilepsy.

Despite an abundance of clues in the vast literature on the mechanistic involvement of astrocytes in epilepsy, there are presently no drugs in the clinic that target these mechanisms. In the near future it is imperative that we continue the development of drugs that specifically target mechanisms that are underlying the etiology of epilepsy and also focus on astrocytes as novel therapeutic targets. So far, pioneering preclinical studies have shown promising results.

Interestingly, recently it was suggested that astrocytes may also be used as biomarkers for epileptogenesis (484, 485). In this review the recently published evidence was reported, supporting the utility of measuring astrocyte activation, the soluble molecules they release, and the associated cognitive deficits during epileptogenesis for early stratification of animals developing epilepsy. Whether this may also be of clinical use needs to be investigated.

AUTHOR CONTRIBUTIONS

QV and LH developed the concept and prepared the first draft. EV and EA provided feedback and contributed to the writing of the manuscript together with QV and LH. All authors read, revised, and approved the final manuscript.

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Astrocytic GABA Accumulation in Experimental Temporal Lobe Epilepsy

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An imbalance of excitation and inhibition has been associated with the pathophysiology of epilepsy. Loss of GABAergic interneurons and/or synaptic inhibition has been shown in various epilepsy models and in human epilepsy. Despite this loss, several studies reported preserved or increased tonic $GABA_A$ receptor-mediated currents in epilepsy, raising the question of the source of the inhibitory transmitter. We used the unilateral intracortical kainate mouse model of temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) to answer this question. In our model we observed profound loss of interneurons in the sclerotic hippocampal CA1 region and dentate gyrus already 5 days after epilepsy induction. Consistent with the literature, the absence of interneurons caused no reduction of tonic inhibition of CA1 pyramidal neurons. In dentate granule cells the inhibitory currents were even increased in epileptic tissue. Intriguingly, immunostaining of brain sections from epileptic mice with antibodies against GABA revealed strong and progressive accumulation of the neurotransmitter in reactive astrocytes. Pharmacological inhibition of the astrocytic GABA transporter GAT3 did not affect tonic inhibition in the sclerotic hippocampus, suggesting that this transporter is not responsible for astrocytic GABA accumulation or release. Immunostaining further indicated that both decarboxylation of glutamate and putrescine degradation accounted for the increased GABA levels in reactive astrocytes. Together, our data provide evidence that the preserved tonic inhibitory currents in the epileptic brain are mediated by GABA overproduction and release from astrocytes. A deeper understanding of the underlying mechanisms may lead to new strategies for antiepileptic drug therapy.

Keywords: temporal lobe epilepsy, hippocampal sclerosis, astrocyte, tonic current, GABA

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HIGHLIGHTS

- Despite massive loss of interneurons, tonic $GABA_A$ receptor-mediated currents are preserved in the sclerotic hippocampal CA1 region and increased in the dentate gyrus.
- Reactive astrocytes in the sclerotic mouse hippocampus display pronounced GABA accumulation.
- Both decarboxylation of glutamate and putrescine degradation may underlie astrocytic GABA accumulation.

INTRODUCTION

Epilepsy is a disorder of the brain characterized by recurrent unprovoked seizures that affects 1–2% of the population worldwide (1). Temporal lobe epilepsy (TLE), the most frequent and severe form of focal epilepsy in adults is particularly difficult to control with antiepileptic therapies. Despite the availability of third-generation antiepileptic drugs (AEDs) a high proportion of TLE patients do not respond adequately to medication (2, 3). The main goal of epilepsy research is, therefore, to identify new therapeutic targets and strategies for the development of more effective and better tolerated AEDs. The most common pathologic finding in patients with TLE is hippocampal sclerosis (HS), histologically characterized by segmental loss of principal pyramidal neurons, synaptic reorganization and reactive astrogliosis in the hippocampus (4). In addition, loss of hippocampal GABAergic interneurons has been described in human TLE (5–9) and in many different animal models (10–13). The consequential shift in the excitation-inhibition balance toward excitation has been hypothesized to represent the primary cause of seizure activity in TLE (11, 14–17). However, this hypothesis has difficulty explaining the fact that epileptic seizures are intermittent and relatively rare events even in patients and animals with severe epilepsy, pointing to the existence of a compensatory mechanism that restores the excitation-inhibition balance to a large extent (18–20). Most recent work suggested that the compensation is accomplished by channel-mediated tonic GABA release from reactive astrocytes (21), a mechanism that was proposed to be relevant also in other neurological disorders, such as Alzheimer's disease, Parkinson's disease or stroke (22–25). According to this scenario, reactive astrocytes aberrantly overproduce and release GABA, which in turn inhibits neuronal excitability and network activity through activation of high affinity, slowly desensitizing extrasynaptic $GABA_A$ receptors ($GABA_A$Rs) (20). In line with this view, evidence from animal models indicate that despite the loss of synaptic inhibition, tonic $GABA_A$R-mediated currents (often termed "tonic inhibition") are preserved or even increased in focal epilepsy (18, 20, 21). Hence, it was suggested that reactive astrocytes suppress network excitability and prevent seizure generation through tonic GABA release, a pathway that could provide an attractive target for the development of new AEDs.

To gain further insight into this highly relevant topic, in the present study we used immunohistochemical and electrophysiological methods to unravel the relationship between the extent of interneuronal loss, tonic inhibition and astrocytic GABA content in the hippocampal CA1 region and dentate gyrus during the early chronic phase of epileptogenesis in the unilateral intracortical kainate mouse model of TLE-HS. The results further support the hypothesis that the preserved tonic inhibition in TLE-HS is mediated by ambient GABA released from reactive astrocytes.

MATERIALS AND METHODS

Animals

Maintenance and handling of animals was according to the local government regulations. Experiments were approved by the North Rhine–Westphalia State Agency for Nature, Environment and Consumer Protection (approval numbers 84-02.04.2012.A212 and 84-02.04.2015.A393). All measures were taken to minimize the number of animals used. Mice were kept under standard housing conditions (12/12 h dark–light cycle, food, and water *ad libitum*). Male FVB (Charles River, Sulzfeld, Germany) or transgenic mice with human GFAP (hGFAP) promoter-controlled expression of EGFP [hGFAP/EGFP (26)] aged 90–120 days were used for the experiments.

Unilateral Intracortical Kainate Injections

We used the TLE animal model previously established (27, 28). Briefly, mice were anesthetized with a mixture of medetomidine (Cepetor, CP-Pharma, Burgdorf, Germany, 0.3 mg/kg, i.p.) and ketamine (Ketamidor, WDT, Garbsen, Germany, 40 mg/kg, i.p.) and placed in a stereotaxic frame equipped with a manual microinjection unit (TSE Systems GmbH, Bad Homburg, Germany). A total volume of 70 nl of a 20 mM solution of kainic acid (Tocris, Bristol, UK) in 0.9% sterile NaCl were stereotactically injected into the neocortex just above the right dorsal hippocampus. The stereotactic coordinates were 2 mm posterior to bregma, 1.5 mm from midline and 1.7 mm from the skull surface. Sham control mice received injections of 70 nl saline under the same conditions. For the analysis of tonic $GABA_A$R currents untreated mice served as controls. After injection, the scalp incision was sutured and anesthesia stopped with atipamezol (Antisedan, Orion Pharma, Hamburg, Germany, 300 mg/kg, i.p.). To reduce pain, mice were subsequently injected for 3 days with carprofen (Rimadyl, Pfizer, Karlsruhe, Germany). Furthermore, 0.25% Enrofloxacin (Baytril, Bayer, Leverkusen, Germany) was administered via drinking water to reduce the risk of infection. Brains of the mice were perfusion fixed with 4% PFA followed by overnight fixation in 4% PFA.

Immunhistochemistry

Tissue Preparation

Adult animals were deeply anaesthesised by intraperitoneal (i.p.) injection with 100–120 µl of a solution containing 80 mg/kg ketamine hydrochloride (WDT) and 1.2 mg/kg xylazine hydrochloride (Sigma-Aldrich, Darmstadt, Germany). After testing the hind paw reflexes, transcardial perfusion was applied with ice-cold PBS (30 ml) followed by ice-cold PFA (30 ml, 4%). The brain was removed and an additional fixation with 4% PFA overnight was performed. Tissue was stored in PBS at 4°C until sectioning.

Staining

Slices from PFA-perfused animals were cut into 40 µm thickness with a vibratome. Each slice was transferred into a well of a 24-well plate and able to freely move during the whole staining procedure. Only dorsal hippocampal slices close to the injection site (1.8–2.2 mm from bregma) were used for staining. To avoid unspecific binding of antibodies, all slices were incubated in blocking solution for 1.5–2 h at room temperature (RT), containing PBS 0.5–1% TritonX-100 for cell membrane permeabilisation and normal goat serum (NGS, 10%). Primary antibodies were diluted in PBS, 0.1% TritonX-100 and 5% NGS and the slices were incubated with overnight shaking at 4°C

(except for GABA staining where slices were incubated for 48 h at RT). The following primary antibodies were used: mouse-anti-S100b (1:200, Abcam, Cambridge, UK), rabbit-anti-GABA (1:2000; ImmunoStar, Hudson, WI, USA), rabbit-anti-GFAP (1:500, DAKO, Hamburg, Germany), mouse-anti-PARV (1:1000, Millipore, Darmstadt, Germany) rabbit-anti-GAD65+67 (1:1000, Sigma-Aldrich, Steinheim, Germany), rabbit-anti-MAO-B (1:500, Sigma-Aldrich, Steinheim, Germany). On the following day every slice was washed three times with PBS for 10 min each, followed by incubation with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 647 (Invitrogen, dilution 1:500 each) in PBS with 2% NGS and 0.1% Triton X-100 for 2 h at RT. After washing them again three times with PBS for 10 min, nuclear staining with Hoechst (1:200, diluted in dH_2O) was performed (10 min, RT). A final washing step was performed and slices were mounted with Aquapolymount (Polysciences, Heidelberg, Germany) on objective slides and covered with cover slips. Before confocal imaging, slides were stored at 4°C overnight.

Confocal Microscopy

Slides were examined using a confocal laser scanning microscope (SP8, Leica, Hamburg, Germany) in either standard or photon counting mode (8 bit) using 20 or 63× objectives. Image resolution was 1,024 × 1,024 pixels taken at a speed of 400 Hz, with a pinhole of 1.2 or 1 au and zoom 0.75 or 1. For detection of Hoechst, a photomultiplier was used, whereas for all other staining hybrid detectors were acquired with the same laser settings. For the 63× immersion objective a motor correction was performed to improve resolution, depth of penetration and signal strength. Z-stacks were taken as 2 μm thick planes.

Quantification of Staining

Immunohistochemical data were quantified using the software Imaris 8.0 (Bitplane, Zürich, Switzerland). 3D images (246 × 246 × 40 μm^3 in size, taken in the CA1 and DG area directly below the injection site) were loaded into the software and co-localization between GFAP and GABA-positive voxels was determined based on an automatic threshold algorithm implemented in the ImarisColoc tool (29), creating a separate fluorescence intensity channel containing only co-localized GFAP- and GABA-positive voxels. The thresholding procedure was applied equally to all images analyzed. Co-localized voxels were subsequently reconstructed creating 3D surface representations (isosurfaces) of the GFAP-positive voxels containing GABA. Additionally, GFAP-negative surfaces with roundish cell bodies were identified as neurons, as confirmed through double staining with antibodies for NeuN and GABA (data not shown). After 3D surface reconstruction, GABA concentration was determined by quantifying total fluorescence intensity of GABA within 3D surfaces representing GFAP/GABA-positive cells. For statistical analysis image data from five animals per group were considered. The number of GABA- and PARV-positive interneurons was counted manually in an area of 246 × 246 × 40 μm^3 or 582 × 582 × 40 μm^3, respectively, in the CA1 and DG area below the injection track on the ipsilateral side and at the same position on the contralateral side. Expression of MAO-B and GAD in astrocytes was quantified based on the same procedure described above for quantification of GABA.

Analysis of Tonic $GABA_AR$ Currents

Neuronal tonic $GABA_AR$ currents were measured in coronal brain slices of 200 μm thickness. For kainate injected mice, slices were obtained 2 weeks post-injection and neurons in both, contra and ipsilateral side, were recorded. Untreated mice were used as controls. Slices were prepared as mentioned before and allowed to recover for at least 1 h prior to the experiments. Patch-Clamp recordings were performed at RT at an upright microscope (Axioskop FS2, Zeiss, Jena, Germany) equipped with a CCD camera (VX45, Optronis, Kehl, Germany), infrared-DIC optics (Eclipse E600 FN; Nikon, Japan) and epifluorescence (Polychrome II, Till Photonics, Martinsried, Germany). Slices were constantly perfused with aCSF containing (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 1.25 NaH_2PO_4, and 26 $NaHCO_3$ (pH 7.4, 305–315 mOsm). Whole-cell recordings were obtained from granule cells located in dentate gyrus and from CA1 pyramidal cell neurons located close to the CA2 region. The holding potential was −70 mV. Patch pipettes with a resistance of 3–5 MΩ were filled with an internal solution (in mM): 130 CsCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 5 BAPTA, 3 Na2-ATP and 5 QX-314 (blocker of voltage gated Na^+ currents) (pH 7.3, 278–285 mOsmol). To isolate tonic $GABA_AR$ currents, focal pressure applications were performed with an Octaflow system (ALA Scientific Instruments, Farmingdale, NY, USA). The different channels of the application system contained either aCSF (initial control) or a blocker cocktail containing D-AP5 (10 μM, Abcam, Cambridge, UK), NBQX (5 μM, Tocris) and CGP52432 (5 μM, Abcam) w/o bicuculline (20 μM, Tocris), duration of application always 30–50 s. Another channel was loaded with the cocktail plus SNAP5114 (100 μM, Tocris, application for 300 s), and finally the SNAP-containing blocker cocktail was supplemented with bicuculline (application for 30 s). The shift in baseline (i.e., tonic inward) current upon bicuculline application (with or without SNAP) was analyzed with Igor Pro 5.03 software (WaveMetrics, Lake Oswego, OR, USA) and Origin 9.1 (OriginLab Corporation, Northampton, MA, USA). Signals were obtained with an EPC800 amplifier (HEKA Electronic, Lambrecht, Germany) and processed by a differential amplifier (DPA-2FS; npi electronic, Tamm, Germany). Spontaneous inhibitory post-synaptic currents (sIPSCs) were analyzed by the software pClamp (Molecular devices, San José, USA). Individual sIPSCs were identified by a template search, representing sIPSCs in their shape and kinetics. The template was generated from the average of several sIPSCs and kept constant for all experiments. The peak amplitude of each identified sIPSC was measured and the mean amplitude of all recorded cells calculated. Signals were digitized with an ITC 16 D/A converter (HEKA) and displayed with TIDA software (HEKA). Signals were filtered at 1 kHz and sampled at 20 kHz.

Statistics

Statistical analyses were performed using Origin (OriginLab, version 9, US) and R software [R Core Team 2020, version

4.0.2, Austria (30)]. Data are displayed as mean ± SD. To test whether the data follow a Gaussian distribution both histograms as well as Q-Q plots, which represent the relationship between the percentiles of the theoretical and empirical distributions, were visually inspected. In addition the data were statistically tested for normality (Shapiro-Wilk test). In case of a deviation from normality data were transformed according to Tukey's ladder of powers prior to conducting statistical analysis. For comparison of two groups a Student's *t*-test was used. More than two groups were compared with one-way analysis of variance (ANOVA) with *post-hoc* Tukey test. For multifactorial data stratified two-way ANOVA was conducted. Spearman's rank correlation coefficient was calculated to assess the correlation between GFAP and astrocytic GABA immunoreactivity. Differences between means were considered as being significant at $p \leq 0.05$. Box plot data represent median (line) and quartiles (25 and 75%; box), whiskers extend to the highest and lowest values within 1.5 times interquartile range.

RESULTS

Loss of Hippocampal Interneurons in the Intracortical Kainate Model of TLE

Loss of GABAergic interneurons, especially those containing parvalbumin (PARV), has been documented in human epilepsy and in several different experimental models of the disease, suggesting that it is critically involved in epileptogenesis. We have previously shown that the unilateral intra*cortical* kainate injection model reliably mimics key morphological and functional features of chronic human TLE-HS (27). However, whether the model also reproduces the reported loss of interneurons has not been investigated so far. We used immunohistochemical staining with antibodies against PARV and GABA to tackle this question. Experiments were performed 5 and 14 days after kainate injection (dpi), time points that represent the onset and the early stage of chronic seizure activity in this model. On the contralateral (non-injected) side, abundant PARV-positive cells were detected in the hippocampal CA1 region and DG at both time points. In contrast, cells displaying PARV-immunoreactivity were virtually absent ipsilaterally (**Figures 1A,B**). Since only a subset of interneurons expresses PARV, we used next anti-GABA antibody to label all types of GABAergic interneurons. Co-staining with the astrocyte marker GFAP revealed an almost complete (∼90%) loss of GABA-positive/GFAP-negative cells in the sclerotic hippocampal CA1 region and a substantial reduction (>50%) in the DG at both time points investigated (**Figure 1C**, representative immune staining are shown in **Figures 3A,B**). The number of contralateral GABAergic interneurons was not different from sham injected controls (**Figure 1C**).

Together, these findings are consistent with other work demonstrating loss of interneurons in epilepsy, and indicate that this pathological process represents a very early event during epileptogenesis.

Tonic $GABA_AR$-Mediated Currents Are Maintained in CA1 Pyramidal Cells and Increased in Dentate Granule Cells in Experimental TLE-HS

The reduced number of GABAergic interneurons in our experimental model prompted us to examine the magnitude of tonic inhibition in hippocampal neurons. As vesicular release from interneurons has been suggested to be the main source of ambient GABA responsible for tonic inhibition (31), one would expect a reduction in the amplitude of these currents in the sclerotic hippocampus. To test this assumption, we performed electrophysiological recordings from CA1 pyramidal neurons and dentate granule cells 14 dpi. Whole-cell patch-clamp recordings were made using a CsCl-based pipette solution at a holding potential of −70 mV in the presence of the ionotropic glutamate receptor antagonists D-AP5 and NBQX and the $GABA_B$ receptor antagonist CGP52432. Tonic current amplitude was calculated as the difference in holding current before and after bicuculline (20 μM) application. Interestingly, tonic inhibition on the ipsi- vs. contralateral sides in kainate injected mice and vs. untreated control animals were not different in CA1 pyramidal neurons (ipsi: 11.4 ± 7.3 pA; contra: 16.3 ± 5.4 pA; control: 12.5 ± 5.4 pA, **Figure 2A**). In dentate granule cells the amplitudes of tonic currents were even higher at the ipsi- vs. contralateral sides and controls (ipsi: 64.7 ± 20.6 pA; contra: 8.98 ± 1.9 pA; control: 8.3 ± 3.8 pA, **Figure 2B**). In line with the above described loss of interneurons, ipsilaterally the frequency of spontaneous inhibitory post-synaptic currents (sIPSCs) before application and after washout of bicuculline was substantially lower compared to the contralateral side and sham controls (**Figures 2C,D**, left graphs). In contrast, sIPSC peak amplitudes were not different between groups, indicating unaltered post-synaptic receptor function (**Figures 2C,D**, right).

Collectively, these data indicate that, despite the loss of GABAergic interneurons and phasic inhibition, ambient GABA levels are preserved or even increased in the ipsilateral hippocampus.

Reactive Astrocytes in the Sclerotic Hippocampus Display Pronounced GABA Accumulation

The preserved or aberrantly increased tonic $GABA_AR$ currents raised the question of the cellular origin of the transmitter. A number of studies have proposed that GABA produced and released by astrocytes significantly contributes to extrasynaptic GABA levels and tonic inhibition, especially under pathological conditions (22, 23, 32–34). To explore whether astrocytic GABA could account for the maintained/increased tonic currents in our TLE model, we performed immunostaining with antibodies against GABA and GFAP in hippocampal slices at different time points after kainate (5, 14, and 28 dpi) or sham injection (14 dpi). In the latter, strong GABA immunoreactivity was mainly seen in neurons while astrocytes were only weakly immunoreactive. Remarkably, in the ipsilateral hippocampus of kainate-treated mice, a strong increase in astrocytic GABA levels (∼8-fold

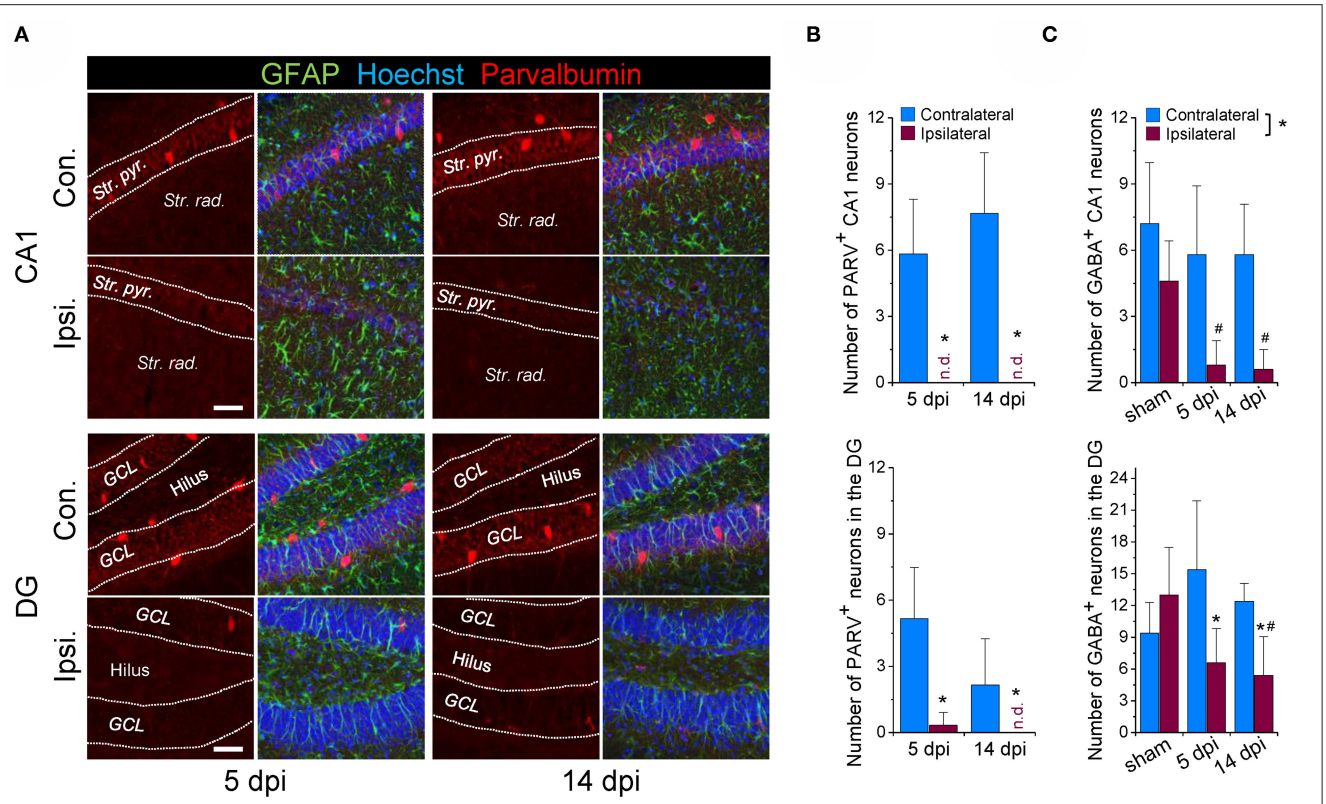

FIGURE 1 | Loss of GABAergic interneurons during the early phase of kainate-induced epileptogenesis. **(A)** Representative confocal images of parvalbumin (PARV) immunoreactivity in the hippocampal CA1 region and dentate gyrus (DG) of mice injected with kainate 5 days and 14 days before. **(B)** Quantification of the number of parvalbumin-positive neurons in an area of 582 × 582 × 40 μm within the hippocampal CA1 and DG region below the injection site. $N = 6$ slices from 3 animals for each time point and area. **(C)** Quantification of the number of GABA-positive neurons (GABA staining shown in **Figure 3**) in the ipsi- and contralateral hippocampus of sham and kainate injected animals. Cells were counted in an area of 246 × 246 × 40 μm within the hippocampal CA1 and DG region below the injection site. $N = 5$ slices from five animals (GABA) for each time point, area and condition. Str. pyr. = Stratum pyramidale, Str. rad. = Stratum radiatum, dpi = days post-injection, n.d. = not detected. *ipsi- vs. contralateral significantly different, #significantly different from sham ($p < 0.05$, stratified two-way ANOVA followed by Tukey's test). Scale bar = 50 μm.

in the CA1 region and ∼14-fold in the DG) was observed already 5 dpi, while maximal accumulation was reached 28 dpi in CA1 (∼70-fold increase) and 14 dpi in the DG (∼55-fold increase; **Figures 3A–C**). Contralaterally, astrocytic GABA was also elevated in both hippocampal regions and at all investigated time points, but compared to the ipsilateral side the increase at the later time points was significantly less (**Figures 3A–C**). GFAP immunoreactivity (the increase of which reflecting astrogliosis) showed a strong elevation already 5 dpi on both sides, which, however, did not increase further during the next 4 weeks (**Figure 3D**). We found a significant positive correlation between the astrocytic GABA content and GFAP immunoreactivity with a correlation factor of $r = 0.65$ ($p < 0.000001$).

In the DG of sham injected animals, 88% of total (astrocytic + neuronal) GABA was found in neurons and merely 12% in astrocytes. Intriguingly, after kainate injection the astrocytic contribution increased to 67% at 5 dpi and reached 90% at 14 dpi (**Figure 3E**). In the CA1 area astrocytic GABA increased from 38% in sham mice to 97% at 5 dpi and 99.5% at 14 dpi in kainate injected mice (not shown).

These data show that during epileptogenesis the loss of GABAergic neurons goes along with a pronounced increase in astrocytic GABA content. Release of GABA from astrocytes might thus mediate tonic inhibition in the epileptic hippocampus.

Astrocytic GABA Synthesis Rather Than Uptake From the Extracellular Space Accounts for GABA Accumulation

Next we investigated potential mechanisms that might underlie astrocytic GABA accumulation. Astrocytes can acquire GABA in different ways: by uptake, reduced degradation or synthesis. To evaluate these potential mechanisms, we blocked glial GABA uptake with the GAT-2/3-specific inhibitor SNAP-5114 and utilized neuronal tonic $GABA_AR$ current as an indirect read-out of extracellular GABA levels. On the contralateral side, the blocker caused the expected increase in tonic current amplitudes in both CA1 and DG (65 and 191% increase, respectively). Ipsilaterally, however, SNAP-5114 had no effect

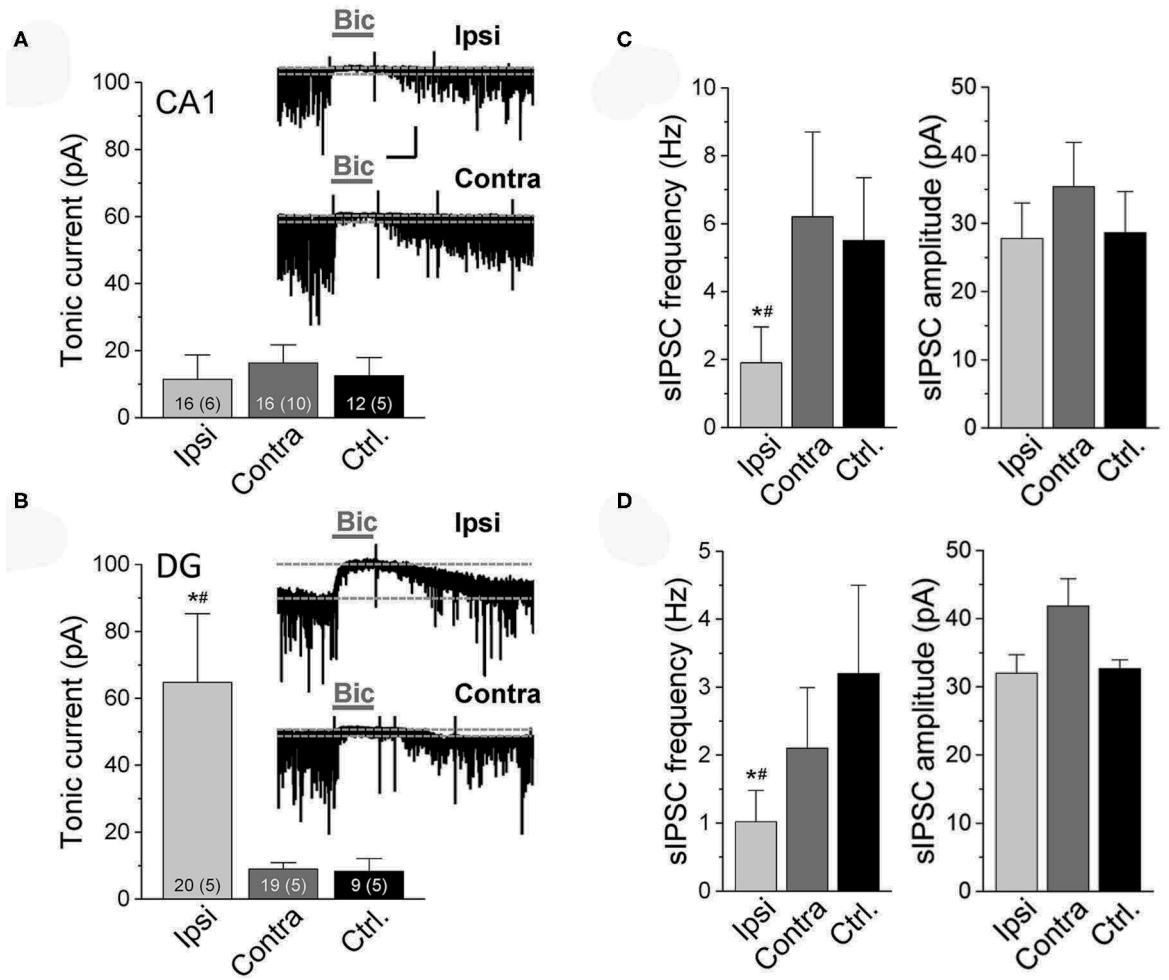

FIGURE 2 | Tonic $GABA_AR$ currents recorded in CA1 pyramidal neurons and dentate granule cells of epileptic mice. Currents were measured in the ipsilateral (Ipsi) and contralateral (Contra) hippocampus of kainate-treated mice and in the hippocampus of untreated control mice (Ctrl.) 2 weeks after kainate injection. Whole-cell recordings were made with a CsCl-based internal solution, holding potential −70 mV. Tonic current amplitudes represent the shift in baseline current produced by bicuculline (Bic, 20 μM). **(A)** Representative traces of $GABA_AR$-mediated currents and their quantification in CA1 pyramidal neurons and **(B)** dentate granule cells. Numbers of experiments and mice (in parentheses) are given in bars. **(C,D)** Frequency and amplitudes of spontaneous inhibitory post-synaptic currents (sIPSCs) recorded before application and after washout of bicuculline in the CA1 and DG, respectively. Numbers of experiments and mice correspond to those in **(A)** and **(B)**. Calibration bars for original traces in **(A)** indicate 20 s and 50 pA and also apply to **(B)**. Error bars represent SD. *significantly different from the contralateral side, #significantly different from controls ($p < 0.05$, one-way ANOVA followed by Tukey's test).

(**Figure 4**), indicating lack of GABA transporter activity. This observation led us to conclude that astrocytic GABA accumulation in the ipsilateral hippocampus is not simply mediated by enhanced uptake from the extracellular space. Since SNAP-5114 had no effect, it is also unlikely that GABA was released from astrocytes through a reversed operation of the glial transporter (23, 34, 35). Astrocytes can synthesize GABA via decarboxylation of glutamate by glutamate decarboxylase (GAD) or through degradation of putrescine mediated primarily by monoamine oxidase B (MAO-B) (33, 36). We performed immunohistochemical analysis using antibodies against MAO-B and two isoforms of GAD, GAD67 and GAD65, to gain information about the expression levels of these enzymes in astrocytes. We have limited this study to the CA1 region because in human and experimental TLE GAD is strongly up-regulated in DG granule cells and mossy fibers, which complicates analysis in this region. In our TLE model, GAD and MAO-B immunoreactivity was significantly increased in GFAP/S100β-positive astrocytes of kainate injected mice as compared to sham injected controls. However, there was no difference in GAD- or MAO-B-immunoreactivity between the ipsi- and contralateral hippocampus of kainate injected mice (**Figure 5**).

Taken together, these results indicate that astrocyte GABA accumulation in epilepsy is mediated by glutamate decarboxylation and monoacetylation of putrescine but not by uptake from the extracellular space.

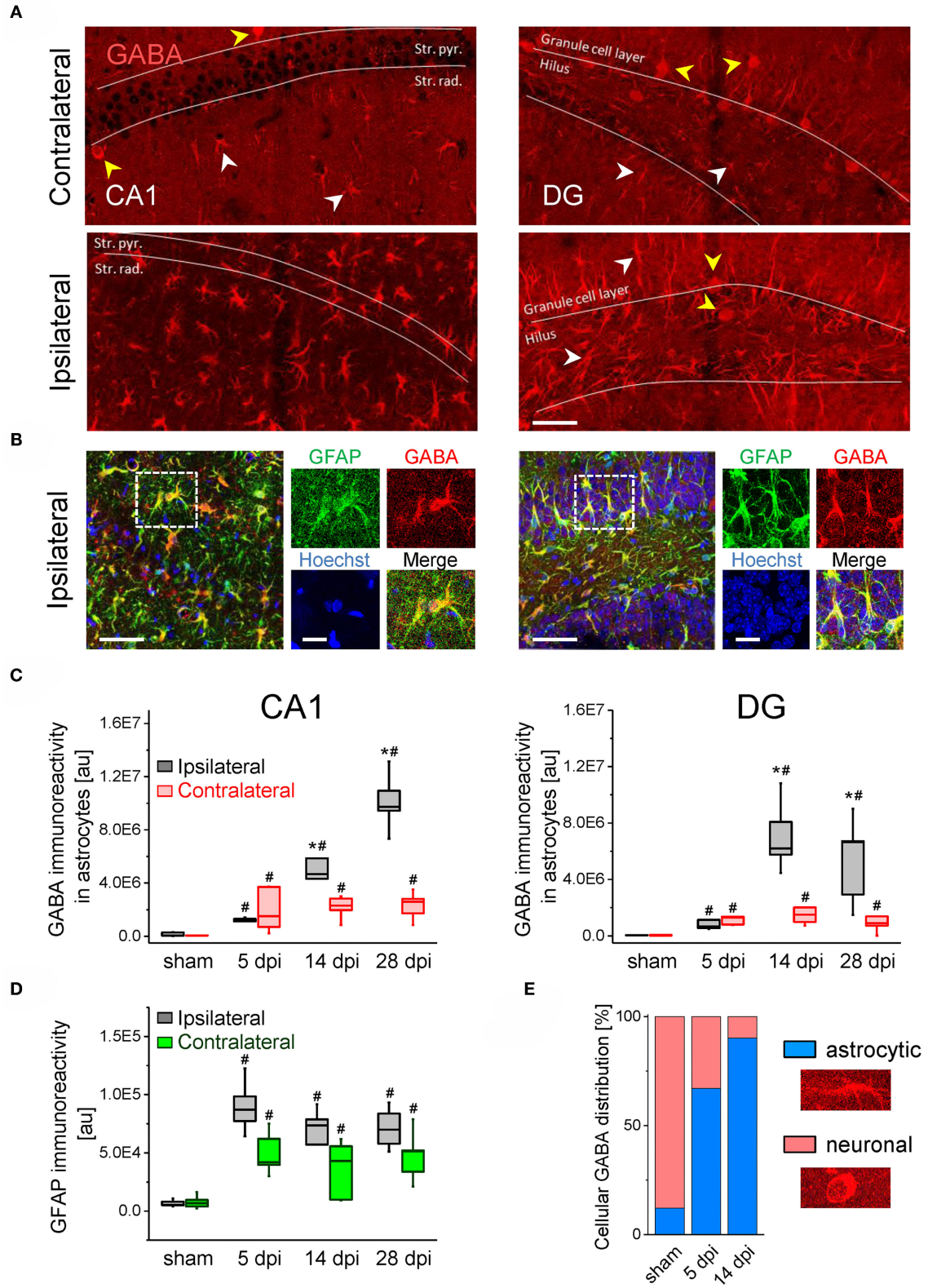

FIGURE 3 | GABA immunostaining in the ispi- and contralateral hippocampus of kainate injected animals. **(A)** Representative confocal images of GABA staining in the hippocampal CA1 region (left panels) and the dentate gyrus (DG, right). Scale bar = 50 μm, white and yellow arrowheads denote GABA immunoreactivity in astrocytes and neurons, respectively. **(B)** GABA (red), GFAP (green) and Hoechst (blue) triple staining in the ipsilateral CA1 region (left panels) and DG (right). Dashed boxes in the

(Continued)

FIGURE 3 | left panels indicate areas enlarged to the right. Scale bars = 50 μm (large panels) and 20 μm (blowups). **(C)** Quantifications of GABA immunoreactivity in GFAP-positive astrocytes in the CA1 region and dentate gyrus at different time points following kainate or sham injection. **(D)** Quantifications of GFAP immunoreactivity in the CA1 region. **(E)** Relative cellular distribution of ipsilateral GABA immunoreactivity in the DG. N = 5 slices from five animals for each condition and time point. Error bars represent SD. *significantly different from the contralateral side, #significantly different from sham ($p < 0.05$, stratified two-way ANOVA followed by Tukey's test and independent samples *t*-test per group).

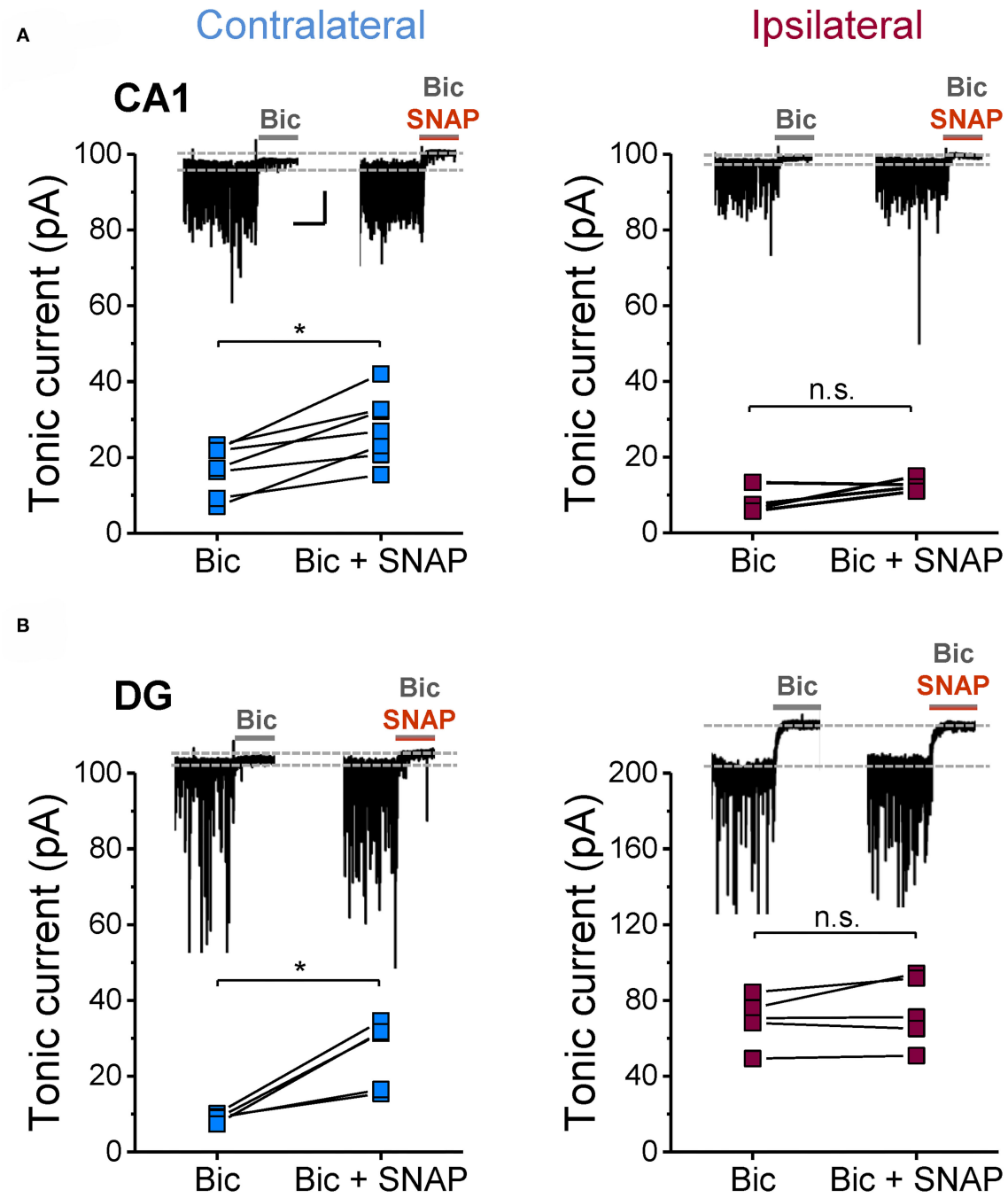

FIGURE 4 | Effect of GAT3 inhibition on tonic inhibitory currents in the hippocampus of epileptic mice. The glial GABA transporter was blocked with SNAP-5114 (100 μM) and consequences on tonic inhibitory currents were tested in ipsi- and contralateral **(A)** CA1 neurons and **(B)** DG granule cells (14 dpi). Inhibition of GAT3 caused an increase in tonic currents on the contralateral but not on the ipsilateral side. Each data set was obtained from at least three mice. Calibration bars for original traces in **(A)** indicate 20 s and 50 pA and also apply to **(B)**. *significantly different ($p < 0.05$; paired *t*-test).

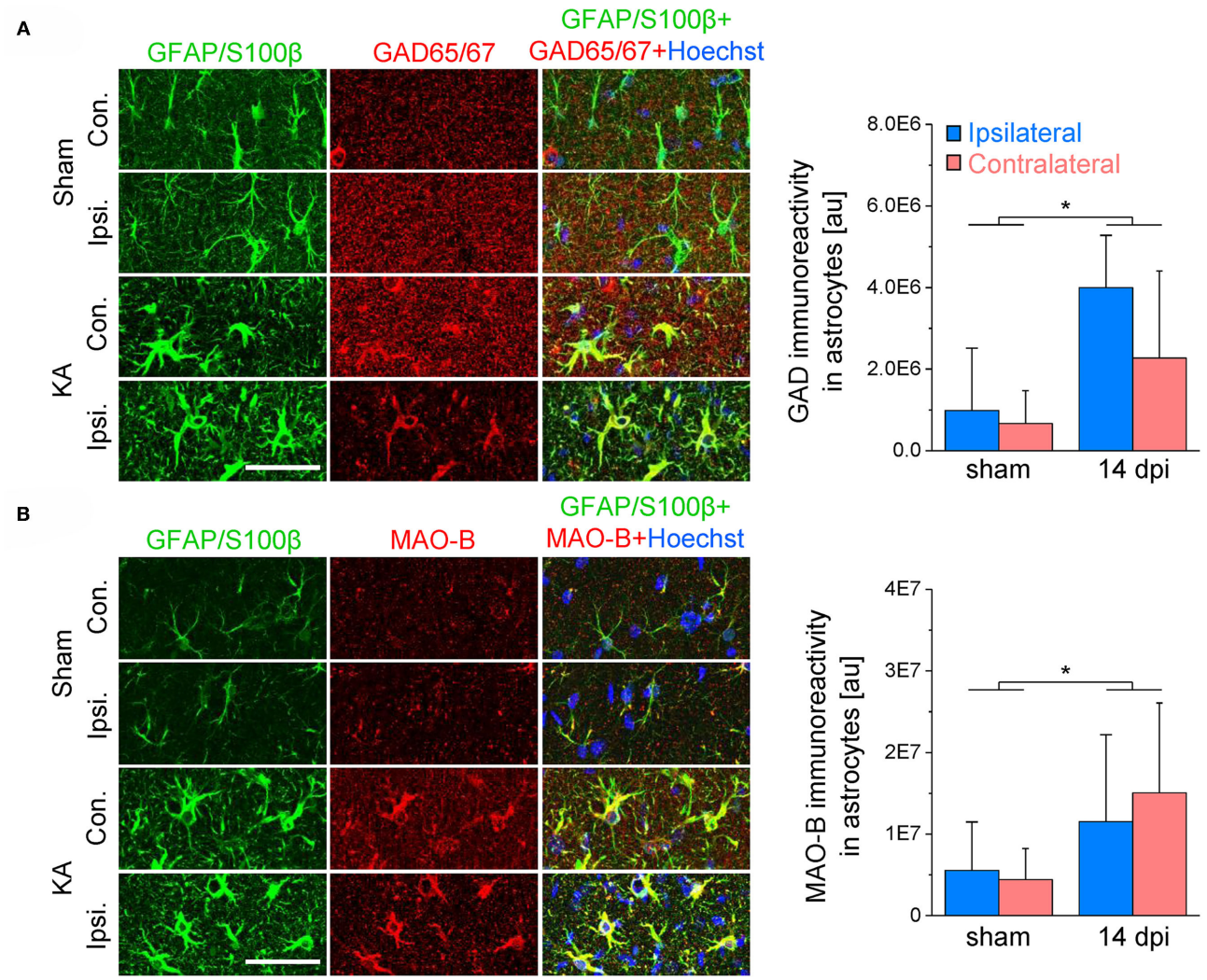

FIGURE 5 | GAD65/67 and MAO-B immunoreactivity in the hippocampal CA1 region 2 weeks after epilepsy induction. **(A)** GAD65/67 (red), GFAP/S100β (green) and Hoechst (blue) staining in the ipsi- and contralateral CA1 region of sham and kainate injected animals. The graph (right) shows the quantification of GAD immunoreactivity in astrocytes. **(B)** MAO-B (red), GFAP/S100β (green) and Hoechst (blue) staining and quantification. $N = 10$ slices from five mice for each condition. Error bars represent SD. *significantly different ($p < 0.05$, two-way ANOVA). Scale bar = 50 μm.

DISCUSSION

In the present study we examined the hypothesis that in the sclerotic epileptic hippocampus, increased GABA release from reactive astrocytes counterbalances the reduced neuronal release, caused by loss of interneurons, resulting in preserved tonic inhibition. Two weeks after epilepsy induction in our model, ipsilaterally we observed severe interneuronal loss, preserved or elevated tonic inhibitory currents in CA1 pyramidal neurons or dentate granule cells and a pronounced increase in astrocytic GABA immunoreactivity. Together with the lack of GABA transporter activity and increased GAD65/67 and MAO-B expression, the most plausible interpretation of our results is that during excessive neuronal activity astrocytes overproduce GABA through *de novo* synthesis and decarboxylation of excess glutamate, which after release into the extracellular space activates tonic $GABA_AR$-mediated currents in excitatory neurons and reduces their excitability (**Figure 6**). In fact, in view of the dramatic loss of interneurons, the question is not why these mice have seizures, but rather why they are seizure-free most of the time. The observed GABA accumulation in reactive astrocytes might reflect a compensatory mechanism aimed to restore excitation-inhibition balance in TLE. However, as speculated earlier (18), the compensation probably generates a less stable network that fails whenever an epileptic seizure occurs. On the other hand, several reports suggested that due to altered expression of the Cl^- transporters NKCC1 and KCC2 (and therefore altered neuronal Cl^- homeostasis), GABA may have excitatory effects in epilepsy (37–40). If true, astrocytic GABA would not counteract but exacerbate

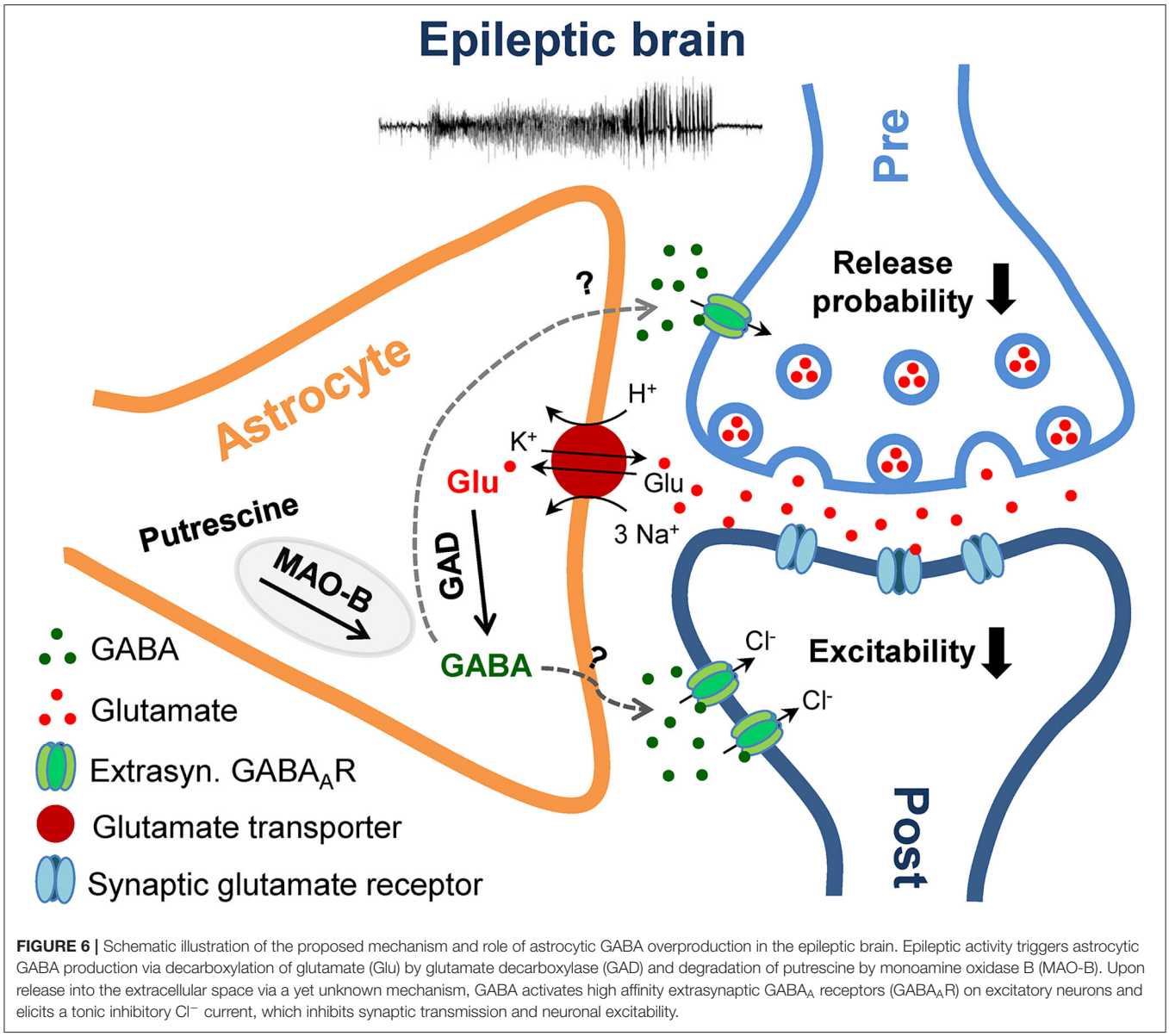

FIGURE 6 | Schematic illustration of the proposed mechanism and role of astrocytic GABA overproduction in the epileptic brain. Epileptic activity triggers astrocytic GABA production via decarboxylation of glutamate (Glu) by glutamate decarboxylase (GAD) and degradation of putrescine by monoamine oxidase B (MAO-B). Upon release into the extracellular space via a yet unknown mechanism, GABA activates high affinity extrasynaptic $GABA_A$ receptors ($GABA_AR$) on excitatory neurons and elicits a tonic inhibitory Cl^- current, which inhibits synaptic transmission and neuronal excitability.

seizures. This scenario is, however, unlikely for several reasons. First, using immunohistochemical analysis we did not observe KCC2 down-regulation in our model (data not shown). Second, in a recent study Pandit et al. (21) suggested that reactive astrocytes release GABA through Bestrophin 1 (Best1) anion channels. Consistent with an anti-epileptic function of astrocytic GABA, in two different TLE models, mice with Best 1 deletion displayed increased seizure susceptibility, an effect that could be reversed by astrocyte-specific re-expression of Best1. Finally, it must be stressed that loss of inhibition with a concomitant excitatory effect of astrocytic GABA would make the occurrence of seizure-free periods difficult to explain. Hence, the most reasonable conclusion that can be drawn from our results is that excessive astrocytic GABA production and release represents a compensatory mechanism in epilepsy.

Loss of Interneurons but Preserved or Increased Tonic Inhibition in the Sclerotic Hippocampus

Loss of interneurons and the consequential impairment of GABAergic inhibition has been regarded as the main cause of seizure activity (16). However, there is still controversy about the extent of the loss and the relative vulnerability of different interneuron subtypes in human epilepsy (5–9). Already at 5 dpi, our immunohistochemical analysis revealed a pronounced loss of PARV-positive and GABA-positive interneurons in the sclerotic CA1 region, and a strong reduction in the ipsilateral dentate gyrus. This early timing and massive extent of cell loss in our intra*cortical* model agrees with data from the intra*hippocampal* kainate TLE model where a similar decline in interneuron numbers was evident already 1–2 dpi (12, 13).

In situ hybridization of GAD67 mRNA (12) or staining against the α1 subunit of the $GABA_AR$ (13) demonstrated that the loss was attributable to cell death and not merely down-regulation of PARV, as suggested previously (41–43). Our GABA staining together with the observed reduction in sIPSC frequency (reflecting reduced synaptic GABA release) supports this view.

Previous work has suggested that GABA spillover from synapses represents the main source of ambient GABA under physiological conditions (31), and thus one would have expected decreased tonic currents after interneuronal loss. However, several studies reported preserved or even increased tonic inhibition in experimental and human TLE (18, 44–50). Consistent with these studies, 14 dpi we detected strongly increased or preserved tonic inhibitory currents in ipsilateral dentate granule and CA1 pyramidal cells, respectively. Pandit and colleagues also reported preserved tonic GABA currents in CA1 neurons 15 days after intracerebroventricular kainate injection, which was preceded by a transient increase at 3 dpi (21). Although this time point was not evaluated in the present study, it is tempting to speculate that an early increase in tonic inhibition might contribute to the suppression of seizures during the latent period in our model.

Astrocytic GABA Production and Release

The preserved/increased tonic GABA currents in epilepsy might be explained either by $GABA_AR$ plasticity or preserved/increased extracellular GABA concentrations (18). Concerning the latter, it has been argued that reactive astrocytes are the main source of ambient GABA in epilepsy (21, 31). In agreement with this view, we detected massive GABA accumulation in reactive astrocytes already 5 dpi in both, the CA1 region and the dentate gyrus. Accumulation was even more pronounced 14 and 28 dpi and positively correlated with GFAP immunoreactivity. Indeed, GABA accumulation in reactive astrocytes has been observed in different brain pathologies, including Alzheimer's disease, Parkinson's disease, stroke and epilepsy (22–25), suggesting that it represents a general feature of the astrocytic reaction. Increasing evidence suggests that astrocyte changes associated with astrogliosis play a detrimental role in epileptogenesis (51–53), a view that is not compatible with an assumed protective effect of astrocytic GABA release. On the other hand, reactive astrocytes might play a dual role in CNS pathologies (54), and whether they exert pro- or antiepileptic effects in epilepsy probably depends on a number of factors, including etiology, timing and severity of epilepsy as well as environmental conditions and interactions with other factors (e.g., inflammatory mediators).

Interestingly, astrocytic GABA was also enhanced contralaterally. Given the absence of interneuron degeneration, it is somewhat surprising that tonic inhibition was not affected on this side. Differences in the extent of GABA increase or, more likely, in astrocytic GABA release might account for this phenomenon. Ictal activity may trigger GABA production on the contralateral side (as indicated by MAO-B and GAD up-regulation), but not affect expression and/or activation of the release machinery. It is also conceivable that astrocytic GABA release occurs only in the absence of GABA uptake. Clearly, the functional significance of astrocytic GABA on the contralateral side remains to be established and requires further studies.

Since the latent period of epileptogenesis in our model lasts about 5 days (27), the increased GABA immunoreactivity detected at the three different time points indicates that astrocyte GABA release influences both development and progression of TLE. The time course of GABA accumulation in our model differs from that reported recently for intracerebroventricular injection where astrocytic GABA peaked 3 dpi and then returned to control level (21). This transient increase implies a role for astrocytic GABA release in inception, rather than progression, of TLE. Differences between models might account for this discrepancy. Indeed, we injected 300 ng kainate into the neocortex just above the right dorsal hippocampus, while Pandit and colleagues applied 100 ng of the drug into the ventricle. In our model, initiation of epileptogenesis should therefore be faster and more focal. Our data are not in line with the concept that GABA was released through reversed operation of astrocytic GABA transporters, as suggested earlier (23, 34). Recent studies provided evidence that reactive astrocytes in epilepsy and other CNS pathologies release GABA through Best1 anion channels (21, 22, 24). Whether this mechanism underlies GABA release in our model remains to be investigated. Our immunohistochemical analysis showed increased immunoreactivity for both GAD and MAO-B, indicating that astrocytic GABA synthesis occurs through multiple routes. Involvement of GAD in the process is at odds with studies reporting that astrocytic GABA production is primarily accomplished by putrescine degradation via MAO-B (22, 24, 25, 33, 55). However, decarboxylation of glutamate via astrocytic GAD may represent an epilepsy-specific mechanism, triggered by the excessive astrocytic glutamate uptake during neuronal hyperactivity. Which of the GAD isoforms (GAD65 or GAD67) is expressed and/or up-regulated in reactive astrocytes cannot be deduced from our analysis. Previous studies have shown that astrocytes in the healthy brain express GAD67 but not GAD65 (56). Although this suggests that overproduction of astrocytic GABA in TLE is mediated by GAD67, a participation of GAD65 cannot be excluded.

CONCLUSION

In this study we show that despite massive interneuron loss, tonic $GABA_AR$ currents are preserved in CA1 pyramidal neurons and increased in dentate granule cells of the sclerotic hippocampus in a chronic TLE model. Furthermore, we gained evidence that GABA overproduction and release from reactive astrocytes represents the main source of ambient GABA responsible for inhibition under this condition. As human and rodent astrocytes display many similar functional properties (57), it is reasonable to assume that this form of inhibition is also involved in genesis and/or progression of human TLE. Hence, molecules that stimulate or improve astrocyte GABA production or release might have effective antiepileptogenic properties.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by LANUV 84-02.04.2012.A212, 84-02-04.2015.A393.

AUTHOR CONTRIBUTIONS

JM designed experiments, acquired, analyzed, and interpreted data. AT and LH acquired, analyzed, and interpreted data. HM analyzed and interpreted data. CS and PB designed and supervised experiments and wrote the manuscript. All authors revised the work critically and approved the manuscript.

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REVIEW
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Role of Astrocytic Inwardly Rectifying Potassium (Kir) 4.1 Channels in Epileptogenesis

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Astrocytes regulate potassium and glutamate homeostasis via inwardly rectifying potassium (Kir) 4.1 channels in synapses, maintaining normal neural excitability. Numerous studies have shown that dysfunction of astrocytic Kir4.1 channels is involved in epileptogenesis in humans and animal models of epilepsy. Specifically, Kir4.1 channel inhibition by *KCNJ10* gene mutation or expressional down-regulation increases the extracellular levels of potassium ions and glutamate in synapses and causes hyperexcitation of neurons. Moreover, recent investigations demonstrated that inhibition of Kir4.1 channels facilitates the expression of brain-derived neurotrophic factor (BDNF), an important modulator of epileptogenesis, in astrocytes. In this review, we summarize the current understanding on the role of astrocytic Kir4.1 channels in epileptogenesis, with a focus on functional and expressional changes in Kir4.1 channels and their regulation of BDNF secretion. We also discuss the potential of Kir4.1 channels as a therapeutic target for the prevention of epilepsy.

Keywords: astrocytes, Kir4.1 channels, spatial potassium buffering, epilepsy, BDNF, glutamate, tripartite synapse

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INTRODUCTION

Epilepsy is a common neurological disease that is characterized by recurrent seizures caused by neuronal hyperexcitation. The current antiepileptic agents predominantly act on neuronal ion channels (e.g., blockers of voltage-gated sodium channels and calcium channels), glutamate receptors [e.g., antagonists of a-amino-3-hydroxy-5-methyl-isoxazolopropionic acid (AMPA) receptors], or GABAergic inhibitory systems (e.g., modulators of the $GABA_A$ receptor/chloride channel complex and inhibitors of GABA transaminase), which aim to suppress excessive neural excitation (1, 2). Therapy with these antiepileptic drugs is effective in about 70% of epilepsy patients, whereas seizure control is not achieved for the remaining 30% of patients (3, 4). Thus, there is a high unmet need for novel therapeutic targets or agents to treat refractory epilepsy.

Numerous findings show that astrocytes, the major cell component of glial cells in the central nervous system (CNS), actively regulate the excitability and plasticity of neurons by forming tripartite synapses in conjunction with presynaptic and postsynaptic neural components (5–10). Specifically, astrocytes regulate ion homeostasis and extracellular space volume, metabolize neurotransmitters (e.g., glutamate, GABA, and glycine), and secrete various neuroactive molecules including gliotransmitters [e.g., glutamate, D-serine, adenosine 5′-triphosphate (ATP)], neurotrophic factors [e.g., brain-derived neurotrophic factor (BDNF)

and glia-derived neurotrophic factor (GDNF)], and cytokines [e.g., tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)] (11–13). Among these functions of astrocytes, a spatial buffering system for potassium ions (K^+) plays an important role in the maintenance of neuronal excitability, which transports excessive extracellular K^+ secreted from excited neurons to sites with lower K^+ concentrations (e.g., microcapillaries) (14–18). This potassium clearance mechanism is primarily mediated by astrocytic inwardly rectifying potassium (Kir) channels containing Kir4.1 subunits (Kir4.1 channels) (16–21).

In this review, we introduce the current understanding regarding the pathophysiological role of astrocytic Kir4.1 channels in the development of epilepsy (epileptogenesis). Further, we discuss the potential of Kir4.1 channels as a therapeutic target for the prevention of epilepsy.

SPATIAL POTASSIUM BUFFERING AND ASTROCYTIC KIR4.1 CHANNELS

Extracellular K^+ levels are critical for determining the resting membrane potential of neurons and are normally maintained at ca. 3–5 mM (22). Physiological neural activity leads to an elevation of <1 mM in extracellular K^+ concentration (23). Increases of 10–12 mM K^+ in ceiling levels are induced during excessive neural activity due to electrical stimulation (24). Astrocytes rapidly transport K^+ from synapses, where K^+ is secreted from neurons during the repolarization phase, to regions with lower K^+ levels (e.g., microcapillaries) by coupling into a syncytium through gap junctions (**Figure 1**) (14–21, 25). This astrocytic K^+ clearance mechanism, known as "spatial potassium buffering," is vital for maintaining K^+ homeostasis and preventing neural hyperexcitability during normal brain function. In addition, spatial potassium buffering is known to be linked to glutamate uptake via glutamate transporters [e.g., excitatory amino acid transporters 1 (EAAT1) and EAAT2] and water transport via aquaporin-4 (AQP4) by astrocytes (26–31). Moreover, both connexin30 and connexin43 in astrocytic gap junctions were shown to play a critical role in normal K^+ redistribution, using double knockout techniques in mice (32, 33).

The influx of K^+ into astrocytes is mainly mediated by Kir channels containing Kir4.1 and Kir5.1 subunits, which are highly expressed in astrocytes and retinal Müller cells (16–21, 34–39). Kir4.1 subunits have two transmembrane (TM) domains with an extracellular ion selectivity filter, including the GYG signature sequence, which construct Kir channels by forming tetramers (**Figure 2**) (25, 39). Two types of Kir4.1-containing Kir channels (Kir4.1 channels), the homo-tetramer of Kir4.1 and the hetero-tetramer of Kir4.1 and Kir5.1, conduct large inward K^+ currents at potentials negative to K^+ equilibrium potential (E_K) and moderate outward K^+ currents at those positive to E_K (**Figure 2**) (25, 39, 40).

Pharmacological studies have shown that among CNS agents, several antidepressants reversibly inhibited K^+ currents via Kir4.1 channels in a subunit-dependent manner. Tricyclic antidepressants (TCAs) such as nortriptyline, amitriptyline, desipramine, and imipramine, blocked Kir4.1 channels in a voltage-dependent manner, while selective serotonin reuptake inhibitors (SSRIs) including fluoxetine and sertraline inhibited Kir4.1 channels in a voltage-independent manner (**Figure 2**) (41–44). The inhibitory effects of antidepressants for Kir4.1 channels were achieved at concentrations considered to be within a range of brain concentrations for clinical treatment of depression. Antidepressant treatment is reported to increase the risk of seizure incidence (45, 46), which may be due to antidepressant drug actions on Kir4.1 channels.

Alanine-scanning mutagenesis studies on the antidepressant-Kir4.1 channel interaction demonstrated that these antidepressant agents specifically blocked the Kir4.1 channel pore (47). Two amino acids, T128 and E158, on pore and TM-2 helices respectively, can bind to antidepressants. Recently, anti-malarial agents such as quinacrine and chloroquine, and the anti-protozoal agent, pentamidine, have also been shown to inhibit Kir4.1 channels by binding to T128 and E158, similar to antidepressant agents (48–50). Although few reports are available on drug-Kir4.1 interaction, information about structure-based action on Kir4.1 channels is important for designing novel treatment compounds for epilepsy and reducing the potential of seizure side effects.

KIR4.1 CHANNELS IN EPILEPSY PATIENTS

Mutations in the human *KCNJ10* gene encoding Kir4.1 were reported to cause the epileptic disorders known as "EAST" (Epilepsy, Ataxia, Sensorineural deafness and Tubulopathy) and "SeSAME" (Seizures, Sensorineural deafness, Ataxia, Mental retardation, and Electrolyte imbalance) syndrome (OMIM 612780) (51–53). Patients with EAST/SeSAME syndrome initially manifest generalized tonic-clonic seizures (GTCSs) within a few months after birth and are treated with anticonvulsant agents such as valproate and phenobarbital. The *KCNJ10* mutations responsible for EAST/SeSAME syndrome have been shown to be T57I, R65P, R65C (cytoplasmic end of TM-1), F75L, F75C, G77R (TM-1), V91Gfs*197, F119Gfs*25, C140R (extracellular loop between TM-1 and TM-2), T164I, A167V (TM-2), R175Q, R199X, R240H, V259*, G275Vfs*7, and R297C (C-terminal domain) (51–64). These homozygous or compound heterozygous mutations disrupted Kir4.1 channel function to varying degrees from completely to moderately. Moreover, novel loss-of-function mutations (I60T, I60M, G163D, R171Q, A201T, I209T, and T290A) in *KCNJ10* were identified in patients with atypical EAST/SeSAME syndrome lacking one or more core clinical manifestations (65–69). In contrast, heterozygous gain-of-function mutations (R18Q, V84M, and R348H) in *KCNJ10* caused autism spectrum disorders with spastic seizures and intellectual disability (70–72).

Electrophysiological investigations demonstrated that Kir currents were significantly reduced in hippocampal specimens from refractory temporal lobe epilepsy (TLE) patients, using patch-clamp techniques (73–75). The impairment of glial K^+ uptake sensitive to Ba^{2+}, a blocker of Kir channels, was also in sclerotic hippocampal slices from patients with epilepsy (76, 77).

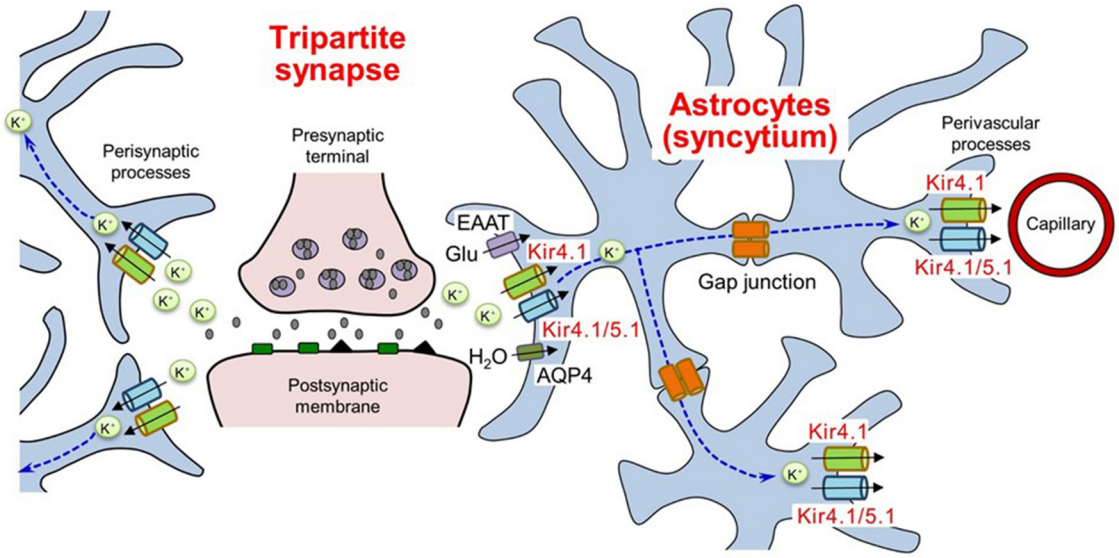

FIGURE 1 | Spatial K^+ buffering of astrocytes in tripartite synapses. Astrocytes uptake extracellular K^+ secreted from neurons and release K^+ in regions with lower K^+ levels by coupling into a syncytium through gap junctions. The K^+ buffering mechanism is corelated with glutamate uptake and water transport by astrocytes. EAAT, excitatory amino acid transporters; AQP4, aquaporin 4. Modified from Ohno et al. (25).

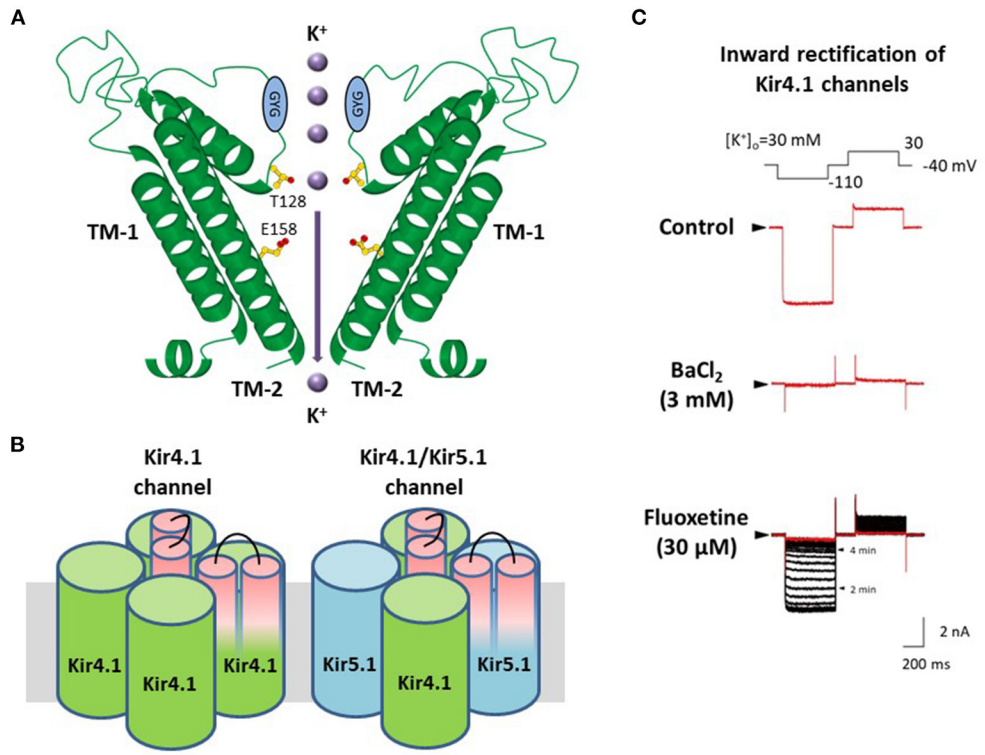

FIGURE 2 | Molecular structure and properties of Kir4.1 channels. **(A)** Kir4.1 subunits have two transmembrane (TM) helices with one extracellular loop, including the GYG signature sequence of the K^+ selectivity filter. **(B)** Kir4.1 subunits construct two types of channels, the homo-tetramer of Kir4.1 and the hetero-tetramer of Kir4.1 and Kir5.1. **(C)** Kir4.1 channels (homo-tetramer of Kir4.1) conduct large inward and relatively small outward K^+ currents. Selective serotonin reuptake inhibitors, fluoxetine, inhibit Kir4.1 channel currents. Modified from Ohno et al. (25).

Furthermore, astrocytic Kir4.1 expression has been shown to decrease in the sclerotic hippocampus of TLE patients (78–80). Additionally, flavoprotein fluorescence imaging, visualizing neuronal activities without exogenous dyes in living tissues taken from epilepsy patients, showed that epileptiform activities propagated from the subiculum of the hippocampus with sclerosis, where the Kir4.1 expression of astrocytes was markedly down-regulated (81). In refractory partial epilepsy pathologically diagnosed as "focal cortical dysplasia type 1," Kir4.1 expression was decreased in the epileptogenic regions where direct current (DC) shifts were detected using wide-band electroencephalography (EEG) recordings (82). These DC shifts preceding conventional ictal pattern and high frequency oscillations (HFOs), known as "active DC shifts," were suggested to reflect the extracellular K^+ accumulation caused by the dysfunction of astrocytic potassium buffering, which can be EEG biomarkers for the epileptic zone (82). Therefore, Kir4.1 channel dysfunction affected by gene mutations or expressional down-regulation is likely to be involved in the pathogenesis of human epileptic disorders.

KIR4.1 CHANNELS IN ANIMAL EPILEPSY MODELS

Kir4.1 homozygous deletion in mice reduced body weight gain and caused progressive weakness by postnatal day (P) 8–10, although heterozygous mice showed no pathological behavior (83, 84). Subsequently, Kir4.1 knockout mice exhibited jerky movements and severe deficits in controlling voluntary movements, posture, and balance, and consequently died by P24. In addition, studies using conditional knockout techniques have reported that mice with conditional knockout of astrocytic Kir4.1 developed pronounced body tremor, ataxia, and stress-induced GTCSs, which were suggested to be involved in astrocytic membrane depolarization and impaired uptake of extracellular K^+ following neural activity (**Table 1**) (30, 85, 86). Moreover, Kir4.1 conditional knockout also reduced glutamate uptake by astrocytes (30). This impairment of glutamate clearance resulted from the dysfunction of EAATs due to membrane depolarization in astrocytes (29–31, 87, 88).

Numerous studies using animal models of epilepsy showed that astrocytic Kir4.1 expressional changes were involved in seizure induction and susceptibility. Specifically, Kir4.1 expression was significantly reduced in Noda epileptic rats (NER), a hereditary epilepsy model (**Table 1**) (89). NER exhibited frequent spontaneous GTCSs associated with two genetic loci, chromosome (Chr) 1q32-33 and Chr5q22, including *cholecystokinin B receptor (Cckbr)*, *suppressor of tumorigenicity 5 (St5)*, and *PHD finger protein 24 (Phf24)* (90–95). In NER, Kir4.1 expression was region-specifically reduced in the amygdala, where the expression of Fos protein, a biological marker of neural excitation, significantly elevated (89). Moreover, *Leucine-Rich Glioma-Inactivated 1 (Lgi1)* mutant rats, a model of human autosomal dominant lateral temporal lobe epilepsy (ADLTE), showed reduced astrocytic Kir4.1 expression in specific regions, including both the lateral and medial temporal lobes, after the acquisition of audiogenic seizure susceptibility (**Table 1**) (96). In these regions, neural hyperexcitation during seizures was confirmed using Fos immunohistochemical techniques (97). Auditory stimuli for seizure induction consisted of sound stimulation twice, priming stimulation at P16 and test stimulation at 8 weeks. Priming stimulation induces epileptogenesis caused by *Lgi1* mutation without spontaneous seizure phenotypes (96, 98). Interestingly, the Kir4.1 expression in astrocytes was reduced during the time-course of epileptogenesis before application of test stimulation at the age of 8 weeks in *Lgi1* mutant rats (96). These findings indicate that the dysfunction of Kir4.1 channels is involved not only in evoking seizure generation, but also in chronic development of epilepsy (epileptogenesis).

Furthermore, the *Kcnj10* single nucleotide polymorphism (SNP) with T262S variation that disrupts Kir4.1 channel activity has been identified as the mutation responsible for seizure susceptibility of DBA/2 mice (**Table 1**) (99, 100). A rodent epilepsy model induced by fluid percussion injury or albumin injection also exhibited down-regulation of Kir4.1 expression in regions related to seizure foci (**Table 1**) (101, 102). Kir4.1 expression was transiently reduced after status epilepticus (SE) in temporal lobe epilepsy (TLE) models induced by electrical stimulation, although the expression of Kir4.1 returned to the normal level 1 week after SE (**Table 1**) (103). In contrast to epilepsy models with convulsive seizures, no changes in Kir4.1 expression were detected in Groggy rats, an absence epilepsy model (**Table 1**) (104). In addition, hyperglycemia has been reported to reduce Kir4.1 expression and disrupt the clearance of both K^+ and glutamate using astrocyte primary cultures (105). Type 2 diabetic mice (db/db) also showed down-regulation of Kir4.1 expression and dysfunction in K^+ intake that were associated with hippocampal neural hyperexcitability (**Table 1**) (106). These studies may explain the epileptic predisposition of type 2 diabetes patients (107, 108).

ASTROCYTIC KIR4.1-BDNF SYSTEM IN EPILEPTOGENESIS

BDNF is a member of the neurotrophin family essential for the normal development and function of the CNS. Specifically, BDNF regulates cell survival, neurogenesis, neuronal sprouting, synaptic plasticity, and reactive gliosis by binding to tropomyosin-related kinase (Trk) receptors, especially TrkB receptors (109–112). The neurotrophic properties of BDNF potentially produce therapeutic effects for neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease, and Huntington's disease) and neuropsychiatric diseases (e.g., depression and bipolar disorder) (113–119). However, elevated expression of BDNF is known to be involved in the pathogenesis of epilepsy in various animal models and human brains (111, 120, 121). In addition, inhibition of BDNF/TrkB signaling has been shown to suppress the development of epilepsy in animal models (122–126).

While the expressional levels of BDNF were higher in neurons, astrocytic BDNF expression and BDNF/TrkB signaling have also

TABLE 1 | Pathophysiological changes in Kir4.1 channels in animal epilepsy models.

Animal model	Functional and expressional changes in Kir4.1	Pathological behaviors and seizure types
Conditional knockout mice of astrocytic Kir4.1	Dysfunction of Kir4.1 channel Impaired uptake of extracellular K^+ and glutamate	Body tremor, ataxia, stress-induced GTCSs, premature death
Noda epileptic rats (NER)	Down-regulation of Kir4.1 expression in the amygdala	Spontaneous GTCSs
Lgi1 mutant rats (ADLTE model)	Down-regulation of Kir4.1 expression in the temporal lobe after development of audiogenic epilepsy	Audio-induced GTCSs
Seizure susceptible DBA/2 mice	*Kcnj10* SNP with T262S variation Dysfunction of Kir4.1 channel Impaired uptake of glutamate	Increased seizure susceptibility
Trauma-induced epilepsy rats	Down-regulation of Kir4.1 expression in the cerebral cortex	Spontaneous partial seizures of cerebral cortex origin
Albumin-induced epilepsy rats	Down-regulation of Kir4.1 expression in the hippocampus exposed to albumin	Increased seizure susceptibility due to hippocampal hyperexcitability
Electrical stimulation-induced TLE rats	Transient reduction of Kir4.1 expression in the temporal cortex 24 hours after SE	No assessment
Groggy rats (absence epilepsy model)	No change in Kir4.1 expression	Absence-like seizures, ataxia
db/db mice (type 2 diabetic model)	Down-regulation of Kir4.1 expression in the hippocampus	Hippocampal hyperexcitability

been shown to contribute to brain functions under physiological and pathophysiological conditions (127–130). A recent study demonstrated that BDNF overexpression in astrocytes caused neuronal hyperexcitability and cell death, and deteriorated the phenotypes in lithium pilocarpine-induced TLE models, which were suggested to be mediated by astrocytic TrkB receptors, rather than neural TrkB receptors (131).

Astrocytic Kir4.1 channels have been shown to modulate BDNF expression using astrocyte primary cultures (25, 44, 132). Several antidepressant agents (e.g., imipramine and amitriptyline), which reportedly inhibited Kir4.1 channels in a subunit-specific manner (41, 43, 47), facilitated the expression of BDNF in astrocytes (133–135). Furthermore, the relative potencies of antidepressant agents for BDNF induction were consistent with those for the blockade of Kir4.1 channels, but not for the inhibition of 5-HT reuptake (43, 44). In addition, Kir4.1 knockdown by small interfering RNA (siRNA) transfection significantly elevated BDNF expression in astrocytes, which was suppressed by a MEK1/2 inhibitor, but not by a p38 MAPK inhibitor or a JNK inhibitor (44). These results suggest that the reduced function of Kir4.1 channels facilitates BDNF expression in astrocytes by activating the Ras/Raf/MEK/ERK pathway (**Figure 3**) (25, 44, 132). This hypothesis was supported by previous studies showing that the Ras/Raf/MEK/ERK signaling pathway regulates the transcription of BDNF and other survival/plasticity genes through interaction with cyclic AMP response element binding protein (CREB) (136, 137). It is therefore likely that Kir4.1 channels play a key role in modulating epileptogenesis by controlling not only the extracellular K^+ and glutamate levels in synapses, but also the BDNF expression in astrocytes. The astrocytic Kir4.1-BDNF system is expected to serve as a novel target for the treatment of epilepsy, especially epileptogenesis.

KIR4.1 CHANNELS AS A NOVEL THERAPEUTIC TARGET FOR PREVENTION OF EPILEPSY

Based on the potential role of astrocytic Kir4.1 channels in epileptogenesis, normalizing the down-regulation of astrocytic Kir4.1 channel expression during epileptogenesis can be a therapeutic strategy to prevent epilepsy. We recently showed that repeated treatments with antiepileptic drugs (valproate, phenytoin, and phenobarbital), which are effective for convulsive seizures, commonly elevated the astrocytic Kir4.1 expression in the limbic region (138). These antiepileptic drugs have previously showed inhibitory effects on kindling development in animal models (139–141), in which the elevated expression of Kir4.1 channels may contribute to the prophylactic effect of these drugs. Moreover, we have shown that valproate prevented audiogenic epileptogenesis in *Lgi1* mutant rats by elevating the down-regulated Kir4.1 expression during epileptogenesis in a dose-dependent manner (96). Although further studies are required to reveal the mechanisms underlying the Kir4.1 pathogenic changes in epileptogenesis, these findings support the notion that astrocytic Kir4.1 channels can be therapeutic targets for prevention of epilepsy. Specifically, novel compounds positively modulating astrocytic Kir4.1 channels are expected to have potential for treatment of epilepsy and epileptogenesis. Although no information on the structure-activity relationship for Kir4.1 channel stimulators is available, gain-of-function mutations of the *KCNJ10* gene (e.g., R18Q in N-terminus and V84M in TM1 region) reported in patients with autism spectrum disorders may give hints for new drug discovery (70, 72). In addition, retigabine (ezogabine), an antiepileptic drug for focal onset seizures, may also give information since it primarily acts on neural KCNQ2-5 (K_v7.2-7.5) ion channels as a positive allosteric modulator (142).

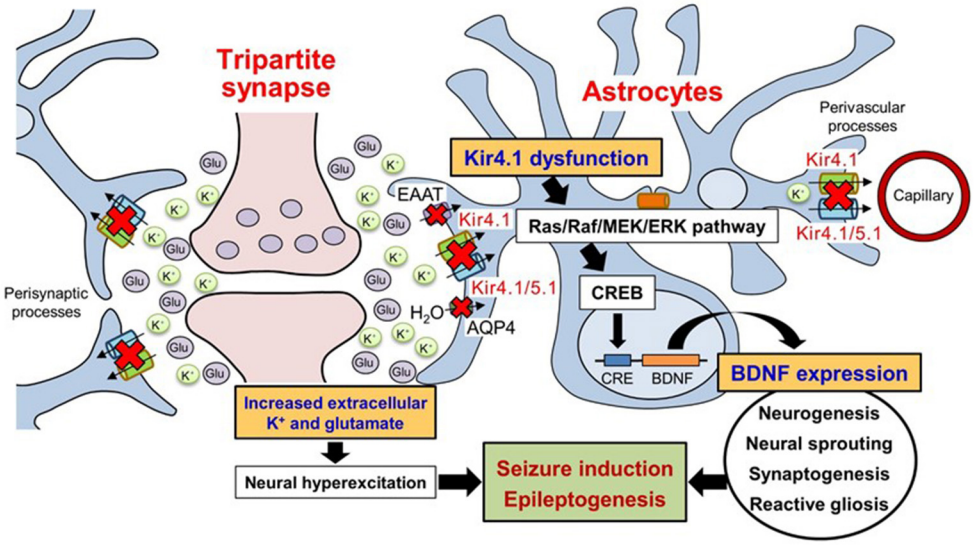

FIGURE 3 | Schematic drawing illustrating the effects of Kir4.1 dysfunction on neural hyperexcitation and astrocytic BDNF expression. Dysfunction (genetic mutation, down-regulated expression, and pharmacological blockade) of Kir4.1 channels increases extracellular K^+ and glutamate at synapses and causes neural hyperexcitability. The dysfunction of Kir4.1 channels activates the Ras/Raf/MEK/ERK signaling pathway and facilitates BDNF expression in astrocytes. Based on these changes, astrocytic Kir4.1 channels play important roles in modulating seizure induction and epileptogenesis. Modified from Ohno et al. (25).

CONCLUSION

Astrocytic Kir4.1 channels play a critical role in the regulation of brain homeostasis and neural excitability. Evidence is accumulating that dysfunction of astrocytic Kir4.1 channels is involved in epileptogenesis in both epilepsy patients and animal epilepsy models. Moreover, the reduced activity of Kir4.1 channels elevates the levels of extracellular K^+ and glutamate at tripartite synapses and facilitates astrocytic BDNF expression, which can promote the development of epilepsy. Although data are limited, the approach to restore Kir4.1 down-regulation during epileptogenesis was actually effective to prevent the development of epilepsy in an animal model of epilepsy. Thus, the Kir4.1-BDNF system in astrocytes is expected to serve as a novel therapeutic target for epilepsy. especially epileptogenesis.

AUTHOR CONTRIBUTIONS

MK, AI, and YO designed and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Seizure-Induced Acute Glial Activation in the *in vitro* Isolated Guinea Pig Brain

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Introduction: It has been proposed that seizures induce IL-1β biosynthesis in astrocytes and increase blood brain barrier (BBB) permeability, even without the presence of blood borne inflammatory molecules and leukocytes. In the present study we investigate if seizures induce morphological changes typically observed in activated glial cells. Moreover, we will test if serum albumin extravasation into the brain parenchyma exacerbates neuronal hyperexcitability by inducing astrocytic and microglial activation.

Methods: Epileptiform seizure-like events (SLEs) were induced in limbic regions by arterial perfusion of bicuculline methiodide (BMI; 50 μM) in the *in vitro* isolated guinea pig brain preparation. Field potentials were recorded in both the hippocampal CA1 region and the medial entorhinal cortex. BBB permeability changes were assessed by analyzing extravasation of arterially perfused fluorescein isothiocyanate (FITC)–albumin. Morphological changes in astrocytes and microglia were evaluated with tridimensional reconstruction and Sholl analysis in the ventral CA1 area of the hippocampus following application of BMI with or without co-perfusion of human serum albumin.

Results: BMI-induced SLE promoted morphological changes of both astrocytes and microglia cells into an activated phenotype, confirmed by the quantification of the number and length of their processes. Human-recombinant albumin extravasation, due to SLE-induced BBB impairment, worsened both SLE duration and the activated glia phenotype.

Discussion: Our study provides the first direct evidence that SLE activity *per se* is able to promote the activation of astro- and microglial cells, as observed by their changes in phenotype, in brain regions involved in seizure generation; we also hypothesize that gliosis, significantly intensified by h-recombinant albumin extravasation from the bloodstream to the brain parenchyma due to SLE-induced BBB disruption, is responsible for seizure activity reinforcement.

Keywords: blood brain barrier, inflammation, albumin, microglia, astrocytes

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INTRODUCTION

Blood brain barrier (BBB) dysfunction has been associated with disturbances of neural function in the central nervous system (CNS). A compromised BBB is often found in epileptic brain tissue obtained from epilepsy surgery (1, 2) and in patients with post-traumatic epilepsy (3) as well as in pharmacological models of status epilepticus (4). Experimental BBB leakage during intense seizures and the associated extravasation of serum albumin have been recognized as important contributors to glial dysfunction and epileptogenesis (5–7). Serum albumin binds the transforming growth factor ß (TGF-ß) receptor II in astrocytes and activates a transcriptional response resulting in a cascade of events culminating in the generation of epileptiform discharges (8, 9). Accumulating data from human and animal studies support the notion that glial cells make an important contribution to the pathogenesis of neurological diseases (10–12). Astrocytes are indispensable for proper brain development, playing fundamental roles in promoting formation and function of synapses, maintaining ion, neurotransmitter, water and ATP homeostasis and modulating neuronal signaling (13–16). Astrocytes can become reactive and develop a gliosis-like state where inflammation processes are triggered and up-regulated in a positively-feedback loop after brain injury and disease (17, 18), having a key role in in the generation and spread of seizure activity (11, 19–21).

Through dedicated molecular cascades, astrocytes (i) protect neurons against glutamate excitotoxicity by removing and recycling this neurotransmitter released during neuronal activity from the extracellular space; (ii) remove extracellular activity-dependent potassium accumulation; (iii) reduce the subsequent neuronal depolarization and hyperactivity (22, 23). Astrocytes also represent an important source of pro-inflammatory mediators and have been shown to initiate and regulate many immune-mediated mechanisms in the CNS (24–26). Changes of astrocytic receptors, transporters, ion channels and intracellular proteins are present in almost all forms of epilepsy (27). Accordingly, modified astroglial functioning is a key element leading to a reduction in: (i) expression of potassium inward-rectifying channels (Kir4.1) and water channels (aquaporin 4, AQP4) resulting in impaired potassium $[K^+]_o$ buffering, $[K^+]_o$ accumulation and consequent neuronal depolarization and seizures (28, 29); (ii) gap junction expression, with consequent alteration of spatial buffering of small molecules (e.g., K^+) (30, 31); (iii) glutamate uptake, favoring brain excitability increase (11).

Microglial cells are brain-resident macrophage-like cells that contribute to innate immune system mechanisms and respond early to CNS injuries (32). Accordingly, their reaction to damage can be either detrimental or protective (33). In a resting state, microglial cells feature a small cell body with vastly ramified processes (surveilling microglia). After a pathological challenge, microglial cells acquire amoeboid-like shape somata with almost no processes and achieve phagocytic properties (32). Recently, resident microglial cells have been implicated in driving astrocytes reactivity (34, 35) contributing to neuronal hyperexcitability and neurodegeneration (36, 37) and to the process of epileptogenesis in human and animal models of epilepsy (38, 39). Serum albumin-activated microglia releases pro-inflammatory cytokines [TNF-α; (40, 41)] and interacts with the damage-associated molecular patterns [DAMPs; (42)], contributing to astrocytes activation, brain inflammation and seizure recurrence (24, 40, 43). Seizures by themselves can induce brain inflammation and gliosis independent from blood-borne molecules, mediated by the synthesis and release of IL-1β that promotes BBB disruption (44).

In this study, we aim to investigate more in detail the effects of seizure activity on glial response, focusing on the morphological changes characterizing reactive glial cells. We also investigate if brain parenchyma exposure to serum albumin worsens glial cells reactivity and, as consequence, favors brain excitability and seizure recurrence. To verify these hypotheses, we induced pharmacological seizures in the *in vitro* isolated guinea pig brain (44, 45), a preparation that retains the physiological interactions between neurons, glia and vascular compartments (BBB included) in a condition close to *in vivo* (46, 47). In this isolated preparation, seizure-induced inflammatory responses can be analyzed in the absence of peripheral immune cells or blood-derived molecules.

MATERIALS AND METHODS

Procedures involving animals and their care were conducted in accordance with the ethically approved institutional guidelines that are in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, Official Journal L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All efforts were made to minimize the number of animals used and their suffering. Brains were isolated from young adult Hartley guinea pigs (150–200 g; Charles River Laboratories, Comerio, Italy) according to the standard technique described in detail elsewhere (46, 48). After barbiturate anesthesia the brain was carefully isolated and transferred to the incubation chamber. The basilar artery was cannulated with a polyethylene cannula to ensure arterial perfusion with a saline solution (composition: NaCl, 126 mM, KCl, 3 mM, KH_2PO_4, 1.2 mM, $MgSO_4$, 1.3 mM, $CaCl_2$, 2.4 mM, $NaHCO_3$, 26 mM, glucose, 15 mM, 3% dextran M.W. 70,000) oxygenated with a 95% O_2-5% CO_2 gas mixture (pH 7.3). This solution was arterially perfused at a rate of 6.5 ml/min via a peristaltic pump (Gilson Minipulse, Villiers Le Bel, France). Brain isolation was performed at low temperature (15°C) and experiments were carried out at 32°C, to maintain the isolated brain under hypothermic anesthesia. Human recombinant albumin (h-ALB; Sigma-Aldrich, Italy; 1 gr/250 ml) and bicuculline methiodide (Sigma-Aldrich, Italy) were applied by arterial perfusion (49, 50).

Induction of Epileptiform Activity

In a first set of experiments, epileptiform seizure-like events (SLEs) were induced by arterial perfusion of the $GABA_A$ antagonist BMI (50 μM; $n = 4$) and a second BMI perfusion was applied 90 min after the first one (**Figure 1**, protocol B). In

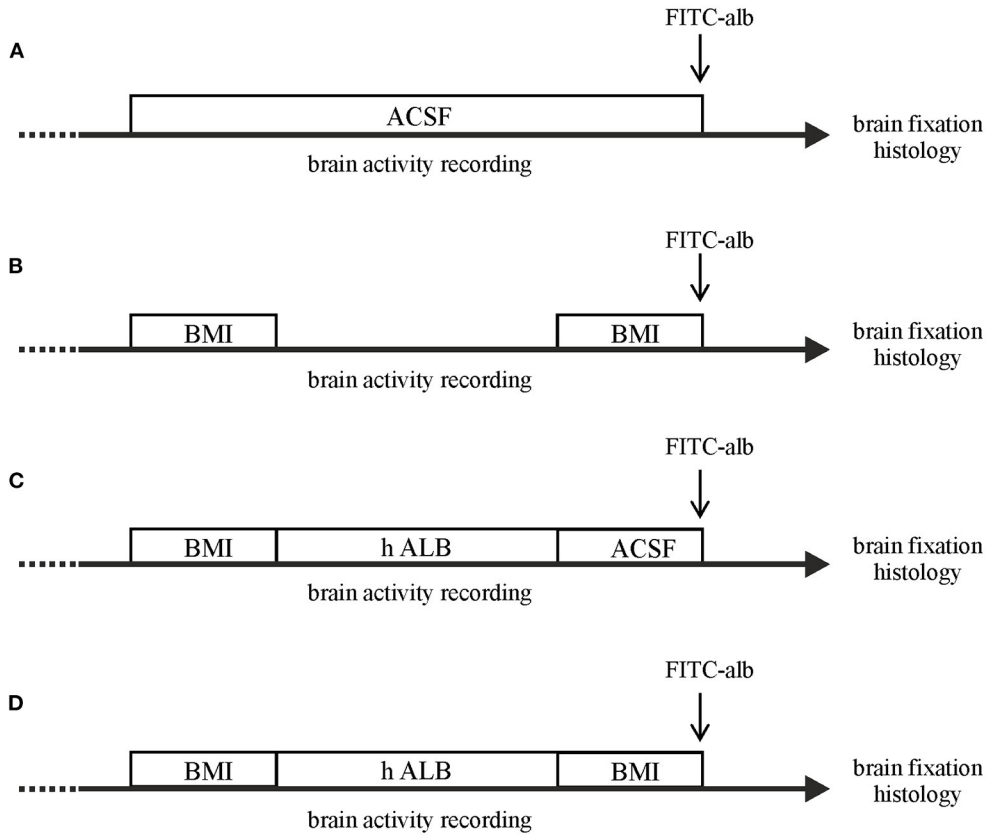

FIGURE 1 | Schematic drawing of the different experimental protocols utilized in the study. The *in vitro* brains were arterially perfused with: **(A)** control saline solution; **(B)** a double 3-min bolus of BMI; **(C)** 30-min perfusion with h-recombinant albumin (h-ALB) after the birst bolus of BMI; **(D)** 30-min perfusion with h-recombinant albumin (h-ALB) interposed between two boli of BMI. At the end of each experiment the brain was perfused with FITC-albumin for 4 min to evaluate BBB leakage. Each experimental protocol lasted 4 h.

a second set of the experiments, h-ALB (4 g/L, 329 mOsm; $n = 3$) added to control solution was arterially perfused for 30 min after the first bolus of BMI (**Figure 1**, protocol C). In a third set of the experiments, h-ALB ($n = 4$) added to control solution was arterially perfused for 30 min, between the two BMI applications (**Figure 1**, protocol D), just after the recovery of the first SLE. Brains were maintained *in vitro* for 4 h. In control experiments, brains were perfused only with control saline solution (**Figure 1**, protocol A; $n = 4$). Two control brains were perfused with FITC-albumin at the end of the experiment.

Electrophysiology

To test brain viability during the experiments, simultaneous extracellular recordings were performed in the piriform cortex (PC), medial entorhinal cortex (mEC), and the CA1 hippocampal region with glass micropipettes filled with a 0.9% NaCl solution (2–5 MOhm input resistance) during stimulation of the lateral olfactory tract with bipolar twisted-wire silver electrodes (51, 52).

Evaluation of BBB Permeability

The morphological and functional integrity of the BBB in the *in vitro* isolated guinea pig brain preparation has been previously demonstrated (47). We assessed the presence of BBB breakdown in isolated brains by perfusing fluorescein-isothiocyanate (FITC)–albumin (50 mg/10 ml, Sigma-Aldrich, Italy; $n = 9$) for 4 min immediately before the brains were fixed for histologic analysis. Control brains ($n = 4$) were maintained *in vitro* for a comparable time as experimental brains. The brief FITC-alb perfusion at the end of the experiment was utilized as a fluorescent marker of protein extravasation; the prolonged non-fluorescent h-ALB perfusion was used to enhance tissue excitability.

Immunohistochemistry

At the end of the electrophysiological experiments, brains were removed from the recording chamber and were fixed by immersion into 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1M, pH 7.4) for at least 24 h; 50 μm thick coronal sections were cut by vibratome (VT 1000S Leica, Heidelberg, Germany) throughout the extension of the hippocampus (plates A5.4–A7.4 of guinea pig brain atlas by Luparello). Sections collected on gelatin-coated slides were mounted in Fluorosave (Calbiochem, San Diego, CA, U.S.A.), and were cover-slipped. Two sections corresponding to plates A5.40

and A5.76 were collected for each brain to assess the intraluminal vs. extravascular FITC–albumin fluorescein signal. Slide-mounted sections were examined with a laser scanning confocal microscope using excitation of 488 nm (Laser Ar). Quantification of parenchymal FITC–albumin was performed in the hippocampal formation. In each brain, three high -power non-overlapping fields per section were acquired bilaterally at 10x magnification. Laser intensity was set at 30–35% power. Gain and photomultiplier were kept constant during acquisition of all images. As index of BBB damage, the area (number of pixels) occupied by the extravascular parenchymal FITC signal was quantified. Data obtained were used for statistical analysis. Values for experimental groups were expressed as percentage of the mean leakage area in the control group (defined as 100%). A standardized protocol was used for histochemical staining: in short, after endogenous peroxidase inactivation (3% H_2O_2 in PBS) and non-specific antigen binding sites blocking (1% BSA/0.2% Triton-X 100 in PBS), free-floating sections were incubated overnight at 4°C with the desired primary antibody in 0.1% BSA/0.2% Triton-X 100 at 4°C. On the subsequent day, sections were incubated for 75 min in the correspondent secondary antibody diluted in 0.1% BSA. Tissue was washed in PBS 3 times and then rinsed, mounted, dehydrated, and cover-slipped with fluorsave (Calbiochem, San Diego, CA, USA). For tridimensional reconstruction of microglial cells, immunofluorescence for ionized calcium-binding adapter molecule 1 (Iba-1 1:200 – Merck- Millipore, Darmstadt, Germany) and DAPI (1:5,000) conjugated with cy3 (1:600 – Neomarker-Invitrogen, Fremont, CA, USA) was performed. Regarding astrocytes, polyclonal rabbit anti-glial fibrillary acid protein (GFAP 1:500 - DAKO, Glostrup, Denmark) counterstained with DAPI (1:5,000) and coupled with alexa 594 (1:500 – Neomarker-Invitrogen, Fremont, CA, USA) was used.

Morphometric Analysis of Glial Cells

For tridimensional reconstruction of glia, two coronal sections *per* animal were stained for Iba-1 and DAPI (cell nuclei) for microglial cells or GFAP and DAPI for astrocytes, as described before. Sections were visualized using a Leica SP8 Confocal (Leica Microsystems, Germany), applying LASX software (version 3.1.5.1). Previews of the whole section in widefield (10X/0.3 dry) using the DAPI channel were taken to choose areas of interest in the ventral CA1 *stratum radiatum,* that was further acquired at a higher resolution with the confocal mode. Two channel (Iba-1/GFAP and DAPI) Z-stack images (Z-step intervals of 0.3 μm) were acquired using a 63X/1.4 oil objective and a DFC365 FX CCD Camera (Leica) with a x-y sampling of 72 nm. Cells were eligible for reconstruction if the following criteria were met: (i) the Iba-1/GFAP positive cell coincided with a single DAPI-stained nucleus; (ii) the cell did not present truncated processes; (iii) the cell could be singled out from neighboring cells to ensure correct reconstruction. A total of 75 cells (5 ROIs *per* animal) were selected for reconstruction performed using simple neurite tracer plugin available in FIJI-ImageJ software (v2.0.0), an open-source tool previously described to effectively assess tridimensional morphology of neurons and glial cells (53). Glia morphometric properties were evaluated by quantifying the number of processes, total length (in μm), sum of intersections; Sholl analysis was also performed to identify the number of intersections at radial intervals of 2 μm starting from the central point of the soma, as a measure of the complexity of glial cells ramifications and branches.

Statistical Analysis

Quantitative results were analyzed using Student *t* and Mann–Whitney tests and ANOVA. The normal distribution of samples was checked with Shapiro–Wilks test and the homogeneity of variances was evaluated with *F* test. When the equal variance criterion was violated, the Welch correction was used. The Mann–Whitney non-parametric test was chosen when data were not normally distributed. Otherwise, Student *t*-test was used. All statistical tests were performed in Origin 9.0 (OriginLab Corporation, Northampton, MA, USA), except the morphology experiments for which statistical analysis was performed using Prism 8.2 (GraphPad Software Inc., San Francisco, CA, USA). The format of Student *t*-test results is: $t(\mathrm{df}) = t$ statistic, p significance value. The format of Mann–Whitney test results is: $U(\mathrm{n1}, \mathrm{n2}) = \mathrm{x}$, $p \leq$ significance value. The format for ANOVA test was $F(\mathrm{df}) = F$, p significance value. The tests are two-sided and confidence interval was set at 95% (0.95) so that the difference between means was considered statistically significant at *p*-values of <5% (0.05), 1% (0.01), and 0.1% (0.001). Data are shown as mean ± standard deviation (SD).

RESULTS

Experiments were performed in 15 isolated guinea pig brains. Control condition brains were maintained *in vitro* with control solution for 4 h before perfusing FITC-alb (**Figure 1A**). The reasoning behind the 4 h timeline was due to technical issues. The isolated *in vitro* brain takes 90 min (0.2°C/ min) to reach 32°C, which is the optimal temperature for the experiments to be carried on. Subsequently, LOT-evoked potentials were induced to verify the viability of the preparation throughout the experiment and assess the position of depth electrodes. The infusion of the first bolus of bicucculline followed. A second perfusion of bicuculline was applied 90 min after the first and the recording were carried on for 30 min. In the end, the brain was perfused for 4 min with FITC albumin. As expected, no SLEs were observed in control experiments ($n = 4$). SLEs were induced by arterial perfusion of BMI (50 μM) for 3 min. The first BMI application evoked a 13.5 ± 2.6 min SLEs in the limbic region, recorded in the hippocampus (area CA1; left trace in **Figure 2A**) and in the mEC. A second BMI perfusion applied 90 min after the first one (**Figure 1B**) induced SLEs lasting 12.1 ± 3.4 min ($n = 4$; see **Figure 2C**). This protocol induced significant brain extravasation of FITC-albumin compared to control animals (**Figures 3A,B**; $t(18) = -5, 5$; $p < 0.001$ with two samples Student *t*-test). The increase in BBB permeability induced by a first SLE allowed extravasation of later perfused FITC-alb (49). Therefore, we perfused 4 g/L h-ALB via the basilar artery for 30 min immediately after the occurrence of the first BMI-induced SLE ($n = 4$; **Figure 1C**) and 60 min before the second BMI bolus ($n = 4$; **Figure 1D**). The perfusion of h-ALB increased both SLE

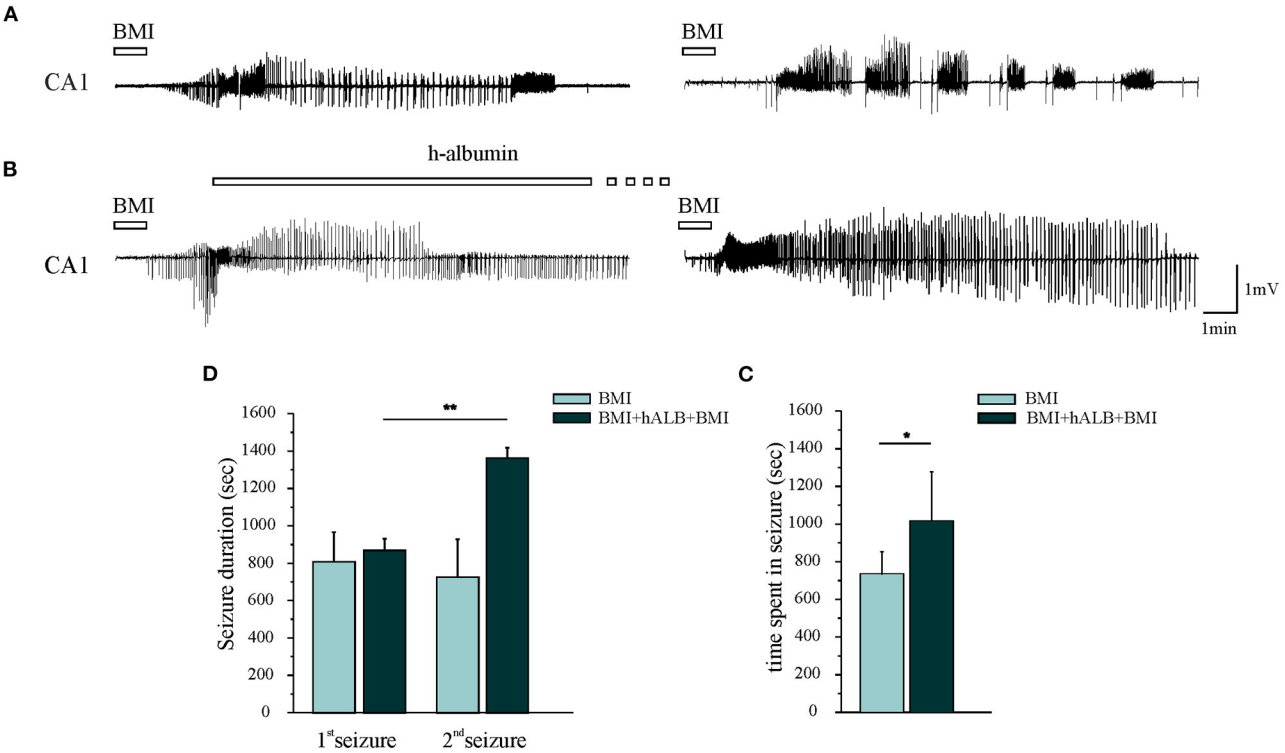

FIGURE 2 | Epileptiform discharges recorded in CA1. **(A)** The first and the second 3-min application of BMI induced one seizure-like event (SLE). **(B)** 30-min h-recombinant albumin (h-ALB) perfusion after the first BMI-induced SLE (left trace) significantly increased the duration of the second BMI-induced SLE (right trace). **(C)** Mean SLE durations induced by the first (1st SLE) and the second application of BMI with and without h-recombinant albumin application (2nd SLE). **(D)** Total time spent in seizure after the application of the experimental protocols B and C, respectively. *$p < 0.05$; **$p < 0.01$ with Student *t*-test.

duration induced by the second BMI perfusion (**Figures 2B,C**; 22.7 ± 0.9 min; t(5) = −4, 2; $p < 0.01$ with two sample student *t*-test) and the total time spent in SLE (**Figure 2D**; $t(12) = -2, 4$; $p < 0.05$ with two sample student *t*-test) compared to the experiments without h-ALB perfusion between the two BMI tests. As expected, the extent of BBB leakage, assessed by measuring the area of FITC-alb extravasation, was up to 3-fold larger after BMI + hALB + BMI perfusion compared to BMI only (**Figures 3A,B**; $F(2) = 60$; $p < 0.001$ with ANOVA). Also, the extent of BBB leakage induced by application of BMI +hALB was lower compared to BMI+hALB+BMI (**Figures 3A,C**; $t(23) = 2, 3$; $p < 0.05$ with two samples Student *t*-test).

To exclude unspecific effects, in 2 experiments h-ALB was perfused via the basilar artery for 30 min without BMI. H-ALB perfusion alone was unable to spontaneously evoke ictal discharge (data not shown). Afterwards, we evaluated the influence of SLE activity either alone (BMI) or in combination with h-ALB (BMI + hALB) on the reactive state of GFAP immunostained astrocytes (**Figure 4**) and IBA-1 stained microglial cells (**Figure 5**) analyzed in the CA1 hippocampal field, where epileptiform activity was recorded. In order to better investigate the role of serum albumin on glial dysfunction and BBB damage, in a separate set of experiments we also studied the effect of h-ALB after the first BMI-induced SLE. In this case, at the end of the h-ALB treatment, the isolated brain was perfused with perfusion solution until the end of the experiment ($n = 3$). Sholl analysis was used to quantify the number of intersections at radial intervals of 2 μm starting from the soma of glial cells (**Figures 4B,C**, **5B,C**). As summarized in **Figure 4C**, the number of intersections counted in CA1 astrocytes from guinea pig brains submitted to BMI, BMI + hALB and BMI + hALB +BMI was higher than control brains ($F(3) = 115.9$; $p < 0.001$ with ANOVA). Representative astroglia typical of the four experimental conditions are illustrated in **Figure 4A**. Furthermore, astrocytes had a higher number of processes (**Figure 4D**), total length of their processes (**Figure 4E**), and sum of intersections in Sholl analysis (**Figure 4F**) in BMI, BMI + hALB, and BMI + hALB + BMI in comparison to CT animals ($F(3) = 65.18$, $F(3) = 119.3$, and $F(3) = 115.9$, respectively; $p < 0.001$ with ANOVA). Interestingly, there was a consistent increase in all three parameters when comparing BMI against BMI + hALB + BMI (**Figures 4D–F**), indicating that the astrocitic morphological changes that occured in the presence of SLEs only (BMI) worsened in the BMI + hALB + BMI protocol.

Lastly, microglial morphology was also assessed (**Figure 5**) using the same methodology employed for astrocyte reconstruction. Sholl analysis revealed that microglia in BMI, BMI + hALB, BMI + hALB + BMI animals had a lower number of intersections when compared to control animals ($F(3) = 99.2$; $p < 0.001$ with ANOVA; **Figures 5B,C** – representative

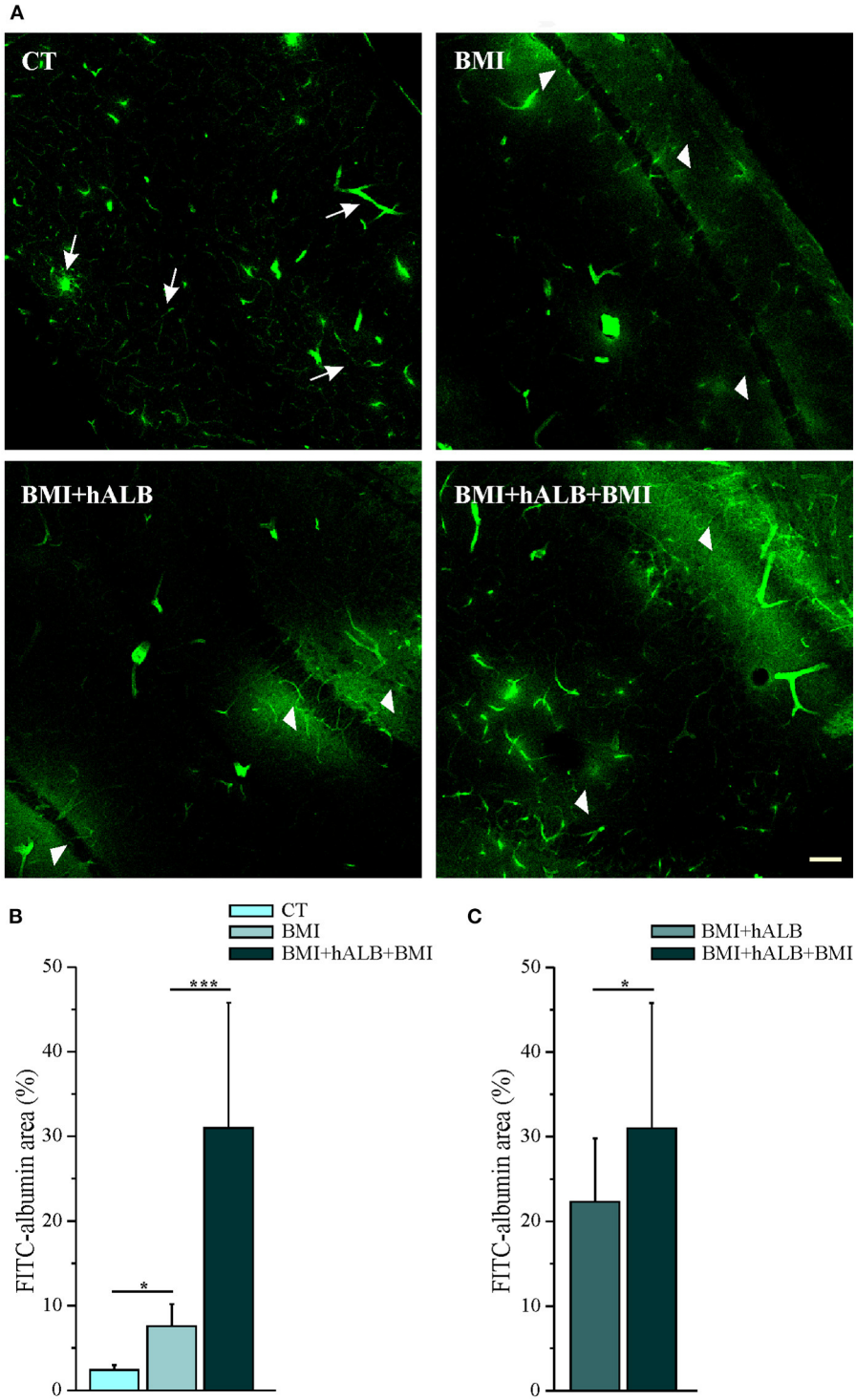

FIGURE 3 | Quantification of parenchymal Fluorescein isothiocyanate (FITC)–albumin leakage. **(A)** Representative photomicrographs of intraparenchymal FITC–albumin signal in the limbic area in control solution (top, left), BMI (top, right), BMI + h-ALB (bottom, left), and BMI + h-ALB + BMI (bottom, right) treated brains. Control sections show intraluminal signal with scattered perivascular spots (white arrow). Areas of FITC–albumin parenchymal extravasation around vessels (white arrowheads) after the second pulse of BMI alone or co-perfused with h-recombinant albumin, showed as FITC–albumin parenchymal leakage is broader after the application of protocol C and D (see **Figure 1**). **(B,C)** Quantification of parenchymal FITC–albumin leakage in the experimental conditions. FITC-albumin leakage has been evaluated as spot area (number of pixels) and it is expressed as percentage of values vs. control experiments $^{*}p < 0.05$; $^{***}p < 0.001$ with ANOVA and Student *t*-test. Calibration bar = 100 μm.

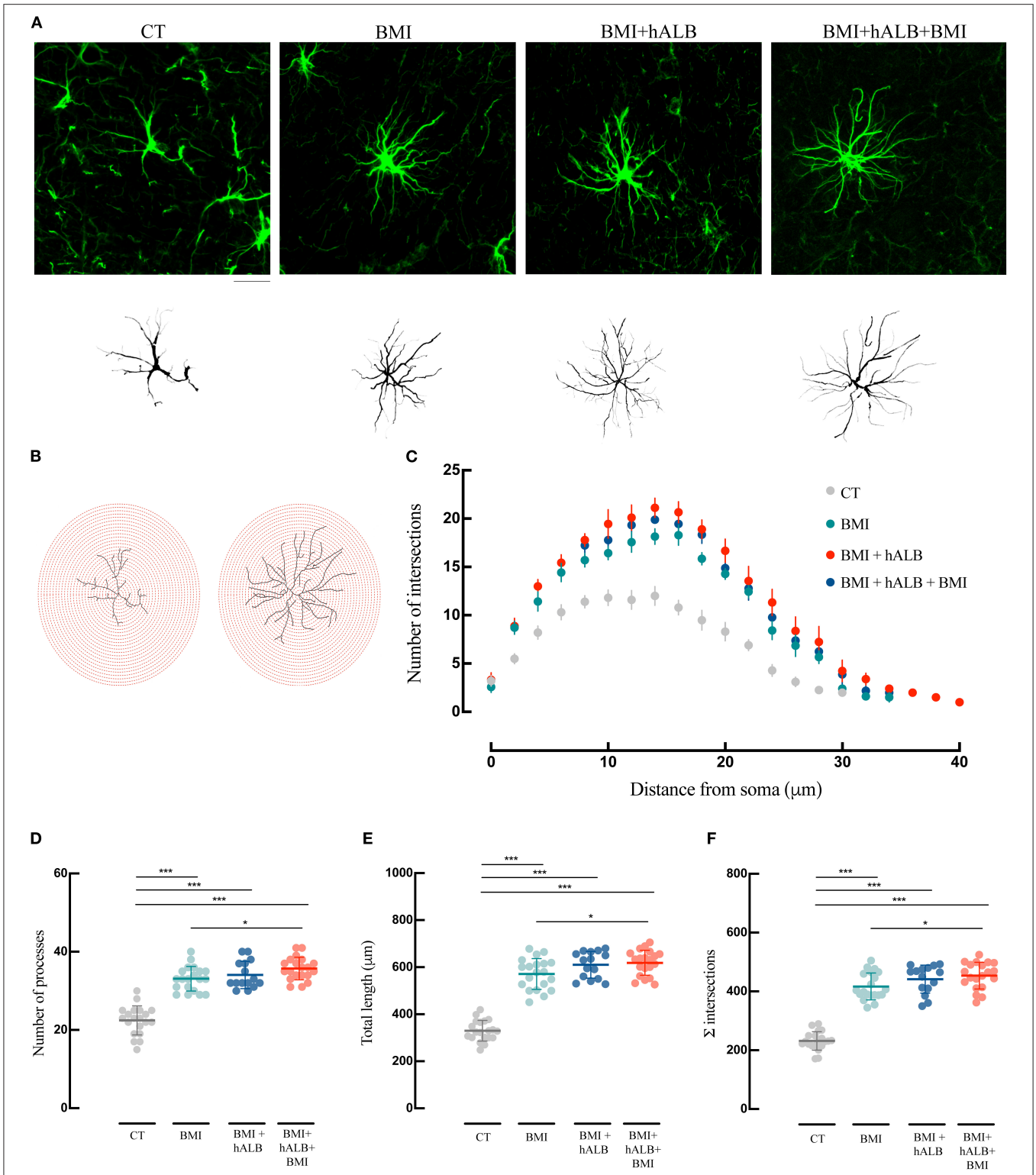

FIGURE 4 | Morphological analysis and reconstruction of astrocytes in ventral hippocampal CA1. **(A)** Representative morphologies of astrocytes in GFAP immunofluorescence coronal sections of the ventral CA1 hippocampal region are shown for CT, BMI, BMI+hALB, and BMI+hALB+BMI; the correspondent reconstruction of the astrocyte is illustrated in the lower part of the panel. Calibration bar = 20 μm. **(B)** Representative Sholl analysis setting of a manually reconstructed astrocyte from CT (left) and BMI+hALB+BMI (right). The circles centered around the soma are separated by radial intervals of 2 μm. **(C)** Number of intersections *per* 2 μm radius plotted against the distance from the cell soma in CT, BMI, BMI+hALB, and BMI+hALB+BMI animals. **(D)** Number of processes *per* 2 μm radius of astrocytes in CT ($n = 20$ cells), BMI ($n = 20$ cells), BMI+hALB ($n = 15$ cells), and BMI+hALB+BMI ($n = 20$ cells) animals. **(E)** Total length in μm of

(Continued)

FIGURE 4 | astrocytes in CT ($n = 20$ cells), BMI ($n = 20$ cells), BMI+hALB ($n = 15$ cells), and BMI+hALB+BMI ($n = 20$ cells) guinea pigs. **(F)** Sum of intersections of astrocytic cells CT ($n = 20$ cells), BMI ($n = 20$ cells), BMI+hALB ($n = 15$ cells), and BMI+hALB+BMI ($n = 20$ cells) animal groups. *$p < 0.05$; ***$p < 0.001$ with ANOVA test. All experiments were done in 5 cells *per* animal.

panel in **Figure 5A**). Moreover, microglia consistently had lower number of processes (**Figure 5D**), total length of their processes (**Figure 5E**) and sum of Sholl-analysis intersections (**Figure 5F**) in BMI, BMI + hALB, and BMI + hALB + BMI animal cohorts, in comparison to control brains ($F(3) = 71.18$, $F(3) = 225.9$ and $F(3) = 99.2$, respectively; $p < 0.001$ with ANOVA). Similar to astrocytes, when BMI was coupled with h-ALB + BMI, the morphology of microglia had a more activated phenotype when compared to BMI alone (**Figures 5D–F**). Additionally, comparing microglia cells that had the same seizure profile (single seizure induced by BMI), cells that were perfused with hALB as well had a higher gliosis-like phenotype when compared to BMI only protocol (BMI vs. BMI + hALB in **Figures 5D–F**). In sum, our data demonstrate a worsening of the astro and microgliosis state when major seizure activity is in coallition with h-ALB extravasation into the brain. However, h-ALB by itself is also able to induce major gliosios (to a less extent than the previous mentioned protocol).

DISCUSSION

A growing body of evidence supports gliosis as a primary factor in the pathogenesis of neurological diseases (54, 55). Data from human and animal studies support the notion that glial cells contribute to the control of neuronal function under both physiological and pathological conditions (11, 12, 56) and respond to changes in normal physiology of the CNS by establishing and coordinating response to disease resulting in gliosis (57). Recent evidences from experimental models of epilepsy and drug-resistant forms of human epilepsy suggest that epilepsy is often accompanied by astrocytes and microglia phenotypic and functional alterations (12, 21, 58).

We previously demonstrated that pharmacologically-evoked SLEs in the *in vitro* guinea pig brain induce IL-1β expression in perivascular astrocytes and compromise BBB permeability (44, 49). Our data confirmed that serum albumin entering into the brain through an impaired BBB contributed to the generation of sustained epileptiform activity (49). In the present study we investigated the role of serum albumin extravasation into brain parenchyma following seizure-induced BBB damage in enhancing reactive gliosis without the contribution of any blood-borne molecules/cells, since our guinea pig brain preparation is maintained in isolation. The BBB is involved in almost all pathologies of the CNS (59–61). Its alterations can compromise the fundamental processes which govern brain functions. Serum albumin extravasation into brain parenchyma following BBB integrity loss is reported to lead to glial activation and alterations in the extracellular milieu around neurons (8, 62). Normal brain albumin concentration is much lower (35–50 microg/mL) than blood albumin concentration, that ranges from 35 to 50 mg/mL (63, 64). Thus, BBB opening has the potential to expose brain cells to high levels of albumin (65). The contribution of serum albumin in astrocytes activation is supported by several studies showing induction of calcium signaling and DNA synthesis in astrocytes (66, 67). One pivotal mechanism involved in these effects is the albumin-mediated activation of the *transforming growth factor beta receptor II* (TGF-βR); recent studies demonstrated that serum albumin leaks into brain parenchyma through a dysfunctional BBB to bind astrocytic TGF-βR activating TGF-β signaling (8, 9, 68, 69). This cascade of events leads to astrocytes Kir4.1 downregulation and to their consequent failure to buffer extracellular K^+ and glutamate, that culminates in the synthesis of inflammatory molecules and increase brain excitability (9, 56). Accordingly, blockade of Kir4.1 in glia with cesium has been demonstrated to promote seizure like activity (70). Furthermore, activation of TGF-β signaling by albumin induced rapid and persistent up-regulation of genes related to inflammation (9). BBB impairment also easily allows microglia to be exposed to high concentrations of albumin. Even though the effects of albumin on cells in the brain have mainly been investigated in astrocytes, several studies support the pathological role of microglial activation by albumin (63, 69, 71). Since albumin can activate microglia, which in turn can activate astrocytes and exacerbate reactive pathways (40, 41), it is of the upmost interest to understand which signaling cascades are activated in microglia exposed to serum albumin after BBB damage. Hooper and colleagues demonstrated that microglia respond to serum albumin by increasing intracellular calcium *via* Src tyrosine kinases, which successively leads to glutamate and TNF-α release (63, 65).

In our experiments, the concentration of albumin perfused in the arterial system of the *in vitro* guinea pig brain (4 mg/ml) falls within the range associated with BBB damage occurring in a pathology associated condition (65). The changes observed in our acute ictogenic model confirmed that astro- and microglial cells promptly respond to seizure activity. BMI-evoked SLE determined changes in astrocytic and microglial morphological phenotype toward a more activated state. SLEs-induced microglia adopts an amoeboid shape, starting from a ramified structure in the control brains (72, 73), while astrocytes express a hypertrophic phenotype with longer processes compared to control condition (74). Interestingly, seizure pattern, duration and astro- and microgliosis were exacerbated when SLE activity was combined with the perfusion of h-albumin. Our data support the hypothesis that albumin increases SLE activity in limbic areas by directly inducing a reactive state in both astrocytes and microglia. Whether acute gliosis represents an early possible defensive mechanism triggered by seizure activity or their activation is actively involved in the epileptogenic process cannot be answered in our acute experimental conditions. However, *in vivo* studies performed in our laboratory in the intrahippocampal

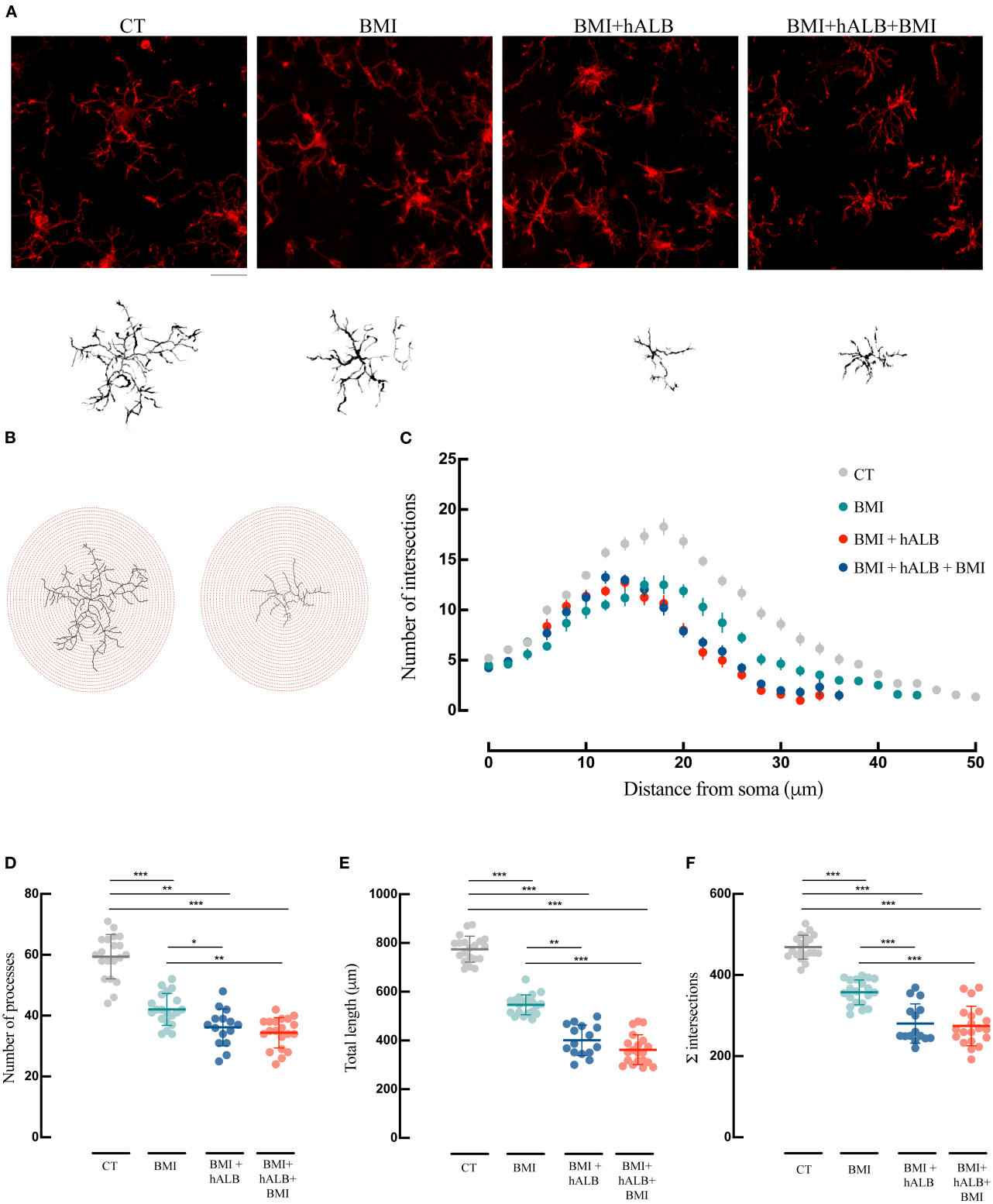

FIGURE 5 | Morphological analysis and reconstruction of microglia in ventral hippocampal CA1. **(A)** Representative morphologies of microglial cells in Iba-1 immunofluorescence coronal sections of the ventral CA1 hippocampal region are represented for CT, BMI, BMI+hALB, and BMI+hALB+BMI; the correspondent reconstruction of the microglial cell is illustrated in the bottom part of the panel. Calibration bar = 20 μm. **(B)** Representative Sholl analysis setting of a manually reconstructed microglia cell from CT (left) and BMI+hALB+BMI (right). The circles centered around the soma are separated by radial intervals of 2 μm. **(C)** Number of

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FIGURE 5 | intersections *per* 2 μm radius plotted against the distance from the cell soma in CT, BMI, BMI+hALB, and BMI+hALB+BMI animals. **(D)** Number of processes *per* 2 μm radius of microglia cells in CT ($n = 20$ cells), BMI ($n = 20$ cells), BMI+hALB ($n = 15$ cells), and BMI+hALB+BMI ($n = 20$ cells) guinea pigs. **(E)** Total length in μm of microglia cells in CT ($n = 20$ cells), BMI ($n = 20$ cells), BMI+hALB ($n = 15$ cells), and BMI+hALB+BMI ($n = 20$ cells) animal groups. **(F)** Sum of intersections of microglia cells in CT ($n = 20$ cells), BMI ($n = 20$ cells), BMI+hALB ($n = 15$ cells) and BMI+hALB+BMI ($n = 20$ cells) animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ with ANOVA test. All experiments were done in 5 cells *per* animal.

kainic acid (KA) model suggest that seizures, gliosis and BBB damage contribute to epileptogenesis at the site of kainic acid injection, but not in regions remote from the injection site. Even though gliosis was still present at an early phase and seizure activity was present in both regions, no detrimental markers of brain damage were detected (75). In the same animal model, genes associated with inflammatory response (IL1-β and COX-2), brain activity (c-FOS) and oxidative stress (HO-1) were early upregulated exclusively in the KA-injected hippocampus during the acute phase and remained upregulated 1 month post-KA injection. Interestingly, only genes linked to glial function (AQP4 and Kir4.1) were upregulated 3 days post-KA (but not after 1 month) in regions remote from the kainic acid injection site that also generated epileptiform discharges. In these regions late damage did not develop (Vila Verde in press on Neurophatolo Appl Neurobiol). It can be hypothesized that early after seizure occurrence transient gliosis could helping neurons to cope seizure activity preventing neuronal damage, whereas in regions in which seizures are coupled with the excitotoxic effects of kainic acid, persistent gliosis may have nefarious effects to the brain. It can therefore be expeculated that, early after seizure occurrence, transient gliosis may help neurons cope with seizure activity preventing neuronal damage development, whereas in regions in which seizures are coupled with the excitotoxic effects of kainic acid, persistent gliosis induces permanent nefarious effects in the brain.

In conclusion, the present study reinforces our previous observation that in an *in vitro* acute model of ictogenesis seizure activity *per se* enhances BBB permeability in brain regions involved in seizure generation and that extravasation of albumin into brain parenchyma increases seizure activity in those regions affected by BBB impairment (44, 49). We demonstrate for the first time simultaneous morphological phenotype changes in both astrocytes and microglia due to seizure activity. Our data strongly suggest seizure-induced BBB breakdown and the consequent albumin extravasation leads to astrocytes and microglia reactivity and eventually to reinforce seizure activity by increasing its duration. Further studies are required to recognize when astro- and microgliosis might help or harm the brain in our experimental conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Organismo Preposto al Benessere Animale - OPBA Fondazione Istituto Neurologico C. Besta Via Celoria 11 20133 Milano.

AUTHOR CONTRIBUTIONS

DV and LL: conception, design of the study, acquisition, and analysis of data. DV, LL, and MC: drafting a significant portion of the manuscript or figures. All authors contributed to the article and approved the submitted version.

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Multiple Disruptions of Glial-Neuronal Networks in Epileptogenesis That Follows Prolonged Febrile Seizures

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Background and Rationale: Bi-directional neuronal-glial communication is a critical mediator of normal brain function and is disrupted in the epileptic brain. The potential role of aberrant microglia and astrocyte function during epileptogenesis is important because the mediators involved provide tangible targets for intervention and prevention of epilepsy. Glial activation is intrinsically involved in the generation of childhood febrile seizures (FS), and prolonged FS (febrile status epilepticus, FSE) antecede a proportion of adult temporal lobe epilepsy (TLE). Because TLE is often refractory to treatment and accompanied by significant memory and emotional difficulties, we probed the role of disruptions of glial-neuronal networks in the epileptogenesis that follows experimental FSE (eFSE).

Methods: We performed a multi-pronged examination of neuronal-glia communication and the resulting activation of molecular signaling cascades in these cell types following eFSE in immature mice and rats. Specifically, we examined pathways involving cytokines, microRNAs, high mobility group B-1 (HMGB1) and the prostaglandin E2 signaling. We aimed to block epileptogenesis using network-specific interventions as well as *via* a global anti-inflammatory approach using dexamethasone.

Results: (A) eFSE elicited a strong inflammatory response with rapid and sustained upregulation of pro-inflammatory cytokines. (B) Within minutes of the end of the eFSE, HMGB1 translocated from neuronal nuclei to dendrites, *en route* to the extracellular space and glial Toll-like receptors. Administration of an HMGB1 blocker to eFSE rat pups did not decrease expression of downstream inflammatory cascades and led to unacceptable side effects. (C) Prolonged seizure-like activity caused overall microRNA-124 (miR-124) levels to plunge in hippocampus and release of this microRNA from neurons *via* extra-cellular vesicles. (D) Within hours of eFSE, structural astrocyte and microglia activation was associated not only with cytokine production, but also with activation of the PGE_2 cascade. However, administration of TG6-10-1, a blocker of the PGE_2 receptor EP2 had little effect on spike-series provoked by eFSE. (E) In contrast to the failure of selective interventions, a 3-day treatment of eFSE–experiencing rat pups with the broad anti-inflammatory drug dexamethasone attenuated eFSE-provoked pro-epileptogenic EEG changes.

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Conclusions: eFSE, a provoker of TLE-like epilepsy in rodents leads to multiple and rapid disruptions of interconnected glial-neuronal networks, with a likely important role in epileptogenesis. The intricate, cell-specific and homeostatic interplays among these networks constitute a serious challenge to effective selective interventions that aim to prevent epilepsy. In contrast, a broad suppression of glial-neuronal dysfunction holds promise for mitigating FSE-induced hyperexcitability and epileptogenesis in experimental models and in humans.

Keywords: epilepsy, microRNA, microglia, astrocyte, cytokines, neuroinflammation, high mobility group box 1, prostaglandins

INTRODUCTION

Febrile seizures (FS) are the most common seizure type in infants and young children and prolonged FS [febrile status epilepticus (FSE)] is associated with an increased risk of epilepsy in later life (1–6). The effects of FSE on neuronal structure and function are manifold and include persistent aberrant expression of critical neuronal genes, altered dendritic complexity and the development of abnormal excitatory synapses (7–11). However, epileptogenesis following FSE is complex. Genetic factors play a critical role in determining susceptibility to FSE and progression of epileptogenesis following FSE (3, 12–14). Mechanistically, glial cells are activated by prolonged FS and their release of inflammatory mediators may contribute to the pathogenesis of epilepsy (15, 16). Indeed, pre-clinical models of prolonged FS have identified rapid and persistent microglial and astrocyte activation following FSE and persistent upregulation of pro-inflammatory molecules including cytokines and prostaglandins (17–22).

The neuronal and glial response to human and experimental FSE and subsequent contribution to epileptogenesis are likely interdependent and involve a three-way neuronal-microglial-astroglial communication, activation and feedback (23, 24). Neuronal-glial cross-talk can take place in many ways and influence function accordingly (25–29). Recently, miRNAs were identified as strong candidate mediators of the communication among a number of glial cell types and neurons: miRNAs are abundant components of extra-cellular vesicles (ECVs), which upon generation in one cell type are released and can traverse the extracellular space and enter cells of other types (30–32). This type of intercellular communication takes place bidirectionally between neurons and glia (both microglia and astrocytes) and also between microglia and astrocytes (30–34). ECV miRNA content was recently profiled during epileptogenesis and found to differ significantly in mouse brain following prolonged seizures (35–37). Once miRNA containing ECVs dissolve and release their cargo, miRNAs may influence the cellular machinery of their new host cell (38–40). Specifically, because miRNA modulate protein translation by repressing target mRNAs, they can provoke large scale changes in the repertoire of genes translated. In the context of epilepsy and epileptogenesis, the cellular origin of ECVs released during epileptogenesis and how they influence the function of recipient cells remains unknown.

miRNA transported *via* ECV are just one of a host of communication networks among neurons and glia that are initiated by epilepsy-inciting events including FSE. FSE rapidly initiates the subcellular translocation of the damage-associated molecular pattern (DAMP) molecule HMGB1 and subsequent cellular release from neurons which may influence inflammatory mediators on surrounding cells including Toll-Like receptors (TLRs) and RAGE receptors (16, 41, 42). The translocation of HMGB1 from the nucleus to the dendritic compartment of cells occurs rapidly following eFSE (43). This release and interaction may activate microglia and initiate large-scale inflammatory responses (20, 41, 43–47). Structural and molecular changes in microglia occur rapidly and persist throughout epileptogenesis following eFSE but how FSE induces the activated microglia phenotype remains elusive. However, the rapid neuronal DAMP molecular cascade represents an enticing target for intervention because it may initiate microglial and astrocytic activation and inflammatory consequences.

A number of cytokines have been shown to be involved in both human FSE (15) and experimental FSE and its consequences. These include interferon (48), Il-1β (49, 50) and others. In other types of prolonged seizures, prostaglandin and specifically EP2 have been implicated (51–53). Yet, the effects of FSE and other epilepsy-inciting events on neuronal-glial communication remain understudied, and which lines of communication fail or are enhanced during epilepsy development is unclear. A greater understanding of neuron-glial communication is critical because the mediators represent potential therapeutic targets. In the present study we use a multi-pronged approach to interrogate several neuronal-glial interactions and determine how they are affected by eFSE. We then tested whether intervention in these processes ameliorates eFSE-related development of spontaneous seizure development (epileptogenesis).

METHODS

Experimental Overview

The goal of the study was to identify the mechanisms by which eFSE triggers activation of glial cells in the brain and whether these signals originate in neurons. We also sought to explain the role of inflammation in the epileptogenesis that follows eFSE and whether targeting inflammation (either *via* targeted or global mechanisms) can block or ameliorate the subsequent epilepsy

(**Figure 1**). To achieve these goals, we used a combination of *in vivo* models of FSE as well as *in vitro* hippocampal slice cultures in which we induce seizure-like events. Four experiments were conducted.

Experiment 1 characterized the inflammatory cascade elicited by eFSE at a number of important timepoints using qPCR quantification. We also measured and demonstrate the rapid subcellular translocation of the HMGB1 signaling protein which provokes microglial activation by binding to surface TLRs and RAGE receptors using immunohistochemical methods. Finally, in experiment 1 we examined whether blocking HMGB1 interaction with TLRs can reduce eFSE evoked inflammation.

Experiment 2 used organotypic hippocampal slice cultures to examine the mechanism by which the neuronal-specific miR-124, mediates microglial activation following prolonged neuronal activity. To determine whether miR-124 is released from neurons we measured the levels of miR-124 in culture media following seizure-like events using qPCR quantification. We also isolated ECVs and measured the levels of encapsulated miR-124 following seizure-like events to understand whether neuronal release is facilitated *via* ECVs.

Experiment 3 examined the effect of *post-hoc* EP2 receptor antagonism using TG6-10-1 (an EP2 receptor antagonist) on spike series development. eFSE rats [postnatal day (P)10-11] received three doses of either vehicle or TG6-10-1 at 4, 22, and 30 h post eFSE. EEG electrodes were implanted on P21 and recording began 4 days later following recovery from surgery. Recording was performed over 60 days.

Experiment 4 examined the effects of DEX on microglia and astrocyte activation and number and on the development of spike series (a measure of aberrant hippocampal excitability). Immediately following eFSE cessation, control and eFSE rats received injection of either vehicle or DEX. Hippocampal microglial activation and number were assessed using P2Y12 staining while astrocytes were identified using GFAP as a marker. As before, EEG was recorded from P21 over the course of 60 days to identify the emergence, frequency, and duration of spike series.

Animals

Male Sprague Dawley rats were used for both *in vivo* and *in vitro* experiments. All experiments were performed with approval from the institutional animal care committee and conformed to National Institute of Health guidelines. Group sizes were determined *a priori* and animals were randomly assigned to experimental groups.

Prolonged Febrile Seizure Induction

Experimental febrile status epilepticus (eFSE) was induced in male Sprague Dawley rats on post-natal day 10 or 11 (P10-11) as previously described (22, 50, 54). Briefly on P10-11 rats were weighed and pre-procedure weight was recorded. To prevent hyperthermia-related burns a thin wooden splint was attached to the tail and wrapped with a gauze. Ears and paws were then covered with a hydrating glycerin-based ointment. In pairs, rats were then placed in a 3 L flask and a warm stream of air was directed at them from a fixed height to elevate core body temperature gradually. The beginning of seizure was recorded as the onset of freezing behavior followed by chewing automatisms. Upon seizure onset a delicate rectal thermometer probe was used to measure core body temperature every 2 min to ensure body temperature remained between 39.5 and 41°C. Core body temperature was maintained at an elevated temperature for 60 min. Rats were then briefly immersed in room temperature water to restore normal body temperature and returned to home cages.

Treatment With HMGB1 Box A

To block the pro-inflammatory action of HMBG1 on TL and RAGE receptors, we gave a cohort of animals intracerebroventricular (ICV) infusions of HMGB1 Box A, a direct antagonist. There were four groups studied: Naïve Control (Naïve-Ctrl; no eFSE, no infusion; $n = 8$), Control Vehicle (Ctrl-Vehicle; $n = 5$), eFSE-Vehicle ($n = 7$), and eFSE HMGB1 Box A (eFSE-Box A, $n = 7$). The vehicle and HMGB1 Box A groups, immediately following eFSE, were given ICV injections of 2.5 μl of either vehicle (sterile 0.9% saline) or HMGB1 Box A (3 μg/μl in sterile normal saline, for 7.5 μg/hemisphere), ICV dose of HMGB1 Box A was administered to each hemisphere, and then pups were sacrificed and hippocampal and amygdalar tissue samples collected either 3 or 6 h after infusion to measure HMGB1 signaling cascades. A third experimental cohort was generated to examine the longer-term inflammatory effects of HMGB1 Box A treatment at 24 h after eFSE. This experiment was aborted because the majority of rats that received HMGB1 Box A treatment were found to have rectal bleeding the following day, a side effect which did not occur following eFSE alone or with ICV-vehicle treatment.

Treatment With TG6-10-1

TG6-10-1 was generously provided for this study by Professor Ray Dingledine and Emory University and used to block the Prostaglandin EPE2 Receptor. The TG6-10-1 was dissolved to a concentration of 0.25 mg/mL in the vehicle, a mixture of 10% dimethyl sulfoxide (DMSO) (molecular biology grade, Sigma-Aldrich, St. Louis, MO), 50% polyethylene glycol 400 (Sigma-Aldrich, St. Louis, MO), and 40% ultra-pure water (HyClone Water, ThermoFisher Scientific, Hampton, NH). All animals received 20 μl intraperitoneal injections of either the drug or the vehicle at 4, 22, and 30 h after the start of eFSE (Ctrl + Veh $n = 5$; eFSE + Veh $n = 7$; eFSE + drug $n = 9$).

Dexamethasone Administration

Immediately following eFSE cessation, rats randomized into the dexamethasone group (Ctl-DEX, eFSE-DEX) were given an intra-peritoneal (i.p.) injection of dexamethasone (3 mg/kg; Sigma-Aldrich). Aldosterone was also administered with dexamethasone *via* sub-cutaneous (s.c) injection (0.2 μg/100 mg; Acros Organics) to provide mineralocorticoid functional support (55). This dose had been shown to fully suppress inflammation and employed a short-tapered treatment course to avoid many of the side effects (56–58). Rats received tapering doses of DEX (together with aldosterone) over 48 h as follows: 1.5 mg/kg DEX 24 h after eFSE; 0.75 mg/kg DEX 48 h after eFSE. Aldosterone was continued for the next 4 days (7 treatments total), to provide

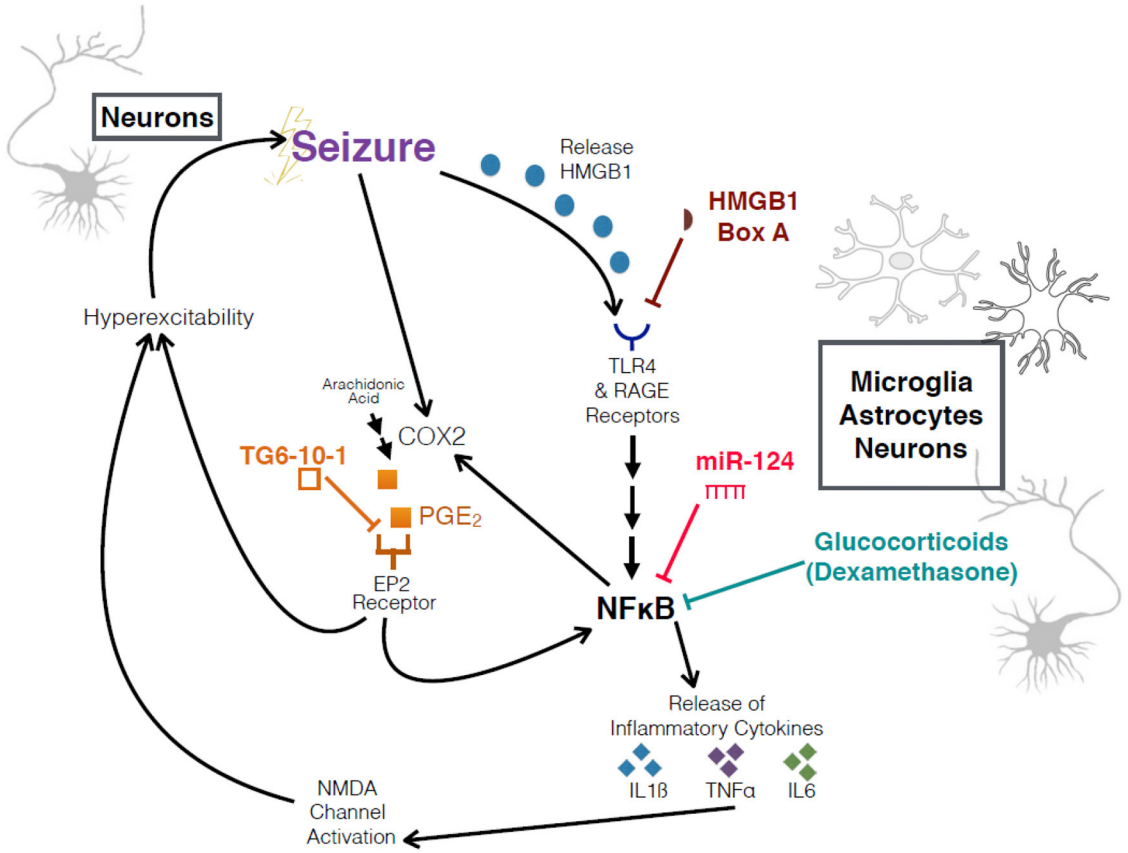

FIGURE 1 | Schematic of interrogated pathways and experimental interventions. Overview of relationship between seizures, HMGB1, COX2, the inflammatory cascade, and an increase in hyperexcitability. HMGB1 Box A and TG6-10-1 are both competitive antagonists at their respective receptors.

mineralocorticoid support during the potential suppression of the rats' adrenals. Vehicle controls consisted of i.p. saline. For the analyses reported here, group sizes were between 5 and 8.

Electroencephalogram (EEG) Recording and Analysis

Following eFSE and respective treatments, bilateral hippocampal electrodes to bilateral dorsal hippocampus were surgically implanted (AP: −2.2 mm, LR: ±1.9 mm, Depth: 2.7 mm from bregma). Four days after recovery from surgery, Video-EEG recording began, using a tethered system with synchronized video recording (PowerLab data acquisition hardware, Bio Amplifiers, and LabChart 7 and 8 software, AD Instruments). Rats were recorded on a rotating basis for up to 60 days, with each recording period lasting at least 48 h. After recording, the EEG recording data and videos were analyzed for aberrant spike series by experienced investigators without knowledge of treatment groups.

The criteria used to identify spike series from the EEG data were: (1) Spikes must be clearly detectable from the background, with peak duration between 20 and 70 ms. (2) The spikes must have a biphasic shape with an amplitude of each peak at least two times higher than the baseline. (3) The slow waves occurring before the peaks need to be stable without any exhibiting significant movements (i.e., normal grooming) that would produce excessive artifacts. (4) At least four adjacent spikes with stable baseline are required to form a single spike series. (5) Excluded artifact based on video recording when animal was eating or scratching.

Organotypic Hippocampal Slice Culture

Organotypic hippocampal slice cultures were prepared and maintained as previously described (59). Briefly we followed guidelines previously established using the interface method (60). Slices were prepared from rat pups on postnatal day 8 [Day *in vitro* 0 (DIV0)]. Rat pups were decapitated, brains removed, and hemispheres were separated. Three hundred micrometer thick slices were prepared on a McIlwain chopper and the hippocampus was dissected on ice cold prep media [MEM (Gibco), L-Glutamine (Gibco), Hepes Buffer (Fisher Scientific), Magnesium sulfate (Sigma), and cell culture grade water (GE Healthcare)] in a laminar flow hood. Slices were then maintained on 0.4 μm, 30 mm diameter cell culture inserts (Merck Millipore, Cork Ire) in six well plates with media containing MEM, HBSS

(Gibco), L-Glutamine, Magnesium sulfate, Sodium bicarbonate (Gibco), Hepes, heat inactivated horse serum (Gibco), ascorbic acid (Sigma) and cell culture grade water. Slices were maintained in a CO_2 enriched incubator (Thermo). On DIV 2 seizure-like activity was induced by transferring inserts to plates containing media supplemented with 6 μM KA (Abcam) and incubated for 3 h before being transferred to a new plate containing fresh media. Slices were harvested and media collected for analysis at 24 h post-seizure-like activity. Each well contained three slices, each from different pups. Slices from each well were pooled to make one sample. Each group contained three samples.

Exosome Purification

Exosomes were isolated from 1 ml of media using the ExoQuick kit according to the manufacturer's instructions with modifications for volume differences. Briefly, the collected media was centrifuged to pellet cellular debris. The supernatant was removed to a fresh tube and Exoquick solution was added. The tubes were inverted and then incubated at 4°C overnight. Exosomes were then pelleted by centrifugation. The supernatant was aspirated and the exosome containing pellet was resuspended in lysis buffer. Hundred percentage of ethanol was added to the resuspended exosomes and the sample was transferred to an Exoquick RNA spin column. Exosome-derived RNA binds to the column and was washed and purified further with ethanol-based washes. The column was then transferred to a collection tube and RNA was captured by passing elution buffer through the column. The eluted RNA quantity and quality was measured using a nanodrop and stored at −80°C until required for downstream analysis.

qPCR

Gene expression was measured as previously described (61). Using RNase free-dissection tools whole hippocampi were removed from rats and immediately stored at −80°C until use. Total RNA was isolated using the mirVana RNA isolation kit without small RNA enrichment according to the manufacturer's protocol. Quality and quantity of RNA was measured using a nanodrop with 260:280 and 260:230 values between 1.8 and 2.2 being considered acceptable for downstream analysis. cDNA libraries were then generated using a random hexamer reverse transcriptase approach. Analysis of the PCR product was performed using SYBR Green quantification on a Roche Lightcycler 96 well plate. Samples were analyzed in triplicate and normalized to a housekeeping gene using specific axon-spanning primers. For miRNA quantification and from exosomes specific stem loop primers were used for cDNA synthesis (ThermoScientific rno-miR-124-3p assay ID 001182). When comparing miRNA levels from exosomes miRNA levels were normalized to a spike in control. Quantification was performed using the ΔΔCt method.

Immunohistochemistry and Cell Counting

For all procedures, rats were deeply anesthetized with pentobarbital and transcardially perfused with 4% paraformaldehyde (PFA) at desired time points post eFSE. Brains were removed and post-fixed in 4% PFA for 90 min. Brains were then cryoprotected in 30% sucrose, rapidly frozen and stored at −80°C. Thirty-micron sections of dorsal hippocampus were obtained on a cryostat and stored in anti-freeze at 4°C until use. Serial sections were blocked in 10% normal goat serum and 0.03% Triton-x in 1x PBS for 1 h at 4°C. Primary antibodies were incubated in 4% Normal Goat Serum with 0.03% Triton-x overnight at 4°C. The following antibodies were used: rabbit anti-HMGB11:1,000 (Abcam), mouse anti-GFAP 1:3,000 (Millipore), mouse anti-IBA1 or P2Y12 1:4,000 (Wako). Sections were washed with 1x PBS and the reaction product was visualized using a 3,3′-diaminobezidine. Colocalization of cell markers with HMGB1 was achieved by co-incubating rabbit anti-HMGB1 1:1,000 (Abcam) with the following antibodies: mouse anti-NeuN (Chemicon), mouse anti-GFAP 1:3,000 (Millipore) and mouse anti-CD11b (ABD Serotec). After 24-h incubation, sections were washed in 1x PBS and then incubated in the appropriate secondary antibodies conjugated to Alexa Flour 568 or Alexa Flour 488. Colocalization was visualized using confocal microscopy.

For quantification of activated microglia and astrocytes, group sizes were 5–8. Assessment of cell numbers in hippocampal CA1, CA3 and hilus was achieved by boxing off 1,000 by 500 μM areas of each studied region. First, all P2Y12 or IBA1+ (or GFAP) positive cells in the defined region were counted and compared among groups. Activated microglia were identified as IBA1+ cells that had large, dark soma and few short processes that were thicker in appearance than non-activated microglia. Microglia activation is presented as the percent change in activated microglia over the total number of P2Y12 or IBA+ cells. Quantification of GFAP+ cells was achieved by using the same hippocampal regions and area delineations. GFAP+ cells were counted and compared among groups.

Group sizes for the HMGB1 translocation studies were three per group per timepoint. Two representative and matching section per animals were employed. HMGB1 quantification was accomplished by counting all HMGB1+ cells in a 100 by 500 μM region of CA1. Translocation of HMGB1 protein was identified by the presence of immunoreactivity outside the nucleus, i.e., in the somatic cytoplasm and in the processes of cells. Change in HMGB1 translocation is presented as the percent change in translocated cells over the total number of HMGB1+ cells.

Statistical Analyses

All analyses were performed without knowledge of treatment assignment. Comparisons of two groups were analyzed using standard *t*-test while analyses comprising more than two groups were performed using one- or two-way ANOVA. Outliers for all qPCR data were analyzed based on Rout Test Q = 5% and removed prior to the completion of any analysis. Statistical significance was set at less than or equal to 0.05.

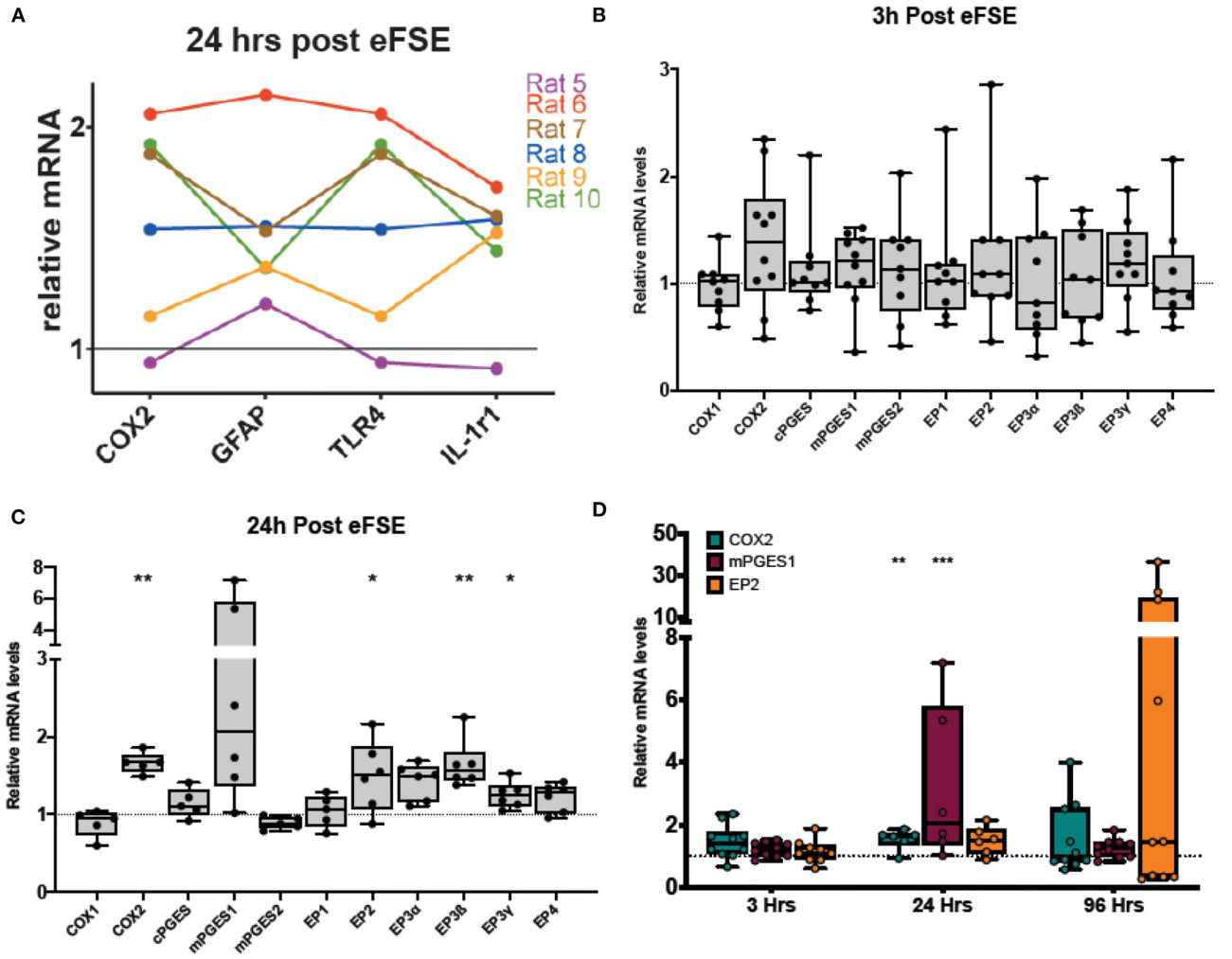

FIGURE 2 | Rapid and persistent activation of inflammatory cascades following eFSE. **(A)** Twenty-four hours after eFSE, COX2, GFAP, TNFα, and IL-1r1 levels were significantly increased at the mRNA level. When the expression profile of an individual rat is examined across these mediators, trends in expression appear. **(B)** Examination of inflammatory mediators including those involved in the prostaglandin-mediated inflammatory pathway at early timepoints suggests the inflammatory cascade is rapidly induced by eFSE as early as 3 h post-insult in a subset of rats but not all. **(C)** Examination of the same set of inflammatory mediators at 24 h showed persistent dysregulation and significant disruption of COX2 and downstream signaling components. **(D)** qPCR analysis of COX2, mPGES1 and EP2 at 96 h post eFSE demonstrates persistent disruption of both COX2 and EP2 in a subset of rats (~40%) consistent with our previous findings. **(A)** Adapted from (20). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

Increased Expression of Inflammatory Cytokines, Including Prostaglandins, Following eFSE

Our previous work has revealed that a subset of rat pups that undergo eFSE have a coordinated increase in a wide variety of inflammatory markers (**Figure 2A**). These same rats also had amygdalar MRI signal changes which predicted later epilepsy (20). In addition to traditional cytokines, we measured the enzyme COX2 because it was the most strongly correlated with the T_2 signal changes and had the most long-lasting increase in expression, encouraging further work investigating the COX2-prostaglandin pathway.

To investigate the COX2-prostaglandin inflammatory cascade, we measured the expression of mRNA levels of COX1, COX2, the three prostaglandin E synthase enzymes (cPGES, mPGES1, and mPGES2), as well as the prostaglandin E_2 (PGE_2) receptors (EP1, EP2, EP3α, EP3ß, EP3γ, EP4). At 3 h following eFSE, there was no significant increase in expression of the cascade (**Figure 2B**, unpaired *t*-test, $p > 0.05$ for all). At 24 h after eFSE, we found significantly increased expression of COX2, EP2, EP3ß, EP3γ, with a subset of the rats having markedly high expression of mPGES1 (**Figure 2C**, unpaired *t*-test, COX2: $p = 0.002$, t = 4.21, df = 9; mPGES1: $p = 0.07$, t = 2.01, df = 10; EP2: $p = 0.04$, t = 2.33, df = 10; EP3ß: $p = 0.003$, t = 4.48, df = 10, EP3γ: $p = 0.01$, t = 3.024, df = 10). These are all elements of the PGE_2 pathway, which has been investigated for

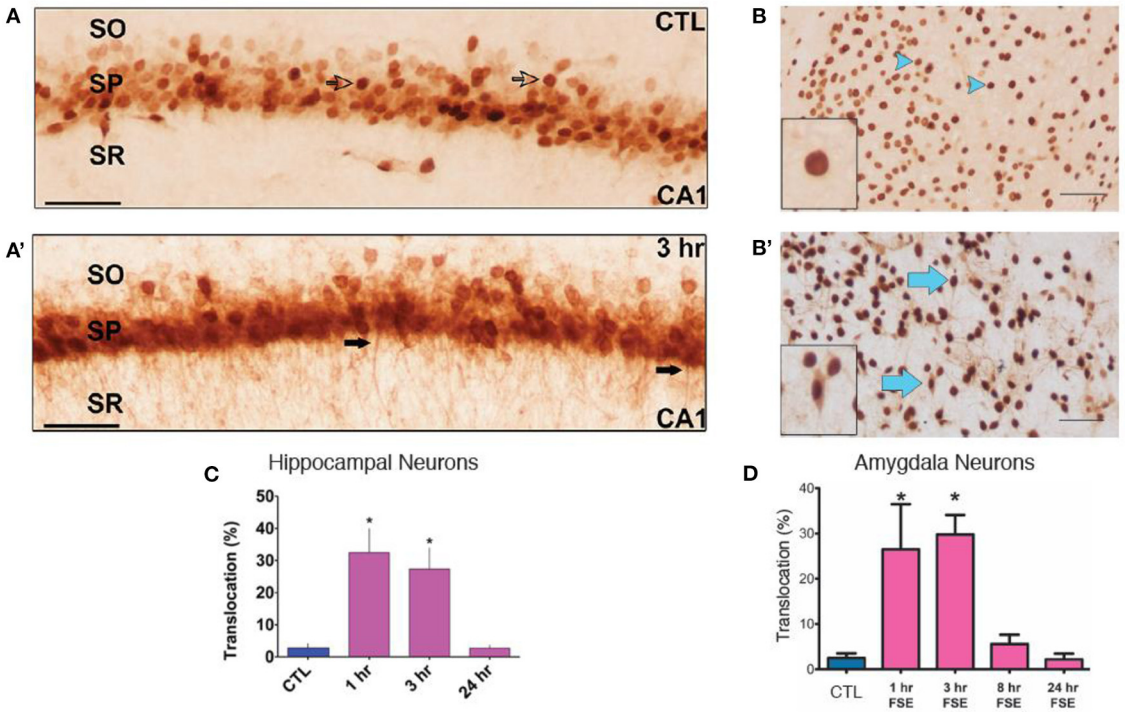

FIGURE 3 | HMGB1 Translocation in the Amygdala and Hippocampus following experimental Febrile Status Epilepticus (eFSE). **(A)** In control (CTL) animals, HMGB1 is confined to the nucleus of cells (open arrows) in area CA1 of dorsal hippocampus. **(A′)** Three hours after the end of eFSE, HMGB1-IR is seen in the processes of neurons (closed arrows), indicating translocation of HMGB1 from the nucleus. **(B,B′)** Differences in HMGB1 localization between normothermic control (NT-C) and experimental FSE rats. Arrowheads indicate nuclear HMGB1 and arrows indicate cytoplasmic (translocated) HMGB1. **(C)** Quantification of HMGB1 immunocytochemistry as the percentage of HMGB1-IR cells with translocation over total HMGB1-IR cells shows a significant increase in the percentage of HMGB1 translocation 1 and 3 h after eFSE with a return to control conditions by 24 h. Data are presented as the mean ± SEM. Scale bars, 100 μm. *Statistically significant at $p < 0.05$. CTL, Control; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. **(A,A′,C)** Adapted from (20). **(B,B′,D)** Adapted from (43), and $n = 3$ per group per timepoint.

its role in epileptogenesis, specifically the EP2 receptor. Thus, we next analyzed the expression of COX2, mPGES1, and EP2 at 96 h after eFSE. While not statistically significant when grouped together, a subset of rats had very high expression of EP2 at 96 h, much higher levels than any of the inflammatory cytokines previously analyzed (**Figure 2D**, EP2: $p = 0.21$, $t = 1.31$, $df = 13$), This selective increase in a subset of animals across inflammatory markers (**Figures 2B–D**) is consistent with prior findings in the animal studies of eFSE (20) and with human data that only a subset of children are at risk of developing epilepsy following prolonged febrile seizures (1, 3, 5). Thus, these findings encouraged investigation of inflammation and EP2 blockade to prevent the development of abnormal hyperexcitability.

HMGB1, a Potent Antecedent of Cytokine Expression Rapidly Translocates During eFSE, but Its Blockade Does Not Decrease Inflammation

In view of the robust increase of cytokine expression rapidly after eFSE, we examined the putative upstream mechanisms. Translocation of the neuronal danger signaling molecule HGMB1 from the nucleus to the cytoplasm and its release into the extracellular space is known to initiate cytokine expression by activating TLRs on glial cells (25, 62–64). Indeed, translocation of HMGB1 from the nucleus into the cytoplasm of neurons in both the amygdala and hippocampus of rats that underwent eFSE was found in prior work (**Figures 3A,A′,B,B′**). There was a significant increase in translocation 2 and 4 h after the start of eFSE within the amygdala at 1 and 3 h after eFSE but had returned to normal by 8 h (**Figure 3D**) (43). This same temporal pattern was seen in the hippocampi of rats that experienced eFSE (**Figure 3C**) (20).

Based on this, we investigated if HMGB1 blockade in neurons would decrease eFSE-provoked microglial activation and cytokine expression. Following eFSE, we administered ICV injections of HMGB1 Box A, a direct HMGB1 antagonist. We found that at 3 h after eFSE, HMGB1 Box A treatment did not significantly reduce the expression of pro-inflammatory cytokines including IL1ß, COX2, TNFα, and Inhibitor κB-α (IκB-α; a measurement of NFκB activity, a direct downstream target of HMGB1) (**Figures 4A–H**, one-way ANOVA with Bonferroni correction for multiple comparisons, IL1ß: naïve-ctrl vs. eFSE-Box A $p = 0.003$, ctrl-veh vs. eFSE-Box A p

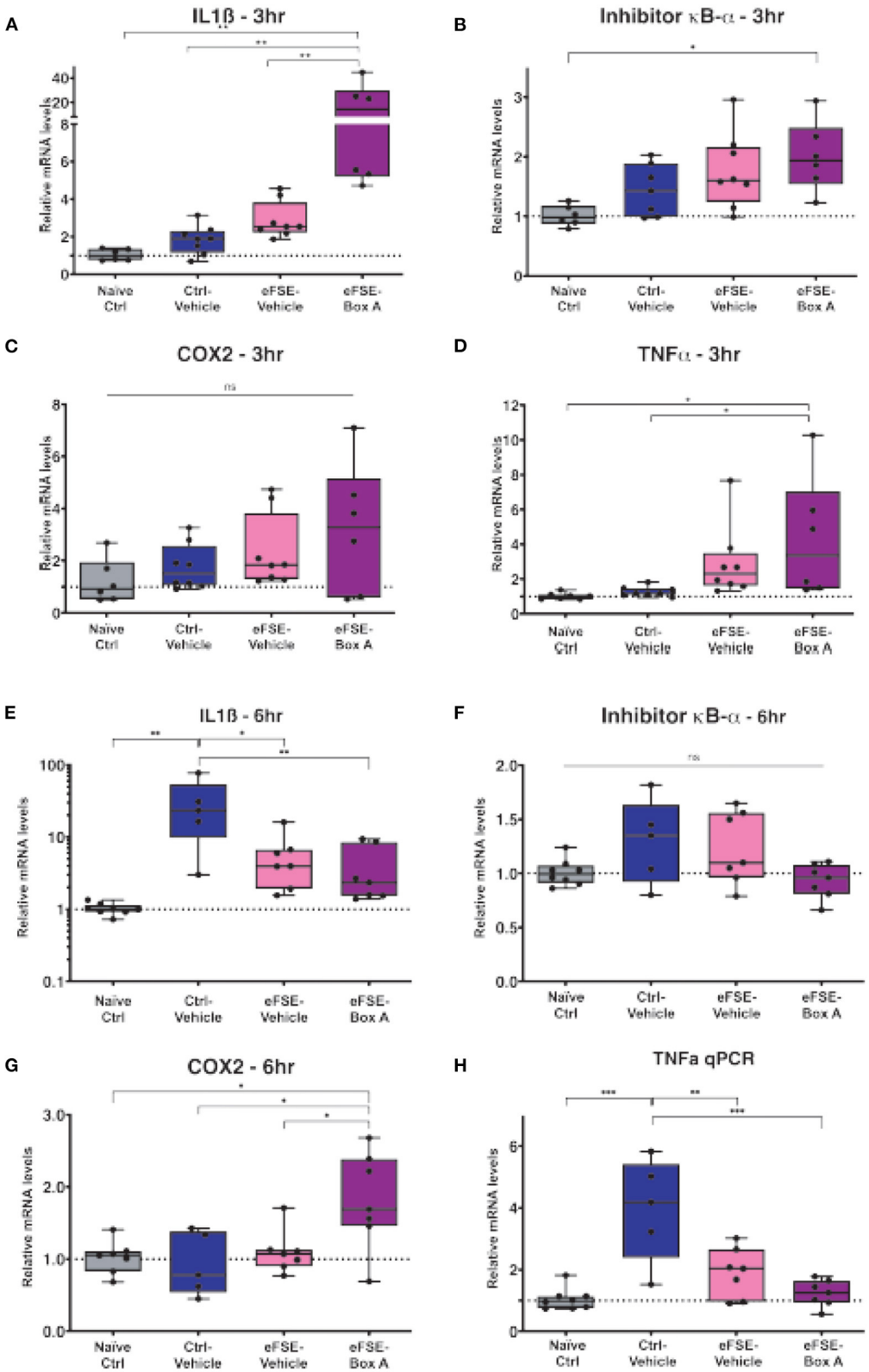

FIGURE 4 | HMGB1-inhibition does not reduce eFSE-evoked inflammation. **(A–D)** qPCR analysis of pro-inflammatory cytokines at 3 h post eFSE with or without HMGB1 inhibition. HMGB1 inhibition actually increased levels of pro-inflammatory cytokines. **(E–H)** qPCR analysis of pro-inflammatory cytokines 6 h post eFSE and treatment with veh or Box A. While infusion of Veh alone even in control rats was sufficient to induce inflammation Box A delivery was not effective at blocking inflammatory signaling post eFSE. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

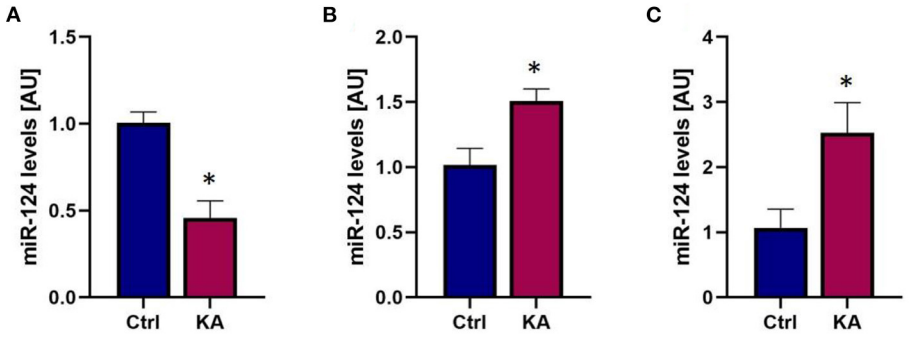

FIGURE 5 | Seizure-like activity reduces total miR-124 levels but promotes miR-124 packaging into ECVs and release. **(A)** qPCR analysis reveals rapid reduction of miR-124 levels in organotypic hippocampal slices following seizure-like events. **(B)** miR-124 levels in culture media increase following seizure-like events. **(C)** Analysis of miR-124 levels from exosomes isolated from culture media shows enrichment of miR-124 in exosomes following seizure-like events. $^*p < 0.05$.

= 0.003, eFSE-veh vs. eFSE-Box A $p = 0.005$; IκB-α: naïve-ctrl vs. eFSE-Box A $p = 0.014$; COX2: all adjusted $p > 0.05$; TNFα: naïve-ctrl vs. eFSE-Box A $p = 0.04$). Indeed, rather than suppressing cytokine expression, HMGB1 Box A actually increased expression of several inflammatory molecules (**Figures 4A,B,G**). Notably, administration of HMGB1 Box A to immature rats led to rectal bleeding in some rats at the 24 h timepoint, leading to a termination of this line of work. The lack of success in blocking pro-inflammatory cytokines combined with unacceptable side effects led us to conclude that interfering with HMGB1 *via* the direct antagonist HMGB1 Box A was not an encouraging candidate to prevent the long-term consequences of eFSE.

MiR-124 Is Released From Neurons via ECVs Following Seizure-Like Activity

Inter-cellular communication can take place when ECVs containing miRNAs originating from one cell type are released and taken up by a recipient cell (30, 38, 65, 66). The released ECV cargo can then influence its new environment. miRNA-124 is produced almost exclusively in neurons in response to intense activity such as seizures (61, 67), yet has been reported to influence microglial activity and cytokine release (66). To test whether miRNA-mediated neuronal-glia communication may occur following seizures we induced seizure-like activity in organotypic hippocampal slice cultures to approximate the type of seizure activity induced by eFSE. We used kainic acid KA at a dose which does not induce cell death (59), and that results in prolonged seizure-like network activity for the duration of exposure (68). We then measured the levels of miR-124 in slices and in the surrounding medium to measure exported miRNA levels (**Figures 5A,B**). We found a rapid reduction in miR-124 levels in the hippocampal tissue, recapitulating our prior findings both *in vivo* and *in vitro* (61). Notably, miR-124 was readily detected in the medium. To determine whether the mechanism of release was *via* ECV expulsion, we then isolated ECVs from the medium and quantified miRNA content. We found that miR-124 was enriched within ECVs, and particularly from slice cultures treated with KA (**Figure 5C**).

These findings supported the impetus to consider miR-124 as a potential intercellular communicator between neurons and glia that might play a role in the epileptogenesis that follows eFSE (61).

Blockade of Prostaglandin E_2 (PGE_2) at the EP2 Receptor Does Not Prevent Aberrant Hyperexcitability

As mentioned above, the neuroinflammatory molecules whose expression was induced by eFSE included members of the prostaglandin family, and especially the EP2 receptor. Therefore, we probed the potential contribution of PGE2, and specifically its signaling *via* the EP2 receptors (51, 53, 69). The small molecule inhibitor, TG6-10-1, is a direct antagonist of PGE_2 at the EP2 receptor developed by Dingledine et al. (51). We administered TG6-10-1 to rats that had undergone eFSE at 4, 22, and 30 h post-eFSE and then recorded video-EEG from hippocampal electrodes to analyze spike series, a sign of aberrant hyperexcitability and precursor to epilepsy (22) (experimental design, **Figure 6A**). Consistent with previous work, none of the control rats that were recorded had spike series. There was a subset of rats within both the rats that underwent eFSE and received vehicle injections (eFSE-Veh) and those that received TG6-10-1 injection (eFSE-TG6) that had measured spike series. When analyzed, there was no effect of TG6-10-1 treatment on the age at onset of spike series (**Figure 6B**, unpaired *t*-test, $p = 0.61$), the number of spike series within the first week of recording (**Figure 6C**, one-way ANOVA with Bonferroni's correction for multiple comparison, eFSE-Veh vs. eFSE-TG6, $p > 0.99$), total spike series in 60 days of recording (**Figure 6D**, one-way ANOVA with Bonferroni's correction for multiple comparison, eFSE-Veh vs. eFSE-TG6, $p > 0.99$), or duration of spike series (**Figure 6E**, one-way ANOVA with Bonferroni's correction for multiple comparison, eFSE-Veh vs. eFSE-TG6, $p = 0.18$). This led us to definitively conclude that short term treatment with TG6-10-1 does not decrease the development of abnormal hyperexcitability following eFSE, and thus, is unlikely to prevent the development of eFSE-induced epilepsy.

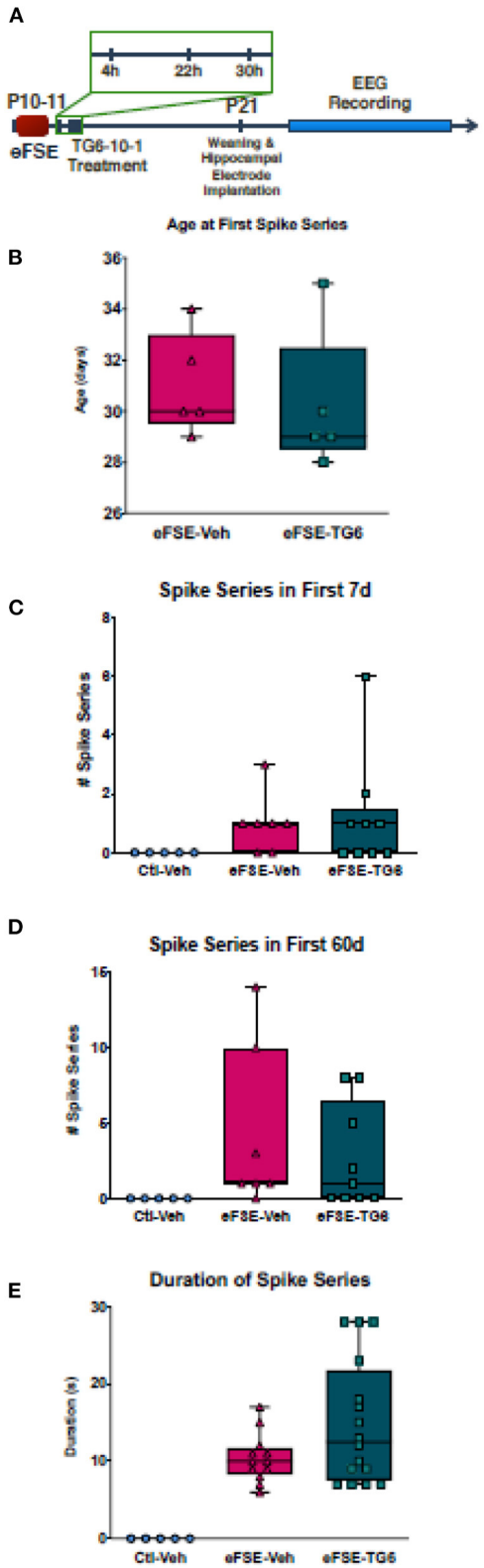

FIGURE 6 | TG6-10-1 treatment does not ameliorate or alter eFSE-evoked epileptogenesis. **(A)** Schematic of experimental design. TG6-10-1 treatment failed to alter the latency to onset of spike series post eFSE **(B)**, the total number of aberrant EEG spikes recorded in the first 7 days **(C)**, the first 60 days **(D)** nor the duration of each spike series **(E)**.

Global Suppression of Neuroinflammation Abrogates Pro-epileptogenic Neuronal Changes Following eFSE

The experiments described above suggested that targeting individual neuron-glia communication cascades to eliminate eFSE-induced epileptogenesis would not be successful. Yet, the evidence for contribution of neuroinflammatory mediators to epileptogenesis is strong. To address this conundrum, we investigated whether a global anti-inflammatory drug would decrease the aberrant hippocampal network hyperexcitability that follows eFSE. We first examined whether dexamethasone (DEX), a synthetic glucocorticoid, given for 3 days following eFSE attenuated glial proliferation. DEX treatment following eFSE decreased the density of P2Y12+ microglia [**Figures 7A–D**, adapted from (22), two-way ANOVA; main effect of DEX, $p = 0.016$]. The drug had no effect on astrocyte number which was unaffected by the eFSE [**Figures 7E–H**, adapted from (22), two-way ANOVA; main effect of eFSE, $p = 0.30$; DEX $p = 0.43$]. We then utilized EEG to determine if DEX attenuated the eFSE-induced aberrant hyperexcitability, measured as spike series. Spike series classically precede the development of spontaneous seizures in eFSE rats that go on to develop epilepsy [(50, 70–72); see (22)]. Therefore, spike series can be used as a measure of abnormal hippocampal excitability in this context. DEX treatment reduced the proportion of rats with recorded spike series in as compared to their vehicle-eFSE littermates (**Figure 7I**, unconditional Barnard's exact test, control vs. eFSE-VEH, $p = 0.0062$; eFSE-VEH vs. eFSE-DEX, $p = 0.031$; control vs. eFSE-DEX, $p = 0.37$). DEX also reduced the mean number of spike series per rat (**Figure 7J**, Kruskal–Wallis ANOVA: mean rank difference, -8.083; $p < 0.01$; Dunn's multiple-comparisons test, eFSE-DEX vs. controls: mean rank difference, -1.53; $p = 0.999$; eFSE-VEH vs. eFSE-DEX: mean rank difference, 6.55; $p = 0.067$). DEX attenuated the mean spike series frequency (spike series/hour recorded) bringing the level down to that seen in controls (**Figures 7K,L**, CTL mean, 0 ± 0; eFSE-VEH mean, 0.029 ± 0.06; K-W ANOVA: K-W statistic, 9.71; mean rank difference, -8.29; $p < 0.01$; Dunn's multiple-comparison test, eFSE-DEX vs. eFSE-VEH: mean rank difference, 6.95; $p = 0.046$; eFSE-DEX vs. controls: mean rank difference, -1.35; $p = 0.99$) [**Figures 7I–K**, adapted from (22)].

DISCUSSION

The current study identifies a critical role for neuro-glial communication in epilepsy development caused by eFSE. This includes the rapid translocation of HMGB1 from neurons, which then initiates microglial activation by binding to glial surface TL and RAGE receptors. We also identify for the first time a direct release of neuronal miRNA-124 from neurons in response to aberrant network activity, which provides a mechanism for the previously suspected role for neuronal miRNAs in seizure-induced microglial activation (61). We attempted to ameliorate epileptogenesis following eFSE by targeting individual components of the inflammatory cascade, specifically HMGB1 and the EP2 receptor, but found that this approach was unable to block the development of inflammation and hyperexcitability,

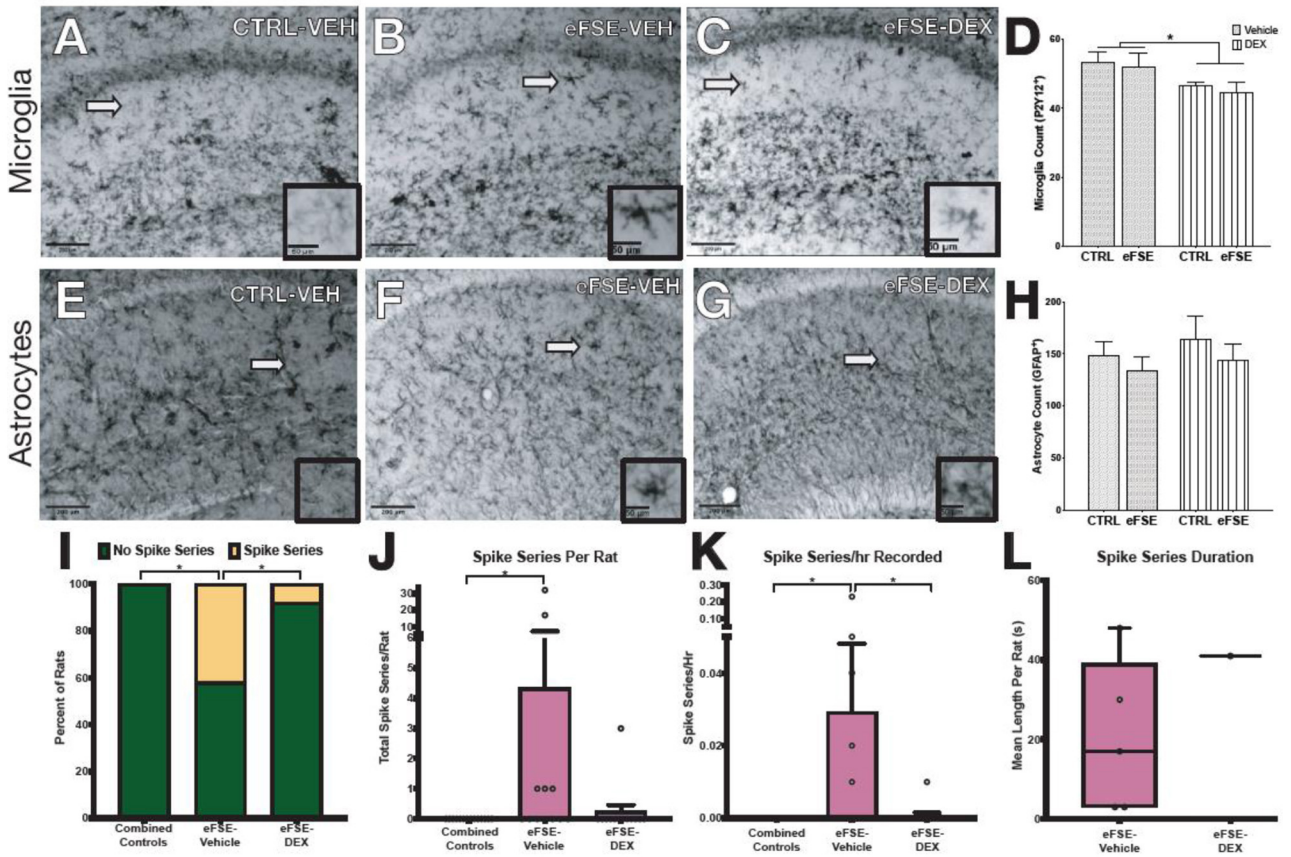

FIGURE 7 | Dexamethasone reduces inflammation following SE and epileptogenesis-associated spike-series. **(A–D)** Representative photomicrographs of hippocampal microglia staining with P2Y12 in control-Veh **(A)**, eFSE-Veh **(B)**, and eFSE-DEX **(C)** plus quantification **(D)**. Hippocampal microglial counts revealed a significant reduction in the number of activated microglia in eFSE rats treated with DEX as compared to control rats treated with Veh or Dex or eFSE rats treated with Veh. No effects were detected when astrocytes were analyzed **(E–H)**. **(I)** DEX treatment significantly reduced the number of rats who developed electrographic spike series after eFSE. Overall DEX treatment reduced the total number of spike series identified in rats following eFSE **(J)**, the frequency of spike series recorded per hour **(K)**, and the duration of spike series which were detected **(L)**. Group sizes: $n = 5–8$, the figure is adapted from (22). $^*p < 0.05$.

respectively. We therefore took a more global approach and blocked inflammation using DEX and found that this blunt force approach could in fact prevent the development of hippocampal hyperexcitability following eFSE. Together our findings highlight the complexity of inter-cellular communication following eFSE and show that a hammer rather than scalpel approach may be required to overcome the complexities of eFSE-induced epileptogenesis and to prevent the emergence of epilepsy.

We have previously seen a coordinated inflammatory response following eFSE, with rapid and persistent increase in pro-inflammatory cytokine levels including elevated IL1β, TNFα, COX-2, and Il-6 (20). Here we examined and found marked activation of pro-inflammatory cytokines but also the prostaglandin inflammatory cascade after eFSE. We observed persistently elevated EP2 receptor levels in a subset of eFSE rats. The EP2 receptor is upregulated in several animal models of epilepsy and prolonged seizures including the pilocarpine model of epilepsy and following pentylenetetrazole (PTZ) and diisopropofluorophosphate (DFP)-induced seizures (73, 74). FSE results in epilepsy in about 40% of cases (6, 75), and we see the same penetrance in rodent models of eFSE (43). Here, we see persistent activation of the prostaglandin in only a subset of rats suggesting that inflammatory processes are intrinsically linked to epilepsy development.

Our recent studies have found that eFSE rapidly promotes HMGB1 translocation from neuronal nuclei to the cytoplasm and the surrounding extracellular space where it binds surface TL and RAGE receptors on neighboring microglia, initiating an inflammatory response (43). Inhibiting HMGB1 signaling with Box A has previously been shown to be anti-convulsant when administered before a seizure challenge (25) to adult rodents; others reported that intra-nasal administration of recombinant HMGB1 reduced seizure threshold and promoted hyperthermia-induced seizures (46). In view of the above work, we sought to block HMGB1 signaling to reduce eFSE-evoked microglial activation and inflammation. We found that, within 3 h, HMGB1 antagonism with Box A actually increased the levels of specific pro-inflammatory cytokines including Il-1ß and COX2 which

are known to contribute to eFSE-evoked epileptogenesis. These unexpected findings suggest that HMGB1 blockade may activate alternative or compensatory inflammatory pathways which in this case resulted in an even greater inflammatory response. This seems further plausible given the unacceptable side effects we detected at later time points in young rats treated with this drug.

While it is accepted that neuronal-glial communication plays a key role in the epileptogenesis evoked by many epilepsy-inciting events including FSE, the mechanisms by which neurons and glia communicate during epilepsy development are relatively unknown. Extracellular vesicles including exosomes have emerged as critical mediators of cell-cell communication and are enriched in the CNS. Detailed tracing studies now exist which follow ECV export from originating cells and subsequent uptake by surrounding cells (30, 76). Recent studies have profiled exosome miRNA content during epileptogenesis and found gross changes in exosomal miRNA content at specific disease stages (35–37). Our previous studies found that miR-124, despite residing in neurons could influence the inflammatory response when introduced to microglia (61). Here we find that seizure-like events promote miR-124 inclusion in ECVs which are released by neurons and may enter neighboring cells. Due to the neuronal specific nature of miR-124 we can conclude that ECVs containing miR-124 originate in neurons. We specifically used a non-excitotoxic dose of KA to evoke seizure-like events which suggests that ECV and miR-124 release from neurons is not due to seizure-induced neuronal apoptosis or necrosis. Instead miR-124 containing ECVs are actively exported from viable yet stressed neurons and may influence surrounding cells. Thus, we implicate miR-124 as a critical regulator of neuronal-glial crosstalk during epileptogenesis.

Having identified EP2 as heavily involved in the inflammatory milieu following eFSE we next sought to block EP2 using a highly specific inhibitor as a potential anti-epileptogenic approach (51). Surprisingly we found that inhibition of the EP2 receptor had no effect on the emergence of abnormal hyperexcitability induced by eFSE and likely would be ineffective at blocking epileptogenesis. Previous studies utilizing EP2 inhibition have successfully ameliorated the long-term effects of status epilepticus (52). These studies however have been mostly performed in adult models of epilepsy and this is further evidence that targeting individual inflammatory pathways in juvenile epilepsy may activate alternative inflammatory pathways in response or be insufficient to have meaningful effects on epilepsy development.

To test this, we next investigated whether using a less specific anti-inflammatory agent may ameliorate or alter eFSE-evoked epileptogenesis. We used the glucocorticoid dexamethasone (DEX) which effectively and broadly dampens inflammation and is used to treat many inflammation-related diseases. We found that administration of DEX following eFSE blocked microglial activation and proliferation in the hippocampus and also led to spike series development in much fewer animals, with most DEX treated rats not developing spike series at all. This suggests that anti-inflammatory approaches, at least for eFSE-induced epilepsy might be better treated with broad-acting anti-inflammatory drugs like DEX rather than those which target individual receptors or cytokines. It is also conceivable that etiology plays a large role and anti-epileptogenic strategies must be designed with this in mind.

Together our findings suggest that eFSE in rodents leads to multiple and rapid disruption of interconnected glial-neuronal networks with a likely role in epileptogenesis. The intricate, cell-specific and homeostatic interplays among these pathways constitute a serious challenge to effective selective interventions that aim to prevent epilepsy. In contrast, a broad suppressive measure of glial-neuronal dysfunction holds promise for mitigating FSE-induced hyperexcitability and epileptogenesis in experimental models and in humans.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of California-Irvine Animal Care Committee.

AUTHOR CONTRIBUTIONS

GB, MG-C, KP, and RL performed experiments. TB, GB, and MG-C designed experiments. GB, MG-C, and TB wrote the paper. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Dysregulation of Ambient Glutamate and Glutamate Receptors in Epilepsy: An Astrocytic Perspective

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Given the important functions that glutamate serves in excitatory neurotransmission, understanding the regulation of glutamate in physiological and pathological states is critical to devising novel therapies to treat epilepsy. Exclusive expression of pyruvate carboxylase and glutamine synthetase in astrocytes positions astrocytes as essential regulators of glutamate in the central nervous system (CNS). Additionally, astrocytes can significantly alter the volume of the extracellular space (ECS) in the CNS due to their expression of the bi-directional water channel, aquaporin-4, which are enriched at perivascular endfeet. Rapid ECS shrinkage has been observed following epileptiform activity and can inherently concentrate ions and neurotransmitters including glutamate. This review highlights our emerging knowledge on the various potential contributions of astrocytes to epilepsy, particularly supporting the notion that astrocytes may be involved in seizure initiation via failure of homeostatic responses that lead to increased ambient glutamate. We also review the mechanisms whereby ambient glutamate can influence neuronal excitability, including via generation of the glutamate receptor subunit GluN2B-mediated slow inward currents, as well as indirectly affect neuronal excitability via actions on metabotropic glutamate receptors that can potentiate GluN2B currents and influence neuronal glutamate release probabilities. Additionally, we discuss evidence for upregulation of System x_c^-, a cystine/glutamate antiporter expressed on astrocytes, in epileptic tissue and changes in expression patterns of glutamate receptors.

Keywords: glutamate homeostasis, System x_c^-, epilepsy, astroglia, metabotrophic glutamate receptor, NMDAR

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INTRODUCTION

The critical roles of astrocytes in supporting the healthy development and maintenance of a mature brain has been firmly established in the last few decades. It is well-appreciated that an imbalance between excitatory and inhibitory neurotransmission causes hyperexcitability in neuronal circuitry and underlies the processes of ictogenesis and epileptogenesis. Given the important functions that glutamate serves in excitatory neurotransmission, understanding the mechanisms regulating glutamatergic drive under physiological and pathological states provides critical insights into devising strategies to maintain glutamate homeostasis. Since 1-in-3 epilepsy patients are pharmacoresistant to currently available antiseizure drugs, that mainly target neuronal mechanisms (1, 2), elucidating the astrocytic processes involved in seizure generation and epileptogenesis in detail may help identify new targets to treat intractable forms of epilepsy.

This review serves to highlight the emerging dynamic processes that astrocytes undergo in epilepsy, in support of the notion that astrocytes play a critical role in seizure generation via homeostatic responses such as the increase in ambient glutamate through a reduction in extracellular space (ECS) following activity-dependent astrocytic potassium uptake and buffering (**Figure 1-1**). Importantly, we also review the mechanisms in which increased ambient glutamate can directly influence neuronal excitability, via generation of the glutamate receptor subunit GluN2B-mediated slow inward currents (SICs), as well as indirectly affect neuronal excitability via actions on metabotropic glutamate receptors (mGluRs) that can potentiate GluN2B currents, alter extracellular glutamatergic clearance, and influence neuronal glutamate release probabilities (**Figure 1-2**). Additionally, evidence of upregulation of System x_c^- (SXC), a cystine/glutamate antiporter expressed on astrocytes, in epileptic tissue and changes in expression patterns of glutamate receptors have provided insights in how astrocytic dysregulation can contribute to seizure generation and epileptogenesis (**Figure 1-3**).

GLUTAMATE SYNTHESIS, RELEASE, AND REUPTAKE

Before discussing the mechanisms whereby astrocytes regulate glutamatergic neurotransmission, it is important to understand the fundamental functions astrocytes play in CNS glutamate homeostasis. Glutamate or glutamic acid is a ubiquitous biological molecule serving multiple functions—mainly as an amino acid for protein synthesis, as a principal excitatory neurotransmitter in the mammalian CNS and as a source of energy (3). The use of an abundant amino acid as a neurotransmitter poses a significant challenge in the regulation of available glutamate in the CNS, since excess extracellular glutamate is highly neurotoxic. The evolution of two remarkable features of the biological system—the blood-brain barrier (BBB) and the astrocytic specializations—has solved the problem of potential "spillover" of peripheral glutamate into the CNS as a neurotransmitter. Interestingly, in both cases astrocytes are involved to the extent that glial dysfunctions can alter glutamate homeostasis and cause excitotoxicity and neuronal hyperexcitability.

The BBB is impermeable to glutamate (4), which is critical in preventing the flooding of the CNS by peripheral glutamate within the vasculature. However, this system also necessitates autonomous turnover of glutamate in the brain. The role of astrocytes in *de novo* synthesis of glutamate in the brain is well-established in literature (5). Astrocytes specializations, namely exclusive expression of pyruvate carboxylase and glutamine synthetase, allow them to serve critical functions in CNS glutamate homeostasis (6, 7). Since pyruvate carboxylase is essential for the synthesis of oxaloacetate that is subsequently utilized in the synthesis of α-ketoglutarate and glutamate, astrocytes are the only cell type in the brain capable of *de novo* synthesis of glutamate by the oxidative metabolism of glucose. Although one study recently identified another source of glutamate in the brain where glutamate is synthesized by neurons from blood urocanic acid (8), more studies are required to validate it as well as to estimate the relative contribution of neurons in total glutamate synthesis in the brain. Glutamate is subsequently amidated by glutamine synthetase into glutamine, which enters the glutamate-glutamine cycle between astrocytes and neurons.

After release into the synaptic cleft, excessive glutamate must be cleared quickly to prevent neurotoxicity. There are no extracellular enzymes that can neutralize glutamate (9), however, astrocytes can rapidly clear glutamate from the synaptic clefts via astrocytic processes that completely enclose many glutamatergic synapses. These astrocytic processes also express highly efficient excitatory amino acid transporters (10) (EAATs) that take up 80% of extracellular glutamate in the CNS (5) (**Figure 1-1a**). Once inside the astrocyte, it is estimated that ∼85% of glutamate is converted into glutamine and returned to neurons, while the remaining ∼15% is metabolized to α-ketoglutarate and further oxidized through the tricyclic acid cycle for energy production. This helps to cover the energy costs of glutamate handling as both pyruvate carboxylase and glutamine synthetase catalyze energy-dependent reactions (3). Insights into the synthesis and regulation of glutamate in the CNS serve to highlight the role that astrocytes play in health and to set up the potential consequences of disrupting astrocytic glutamate homeostasis in the pathology of epilepsy.

ASTROCYTIC REGULATION OF AMBIENT GLUTAMATE VIA VOLUMETRIC SHIFTS

Several recent reviews have emphasized the relationship between cerebral edema, astrocytic swelling, and epilepsy, due to a strong link between the volume reduction of the ECS hyperexcitability (11, 12). ECS is a narrow space between CNS cells that serves as a reservoir of water, ions, and signaling molecules to maintain ionic and water homeostasis. Interestingly, ECS reduction is one of the common mechanisms of generating hyperexcitability in several forms of epilepsies and astrocytes play key role in ECS volume regulation.

Astrocytes can significantly alter the volume of the CNS ECS due to their expression of the bi-directional water channel, aquaporin-4 (AQP4), which is enriched at perivascular endfeet (13). Neuronal activity-dependent astrocytic swelling, following potassium uptake and buffering, is an important mediator in the reduction of ECS (**Figure 1-1b**). Indeed, measurements of the ECS before and during bath application of picrotoxin, a $GABA_A$ receptor antagonist, to induce epileptiform activity revealed that brief epileptiform discharges rapidly decreased the median ECS width, measured as the width of interstitial space separating neural structures, by over 50% (14). Rapid ECS shrinkage can inherently concentrate ions and neurotransmitters including glutamate, explaining elevated extracellular glutamate during seizures in epilepsy patients (15). ECS shrinkage due to astrocyte swelling in hypoosmolar conditions has been shown to be sufficient in evoking large excitatory slow inward currents (SICs) in neurons (16). Conversely, studies in AQP4-knockout animals

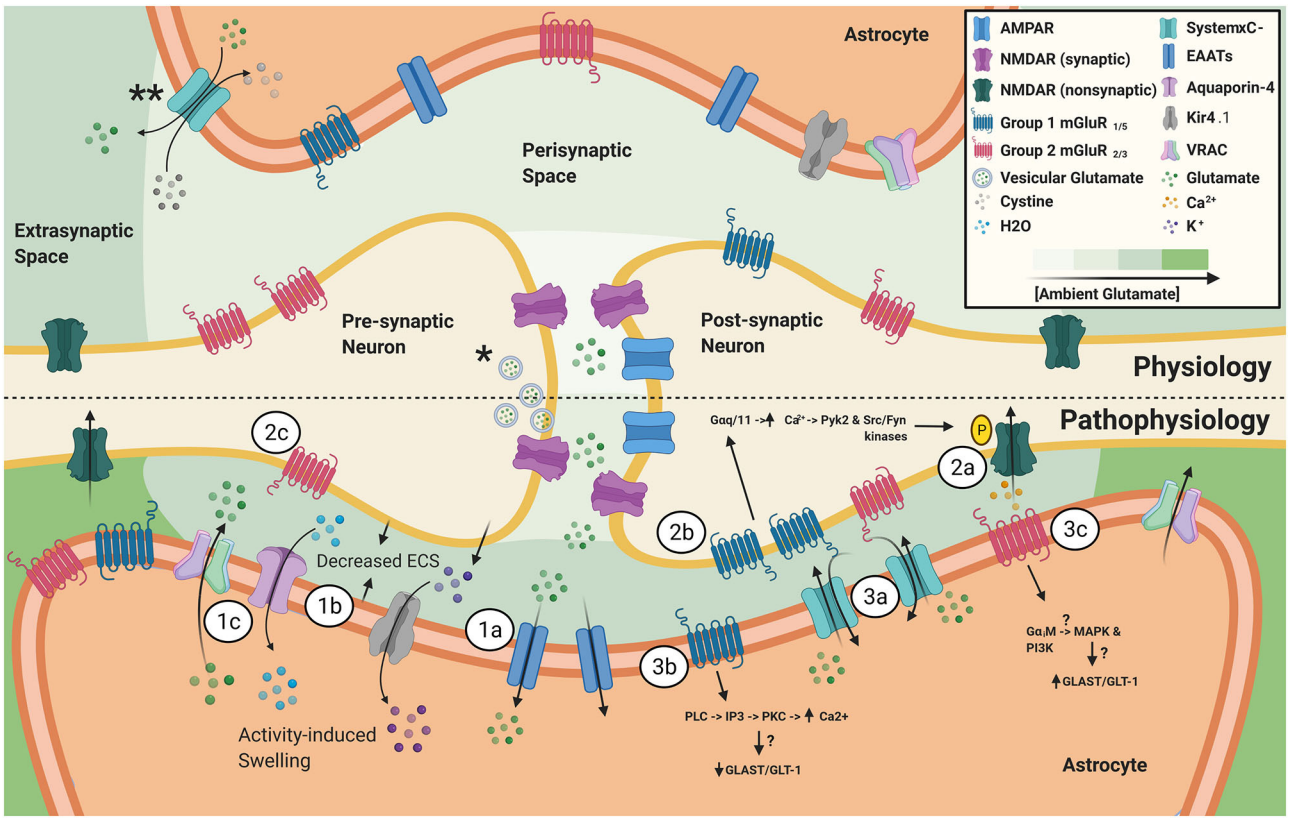

FIGURE 1 | *Vesicular glutamate release during action potentials is the primary source of synaptic glutamate. **SXC, primarily expressed on astrocytes, is a major source of ambient, extrasynaptic glutamate. Ambient glutamate concentration around the synapse, after EAAT activity, follows a gradient with the lowest level in the synaptic cleft to the highest in the extrasynaptic compartment. **(1)** Astrocyte homeostatic responses to increased activity from hyperexcitable neurons. **(1a)** Increased vesicular glutamate release from hyperexcitable neurons leads to increased astrocytic EAAT activity. **(1b)** Elevated neuronal activity also causes release of K^+, in attempts to maintain homeostatic neuronal resting membrane potential. Next, astrocytic buffering of extracellular K^+ through elevated Kir4.1 activity, which is accompanied by increased H20 uptake through aquaporin-4, ultimately results in activity-induced astrocytic swelling and reduction in ECS. **(1c)** Astrocytic swelling leads to activation of VRAC and release of glutamate and other gliotransmitters into the ECS. **(2)** Pathophysiological effects of increased activity and changes in expression of neuronal extrasynaptic glutamate receptors. **(2a)** Activation of N2B-containing NMDARs leads to the generation of slow, depolarizing currents. **(2b)** Elevated expression and activity of group 1 mGluRs in epilepsy has been linked to increased NMDAR-mediated currents via a mechanism involving Ca^{2+}-calmodulin dependent tyrosine phosphorylation of NMDAR subunits NR2A/B. **(2c)** Presynaptic group 2 mGluRs have been shown to inhibit glutamate and GABA release. Tissue from epileptic patients and animal models have revealed decreased $mGluR_{2/3}$ expression, which can contribute to a pro-epileptic brain state. (3) Changes in astrocytic glutamatergic protein expression in epilepsy. **(3a)** SXC expression has been found to be elevated in human epileptic tissue, as well as various epilepsy animal models. SXC activity leads to the release of glutamate from astrocytes. **(3b)** Animal models of epilepsy have revealed that persistent upregulation of astrocytic $mGluR_5$ was a reliable indicator of epileptogenesis. $mGluR_5$ activation leads to altered GLAST/GLT-1 expression and induces NR2B-dependent NMDAR mediated neuronal currents. **(3c)** Upregulation of $mGluR_3$ has been reported in epilepsy animal models and experimental activation of group 2 mGluRs in cultured astrocytes was shown to upregulate GLAST/GLT-1 expression, suggesting that a balance of group 1 and group 2 mGluRs on astrocytes is important in maintaining homeostatic extracellular glutamate.

have revealed that these mice have larger ECS, slower potassium kinetics and are more resistant to seizure generation using pentylenetetrazole (PTZ) (17–19), a $GABA_A$ receptor antagonist.

Another way astrocytes regulate water homeostasis and consequently ECS volume is the volume-regulated anion channel (VRAC), which are typically activated through hypotonicity-induced cell swelling (20, 21). VRAC belongs to the leucine-rich repeat-containing 8 (LRRC8) family of proteins (22, 23) and consists of a heterogenous mix of LRRC8 proteins of which LRRC8A, also known as Swell1, is an essential subunit (24). Upon astrocytic swelling, VRAC is activated and allows the release of chloride ions and other osmolytes, including glutamate (**Figure 1-1c**). This in turn generates an osmotic gradient that drives water out of the astrocyte. Using astrocyte specific *Swell1* knockout mice and VRAC inhibitors, recent studies have identified that VRAC-mediated glutamate release can modulate NMDAR-mediated tonic currents, as well as affect neuronal excitatory vesicle release probabilities (25, 26).

These processes support the hypothesis that impairment in astrocyte functions may play a critical role in initial seizure generation by increasing physiologically relevant ambient glutamate through (1) a reduction of ECS, following astrocytic potassium uptake and buffering, (2) glutamate release through VRAC or (3) a dysregulated state combining both mechanisms.

ASTROCYTIC REGULATION OF AMBIENT GLUTAMATE VIA SXC

Studies investigating the physiological role(s) of ambient glutamate [reviewed in (27)] have identified several diverse functions such as involvement in sleep-wakefulness cycles, synaptic plasticity, and the ability to influence neuronal resting membrane potentials via induction of NMDAR-mediated inward currents. However, the processes that maintain and regulate levels of ambient glutamate continue to be elucidated (27). Another important source of ambient, extracellular glutamate is via the sodium independent activity of the cystine/glutamate antiporter SXC. SXC is a covalently coupled heterodimeric protein complex comprised of the 4F2 heavy chain, *SLC3A2,* linked to the cystine/glutamate exchanger (xCT), *SLC7A11*. Notably, while xCT is abundantly expressed in cells throughout the body, examination of SXC knockout mice ($xCT^{-/-}$) mice revealed that in the CNS, xCT is exclusively expressed in astrocytes, and absent in neurons, oligodendrocytes, and microglia (28). xCT plays a major role in the modulation of ambient extracellular glutamate as pharmacological inhibition of SXC, using (S)-4-carboxyphenylglycine (S-4-CPG), resulted in a 60% reduction in extrasynaptic glutamate in the nucleus accumbens (29). Although S-4-CPG is known to inhibit type 1 mGluRs, this study found that use of the type 1 mGluR antagonist (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA) did not result in changes in ambient glutamate. This finding was confirmed recently using antisense xCT which similarly decreased extracellular glutamate in the nucleus accumbens (30). Additionally, $xCT^{-/-}$ mice have around 30% less hippocampal ambient glutamate (31).

In tumor-associated epilepsy, overexpression of SXC in glioblastomas and the corresponding increase in peritumoral glutamate levels have been directly associated with the development of seizures and poor patient survival (32–34). These studies also found that inhibition of SXC via sulfasalazine (SAS), an FDA approved drug used to treat inflammatory bowel disease, reduced epileptiform activity and seizure frequency both *in vitro* and *in vivo* in glioblastoma models. In addition to tumor-associated epilepsy, resected human epileptic tissue from patients with temporal lobe epilepsy (TLE) also had an elevated expression of SXC (35) (**Figure 1-3a**). We have also shown recently that SAS could significantly decrease the frequency and/or amplitude of evoked excitatory postsynaptic currents in multiple *in vitro* models of hyperexcitability (36). Furthermore, co-application of SAS with topiramate, an FDA-approved anti-seizure drug, further decreased epileptiform activity synergistically compared to topiramate alone.

The role of SXC in neuronal hyperactivity via ambient glutamate regulation is further supported by recent *in vivo* studies on $xCT^{-/-}$ mice showing delayed epileptogenesis and reduced seizures in the self-sustained status epilepticus (SSSE) and pilocarpine induced status epilepticus models (37). Additionally, this study found decreased micro- and astrogliosis in $xCT^{-/-}$ mice after SSSE. In contrast, wildtype mice that underwent pilocarpine-induced status epilepticus had significantly increased xCT expression during latent phase of epileptogenesis. Using another strain of SXC knockout mice, which have a spontaneous deletion in the xCT gene in subtle gray mice ($xCT^{sut/sut}$), it was found that $xCT^{sut/sut}$ mice were significantly resistant to epileptic kindling compared to wildtype mice. Additionally, western blot analysis of plasma membrane proteins found that cortical, but not hippocampal, surface GluA1 expression was significantly decreased in $xCT^{sut/sut}$ mice (38).

In contrast to the above studies that show a pivotal role of SXC in hyperexcitability, a recent study using the Theiler's Murine Encephalomyelitis Virus (TMEV) model of viral-induced epilepsy found that $xCT^{-/-}$ mice were not protected against this form of epilepsy and had similar number and severity of behavioral seizures (39). The lack of difference in TMEV-induced seizures between $xCT^{-/-}$ and WT mice can be possibly explained by the fact that the proinflammatory cytokines, tumor necrosis factor-α and interleukin-6, are known as the major drivers of hyperexcitability and seizures in this epilepsy model (40, 41). Taken together, these above cited studies establish SXC as a potential astrocytic drug target. Clearly astrocytes have the capabilities to modulate ambient extracellular glutamate, yet whether astrocytic SXC exerts a direct, pro-epileptic effect by modulating ambient glutamate in acquired epilepsies requires further study.

MECHANISMS OF ASTROCYTE-DERIVED GLUTAMATE CONTRIBUTING TO NEURONAL HYPEREXCITABILITY

So far, we have discussed that astrocytes not only prevent excitotoxic glutamate accumulation in the ECS, but also serve as a source of extracellular glutamate under specific conditions. However, important questions remain. How might astroglial glutamate contribute to neuronal activity, and is it sufficient to trigger neuronal hyperactivity? Additionally, how might different types of glutamate receptors at tripartite synapses and extrasynaptic spaces respond to fluctuations in ambient glutamate?

The notion of glial-neuronal crosstalk via gliotransmission has been extensively studied in the last two decades. Regardless of the mechanisms, a wealth of evidence has demonstrated that astrocytes can sense neuronal activity and respond through the release of gliotransmitters including ATP, D-serine, and glutamate (42, 43). Research into the origin and consequences of neuronal SICs revealed that these currents are generated via astrocyte-derived glutamate acting on extrasynaptic GluN2B subunit-containing N-methyl D-aspartate type of glutamate receptors (44, 45) (NMDARs) (**Figure 1-2a**). Extrasynaptic NMDARs are thought to have increased GluN2B-containing heterodimers, suggesting a spatially specific function. Indeed, GluN2B-containing NMDARs have a higher affinity for glutamate compared to GluN2A-containing NMDARs (46), which may facilitate sensing glutamate in the extrasynaptic space that has far lower extracellular glutamate compared to an active synapse.

SICs can be synchronized in multiple neurons over 100 microns apart in the hippocampus, raising the possibility

of astrocytic synchronization of neuronal hyperactivity in epilepsy. Interestingly, blockade of glutamate uptake and exocytotic glutamate release increased the frequency and amplitude of these NMDAR-mediated SICs, suggesting that increases in ambient glutamate could play a physiologically relevant role (45). A study investigating the actions of extrasynaptic glutamate determined that SXC-mediated glutamate release preferentially activates extrasynaptic GluN2B-containing NMDARs (47). Similarly, another study determined that the glutamate responsible for generating SICs occur independently from exocytotic Ca^{2+}-dependent glutamate release (48). Notably, a paper examining the sensitivities of glutamate receptors to glutamate predicted the percentage of glutamate receptors that would remain activated at increasing levels of ambient extracellular glutamate (49). This paper succinctly demonstrates that NMDARs and mGluRs would be preferentially activated by small, local fluctuations in astrocytic glutamate release as they are activated by glutamate concentrations around 1–30 μM, while α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type of glutamate receptors (AMPARs) are only activated in the presence of 100–3,000 μM of glutamate. Therefore, it is evident that astrocytes possess the molecular machinery necessary to significantly alter the concentration of extrasynaptic ambient glutamate, which can act on NMDARs to induce neuronal SICs that can contribute to hyperexcitability.

METABOTROPIC GLUTAMATE RECEPTORS AND EPILEPSY

Metabotropic glutamate receptors (mGluRs), which are coupled to G protein-coupled pathways and G protein-independent pathways, are another class of glutamatergic receptors whose dysregulation have been implicated in the pathology of epilepsy. Unlike ionotropic glutamate receptors, mGluR activation can trigger long-term changes in cellular signaling by regulating the expressions of various homeostatic and glutamatergic proteins in neurons and glial cells (50). Currently, eight mGluR subtypes have been described that fit into three subgroups (51, 52). Group 1 mGluRs consists of mGluR1 and mGluR5 and are coupled to $G\alpha_{q/11}$, which stimulates the release of Ca^{2+} from intracellular stores upon activation. Group 1 mGluRs are thought to be distributed primarily on post-synaptic neurons, in the perisynaptic zone (53, 54). Using the kainic acid (KA) rat model of TLE, one study has reported upregulation of neuronal mGluR1 in rodent hippocampi, in addition to, mGluR1 upregulation in human TLE tissue (55). Importantly, neuronal mGluR1 activation was shown to potentiate NMDAR-mediated currents via a mechanism involving Ca^{2+}, calmodulin and Src-dependent activation of proline-rich tyrosine kinase leading to tyrosine phosphorylation of NMDAR subunits GluN2A/B (56) (**Figure 1-2b**). A study investigating the efficacy of mGluR1 inhibition in epilepsy found that mGluR1 inhibition decreased PTZ-induced seizures, and this effect could be prevented by adding mGluR1 agonists (57).

mGluR5 is also expressed on cortical and hippocampal astrocytes, primarily during development (58), however, the reappearance of mGluR5 expression has been observed in astrocytes of specific epilepsy animal models, and human epileptic tissue (59–61). Intriguingly, mice with persistent astrocytic mGluR5 expression during the latent period reliably went on to develop epilepsy, whereas mice with only transient mGluR5 expression did not (59) (**Figure 1-3b**). Additionally, epileptic mice with astrocytic mGluR5 knocked out displayed lowered glutamate uptake kinetics during high-frequency stimulation compared to epileptic wildtype mice. Downstream effects of astrocytic mGluR5 activation include increased Ca^{2+}-dependent, GluN2B-subunit containing NMDAR-mediated neuronal currents (61). *In vivo* loading of BAPTA-AM, a Ca^{2+}chelator, selectively into astrocytes was found to be neuroprotective and significantly reduced the amount of Fluoro-Jade B labeling of dying neurons. Together, these findings suggest that reappearance of astrocytic mGluR5 may be a useful biomarker for active epileptogenesis, as well as a contributor to epileptogenesis by potentiating GluN2B-mediated inward neuronal currents.

Group 2 mGluRs consist of mGluR2 and mGluR3, while Group 3 mGluRs consist of mGluR4, mGluR6, mGluR7, and mGluR8. Both Group 2 and Group 3 inhibit adenylyl cyclase activity through $G\alpha_i$ activation and are thought to be largely distributed on pre-synaptic neurons, where they act to inhibit glutamate and GABA release (62, 63). Additionally, mGluR3 has been reported to be expressed on post-synaptic neurons and astrocytes (64–66). Using the pilocarpine TLE animal model, studies have shown that neuronal mGluR2/3 expression in the hippocampus (67, 68) and cortex (69) are decreased in epilepsy. Additionally, epileptic tissue from patients with TLE also have decreased mGluR2/3 expression (68) (**Figure 1-2c**). As pre-synaptic Group 2 mGluRs are thought to inhibit glutamate release, down-regulation of these receptors could promote glutamate release and neuronal hyperexcitability. Indeed, studies using Group 2 mGluR agonists have been shown to be neuroprotective in the models of absence epilepsy and the epilepsy models induced through amygdala kindling (57, 70). Conversely, Group 2 mGluR antagonists were shown to be pro-epileptic in an absence epilepsy model (71). Notably, these researchers also found that a mGluR1 potentiator, 9H-xanthene-9-carboxylic acid(4-trifluoromethyl-oxazol-2-yl)amide (SYN119), played a protective role against spike and wave discharges in a rat model of absence epilepsy (72).

One study investigating the localization and changes in mGluR3 and mGluR5 expression after hippocampal injury via KA-induced seizures found that mGluR3 mRNA was exclusively upregulated in astrocytes and oligodendrocytes (**Figure 1-3c**). Additionally, GFAP-positive astrocytes were found to be persistently upregulated from 2 days to 12 weeks post hippocampal injury (73). Although mGluR5 mRNA was not found to be upregulated in astrocytes, other studies have reported upregulated mGluR5 protein levels in animal models

TABLE 1 | Pharmacotherapies targeting glutamate signaling for epilepsy.

Drug	Brief mode of action	Effects on glutamatergic system	Effects on seizures	Developmental Stage	Reference
Perampanel	Noncompetitive selective AMPAR antagonist	↓ AMPAR-mediated fast excitatory neurotransmission	↓ focal and generalized tonic-clonic seizures	FDA-approved in 2012	(79)
Topiramate	AMPAR/KAR inhibitor; multiple other mechanisms	↓ excitatory neurotransmission	↓ focal and generalized convulsive seizures	FDA-approved in 1995	(80)
Felbamate	NMDAR inhibitor; multiple other mechanisms	↓ excitatory neurotransmission	↓ focal and generalized convulsive seizures	FDA-approved in 1993	(81)
Ketamine	NMDAR antagonist	↓ NMDAR-mediated excitatory neurotransmission; potentially neuroprotective	efficacious against refractory status epilepticus	Under clinical trial	(82)
Gabapentinoids (Gabapentin, Pregabalin)	Blocker of α2δ subunit of voltage-gated Ca^{2+} channel	↓ release of glutamate	↓ focal seizures (gabapentin, pregabalin)	FDA-approved (Gabapentin, 1993; Pregabalin, 2004)	(83)
		↓ excitatory synaptogenesis	↓ generalized convulsive seizures (gabapentin)		(80)
		inhibits surface trafficking and synaptic targeting of NMDAR			
Levetiracetam	Synaptic vesicle glycoprotein 2A (SV2A) modulator	↓ release of glutamate, ↑ synaptic depression	↓ focal and generalized tonic-clonic seizures; potentially antiepileptogenic	FDA-approved in 2000	(84, 85)
Brivaracetam	SV2A modulator (more selective than levetiracetam)	↓ release of glutamate, ↑ synaptic depression	↓ focal and generalized tonic-clonic seizures	FDA-approved in 2016	(84, 85)
17AAG	HSP90β inhibitor	inhibits internalization and proteosomal degradation of GLT-1	↓ seizures in intrahippocampal kainate model of epilepsy	Investigational	(86)
		↑ glutamate clearance from ECS			
Ceftriaxone	GLT-1 transcriptional activator	↑ glutamate clearance from ECS	↓ frequency and duration of post-traumatic seizures	Investigational	(87)
		↓ excitotoxic loss of inhibitory interneurons			(88)
		↑ intracellular glutathione and ↓ oxidative stress			(89)
Sulfasalazine	System x_c^- transporter inhibitor	↓ extracellular level of glutamate	↓ seizures in a murine model of tumor-associated epilepsy	Investigational	(32)
		↑ intracellular glutathione and ↓ oxidative stress			
LY367385, LY339840	mGluR1 antagonist	↓ excitatory neurotransmission	Potent anticonvulsant activity in animal models of seizures	Investigational	(90, 91)
		↓ glutamate release from presynaptic terminals and perisynaptic astrocytic processes			(92)
MPEP	mGluR5 negative allosteric modulator	↓ excitatory neurotransmission	Potent anticonvulsant activity in animal models of seizures	Investigational	(93, 94)
		↓ glutamate release from presynaptic terminals and perisynaptic astrocytic processes			(92)
S-4C3HPG	mGluR1 antagonist, mGluR2 agonist	↓ glutamate release and neurotransmission	Protects against audiogenic seizures in DBA/2 mice	Investigational	(95)
			Suppresses PTZ and DMCM-induced seizures		(96)

(Continued)

TABLE 1 | Continued

Drug	Brief mode of action	Effects on glutamatergic system	Effects on seizures	Developmental Stage	Reference
2R,4R-APDC	Group 2 mGluR agonist	↓ glutamate release and neurotransmission	Enhance seizure threshold in a rat model of amygdala kindling	Investigational	(97)
DCG-IV	Group 2 mGluR agonist	↓ glutamate release and neurotransmission	↓ kainate and amygdala kindling-induced seizures	Investigational	(98, 99)
JNJ-42153605, JNJ-40411813, JNJ-46356479	mGluR2 positive allosteric modulator	↓ glutamate release and neurotransmission	Anticonvulsant effect in the mouse 6-Hz and corneal kindling models Enhances antiseizure efficacy of levetiracetam	Investigational	(100, 101)
LY404039	Group 2 mGluR agonist	↓ glutamate release and neurotransmission	Anticonvulsant effect in a model of 6 Hz psychomotor seizures Enhances antiseizure efficacy of levetiracetam	Investigational	(100)

17AAG, 17-allylamino-17-demethoxygeldanamycin; HSP90β, Heat shock protein 90β; MPEP, 2-methyl-6-phenylethynyl-pyridine; S-4C3HPG, S-4-carboxy-3-hydroxyphenylglycine; PTZ, pentylenetetrazol; DMCM, methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate; 2R,4R-APDC, 2R,4R-1-aminocyclopentane dicarboxylic acid; DCG-IV, 2S,2′R,3′R-2-(2′,3′-dicarboxycyclopropyl)glycine.

of epilepsy (74, 75). To understand how changes in astrocytic mGluR3 or mGluR5 activity may affect seizure generation, one study looked at the effects of adding Group I and Group II modulators to cultured astrocytes (76). This paper found that astrocytic Group 1 mGluR activation, via the use of (S)-3,5-dihydroxyphenylglycine [(S)-3,5-DHPG], led to decreased expression of glutamate transporters (GLAST and GLT-1), and that this effect could be antagonized using a selective mGluR5 antagonist, MPEP. Conversely, astrocytic Group II mGluR activation, using DCG-IV, resulted in upregulation of GLAST and GLT-1 expression, and this effect could be abolished using the Group II antagonist EGLU. As mGluR2 has not been found to be expressed by astrocytes, the authors concluded that the effects of Group II modulation in these experiments was attributable to astrocytic mGluR3 activity.

Questions remain and more work certainly needs to be done to further elucidate the role of mGluRs in epilepsy, however, it appears evident that mGluR expression and activity are significantly altered both in animal models and human epileptic tissue in ways that contribute to hyperexcitability and epileptogenesis. Although ionotropic and metabotropic glutamate receptors differ fundamentally in their structure and downstream effectors, NMDARs and mGluRs share the ability to sense changes in ambient glutamate and potentiate neuronal inward currents.

DISCUSSION

Supporting the notion that astrocytes are critically involved in the initiation of seizures through dysregulation of ambient glutamate, a recent *in silico* study found that increased ambient glutamate, either through increased astrocytic glutamate release or decreased uptake, was sufficient to initiate synchronous epileptiform-like discharges from neurons (77). Additionally, the use of transparent zebrafish combined with two-photon calcium imaging and local field potential recordings allowed investigators to monitor the activity and connectivity of thousands of neurons and glia revealing new insights into the role glia may play in seizure generation (78). Using PTZ, a GABAaR antagonist, to initiate seizures investigators found that pre-ictal neuronal activity did not significantly increase in synchrony, however, radial glia exhibited a significant increase in synchrony during the pre-ictal period followed by neuronal bursts. These findings support the idea that the local, pro-epileptic glial responses presented throughout this review can culminate in network-level events that can ultimately lead to neuronal hyperexcitability synchronization and seizure initiation.

Knowing that current anti-seizure therapeutics are ineffective for 1-in-3 people living with epilepsy (1, 2), elucidating the astrocytic processes involved in epileptogenesis may help identify new therapeutic targets that offer relief to patients with intractable epilepsy. **Table 1** summarizes current FDA-approved and investigational pharmacotherapies targeting glutamate signaling for epilepsy. In summary, astrocytes exclusively possess the enzymatic activity to generate *de novo* glutamate in the CNS, as well as the molecular machinery to determine ambient glutamate through (1) a reduction of ECS, (2) glutamate release through SXC or VRAC or (3) a dysregulated state combining these mechanisms. Importantly, ambient glutamate can directly influence neuronal excitability, via generation of GluN2B-mediated SICs, as well as indirectly affect neuronal excitability via actions on mGluRs that can potentiate GluN2B-mediated currents, alter extracellular glutamatergic clearance, and influence neuronal glutamate release probabilities. Although inherent challenges exist in

ubiquitously targeting glutamatergic mechanisms in the CNS, it appears clear that astrocytes are uniquely positioned to act as a master regulator of glutamate in health, and when dysregulated, can mediate pro-epileptic changes that warrant further investigation in hopes of unveiling novel therapeutic targets.

AUTHOR CONTRIBUTIONS

OA did an exhaustive literature search, generated a complete draft of the review, and prepared the figure. DP contributed written material and prepared **Table 1**. DP, BT, and HS also reviewed the literature, provided detailed comments and edits to the review, figure, and table. All authors contributed to the article and approved the submitted version.

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REVIEW
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Astroglial Glutamine Synthetase and the Pathogenesis of Mesial Temporal Lobe Epilepsy

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The enzyme glutamine synthetase (GS), also referred to as glutamate ammonia ligase, is abundant in astrocytes and catalyzes the conversion of ammonia and glutamate to glutamine. Deficiency or dysfunction of astrocytic GS in discrete brain regions have been associated with several types of epilepsy, including medically-intractable mesial temporal lobe epilepsy (MTLE), neocortical epilepsies, and glioblastoma-associated epilepsy. Moreover, experimental inhibition or deletion of GS in the entorhinal-hippocampal territory of laboratory animals causes an MTLE-like syndrome characterized by spontaneous, recurrent hippocampal-onset seizures, loss of hippocampal neurons, and in some cases comorbid depressive-like features. The goal of this review is to summarize and discuss the possible roles of astroglial GS in the pathogenesis of epilepsy.

Keywords: epilepsy, epileptogenesis, glutamine synthetase, astrocyte, epilepsy network, mesial temporal lobe epilepsy

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INTRODUCTION

Astrocytes have historically been thought to serve a primarily structural role by supporting surrounding neurons (1). Over the last 40 years, however, a growing body of evidence has suggested that astrocytes serve important roles in normal brain function, and are critical for axonal growth, energy metabolism, neurotransmitter homeostasis and water/electrolyte balance (2–11). Moreover, abnormal astrocyte function has been postulated to contribute to the pathogenesis of a wide range of neurological and psychiatric disorders (12–17).

Following an acute central nervous system (CNS) injury, astrocytes undergo several morphological and functional changes. These "reactive astrocytes" are present in several pathological conditions While reactive astrocytes were originally thought to reflect scar tissue in response to neuronal injury and loss, recent studies have suggested that reactive astrocytes may in fact play important roles in the causation of many disorders, including epilepsy (18–20).

Glutamine synthetase (GS, also known as glutamate-ammonia-ligase, EC 6.3.1.2), an enzyme that is highly abundant in astrocytes, is of particular interest due to its roles in health and disease (21). Systemic mutations of the GS gene have been associated with brain malformations, seizures, multiorgan failure, and early death (22, 23). Studies have further suggested that acquired GS deficiencies in discrete areas of the brain might play a causative role in various neurological disorders and psychiatric conditions including Alzheimer's disease, hepatic encephalopathy,

suicide/depression schizophrenia, and epilepsy (24–32). The goal of this review is to discuss the significance of GS in the pathogenesis of focal epilepsies, particularly mesial temporal lobe epilepsy (MTLE), which is one of the most common types of medication-refractory epilepsies in humans (26, 32–35).

GLUTAMINE SYNTHETASE AND NORMAL PHYSIOLOGY

GS serves important roles in nitrogen metabolism, acid-base homeostasis, and cell signaling in many species of prokaryotes and eukaryotes (36–38). GS is thought to eliminate or reduce the toxic effects of glutamate and ammonia in the mammalian CNS by metabolizing these compounds (39–41). Moreover, multiple physiological processes, including synthesis of glutamate and GABA, synthesis of proteins, and osmoregulation, rely on a steady supply of glutamine (42). Because GS is the only enzyme capable of synthesizing large amounts of glutamine in the human body, changes in its expression and activity are expected to have significant consequences for normal physiology.

GS and the Glutamine-Glutamate-GABA Cycle

After its release from the presynaptic neuron, neurotransmitter glutamate interacts with receptors in the postsynaptic membrane, followed by its removal from the synapse into astrocytes. After uptake into astrocytes, glutamate may be converted to glutamine via glutamine synthetase (**Figure 1**). Glutamine can subsequently be transferred from astrocytes to glutamatergic neurons (44, 45). Once in the glutamatergic neuron, the mitochondrial enzyme phosphate-activated glutaminase (PAG) converts glutamine to glutamate (46, 47), which is concentrated in synaptic vesicles and subsequently released into the extracellular space in response

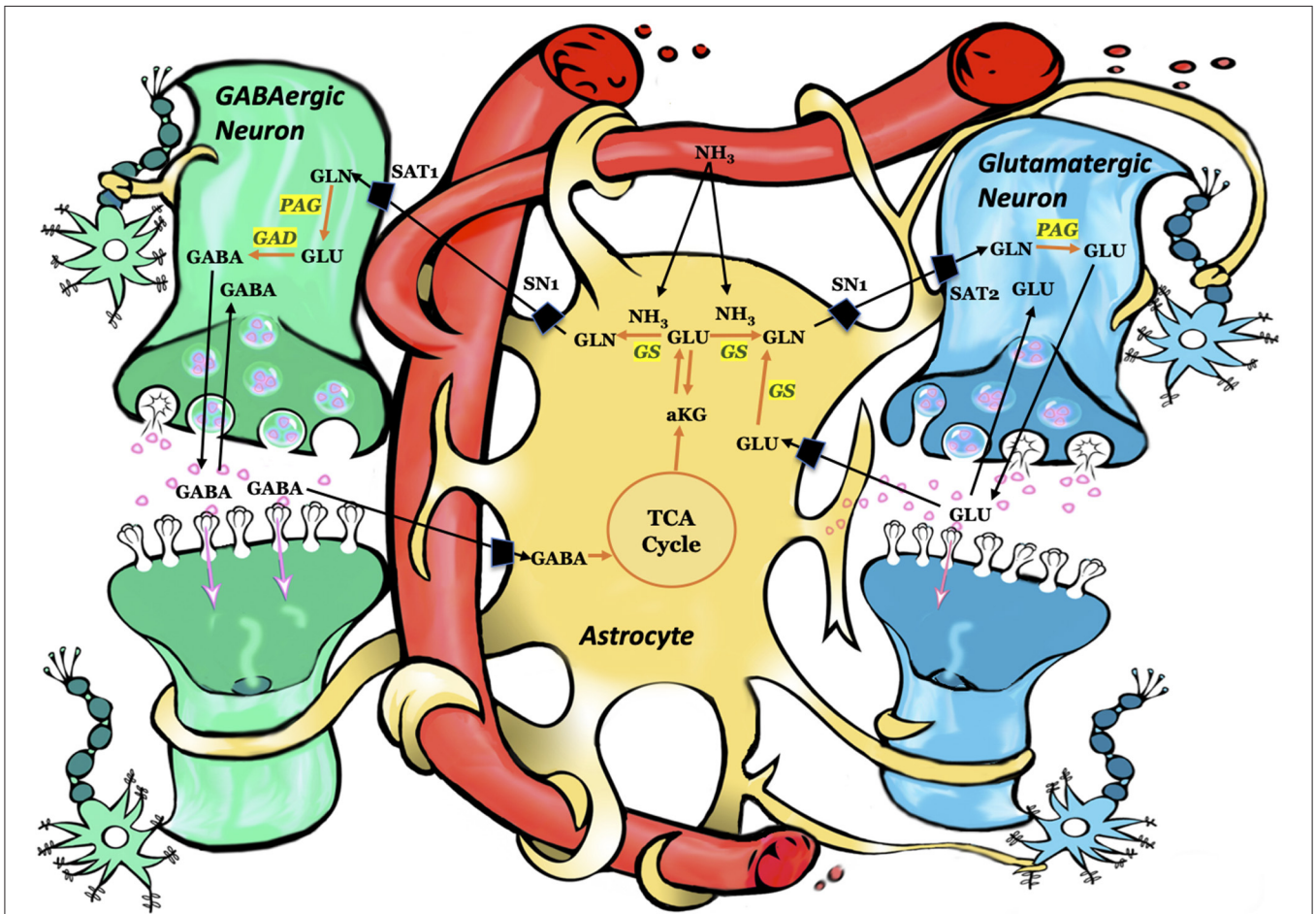

FIGURE 1 | Schematic representation of key pathways involving astrocytes, glutamatergic neurons, and GABAergic neurons. Each arrow signifies several reactions. Using ammonia, glutamine synthetase converts glutamate to glutamine. Subsequently, glutamine is taken up by the adjacent neurons and converted to glutamate or GABA. Astrocytes take up the synaptic glutamate (glutamine–glutamate cycle) or GABA (glutamine–glutamate–GABA cycle) and converts these neurotransmitters to glutamine via glutamine synthetase. GLN, glutamine; GLU, glutamate; GABA, gamma-aminobutyric acid; aKG, alpha ketoglutarate; TCA, tricarboxylic acid cycle; GS, glutamine synthetase; PAG, phosphate-activated glutaminase; GAD, glutamic acid decarboxylase; SN1, system N transporter 1; SAT1, system A transporter and SAT2, system A transporter 2. Figure adapted with permission from (43).

to an action potential. The glutamate is either taken up by astrocytes and converted back to glutamine via GS or taken up by inhibitory neurons and metabolized to GABA, which is used during inhibitory neurotransmission (**Figure 1**). Astrocytic glutamine can be taken up by GABAergic neuron as precursor for the inhibitory neurotransmitter γ-aminobutyric acid (GABA) via glutamate (48, 49). While most of the released GABA is taken up by the pre-synaptic neuron, some enters astrocytes where it is converted back to glutamine (50). Thus, astroglial GS is crucial for both excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission (51).

GS and Ammonia Detoxification

The human body contains large amounts of ammonia, produced mainly by the action of bacterial enzymes on colonic content and from the hydrolysis of glutamine in the small and large intestinal cells. While the gut-derived ammonia is mostly metabolized by the liver, via the urea cycle and the GS reaction, a small amount of ammonia (10–30 μmol/L) remains in the plasma under normal conditions. Much higher plasma ammonia concentrations are detected in pathological conditions such as liver disease and urea cycle disorders. Because ammonia is neurotoxic and easily crosses the blood-brain-barrier, an efficient mechanism for clearing brain ammonia is essential (52). Astroglial GS is critical for such clearance, because the CNS lacks a functional urea cycle (52). GS utilizes ammonia, that is taken up by astrocytes, to convert glutamate to glutamine (**Figure 1**). The important role of astrocytes in this process is underscored by the presence of astrocytic end-feet surrounding the brain endothelial cells, which serve as a metabolic buffer between the blood and the brain, thereby reducing the toxic load of ammonia on neurons (53–55).

GLUTAMINE SYNTHETASE AND MESIAL TEMPORAL LOBE EPILEPSY

Glutamate is the predominant excitatory neurotransmitter in the adult brain, and perturbed extracellular brain glutamate levels have been implicated in the pathogenesis of epilepsy, particularly MTLE. Notably, extracellular glutamate is chronically elevated in the epileptogenic hippocampus (the seizure onset area) in human patients with MTLE, as ascertained by simultaneous depth electrode EEG and *in vivo* brain microdialysis (56–59). In addition, during seizure activity, a six-fold increase in the extracellular hippocampal glutamate above the basal (chronic) level was observed, followed by a slow decline to basal levels over a period of several minutes (60). Furthermore, many animal studies have suggested a causational relationship between increased brain glutamate signaling and epilepsy (39, 61, 62). Therefore, it is possible that an excessive amount of extracellular glutamate in the seizure onset area of the brain acts as a central metabolic cause of the neuronal loss and spontaneous seizures associated with MTLE (39, 61, 62).

Because GS is thought to be critical for glutamate metabolism following uptake into astrocytes, deficiency in this enzyme has been postulated as a possible basis for the increased glutamate observed in the extracellular fluid of the epileptogenic areas of the brain (26, 62). Moreover, isotopic tracer (C^{13}) studies suggest that a slowing of the glutamate-glutamine cycle metabolism in the epileptogenic hippocampus is responsible for the accumulation and reduced clearance of glutamate in MTLE (63). Given the essentiality of GS in the glutamate-glutamine cycle, we and others sought to quantify activity of astroglial GS in the epileptogenic hippocampus in human patients with MTLE (26, 32). Intriguingly, the protein content and functional activity of GS was reduced by ∼40% in subfields of the epileptogenic hippocampal formation in patients with MTLE and concomitant mesial temporal sclerosis (26, 32). Similarly, GS deficiency in the amygdala occurs in some patients with neocortical epilepsies (64) and in the tumor tissue of patients with malignant gliomas and secondary epilepsy (65).

Furthermore, several other studies support the idea that GS deficiency is a causative or contributing factor in some types of epilepsies besides MTLE. In the first three known cases of congenital, homozygous mutations in the GS gene, the patients had severe brain malformations and epileptic seizures (22, 66). Two of the patients died shortly after birth (22). Similarly, the high morbidity and mortality rates that accompany genetic GS deficiencies in humans are also observed in transgenic mouse models. For example, animals with prenatal excisions of the GS gene in all cell types do not survive past early embryonic development (67), and mice with selective gene deletions in GFAP-positive astrocytes survive until postnatal day 3 (68). However, mice with selective deletions in the neocortex and hippocampus are born without any apparent malformations but develop neurodegeneration and spontaneous seizures that seem to increase in frequency with age (34).

A common approach to assess the effect of GS deficiencies in specific brain regions involves the use of methionine sulfoximine (MSO). MSO impedes the ability of GS to catalyze the conversion of glutamate and ammonia to glutamine by irreversibly binding to the catalytic sites of GS (69). In one study, a continuous infusion of MSO into the right hippocampal formation of normal adult Sprague-Dawley rats was compared to a continuous infusion of normal saline into the same region of a separate group of rats. The animals were monitored by continuous video-intracranial electroencephalogram (EEG) recordings for several weeks and the brains were analyzed for GS activity. MSO resulted in a reduction of GS activity in the infused hippocampal formation of ∼80% (28), and the animals displayed repetitive seizures that began several hours after the onset of infusion. The initial repetitive seizures, which lasted between 24 and 48 h, were followed by a quiet period of variable length before spontaneous, recurrent seizures commenced (70). The MSO-treated animals sometimes exhibited glial proliferation and patterned neuron loss in the infused hippocampal formation, similar to that of human MTLE (28, 33).

Another study analyzed whether the neuroanatomical site of GS inhibition is an important determinant for the epileptogenic process and for the epileptic phenotype. MSO was infused unilaterally into different limbic regions of adult rats, including the angular bundle, the deep entorhinal cortex, area CA1, the molecular layer of the subiculum, the hilus of the dentate gyrus, the lateral ventricle, and the central nucleus of the amygdala

(71, 72). Recurrent seizures were observed in all animals infused with MSO into the brain tissue, and the seizures increased in severity (Racine grade) over a period of several weeks with variations in the seizure frequency and severity patterns between brain regions. Moreover, animals infused with MSO into the central nucleus of the amygdala displayed recurrent seizures with depressive-like behaviors, as observed by a reduction of sucrose consumption in the sucrose preference test (72). Collectively, these studies suggest that the neuroanatomical site of GS inhibition affects the epileptogenic process as well as the overall phenotype of the disease.

GLUTAMINE SYNTHETASE AND EPILEPSY NETWORKS

The prevalent idea that the neuroanatomical and electrophysiological substrates of focal epilepsies occur only in a circumscribed brain region, the seizure focus, has been questioned more recently (73–77). Many clinical and EEG studies have suggested that large-scale, aberrant "brain networks" play key roles in seizure initiation and propagation, and that aberrant network activity may be present even during the time between seizures (76–85).

Albright et al. analyzed changes in neuronal networks during epileptogenesis in the intrahippocampal MSO-infusion model of MLTE (86). Intracranial EEG recordings and c-Fos immunohistochemistry were used to record seizure-associated neuronal activation at different stages during epileptogenesis. It was found that low-grade seizures during the earliest stages of epileptogenesis activated neurons in the entorhinal-hippocampal territory, the basolateral amygdala, the piriform cortex, the midline thalamus, and the anterior olfactory area. However, during later stages of epileptogenesis, when the seizures were more severe, neuronal activation was evident in extensive areas of the brain, such as the neocortex, the bed nucleus of the stria terminalis, the mediodorsal thalamus, and the central nucleus of the amygdala. These areas were activated in addition to the areas activated during early epileptogenesis.

In another study, whole brain diffusion tensor imaging (DTI) was used to assess any structural changes during early and late epileptogenesis in the hippocampal MSO-infusion model of MTLE (33). There were significant changes in fractional anisotropy (FA) in multiple brain regions in MSO-infused vs. phosphate buffered saline (PBS)-infused control animals. The changes in FA were markedly different in early epileptogenesis compared to late epileptogenesis, suggesting that inhibition of GS in one hippocampal formation results in structural changes that affect multiple brain regions and change over a period of several weeks (33, 71, 86).

CONCLUSION

An increasing number of studies in humans and animal models have linked astroglial GS dysfunction to the pathogenesis of focal epilepsies, particularly MTLE. Even very small and circumscribed deficiencies in GS affecting a subfield of the hippocampal formation or a nucleus of the amygdala can lead to epileptic seizures and comorbid, depressive-like features in laboratory animals. Moreover, such small deficiencies result in widespread and progressive changes in neuronal network activation and in the structure of the brain. While the mechanism by which GS dysfunction causes epilepsy remains unclear, several scenarios are possible such as reduced clearance of extracellular brain glutamate and ammonia, glutamine deficiency, and perturbed glutamatergic and GABAergic neurotransmission. Further studies are needed to determine the causes of astroglial GS deficiency so that more effective treatments can be developed to prevent such deficiencies and combat the development and progression of GS-associated epilepsies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Initiation of Experimental Temporal Lobe Epilepsy by Early Astrocyte Uncoupling Is Independent of TGFβR1/ALK5 Signaling

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Blood–brain barrier (BBB) dysfunction following brain insults has been associated with the development and progression of focal epilepsy, although the underlying molecular mechanisms are not fully elucidated yet. Activation of transforming growth factor beta (TGFβ) signaling in astrocytes by extravasated albumin impairs the ability of astrocytes to properly interact with neurons, eventually leading to epileptiform activity. We used the unilateral intracortical kainate mouse model of temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) to gain further insights into the role of BBB leakage in *status epilepticus* (SE)-induced epileptogenesis. Immunohistochemical examination revealed pronounced albumin extravasation already 4 h after SE induction. Astrocytes were virtually devoid of albumin immunoreactivity (IR), indicating the lack of uptake by this time point. Inhibition of the TGFβ pathway by the specific TGFβ receptor 1 (TGFβR1) kinase inhibitor IPW-5371 did not prevent seizure-induced reduction of astrocytic gap junction coupling. Thus, loss of coupling, which is thought to play a causative role in triggering TLE-HS, is most likely not mediated by extravasated albumin. Continuous telemetric EEG recordings and video monitoring performed over a period of 4 weeks after epilepsy induction revealed that inhibition of the TGFβ pathway during the initial phase of epileptogenesis slightly attenuated acute and chronic epileptiform activity, but did not reduce the extent of HS. Together, these data indicate that albumin extravasation due to increased BBB permeability and TGFβ pathway activation during the first hours after SE induction are not significantly involved in initiating TLE.

Keywords: blood–brain barrier dysfunction, albumin extravasation, temporal lobe epilepsy, gap junctional coupling, astrocyte, transforming growth factor beta

KEY POINTS

- Strong albumin extravasation, but no astrocytic uptake, was detected 4 h after epilepsy induction.
- Seizure-induced reduction of astrocytic gap junction coupling was independent of TGFβR1/ALK5 signaling.
- Inhibition of TGFβR1/ALK5 signaling during early epileptogenesis slightly attenuated epileptiform activity but did not prevent the development of hippocampal sclerosis in experimental temporal lobe epilepsy.

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INTRODUCTION

Epilepsy is often preceded by epileptogenic brain insults including traumatic brain injury, febrile seizures, or stroke (1–3). Impairment in blood–brain barrier (BBB) integrity is often associated with these insults and also occurs in the sclerotic hippocampus of patients with temporal lobe epilepsy (TLE) and corresponding animal models (4–7). Blood–brain barrier leakage leads to extravasation of the serum protein albumin into the brain parenchyma, which promotes epileptiform activity both *in situ* and *in vivo* (6, 8–10). Interestingly, serum albumin was proposed to be endocytosed into astrocytes *via* binding to transforming growth factor beta (TGFβ) receptors and to activate TGFβ signaling pathways (5, 6, 8). Albumin-induced TGFβ signaling in astrocytes in turn provokes several physiological changes in these cells associated with the development of epilepsy, including excitatory synaptogenesis, impaired K^+ and glutamate buffering, and production of pro-inflammatory cytokines (6, 11–13). Previously, we could demonstrate that disruption of astrocytic gap junction coupling and concomitant impairment of extracellular K^+ buffering precede neuronal cell death in kainate-induced TLE, pointing toward a causative role in epileptogenesis (14). However, the underlying signaling pathway remains ill-defined. Interestingly, our previous work showed that albumin injected into the lateral ventricle is transported into hippocampal astrocytes leading to impaired astrocytic gap junction coupling 24 h after albumin injection (15). This was further supported by other findings demonstrating significant downregulation of transcripts for both astroglial gap junction-forming connexins, 24 h after albumin treatment in the rat brain (5, 11, 16). This suggests that albumin uptake into astrocytes promotes gap junction uncoupling *via* activation of TGFβ signaling, leading to impaired spatial buffering of extracellular K^+ and thereby promoting epileptic activity. To test this hypothesis, here we assessed in a mouse model of TLE whether aberrant albumin-mediated TGFβR1/ALK5 signaling upon kainate-induced *status epilepticus* (SE) induces astrocyte uncoupling and epileptogenesis. We took advantage of a novel TGFβR1 kinase inhibitor, IPW-5371, which effectively reduced TGFβ signaling in the hippocampus of mice receiving intraventricular infusion of albumin (17). Immunohistochemistry, tracer diffusion studies, and continuous telemetric electroencephalography (EEG) were combined to assess the consequences of early inhibition of TGFβR1/ALK5 signaling on astrocytic coupling, seizure activity, and the development of HS.

MATERIALS AND METHODS

Animals

Male C57B6/J (Charles River, Sulzfeld, Germany, or bred in-house) mice aged 90–120 days were used for the experiments. Maintenance and handling of animals was performed according to local governmental regulations. Experiments were approved by the North Rhine–Westphalia State Agency for Nature, Environment and Consumer Protection (approval number 84-02.04.2015.A393). All measures were taken to minimize the number of animals used. Mice were kept under standard housing conditions (12 h/12 h dark–light cycle) with food and water provided *ad libitum*.

Unilateral Intracortical Kainate Injection and Implantation of Telemetric Electroencephalography Transmitters

We employed the TLE-HS animal model as described previously (13, 18). Briefly, mice were anesthetized with a mixture of medetomidine (Cepetor, CP-Pharma, Burgdorf, Germany, 0.3 mg/kg, i.p.) and ketamine (Ketamidor, WDT, Garbsen, Germany, 40 mg/kg, i.p.) and placed into a stereotaxic frame equipped with a manual microinjection unit (TSE Systems GmbH, Bad Homburg, Germany). A total volume of 70 nl of a 20-mM solution of kainate (Tocris, Bristol, UK) in 0.9% sterile NaCl was stereotactically injected into the neocortex just above the right dorsal hippocampus. The stereotactic coordinates were 2 mm posterior to bregma, 1.5 mm from midline, and 1.7 mm from the skull surface. Sham control mice received injections of 70 nl saline under the same conditions. Directly after kainate injection, two drill holes were made at 1 mm posterior to the injection site and 1.5 mm lateral from midline for insertion of two monopolar leads required for electrographic seizure detection. Telemetric transmitters [TA10EA-F20 or TA11ETA-F10; Data Sciences International (DSI), St. Paul, MN, USA] were implanted subcutaneously into the right abdominal region, and both monopolar leads were inserted ∼1 mm into the cortex. Attached leads were fixed to the skull using superglue and then covered with dental cement. Subsequently, the scalp incision was sutured and anesthesia stopped with atipamezol (Antisedan, Orion Pharma, Hamburg, Germany, 300 mg/kg, i.p.). To reduce pain, mice were injected for 3 days with carprofen (Rimadyl, Pfizer, Karlsruhe, Germany). Moreover, 0.25% enrofloxacin (Baytril, Bayer, Leverkusen, Germany) was administered *via* drinking water to reduce risk of infection. After surgery, mice were returned to clean cages and placed on individual radio receiving plates (RPC-1; Data Sciences International, New Brighton, MN, USA), which capture data signals from the transmitter and send them to a computer using the Ponemah software (Version 5.2, Data Sciences International) to convert the digital output of the receiver into a calibrated analog output. A video surveillance system (Bascom, Düsseldorf, Germany) was used to monitor behavioral seizure activity. Electroencephalography recordings (24 h/day, 7 days/week) were started immediately after transmitter implantation and continued for 28 days following the induction of SE.

Electroencephalography Analysis

Electroencephalography data were analyzed using NeuroScore (version 3.3.1) software (Data Sciences International) as described previously (19). Briefly, seizure frequency and duration as well as spike numbers was determined using the spike train analysis tool implemented in NeuroScore with the following criteria: threshold value = 7.5 × SD of the baseline (i.e., activity during artifact- and epileptiform-free epochs) – 1,000 μV, spike duration = 0.1–50 ms, spike interval = 0.1–2.5 s, minimum train duration = 30 s, train join interval = 1 s, and minimum

number of spikes = 50. Prior to spike analysis, recordings were high pass filtered at 1 Hz. Electroencephalography recordings were additionally verified by manual screening. Fast Fourier transformation (FFT) was performed to derive absolute δ (0.5–4 Hz), θ (4–8 Hz), α (8–13 Hz), β (13–30 Hz), and γ (30–50 Hz) power values during SE and the chronic phase, which were subsequently normalized to baseline activity prior to conducting statistics. The number of spontaneously generalized seizures during the chronic phase was determined manually by two experienced experimenters.

IPW-5371 Treatment

IPW-5371 was prepared as a suspension formulation (2 mg/ml IPW-5371 in 0.5% methylcellulose dissolved in 0.9% NaCl and Tween® 80) and applied once per 16–24 h and again ∼15 min prior to kainate application (i.p. injection, 20 mg/kg). Daily i.p. injections of IPW-5371 at 20 mg/kg for 2 days effectively reduce TGFβ signaling in the hippocampus of mice receiving intraventricular infusion of albumin (17). As a control, mice received vehicle (saline) injections under the same conditions.

Electrophysiology and Biocytin Loading of Astrocytes

Mice were anesthetized with isoflurane (Piramal Healthcare, Morpeth, UK) and decapitated. Next, brains were quickly removed and 200-μm-thick coronal slices were cut on a vibratome (VT1000S, Leica, Wetzlar, Germany) in an ice-cold preparation solution containing the following (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 glucose, and 75 sucrose, equilibrated with carbogen (5% CO_2/95% O_2, pH 7.4). After storage of slices (15 min, 35°C) in preparation solution, the slices were transferred to a solution containing the following (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 1.25 NaH_2PO_4, and 26 $NaHCO_3$ and gassed with carbogen to stabilize pH at 7.4 [artificial cerebrospinal fluid (aCSF)]. To aid in the identification of astrocytes in the tissue, aCSF was supplemented with SR101 (1 μM, Sigma Aldrich, S7635, Steinheim, Germany; incubation 20 min, 35°C) (20). After SR101 staining, slices were transferred to aCSF and kept at room temperature (RT) for the duration of the experiments. For recordings, slices were transferred to a recording chamber and constantly perfused with aCSF. Patch pipettes fabricated from borosilicate capillaries with a resistance of 3–6 MΩ were filled with a solution containing the following (in mM): 130 K-gluconate, 1 $MgCl_2$, 3 Na_2-ATP, 20 HEPES, 10 EGTA, and biocytin (0.5%, Sigma Aldrich) (pH 7.2, 280–285 mOsm). For the analysis of gap junction coupling, whole-cell patch clamp recordings of SR101-positive astrocytes were performed during which astrocytes were filled with biocytin (20 min, RT). In addition to SR101 staining, astrocytes were identified by their characteristic morphology, small soma size, passive current–voltage relationship, and a resting membrane potential close to the Nernst potential for K^+. Current signals were amplified (EPC 8, HEKA Electronic, Lambrecht, Germany), filtered at 3 or 10 kHz, and sampled at 10 or 30 kHz (holding potential −70 mV). Online analysis was performed with TIDA 5.25 acquisition and analysis software for Windows (HEKA) and Igor Pro 6.37 software (WaveMetrics, Lake Oswego, OR, USA). Voltages were corrected for liquid junction potentials. Only recordings matching the following criteria were included in the analysis: (i) resting potential negative to −60 mV, (ii) membrane resistance ≤10 MΩ, and (iii) series resistance ≤20 MΩ.

Immunohistochemistry

Tissue Preparation

Animals were deeply anesthetized by i.p. injection with 100–120 μl of a solution containing 80 mg/kg ketamine (Ketamidor, WDT, Garbsen, Germany) and 1.2 mg/kg xylazine hydrochloride (Sigma-Aldrich). After checking for hind paw reflexes, transcardial perfusion was applied with ice-cold PBS (30 ml) followed by 4% ice-cold PFA in PBS (30 ml). Brains were removed and stored overnight in 4% PFA-containing solution and subsequently stored in PBS at 4°C until slicing. Brains were cut into 40-μm-thick coronal slices using a Leica VT1200S vibratome (Leica Microsystems).

Staining

Immunohistochemistry was performed using free-floating slices kept in 24-well plates. Only slices from the dorsal hippocampus close to the injection site were used for staining. For membrane permeabilization and blocking of unspecific epitopes, slices were incubated (2 h, RT) with 0.5% Triton X-100 (or 2% for staining of biocytin-filled astrocytes) and 10% normal goat serum (NGS) in PBS. For immunostaining of albumin, no serum was applied during blocking and permeabilization steps. Slices were subsequently incubated overnight with primary antibody solution containing PBS on a shaker at 4°C. The following primary antibodies were applied: rabbit anti-GFAP (1:500, DAKO, Z0334, Hamburg, Germany), goat anti-albumin (1:200, Abcam, ab19194, Berlin, Germany), and mouse anti-NeuN (1:200, Merck Millipore, MAB377, Darmstadt, Germany). On the following day, slices were washed three times with PBS for 10 min each, followed by incubation with secondary antibodies conjugated with Alexa Fluor® 488, Alexa Fluor® 647, or streptavidin-conjugated Alexa Fluor® 647 (1:500 or 1:600, respectively, Invitrogen, Karlsruhe, Germany) in PBS (2% NGS, 1.5–2 h, RT). For staining of NeuN, slices were incubated with goat anti-mouse biotin (1:500, Dianova, AB_2338557, Hamburg, Germany) prior to incubation with streptavidin-conjugated Cy3 antibody (1:300, Sigma Aldrich, S6402; 1 h, RT). After washing the slices again three times with PBS (10 min), nuclear staining with Hoechst (1:200, diluted in dH_2O) was performed (10 min, RT). A final washing step (3× PBS, 5 min each) was performed and slices were mounted with Aqua-Poly/Mount (Polysciences, Heidelberg, Germany) on objective slides and covered with coverslips. Slides were stored at 4°C before confocal imaging.

Confocal Microscopy

Slides were imaged using a confocal laser scanning microscope (SP8, Leica, Hamburg, Germany) at 8 bit using 20× [numerical aperture (NA): 0.75], 40× (NA: 1.1), and 63× (NA: 1.2) objectives. Image resolution was set at 1,024 × 1,024 pixels recorded at a speed of 400 Hz, with a pinhole size of 1 airy unit (AU) and a digital zoom of 1 (albumin extravasation), 1.2

(GJ coupling), or 2 (colocalization). Standard photomultiplier tubes were used for the detection of fluorescent signals. Laser and detector settings were applied equally to all images acquired. Z-stacks were taken at 2 μm (albumin extravasation and GJ coupling) or 0.3 μm (colocalization) intervals.

Quantification of Immunostainings

Immunohistochemical stainings were quantified either using Fiji/ImageJ (21) or Imaris 8.0 software (Bitplane, Zürich, Switzerland).

Albumin Extravasation

Albumin extravasation in the parenchyma was estimated by measuring the fluorescent intensity in the albumin channel in maximum intensity projections (MIP) using the image processing package Fiji. Initially, images were background subtracted using the rolling ball algorithm implemented in Fiji, with a radius set at 50 pixels (22). Albumin immunoreactivity (IR) was subsequently determined by quantifying the average pixel intensity in the albumin channel within the imaged field of view (290 × 290 × 20 μm).

Colocalization of Albumin and GFAP

Albumin content was quantified in albumin/GFAP double-stained hippocampal sections of C57B6J mice injected with kainate 4 h prior to brain perfusion. Images were analyzed equally applying a custom-written macro in Fiji software. Fluorescent intensity analysis was performed in regions of interest (ROIs) of 92 × 92 × 10 μm^3. In a first step, the $GFAP^+$ image was median filtered (5 pixel radius) and subsequently converted into a binary image applying the Triangle threshold algorithm implemented in Fiji (23). Next, the binary $GFAP^+$ images and the $albumin^+$ images were multiplied to derive albumin signal intensity in $GFAP^+$ pixels. Within this multiplied image, the average pixel intensity value of albumin in $GFAP^+$ pixels was measured and subsequently summed up across all focal plains to obtain albumin contents in $GFAP^+$ cells. To determine albumin content in $GFAP^-$ pixels, the GFAP image was preprocessed applying filtering and thresholding steps as described above. Next, the binarized $GFAP^+$ image was subtracted from the $albumin^+$ image to obtain albumin signal intensity in $GFAP^-$ pixels. Average signal intensities were subsequently summed up across all focal plains for statistical comparison.

Coupling Efficiency in Biocytin-Filled Astrocytes

Coupling efficiency was determined by manual counting of $biocytin^+$ cells using the cell counter plugin for Fiji and compared between injected (ipsilateral) and non-injected (contralateral) hemispheres. Another observer blinded to the experimental conditions recounted images of biocytin-filled astrocytes, and cell counts were subsequently averaged across both counts prior to statistical analysis.

Hippocampal Sclerosis

The extent of hippocampal sclerosis (HS) was estimated based on the quantification of three parameters: (i) extent of granule cell dispersion (GCD) in the dentate gyrus (DG), (ii) shrinkage of the CA1 *stratum radiatum*, and (iii) number of pyramidal neurons in CA1 *stratum radiatum*. All three parameters were estimated in MIPs (1,163 × 1,163 × 40 μm^3). Granule cell dispersion quantification was performed as described previously (19). Briefly, GCL width was measured at four positions indicated as T1–T4. T1 and T2 were measured along a vertical line connecting the upper and lower cell layers of the DG, T3, and T4 at a distance halfway between the vertical line and the tip of the hilus. The average of the four values was used as an estimation of GCD. Shrinkage of the *stratum radiatum* was determined by drawing a vertical line connecting the pyramidal and molecular layer, above the peak of the DG granule cell layer. The length of the vertical line served as an indication of the remaining width of the *stratum radiatum*. Both GCL and *stratum radiatum* width were quantified using Fiji software. Finally, the number of pyramidal neurons in the CA1 region was quantified using the automated spot detection algorithm implemented in Imaris 8.0 within a 360 × 120 × 40-μm^3 ROI placed within the CA1 pyramidal layer just above the peak of the DG granule cell layer.

Statistical Analysis

Statistical analyses were performed using R software (R Core Team 2020, version 4.0.2, Austria) (24). Data are displayed as mean ± SD or as box plots representing median (line) and quartiles (25^{th} and 75^{th} percentile) with whiskers extending to the highest and lowest values within 1.5 times the interquartile range (IQR). Prior to statistical analysis, data were checked for normality by inspection of histograms as well as by statistically testing for normality using a Shapiro–Wilk test. Levene's test was performed to check for homogeneity of variance between groups. In case of a significant deviation from normality, data were transformed according to Tukey's ladder of powers (25) prior to conduction of statistical tests or by performing the appropriate non-parametric test. For comparison of two groups, Student's *t*-test or Wilcoxon-rank sum test was used. More than two groups were compared with one-way analysis of variance (ANOVA) followed by *post-hoc* Tukey test or using Kruskal–Wallis test with Dunn's *post-hoc* test. For multifactorial data, two-way ANOVA was conducted. Kaplan–Meier estimates were compared using a log-rank test. Differences between means were considered significant at $p < 0.05$.

RESULTS

Strong Albumin Extravasation but Negligible Astrocytic Uptake 4 h After *status epilepticus* Induction

Disruption of the BBB accompanied by albumin extravasation occurs in human and experimental epilepsy, and there is growing evidence that this represents not only a pathological consequence but also a causative factor in epileptogenesis (26, 27). To shed further light on this important topic, we used an experimental mouse model, unilateral intracortical kainate injection, that closely mimics human TLE-HS in terms of seizure types, neuropathological changes, and pattern of epileptogenesis (14). In this model, we first examined the extent of albumin

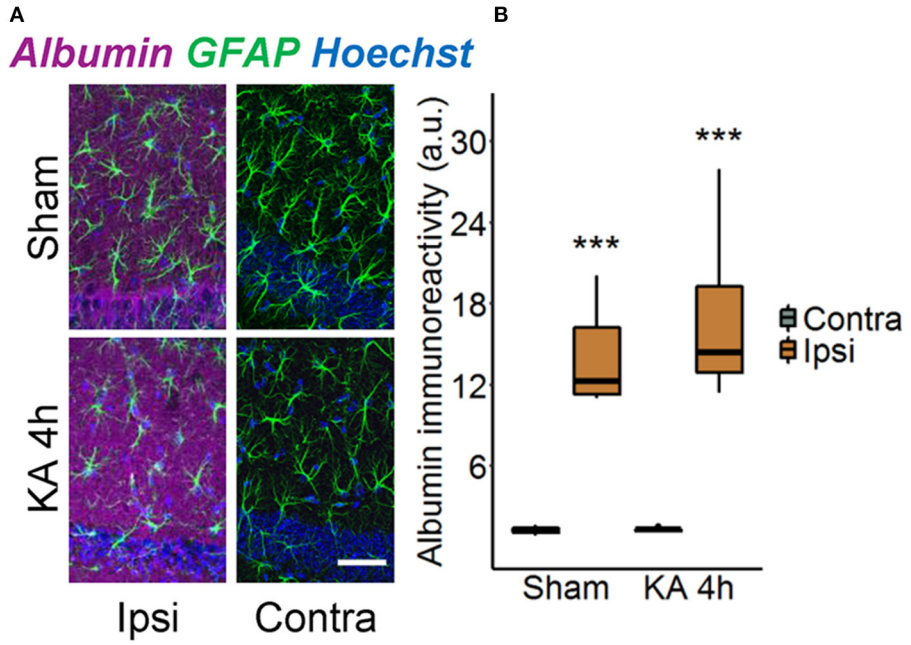

FIGURE 1 | Ipsilateral albumin extravasation 4 h after *status epilepticus* (SE) induction. **(A)** Representative maximum intensity projections depicting albumin (magenta), GFAP (green), and Hoechst (blue) labeling in ipsi- and contralateral hippocampal slices of sham- and kainate-injected mice. Scale bar: 50 μm. **(B)** Quantification of the staining revealed substantially higher ipsilateral vs. contralateral albumin extravasation after injection of both kainate and saline. Box plots represent median and quartiles. ***$p < 0.001$ vs. contralateral (two-way ANOVA). $N = 9$ slices from three mice/group. KA, kainate; au, arbitrary unit.

extravasation 4 h after kainate injection, a time point preceding neuronal cell death and the onset of spontaneous seizure activity (14). Immunohistochemical staining revealed strong extravascular albumin IR in the hippocampal CA1 region of the dorsal ipsilateral hippocampus (**Figure 1**, $p < 0.001$, two-way ANOVA). On the contralateral side, albumin was largely confined to blood vessels, indicating that BBB breakdown was restricted to the ipsilateral hippocampus. Surprisingly, we also detected a significant increase of albumin IR in the hippocampus of sham-injected mice. However, whereas albumin extravasation in epileptic mice persisted for months (26), it was a transient event in sham-injected mice and disappeared within 5 days after injection (data not shown).

Previous works demonstrated that TGFβ receptor-mediated uptake of extravasated serum albumin into astrocytes is involved in epileptogenesis (6, 16). We therefore examined astrocytic albumin contents in albumin/GFAP double-stained hippocampal sections injected with kainate 4 h before (**Figure 2A**). Quantification revealed only faint albumin IR (<1%) in GFAP-positive compared with GFAP-negative pixels in the hippocampal CA1 region of both hemispheres (**Figure 2B**), indicating negligible astrocytic albumin uptake even in regions of high extravasation. Albumin IR was slightly higher in ipsilateral compared with contralateral astrocytes (**Figure 2B**, $p = 0.016$, two-way ANOVA), but because this effect was extremely small, its biological relevance is unclear. In conclusion, these results show that 4 h after epilepsy induction, uptake of extravasated albumin by astrocytes is negligible.

Seizure-Induced Disruption of Astrocytic Coupling Is Independent of TGFßR1 Signaling

Disruption of astrocytic gap junctional communication is a characteristic feature of the sclerotic hippocampus of TLE patients and animal models. This astrocytic dysfunction and the consequential accumulation of extracellular K^+ was detected already 4 h after intracortical kainate injection, leading to the suggestion that it plays a causative role in the pathogenesis of TLE (14). In another study, we demonstrated that intracerebroventricularly injected albumin is taken up by astrocytes and reduces their gap junctional coupling (15). Therefore, despite negligible astrocytic albumin uptake, the question arose whether activation of TGFβR1 by extravasated albumin mediates astrocyte uncoupling at this early stage of epileptogenesis. To address this question, we used a specific TGFβR1/ALK5 kinase inhibitor, IPW-5371, which has been shown to cross the BBB and to effectively reduce TGFβ signaling (17). IPW-5371 was injected i.p. (20 mg/kg) 1 day and 15 min prior to kainate application, and gap junction coupling was assessed 4 h later by biocytin filling of individual hippocampal astrocytes. The results show that IPW-5371 pretreatment did not prevent seizure-induced astrocyte uncoupling (**Figure 3**).

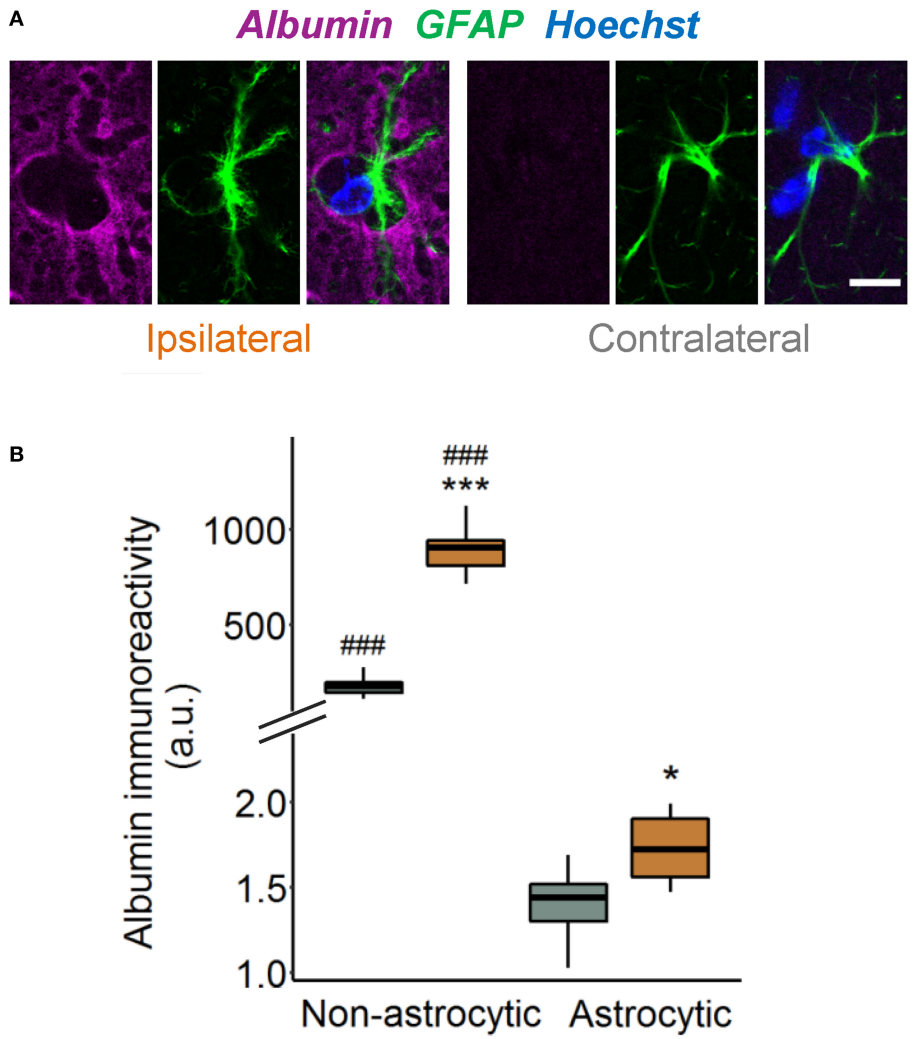

FIGURE 2 | Tiny astrocytic albumin uptake 4 h after KA injection. **(A)** Representative images depicting one focal plane of combined albumin (magenta), GFAP (green), and Hoechst (blue) staining in the hippocampal CA1 *stratum radiatum* of ipsi- and contralateral slices, 4 h after kainate injection. For the purpose of illustration, images are background subtracted and adjusted for brightness. Scale bar: 10 μm. **(B)** Albumin immunoreactivity increases in both GFAP-positive and GFAP-negative pixels on the ipsi- vs. contralateral hemisphere, although the increase in astrocytes is orders of magnitude lower than outside the cells. Box plots represent median and quartiles. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. contralateral, $\#\#\#p < 0.001$ vs. astrocytic (two-way ANOVA). $N = 9$ slices from three mice. au, arbitrary unit.

Indeed, the number of biocytin-positive cells was reduced by 45% in the hippocampus of the injected hemisphere (ipsi 70 ± 26 vs. contra 128 ± 34 cells, mean ± SD, $p = 0.0015$, independent samples *t*-test), which corresponds to data from epileptic mice with undisturbed TGFβR1 signaling (14). Thus, TGFβ signaling appears not to be responsible for, or involved in, astrocyte uncoupling in experimental TLE.

IPW-5371 Pretreatment Slightly Attenuates Acute and Chronic Epileptiform Activity but Has no Effect on the Development of HS in Experimental TLE

Blood–brain barrier breakdown is implicated in the development of epilepsy through a mechanism involving astrocytic TGFβR1/ALK5 signaling (6, 9, 16, 28). To gain a deeper insight into this relationship, we investigated consequences of TGFβR1 kinase inhibition during the initial phase of epileptogenesis on acute and chronic electrographic epileptiform activity and histopathological changes in our TLE model. Mice were injected with IPW-5371 as described above and implanted with telemetric transmitters directly after kainate injection. Electroencephalography recording and video monitoring were subsequently performed continuously (24 h/day) over a period of 4 weeks. Quantification of SE, which in our model is manifested by a series of convulsive seizures lasting up to 6 h, was performed in three different ways: (a) by examining the number and duration of seizures and time spent in ictal activity during the first hour of recording, (b) by counting the number of EEG spikes with amplitudes exceeding baseline

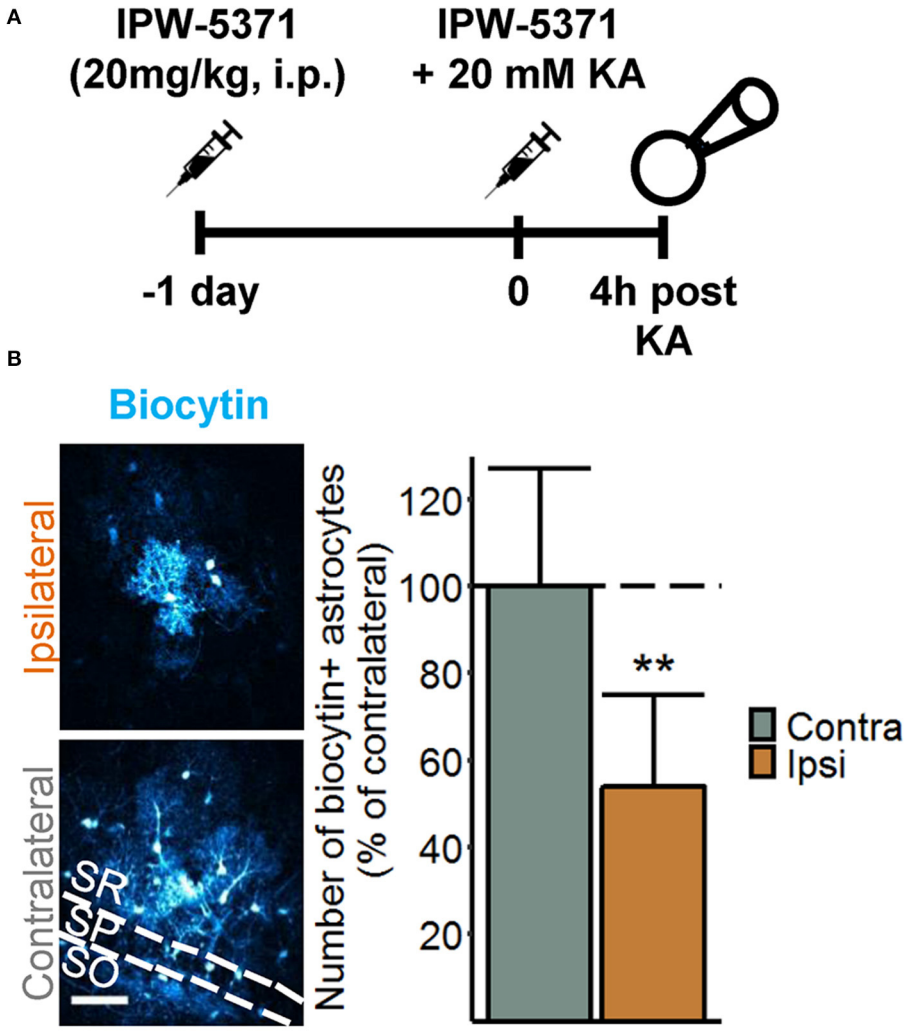

FIGURE 3 | Seizure-induced loss of astrocytic gap junctional communication is not mediated by TGFßR1 signaling. **(A)** Injection scheme for the biocytin diffusion studies. IPW-5371 was injected intraperitoneally (i.p.) once per day at 20 mg/kg for two consecutive days prior to SE induction *via* intracortical kainate injection. Four hours after kainate injection, animals were sacrificed and gap junctional coupling between hippocampal astrocytes was visualized by the intercellular spread of biocytin, which was included in the patch pipette solution during whole-cell patch clamp recordings (20 min). **(B)** Representative maximum intensity projections depicting biocytin-filled astrocytes labeled with streptavidin-conjugated AlexaFluor® (AF) 647 in hippocampal CA1 *stratum radiatum* of the ipsi- and contralateral hemispheres (*left*). Scale bar: 50 μm. The number of biocytin-positive astrocytes was significantly reduced on the ipsi- vs. contralateral hippocampus of IPW-5371-pretreated mice (*right*). Data represent mean ± SD. $^{**}p < 0.01$ (independent samples *t*-test). $N = 9$ slices/condition from three mice. KA, kainate; SR, *stratum radiatum*; SP, *stratum pyramidale*; SO, *stratum oriens*.

activity at least 7.5-fold during the first 6 h of recording, and (c) by comparison of the spectral power in the γ range after FFT of the EEG data. Notably, activity during epileptiform-free periods did not differ between experimental groups (γ power baseline: kainate 2.72 ± 1.36 nV^2 vs. IPW + kainate 3.38 ± 0.97 nV^2, $p = 0.29$, independent samples *t*-test; 7.5 × SD of baseline: kainate 196.61 ± 37.65 vs. IPW + kainate 222.75 ± 31.6, $p = 0.136$, independent samples *t*-test). We found no differences between kainate mice treated with IPW-5371 or vehicle regarding the number of seizures or seizure duration within the first hour of SE (**Figure 4A**, $p = 0.67$ and $p = 0.076$, respectively, Mann–Whitney *U*-test). As mentioned earlier (19), this type of analysis is only possible within the initial period of SE. For quantification of the entire SE, spike, and spectral analyses were extended to 6 h. While there was no difference in the number of spikes per minute during this period ($p = 0.11$, Mann–Whitney *U*-test), the normalized γ band power was significantly reduced in IPW-5371- vs. vehicle-treated kainate mice (**Figures 4B,C**, $p = 0.037$, Mann–Whitney *U*-test). These data indicated that TGFßR1 kinase inhibition interferes with acute seizure activity following kainate administration, specifically attenuating high-frequency activity in the γ range. The duration of the subsequent seizure-free (latent) phase was not influenced by IPW-5371 treatment (**Figure 4A**, right graph). Spontaneous generalized seizures

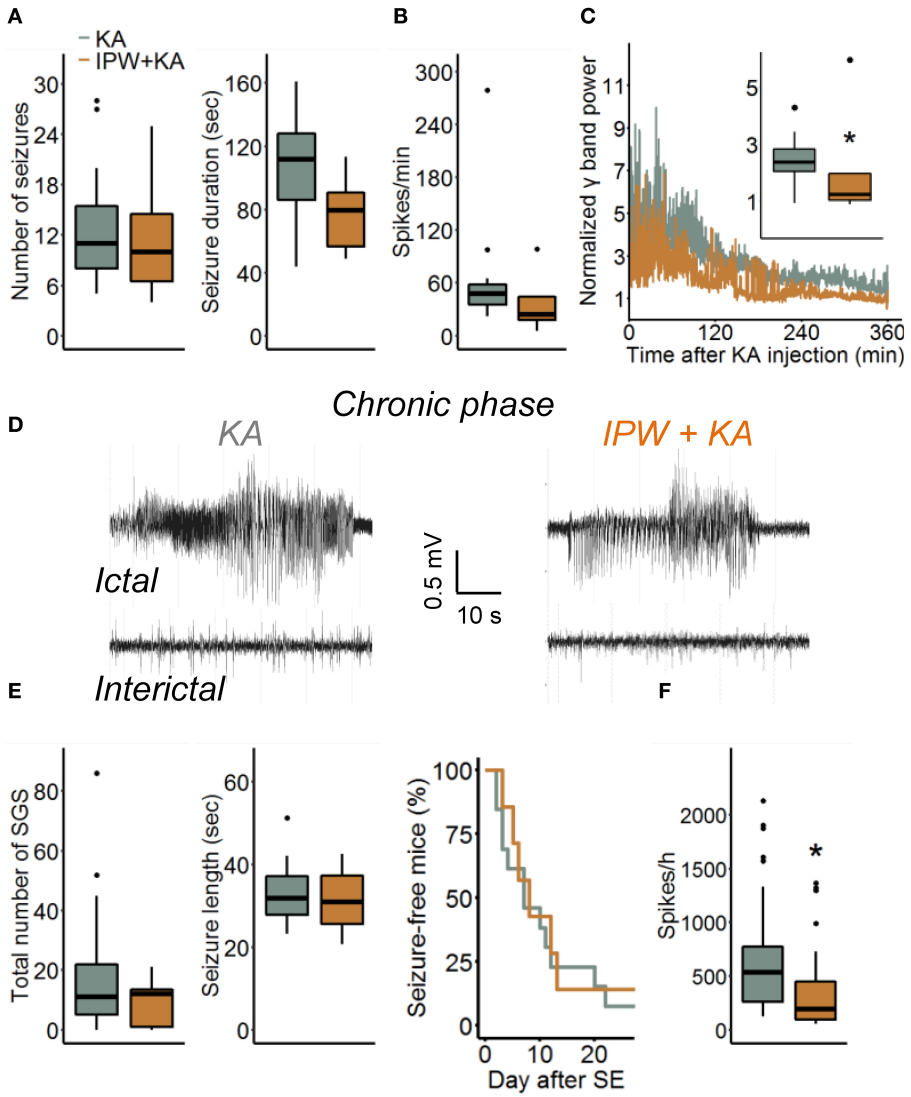

FIGURE 4 | Kainate-induced acute and chronic epileptiform activity in IPW-5371-pretreated mice. **(A)** Number and duration of seizures during the first hour of kainate-induced SE did not differ between IPW-5371- and vehicle-treated kainate mice. **(B)** Severity of SE determined by counting the number of EEG spikes exceeding 7.5-fold baseline activity during the first 6 h after kainate injection. Spike activity was not affected by IPW-5371. **(C)** Spectral analysis yielded significantly lower γ band power in IPW-5371- vs. vehicle-treated kainate mice. **(D)** Representative EEG traces depicting spontaneous generalized seizures (*ictal*) and interictal spiking activity (*interictal*) in IPW-5371-treated and control mice. **(E)** Total number and length of spontaneous generalized seizures (SGS) in IPW-5371- and vehicle-treated kainate mice. Neither the number nor the length of SGS was significantly different between the experimental groups (left graph). The length of the latent phase was also similar in both groups (right graph). **(F)** The number of spikes/hour during the entire 4 weeks of recording was slightly but significantly reduced in IPW-5371- vs. vehicle-treated kainate mice. Box plots represent median and quartiles. *$p < 0.05$ (independent samples *t*-test). $N = 7$ (kainate + IPW-5371) and 13 (kainate) mice. KA, kainate.

(**Figure 4D**) were seen in 92.3% (12 of 13) of vehicle-treated and 85.7% (6 of 7) of IPW-5371-treated kainate mice ($p = 0.73$, log-rank test). As shown in **Figure 4E** (left graph), the total number of spontaneous generalized seizures during the 4 weeks of recording hardly differed between the conditions ($p = 0.32$, Mann–Whitney *U*-test). Likewise, the duration of individual generalized seizures was not different between the experimental groups (**Figure 4E**, right graph, $p = 0.6$, independent samples *t*-test). Importantly, during the entire recording period, the number of epileptic spikes per hour was significantly lower in the IPW-5371 group, indicating reduced total (e.g., ictal + interictal) activity (**Figure 4F**, $p = 0.023$, Mann–Whitney *U*-test). This

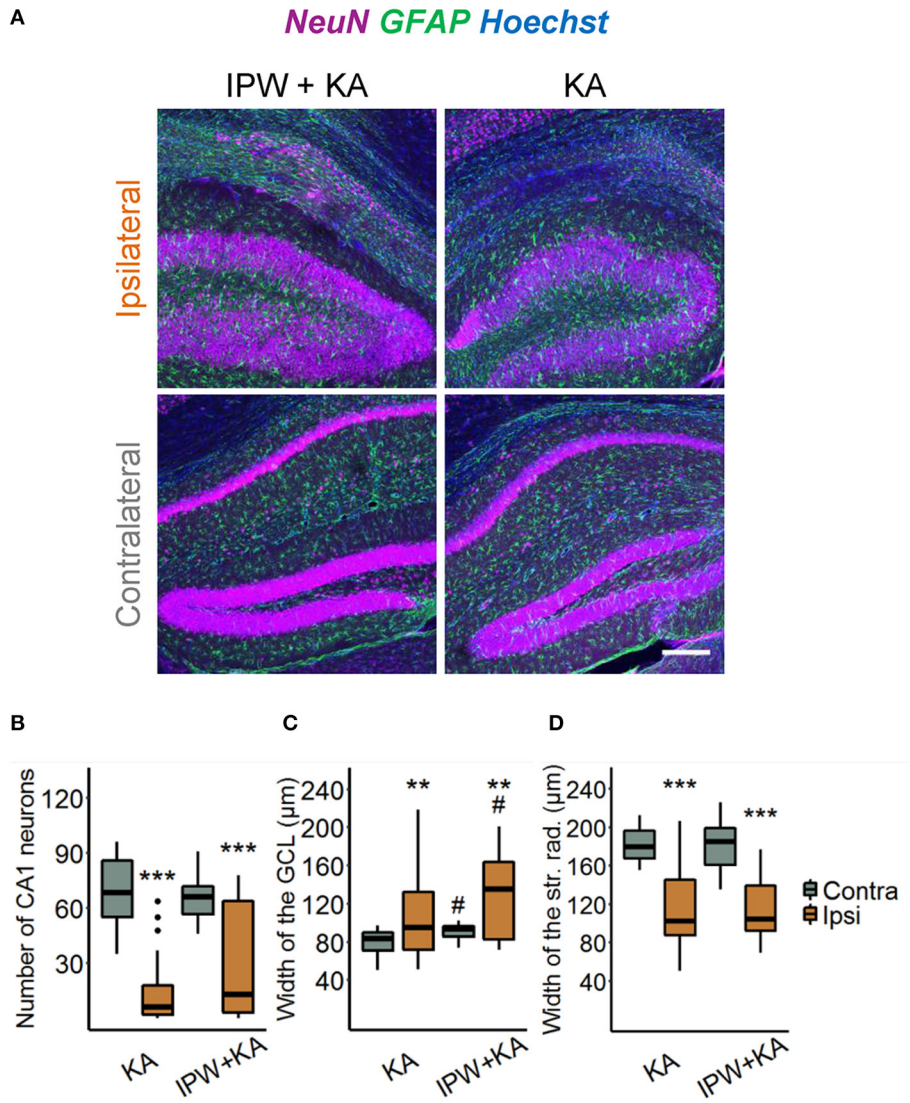

FIGURE 5 | IPW-5371 treatment has no effect on the development of HS in experimental TLE. **(A)** Representative maximum intensity projections of combined NeuN (magenta), GFAP (green), and Hoechst (blue) staining in ipsi- and contralateral hippocampal slices from IPW-5371- and vehicle-treated kainate mice 1 month after kainate injection. Scale bar: 200 µm. Hippocampi of both IPW-5371- and vehicle-treated kainate mice displayed similar **(B)** pyramidal cell loss in the CA1 region, **(C)** GCD in dentate gyrus, and **(D)** shrinkage of the CA1 *stratum radiatum*. Data display box plots representing median and quartiles. ***$p < 0.001$ vs. contralateral, **$p < 0.01$, #$p < 0.05$ vs. control (two-way ANOVA). $N = 29$–31 slices from six animals/condition. CA, cornu ammonis; GCL, granule cell layer; str. rad., *stratum radiatum*.

finding was, however, not reflected by spectral analysis, since no difference was found in any of the frequency bands (data not shown). To assess a potential influence of the surgical procedure itself on seizure activity, brain activity was continuously EEG monitored for 9 days in a group of control mice that had received intracortical injection of 70 nl sterile NaCl (0.9%) instead of kainate. No seizures could be detected under these conditions ($n = 6$, data not shown).

We next explored the effect of IPW-5371 treatment on the development of HS. Hippocampal slices were stained with antibodies directed against NeuN, GFAP, and Hoechst 4 weeks after epilepsy induction (**Figure 5A**). Three hallmarks of HS—degeneration of CA1 pyramidal neurons, shrinkage of the CA1 region, and GCD—were evaluated. The extent of neurodegeneration was determined by counting the number of NeuN-positive cells in an area of 360 × 120 × 40 µm^3 within the CA1 *stratum pyramidale* underneath the injection side and at the same position on the contralateral side. The data show that the ipsilateral loss of NeuN-positive cells was similar in IPW-5371- vs. vehicle-treated kainate mice (19.69 ± 92.42 vs. 8.76 ± 23.35% of contralateral; $p < 0.001$, two-way ANOVA, **Figure 5B**). Likewise, neither GCD (148.69 ± 61.71 vs. 114.49 ± 73.69% of contralateral, $p = 0.0013$, two-way ANOVA) nor CA1 shrinkage (56.49 ± 25.41 vs. 56.86 ± 32.17% of contralateral, $p <$

0.001, two-way ANOVA) was attenuated by IPW-5371 treatment (**Figures 5C,D**). Unexpectedly, kainate mice pretreated with IPW-5371 displayed increased GCD compared with vehicle-treated kainate mice both ipsi- and contralaterally ($p = 0.014$, two-way ANOVA). Taken together, these data reveal that inhibition of the TGFβR1/ALK5 pathway with IPW-5371 slightly reduces acute and chronic kainate-induced epileptiform activity but does not prevent the development of HS.

DISCUSSION

Our data confirm that leakage of albumin through a compromised BBB represents an early event and possibly one of the causative factors in epileptogenesis. Indeed, prominent albumin IR is observed in the brain parenchyma as early as 4 h after SE induction, preceding most of the epilepsy-associated histopathological alterations in our experimental model (14). This result is not surprising, as it is known from various animal models of seizures and epilepsy that BBB opening occurs within a few minutes after seizure induction [for review, see (27, 29)]. Although previous work concluded that BBB leakage in epileptogenesis is transient and lasts only a few days, more recent studies observed the dysfunction also in chronic human and experimental epilepsy, indicating that it may also contribute to the progression of the disorder (5, 26, 29–33). In agreement with this view, we detected albumin extravasation also 5 days and 3 months after SE, triggered by intracortical kainate injection (26). A surprising finding of the present study was the relatively high hippocampal albumin extravasation in sham-injected mice. Apparently, in this model, initial BBB leakage is not solely evoked by kainate-induced seizure activity but may also be caused by the injection itself. Because in this model damage to the hippocampus is avoided by intracortical injections, the phenomenon may arise from local tissue pressure changes and/or inflammatory processes evoked by the injected saline. The fact that extravasated albumin was only transiently seen in sham controls indicates that seizure activity is a prerequisite for long-lasting BBB opening. On the other hand, these control experiments provide evidence that transient albumin extravasation is not sufficient to cause neuronal hyperactivity or neuronal damage, since we have never observed seizures or histopathological changes in sham-injected mice.

A number of reports have proposed that extravasated albumin exerts its epileptogenic effects by altering essential astrocytic functions, such as their ability to buffer K^+ and glutamate (11, 27, 34). Mechanistically, TGFβ receptor-mediated albumin uptake into astrocytes was proposed to mediate changes in gene expression responsible for these functional alterations (6, 8, 9, 15). However, some studies reported proepileptic effects of extravasated or injected albumin in the absence of astrocytic albumin IR, raising the question of whether uptake is really required for the albumin effects (13, 35). Interestingly, despite the lack of astrocytic albumin, Bankstahl and colleagues observed reduced GFAP and AQP4 IR in albumin-positive hippocampal regions during the early phase of pilocarpine-triggered epileptogenesis, showing that extravasated albumin can indeed influence astrocyte function without being taken up (35). Therefore, the lack of astrocytic albumin uptake found in our model does not exclude the possibility that albumin influences epileptogenesis *via* these cells.

Brain exposure to serum albumin impedes extracellular K^+ ($[K^+]_o$) buffering by reducing the expression of astrocytic inward rectifying K^+ channels (Kir 4.1) and gap junction proteins (6, 11, 15). According to the spatial K^+ buffering concept, excessive extracellular $[K^+]_o$ released during neuronal activity is passively taken up by astrocytes through Kir4.1 channels, and then redistributed through the gap junction-coupled astrocytic network to be released at regions of lower $[K^+]_o$ (36). Consequently, loss of Kir4.1 expression or astrocytic coupling would result in accumulation of $[K^+]_o$, neuronal depolarization, and a lowered threshold for seizure generation. We have previously demonstrated Kir4.1 downregulation in chronic human TLE (37, 38) as well as reduced astrocytic coupling and impaired K^+ clearance 4 h after SE induction in the intracortical kainate injection model of TLE (14). Since strong albumin extravasation was also found at this time point, it was reasonable to assume that albumin mediated the uncoupling. Our experiments with IPW-5371 do not support this hypothesis, although there is still the possibility that albumin affects coupling *via* a TGFβ-independent pathway. Moreover, because we did not examine Kir4.1 expression in this study, we cannot rule out that impaired K^+ buffering in this model is mediated, at least in part, by albumin-induced downregulation of Kir4.1. Indeed, reduction of Kir4.1 expression may occur already 2 h after intracerebroventricular albumin injection (13), while downregulation of gap junction proteins and reduced interastrocytic coupling were demonstrated 24 h following exposure to albumin (11, 15). Disruption of astrocytic coupling in epileptogenesis is likely determined by different mechanisms at different time points during and after SE, and extravasated albumin may be involved at later time points of epileptogenesis.

Several studies provided convincing evidence that BBB leakiness contributes to epileptogenesis through albumin extravasation and activation of the TGFβ pathway (6, 8, 9). This work revealed that albumin and TGFβR1 possess similar proepileptic effects that are prevented by TGFβ pathway inhibition (9, 16). Our results show that early TGFβR1 inhibition only marginally affects the development of TLE, implying that BBB disruption and albumin extravasation over longer periods are required to crucially influence the process. Indeed, experimental opening of the BBB or albumin infusion over days was necessary to induce spontaneous seizures (6, 9, 10), while transient (short-term) hippocampal exposure to albumin, evoked by a single intracerebroventricular injection, was not sufficient to trigger seizures (13). According to its published pharmacokinetics, IPW-5371 effectively inhibits TGFβR1/ALK5 signaling for about 24 h (17). Future experiments are needed to reveal whether long-term inhibition (e.g., over the entire latency period, which lasts on average 5 days in this model) would completely suppress epileptogenesis. It must be stressed, however, that our data do not provide information about

whether TGFβ signaling is activated by extravasated albumin and whether its proepileptic action is caused by changes in astrocytic function. Not only astrocytes but virtually all cell types in the brain, including neurons, microglia, and endothelial cells, produce TGFβ and possess TGFβ receptors (39). Our immunostaining indicated neuronal albumin uptake (at 24 h, but not yet 4 h post kainate, **Supplementary Figure 1**), an observation that matches several other studies (5, 13, 15, 40). Therefore, it would also be possible that albumin directly affects neuronal activity *via* TGFβ signaling. Independent of this, extravasated albumin seems to affect neuronal excitability without influencing the strong histopathological alterations characteristic of HS. This result is consistent with previous research that found no evidence for albumin-induced neurodegeneration (10, 13, 34, 35). Unexpectedly, we observed even more pronounced GCD in IPW-5371-pretreated mice, both ipsi- and contralaterally. How TGFβR1 signaling regulates seizure-induced GCD remains to be elucidated in future experiments.

CONCLUSION

We show, in a mouse model closely resembling human TLE with HS, that albumin extravasation into the brain parenchyma arises very soon after SE induction. At this early stage, albumin is not yet taken up by astrocytes and uncoupling is not the result of albumin-stimulated TGFβR1/ALK5 signaling. Whether the latter is involved in the complete loss of coupling seen in chronic experimental and human epilepsy remains to be investigated. Inhibition of TGFβ signaling during the first hours of kainate-induced SE only slightly affected epileptogenesis in our TLE model, suggesting that longer-lasting albumin extravasation is necessary to critically alter the pathological process. Our results provide new insights into the role of BBB dysfunction and the development of TLE, which may help in identifying new targets for antiepileptogenic strategies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by North Rhine–Westphalia State Agency for Nature, Environment and Consumer Protection (approval number 84-02.04.2015.A393).

AUTHOR CONTRIBUTIONS

CS and PB designed and supervised the experiments, LH and PB performed and analyzed the experiments. All authors wrote the manuscript, contributed to the article, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.660591/full#supplementary-material

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REVIEW
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The Role of Neuroinflammation in Post-traumatic Epilepsy

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Post-traumatic epilepsy (PTE) is one of the consequences after traumatic brain injury (TBI), which increases the morbidity and mortality of survivors. About 20% of patients with TBI will develop PTE, and at least one-third of them are resistant to conventional antiepileptic drugs (AEDs). Therefore, it is of utmost importance to explore the mechanisms underlying PTE from a new perspective. More recently, neuroinflammation has been proposed to play a significant role in epileptogenesis. This review focuses particularly on glial cells activation, peripheral leukocytes infiltration, inflammatory cytokines release and chronic neuroinflammation occurrence post-TBI. Although the immune response to TBI appears to be primarily pro-epileptogenic, further research is needed to clarify the causal relationships. A better understanding of how neuroinflammation contributes to the development of PTE is of vital importance. Novel prevention and treatment strategies based on the neuroinflammatory mechanisms underlying epileptogenesis are evidently needed.

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SEARCH STRATEGY

Search MeSH Terms in pubmed: "["Epilepsy"(Mesh)] AND "Brain Injuries, Traumatic"[Mesh]". Published in last 30 years. 160 results were founded. Full text available:145 results. Record screened manually related to Neuroinflammation and Post-traumatic epilepsy. Then finally 123 records were included.

Keywords: post-traumatic epilepsy, traumatic brain injury, neuroinflammation, epileptogenesis, immunotherapy

INTRODUCTION

Epilepsy is a chronic neurological disease that is characterized by recurrent, transient and episodic discharge of neurons in the brain. The recurrent and frequent seizures seriously affect patients' life quality and cause a substantial economic burden to society and family (1). Post-traumatic seizure (PTS) is one of the severe consequences of brain trauma, with an incidence ranging from 4 to 53% (2, 3). According to different latency from injury to seizure onset, PTS can be divided into immediate (<24 h), early (1–7 d), or late (>1 week) seizures, and only the recurrent late seizures can be called post-traumatic epilepsy (PTE) (4, 5). Therefore, PTE can be generally defined as unprovoked and recurrent seizures that occur more than 1 week after traumatic brain injury (TBI), accounting for as high as 20% of acquired epilepsy and 4% of all patients with epilepsy (6). Injury severity, age, and surgical methods after trauma are important risk factors for developing

PTE. Moreover, we should also consider other factors such as hypoxia, hyperthermia, intracerebral bleeding, infection, or status epilepticus (SE) combining TBI that may increase PTE risk.

The pathogenesis of PTE is not yet clear. A growing body of evidence from clinical and experimental studies suggested the involvement of neuroinflammation in the process of epileptogenesis post TBI (7–9). Acute and early epileptic attacks may be a direct response to brain injury: Epidural and subdural hematoma, cerebral edema and brain contusion occur at the time of head impact can compress and stimulate the focal damaged tissue, which may cause blood-brain barrier (BBB) breakdown and reduce the threshold of seizure (10, 11). In contrast, the late onset is mediated by several factors including, but not limited to: generation of oxygen free radicals, abnormal release of excitotoxicity neurotransmitters, neuroimmune abnormalities caused by the inflammatory response, and neural network remodeling consisting of neurogenesis and neurodegeneration. The complexity of mechanisms and the severity of injury are the leading causes of the different outcomes and prognosis of PTE patients. Neuroinflammation is a crucial component of the epileptogenesis following TBI, and is also a promising target for treatment. Since neuroinflammatory mechanisms can be harmful or beneficial, it is necessary to have a good understanding of the timing and complexity of the immune response after TBI before developing immunomodulatory therapies to develop new preventative treatments of PTE.

NEUROINFLAMMATION SECONDARY TO TBI DRIVING PTE

TBI is one of the common emergencies in neurosurgery with high rates of mortality and disability. There is increasing evidence that TBI can cause direct and immediately impacts and evolves over time, contributing to long-term sequelae, such as behavioral disturbances, epilepsy and neurodegenerative disorders (12). The pathological mechanisms are characterized by a robust immune response, including BBB damage, activation of glial cells, infiltration of peripheral leukocytes, and release of pro- and anti-inflammatory cytokines (IL-1β, HMGB1 TGF-β, TNF-α, etc.). Over time, from months to years, neurogenesis and neuroplasticity caused by injury help repair and regeneration, and an ongoing chronic neuroinflammation promotes neurodegeneration. These pathological processes lead to excessive excitation of neurons and ultimately drive PTE development (**Figure 1**).

The immune system is considered to react to injury with a two-phase response: innate immunity and adaptive immunity, the latter characterized by antigen-specificity and "remember" ability, which plays a vital role in our defense against pathogens. Innate immune response secondary to damaged or infected central nervous system (CNS) is mediated partly by damage- or pathogen-associated molecule patterns (DAMPs and PAMPs) (13). By interacting with "danger" sensors-pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) like receptors (NLRs), and scavenger receptors, pathogens and danger signals can act to initiate the innate immune response. Innate immune cells (e.g., phagocytes, granulocytes and T lymphocytes et al) can then release cytokines and chemokines that amplify the inflammatory cascade. Adaptive (or acquired) immunity is a more targeted response, which develop immunological memory to a specific pathogen in an initial response. CD8 and CD4 T lymphocytes recognize major histocompatibility complex (MHC) and play cytotoxic, helper or regulatory roles. On the other hand, B lymphocytes can produce immunoglobulins and participate in T cell activation (14).

Inflammasome, a multi-protein complex assembled by cytosolic receptors, is an essential component of the innate immune system and upon activation can be involved in the production of pro-inflammatory cytokines. NLRP1, NLRP3, NLRC4, and AIM2 are the most concerned inflammasomes in brain. Inflammasomes, especially NLRP3, can recognize DAMPs or PAMPs, recruit and activate pro-inflammatory protease Caspase-1. Activated Caspase-1 cleaves the precursors of IL-1β and IL-18 into their mature forms, which plays a vital role in promoting sterile immune response following TBI (15, 16). In addition to inducing cytokine release, activation of inflammasomes can also mediate pyroptosis, a form of necrotic cell death (17). Adamczak et al. (18) found that inflammasome components caspase-1, ASC and NLRP-1 were significantly elevated in the cerebrospinal fluid (CSF) of patients with moderate or severe brain trauma, and the levels of these proteins were correlated with unfavorable outcomes. One study showed that administration of anti-ASC antibodies in a mouse of fluid-percussion injury (FPI) model reduced capase-1 activation and IL-1β generation, resulting in a decrease in brain lesion volume. These data indicate that inflammasome proteins might serve as potential biomarkers to assess inflammation and TBI severity.

Microglial Activation

Microglia are sentinel cells of the CNS and are often the first responders to brain damage. Under physiological conditions, microglia are resting, capable of sensing inflammatory signals, promoting neuronal survival and synaptic remodeling, thus playing an "immune surveillance" role. After TBI, microglial cells can be activated rapidly and sustained for several years in the brain (19). Microglia response to brain trauma has several

Abbreviations: AD, Alzheimer's disease; AEDs, Antiepileptic drugs; AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazoleproprionic Acid; AQP4, Aquaporin-4; BBB, Blood-brain barrier; CNS, Central nervous system; CSF, Cerebrospinal fluid; CTE, Chronic traumatic encephalopathy; DAMPs and PAMPs, Damage- or pathogen-associated molecule patterns; FPI, Fluid-percussion injury; FS, Febrile seizures; GFAP, Glial fibrillary acidic protein; GLT, Glutamate transporter; Glu, Glutamate; Gn, Glutamine; GS, Glutamine synthetase; ICE, IL-converting enzyme; IGF1, Insulin-like growth factor 1; IL-1, Interleukin-1; KA, Kainic acid; MAPK, Mitogen-activated protein kinase; MHC, Major histocompatibility complex; MMP9, Matrix metalloproteinase 9; mTBI, mild traumatic brain injury; NFTs, Neurofibrillary tangles; NF-κB, Nuclear factor kappa-B; NGF, Nerve growth factor; NLRs, Nucleotide-binding oligomerization domain like receptors; NMDA, N-methyl-D-aspartate; NO, Nitric oxide; PP2A, Protein phosphatase 2A; PRRs, Pattern recognition receptors; PTE, Post-traumatic epilepsy; PTS, Post-traumatic seizure; RAGE, Receptor for advanced glycation end products; ROS, Reactive oxygen species; SE, Status epilepticus; SNPs, Single nucleotide polymorphisms; TBI, Traumatic brain injury; TLE, Temporal lobe epilepsy; TLRs, Toll-like receptors.

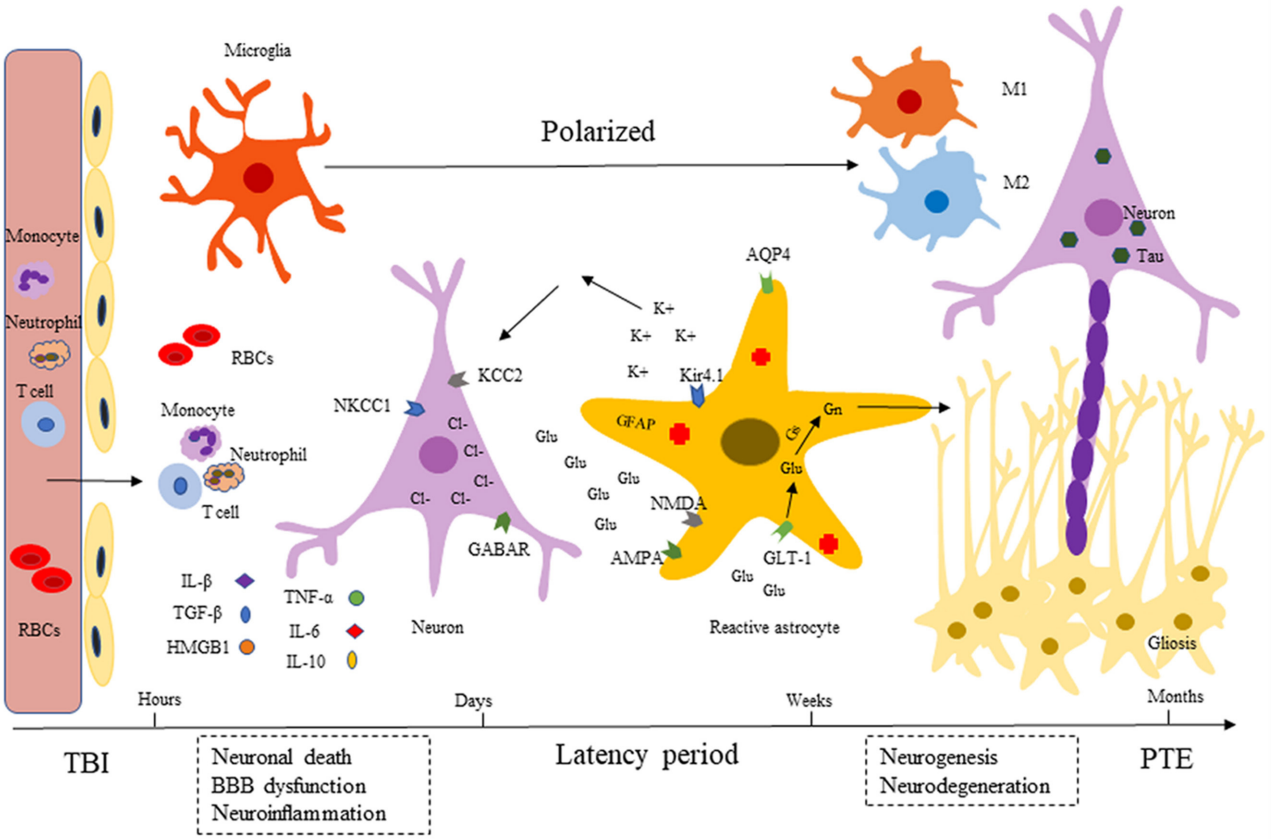

FIGURE 1 | Development of post-traumatic epilepsy (PTE). After TBI, there are a series of immune inflammatory reactions including BBB dysfunction (endothelial cells destruction), microglia and astrocytes activation, neuroinflammatory factors release (IL-1β, HMGB1 TGF-β, TNF-α, etc.). Released inflammatory cytokines can recruit neutrophils and monocytes into injured tissues, expanding the inflammatory cascade. Over time (months to years), injury-induced neurogenesis and neuroplasticity help repair and regeneration, persistent chronic neuroinflammation promotes neurodegeneration (Tau accumulation). These pathological processes lead to excessive excitement of neurons, which eventually causes repeated seizures.

phases, including morphological transformation, proliferation, migration, phagocytosis and chemokines or cytokines release (19, 20). After acute classical activation, microglia can release various inflammatory mediators, including IL-1β, IL-6, TNF-α, nitric oxide (NO), metalloproteinases, and reactive oxygen species (ROS). The production of these inflammatory mediators can promote the immune response to TBI by increasing BBB permeability and facilitating peripheral immune cell recruitment. The researchers found that minocycline specifically inhibited the activation of microglia and monocytes, which could reduce the volume of trauma foci and improve the neurological outcome of the mice (21). However, it is worth noting that microglia are highly plastic cells, and how they promote sterile immune response also rely on their activation state, type of damage, and interaction with neighboring cells, etc. Their effect on TBI should be considered in terms of time and context. In the same damaged brain tissue, microglia may be in different functional states, which are determined by the expression of molecules such as adhesion, maturation, effector, and among others. The conventional wisdom is that microglia, stimulated by injury, can be activated and polarized into two major phenotypes: M1-like (classical activation) and M2-like (alternative activation).

M1-like activated microglia can express IL-1, HMGB1, TNF-α and other cytokines that promote inflammatory reaction, which is involved in PTE occurrence. In contrast, inflammation mediators secreted by M2-like microglia can downregulate the responses of M1-like microglia, thus potentially inhibiting epileptogenesis. Microglial polarization may be a critical contributor that is directly associated with the pathological prognosis of epileptogenesis. Promoting and maintaining M2-like microglial phenotype after brain injury could be a potential preventive strategy for PTE. However, the current research on M1 and M2-like microglial polarization after TBI is still in the preliminary stage, and the correlation of the findings with epilepsy is even less known. Benson et al. (22) compared microglial polarization in two different acquired temporal lobe SE models, the results showed that pilocarpine-induced SE expressed both M1 and M2 markers, but only M1 markers were upregulated in kainate-induced SE model, which may explain why kainate-induced seizures attack more frequently.

Therajaran et al. (23) summarized the possible epileptogenic mechanisms induced by microglial cell polarization, which mainly included altering excitation/inhibition balance, extracellular matrix, oxidative stress regulation, synaptic remodeling and neurodegeneration. In recent years, Hickman et al. (24) have used single-cell sequencing to analyze the gene expression of microglia in aging, and found that microglial polarization representing a mixed and complex state even in the normal physiological aging process. Therefore, oversimplifying microglia polarization into the M1/M2 phenotype does not reflect the microglia/macrophage functional polymorphism in the complex diseases. The occurrence of PTE is result from a combination of multiple factors, and the underlying mechanism remains to be further explored.

Reactive Astrocytes

Astrocytes are the most widely distributed type of cells in the mammalian brain, and they are not only playing a supporting and isolating role in the CNS, but also participate in pathophysiological processes such as synaptic transmission, neuroimmunity, maintenance of internal environment, and promotion of nerve tissue repair and regeneration (25). Astrocytes play an important role in controlling cerebral blood flow and prevent harmful substances from entering the brain by participating BBB formation with capillary endothelial cells and basal membrane through their terminal foot process. In TBI early stage, astrocytes exert neuroprotective effects by ingesting glutamate(Glu) through glutamate transporters (GLTs), inactivating oxygen free radicals, regulating Na+/K+ balance, and secreting neurotransmitters (26). Within a period after TBI, astrocytes can be activated as reactive astrocytes, which are characterized by high expression of glial fibrillary acidic protein (GFAP), hypertrophy of cell bodies, and extension of primary processes, etc. Astrocytes participate in inflammation through the HMGB1-receptor for advanced glycation end products (RAGE) signaling pathway, thereby activating nuclear factor kappa-B (NF-κB) signaling transduction (27). Reactive astrocytes can also secrete matrix metalloproteinase 9 (MMP9), affecting the integrity of BBB after TBI (28). The glial scar formed by the reactive hyperplasia of glial cells after brain injury, restricts axonal regeneration and functional connections, thereby impeding nerve recovery. Studies have found that astrocytes interact with microglia and other immune cells to produce cytokines, such as insulin-like growth factor 1 (IGF1) and nerve growth factor (NGF), which promote the healing following TBI (29).

Epilepsy has long been thought to be a specific neuronal disease caused by changes within neurons, but novel evidence challenges us to consider that astrocytes also play a non-negligible role in acquired epilepsy. However, the relationship between reactive astrocytes and PTE is unclear. High extracellular potassium and Glu levels after TBI may be the main reasons for seizures induced by altered homeostasis of astrocytes. The homeostasis of extracellular K+ is crucial for regulating neuronal excitability. Kir4.1 is an inward rectifying potassium channel highly expressed in astrocytes of the CNS, buffering excessive spatial potassium load and maintaining the dynamic balance of K+ in the neuronal environment (30). Insufficient K+ buffering and severe seizures were observed in mice with conditional Kir4.1 knockout (31, 32). Similarly, it has been found that TBI injury can lead to loss or down-regulation of Kir4.1 channel of astrocytes (33) and induce PTE (34). A series of studies showed aquaporin-4 (AQP4) and Kir4.1 are co-expressed in astroglial end feet, where Kir4.1 regulates the buffering on extracellular K+ and AQP4 mediates water homeostasis. AQP4 and Kir4.1 co-regulate extracellular interstitial water and electrolyte balance and play a pivotal role in neuronal excitability. Studies have shown that if the number of AQP4 channels is reduced, the ability of Kir4.1 to clear extracellular K+ will be weakened, and the excitability of neurons will be increased, thus triggering seizures (35). The AQP4 and Kir4.1 channels of astrocytes may be new potential targets for the treatment of epilepsy.

Altered neurotransmitter metabolism in astrocytes may also contribute to epileptogenesis. Under normal conditions, the GLTs (mainly GLT1) on astroglial membrane can quickly remove the excess Glu in extracellular space and reduce the excitatory toxicity. Excitotoxic Glu can be converted into non-toxic glutamine (Gn) under the action of glutamine synthetase (GS) after Glu uptake into glial cells. Gn is a substrate for neuronal synthesis of Glu and GABA in neurons. Blocking GS can lead to GABA inhibitory postsynaptic potential. Brain injury results in increased concentrations of extracellular Glu, which can not only overstimulate glutamate receptors such as α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) and *N*-methyl-D-aspartate (NMDA), but also affect the function of GLT-1 in astrocytes. Osteen et al. (36) demonstrated that the increased excitability of neurons that survived in injury was related to the long-term activation of NMDA subunit receptors, especially NR2B. Samuelsson et al. (37) discovered that the level of GLT-1 was temporarily decreased in the epilepsy model induced by injecting ferrous chloride, which restricted the uptake of Glu and led to seizures. The results indicated that astrocytic Glu transporter may be one of epileptogenesis mechanism after trauma.

New research point to a direct effect of reactive astrocytes in regulating neuronal function by weakening the inhibition of GABA receptors (38). Two antagonistically acting neuronal chloride (Cl^-) transporters NKCC1 and KCC2 establish the transmembrane gradient for Cl^-, which serves as the premise for inhibitory effect of GABA receptors. NKCC1 transports Cl^- into the neuron cell body across the membrane to maintain high intracellular Cl^-, while KCC2 shunts Cl^- out of the cell to lower intracellular Cl^- concentration. Wang et al. (39) pointed out that NKCC1 expression was up-regulated after TBI, which is responsible for the intracellular Cl^- concentration, and gene knockout of NKCC1 or NKCC1 inhibitor bumethanide could reduce seizure frequency. However, a specific KCC2 agonist is not yet available for clinical practice. Besides, long-term chronic epileptic seizures cause astrocytic hyperplasia to participate in hippocampal sclerosis, which will affect the normal physiological regulatory function of brain and play a role promoting epileptogenesis.

Peripheral Immune Cells

sThe inflammatory response after TBI is not restricted to the CNS. Peripheral cells, such as neutrophils and T lymphocytes, monocytes and macrophages, can infiltrate into the brain through the broken BBB, further complicating the primary injury and local inflammatory response. After activation of CNS resident immune cells, neutrophils are among the first peripheral cellular responders to arrive in the injured brain with just a few hours (40). Neutrophils can permeate through BBB under the induction of cytokines (e.g., TNF-α, IL-1β), chemokines (e.g., CXCL1, 2, 3) and purines, releasing a series of proteases that destroy microvessels and subsequent aggravate BBB destruction. Activated neutrophils are also ROS producer, facilitating oxidative stress and thereby neurodegeneration secondary to TBI (41, 42). While the above data suggest that neutrophils are predominantly detrimental, it is worth noting that neutrophils can also play a beneficial role in promoting neurological recovery after injury. Future research is needed to determine how neutrophils influence wound-healing. Neutrophils have been shown to affect T-cells, including regulatory T cells, CD8+ T cells, and CD4+ T cells, contributing to adaptive immunity (43). In addition, recruitment of neutrophils after TBI is usually accompanied by the arrival of monocytes that turn into macrophages. Macrophages derived from monocytes often participate in the injury response together with yolk-sac derived resident myeloid cells such as microglia, contributing to tissue repairment and even regeneration. However, sustained activation of proinflammatory macrophages is considered to be deleterious, and may lead to progressive neurodegeneration and dysfunction. The mechanism of monocytes recruitment after TBI relies on the production of local chemokine CCL2. Targeting CCL2/CCR2 chemokine signaling pathway can decrease the number of monocytes, which can reduce lesion size and promote neurological recovery (44, 45). CCR2+ mononuclear macrophages infiltration has also been observed in the epileptic tissues (46). Pharmacological inhibition of CCL2 or CCR2 can suppress lipopolysaccharide-induced seizures (47), suggesting an association between monocyte accumulation and seizure susceptibility. As mentioned above, these peripheral immune responses are involved in neuroinflammation. They may promote epileptogenesis following TBI, but how they modulate vulnerability to seizures has not yet been explored.

Inflammatory Cytokines

More recently, increasing evidence has supported that neuroinflammation plays a causal role in seizure induction and propagation. TBI gives rise to inflammatory cytokines, mainly including IL-1β, TGF-β, HMGB1, TNF-α, IL-6, and IL-10, which may be the critical inflammatory mediators involved in PTS/PTE (**Table 1**). These cytokines can recruit neutrophils and monocytes to infiltrate into damaged tissue, expanding the inflammatory cascade reaction (48, 49). Three key signaling pathways that may mediate the relation between neuroinflammation and epileptogenesis: IL-1β/IL-1R signaling pathway, HMGB1/TLR4 signaling pathway, and TGF-β/albumin signaling pathway (**Figure 2**) (7, 50). These signaling pathways are expected to be more important targets to modulate post-traumatic epileptogenesis.

IL-1β

Interleukin-1(IL-1) is one of the key mediators involved in both focal and diffuse TBI inflammatory response. The proinflammatory factor IL-1β is the most characteristic member of the IL-1 family and is elevated quickly in damaged brain tissue. IL-1β binding to IL-1R can activate the downstream NF-κB, p38 mitogen-activated protein kinase (MAPK), Src family kinases, etc. Through MyD88-dependent or non-dependent signaling pathways, IL-1β/IL-1R initiates intracellular signal transduction in hippocampal neurons (51). Under physiological conditions, IL-1β is undetectable, which can be upregulated within minutes to hours post-TBI and this high level may last for several months (52). IL-1β is a crucial initiator of the immune inflammatory response, and can involve in leukocytes recruitment, other inflammatory factors and chemokines release (53, 54), glial cells activation, and BBB disruption (55). Frugier et al. (52) found that IL-1β mRNA and protein levels significantly increased in patients who died post-injury, IL-1β neutralizing antibody (56, 57), IL-1R1 antagonist (58) or IL-1R1 gene defect (59) could alleviate TBI-induced glial activation, neutrophil infiltration, brain edema and cognitive dysfunction in animal models of TBI.

Elevated levels of IL-1β in CSF/serum/brain tissue are associated with the development of epilepsy (60). In a previous study, Diamond and his colleagues demonstrated that one of IL-1β functional single nucleotide polymorphisms (SNPs), "rs1143634," significantly raised PTE risks. In addition, elevated CSF/serum IL-1β ratio was also associated with increased PTE risks (50). In *in vivo* and *in vitro* experiments, IL-1β have been found to enhance the permeability and current strength of Ca^{2+}, increase intracellular calcium $[Ca^{2+}]_I$ (61), down-regulate of GABA (A) receptor function in hippocampal neurons (62) and inhibit the uptake of Glu by astrocytes (63) through AMPA and NMDA receptors. Consistent with these findings, Semple et al. (64) discovered that IL-1R antagonist could reduce seizure susceptibility 2 weeks after the mice injury, accompanied by reduced hyperplasia of hippocampal astrocytes, and spatial memory was improved 4 months later. Other members of the IL-1 family such as IL-1α have also been reported to up-regulate in brain tissue after TBI (65), but are not related to the prognosis of TBI or epilepsy. Taken together, these data support a key role for IL-1β in epileptogenesis, and implicate IL-1β/IL-1R signaling pathway as a potential target to prevent PTE.

HMGB1

TBI causes the release of DAMPs, such as HMGB1, ATP, heat shock proteins (HSPs), and S100. By interacting with PRRs, or RAGE, DAMPs act to activate intracellular signal transduction and initiate an inflammatory cascade. HMGB1 is a multifunctional protein whose function depends on its subcellular localization. In the nucleus, it plays the role of stabilizing nucleosomes, participating in gene transcription, and regulating DNA replication and repair. As a typical DAMPs, HMGB1 can be passively released by necrotic cells, or be actively secreted to the extracellular after tissue injury, activating the

TABLE 1 | Key inflammatory cytokines involved in post-traumatic seizure/epilepsy.

Factors	Fluid/ Tissue	Time course	Role in neuroinflammation	Signaling pathways	Role in epileptogenesis
IL-1β	CSF/ ECF Tissue	Peak on day 1–2, decrease on day 2–4. Increased above control 6–122 h after injury.	Pro-Inflammatory: Mediates leukocytes recruitment, other inflammatory factors and chemokines release, glial cells activation, and BBB disruption.	IL-1β/IL-1R Downstream: NF-κB, p38 MAPK, Src, etc.	Pro-epileptogenesis: Increases intracellular calcium [Ca2+]i; Down-regulates GABA (A) receptor function; Inhibits the uptake of Glu through AMPA and NMDA receptors; IL-1R antagonist reduces seizure susceptibility.
HMGB1	CSF	Peak on day 1–3, Decrease on day 4–7.	Pro-Inflammatory: As a typical DAMPs, HMGB1 released passively or actively to cytoplasm or extracellular space; Activates the innate immune system and initiates the inflammatory cascade.	HMGB1/TLR4 Downstream: NF-κB, p38 MAPK, etc.	Pro-epileptogenesis: Regulates long-term enhancement and long-term inhibition; Intracerebral injection of HMGB1 accelerates epileptic activity; Phosphorylates the NR2B subunit of NMDA receptor that promotes calcium influx; Blocking HMGB1/TLR4 decreases both seizure duration and frequency.
TGF-β	CSF	Peak on day 1, gradually decrease over 21 days.	Pro-Inflammatory: Mediates BBB disruption.	TGF-β/albumin	Pro-epileptogenesis: TGF-β can be upregulated in amygdale-kindled or SE models; Down-regulates astrocytes Kir4.1 function; Antagonists of TGF-β receptors can reduce and even inhibit such epileptic activity.
TNF-α	CSF/ ECF Tissue	Peaks early on day 1. Increased above control within 17 min of injury.	Dual role: Activates polymorphonuclear leukocytes; releases ROS and various inflammatory mediators; Damages vascular endothelial cells, and aggravates cerebral edema; Inhibits NMDA-mediated calcium influx; Promotes neurotrophin production.	The TNF-α signaling pathway is mediated by two membrane receptors TNFR1(p55) and TNFR2(p75)	Dual role: The p75 pathway is involved in the anti-seizure activity of TNF-α, whereas the pro-seizure effect is mediated by the p55 pathway; The role of TNF-α signaling pathway in epileptogenesis after TBI remains unclear.
IL-6	CSF/ ECF Tissue	Peak on day 1, decline on day 2–3. Increased above control within 17 min of injury.	Dual role: Increases adhesion molecules and chemokines secretion and enhances leukocyte recruitment; Inhibits the production of TNF-α and reduces NMDA-mediated calcium influx.	-	Dual role: IL-6 can be upregulated after limbic status epilepticus; Over-expression of IL-6 results in seizure threshold reduction; Promotes hippocampal GABAergic neurons loss, leading to an increased propensity for seizures.
IL-10	CSF	Peak on day 1, decline on day 2–3. May have second or third peak of lower magnitude.	Anti-Inflammatory: Inhibits proinflammatory cytokine expression; Reduces leukocyte recruitment and accumulation.	-	Anti-epileptogenesis: Eliminates the hypoxia-evoked epileptiform activity; Renders animals more resistant to FS.

innate immune system. TLR4 is the hypothesized receptor of HMGB1 and mainly distributed in neurons and glial cells. Here, we particularly focus on HMGB1/TLR4 axis which recently are known to be implicated in TBI-induced immune inflammatory response and epileptogenesis.

Recent studies have indicated that HMGB1 can regulate long-term enhancement and long-term inhibition of the hippocampus after activation of TLR and RAGE (66) and play a role in synaptic transmission and plasticity of neurons, which may be relevant to epileptogenesis and cognitive dysfunction. Kainate and bicuculline-induced acute and chronic epileptic model showed that HMGB1 was highly expressed in neurons and glial cells at the injured sites of mice, and could be transferred from the nucleus to the cytoplasm and then secreted to the extracellular space, suggesting that it might be involved in the initiation of epilepsy (67). New studies have noted that the ATP-gated ionophilic P2X7 receptor promotes the release of IL-1β and HMGB1 from glial cells by mediating the activation of NALP3 inflammasome, thereby facilitating seizures (68). The direct evidence of HMGB1 involved in PTE was that intracerebral injection of HMGB1 in mice accelerated the acute epileptic activity induced by convulsive drugs and increased the frequency and severity of seizures. HMGB1/TLR4 antagonist or anti-HMGB1 monoclonal antibody significantly increased the epileptogenic threshold, decreased both seizure duration and frequency (67, 69). A study using primary cultured hippocampal neurons found that TLR4

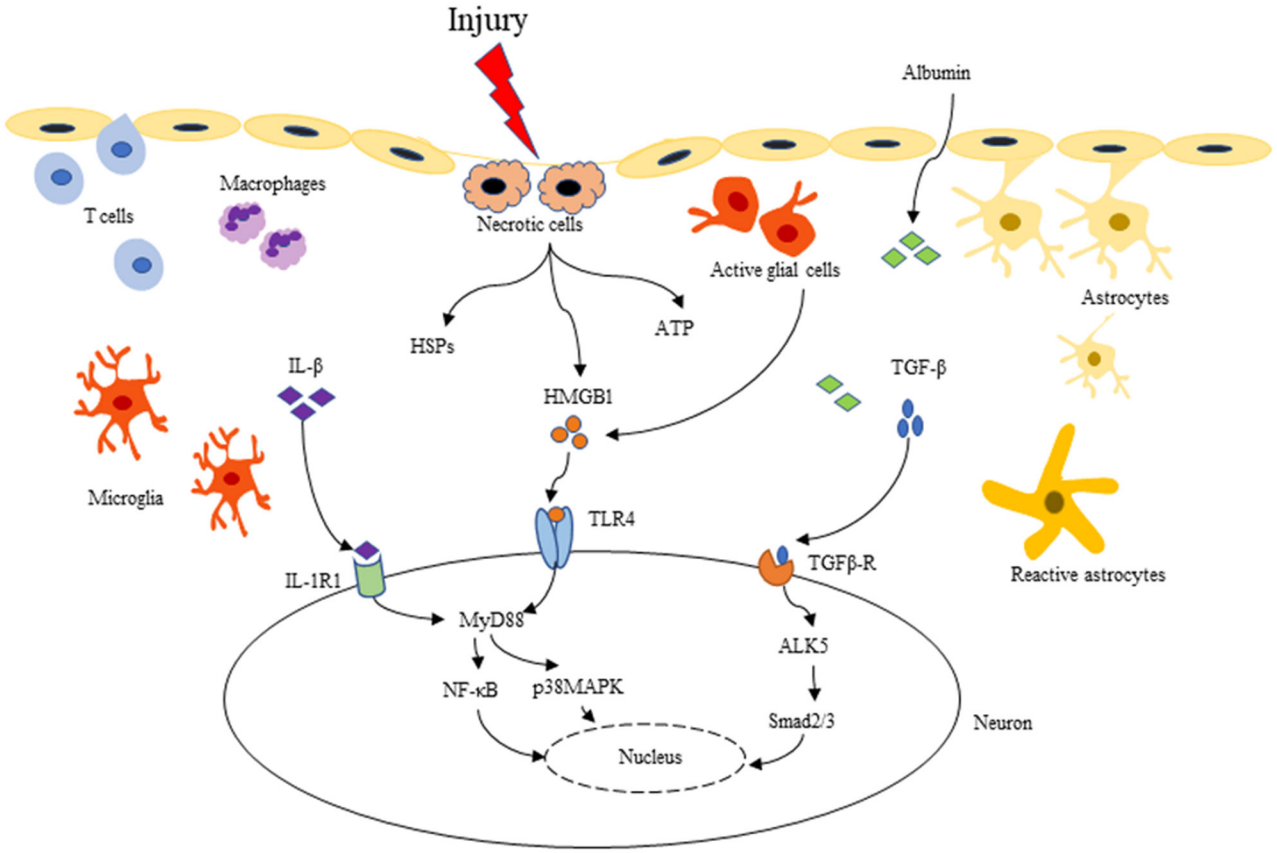

FIGURE 2 | Three key inflammatory signaling pathways related to PTE: IL-1β/IL-1R, HMGB1/TLR4, and TGF-β/albumin pathway. After TBI, BBB is destroyed, as well as microglia and astrocytes are activated. Pro-inflammatory cytokines such as IL-1β, HMGB1 and TGF-β are released into the extracellular matrix. IL-1β binding to IL-1R can activate the downstream NF-κB, p38 MAPK, Src, etc. and initiate intracellular signal transduction through MyD88-dependent or independent signaling pathways. HMGB1 can be passively released by necrotic cells, or be actively secreted to the extracellular from activated microglia and astrocytes, binding to many different types of cell receptors (TLR2/4, RAGE), and activating downstream signaling molecules like the IL-1β/IL-1R signaling pathway. In addition, TBI causes BBB destruction, serum albumin extravasates into the extracellular matrix, activating the TGF-β/ALK5 pathway. These series of inflammatory cascades can lead to increased excitability and synaptic reconstruction, which in turn promotes the development of PTE.

mediated HMGB1 signaling phosphorylates NR2B subunit of NMDA receptor and promotes calcium influx, which is pivotal for inducing excitotoxicity and accelerating epileptogenesis (70). The HMGB1/TLR4 signaling pathway is similar to that of IL-1β/IL-1R, which activates the downstream NF-κB and MAPK through the MyD88-dependent or independent pathways, and promotes the neurovascular dysfunction. Inhibiting TLR-4 can reduce brain edema and IL-6 production post-TBI (71). Overall, there is much accumulating evidence implicating HMGB1/TLR4 signaling pathway in epileptogenesis, however further studies are needed to prove their precise mechanism in PTE.

TGF-β

TGF-β is a multifunctional cytokine involved in many different cellular processes such as cell proliferation, differentiation, adhesion, migration and apoptosis (72, 73). TGF-β can be up-regulated in various CNS diseases, including multiple sclerosis, Alzheimer's disease (AD), stroke, and TBI. Clinical studies found that TGF-β in CSF was significantly elevated 24 h after TBI, and gradually recovered after 21 days, and the level of TGF-β was related to the function of BBB (74). TGF-β is mediated by two TGF-β receptors (TGFβR I and TGFβR II), which phosphorylates downstream Smads protein (Smad 1/5 or Smad 2/3) through ALK1 and ALK5 receptors, activates NF-κB or MAPK, and regulates target gene transcription.

Is TGF-β associated with epileptogenesis? Animal experiments have shown that TGF-β upregulation in neurons of amygdale-kindled rats (75) and in hippocampal astrocytes of SE models (76), supporting the potential role of TGF-β in epileptogenesis. BBB dysfunction is a hallmark of brain injury. TGF-β has also been demonstrated to be involved in microglial activation (77) and pericyte-induced BBB function (78). Increased BBB permeability was found in MRI of PTE patients, which was consistent with the site of epileptogenic foci (79). Studies on the ultrastructure of surgically resected epileptic tissues have confirmed significant anatomical abnormalities in BBB components, including endothelial cells, basement

membranes, and tight junctions (80). van Vliet et al. (81) found a positive correlation between BBB permeability and seizure frequency in the chronic epileptic model, suggesting that BBB dysfunction is conducive to the development of temporal lobe epilepsy (TLE). *In vivo* and *in vitro* experiments (82) have confirmed that exosmic serum albumin post-injury could activate TGF-β/ALK5 pathway of astrocytes, and TGF-β inhibition, SJN2511, could effectively reduce and prevent synaptic remodeling and seizures. Consistent with this finding, Iven et al. (83) also found that TGF-β1 was directly exposed to albumin after BBB destruction, which could cause local cortical dysfunction and induce epileptic discharge. One possible mechanism is local BBB damage leading to serum albumin seep into the cerebral cortex microenvironment. Albumin is uptake by astrocytes through its TGFβR II, and then down-regulate membrane Kir4.1, resulting in an increase in extracellular potassium, which leads to excessive activation of NMDA receptors and causes neuronal hyperexcitability and epileptiform discharge. Antagonists, blocking albumin binding to TGF-β receptors, have been reported to reduce or inhibit such epileptic activity (83). Transcriptome analysis revealed that TGF-β1 induced a similar transcriptional regulation patterns when exposed to serum albumin; Blocking the TGF-β signaling pathway not only reversed the transcriptional response after albumin exposure, but also prevented epileptic activity (84). Therefore, damage to microvessels during TBI may lead to serum albumin extravasation and inflammatory response, which are key steps in PTE development.

TNF-α

TNF-α, the primary subtype of TNF, is one of the critical mediators involved in immune response and neuroinflammation in the CNS, and is known to be released acutely after tissue injury in an active fashion by reactive glial cells, neurons and vascular endothelial cells. TNF-α can activate polymorphonuclear leukocytes, release ROS and various inflammatory mediators, play an important role in secondary brain injury. In addition, TNF-α can directly damage vascular endothelial cells, cause microvascular spasm, increase capillary permeability, and aggravate cerebral edema (85). The TNF-α signaling pathway is mediated by two membrane receptors TNFR1 (also called p55) and TNFR2 (also called p75). TNFR1, which is widely expressed, can be activated by binding to soluble TNF (solTNF) or transmembrane TNF (tmTNF) mediating downstream signaling pathways to initiate apoptosis (86). Compared with TNFR1, TNFR2 expression is limited and mainly released by microglia and endothelial cells known to regulate cell proliferation (87). Current studies have suggested that TNF-α may play a dual role as a pro-inflammatory and anti-inflammatory cytokine, depending on the timing and signaling cascade involved. Scherbel et al. (88) found in TNF-α knockout mice that TNF-α produced in the early stage of TBI may be deleterious, but the lack of TNF may increase neuronal loss and recovery time in the chronic period. TNF-α and IL-1 stimulate astrocytes to produce NGF and IL-10, which may be the basis of the neuroprotective and anti-inflammatory effects of TNF-α (89, 90).

As for the demonstration of a dual role of TNF-α in neuroinflammation, studies have shown that TNF-α has both epileptogenic and antiepileptic effects. In one study, transgenic mice with neuronal overexpression of TNF-α developed seizures and early death (91). Another study demonstrated that mice lacking TNFα receptors were observed to have prolonged seizures (92). The dichotomous role of TNF-α in seizures is thought to be mediated by the different receptors, p55 and p75. Balosso et al. suggested that the p75 pathway is involved in the anti-seizure activity of TNF-α, whereas p55 pathway mediates the pro-seizure effect. However, the mechanism that determines the predominance of these two pathways has not yet been explored, the role of TNF-α signaling pathway in epileptogenesis after TBI remains unclear.

IL-6

IL-6 is a multifunctional factor that can be secreted by several cells in the CNS, including microglia, astrocytes and neurons, and may also play a dual role in neuroinflammation following TBI. IL-6 has been reported to increase adhesion molecules and chemokines secretion, enhancing leukocyte recruitment and acting as a pro-inflammatory cytokine. In contrast, IL-6 can inhibit the production of TNF-α, and reduce NMDA-mediated calcium influx. Swartz et al. (93) showed that IL-6-deficient mice were found to have a slowed healing process following TBI, whereas overexpression of IL-6 resulted in a more rapid recovery by improving re-vascularization of the injury site.

Similarly, there is some evidence also implicated a role for IL-6 in seizure pathologies.

IL-6, IL-1β, and TNF-α were rapidly upregulated 2 h after limbic SE induced by electrical stimulation, peaking at 6 h, which may cause hyperexcitability in epileptic tissue (94). IL-6 was also upregulated in the CSF of patients with newly diagnosed tonic-clonic seizures (95). Elevated IL-6 in the CSF or plasma has been reported to be associated with the severity of epileptic seizures. Transgenic mice over-expression of IL-6 resulted in seizure threshold reduction and hippocampal excitation augment. In fact, IL-6 et al. inflammatory cytokines may promote hippocampal GABAergic neuron loss, leading to an increased propensity for seizures owing to reduced inhibitory interneurons (96).

IL-10

The cytokine IL-10 is a potent anti-inflammatory cytokine, which is found in the CSF of patients with TBI. Intravenous injection of IL-10 significantly reduced proinflammatory cytokine expression (particularly TNF-α and IL-1) and improved neurological outcome in lateral FPI model of rats (89). It has been indicated that IL-10 plays a neuroprotective role by acting on the peripheral immune system and is associated with circulating monocytes which can inhibit leukocyte recruitment and accumulation.

Although few studies have shown that IL-10 regulates susceptibility to seizures after TBI, several animal studies have indicated its anti-seizure effects. For example, IL-10 application was shown to eliminate the hypoxia-evoked epileptiform activity in rat hippocampal slices (97). Another study suggested that IL-10 was genetically related to febrile seizures (FS) in rats, the

seizure threshold temperature in IL-10 treated rats was higher than that in control groups, indicating that IL-10 made animals more resistant to FS (98). The antiepileptic functions of IL-10 are thought to be due to the anti-inflammatory effects of cytokines.

Chronic Neuroinflammation

About a quarter of TBI patients develop progressive neurodegenerative syndromes such as AD, chronic traumatic encephalopathy (CTE), and PTE. The underlying pathogenesis remains unclear, but inflammation has received increased attention from researchers in recent years concerning the pathophysiologic mechanism of various neurodegenerative conditions. As we all know, the pathogenesis of AD is still controversial. There are several hypothetical mechanisms, such as Aβ cascade reaction, Tau hyperphosphorylation, cholinergic hypothesis, etc. However, several lines of evidence suggest that chronic neuroinflammation caused by brain trauma may be a potential factor for AD. Studies in AD models have suggested that neuroinflammatory cytokines and reactive microglia can promote the accumulation and deposition of pathological tau, which may explain the relationship between TBI-induced inflammation and the predisposition to AD (99, 100). But how Aβ induces neuronal hyperexcitability is still unknown (101, 102). Ren et al. (103) explained the possible mechanism through whole-cell recordings of mouse brain slices. They found that Aβ promotes dopamine release in the anterior cingulate cortex, overactivating D1 receptors on interneurons which inhibits GABA release, and then leading to excitatory/inhibitory imbalance. In addition, the accumulation of Aβ has been shown to induce microglial activation and pro-inflammatory mediators release (104). The production of Aβ toxicity after TBI and the disruption of neurotransmitters such as dopamine may have an impact in the development of PTE through inflammatory mechanisms, but this question remains to be further explored. CTE is a progressive neurodegenerative disease associated with repeated head injury, and is most common in athletes and soldiers (105). Studies have shown that activated microglia can last for several years after brain injury, suggesting that a role for a persistent TBI-induced neuroinflammation in CTE development (106). Aungst et al. (107) found that chronic inflammation caused by repeated mild traumatic brain injury (mTBI) can change hippocampal synaptic plasticity, leading to sustained cognitive and neuropsychiatric changes.

In the Kainic acid (KA)-induced acute epilepsy model, the hyperphosphorylated Tau was significantly increased, and the time and location of Tau were consistent with that of mossy fiber sprouting (108). Recent studies have demonstrated that late-onset seizures after TBI are also accompanied by a certain degree of neuronal degeneration and hyperphosphorylated tau (109). Neurofibrillary tangles (NFTs) consisted of Tau were also found in surgical specimens of patients with refractory epilepsy and focal cortical dysplasia, and these Tau tangles are specifically located in the dysplastic area (110, 111). Protein phosphatase 2A (PP2A) appears to be a major serine/threonine protein phosphatase that plays a negative regulatory role in signal transduction, and its increased activity can promote the dephosphorylation of hyperphosphorylated tau (112).

Studies (113) have reported that selenate specifically targets hyperphosphorylated tau, enhances PP2A activity and inhibits seizures in multiple epileptic animal models, suggesting that this may be a new approach to the treatment of PTE. Hippocampal sclerosis is associated with tau protein degeneration in patients with PTE (109). However, there is still no definitive proof showing the hyperphosphorylated tau-based mechanisms in PTE. A comprehensive understanding of the relationship between chronic neuroinflammation and PTE will require more research or more advanced neuroimaging techniques (such as PET imaging) that enable us to study the potential mechanisms of Aβ deposition, tau phosphorylation and microglia/astrocytes activation in neurodegenerative diseases post-TBI.

THERAPEUTIC TARGETS

Although neuroinflammation is increasingly recognized as a critical mechanism in the development of epilepsy, few studies have been conducted on immune-targeted pharmaceuticals of PTE to date. A phase IIA clinical trial showed that the selective IL-converting enzyme (ICE)/Caspase 1 inhibitor VX-765 could effectively alleviate seizures in some patients and continue for a period of time after drug discontinuation (114). Anakinra, an IL-1R1 antagonist, has also been demonstrated to reduce refractory epilepsy (115). The broad-spectrum antibiotic minocycline was reported to inhibit the microglial activation and proinflammatory factors release, reducing the frequency of seizures in patients with drug-resistant epilepsy (116). Given the complex, and variable inflammatory pathways associated with, combinations of anti-inflammatory drugs may be more effective than a single medication. VX-765 and TLR4 antagonist therapy on the IL-1R1/TLR-4 signaling pathway effectively prevented the epilepsy progression and significantly reduced the chronic seizures (117). Similarly, Kwon et al. (118) found in the pilocarpine-induced SE model that a combination of COX2 inhibitor CAY10404 and minocycline was more effective than single drug in reducing neuron damage of the hippocampus CA1 region and spontaneous seizures. As mentioned above, the TGF-β/albumin signaling pathway has also generated interest as an immune-therapeutic target for PTE. Studies have reported that angiotensin II type 1 receptor antagonist losartan can effectively block the TGF-β activation induced by albumin, delay the development of acquired epilepsy and reduce the severity of seizures (119). Inducible nitric oxide synthase (iNOS) is a key mediator of immune activation and inflammation, and its inhibitor, 1,400 W, has shown to inhibit epileptogenesis in rodent models of epilepsy (120–122). It is important to note that some of the drug-resistant epilepsy therapies, such as non-steroidal anti-inflammatory drugs, steroids, cannabinoid drugs, ketogenic diet and vagus nerve stimulation, have also been proved to have an anti-inflammatory effect, but there is still a notable lack of conclusive evidence to delineate these relationships (123, 124). Anti-epileptic therapeutics targeting immune inflammation has shown great potential in preventing and treating PTE, which is worthy of further research.

CONCLUSIONS

PTE is a severe complication of TBI, which significantly affects the quality of life of patients. As PTE is drug-resistant in at least one-third of patients, further research is needed to find novel therapeutic strategies for preventing the development of epilepsy after TBI. Clinical and experimental evidence has emphasized brain neuroinflammation as a key factor contributing to epileptogenesis. This review presents our current understanding of the immune inflammatory response to PTE, including microglial activation, reactive astrocytes proliferation, peripheral immune cells infiltration, inflammatory cytokines release, chronic neuroinflammation and potential therapeutic targets. However, the pathogenesis of PTE is very complex and has not yet been fully elucidated. There are still many unknown areas worth exploring further. One of the most essential areas warranting investigation is the possible inflammatory signaling pathways, especially the TGF-β/albumin signaling pathway. Another field of concern is the relationship between neurodegeneration and PTE. Inhibiting tau phosphorylation by sodium selenite may be a new approach to the treatment of delayed seizures. A better understanding of how the inflammatory response promotes epileptogenesis after TBI is the key to immune-targeted therapy.

AUTHOR CONTRIBUTIONS

LS conducted literature review and wrote the initial draft of the manuscript. WS, HY, and RL made preliminary revision. JW and QW made critical revision. All authors contributed to manuscript revision and approved the submitted version.

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Altered Protein Profiles During Epileptogenesis in the Pilocarpine Mouse Model of Temporal Lobe Epilepsy

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Epilepsy is characterized by recurrent, spontaneous seizures and is a major contributor to the global burden of neurological disease. Although epilepsy can result from a variety of brain insults, in many cases the cause is unknown and, in a significant proportion of cases, seizures cannot be controlled by available treatments. Understanding the molecular alterations that underlie or are triggered by epileptogenesis would help to identify therapeutics to prevent or control progression to epilepsy. To this end, the moderate throughput technique of Reverse Phase Protein Arrays (RPPA) was used to profile changes in protein expression in a pilocarpine mouse model of acquired epilepsy. Levels of 54 proteins, comprising phosphorylation-dependent and phosphorylation-independent components of major signaling pathways and cellular complexes, were measured in hippocampus, cortex and cerebellum of mice at six time points, spanning 15 min to 2 weeks after induction of status epilepticus. Results illustrate the time dependence of levels of the commonly studied MTOR pathway component, pS6, and show, for the first time, detailed responses during epileptogenesis of multiple components of the MTOR, MAPK, JAK/STAT and apoptosis pathways, NMDA receptors, and additional cellular complexes. Also noted are time- and brain region- specific changes in correlations among levels of functionally related proteins affecting both neurons and glia. While hippocampus and cortex are primary areas studied in pilocarpine-induced epilepsy, cerebellum also shows significant time-dependent molecular responses.

Keywords: epileptogenesis, seizures, signaling, pilocarpine, protein phosphorylation, reverse phase protein array

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INTRODUCTION

Epilepsy affects more than 65 million people worldwide, making it the third most common neurological disorder (1). While some cases of epilepsy have a known genetic basis, others, the acquired epilepsies, arise from brain insults, such as traumatic injury, inflammation, tumor, and stroke (2). In many cases, however, the causes are unknown. Importantly, in ∼30% of cases, seizures are not controlled by current therapeutic options.

Epileptogenesis is the process in which a normally functioning brain is transformed to one displaying the excessive and abnormal synchronous neuronal activity of an epileptic brain, manifested as spontaneous recurrent seizures (2, 3). Epileptogenesis is likely a continuous process developing over weeks, months, or even years beyond the initial insult (4–6). Treatments for epilepsy may therefore focus on preventing or controlling seizures after epilepsy is established, or ideally, on interrupting the damaging processes during epileptogenesis and preventing onset of spontaneous seizures all together. Understanding of the molecular processes occurring during epileptogenesis, and discriminating the deleterious from the compensatory, will help in developing novel therapeutics.

The most common focal human epilepsy is Temporal Lobe Epilepsy (TLE), representing ∼60% of focal epilepsy cases. Pilocarpine injection in rodents is considered a good model of TLE because it produces characteristics observed in human epilepsy, including a seizure-free (latent) period prior to onset of recurrent seizures and development of localized lesions within the hippocampus (7, 8). Of interest also, the chronic seizures induced by pilocarpine are largely refractory to anti-seizure drugs, a situation similar to that observed in patients with TLE. The pilocarpine model and other models of acquired epilepsy such as kainate, pentylenetetrazol, and direct electrical stimulation, allow analysis of the molecular processes occurring at specific time points during epileptogenesis, from status epilepticus through the latent period to the stage of chronic spontaneous seizures. Using these models, detailed molecular observations can be made. For example, during the chronic phase after pilocarpine injection, increased levels of the protein for the immediate early gene, cFos, were seen in principal neurons within 15 min of an episode of seizures and, a few hours later, increased Fos protein was present in the cell bodies of interneurons (9). In similar studies, increased phosphorylation of ERK1/2 was seen in a subpopulation of neural progenitors specifically located within the sub-granular zone of the dentate gyrus of hippocampus (10); this disappeared within minutes of seizure onset (11). From the phenotypes caused by single gene mutations (e.g., TSC, tuberous sclerosis), a role for the Mechanistic Target of Rapamycin (MTOR) pathway has been established in epilepsy. MTOR activation, commonly assessed by levels of ribosomal protein S6 phosphorylation (pS6), has been demonstrated during epileptogenesis in rodent models (12–15). More comprehensive information on gene expression abnormalities can be obtained from whole transcriptome studies via RNA microarrays. Hansen et al. found that pilocarpine caused transcriptional changes influencing the MAPK pathway (16). Integrating results from multiple such SE rodent model transcriptome studies, Chen et al. identified common differentially expressed genes; "hub" genes were enriched in those related to inflammatory responses and microglial/macrophage activation (17). A limitation of these latter studies remains that RNA levels do not robustly predict protein levels, and perturbations in levels of protein modifications/activations, critical to driving cellular changes, necessarily remain unknown. Mass spectrometry-based large-scale proteomic studies have also been carried out in rodent models (18–21). Such studies have identified additional candidate proteins and pathways but, notably, failed to identify commonly studied components of the MTOR and MAPK pathways. Thus, knowledge of critical perturbations occurring in rodent models of TLE, and by extension human TLE, remain to be robustly established.

To this end, we have used the moderate throughput technique of reverse phase protein arrays (RPPA). We measured levels of 54 components of major signaling pathways and cellular processes, in hippocampus, cortex and cerebellum at six time points after injection with pilocarpine and induction of status epilepticus. The results include demonstration of complex sequential alterations in levels of components of the MTOR and MAPK pathways and in subunits of the N-methyl-D-aspartate receptor (NMDAR) receptor. They also show correlations among levels of pathway and receptor components in saline treated mice, and how these correlations are lost and altered over time with pilocarpine. Components of the JAK/STAT and apoptosis pathways also show significant time-dependent alterations. While hippocampus, the brain region most affected during pilocarpine-induced epileptogenesis, shows large numbers of perturbations, cortex and cerebellum also display significant and divergent responses in pathways that are critical for brain function. Together, these data present a novel picture of the complexity of protein responses during epileptogenesis. These may provide targets for novel therapeutics aimed at disrupting the epileptogenic processes and preventing development of spontaneous seizures.

MATERIALS AND METHODS

All animal procedures were performed in accordance with the regulations of the institutional animal care and use committees of the University of Colorado Anschutz Medical Campus and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Pilocarpine-Induced Status Epilepticus

A total of 160FVB/NJ mice (all males JAX stock #001800) were purchased from The Jackson Laboratory (Bar Harbor, ME) and received at 5–7 weeks of age. Prior to experiments, littermates (on average, 5) were group housed in temperature- and humidity-controlled rooms on a 12 h light/dark cycle with access to food and water *ad libitum*. All mice were allowed to acclimate to the environment and altitude for 1 week prior to handling. Prior to the start of experiments, mice (18–24 g) were briefly handled once daily for at least 1 week to reduce the stress induced by experimental protocols. On the day of

the injections, mice were transferred to an experimental room, marked, weighed, and allowed to habituate undisturbed for at least 1 h. Entire litters (an average of 5 mice) were randomly assigned to a single treatment/time point group. Only in cases where fewer than 5 mice comprised a single litter were mice from two litters combined for a single treatment/time point. For pilocarpine treated mice, 15 min before the first pilocarpine injection, mice were given an intraperitoneal injection of 1 mg/kg scopolamine methyl bromide (Sigma-Aldrich) to block the peripheral muscarinic effects of pilocarpine. The initial dose of pilocarpine HCl (200 mg/kg; Sigma-Aldrich) was followed, after 1 h, by subsequent doses (100 mg/kg) given at 30 min intervals until the onset of SE, defined as the appearance of repeated behavioral seizures (stage four or higher with at least one seizure being five or higher) according to a modified Racine scale (22, 23). In the majority animals, SE initiated within 1 h of the first pilocarpine injection; thus these animals received a single injection. The maximum number of injections given was three. SE persisted for at least 90 min. Control mice were given injections of saline at identical time intervals. After SE induction, mice were returned to their home room, singly housed, and given free access to water and moistened chow. Cohorts (5 per group) of pilocarpine treated mice were sacrificed after SE, at 15 min (onset), 1 and 6 h, 1 and 5 days, and 2 weeks. Groups of saline-injected mice were sacrificed at the same time points (see **Supplementary Figure 1**).

Tissue Processing and Preparation of Protein Lysates

In order to preserve the endogenous levels of protein phosphorylation, mice were sacrificed by cervical dislocation without anesthetic. The whole brain was rapidly removed, immediately snap frozen in liquid nitrogen, and stored at −80°C. Before lysate preparation, the brains were removed from the freezer and, without thawing, rapidly heated to 95°C under vacuum conditions using the Stabilizor T1 (Denator, AB), as described previously (24). The cortex, hippocampus, and cerebellum were dissected out, weighed, placed in 10 volumes of IsoElectric Focusing (IEF) buffer (8 M urea, 4% CHAPS, 50 mM Tris) and homogenized by sonication with three bursts of 5 s in a Branson Sonic Power Co. (Danbury, CT). Homogenates were centrifuged at 14,000 rpm for 30 min at 4°C to remove debris, and the protein concentration of the cleared supernatant was determined using the 660 nM Protein Assay Kit (Pierce); all sample protein concentrations were within the range of 9–11 mg/ml.

Antibodies and Validation for RPPA

Reverse phase protein arrays (RPPA) require highly specific antibodies. Prior to use, the specificity of each lot of each antibody was verified by Western blot using mouse brain lysates to show that only clean band(s) of explainable size, with no non-specific bands, were present. All secondary antibodies (IgG; anti-goat, rabbit, and mouse) have been shown previously to produce signals that are <5% above local background when incubated with an RPPA slide in the absence of any primary antibody; signals of these levels are too low to be reliably quantitated and were ignored in data analysis. Proteins screened for expression level are listed in **Supplementary Table 1**, with antibody information regarding source, catalog number, and dilution factor.

Array Assembly, Printing, and Staining

Each sample lysate was prepared in three replicates of a 5-point serial dilution, with a 0.8 dilution factor, and 1 buffer control, in a 384-well V-shaped AB gene plate (Thermo Fisher Scientific, Rockford, IL). Samples were printed, in triplicate, onto nitrocellulose-coated glass slides (Grace Bio-Laboratories, Inc., Bend, OR) using an Aushon BioSystems 2470 Arrayer (Aushon BioSystems, Billerica, MA) with 185-μm pins and a single touch. The arrays were produced in two major print runs and slides were stored at 4°C until further use. For protein detection, slides were incubated in blocking solution (3% BSA, Sigma, USA) in TBST (Tris-buffered saline, 0.1% Tween 20) for 4 h, followed by overnight incubation at 4°C with shaking with the primary antibody (antibody dilutions are provided in **Supplementary Table 1**). Detection of the bound primary antibody was performed by incubation with the secondary antibody, Fluorescence Alexa Fluor 555 goat anti-mouse or anti-rabbit or rabbit anti-goat (1:2,000 dilution) (Invitrogen, Carlsbad, CA), for 90 min at room temperature. Slides were washed and dried, and signals were detected by scanning on a PerkinElmer Scan Array Express HT Microarray Scanner (PerkinElmer Inc., MA, USA). For normalization, total protein for each spot was determined by staining three non-sequential slides from each print run with SyproRuby reagent (Invitrogen, CA, USA) following the manufacturer's protocol.

Image Analysis, Quantification, Normalization, and Statistical Analysis

Signals on each slide were quantified using Scan Array Express software (PerkinElmer Inc., MA, USA). After removal of technical outliers, each SyproRuby-normalized protein value was included in the statistical analyses if the level was within its mean ±3 standard deviations. Mean differences between groups were reported as a percent change, assessed using a hierarchical three-level mixed effects model to account for possible correlations and variability between replicates and dilutions within each sample. The Benjamini-Hochberg corrected $p < 0.05$ with a false discovery rate (FDR) of 5% was considered for overall statistical significance across the entirety of the hypotheses. **Supplementary Tables 2–4** contain results (% differences, p-value. and FDR) for all measured proteins in the hippocampus, cortex, and cerebellum for, respectively, the pilocarpine vs. saline, saline vs. saline chronic, and pilocarpine vs. saline-chronic comparisons. To assess possible relationships between levels of all pairs of proteins, Spearman correlation analysis was carried out. Protein values were reduced to one observation per mouse for each brain region of each individual of each treatment group and used to compute Spearman correlation coefficients. Graphs for data from protein pairs with correlation coefficients >0.8 with $P < 0.05$ were manually inspected and those with non-linear relationships were eliminated; the remainder were used to generate correlation network figures. All data analyses

were carried out using SAS® version 9.3 (SAS Institute Inc., Cary, NC). Additional details of quantification and review of data quality and reproducibility were carried out as described previously (25–27).

RESULTS

The goal of these experiments was to determine the time courses of protein expression changes affecting important signaling pathways in a pilocarpine model of acquired epilepsy. Levels of 54 proteins were measured in hippocampus, cortex and cerebellum, at six time points after pilocarpine injection: at ∼15 min (after onset of seizure activity), at 1, 6, and 24 h (acute period), at 5 days (the latent period, with no spontaneous seizure activity), and at 2 weeks (during the chronic period, characterized by spontaneous recurrent seizures). Saline-injected mice served as controls and cohorts were sacrificed at the same time points post-injection as pilocarpine treated mice. Proteins measured included 18 components of the classical MAP kinase pathway (10 to phosphorylation-specific forms and 8 to phosphorylation-independent forms), 16 components of the MTOR pathway (6 to phosphorylation-specific forms), and 8 subunits of ionotropic glutamate receptors (5 for NMDA receptors, including 3 phosphorylation-specific forms, and 3 for AMPA receptor subunits, including one phosphorylation-specific form). Additional proteins included components of apoptosis and JAK/STAT pathways. Not all proteins of interest could be measured because of the requirement for highly specific antibodies, free of significant background signals. A complete list of the proteins analyzed is provided in **Supplementary Table 1**.

Given two treatment groups and six time points, many protein expression comparisons can be made. Three were selected as the most biologically informative. The first, pilocarpine/saline, is the direct comparison of pilocarpine treated to saline treated mice at corresponding time points post-injection. The second, saline/saline-chronic, is the comparison of saline treated mice at each time point to saline treated mice at the chronic time point. No expression changes would be expected to be induced by saline, beyond responses to the stress of handling and injection experience, and these would be expected to dissipate early in the time course. However, saline treated mice were housed in the same room as pilocarpine injected mice and thus were exposed to the stress of seizure experience in their littermates. This "observer" status (or other unknown environmental influences) indeed turned out to be associated with significant protein expression changes throughout the time course. To describe these changes, for the second comparison, we assumed that the saline-chronic expression pattern most closely approximated the expression pattern of untreated mice, because by this time, mice have had 3 weeks to adjust to the post-injection environment. The third comparison, pilocarpine/saline-chronic, describes protein levels in pilocarpine mice at each of the 6 time points relative to those in saline treated mice from the chronic time point. This was chosen to best describe pilocarpine-induced differences in expression from an approximately normal baseline. Data for all proteins in the three brain regions and three comparisons are provided in **Supplementary Tables 2–4**.

Pilocarpine/Saline Comparison

The heatmaps in **Figure 1** compares protein expression, in the three brain regions, between pilocarpine and saline treated mice at corresponding time points. **Table 1** lists the number of proteins whose levels are increased and decreased in each brain region at each time point. In hippocampus (**Table 1** and **Figure 1**), at onset of seizures, there are significant elevations in the levels of 28 proteins, among them, multiple components of the MTOR and MAPK pathways. These include not only the commonly measured pS6 and pERK, but also pMTOR, RAPTOR, AKT, and pAKT, as well as kinases of the MAPK pathway pBRAF and pRSK, and the downstream transcription factor, pELK. Multiple subunits of NMDA and AMPA receptors are also elevated at onset. These initial increases are followed at 1 h by decreases in expression of a total of 20 proteins, 12 of which were initially elevated. This is a bimodal pattern with a second round of increases (in 28 proteins) present at 24 h, followed again by decreases (in 40 proteins) during the latent period. Lastly, during the chronic phase, 8 proteins remain elevated and 2 are repressed compared with saline mice. Among these are pS6 and RAPTOR from the MTOR pathway and BRAF and pERK from the MAPK pathway.

Similar to hippocampus, cortex and cerebellum also showed increased levels of many proteins at onset (20 and 30, respectively; **Table 1** and **Figure 1**), and several were common to those in hippocampus, notably pS6 and pERK, and pMTOR, RAPTOR, BRAF, pRSK, pCASP9, pJNK, pNR1, 2A, and 2B. Cortex does not, however, show the large number of decreased protein levels at 1 h, and has fewer increased proteins at 24 h (only 17 vs. 28 in hippocampus). Cortex does however show a large number (28) of decreases in protein levels during the latent period. Cerebellum was unique in rapidly stabilizing protein expression, showing few changes (only 3–7 in total) at each time point after onset.

Tables 2–4 provide the magnitudes of protein expression differences for components of the MAPK and MTOR pathways, plus apoptosis, and JAK/STAT related proteins in hippocampus, cortex and cerebellum. Of note, in the MAPK pathway, although BRAF, MEK, ERK, and RSK are sequential in the kinase cascade, differences in phosphorylation levels (from saline injected mice) are not necessarily correlated in direction and magnitude. For example, in hippocampus at onset, while pBRAF and pERK levels have increased, the level of the intermediate pMEK has decreased. In cortex at onset, pBRAF and pMEK each increased by ∼25%, but pERK increased by 170%. In general, in both brain regions, while the direction of change is consistent, the magnitude of pERK change tends to be greater than those for pBRAF and pMEK. The ratios of phosphorylated form to total kinase, indicating the ratio of activated kinase, also change with time in a non-uniform fashion. For example, in hippocampus at 24 h, the total ERK level has decreased by 28% while the level of pERK has increased 71%, indicating a 2.4-fold increase in the proportion of activated ERK; in contrast, during the latent period, proportions of pERK/ERK are

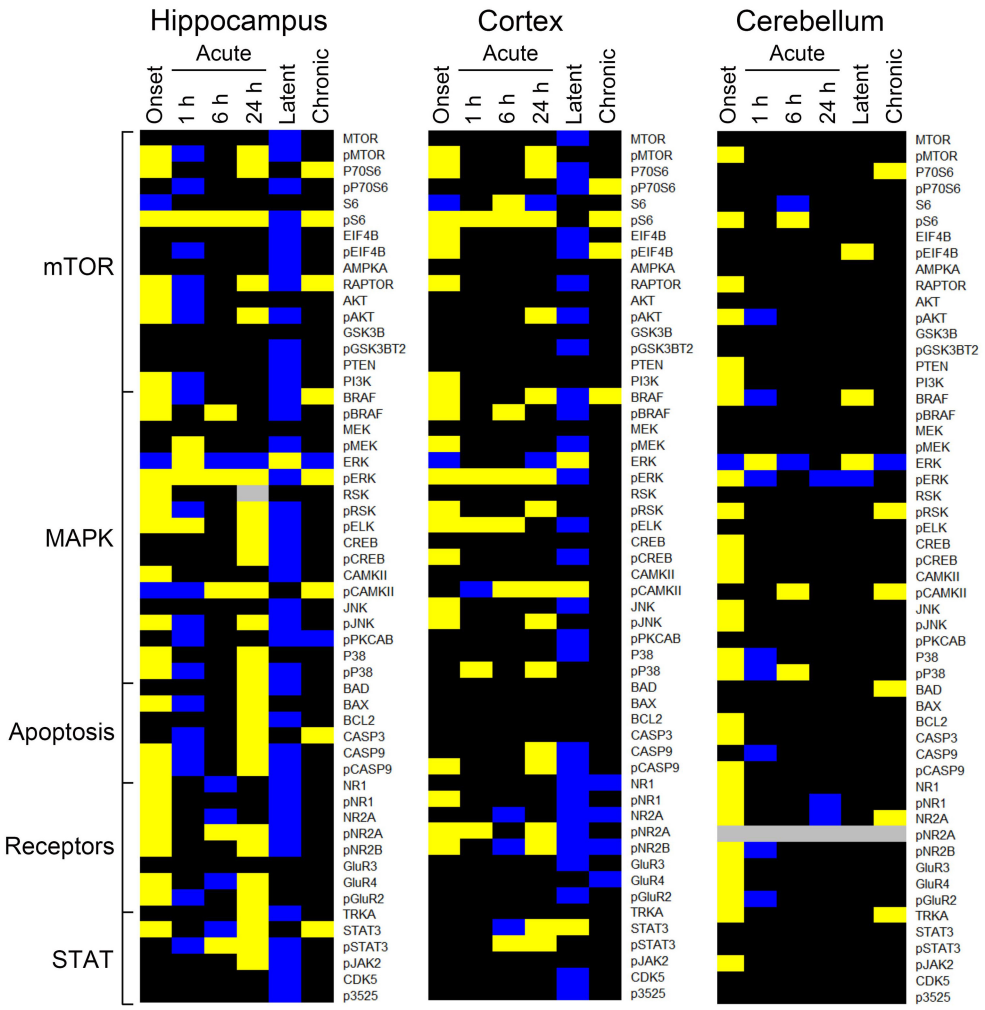

FIGURE 1 | Changes in protein expression during epileptogenesis. Heatmaps show proteins with an average expression level in pilocarpine treated mice significantly different from saline treated mice at the corresponding time points. Only proteins with differences ≥15% as determined by a three level mixed effects model, with $p \leq 0.0005$, and a Benjamini-Hochberg correction for multiple testing applied with a false discovery rate of 0.05 are shown. Data derived from **Supplementary Table 2**. Proteins are grouped by general category. Yellow, increased; blue, decreased; black, no difference; gray, not measured. $n = 5$ for each group, except for chronic pilocarpine where $n = 4$.

decreased by 0.4-fold from those in saline mice. Alternatively, the level of RSK protein is stable after the initial increase at onset, while levels of pRSK range from −32 to +37%. Cortex also shows disproportionate changes in activation; ratios of pBRAF/BRAF do not change as dramatically throughout the time course, while those of pERK/ERK approximately double at onset and 6 h. Large variations during the sub-acute period are seen in levels of pCAMKII, in hippocampus changing from −45% at 1 h to +83% at 24 h, and in cortex, changing from −29 to +102%. Cerebellum does not show great differences in sequential kinase phosphorylation levels, nor time changes in activation ratios. One notable feature common to all three brain regions are the magnitudes of responses of phosphorylated P38. In both hippocampus and cerebellum, pP38 increases by 40% at onset and in cortex, by 47% later, at 24 h.

By the chronic stage, only 4 and 3 of the 17 MAPK components remain abnormal in hippocampus and cortex, respectively, suggesting that, while many proteins are responsive in the early stages post-SE, abnormalities in these proteins largely are not playing roles in the production of chronic seizures. This differs in cerebellum, where 9 MAPK components remain abnormal, in particular pRSK and pCAMKII levels are elevated by 21 and 30%, respectively, and ERK is decreased by 31%.

Observations of the MTOR pathway are qualitatively similar to those of MAPK. In both hippocampus and cortex, levels of total MTOR are largely stable, while levels of pMTOR range from −30 to +27% in hippocampus and −12 to +28% in cortex. While levels of pS6 are constantly elevated in the sub-acute stages, with the largest elevation of any protein, 133 and 175% seen in hippocampus and cortex, respectively, at 24 h, neither levels of S6 nor the precursor kinase, pP70S6, change dramatically. It is

TABLE 1 | Summary of protein expression changes.

Brain region	Onset	1 h	6 h	24 h	Latent	Chronic
Pilocarpine/saline						
HP	28,3 (17)	5,20 (7)	7,5	28,1 (18)	1,40 (23)	8,2
CR	20,2 (4)	5,1	8,3	17,2 (8)	3,29 (11)	4,4
CB	30,1 (13)	1,5	3,2	0,3	3,1	6,1
Saline/saline-chronic						
HP	0,6	5,2	10,0	1,21	9,1	na
CR	0,24 (2)	0,5	1,11	1,16	5,2	na
CB	8,6	18,1	13,1	10,2	18,1	na
Pilocarpine/saline-chronic						
HP	14,1	3,11	14,1	8,6	1,33 (14)	2,8
CR	2,3	5,9	7,5	3,4	1,25 (7)	4,4
CB	30,1 (10)	13,2	11,1	1,1	24,1 (5)	6,1
Pilocarpine/saline-latent						
HP	6,2	3,25 (10)	7,2	3,7	1,49 (23)	1,3
CR	2,6	7,13	5,0	6,6	3,29 (11)	3,5
CB	6,0	5,8	0,1	2,5	3,1	0,2

Number of proteins with significantly increased or decreased levels respectively in each treatment group, per time point, and brain region. Numbers in brackets indicate the numbers of proteins with magnitudes of change ≥25%.

of interest that levels of pMTOR, RAPTOR, and pAKT show the similar magnitudes of increases and decreases through the sub-acute and latent stages in both regions. In cerebellum, there are fewer and smaller changes in MTOR: pS6 is elevated by ∼25% at onset and 6 h, while pEIF4B is uniquely elevated during the latent period. During the chronic phase, pS6 remains elevated in cortex and hippocampus, and pEIF4B uniquely elevated in cortex.

Lastly, **Tables 2**–**4** include proteins involved in apoptosis and JAK/STAT signaling. In hippocampus, the pro-apoptotic proteins, BAD, and BAX, show similar patterns and magnitudes of changes as the anti-apoptotic protein, BCL2 throughout the sub-acute time points. In both hippocampus and cortex, all three proteins are decreased at the latent stage. Cerebellum is unique in abnormal levels in the chronic stage, elevated BAD and BCL2, and decreased BAX. Hippocampus shows the most changes in both CASP9 and pCASP9, with the latter varying ±25 to 30% from onset through the latent stage. Similar to hippocampus, cortex has elevated levels of CASP9 and pCASP9 at 24 h, but repressed levels during the latent period. CASP3 variations have similar patterns to CASP9, but more modest magnitude of change, and remains elevated during the chronic stage in both hippocampus and cerebellum. STAT3 and pSTAT3 both change throughout the entire time course in hippocampus, with pSTAT3 most strongly elevated at 24 h and in the latent period. STAT3 is elevated by 68% at 24 h; it is elevated again, at 55%, during the chronic stage, the largest perturbation at this stage of all proteins and brain regions. Cortex also shows a strong elevation of STAT3, 63% at 24 h.

Figure 2 illustrates the details of expression changes for the NMDA receptor subunits. In hippocampus, at onset, all 5 subunits are elevated, most at ∼25–30%, but NR2A at >55%. By 6 h, levels of 4 are decreased by ∼25% or are not different from those in saline mice. However, now pNR2A is elevated by >60%. During the latent period, all subunits are strongly repressed, but return to control levels during the chronic period. In cortex, only pNR2A shows strong variability, with maxima at 1 and 24 h. Levels of other subunits tend to decrease from highs at onset to levels repressed by 20–40% at the latent time point. Cerebellum shows a much simpler profile; after initial elevated levels, most subunits show few differences from saline controls; only pNR2A and pNR2B are decreased by ∼25% at 1 and 24 h, respectively.

Together these data emphasize that hippocampus is the most affected by pilocarpine and that cortex is also similarly, if less dramatically perturbed. However, the data also show that cerebellum nevertheless displays significant responses in critical signaling pathways, particularly at onset, and that notably, of these proteins, cerebellum displays the largest number of abnormalities chronically.

Saline/Saline-Chronic Comparison

Figure 3 shows heatmaps for protein expression at five time points in saline treated mice compared to the saline chronic time point. Each brain region shows many alterations. In hippocampus (**Figure 3**), there are a large number of decreases (20) at 24 h, among them all measured subunits of NMDA and AMPA receptors, pERK and additional MAPK pathway components. Notably, there are few alterations in the MTOR pathway. In cortex, the majority of decreases occur at onset and 24 h, 24 and 16, respectively. Of the latter, 11 are also repressed in hippocampus, including pNR2A and pNR2B, pMTOR and several MAPK components. In contrast, in cerebellum, the majority of changes are increases, e.g., 18, 10, and 18 are elevated at 1 and 24 h, and during the latent period, respectively. P70S6, GSK3B, and pGSK3B are among those proteins elevated at all time points, and NR2A and BAX are elevated at all time points after onset. Together,

TABLE 2 | Protein changes in hippocampus, MAPK, MTOR, JAK/STAT, and apoptosis pathway components (pilocarpine vs. saline at corresponding time points).

Protein	Onset	1 h	6 h	24 h	Latent	Chronic
MAPK						
BRAF	19%	−23%	–	11%	−25%	19%
pBRAF	26%	–	22%	12%	−15%	–
MEK	–	–	–	–	−14%	–
pMEK	−11%	15%	–	11%	−30%	–
ERK	−37%	39%	−15%	−28%	21%	−25%
pERK	24%	–	13%	71%	−43%	22%
RSK	42%	–	–	–	–	–
pRSK	30%	−16%	−12%	37%	−32%	–
pELK	28%	17%	–	37%	−25%	–
CREB	12%	−10%	–	21%	−26%	–
pCREB	10%	−12%	–	23%	−19%	–
CAMKII	17%	11%	–	9%	−28%	–
pCAMKII	−22%	−45%	21%	83%	12%	38%
JNK	12%	−13%	–	10%	−27%	–
pJNK	34%	−16%	13%	37%	−22%	–
P38	20%	–	–	30%	–	–
pP38	40%	−16%	–	9%	−24%	–
MTOR						
MTOR	–	−14%	–	–	−17%	–
pMTOR	27%	−24%	–	24%	−30%	–
P70S6	18%	−14%	–	46%	−14%	28%
pP70S6	12%	−26%	–	–	−31%	–
S6	−17%	–	–	–	−14%	–
pS6	33%	26%	133%	73%	−32%	44%
EIF4B	11	−13%	–	13%	−24%	–
pEIF4B	12%	−16%	15%	−11%	−26%	–
AMPKA	–	–	–	–	−10%	–
RAPTOR	28%	−19%	–	17%	−35%	20%
AKT	21%	−16%	–	13%	−27%	–
pAKT	23%	−20%	–	26%	−36%	11%
PTEN	8%	10%	–	–	−21%	–
PI3K	21%	−15%	–	–	−17%	–
APOPTOSIS, JAK/STAT						
BAD	14%	−12%	–	16%	−22%	–
BAX	20%	−17%	–	17%	−10%	–
BCL2	12%	−11%	–	21%	−30%	–
CASP3	13%	−18%	–	16%	−13%	21%
CASP9	21%	−19%	14%	23%	−34%	–
pCASP9	31%	−26%	–	28%	−27%	–
STAT3	25%	−14%	−16%	68%	–	55%
pSTAT3	–	−15%	32%	30%	−18%	–
pJAK2	–	–	–	18%	−17%	–
TRKA	13%	–	−12%	25%	−18%	–

*Data extracted from **Supplementary Table 2**. All values are significant as determined by a three-level mixed effects model, with $p \leq 0.005$ and the Benjamini-Hochberg correction for multiple testing, with false discovery rate (FDR) $p \leq 0.05$. Only significant differences $\geq$|10%| are shown.*

these data indicate that the experience of saline treated mice is not totally benign, and that it develops with time. The pattern of changes may be due solely to the injection of saline; however, observing, in close proximity, the trauma of pilocarpine treated littermates may induce stress and be responsible for some responses.

TABLE 3 | Protein changes in cortex, MAPK, MTOR, JAK/STAT, and apoptosis pathway components (pilocarpine vs. saline at corresponding time points).

Protein	Onset	1 h	6 h	24 h	Latent	Chronic
MAPK						
BRAF	17%	–	–	19%	−25%	17%
pBRAF	28%	–	32%	13%	−19%	–
MEK	–	–	–	–	−15%	–
pMEK	24%	−13%	15%	–	−20%	–
ERK	−20%	–	–	−42%	49%	–
pERK	170%	36%	95%	28%	−37%	–
RSK	42%	–	–	–	–	–
pRSK	23%	–	–	22%	–	–
pELK	18%	38%	18%	14%	−19%	−10%
CREB	–	–	–	–	−11%	–
pCREB	17%	–	–	12%	−17%	–
CAMKII	11%	–	–	10%	−12%	–
pCAMKII	–	−29%	62%	102%	26%	–
JNK	23%	–	–	13%	−19%	–
pJNK	22%	–	–	33%	–	–
P38	10%	–	–	–	–	–
pP38	–	29%	–	47%	−14%	–
MTOR						
MTOR	13%	–	–	–	17%	–
pMTOR	28%	–	–	23%	−12%	–
P70S6	17%	–	–	42%	−21%	–
pP70S6	–	–	–	–	−25%	19%
S6	−17%	–	15%	−23%	–	−13%
pS6	32%	87%	175%	67%	–	27%
EIF4B	25%	–	–	14%	−23%	–
pEIF4B	20%	–	–	–	−25	34%
AMPKA	–	–	–	–	–	–
RAPTOR	19%	–	–	15%	−19%	12%
AKT	–	–	–	–	−11%	–
pAKT	11%	–	–	16%	−18%	–
PTEN	–	–	–	–	−11%	–
PI3K	23%	−10%	–	13%	−14%	–
APOPTOSIS, JAK/STAT						
BAD	14%	–	–	–	−13%	–
BAX	–	–	–	11%	−10%	–
BCL2	–	–	–	–	−14%	–
CASP3	–	–	–	10%	−11%	–
CASP9	–	–	–	24%	−25%	–
pCASP9	24%	–	–	24%	−18%	–
STAT3	10%	–	−23%	63%	22%	–
pSTAT3	–	–	22%	18%	–	12%
pJAK2	–	–	–	–	–	–
TRKA	–	–	–	–	−12%	–

*Data extracted from **Supplementary Table 2**. All values are significant as determined by a three-level mixed effects model, with $p \leq 0.005$ and the Benjamini-Hochberg correction for multiple testing, with false discovery rate (FDR) $p \leq 0.05$. Only significant differences $\geq|10\%|$ are shown.*

Pilocarpine/Saline-Chronic Comparison in Cerebellum

Because protein expression in saline injected mice is dynamic, it may be more realistic to compare pilocarpine data at each time point to data from the saline chronic time point, with the assumption that this latter time point most closely resembles expression of untreated mice. We examined data from cerebellum in detail because this brain region showed

TABLE 4 | Protein changes in cerebellum, MAPK, MTOR, JAK/STAT, and apoptosis pathway components (pilocarpine vs. saline at corresponding time points).

Protein	Onset	1 h	6 h	24 h	Latent	Chronic
MAPK						
BRAF	21%	−21%	–	–	16%	–
pBRAF	10%	–	–	–	–	11%
MEK	10%	–	−10%	–	–	–
pMEK	13%	–	–	–	–	–
ERK	−30%	28%	−22%	–	16%	−31%
pERK	24%	−23%	–	17%	−16%	–
RSK	–	−14%	–	–	–	–
pRSK	31%	−12%	–	–	−10%	21%
pELK	–	–	–	–	–	–
CREB	29%	–	–	–	–	13%
pCREB	25%	–	–	–	–	14%
CAMKII	25%	–	–	–	–	13%
pCAMKII	–	–	23%	–	–	30%
JNK	17%	–	–	–	–	–
pJNK	32%	−12%	–	–	–	13%
P38	34%	–	–	–	–	–
pP38	40%	–	22%	–	–	–
MTOR						
MTOR	–	–	–	–	–	–
pMTOR	25%	–	–	–	–	–
P70S6	–	–	–	–	–	19%
pP70S6	–	–	–	–	9%	12%
S6	–	–	−19%	–	–	–
pS6	24%	–	23%	–	–	–
EIF4B	15%	–	–	–	–	–
pEIF4B	–	–	10%	−14%	27%	–
AMPKA	10%	–	–	–	–	10%
RAPTOR	19%	–	12%	–	–	–
AKT		–	–	–	–	–
pAKT	18%	−18%	–	–	–	–
PTEN	18%	–	–	–	–	–
PI3K	17%	–	–	–	–	–
APOPTOSIS, JAK/STAT						
BAD	15%	–	–	–	–	19%
BAX	13%	–	–	–	–	−11%
BCL2	18%	–	–	–	–	–
CASP3	23%	−11%	–	–	–	14%
CASP9	–	−20%	–	–	–	–
pCASP9	23%	–	–	–	10%	11%
STAT3	14%	–	–	–	–	–
pSTAT3	–	14%	–	14%	–	11%
pJAK2	17%	–	–	–	–	–
TRKA	16%	−10%	–	–	–	19%

*Data extracted from **Supplementary Table 2**. All values are significant as determined by a three-level mixed effects model, with $p \leq 0.005$ and the Benjamini-Hochberg correction for multiple testing, with false discovery rate (FDR) $p \leq 0.05$. Only significant differences $\geq$|10%| are shown.*

the greatest number and magnitude of changes in the saline chronic comparison (**Figure 3** and **Supplementary Table 3**). **Table 5** shows the pilocarpine-induced changes in cerebellum when compared with protein levels in the saline-chronic stage. At onset, MAPK pathway responses are not dramatically different from the previous comparison with the exception of P38 and pP38, where both are decreased from 34% and 40 to 12% and 18%. During the latent period, however, now 10 of 17

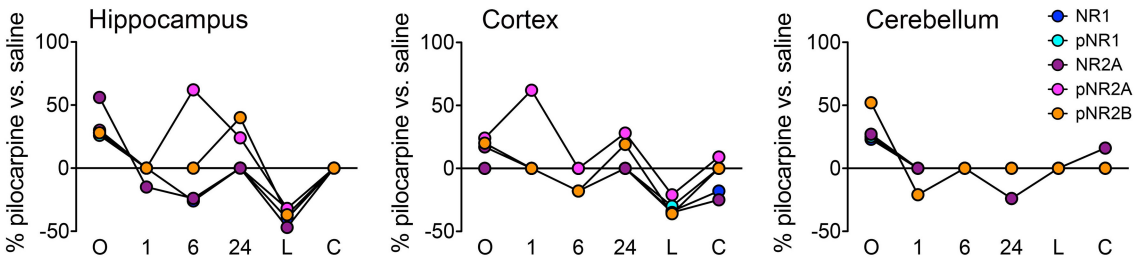

FIGURE 2 | Time course of expression changes for NMDA receptor subunits. Percent changes in protein levels in pilocarpine vs. saline mice at the corresponding time points were obtained using a three-level mixed effects model; data are from **Supplementary Table 2**. $n = 5$ for each group except for chronic pilocarpine where $n = 4$.

MAPK components are altered, where previously only 4 were. More differences are seen in the MTOR pathway, where at onset, MTOR, P70S6, S6, and AKT, that were unaffected, are now elevated between 19–41%, and pS6 and AMPKA are increased to 39 and 19%, respectively, from 24 to 10%. In addition, P70S6, pP70S6, and pS6 that were largely unresponsive throughout the time frame, now remain elevated at most sub-acute times and during the latent period. Indeed, 12 of 14 MTOR pathway proteins are now altered during the latent period, where previously only a single protein, pEIF4B, was. The apoptosis proteins, BAD and BAX are now elevated to ∼25% from ∼14%, and also now remain increased throughout the entire time course. Similarly, STAT3 and pSTAT3 are also increased at more time points and to higher levels. Overall, cerebellum shows more abnormalities during the latent period and appears more similar to hippocampus and cortex.

Correlation Networks

Subsets of proteins measured here interact as components of the same pathway, through crosstalk between pathways, as components of a complex or in modifier-substrate relationships. We therefore investigated possible correlations among levels of individual proteins in saline injected mice to compare their presence or loss in pilocarpine injected mice at corresponding time points (Spearman Correlation analysis). Within each brain region, scatter plots of all pairs of proteins in each treatment group with $r > 0.8$, $p < 0.05$ were manually reviewed and correlations with artifactual significance were eliminated. We then generated networks to illustrate selected relationships. **Figure 4** shows networks involving STAT3, JAK2, and/or apoptosis related proteins, in hippocampus, at onset, 1 h and during the latent period. At onset (**Figure 4A**), in saline mice, levels of phosphorylated subunits if the NMDAR are correlated with each other and with NR1, TRKA, and phosphorylated MTOR pathway components, pS6 and pMTOR. These correlations are absent in pilocarpine mice, where the network instead uniquely includes NMDAR subunits correlated with pSTAT3, pJAK2, and AMPKA. Only a single correlation, between pCAMKII and pNR2B, is common to both treatment groups. By 1 h (**Figure 4B**), most of these relationships are lost. Phosphorylated NMDAR subunits, pSTAT3, and MTOR components are absent. In pilocarpine mice, PTEN now appears as a central hub connected with apoptosis proteins, BAD, BAX, and CASP3, among others that are mostly not present in the saline treated network. At the latent stage (**Figure 4C**), correlations remain predominant in pilocarpine mice. While PTEN remains in the network, now pSTAT3 is correlated with many proteins, directly with BAX, BCL2, pCASP9, and AMPKA, and indirectly with CASP3 and BAD. Saline mice have only a single correlation in common with pilocarpine mice, that between pSTAT3 and AMPKA.

Figure 5 illustrates networks of correlations at the chronic time point among components of the MTOR pathway in hippocampus and cortex. In contrast to the networks in **Figure 4**, correlations in saline mice are dominant. Levels of all components are highly correlated in both brain regions while in pilocarpine mice, there are only three correlations in common with saline mice in hippocampus and five in cortex.

DISCUSSION

The pilocarpine model of acquired TLE provides an opportunity to monitor molecular changes occurring throughout acquired epileptogenesis. Here, the technique of Reverse Phase Protein Arrays was used to assess responses to pilocarpine in signaling pathways and cellular processes known to underlie normal brain function and known or suspected to be involved in epileptogenesis. RPPA is a moderate throughput proteomics technique that is more conservative of sample and more sensitive than Western blots. For example, post-pilocarpine injection, three brain regions were assayed at six time points in 5 mice per time point. With saline injected mice, this resulted in a total of 180 samples, a number impractical for Western blots and mass spectrometric protocols. RPPA also allowed the assay of three replicates of a five point dilution series per sample, thus also providing more reliable measurements. The picture developed here shows complicated perturbations in multiple components of each pathway/cellular process assayed, and loss of normal correlations among functionally related proteins. Responses to pilocarpine are brain region-specific. In particular, while hippocampus and cortex are strongly affected, significant responses are also seen in cerebellum, a region recently receiving attention for contributions to epilepsy but not previously studied with respect to associated molecular processes. The current

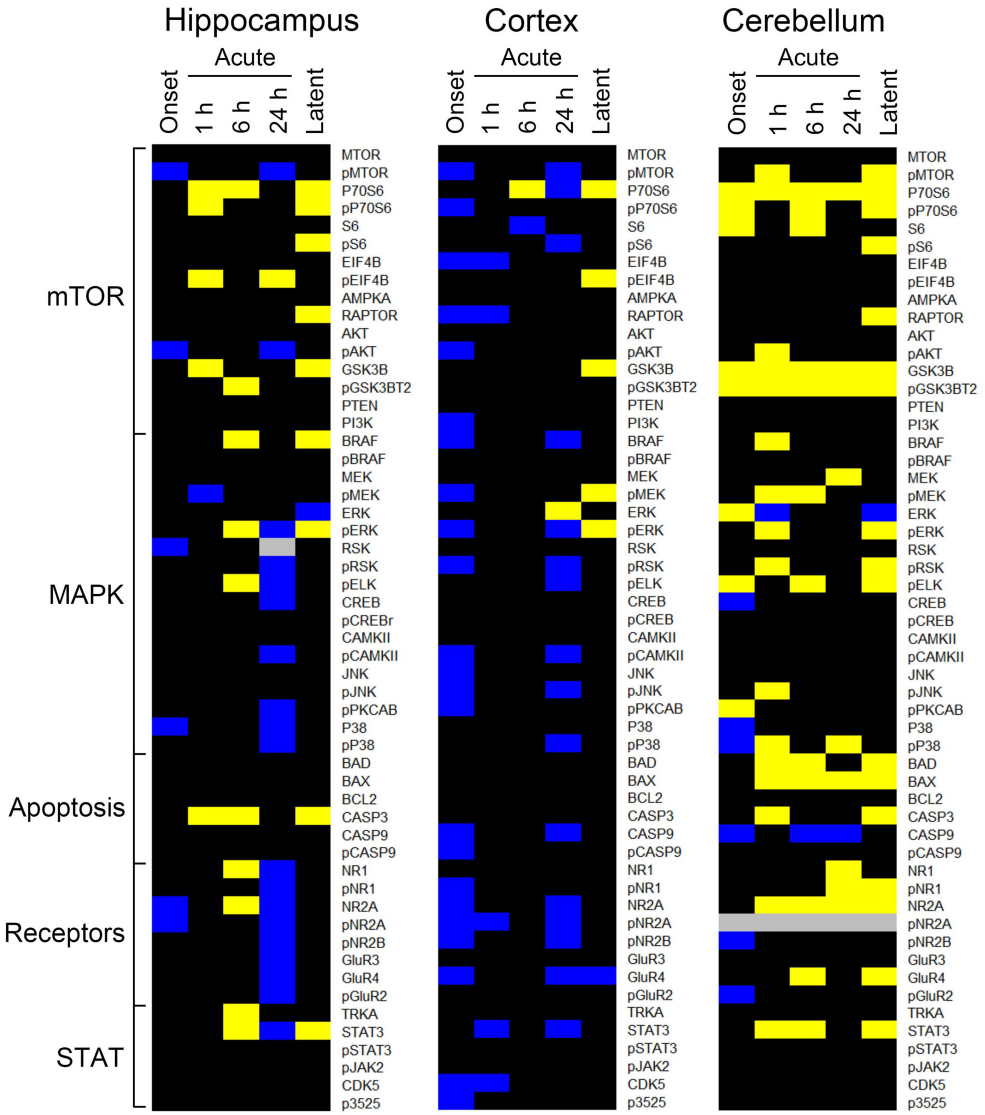

FIGURE 3 | Changes in protein expression in saline-treated mice. Heatmaps show proteins with an average expression level in saline treated mice at the first five time points significantly different from saline treated mice at the chronic time point. Only proteins with differences ≥15% as determined by a three level mixed effects model, with $p \leq 0.0005$, and a Benjamini-Hochberg correction for multiple testing applied with a false discovery rate of 0.05 are shown. Data derived from **Supplementary Table 3**. Proteins are grouped by general category. Yellow, increased; blue, decreased; black, no difference; gray, not measured. $n = 5$ for each group.

report represents an initial study of experiments carried out in only male animals. We recognize that these studies need to also be carried out in females to fully understand the pattern of gene expression associated with acquired epileptogenesis.

Many studies have analyzed rodent models of acquired epilepsy, using pilocarpine as here, or kainate, pentylenetrazole, or direct electrical stimulation. Variations in protocols include, not only the basic methodology, but also the number of time points measured, the brain region(s), and level of tissue resolution. A further complication is measurement of protein phosphorylation, a modification which is known to be labile and sensitive to alteration by both phosphatases and kinases that may be released from cellular sequestration immediately upon animal sacrifice and during tissue processing. Here mice were sacrificed by cervical dislocation without anesthetics, which are known to alter gene expression, and tissues were immediately heat stabilized to inactivate all enzymes involved in protein modification. Thus, results may differ from those in studies where anesthetics were used and/or tissue processing was prolonged due to perfusions. For all these factors, comparing results among studies that used different protocols is not necessarily straightforward.

Results for the MTOR pathway from several studies using western blots and either kainate or pilocarpine-induced SE are

TABLE 5 | Protein changes in cerebellum, MAPK, MTOR, JAK/STAT, and apoptosis pathway components (pilocarpine vs. saline chronic).

Protein	Onset	1 h	6 h	24 h	Latent	Chronic
MAPK						
BRAF	–	–	–	–	15%	–
pBRAF	22%	12%	–	–	10%	11%
MEK	16%	–	−10%	–	12%	–
pMEK	23%	19%	–	12%	–	–
ERK	−15%	–	−29%	–	−16%	−31%
pERK	19%	–	–	−27%	–	–
RSK	14%	–	–	–	–	–
pRSK	24%	–	12%	–	–	20%
pELK	29%	–	–	–	18%	–
CREB	–	−10%	–	–	–	–
pCREB	13%	–	–	–	13%	–
CAMKII	14%	–	–	–	11%	–
pCAMKII	–	–	–	–	–	30%
JNK	–	–	–	–	14%	–
pJNK	23%	–	18%	–	18%	–
P38	12%	−14%	–	–	–	–
pP38	18%	–	29%	–	23%	–
MTOR						
MTOR	19%	13%	–	–	17%	–
pMTOR	14%	–	–	–	19%	–
P70S6	41%	40%	31%	–	20%	19%
pP70S6	–	27%	25%	14%	26%	12%
S6	22%	–	–	27%	–	–
pS6	39%	38%	24%	–	37%	–
EIF4B	–	–	–	–	−11%	–
pEIF4B	–	25%	–	–	28%	–
AMPKA	24%	18%	–	–	11%	10%
RAPTOR	–	–	14%	–	23%	–
AKT	19%	12%	–	–	11%	–
pAKT	13%	–	15%	–	23%	–
PTEN	15%	–	–	–	–	–
PI3K	11%	–	–	–	14%	–
APOPTOSIS, JAK/STAT						
BAD	26%	17%	15%	11%	23%	19%
BAX	25%	18%	15%	12%	19%	11%
BCL2	17%	–	–	–	17%	–
CASP3	18%	–	13%	–	21%	14%
CASP9	–	−21%	–	–	–	–
pCASP9	22%	–	–	–	14%	11%
STAT3	24%	–	–	–	18%	–
pSTAT3	15%	22%	–	12%	–	11%
pJAK2	–	–	–	–	–	–
TRKA	–	–	–	–	–	19%

*Data extracted from **Supplementary Table 4**. All values are significant as determined by a three-level mixed effects model, with $p \leq 0.005$ and the Benjamini-Hochberg correction for multiple testing, with false discovery rate (FDR) $p \leq 0.05$. Only significant differences $\geq|10\%|$ are shown.*

qualitatively consistent with results here. Increases in ratios of pMTOR/MTOR and pAKT/AKT in hippocampus from 2 to 16 h (29) and increases in pS6 in hippocampus and cortex at 1–6 h (12) after SE induced by kainate were replicated here, although a further peak in pS6 reported at 5 days was not observed here. Similar early increases in pS6 in hippocampus and cortex (30 min to 2 h) were also seen after pilocarpine-induced SE (30). MAPK components, pJNK, and pP38, have been reported to increase

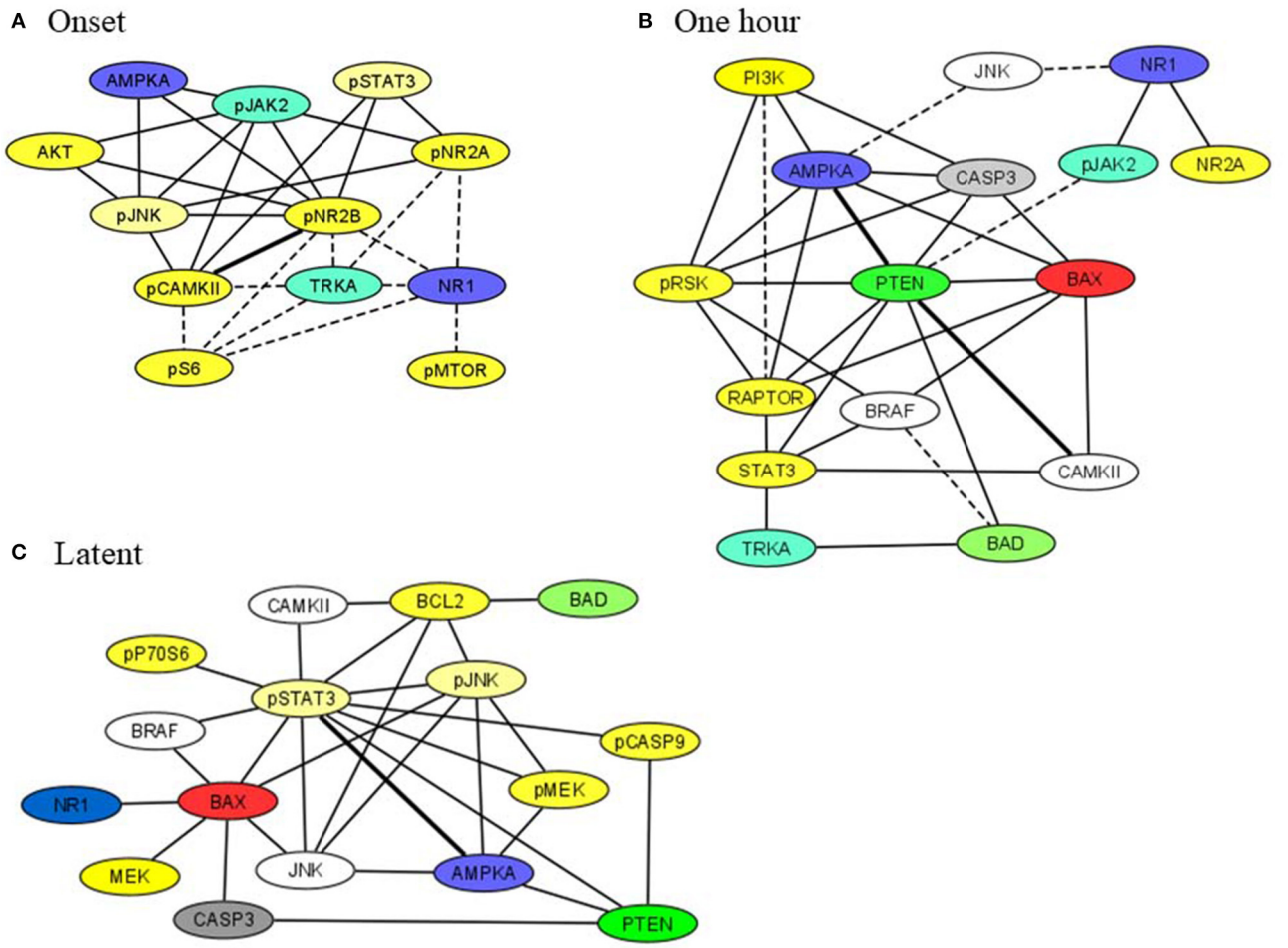

FIGURE 4 | Correlation networks in hippocampus including STAT3, JAK2, and NMDAR subunits at **(A)** onset, **(B)** 1 h, **(C)** latent period. Correlations between protein levels were determined by Spearman Correlation analysis; only correlations with $r > 0.8$, with linear scatter plots and $p < 0.05$ were considered significant and are included. Heavy solid lines, correlations seen in pilocarpine and saline treated mice; thin solid lines, correlations seen only in pilocarpine treated mice; dashed lines, correlations seen only in saline treated mice. Colors indicate proteins present in two or three networks.

in the chronic stage after pilocarpine and pentylenetetrazole SE, respectively (28, 31); here, increases were seen in the sub-acute time points. The former study examined only the CA1 region of hippocampus, which clearly could differ in details that may be masked, by examining whole hippocampus.

Apoptosis related proteins function in neuronal cell death, and its prevention, after acute seizures and can activate a caspase cascade [reviewed in (32, 33)]. Detailed time frames of increases and decreases of BCL2, BAD, BAX, and CASP3 and CASP9 are determined here. SE and neuronal death have also been linked to STAT3 activation (34); here, STAT3 and pSTAT3 show complex patterns in both hippocampus and cortex throughout epileptogenesis, and STAT3 remains high during the chronic phase. These examples show that our results are consistent with prior data, supporting the validity of our datasets overall.

Large scale proteomics studies using 2D gels and/or mass spectrometry can identify hundreds to thousands of proteins and detect differential expression between treatment groups. Commonly, a few tens of differentially expressed proteins have been quantified (18, 19, 35, 36). The advantage of such approaches is that they are unbiased, thus, not limiting protein measurements to a specific hypothesis or pathway focus. None of these large-scale proteomics efforts has identified any of the proteins measured here. One possible reason is that mass spectrometry identifies more highly expressed proteins, unless extensive subcellular enrichment processes are used. Most such studies have applied bioinformatics techniques for search for pathway associations in their datasets. Of interest, this has identified enrichments in the MAPK pathway and inflammation processes.

This work for the first time examines perturbations in multiple pathways in the cerebellum during epileptogenesis. The cerebellum historically has received most attention for sensorimotor control, however increasingly data indicate a role for the cerebellum in cognition [reviewed in (37)]. Evidence for the cerebellum's involvement in epilepsy started with structural

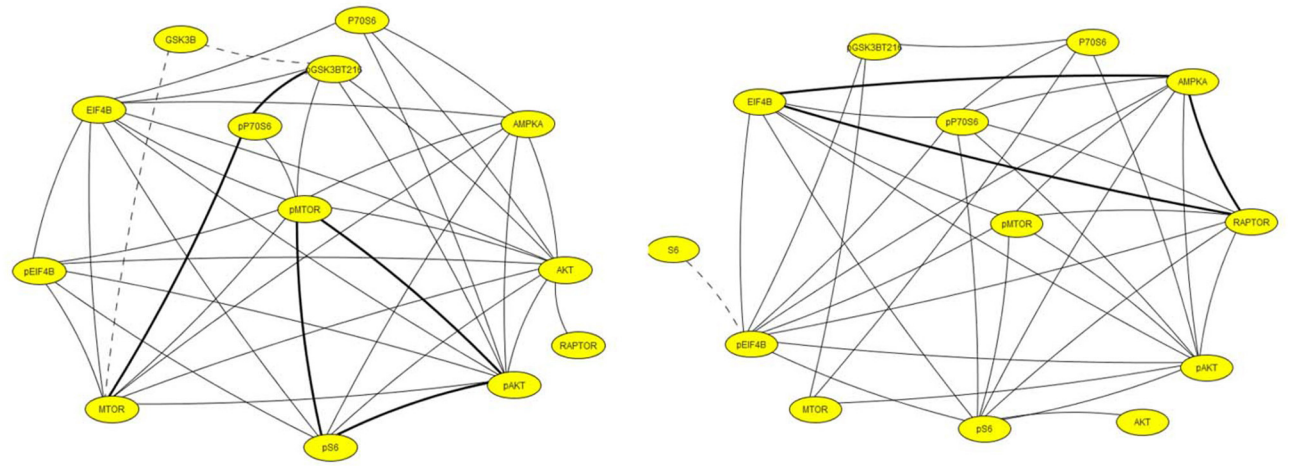

FIGURE 5 | Correlation networks including components of the MTOR pathway in **(A)** hippocampus and **(B)** cortex. Correlations between protein levels were determined by Spearman Correlation analysis; only correlations with $r > 0.8$, with linear scatter plots and $p < 0.05$ were considered significant and are included. Heavy solid lines, correlations seen in pilocarpine and saline treated mice; thin solid lines, correlations seen only in pilocarpine treated mice; dashed lines, correlations seen only in saline treated mice.

abnormalities (38), but now includes functional data from both human patients and rodent models [(39, 40); reviewed in (41)]. At the protein level, Rubio et al. (42) recently showed that levels of BAX and activated CASP9 increased and BCL2 decreased during chronic seizures induced by electrical stimulation. This is consistent with our observations regarding BAX and CASP9 (although not for BCL2). Our data demonstrate that common signaling pathways, MAPK and MTOR, are perturbed at onset of SE as well as through epileptogenesis, to extents similar to those seen in hippocampus and cortex.

The number and nature of the proteins measured allowed generation of correlation networks to provide a novel perspective of molecular events during epileptogenesis. While the functional implications, if any, remain to be determined, two features are notable. First, the hippocampus of pilocarpine treated mice shows uniquely complex networks involving JAK/STAT3 and apoptosis related proteins which evolve during epileptogenesis to contain PTEN. These robust correlations, that are almost entirely absent in saline mice, may contribute to or be reflective of the loss of normal regulation of synaptic activity and the development of highly synchronous neuronal firing underlying spontaneous seizures. Second, and conversely, during the chronic phase, components of the MTOR pathway are highly correlated in both hippocampus and cortex of saline mice. The almost complete absence of these networks in pilocarpine mice is consistent with the known role of mutations of MTOR pathway components in epilepsy. Additional networks can be constructed for hippocampus and cortex, as well as cerebellum; their brain region specificity supports their potential functional significance. Further studies are needed to ascertain the predictive value of these and other correlation networks.

Recent evidence suggests that the signaling pathways involving mTOR and MAPK activation are important regulators of synaptic excitability and might be responsible for epilepsy and the concomitant cognitive impairment (43). Cell-specific mutations of TSC genes induced in both glia and neurons cause epilepsy in mice (44–46). Further, the neuropathological hallmarks of TSC include major morphological and functional changes in glial cells involving astrocytes, oligodendrocytes, NG2 glia, and microglia, as well as in neurons (47), and abnormal function of the mTOR pathway in glial cells is thought to contribute to seizures as well as cognitive co-morbidities in TSC as well as in TLE (48, 49). Specimens surgically removed from people suffering from TLE show increased levels of pMTOR (S2448), pS6 (S235/236), and pS6 (S240/244) that are consistent with the activation of the mTOR pathway. More importantly, a correlation between the increase in the levels of pS6 (S235/236) and the localization of the seizure focus within the mesial temporal lobe structures can be detected (50). Loss of PTEN expression from granule cells of dentate gyrus promotes an increase in S6 phosphorylation associated with numerous cellular abnormalities including hypertrophy, basal dendrites, increased dendritic spine density and ectopically located somas (51). A correlation between mTOR hyperactivity and disease severity has been reported in a particular experimental model of focal malformations (52) produced by *in utero* electroporation of a constitutively active form of Rheb ($Rheb^{CA}$), the canonical activator of mTORC1. In addition to promoting epilepsy and its associated pathologies, $Rheb^{CA}$ overexpression promotes an increase in neuronal size and cell misplacement strongly suggesting that mTOR hyperactivation directly influences seizure severity (52). Excessive mTOR signaling leads to hippocampal hyperexcitability linking mTOR with TLE. Knockout of PTEN,

promotes mTOR-signaling hyperactivation resulting in seizures that also mimic an epileptic phenotype (43). Thus, the relevance of mTOR in the control of excitation/inhibition is crucial for the homeostatic control of neuronal excitability.

The components of the MAPK signaling pathway include the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinases 1-3 (JNK1/2/3), and $p38^{MAPK}$ (a, b, d) (53). The MAPK signaling pathway is massively activated within hippocampus after acute administration of chemoconvulsants like kainate and pilocarpine (54) and was demonstrated here. However, pharmacological blockade of ERK has no effect on the initiation or severity of seizures supporting the notion that ERK activation is due to neuronal excitability rather than the actual cause of seizures (54). A previous study also demonstrated an increase in the phosphorylation of ERK during the acute period (1 and 12 h) post-pilocarpine-induced seizure in both the hippocampus and cortex, suggesting that ERK might be involved in epileptogenesis (55). These results were replicated here, continuing to 24 h. In addition, increased ERK phosphorylation has been reported in the temporal neocortex of patients with intractable epilepsy (56) and linked to persistent activation of MAPK-dependent gene transcription in brain regions with hyperconnected neurons of layer 2/3 (57). ERK also activates the expression of NMDA receptors leading to increased excitability and seizures. As a whole, these observations suggest that, since activation of the MAPK pathway occurs following many protocols for seizure, its activation is the result of seizure activity (43).

Activation of the JAK/STAT pathway has been demonstrated in the hippocampus in animal models of TLE, as well as in patients. Brain injuries leading to TLE (i.e., epileptogenic brain injuries) in rodents persistently alter JAK/STAT pathway activity (58–61). Both the phosphorylation and total expression of STAT3 is increased in tissue samples resected from patients with epilepsy due to hippocampal sclerosis (62), focal cortical dysplasia (62), and tumors (63). The JAK/STAT pathway is a critical cell signaling pathway in glia as well as neurons, and regulates gliogenesis (64), neuronal survival and maturation (65), and neuronal gene expression (66). The current study demonstrates that the activation of the JAK/STAT pathway that has previously been shown to occur acutely in hippocampus after epileptogenic brain insults, persists into the chronic epilepsy stage, and occurs outside of the hippocampus, in areas such as the cortex and cerebellum. Our findings provide further support for the concept that the JAK/STAT may play a pervasive and persistent role in the regulation of multiple mechanisms contributing to epileptogenesis.

In summary, the current findings expand our understanding of time- and brain region- specific changes in expression, activity, and correlations among levels of functionally related proteins affecting both neurons and glia. While hippocampus, the brain region most affected during pilocarpine-induced epileptogenesis, shows large numbers of perturbations, cortex and cerebellum also display significant and divergent responses in pathways that are critical for brain function. The results include demonstration of complex sequential alterations in levels of components of the MTOR, MAPK, JAK/STAT, and apoptosis pathways and in subunits of the N-methyl-D-aspartate receptor (NMDAR) receptor. We further demonstrate correlations among levels of pathway and receptor components in saline treated mice, that are lost over time and replaced with other patterns with pilocarpine.

Epilepsy is not a single disease but rather a constellation of disorders of different etiologies that share the common phenotypic feature of spontaneous seizures. In this manuscript we specifically focus on a preclinical rodent model of acquired epilepsy, with the intent of elucidating the array of molecular pathways that are activated during epileptogenesis and over what time-course. Understanding changes in pathway activation adds to the existent literature on altered gene expression, and provides new information about prospective targets for therapeutic intervention. The current data present a novel picture of the complexity of protein responses during epileptogenesis that may provide targets for novel therapeutics aimed at disrupting the epileptogenic processes and preventing or reducing development of spontaneous seizures. Future interventional studies that modify pathway activation will be required, however, to determine which, if any, of the protein responses here identified play a causal role in epileptogenesis and have potential as a target for disease-modifying therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus.

AUTHOR CONTRIBUTIONS

KG and AB-K conceived, designed and supervised the study, and obtained funding for the study. MA, AC, YC, JC, AT, and MG were involved in the execution of the experiments. MA, AC, and KG were involved in the data acquisition and interpretation. MA, AC, MG, KG, and AB-K were involved in the review and analysis of the data. MA, MG, KG, and AB-K participated in writing and editing the manuscript. All authors approved the final manuscript and are responsible for the work presented in the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.654606/full#supplementary-material

Supplementary Figure 1 | Chronology of the experiments. Male FVB/NJ mice were purchased from Jackson Labs and received at 5–7 weeks of age. Mice were allowed to acclimate to the environment for 1 week prior to the start of experiments. Mice (18–24 g) were briefly handled once daily for about 1 week to reduce the stress induced by experimental protocols. Entire litters (an average of 5 mice) were randomly assigned to a single treatment/time point group. Mice were given an intraperitoneal injection of scopolamine methyl bromide (1 mg/kg) 15 min before the first pilocarpine injection to block the peripheral effects of pilocarpine. The initial dose of pilocarpine HCl (200 mg/kg) was followed, after 1 h, by subsequent doses (100 mg/kg) given at 30 min intervals. The onset of SE was defined as the appearance of repeated behavioral seizures (stage four or higher with at least one seizure being five or higher) according to a modified Racine scale. SE persisted for at least 90 min. Control mice were given injections of saline at identical time intervals. After SE induction, mice were singly housed and given free access to water and moistened chow. Cohorts of 5 mice per group were sacrificed after SE, at 15 min (onset), 1 and 6 h, 1 and 5 days, and 2 weeks (red lines). Groups of saline-injected mice were sacrificed at the same time points.

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Suppression of Microgliosis With the Colony-Stimulating Factor 1 Receptor Inhibitor PLX3397 Does Not Attenuate Memory Defects During Epileptogenesis in the Rat

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Events of status epilepticus (SE) trigger the development of temporal lobe epilepsy (TLE), a type of focal epilepsy that is commonly drug-resistant and is highly comorbid with cognitive deficits. While SE-induced hippocampal injury, accompanied by gliosis and neuronal loss, typically disrupts cognitive functions resulting in memory defects, it is not definitively known how. Our previous studies revealed extensive hippocampal microgliosis that peaked between 2 and 3 weeks after SE and paralleled the development of cognitive impairments, suggesting a role for reactive microglia in this pathophysiology. Microglial survival and proliferation are regulated by the colony-stimulating factor 1 receptor (CSF1R). The CSF1R inhibitor PLX3397 has been shown to reduce/deplete microglial populations and improve cognitive performance in models of neurodegenerative disorders. Therefore, we hypothesized that suppression of microgliosis with PLX3397 during epileptogenesis may attenuate the hippocampal-dependent spatial learning and memory deficits in the rat pilocarpine model of SE and acquired TLE. Different groups of control and SE rats were fed standard chow (SC) or chow with PLX3397 starting immediately after SE and for 3 weeks. Novel object recognition (NOR) and Barnes maze (BM) were performed to determine memory function between 2 and 3 weeks after SE. Then microglial populations were assessed using immunohistochemistry. Control rats fed with either SC or PLX3397 performed similarly in both NOR and BM tests, differentiating novel vs. familiar objects in NOR, and rapidly learning the location of the hidden platform in BM. In contrast, both SE groups (SC and PLX3397) showed significant deficits in both NOR and BM tests compared to controls. Both PLX3397-treated control and SE groups had significantly decreased numbers of microglia in the hippocampus (60%) compared to those in SC. In parallel, we found that PLX3397 treatment also reduced SE-induced hippocampal astrogliosis. Thus, despite drastic reductions in microglial cells, memory was unaffected in the PLX3397-treated groups compared to those in SC, suggesting that remaining microglia may be sufficient to help maintain hippocampal functions. In sum, PLX3397 did not improve

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or worsen the memory deficits in rats that sustained pilocarpine-induced SE. Further research is required to determine whether microglia play a role in cognitive decline during epileptogenesis.

Keywords: microglia, pilocarpine, epileptogenesis, hippocampus, memory, astrocytes, status epilepticus, complement-immunological terms

INTRODUCTION

Temporal lobe epilepsy (TLE) is a type of focal epilepsy that is commonly drug-resistant (1) and is highly comorbid with cognitive deficits (2). Cognitive comorbidities, including deficits in memory, attention, and executive function have been reported in 40–95% of patients with TLE (2). Unfortunately, currently available anti-seizure mediations do not address cognitive defects and in some cases can aggravate intellectual decline in individuals with epilepsy (3), supporting the need for novel therapies. In TLE, the hippocampus is often damaged as evidenced by the presence of extensive gliosis and severe neuronal loss, also known as hippocampal sclerosis. These disruptions to the hippocampal circuitry interfere with essential functions such as processing and consolidation of short- and long-term memories thereby resulting in learning and memory dysfunctions (2). Extensive research in animal models of acquired TLE, typically induced with a single episode of status epilepticus (SE), a long-lasting seizure (>1 h), support that SE-induced hippocampal injury in an otherwise healthy system contributes to epileptogenesis, as well as memory decline (4); though the mechanisms underlying memory loss after SE and TLE are still not fully understood. Our previous studies point to microglial cells, the resident immune cells of the brain, as potential mediators of learning and memory defects during SE-induced epileptogenesis (5–9).

Microglial proliferation and accumulation, microgliosis, in the hippocampus have been widely reported in human and experimental models of TLE, among other epilepsies (9, 10). Previously, we found a robust increase in microgliosis within the hippocampal CA1 region that peaked at 2 weeks post-SE (7, 11) and correlated with the development of hippocampal-dependent spatial learning and memory impairments (5, 6, 8). These microglia were characterized by bushy/amoeboid reactive morphologies (11) and activation of the mechanistic target of rapamycin (mTOR) signaling cascade (8). Importantly, we found that inhibition of mTOR signaling with the drug rapamycin during SE-induced epileptogenesis suppressed microgliosis and attenuated the associated memory loss (8). While this evidence suggests that reactive microglial cells may be active participants underlying cognitive dysfunctions in SE and TLE, mTOR signaling cascade is expressed in neurons and astrocytes (12), thereby indicating that these other cell types may also play a role in this pathophysiology.

Therefore, to specifically study the role of SE-induced microgliosis on cognitive decline we focused on the colony-stimulating factor 1 receptor (CSF1R) because CSF1R was identified by the computational casual reasoning analytical framework for target discovery as a potential anti-epileptic target (13). CSF1R is part of a family of receptors that are responsible for regulating microglial proliferation, survival, motility, and adhesion (14–16). As opposed to mTOR signaling, which is highly conserved across species and is ubiquitously found in numerous cell types (12), CSF1R is mainly expressed in microglia (14–16). In fact, mutations in this CSF1R receptor are associated with the loss of microglia and development of epilepsy in humans (17). In addition, inhibition of CSF1R with Plexxikon (PLX) 3397, or its analogs, have been shown to reduce/deplete the population of microglial cells as well as to improve behavioral cognitive performance in pre-clinical models of neurodegenerative disorders (16, 18–23). Therefore, in this study, we tested the hypothesis that suppression of microgliosis through inhibition of CSF1R signaling with PLX3397 during epileptogenesis may attenuate the hippocampal-dependent spatial learning and memory deficits in the rat pilocarpine model of SE and acquired TLE.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (150–200 grams) (Envigo) were housed (one animal per cage) at the Psychological Sciences Building at ambient temperature (22°C) with diurnal cycles of a 12-h light and 12-h dark (8:00–20:00), with access to unlimited food and water. Rats were weighed and handled daily. Animal procedures followed institutional and NIH guidelines and were approved by the Purdue Institutional Animal Care and Use Committee (Protocol #1309000927).

Induction of SE

Pilocarpine was used to induce SE as previously described Schartz et al. (6), Schartz et al. (7), and Brewster et al. (8). Briefly, all rats were given an intraperitoneal (i.p.) injection of scopolamine methylbromide (1 mg/kg) 30 min (min) prior pilocarpine hydrochloride (280–300 mg/kg; i.p.) ($n = 46$) (SE rats) or a similar volume of saline (sham-treated controls, $n = 26$). The Racine scale (24) was used to score seizure stages from which stage 6 (rearing and falling) indicated the start of SE ($n = 28$). Following 1 h of SE, all rats were given diazepam (10 mg/kg; i.p.) and kept on heating pads. Chow was supplemented with chocolate Ensure and fruit loops for 1–3 days post-SE. Rats that did not reach SE were removed from the study (non-SE, $n = 16$). Two rats died during SE.

PLX3397 Treatment

PLX3397 was mixed in rodent standard chow (SC) (455 mg PLX3397/kg of diet) (Envigo, Madison, WI) (National Research Council (US) Subcommittee on Laboratory Animal Nutrition 1995) to achieve a dose of 50 mg/kg body weight per day, which

was found to be successful in reducing microglia in rats (25). A total of 27 rats received the PLX diet (Control, $n = 13$; SE, $n = 14$). Regular chow (SC) was given to 27 rats (Control, $n = 13$; SE, $n = 14$). After microglial counts were performed, animals on PLX diet that did not show reduced microgliosis compared to their respective control SC groups were deemed non-responders. Five rats from the SE+PLX group did not show decreases in the numbers of microglia and were classified as non-responders (SE+PLX Non-responder; **Supplementary Figure 1**). Therefore, these animals along with their tissues were not included in the behavioral, histological, or biochemical analyses. All control rats in the PLX diet showed reduced microglial populations relative to those in the C+SC group.

Novel Object Recognition (NOR)

The NOR test was performed 2 weeks after SE following as previously described (5, 6). Rats were first acclimated in an adjacent dark room for 30 min before placed in the NOR test chamber (11.5 × 5.75 × 6 inches) for habituation (20 min). Twenty-four hours after habituation, NOR trials 1 and 2 were performed under red light conditions. In trial 1, rats were placed for 5 min in the testing chamber containing 2 identical objects. Two hours later, in trial 2, rats were placed for 5 min in the same chamber containing a familiar object from trial 1 and a novel object. The position of the novel and familiar objects in trial 2 was alternated to counterbalance potential side preferences. The Any-maze video-tracking system V4.99 (Wood Dale, IL) was used to record the NOR trials. The objects' exploration was measured when the rats' nose was within 1 cm from the objects. Time spent exploring the objects was determined by investigators blinded to experimental groups. The recognition index (RI) determined the difference in exploration time between the same object in trial 2 (familiar) and trial 1 relative to the total exploration time during both trials [(Trial1-Trial2)/Trial1+Trial2)]. NOR testing was completed in 4 cohorts ($N = 10$/group; sample size was determined a priori by G*Power, on a power of 80% and rats were selected randomly), three rats in the SE+PLX group were removed for non-response to PLX treatment following microglial counts, and two rats in SE+SC group were removed due to outliers in NOR index test (C+SC, $n = 10$; C+PLX, $n = 10$; SE+SC, $n = 8$; SE+PLX, $n = 7$).

Barnes Maze (BM)

BM was performed after completion of the NOR test (16–20 days after SE), following previously described protocols (5, 6). Briefly, on BM day 1 (habituation), rats were trained to find and enter the escape box as follows: (1) rats were placed directly into the escape box; (2) rats were placed next to the escape box and gently nudged into the box; (3) rats were placed in the center of the platform and guided to the escape box through an open tunnel. On BM training days 1–4, rats were tested in 4 trials/day (15 min between trials) to find the escape box within 3 min or were gently guided into the box after the 3 min expired. On BM day 5 the escape box was removed, the holes were covered, and the rats were tested for their ability to find the location of the covered target hole within 90 s (probe trial). Any-maze video tracking system V4.99 (Wood Dale, IL) was used to record and measure the time to find the escape box during days 1–4 (escape latency), and time spent over the target covered hole during the probe trial. BM testing was completed in 4 cohorts ($N = 10$/group; sample size was determined a priori by G*Power, on a power of 80% and rats were selected randomly), three rats in the SE+PLX group were removed for non-response to PLX treatment following microglial counts (C+SC, $n = 10$; C+PLX, $n = 10$; SE+SC, $n = 10$; SE+PLX, $n = 7$).

Perfusion

Rats were deeply anesthetized with a lethal dose of Beuthanasia (200 mg/kg) and perfused with ice-cold 1X phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4). The brain was removed and hemispheres separated. One hemisphere was post-fixed with 4% paraformaldehyde (PFA) (Thermo Fisher Scientific, Rockville, IL) for immunostainings. The hippocampus was dissected from the other hemisphere, and frozen at −80°C for protein extraction and immunoblotting.

Immunohistochemistry (IHC)

Brain tissues fixed in PFA for 48 hours at 4°C were cryoprotected in 30% sucrose (Thermo Fisher Scientific) (diluted in 1X PBS), frozen in dry ice, and stored at −80°C until used. Coronal sections (20 μm) between the bregma coordinates: −3.00 mm to −5.28 mm were obtained using a Leica CM1860 cryostat and stored in 1XPBS + 0.1% sodium azide at 4°C until used. IHC was done in free-floating sections exactly as previously described (7, 11). Primary antibodies: anti-rabbit IBA1; Secondary antibodies: biotinylated goat anti-rabbit (1:1K; BA-1000, Vector Laboratories, Burlingame, CA) for 1 h at RT. Immunosignal was developed using the ABC Avidin/Biotin complex solution and DAB Peroxidase (HRP) Substrate Kit, 3,3′-diaminobenzidine (SK-4100, Vector Laboratories), and visualized using a Leica DM500 microscope. Images were captured with a high-resolution digital camera (Leica MC120 HD) with 4X and 40X objectives using the LAS4.4 software. Three to six brain sections per rat were analyzed.

Microglia Counts

Cells were counted, exactly as previously described Wyatt-Johnson et al. (11), from the hippocampal CA1 region, and all IBA1-positive cells within the entire 40X image were counted. For this experiment, IBA1-positive cells included both microglia and macrophages referred to throughout this paper as microglia (26). Exclusion guidelines were set: (1) 25% or more of the IBA1-positive cells were located outside the boundaries of the counting region; (2) region of the tissue was broken; (3) stain was too light to visualize. Three distinct, non-overlapping images in CA1 region, 3–6 sections per rat were analyzed. Cell counts were performed, three rats in the SE+PLX group were removed for non-response to PLX treatment (C+SC, $n = 13$; C+PLX, $n = 13$; SE+SC, $n = 14$; SE+PLX, $n = 9$).

Western Blot (WB)

Hippocampi were homogenized in ice-cold 1XPBS and processed for immunoblotting as previously described Schartz et al. (6)

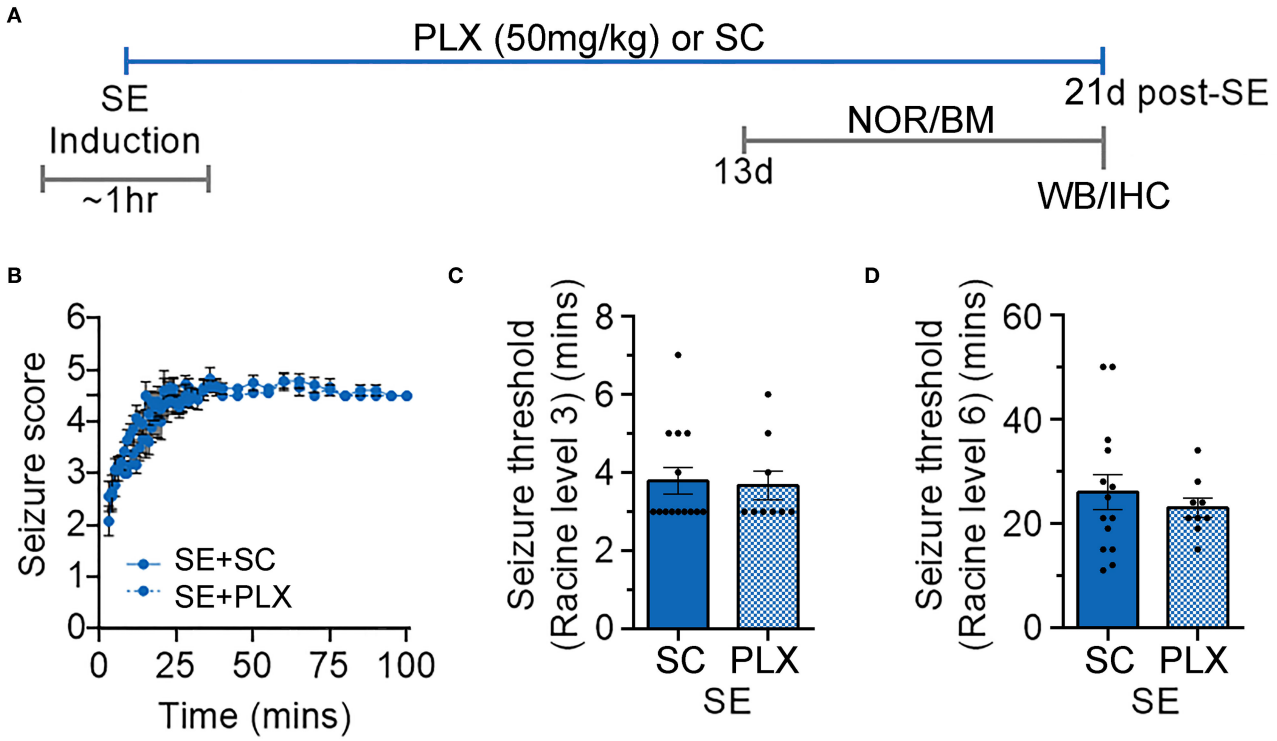

FIGURE 1 | Timeline of induction of status epilepticus (SE) and experimental design. **(A)** Diagram with the timeline of the experimental design with treatments with PLX3397 in chow (PLX; 50 mg/kg per day) or standard chow (SC) alone. Different groups of rats were given PLX or SC starting immediately after SE induction (day 0) to up to 20 days thereafter. Novel object recognition (NOR) and Barnes maze (BM) were performed between days 13 and 20. Brain tissues were then collected for histological and biochemical analyses on experimental day 21. **(B)** Behavioral seizures were monitored for 100 min after SE induction and scored according to the Racine scale (1: rigid posture, mouth moving; 2: tail clonus; 3: partial body clonus, head bobbing; 4: rearing; 4.5: severe whole body clonic seizures while retaining posture; 5: rearing and falling; 6: tonic-clonic seizure with jumping or loss of posture). **(C)** Time to first seizure (level 3). **(D)** Time to SE (level 6). Data analyzed by Kolmogorov-Smirnov test **(B)** and student's *t*-test **(C,D)**. Data are shown as mean ± SEM, SE+SC, $n = 14$; SE+PLX, $n = 9$.

and Brewster et al. (8). The Bradford Protein Assay (Bio-Rad, Hercules, CA) was used to determine protein concentration. Samples were diluted with Laemmli buffer (0.25 M Tris, pH 6.8, 6% sodium dodecyl sulfate (SDS), 40% sucrose, 0.04% Bromophenol Blue, 200 mM Dithiothreitol), separated via SDS-PAGE in Tris-glycine gels (7, 10, or 15%), and transferred to polyvinylidene fluoride membranes (Cat# 88518, GE Healthcare, Chicago, IL). Then, membranes were blocked with 5% non-fat milk diluted in 1XPBS+ 0.1% Triton at RT for 1 h on a rocking platform. Membranes were incubated with primary antibodies anti-rabbit CX3CR1 (1:1K; ab8021; Abcam, Cambridge, United Kingdom), anti-mouse GFAP (1:50K; #3670; Cell Signaling), anti-goal C3 (1:500; 855730; MP Biomedical, Solon, OH), or anti-mouse Beta-Actin (1:5K; #3700S; Cell Signaling) at 4°C overnight. Following multiple washes in 1XPBS with 0.1% Tween, membranes were incubated with HRP-linked secondary antibodies anti-rabbit (1:2K; ab205718; Abcam), anti-mouse (1:2K; ab205719; Abcam), or anti-goat (1:5K; AP180P; Millipore, Burlington, MA). Immunoreactive bands were visualized using enhanced chemiluminescence prime western blotting detection reagent (GE Healthcare), captured on Double Emulsion Blue Autoradiography Film (BX57, MIDSCI, St. Louis, MO). Membranes were stripped from primary antibodies using stripping buffer (25 mM glycine, pH 2.0, 10% SDS) for 2 h at RT then washed in 1XPBS with 0.1% Tween and re-blotted with primary antibody as described above.

Densitometry Analysis for Western Blot

The relative pixel density of each of the immunoreactive bands was measured with the Image J software V1.49 (NIH) (8). Background signal was recorded and subtracted from the immunoreactive bands. Proteins of interest were normalized to the loading control in the same lane/sample. Only the animals where PLX treatment was deemed successful, were included in the analysis (C+PLX, n=13; SE+PLX, $n = 9$), and any samples that had artifacts such as bubbles or smudges in the immunoblots were not included in the final analysis (CX3CR1 & GFAP: C+SC, $n = 13$; C+PLX, $n = 11$; SE+SC, $n = 14$; SE+PLX, $n = 9$; C3 & iC3b: C+SC, $n = 9$; C+PLX, $n = 7$; SE+SC, $n = 8$; SE+PLX, $n = 8$).

Statistical Analysis

G*Power was used for a priori analysis to determine sample sizes with previously collected data based on a power of 80%. Student's *t*-test was used to analyze comparisons between two groups. Two-way ANOVA with Tukey's multiple comparisons

was used to compare 4 groups (C+SC, C+PLX, SE+SC, and SE+PLX). Three-way mixed effects model (days/time vs. condition vs. treatment) with Tukey's multiple comparisons test was used for bodyweight comparisons. Three-way ANOVA (condition vs. treatment vs. training block) with Tukey's multiple comparison test was used to analyze BM. Kolmogorov-Smirnov test was used to analyze the non-normally distributed Racine scale data. The Kruskal-Willis test with Dunn's multiple comparisons was used to analyze the non-normally distributed microglial morphological data and the Racine scale data from the SE+PLX Non-responders. A one-way ANOVA with Dunnett's multiple comparison test was used to analyze the differences between seizure threshold and microglial responses between the SE+PLX Non-responders, SE+SC and SE+PLX responders groups (**Supplementary Figure 1**). Two-way ANOVA with Dunnett's multiple comparison test was used to analyze Non-responders weight changes compared to the SE+SC and SE+PLX groups (**Supplementary Figure 1**). Outliers were determined with ROUT with the maximum desired false recovery rate set to 1%, if an outlier was removed in one test, it was removed in all other dependent tests, this only occurred with the NOR analysis. Statistical significance was set at $\alpha < 0.05$. Data values were reported as mean ± standard error of the mean (SEM). GraphPad Prism 6 software was used for statistical analyses. Figures were generated using Adobe Photoshop (CS6) and Biorender.com.

RESULTS

SE and control rats were randomly assigned to different groups fed with either a rodent standard chow (SC) or chow with PLX3397 (50 mg/kg) for 20 days (C+SC, C+PLX, SE+SC, and SE+PLX). Rats were then exposed to a series of behavioral tests including NOR and BM, followed by brain tissue collection for histological or biochemical processing (**Figure 1A**). During SE inductions the seizures, which were scored according to the Racine scale (24), were not different between rats assigned to either diet [Kolmogorov-Smirnov test, $D = 01556$, $p = 0.6476$] (**Figure 1B**). No differences were found in the time to reach level 3 (first seizure) [student's *t*-test, $t_{(21)} = 0.2318$, $p = 0.8190$; **Figure 1C**], or in the time to level 6 (SE) [student's *t*-test, $t_{(21)} = 0.6675$, $p = 0.5117$; **Figure 1D**]. These data indicate that rats assigned to the CS or PLX treatments reached similar SE severities.

PLX Does Not Attenuate SE-Induced Weight Loss

Immunomodulating drugs have been shown to promote weight recovery after SE (5, 27, 28). Therefore, to determine if PLX treatment has an effect on bodyweight, we monitored the rats' weight daily during this study (**Figure 2**). All rats had similar weights prior to pilocarpine or saline injections (day 0). One day after SE, all SE rats lost weight while control rats gained weight (SE+SC: −3.21 ± 3.88 g; SE+PLX: −7.33 ± 2.36 g; C+SC: +8.34 c± 0.75 g; C+PLX: 4.07 ± 0.99 g). Thereafter, all rats gained weight over time [Mixed-effects ANOVA, $F_{(20,900)} = 911.1$, $p < 0.0001$] [C+SC: 4.93 ± 0.46 g/day (Mean ± SEM); SE+SC: 6.20 ± 0.69 g/day; C+PLX: 5.19 ± 0.46 g/day; SE+PLX: 5.807 ± 1.04 g/day]. However, between 1 and 7 days after SE, the bodyweight of the SE+SC and SE+PLX rats remained significantly lower relative to the C+SC or C+PLX groups ($p < 0.05$). On day 21, the body weight was similar in all groups ($p = 0.99$). Taken together, these data suggest that SE resulted in a transient decrease in body weight that normalized by 3 weeks after SE, and that PLX had no effect on the weight gain of either control or SE rats.

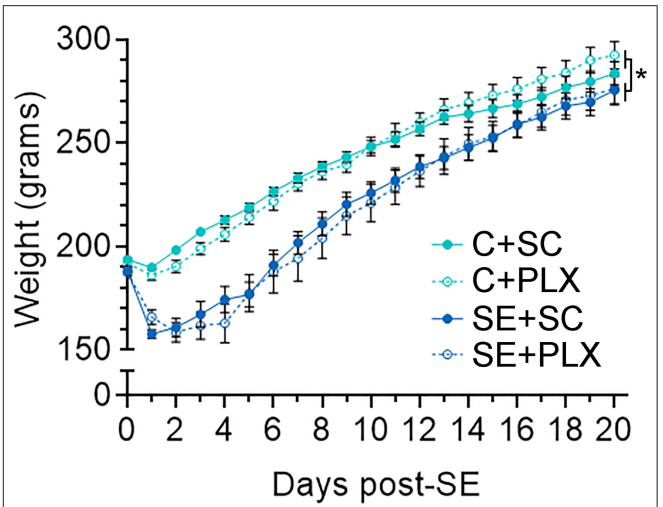

FIGURE 2 | PLX3397 (PLX) treatment does not alter body weight gain in control or SE rats. Graph shows the daily boy weight of rats (days 0–21) from the control and SE groups fed with either chow with PLX or standard chow alone (SC). Data are shown as mean ± SEM. *$p < 0.05$ by a three-way mixed effects model with Tukey's multiple comparisons test. C+SC, $n = 13$; C+PLX, $n = 13$; SE+SC, $n = 14$; SE+PLX, $n = 9$.

PLX Treatment Does Not Alter Recognition Memory in Rats

SE is often followed by memory impairments which parallel microgliosis in the hippocampus (5, 6, 8). Previously, we found that administration of the drug rapamycin during SE-induced epileptogenesis suppressed microgliosis and attenuated hippocampal-dependent learning and memory deficits (8). Thus, to further assess the role that microgliosis may play in the SE-induced cognitive decline we used NOR (**Figure 3**) and BM (**Figure 4**) to test recognition and spatial memory, respectively, in control and SE rats treated with SC or PLX. NOR was performed 2 weeks post-SE, when cognitive impairments are evident (5, 6, 8), and when spontaneous behavioral seizures occur with a frequency of ∼1.5 seizures per 48 h (2–3 weeks after SE) (6). Following the familiarization trial 1, rats were exposed to a familiar and a novel object in trial 2 (**Figure 3A**). We found that rats in the C+SC or C+PLX groups spent significantly more time exploring the novel object compared to the familiar object [C+SC: $t_{(18)} = 6.750$, $p < 0.0001$; C+PLX: $t_{(18)} = 8.180$, $p < 0.0001$]. In contrast, rats in the SE+SC or SE+PLX groups spent similar amounts of time exploring both objects [SE+SC: $t_{(14)} = 0.3224$, $p = 0.7519$; SE+PLX: $t_{(12)} = 1.934$, $p = 0.0770$] (**Figure 3B**), suggesting that these SE rats did not remember

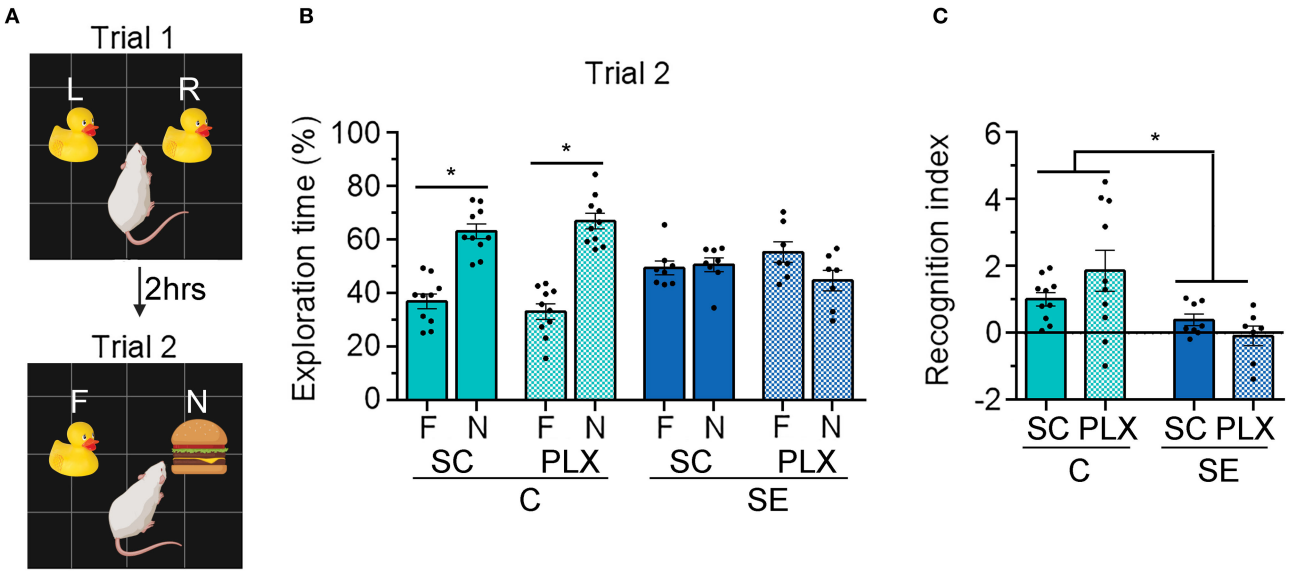

FIGURE 3 | PLX3397 (PLX) treatment after status epilepticus (SE) does not attenuate recognition memory impairments assessed with the novel object recognition (NOR) test. **(A)** Representative illustration of the NOR test trials 1 and 2. **(B)** Percentage exploration time of the familiar (F) and the novel objects (N) in trial 2 is shown. **(C)** Recognition index of the familiar objects in trials 1 and 2 [(F1-F2)/(F1+F2)] is shown. Data are shown as mean ± SEM. *$p < 0.05$ by student's *t*-test **(B)** and two-way ANOVA with Tukey's multiple comparisons test **(C)**. SC, standard chow. C+SC, $n = 10$; C+PLX, $n = 10$; SE+SC, $n = 8$; SE+PLX, $n = 7$.

the familiar object from trial 1. In addition, we determined the RI, which indicates the extent to which the familiar object was recognized in trial 2. The C+SC and C+PLX groups had significantly greater RI than the SE+SC or SE+PLX groups [Main effect, two-way ANOVA, $F_{(1,30)} = 15.32$, $p = 0.0005$] (**Figure 3C**), suggesting that the control animals treated with CS or PLX had greater memory of the familiar objects compared to both SE groups. Taken together these findings indicate that SE provokes memory defects and that PLX treatment had no effect on the recognition memory of either control or SE rats.

PLX Treatment Does Not Alter Hippocampal-Dependent Learning and Memory in Rats

Following NOR, rats were tested for hippocampal-dependent spatial learning and memory using the BM (**Figure 4**). Rats were tested for their ability to find an escape hole (latency to target) in a circular platform by relying on spatial navigation cues (**Figure 4A**). During the first block, the latency to reach the target was similar in all groups ($p > 0.9999$) (**Figure 4B**). Thereafter, the latency to target decreased with each subsequent block [main effect blocks, three-way ANOVA, $F_{(15,528)} = 4.485$, $p < 0.0001$], suggesting general learning of the target location over time. However, rats from the C+SC and C+PLX groups showed a significant decrease in latency to target when compared to the SE+SC or SE+PLX groups [main effect condition, three-way ANOVA, $F_{(1,528)} = 410.4$, $p < 0.0001$], suggesting a SE-induced learning deficit that was not attenuated with PLX. On the probe trial to test memory retention (**Figures 4C,D**), the C+SC and C+PLX groups spent significantly more time over the covered target hole when compared to the SE+SC and SE+PLX groups [two-way ANOVA, $F_{(1,33)} = 11.74$, $p = 0.002$], suggesting a memory deficit in both SE groups that was not attenuated with PLX. Taken together, these findings confirm that SE-induces deficits in hippocampal-dependent spatial learning and memory that are evident during epileptogenesis, and that PLX treatment did not have an effect on cognitive functions in control or SE rats.

PLX Treatment Attenuated SE-Induced Microgliosis in the Ca1 Hippocampus

Microgliosis is a hallmark of epileptogenesis and has been thoroughly characterized in experimental models of SE (9). Previously, we found that microgliosis peaked in the hippocampal CA1 region between 2 and 3 weeks after SE (7, 11). Thus, following behavioral tests, we determined the extent to which PLX suppressed microgliosis in this brain region, as a representative area, by performing counts of IBA1-positive microglial cells (**Figure 5**). Consistent with our previous reports, significantly increased numbers of microglial cells were evident in the SE+SC group when compared to the C+SC group [$F_{(1,45)} = 43.22$, $p < 0.0001$]. Compared to these groups, the number of microglial cells was significantly reduced in both C+PLX and SE+PLX groups [interaction, two-way ANOVA $F_{(1,45)} = 11.96$, $p = 0.001$]. The number of microglial cells was reduced by 60% in the C+PLX group compared to the C+SC group [student's *t*-test, $t_{(24)} = 8.148$, $p < 0.0001$]. Similarly, the number of microglial cells was reduced by 60% in the CA1 region of the SE+PLX group relative to the SE+SC group [$t_{(21)} = 3.858$, $p = 0.0009$]. In addition, there was a sub-group of rats within the SE group that did not respond to the PLX treatment [one-way ANOVA $F_{(2,25)} = 20.40$, $p < 0.001$] (**Supplementary Figure 1**).

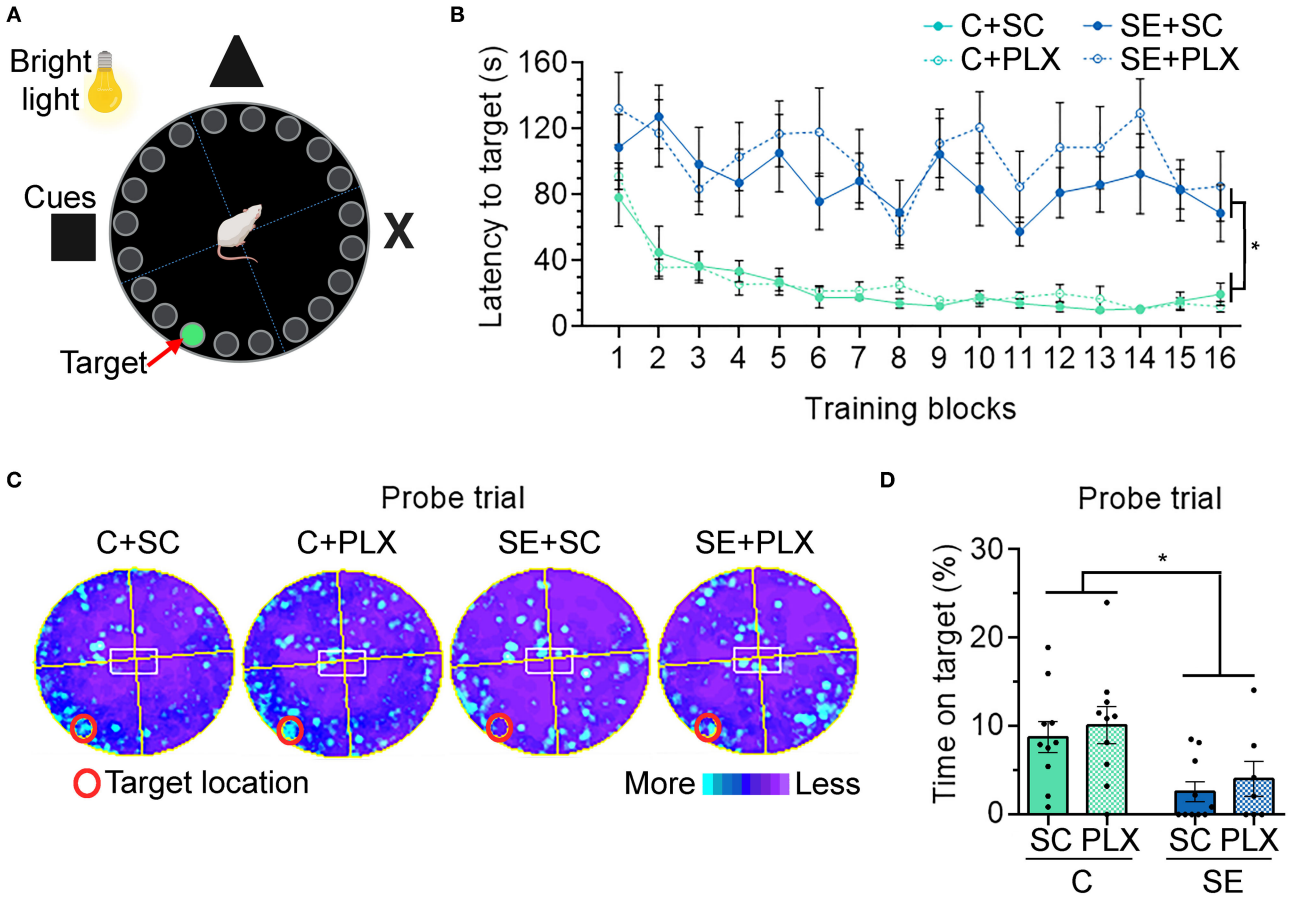

FIGURE 4 | PLX3397 (PLX) treatment after status epilepticus (SE) does not attenuate hippocampal-dependent spatial learning and memory deficits assessed with the Barnes maze (BM). **(A)** Representative illustration of the BM. **(B)** Graph shows the latency to find the target hole on the BM platform during training days 1–4 (4 trials/day). **(C)** Heat maps are shown as the average time spent in each location during the probe trial test. Red circle indicates the target location on the platform (covered hole). **(D)** Percentage of time spent on the target during the probe trial. Data are shown as mean ± SEM. *$p < 0.05$ by three-way ANOVA with Tukey's multiple comparisons **(B)** and two-way ANOVA with Tukey's multiple comparisons **(D)**. SC, standard chow. C+SC, $n = 10$; C+PLX, $n = 10$; SE+SC, $n = 10$; SE+PLX, $n = 7$.

The number of microglial cells was significantly higher in the SE+PLX Non-responder group compared to the SE+PLX responder group (by ∼300%; $p < 0.0001$) or the SE+SC (by 60%; $p = 0.0033$; **Supplementary Figure 1**). Together these data indicate that a 3-week treatment with PLX mixed in chow was effective at reducing the SE-induced microglial proliferation in the CA1 hippocampal region.

Next, we determined the effects of PLX on the morphology of the remaining microglial cells because following SE hippocampal microglia display an array of different shapes that evolve during epileptogenesis (**Figure 6**) (11). We found that PLX treatment altered the morphology of remaining microglial cells within the CA1 hippocampi of rats from both C+PLX and SE+PLX groups. We organized microglia into five distinct shapes: ramified, hypertrophic, bushy, amoeboid, and rod morphologies, based on the diameter of the cell, and the length and thickness of the processes (**Figure 6A**) (11). In the C+SC group, there was a significant difference among these microglial morphologies [Kruskal-Wallis test, $H_{(5,65)} = 51.07$, $p < 0.0001$], with higher numbers of ramified microglia compared to the other four morphologies (**Figures 6B–G**). In contrast, in the C+PLX group, the shapes of the remaining microglia were mainly ramified and hypertrophic [Kruskal-Wallis test, $H_{(5,65)} = 45.41$, $p < 0.0001$], suggesting that PLX had an effect on the remaining microglia. Consistent with our previous findings, the SE+SC group had increased numbers of microglial cells with bushy and amoeboid shapes compared to ramified, hypertrophic, or rod [Kruskal-Wallis test, $H_{(5,65)} = 53.34$, $p < 0.0001$]. In the SE+PLX group, microglial morphologies were mainly hypertrophic, bushy, or amoeboid [Kruskal-Wallis test, $H_{(5,50)} = 13.88$, $p = 0.008$]. Overall, PLX had an effect on the morphology of remaining microglia in both control and SE groups, with a shift to a larger population of cells with hypertrophic shapes suggesting the possibility of a change in the functional status of the remaining microglial cells.

PLX Attenuated SE-Induced Astrogliosis in the Hippocampus

To further assess the effects of PLX treatment on microglia we determined levels of the CX3C chemokine receptor 1

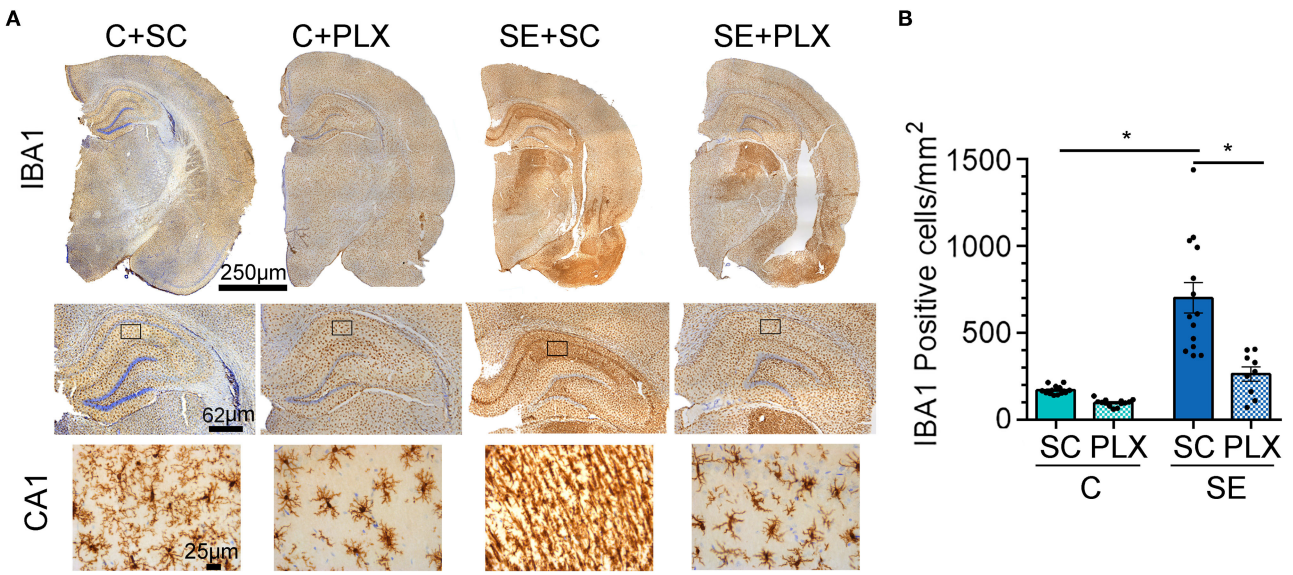

FIGURE 5 | PLX3397 (PLX) treatment after status epilepticus (SE) reduces microgliosis in the hippocampus. **(A)** Representative IBA1 (brown) and Nissl-stained cellular nuclei (blue) are shown at low magnification for one hemisphere (4x) and higher magnetization for the hippocampus (20X) and associated CA1 stratum radiatum (sr) region (boxed- 40X). **(B)** Quantification of IBA1 positive cells per mm^2 in CA1 sr. Data are shown as mean ± SEM. *$p < 0.05$ by two-way ANOVA with Tukey's multiple comparisons. SC, standard chow. C+SC, $n = 13$; C+PLX, $n = 13$; SE+SC, $n = 14$; SE+PLX, $n = 9$.

(CX3CR1) which is expressed in microglia (29, 30). In parallel, we determined levels of the complement protein C3 and its biologically active fractions because these are produced/released by astrocytes in response to microglial signals (31, 32) and are increased by SE (5, 6, 32). We found significantly increased levels of CX3CR1 protein in hippocampi of SE+SC rats when compared to the C+SC group [**Figure 7B**, main effect condition, two-way ANOVA, $F_{(1,42)} = 17.97$, $p = 0.0001$] (C+SC vs. SE+SC, $p = 0.001$). However, there was no significant difference in the protein levels of CX3CR1 between the SE+SC and SE+PLX groups ($p = 0.26$), or between C+SC and C+PLX groups ($p = 0.92$). While PLX reduced the number of microglial cells in CA1 (**Figure 5**), it did not alter the levels of this fractalkine receptor suggesting the possibility that there may be a compensatory increase in CX3CR1 expression in the remaining microglial cells in the SE+PLX group. Next, we determined the protein levels of the glial fibrillary acidic protein (GFAP) found in astrocytes, as well as the protein levels of the complement C3 fractions C3bα and iC3b. We found a significant increase in the levels of GFAP, C3bα, and iC3b in hippocampi of SE+SC rats when compared to the C+SC group (GFAP: $p = 0.003$; C3bα: $p = 0.02$; iC3b: $p = 0.006$). While PLX treatment significantly decreased the GFAP protein levels in the SE+PLX group when compared to the SE+SC group ($p = 0.04$), it did not alter GFAP levels in the C+PLX group relative to the C+SC group ($p = 0.99$) (**Figure 7C**). PLX treatment did not alter the protein levels of C3bα (**Figure 7D**) or iC3b (**Figure 7E**) in either the C+PLX or SE+PLX groups when compared to C+SC and SE+SC, respectively (C3: SE+PLX vs. C+SC, $p = 0.48$; SE+PLX vs. SE+SC, $p = 0.41$; iC3b: SE+PLX vs. C+SC, $p = 0.99$; SE+PLX vs. SE+SC, $p = 0.47$). These data suggest that in addition to reducing SE-induced microgliosis PLX treatment reduced astrogliosis in hippocampi of SE rats.

DISCUSSION

To understand the contribution of microglial cells to the cognitive dysfunction associated with epileptogenesis we used a dietary treatment with the drug PLX3397 to decrease the population of microglial cells during this critical period. The main findings of this study are that the SE-triggered cognitive defects were not resolved with PLX3397 treatment (**Figures 3**, **4**), even though PLX3397 significantly reduced both SE-induced microgliosis (**Figure 5**) and astrogliosis in the hippocampus (**Figure 7C**).

The use of new pharmacological tools such as PLX3397 and its analogs, that specifically suppress CSF1R signaling to interrupt microglial survival/proliferation and reduce their population, implicate these cells as essential players underlying memory defects in preclinical models of neurodegeneration (19–23). For instance, reduction in the population of microglial cells with a PLX3397 or PLX5622 diet restored contextual memory in mouse models of Alzheimer's disease (AD) (19, 20), improved spatial memory in aged mice (22), and attenuated radiation-induced cognitive deficits in mice (21). Based on this evidence we expected that reductions of SE-induced microgliosis with PLX3397 during epileptogenesis would improve cognitive functions. However, despite significant reductions in the numbers of microglia triggered by the PLX3397 diet in both control and SE rats (**Figure 5**), cognitive behaviors remained

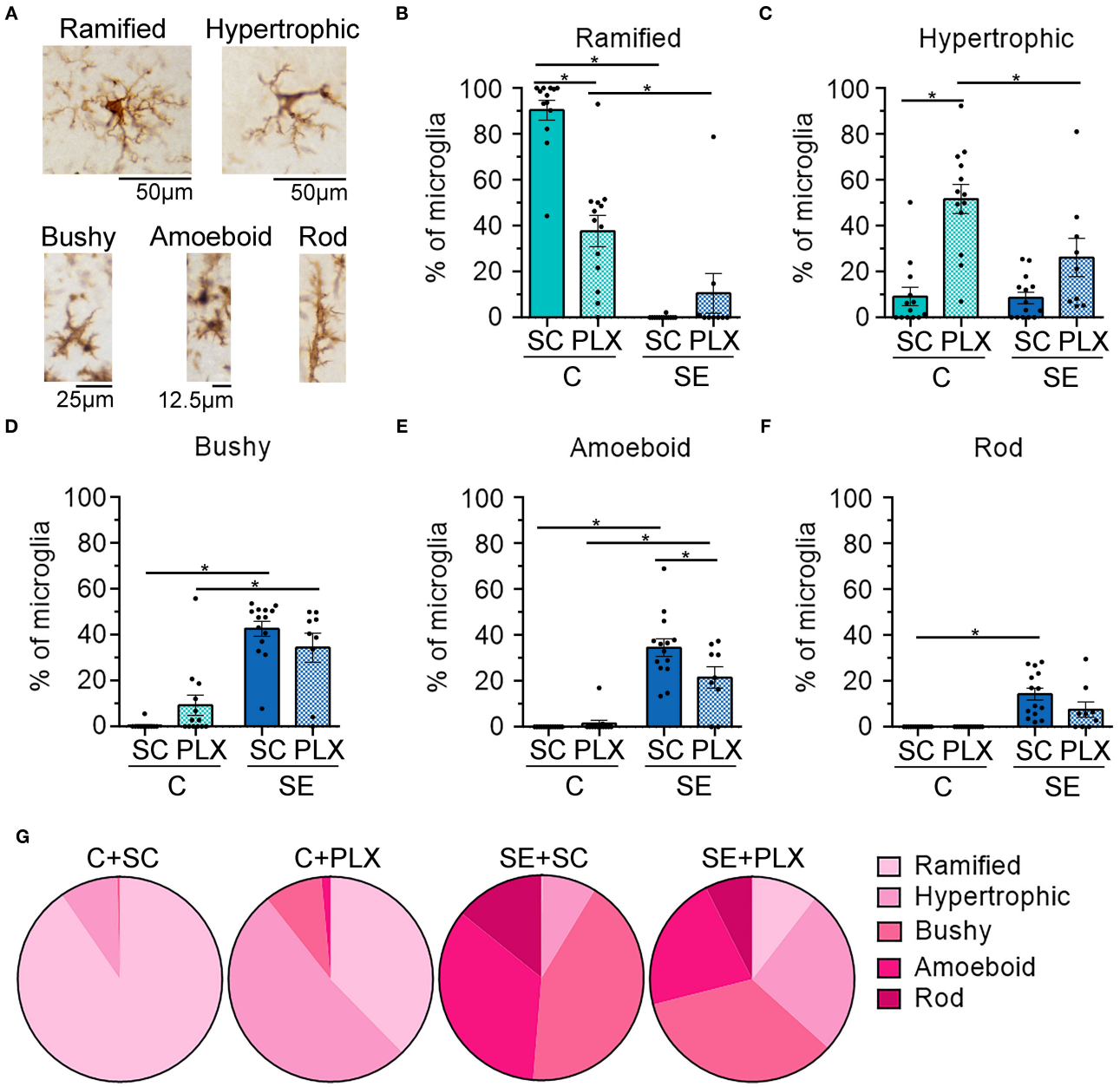

FIGURE 6 | PLX3397 (PLX) treatment alters the morphology of microglia in control and SE rats. **(A)** Representative images of different microglial (brown) morphologies observed in the hippocampus. **(B–F)** Morphological breakdown of each group as ramified **(B)**, hypertrophic **(C)**, 3-bushy **(D)**, amoeboid **(E)**, and rod **(F)**. **(G)** Pie charts are shown the microglial morphologies in each treatment group. *$p < 0.05$ by Kruskal-Wallis test with Dunn's multiple comparisons. Data are shown as mean ± SEM. SC, standard chow. C+SC, $n = 13$; C+PLX, $n = 13$; SE+SC, $n = 14$; SE+PLX, $n = 9$.

similar to the rats on regular chow (**Figures 3**, **4**). This suggests the possibility that the remaining microglia are sufficient to help maintain circuit functions, or that the roles of microglia vary in different disorders, such as in epilepsy which is characterized by neuronal hyperactivity and seizures.

The presence of reactive microglia with an array of morphologies and cytokine profiles with diverse spatiotemporal profiles have been widely described in response to SE as well as in chronic epilepsy (9, 10, 33). However, whether this microgliosis is beneficial or detrimental during epileptogenesis or in established epilepsy is still unresolved. In some models of acquired epilepsies, as well as in models of brain injury by trauma or stroke, drastic reductions in microglial populations with CSF1R inhibitors were shown to either improve or aggravate pathological consequences (13, 34–41). In models of SE and acquired epilepsy, suppression of CSF1R signaling was associated

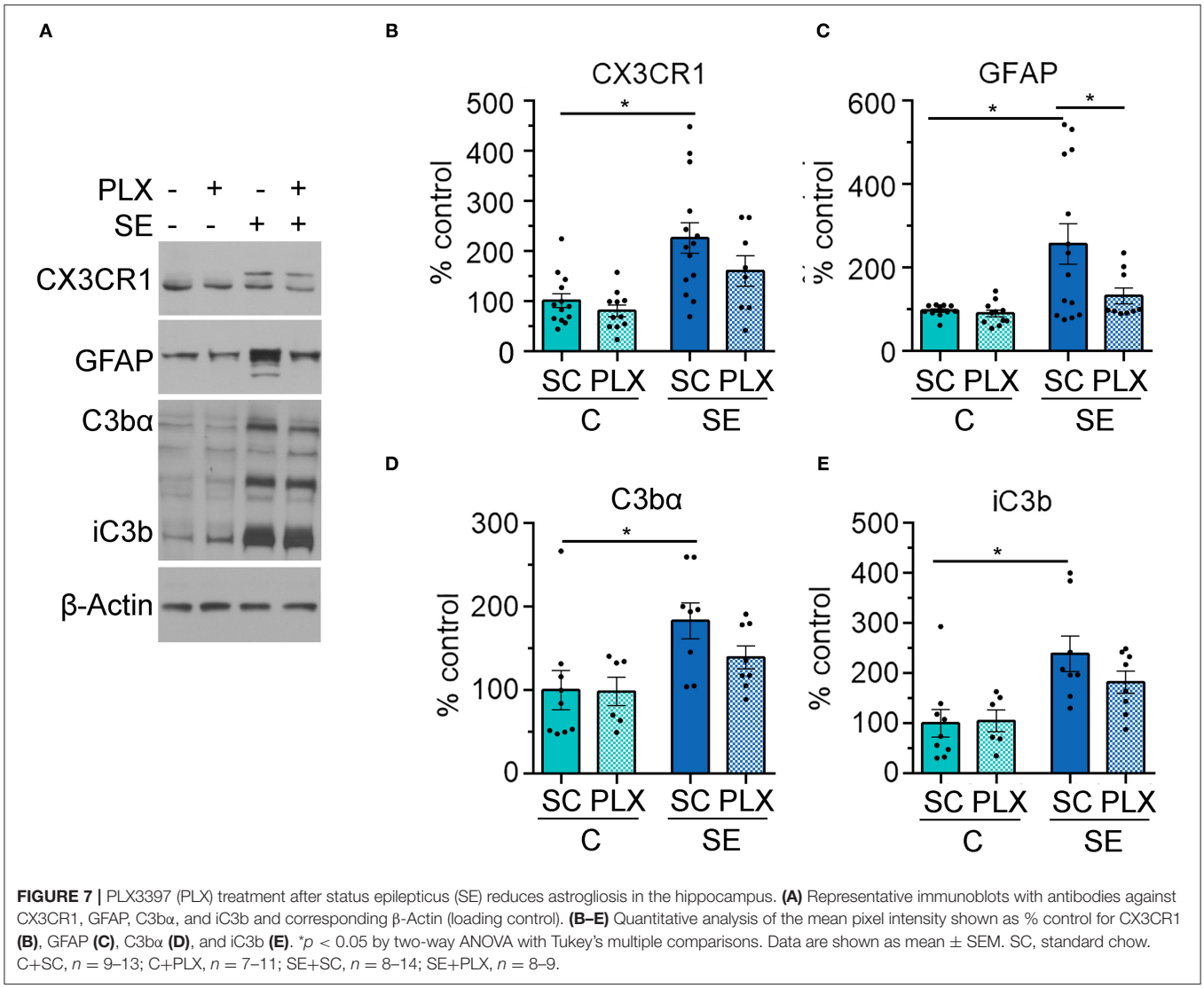

FIGURE 7 | PLX3397 (PLX) treatment after status epilepticus (SE) reduces astrogliosis in the hippocampus. **(A)** Representative immunoblots with antibodies against CX3CR1, GFAP, C3bα, and iC3b and corresponding β-Actin (loading control). **(B–E)** Quantitative analysis of the mean pixel intensity shown as % control for CX3CR1 **(B)**, GFAP **(C)**, C3bα **(D)**, and iC3b **(E)**. *$p < 0.05$ by two-way ANOVA with Tukey's multiple comparisons. Data are shown as mean ± SEM. SC, standard chow. C+SC, $n = 9$–13; C+PLX, $n = 7$–11; SE+SC, $n = 8$–14; SE+PLX, $n = 8$–9.

with decreases in SE-induced neuronal loss and seizure frequency (13, 34) but also with an exacerbated acute seizure response to kainate (40, 41) and increased seizure frequency in epileptic mice (40). In a mouse model of Theiler's murine encephalomyelitis virus-induced epilepsy microglia depletion intensified the seizure severity and resulted in fatal encephalitis (39). This body of evidence along with the finding the lack of microglia due to homozygous mutations in the CSF1R gene are associated with brain malformations, developmental delay, and epilepsy in humans (17), and new findings that microglia directly control neuronal activity through a negative feedback mechanism (41), suggest that microglia may be important to prevent and/or control the generation of epileptic networks. We speculate that the long-lasting suppression of microgliosis after SE in our study may have allowed or perhaps exacerbated epileptogenesis and worsen memory dysfunction, though it is also possible that PLX treatment may slow or prevent epileptogenic processes. Therefore, a limitation of our study is that we did not determine the impact of PLX3397 or a combination treatment of PLX3397 with an anti-seizure drug during the period of epileptogenesis using electroencephalographic (EEG) recordings. These would aid in differentiating the potential role the experimental treatments (PLX3397 and SC) have on the extent of hippocampal microgliosis, spontaneous seizure duration and frequency, and the cognitive defects within the same animals. In addition, EEG recordings could help determine the relation between seizure development and cognitive decline, as seizures can have profound consequences on the behaviors evaluated in the NOR and BM test. Our future studies will investigate these aspects.

Consistent with previous studies (13, 22), we found that the PLX3397 diet promoted a decrease of ∼60% in the population of microglial cells both in control and SE-treated rats. We focused on the CA1 hippocampus as a representative brain area because we found the most robust SE-induced increases in microgliosis in this region (6–9) and because these microglial changes closely paralleled memory loss (5–7). In a mouse model of AD, treatment

with a CSF1R inhibitor reduced microgliosis by 80% in the hippocampus, cortex, and thalamus (19), while in healthy aging mice, this effect was close to 99% (16, 22, 42). Other studies also support that the extent of microglial suppression with the CSF1R inhibitor is consistent when given under physiological conditions but highly variable in neurological disorders including AD, Parkinson's disease, ischemia, and epilepsy (13, 19, 25, 43) which suggest that the activation of CSF1R signaling or the levels of CSF1 may be differently altered under variable pathological conditions, including during the period of epileptogenesis. A limitation of our study is that we were not able to measure the extent of potential alterations in CSF1R expression or its phosphorylation/activation status during epileptogenesis due to the lack of antibodies with appropriate specificity for the rat tissues. Spatial and temporal information about the status of CSF1R signaling would be necessary to determine the extent of the contribution of this pathway to SE-induced microgliosis and the neuropathology and pathophysiology of epilepsy. This information would help guide the selection of a relevant time window along with an appropriate dose-response test to assess different levels of microglial suppression with pharmacological tools such as PLX3397 or its analogs during epileptogenesis and in chronic epilepsy.

Thus, it is not known how exactly SE impacts the activation of CSF1R signaling in microglia and which specific functions are altered at the time point evaluated in this study. The decrease in microgliosis indicates that PLX3397 suppressed the survival and proliferation of these cells. However, we also found a shift in the morphology of the remaining microglia. In both control and SE groups, treated with PLX3397 an increase in the abundance of hypertrophic microglia suggests that the function of these remaining cells may be changed. This is further supported by the lack of changes in the hippocampal protein levels of CX3CR1 between PLX3397- and regular chow-treated groups despite a reduction of 60% in the total numbers of microglia in the hippocampus, which suggest a potential compensatory increase in the expression of this receptor in the remaining microglia. While it is not definitively known how different microglial shapes associate with specific functions some studies support that bushy/amoeboid microglia may be highly phagocytic due to the presence of high levels of phagocytic lysosomal markers (44–47) and that hypertrophic microglia may be inflammatory (48, 49).

Interestingly, we found that PLX3397 also reduced the SE-induced increases in the hippocampal protein levels of GFAP suggesting a decrease in astrogliosis (**Figure 7**). This finding further supports that these two cell types communicate which each other under pathological conditions (50). Microglia and astrocytes communicate through a number of molecules including complement proteins (32, 50), which are upregulated in epilepsy (9). Reactive microglia produce and release the complement protein C1q which in turn triggers astrocytes to release C3a which then binds to its receptor (C3aR) in astrocytes, thereby producing a regulatory loop (32, 50). Although PLX3397 reduced astrogliosis, the levels of C3 protein in whole hippocampal homogenates remained unchanged. This suggests the possibility that other pathways or cell types may be regulating C3 levels in response to SE, or that the immunoblot approach used in our study missed changes that may be regional within the hippocampal formation, as recently shown by Wei et al. (32). Furthermore, due to the tight association between SE-induced increases in C3 complement activation and memory decline following pilocarpine-induced SE in rats (5, 6) we speculate that the high levels of C3 still present in both SE groups (PLX3397 and standard chow) may be modulating the memory loss.

In sum, our study shows that dietary treatment with the CSF1R inhibitor PLX3397 during the period of SE-induced epileptogenesis in the rat does not alter memory functions even though microglial numbers were significantly reduced. Our findings do not support or reject pro- or anti-epileptogenic roles for microglia during SE-induced epileptogenesis, only that reducing microgliosis by 60% in the hippocampus was not sufficient to attenuate SE-induced memory defects. Therefore, the answer to whether microgliosis itself underlies cognitive decline during epileptogenesis is still in need of further investigation. Future studies will focus on a comprehensive characterization of CSF1R receptor expression and activation status needed to understand its contribution to microglial proliferation and function in epilepsy, and to determine whether a full depletion of microglia is necessary to attenuate or prevent epileptogenesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Purdue Institutional Animal Care and Use Committee (Protocol #1309000927).

AUTHOR CONTRIBUTIONS

SW-J and AB contributed to idea development and experimental design of the study. SW-J conducted experiments, analyzed data, made figures, and wrote the first draft of the manuscript. AS performed behavioral and biochemical experiments and analyzed behavioral data. KS performed biochemical experiments and analyzed behavioral data. AB wrote sections of the manuscript. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.651096/full#supplementary-material

Supplemental Figure 1 | PLX3397 in chow (PLX; 50 mg/kg per day) did not suppress microgliosis in five rats that sustained the same level of pilocarpine-induced status epilepticus (SE) and had similar body weights. **(A)** Behavioral seizures were monitored for 100 minutes after SE induction and scored according to the Racine scale (1: rigid posture, mouth moving; 2: tail clonus; 3: partial body clonus, head bobbing; 4: rearing; 4.5: severe whole body clonic seizures while retaining posture; 5: rearing and falling; 6: tonic-clonic seizure with jumping or loss of posture). Three SE groups are shown: SE +standard chow (SC) (SE+SC), SE+PLX, and SE+PLX Non-responder (Non or N). **(B)** Time to first seizure (level 3). **(C)** Time to SE (level 6). **(D)** Graph shows the daily body weight of rats (days 0-21) from all SE groups. **(E)** Quantification of IBA1 positive cells per mm^2 in CA1 sr. Data analyzed by Kruskal-Willis test with Dunn's multiple comparisons **(A)**, one-way ANOVA with Dunnett's multiple comparisons **(B,C,E)**, and two-way ANOVA with Dunnett's multiple comparisons **(D)**. Data are shown as mean ± SEM. SE+SC, $n = 14$; SE+PLX, $n = 9$; SE+PLX Non-Responder, $n = 5$. $^*p < 0.05$.

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REVIEW
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From Physiology to Pathology of Cortico-Thalamo-Cortical Oscillations: Astroglia as a Target for Further Research

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The electrographic hallmark of childhood absence epilepsy (CAE) and other idiopathic forms of epilepsy are 2.5–4 Hz spike and wave discharges (SWDs) originating from abnormal electrical oscillations of the cortico-thalamo-cortical network. SWDs are generally associated with sudden and brief non-convulsive epileptic events mostly generating impairment of consciousness and correlating with attention and learning as well as cognitive deficits. To date, SWDs are known to arise from locally restricted imbalances of excitation and inhibition in the deep layers of the primary somatosensory cortex. SWDs propagate to the mostly GABAergic nucleus reticularis thalami (NRT) and the somatosensory thalamic nuclei that project back to the cortex, leading to the typical generalized spike and wave oscillations. Given their shared anatomical basis, SWDs have been originally considered the pathological transition of 11–16 Hz bursts of neural oscillatory activity (the so-called sleep spindles) occurring during Non-Rapid Eye Movement (NREM) sleep, but more recent research revealed fundamental functional differences between sleep spindles and SWDs, suggesting the latter could be more closely related to the slow (<1 Hz) oscillations alternating active (Up) and silent (Down) cortical activity and concomitantly occurring during NREM. Indeed, several lines of evidence support the fact that SWDs impair sleep architecture as well as sleep/wake cycles and sleep pressure, which, in turn, affect seizure circadian frequency and distribution. Given the accumulating evidence on the role of astroglia in the field of epilepsy in the modulation of excitation and inhibition in the brain as well as on the development of aberrant synchronous network activity, we aim at pointing at putative contributions of astrocytes to the physiology of slow-wave sleep and to the pathology of SWDs. Particularly, we will address the astroglial functions known to be involved in the control of network excitability and synchronicity and so far mainly addressed in the context of convulsive seizures, namely (i) interstitial fluid homeostasis, (ii) K^+ clearance and neurotransmitter uptake from the extracellular space and the synaptic cleft, (iii) gap junction mechanical and functional coupling as well as hemichannel function, (iv) gliotransmission, (v) astroglial Ca^{2+} signaling and downstream effectors, (vi) reactive astrogliosis and cytokine release.

Keywords: astrocytes, sleep/wake cycle, NREM, network plasticity, cortico-thalamo-cortical oscillations, spike and wave discharges, sleep

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INTRODUCTION

Epilepsy is a highly heterogeneous neurological condition characterized by enduring predisposition to unpredictable pathological discharge of rhythmic activity in the brain networks, which is commonly referred as seizure activity (1). In virtue of the severity and nature of the pathological alteration (abnormal, excessive, or excessively synchronous activation) as well as the cellular and anatomical composition of the affected brain networks, seizures can cause changes in the level of consciousness, behavior, memory, and emotional status. Although the etiology of epileptiform activity is still unknown in half of the cases, understanding the pathological alteration at the basis of the epileptic phenotype may not only be of fundamental therapeutical importance but also provide further insights into the functioning of the affected neural networks in the physiology of the healthy brain. The identification of the molecular and cellular mechanisms underlying physiological oscillations is critical for a full comprehension of their relationship to the respective pathological activity. In this regard, an exceptional case of study is the cortico-thalamo-cortical network, physiologically engaged during sleep and pathologically altered in the context of non-motor (absence) seizures (2).

Absence seizures are transient non-convulsive generalized epileptic events and are also referred as *petit mal* seizures (2, 3). Phenotypically, absence seizures are coupled with sudden and brief impairment of consciousness and lack of responsiveness to external stimuli as well as variable secondary clinical symptoms (e.g., automatisms, atonic, and tonic muscular components etc.) (4, 5). Absence seizures are the sole clinical symptom of childhood absence epilepsy (CAE) but are also associated with several other idiopathic generalized epilepsies (4, 6–11). Although CAE has up to 70% remission rate (7, 12), the gold standard monotherapy, based on ethosuximide and valproic acid, is still ineffective in 30% of the cases (13). Moreover, clinical conditions displaying absence seizures are often associated with severe neuropsychiatric comorbid conditions such as impaired attention, learning, memory and cognition, which are often left unaltered or even worsened by common antiepileptic drugs (14–17).

Although absence seizures display inter- and intraindividual variability (17, 18), they exhibit generalized bilateral 2.5–4 Hz spike and wave discharges (SWDs) with no aura or post-ictal depression (**Figures 1A,B**) (4, 27–29). It is widely accepted that the sharp spike and the slow wave component of SWDs are functionally coupled and correspond to a state of neuronal excitation and silence in the cortico-thalamo-cortical network, respectively (30). Blood oxygenation level-dependent (BOLD) functional magnetic resonance imaging (fMRI) studies in humans consistently showed cortical network engagement in correspondence of and even preceding the appearance of SWDs in electrographic traces as well as an increased interictal synchrony in the sensorimotor cortex (**Figure 1C**) (22, 23, 31–36). Most advancements on the understanding of the cellular and synaptic mechanisms underlying SWDs derive from the extensive use of genetic animal models, particularly the genetic absence epilepsy rats from Strasbourg (GAERS) and Wistar-Albino-Glaxo rats from Rijswijk (WAG/Rij) (20, 37–42) as well as many monogenic mouse mutants (43–45). Although sharing most electrographic and behavioral hallmarks of absence seizures, animal models are characterized by higher SWD frequencies (5–11 Hz) (**Figure 1B**). E*x vivo* multi-site local field potential studies identified the peri-oral primary somatosensory cortex as initiation site of absence seizures in WAG/Rij (46) and GAERS rats (47–50). Notably, this has been proven wrong for the acute pharmacological γ-hydroxybutyric acid (GHB) model (51–54) in mice, where the prefrontal cortex was suggested as the initiation site of SWDs (55). With this in mind and considering the many areas contributing to the cortical pre-ictal BOLD changes of absence seizures, one can probably not identify a unique canonical focal onset or initiation site for absence seizures. Instead, the denomination *cortical initiation network* has been recently proposed (17), thereby settling the long-standing controversy about the SWDs initiation site (56–59). However, the existence of a cortical initiation network does not imply that manipulation of the sole thalamic components of the cortico-thalamo-cortical network is not sufficient to induce SWDs, as it is indeed the case (60–62), or that the wide thalamo-cortical innervation is not crucial for SWDs generalization, as suggested by the existence of subclinical SWDs restricted to the cortical network (48). In particular, the thalamic posterior nucleus plays a crucial role in the generalization of SWDs (61, 63–66). Till recently, *ex vivo* studies performed in different mammalian models identified the hyperexcitability and T-type Ca^{2+} channel-mediated burst activity of glutamatergic thalamo-cortical neurons and GABAergic neurons from the thalamic reticular nucleus (or *nucleus reticularis thalami*, NRT) as the rhythmogenic cortico-thalamo-cortical network mechanism of SWDs (**Figures 1D,E**) (24, 41, 67–71). Nevertheless, recent *in vivo* studies performed in rodents showed that only a small fraction of thalamo-cortical and cortico-thalamic neurons are synchronously active at each SWD cycle and the cellular composition of this neuronal subpopulation changes between subsequent cycles, thus excluding the existence of distinctive neuronal subpopulations (**Figures 1F,G**) (25, 26). This explains why, with SWD progression, the activities of the cortico-thalamic and thalamo-cortical neurons undergo a phase-shift in time (46) since different neuronal subpopulations participate in this excitatory feedback-loop with slightly different kinetics. Moreover, this progressive phase-shift between different subpopulations active at the same time accounts for the overlapping average electrical activity in the cortico-thalamic, thalamo-cortical, and NRT neurons within any SWD cycle. Moreover, although interictal T-type Ca^{2+} channel burst activity in the thalamo-cortical neurons increases right before SWD onset, overall *in vivo* ictal thalamic activity decreases and only cortical and NRT T-type channels are essentials for SWDs (25). Interestingly, all NRT neurons fire within each SWD cycle, even though a fraction of those neurons fires relatively asynchronous tonic spikes rather than T-type Ca^{2+} channel-mediated bursts in phase with the SWDs (**Figures 1E,F**) (25). The enhanced tonic inhibition of thalamo-cortical neurons as well as the increased thalamic GABA level are key aspects of absence seizures

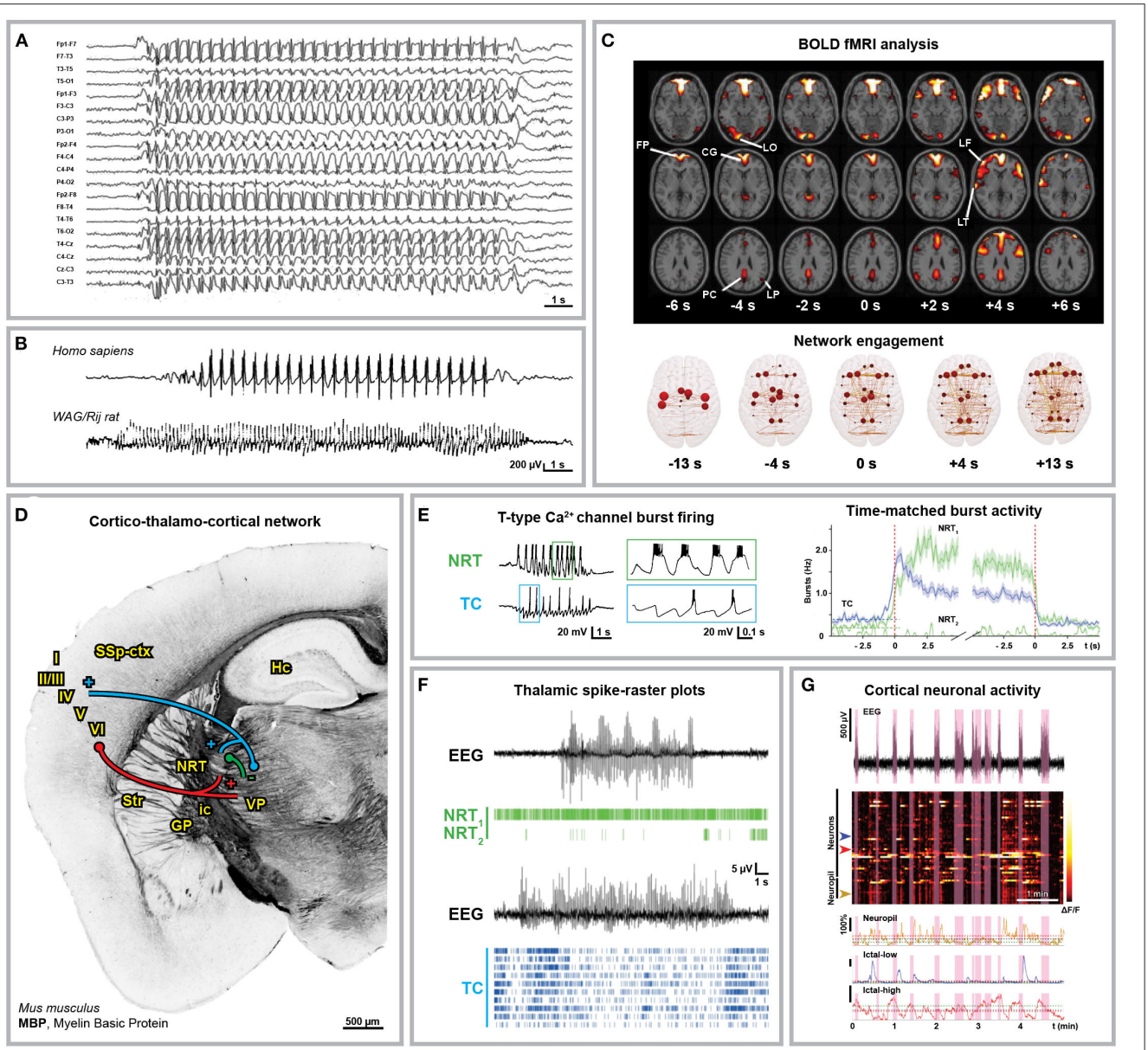


FIGURE 1 | Anatomical and electrophysiological characterization of absence seizures. **(A)** Human electroencephalographical recording (EEG) displaying typical 3 Hz spike-and-wave discharges (SWDs). **(B)** 3 Hz SWDs associated with Childhood Absence Epilepsy (top trace, 8-year-old boy) and 8 Hz SWDs recorded in an adult WAG/Rij rat (bottom trace). **(C)** Blood Oxygenation Level Dependent (BOLD) functional Magnetic Resonance Imaging (fMRI) changes associated with absence seizures before (FP, frontal polar; CG, cingulate; LO, lateral occipital; PC, precuneus; LP, lateral parietal cortex) and after (LF, lateral frontal; LT, lateral temporal cortex) seizure onset (top) and brain network engagement analysis around SWDs events (bottom). **(D)** Anatomical organization of the cortico-thalamo-cortical network in C57BL/6N mice. Myelin Basic Protein (MBP) immunostaining indicates the axonal fiber tracts connecting the network (own data; mouse monoclonal MBP antibody, BioLegend, AB_2564741, Cat. No. 808401, 1:500). Cortico-thalamic excitatory neurons from cortical layers V and VI (red) project to both NRT and thalamus; thalamo-cortical excitatory neurons (blue) project back to cortical layer IV and NRT; NRT GABAergic neurons (green) inhibit the thalamic nuclei. I-VI, cortical layers; GP, globus pallidus; Hc, hippocampus; ic, internal capsule; NRT, nucleus reticularis thalami; SSp-ctx, primary somatosensory cortex; Str, striatum; VP, ventral posterior thalamic nuclei. **(E)** Glutamatergic thalamo-cortical neurons (TC) as well as GABAergic NRT neurons display T-type Ca^{2+} channel-mediated burst firing during SWDs (left, *ex vivo* recording from ferret thalamic slices). **(F)** Spike-time raster plots of two representative NRT neurons (NRT_1, top trace; NRT_2, bottom trace) and 10 TC neurons with time-matched EEG in GAERS rats. The overall TC activity decreases during SWDs and only a small portion of TC neurons fire synchronously. **(G)** 2-Photon laser scanning microscopy of neuronal cortical Ca^{2+} activity in *stargazer* mice during absence seizures. Heatmap of neuronal Ca^{2+} activity shows that only a subpopulation of neurons displays ictal synchronous firing. Modified from **(A)**, (19); **(B)**, (20, 21); **(C)**, (22, 23); **(E)**, (24, 25); **(F)**, (25); **(G)**, (26).

(25, 72–78). Moreover, the fact that SWDs can be induced by the impairment of the cortico-thalamic glutamate release due to deletion of P/Q-type Ca^{2+} channels in the projecting cortical neurons from layer VI could suggest that a balance shift toward GABAergic inhibition more than an absolute increase of GABA levels is the key mechanism of SWD generalization

(79). Additionally, the decreased glutamate release could lead to reduced activity of cortical interneurons, thus contributing to cortical hyperexcitability.

ASTROCYTES CONTRIBUTE TO NETWORK PRIMING AND SYNCHRONIZATION AS WELL AS SWD INDUCTION, PROPAGATION, AND TERMINATION

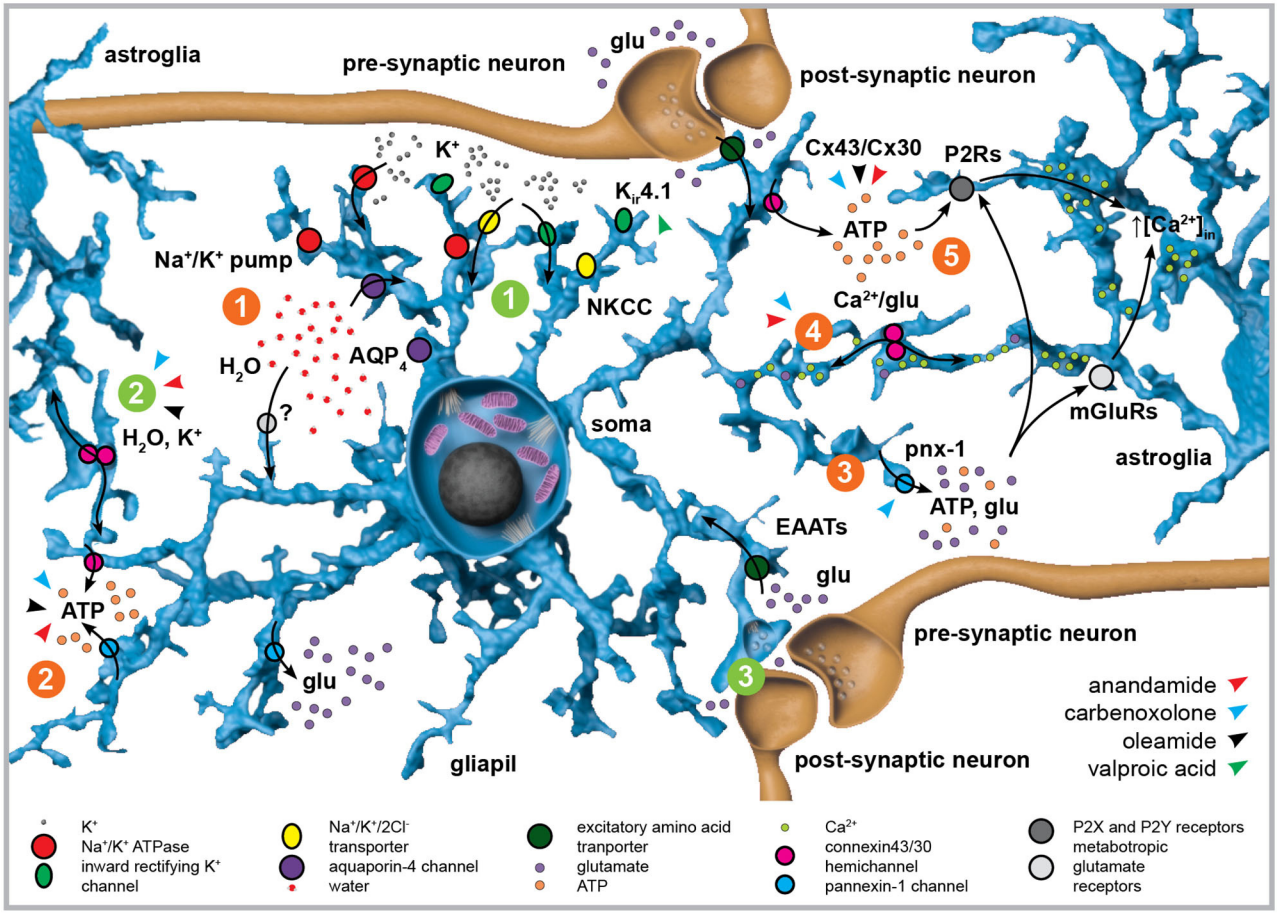
After more than three decades of accumulating evidence, nowadays it is widely established that astroglia constitute a ubiquitous non-neuronal communication system in the brain involved in virtually every physiological and pathological scenario of the central nervous system (80–82). Not only do they support synapses from a mechanical, metabolical as well as functional point of view, but they also participate in synaptic transmission and plasticity, neural network excitability and balance between excitation and inhibition (E/I) as active information integrators and processors (83–86). The contribution of the astroglial network to the pathophysiology of epilepsy encompasses a plethora of different molecular mechanisms which currently represent one of the most fruitful research topics in neuroscience (87–96). Pathological priming mechanisms of the astroglial network ultimately involve either E/I imbalance or enhanced network synchronization (or both simultaneously). Alternatively, astrocytes may influence spatial and temporal propagation of seizures, thus playing a key role in the phenotypical outcome of seizures and their severity and therefore representing a promising target for the development of new non-neurocentric drugs. Most of the recent evidence focuses on astroglial contribution to convulsive epileptic activity. Nevertheless, we discuss in the following the putative involvement of astrocytes in network priming as well as seizure induction and propagation which could have a role in pathological epileptic scenarios including SWDs, as well. We focus on the evidence that links to observations coming from the clinics as well as genetic and pharmacological models of SWDs, aiming to point at specific topics which may be worth further research in the field of SWDs.

The Astroglial Network Controls Extracellular Space Homeostasis Through K^+, Water and Solute Clearance

By means of their close juxtaposition to synapses, their expression of an extraordinary assortment of membrane transporters and receptors as well as their physical and functional coupling through gap junctions (GJs), astroglial networks provide a perfect spatial buffering for neural activity (**Figure 2**) (97–99). Astrocytes are key regulators of the extracellular K^+ concentration. Their high K^+ permeability mediated by inwardly-rectifying K_{ir} and two-pore-domain K_{2P} channels, Na^+/K^+ pumps and $Na^+/K^+/Cl^-$ transporters associated with their extensive GJ coupling enables them to uptake and redistribute excessive extracellular K^+ resulting from neuronal firing (100–103). Astroglial K^+ and glutamate uptake is altered in cultured cortical astrocytes after $K_{ir}4.1$ channel downregulation (104) as well as in astroglial-specific $K_{ir}4.1$ knock-out mice (105, 106). Gain-of-function (107) as well as loss-of-function mutations (108, 109) in the human *KCNJ10* gene encoding $K_{ir}4.1$ have been linked to forms of childhood epilepsies associated with ataxia and cognitive impairment, but not to CAE. Notably, artificially increasing extracellular K^+ concentration *ex vivo* is associated with propagating epileptiform discharges induced by focal optogenetic activation of parvalbumin-expressing interneurons (110). *In vivo* though, K^+ clearance impairment induced by blocking GJ was not sufficient to induce neocortical seizures (111). Interestingly, valproic acid (but not ethosuximide) induces $K_{ir}4.1$ overexpression in the cortex of healthy rats (112). Nevertheless, further research is required to address the actual contribution of $K_{ir}4.1$ overexpression in the anti-absence effects of valproic acid, as well as the putative role of astroglial $K_{ir}4.1$ itself in the development and propagation of SWDs. The combined use of cell-specific conditional knock-out of these channels and pharmacological models of SWDs could shine new light on the topic.

K^+ uptake is associated with cellular swelling due to Na^+/K^+-pump dependent water influx (113). Although the exact molecular mechanisms are still under debate, water fluxes across the astrocytic membrane are associated with K^+ homeostasis and they influence local interstitial osmolarity as well as seizure generation and progression (114–120). Given the accumulating evidence against the predominant contribution of the astroglial water channel aquaporin-4 (AQP4) in water homeostasis (113, 121), the use of AQP4 conditional knock-out as a model of disrupted water homeostasis has been recently challenged. Nevertheless, water homeostasis impairment and the resulting volume and osmolarity dysregulation should affect neural network excitability. Indeed, a recent structural MRI study on CAE showed significant gray matter volume abnormalities in both frontotemporal cortical region and posterior thalami compared to controls (122, 123).

GJ coupling, particularly mediated by connexins Cx30 and Cx43, provides the astroglial network with a high level of intercellular structural, metabolic and functional connectivity, enabling the exchange of ions and small molecules (124–131). In the context of epilepsy, connexins mediate ATP release (into the extracellular space through hemichannels), the spreading of intercellular Ca^{2+} waves (132) and are fundamental in the spatial buffering required for K^+ and water homeostasis as well as glutamate clearance (133, 134). With respect to absence epilepsy, most advancement in unraveling the role of GJs has been obtained employing GJ blockers in well-established genetic animal models (135). The broad-spectrum GJ blocker carbenoxolone (CBX) decreased both amplitude and duration of 4-aminopyridine-induced seizure-like events (SLEs) in thalamocortical slices obtained from mice with spontaneous SWDs (136, 137) as well as the duration of SWDs seen in GAERS rats *in vivo* after systemic application (138). Interestingly,



Anti-epileptic function

1. K^+ clearance through K_{ir} channels, Na^+/K^+ ATPase and $Na^+/K^+/2Cl^-$ tranporter
   Buskila et al., 2019; Pacholko et al., 2020; Wang et al., 2020
2. Gap-junction-mediated K^+ and water spacial buffering
   Wallraff et al., 2006; Bedner et al., 2015
3. Glutamate uptake and clearance at the synaptic cleft
   Coulter & Eid, 2012; Peterson & Binder, 2020

Pro-epileptic function

1. Cell swelling and resulting increased extracellular osmolarity
   Binder & Steinhäuser, 2006; Binder et al., 2006; Lauderdale et al., 2015; Walch et al., 2020
2. ATP release through hemichannels
   Parpura & Verkhrasky, 2012; Lapato & Tiwari-Woodruff, 2018
3. ATP and glutamate release through pannexin-1 channels
   Aquilino et al., 2019; Scemes et al., 2019
4. Intercellular Ca^{2+} spreading through GJs
   Parpura & Verkhrasky, 2012; Kekesi et al., 2015
5. ATP-mediated Ca^{2+} spreading
   Parpura & Verkhrasky, 2012; Nikolic et al., 2020

FIGURE 2 | Astroglial homeostatic control of the extracellular space has opposite effects on epileptogenesis. The astroglial network is responsible for extracellular K^+ uptake by means of inward rectifying K^+ channels (K_{ir}), Na^+/K^+ pump and $Na^+/K^+/2Cl^-$ transporter (NKCC). K^+ clearance is coupled with water uptake through the water channel aquaporin-4 (AQP4) and possibly *via* yet unknown additional pathways. The excitatory amino acid transporters EAAT1 and EAAT2 are responsible for

(Continued)

FIGURE 2 | glutamate uptake. Astroglial connexins Cx43 and Cx30 enable gap-junction (GJ) coupling responsible for spatial ionic and metabolic buffering. Connexin hemichannels as well as pannexin-1 channels (Panx1) mediate glutamate and ATP release in the extracellular space possibly activating astroglial metabotropic glutamate receptors (mGluRs) and purinergic P2X and P2Y receptors (P2Rs), respectively. This, in term, induces intracellular Ca^{2+} increases in the neighboring astroglia. The figure summarizes the pro- and anti-epileptic roles of the mechanisms described above and points to the putative targets of valproic acid and the GJ blockers carbenoxolone, anandamide, and oleamide in this scenario.

in vivo injection of CBX in the NRT of rats with atypical absence seizures and spontaneous SWDs decreased the duration of SWDs (139), whereas no alteration of SWD phenotype was observed if CBX was injected in the posterior thalami of WAG/Rij rats and the *lethargic* mouse genetic model of absence epilepsy (140). Recently, intraperitoneal injection of CBX was associated with absence seizures worsening in WAG/Rij rats (141), hinting at non-obvious and non-trivial differences across the absence epilepsy models. The endocannabinoids anandamide (*N*-arachidonoylethanolamine, ANA) and oleamide (cis-9,10-octadecenoamide, OLE) are specific Cx43 blockers (142, 143). Intracerebroventricular injection of ANA decreased in a dose-dependent manner the recurrence and duration of SWDs, although its mechanism of action likely involves type-1 cannabinoid (CB1) receptor activation (144) or even direct inhibition of T-type Ca^{2+} channels (145). Interestingly, although specific studies addressing the impact of OLE in absence epilepsy are still required, OLE has a sleep-inducing effect and enhances $GABA_A$ receptor-mediated responses, thus possibly affecting the physiological, temporal-spatial pattern of cortico-thalamo-cortical oscillations (146, 147). CBX as well as ANA and OLE block both GJ activity and connexin hemichannels regulating water and solute (notably ATP) exchanges between the intra- and extracellular space, thus challenging the attribution of any observed phenotype to the sole GJ coupling (131, 148). Moreover, regional differences in connexin isoform expression may be at the basis of different contributions of GJ and hemichannel inhibition in different neural networks, and thus the net phenotypical outcome of the pharmacological manipulation (149). CBX is also known to block pannexin-1, which bears significant topological and pharmacological similarities with the connexins and forms single-membrane channels which have been linked to network hyperexcitability and hypersynchronization by mediating both ATP and glutamate release (150, 151). The use of antibodies or small peptides targeting specific amino acid sequences of different connexins (152–155) could shed new light into the differential contribution of GJ coupling and hemichannel function as well as into the role of different connexin isoforms and pannexin-1 channels in the generation and propagation of SWDs. Finally, ANA, but not OLE, can block Ca^{2+} wave propagation in astrocytes, which has to be taken into consideration in the interpretation of the results (142, 143).

In summary, astroglial networks contribute to the imbalance of neural excitation/inhibition through K^+ and neurotransmitter (glutamate but also GABA) clearance under physiological conditions, thus counteracting network priming through aberrant shifts in the E/I balance possibly leading to network synchronization. Astrocytes rely on their extensive GJ coupling enabling effective spatial ionic, osmotic, and functional buffering. GJ hemichannels as well as pannexin-1 channels may be responsible for augmented synchronous activity through ATP and glutamate release and following Ca^2 spreading throughout the astroglial network. So far, we are still missing evidence for linking the astroglial fine-tuning of the extracellular ion and transmitter homeostasis to SWDs. However, as it is the case for other kinds of epileptiform activity, their role in regulating such network excitability is very likely.

Astrocytes Are Actively Involved in Network Dynamics and E/I Balance Through Neurometabolic Coupling, Neurotransmission Modulation and Gliotransmission

Astrocytes do not only contribute to neural excitability and functioning by responding to neurotransmitter release and modification of extracellular ionic composition, they are also actively involved in neurotransmitter uptake and release, thus having a direct control of E/I balance (**Figure 3**) (86, 156–158). One of the key features of absence epilepsy are altered GABA levels (72, 159) and GABAergic tonic and phasic inhibition in the cortico-thalamo-cortical network (25, 73). In both GAERS rats and *stargazer* mice, astroglial GABA transporter GAT-1 malfunction leads to increased GABA levels in the thalamus resulting in altered tonic inhibition of $GABA_A$ receptors on the thalamo-cortical neurons (72, 74, 75, 77). Notably, a number of human mutations in *SLC6A1* encoding GAT-1 leads to reduced GABA transport activity, and some of the mutations are associated with CAE or clinical conditions associated with absence seizures (160–164). Moreover, GABA released by astrocytes was proven to activate $GABA_A$ receptors on the membrane of thalamocortical neurons in rodents (165) and blocking astroglial GATs increased extrasynaptic $GABA_A$ receptor-mediated tonic inhibition (166). On the other hand, thalamic astrocytes express $GABA_A$ receptors themselves (167), whose specific role has not been fully resolved yet. Neuronal presynaptic $GABA_B$ receptor expression and function is impaired in the neocortex of WAG/Rij rats, possibly contributing to network hyperexcitability (168, 169). There is plenty of evidence that $GABA_B$ receptors contribute to network priming in absence seizures facilitating thalamo-cortical burst firing, as supported by the exacerbation of SWDs after baclofen or GHB treatment (170–176). Interestingly, the activation of extrasynaptic $GABA_B$ receptors require GABA spillover resulting from an intense GABAergic stimulation, which is in accordance with a predominant role of astrocytic GAT-1 in regulating SWDs, given its expression in close proximity of neuronal

FIGURE 3 | Regional specific imbalance of E/I at the basis of absence seizures. The main components of the cortico-thalamo-cortical network (SSp-ctx, primary somatosensory cortex; NRT, nucleus reticularis thalami; thalamic nuclei) display regional specific shifts toward either excitation or inhibition associated with absence seizures. The figure summarizes the pro- (+) and anti- (-) epileptic effects of specific alterations of the regional E/I balance in the pathological phenotype of absence seizures. A1R, adenosine receptor type 1; EAAT1/2, excitatory amino acid transporters 1/2; CB1, cannabinoid receptor type 1; GABA, γ-aminobutyric acid; GABA$_B$R, metabotropic GABA$_B$ receptor; GAT-1, GABA transporter 1; mGluR1/5, metabotropic glutamate receptors 1/5; TC, thalamo-cortical; TCA, tricarboxylic acid cycle; Y2R, neuropeptide Y receptor type 2.

synapses compared to a more distal location of GAT-3 (175). A further level of complexity is given by the fact that astrocytes themselves express GABA$_B$ receptors and their activation leads to downstream Ca^{2+} signaling and possibly gliotransmission as shown in the thalamus upon local *ex vivo* baclofen and GHB application (177).

As expected, the injection of the GAT inhibitor tiagabine in the thalamus enhances SWDs (178), while its injection in the somatosensory cortex suppresses SWDs, as does the injection of positive allosteric modulators of glutamate metabotropic receptors mGluR1 and mGluR5 in both somatosensory cortex and thalamus (178). A line of experimental evidence suggests that possibly all metabotropic glutamate receptors, including the mGluR2/3 and mGluR5 expressed on the astroglial membrane, are involved in SWDs through modulation of NMDA receptors and GABA uptake (178–182). Indeed, a subpopulation of astrocytes in the thalamus expresses mGluR5 and respond to cortico-thalamic glutamatergic afferents *via* intracellular Ca^{2+} oscillations (183). Therefore, it is very likely that astrocytes contribute to SWD phenotype by processing glutamate signaling. Astroglial glutamate transporters EAAT1 (GLutamate ASpartate Transporter, GLAST) and EAAT2 (GLutamate Transporter-1, GLT-1) (184, 185) as well as astroglial glutamine-glutamate-GABA cycle impairment (186, 187) have already been associated with the development of various forms of epileptic activities. GAERS rats display decreased protein expression of both astroglial GLT-1 and GLAST proteins before the development of absence seizures (188). Notably, GLAST is overexpressed at the mRNA level, possibly due to a compensatory mechanism of gene transcription (189). Moreover, excessive neuronal firing is known to induce astroglial swelling and subsequent glutamate release (190). This may add a further level of complexity in the already complex temporal firing dynamics of the thalamo-cortical neurons and NRT neurons both ictally and at interictal-to-ictal transitions (25). Although not yet proven in the context of SWDs, astrocytes possess the extraordinary capability of converting intensive glutamatergic neuronal activity into tonic inhibition, by coupling the glutamate/Na^+ symport with the glutamine and GABA/Na^+ symport (191). Notably, the only ATP expenditure associated with this process relies on the replenishment of the intracellular GABA storage since the driving force of the glutamine and GABA release is the re-establishment of the physiological Na^+ homeostasis altered by the glutamate/Na^+ symport. Finally, a comprehensive study of metabolic alterations in GAERS rats provides further insight into the cortical and thalamic astroglial contribution to the pathology of SWDs. Most strikingly, cortical astroglial metabolism and glutamine-glutamate-GABA cycle are enhanced in GAERS rats, leading to increased glutamate and glutamine levels and decreased GABA labeling (192). Interestingly, the expression of astroglial glutamate dehydrogenase is increased, in the cortex before the development of absence seizures

and in the thalamus before and after the development of absence seizures, thus possibly leading to a decreased glutamate availability and a shift to the thalamic GABAergic inhibition fundamental for the generalization of SWDs (193). In line with this hypothesis, the intraperitoneal injection of branched-chain amino acids and α-ketoisocaproate pushing the chemical equilibrium toward the synthesis of glutamine led to decreased thalamic glutamate levels and the worsening of absence seizures (194). Moreover, a gain-of-function mutation of the glutamate dehydrogenase gene leading to aberrant glutamate availability and hyperammonemia has been associated with myoclonic absence epilepsy (195). Although further research in the field of absence epilepsy is still required, this evidence supports the role of astrocytic metabolism and glutamine-glutamate-GABA cycle in providing adequate energy supply and network homeostasis required for epileptic activity generation and propagation (94, 196).

In situ hybridization and Western blot analysis showed reduced levels of CB1 receptor mRNA and protein in the NRT and of the CB1 receptor in the thalamus of WAG/Rij rats at the protein level, thereby suggesting an impaired depolarization-induced CB1-mediated suppression of inhibition (197). Indeed, acute systemic injection of the synthetic CB1 receptor agonist WIN55,212-2 resulted in a transient reduction in SWDs frequency, however surprisingly followed by an increase in SWD duration in subchronic treatment (144, 197–199). Since the beneficial effects of the endocannabinoid ANA, previously described, last longer than the transient reduction in SWD frequency induced by the synthetic CB1 agonist and since ANA does indeed shorten SWDs, its mechanism of action is likely not only dependent on CB1 activation but a more complex molecular process (144).

The release of ATP through connexin and pannexin-1 hemichannels and the resulting spread of Ca^{2+} waves largely contribute to the astrocyte-mediated purinergic signaling in epilepsy (200). However, the net impact on the neural network is often context-dependent and may include the conversion of ATP into adenosine. Adenosine levels depend on extracellular ectonucleotidases as well as on the astroglial adenosine kinase (ADK) and its contribution encompasses antiepileptic A1 receptor-mediated as well as proepileptic A2 and A3 receptor-mediated effects (200–203). Once again, most research results have been derived from the analysis of convulsive seizures. Nevertheless, there is a number of evidence suggesting that purinergic signaling is altered in SWDs, too. To which extent this is related to astroglial contribution is still elusive. With respect to SWDs, GAERS rats show lower expression of A1 receptors in the NRT (204) and WAG/Rij rats are characterized by altered expression of A2A receptors in the somatosensory cortex, NRT and thalamus (205). Absence epileptic activity in WAG/Rij rats increases after activation of A2A receptors directly by the specific synthetic agonist 2-[4-(-2-carboxyethyl)-phenylamino]-5′-N-ethylcarboxamido-adenosine (CGS21680) (205) or indirectly after intraperitoneal injection of guanosine (206) as well as of adenosine (207). Conversely, acute caffeine administration, which is a mixed non-specific A1 and A2A receptor antagonist, reduced both amplitude and duration of SWDs in GAERS rats (208). However, the administration of the specific A1 antagonist 1,3.dipropyl-8-cyclopentylxanthine (DPCPX) in WAG/Rij rats had a proepileptic effect on SWDs (209). Notably, a duplication in the chromosomal region containing the gene coding for the extracellular catabolic enzyme adenosine deaminase was associated with a case of early-onset absence epilepsy, possibly leading to an impairment in adenosine homeostasis (210, 211).

The neuropeptide Y (NPY) released by thalamic neurons promotes phase-specific long-term depression of neuronal excitability in the NRT as well as in the thalamus itself and thus possibly contributing to thalamocortical synchronization and the altered dynamics of T-type Ca^{2+} channel-mediated bursting activity in the thalamic nuclei (212). Interestingly, valproic acid treatment increases thalamic levels of NPY mRNA in GAERS rats (213). Moreover, NPY intracerebroventricular injection as well as focal administration of NPY in the somatosensory cortex of GAERS rats had a strong antiepileptic effect mediated by the NPY receptor Y2 (214–216). This was confirmed by the analysis of specific NPY receptor knock-out mice (217, 218) and injection of the specific Y2 receptor agonist Ac[Leu (28, 31)] NPY24-36 and the specific Y2 receptor antagonist BIIE0246 in GAERS rats (215). Notably, viral overexpression of NPY as well as the mRNA of its receptor Y2, both in thalamus and somatosensory cortex of GAERS rats, reduced the number of seizures and the time spent in seizure activity (219). Since astrocytes produce (220) and release (221) NPY and express NPY receptors, including Y2 receptor (222, 223), one can imagine that astrocytes may play a role in NPY signaling in the pathophysiology of cortico-thalamo-cortical networks.

Alterations of astroglial neurometabolic coupling and contribution to the glutamine-glutamate-GABA cycle may be at the basis of SWDs, possibly through enhanced metabolism and glutamate presentation to cortical neurons. Moreover, astroglial control of extracellular neurotransmitter level, based on the expression of glutamate and GABA transporters (EAATs and GATs, respectively) and receptors (both metabotropic and ionotropic) and direct and indirect release of glutamate and GABA, plays a fundamental role in maintaining the E/I balance in the cortex, thalamus and NRT. Astroglial ATP release and subsequent adenosine production seem to have context-dependent effects on neural excitability, but generally in line with observations derived from convulsive seizures pointing at an antiepileptic and proepileptic role of A1 and A2 receptors, respectively. Shifts in the E/I toward inhibition in the thalamus (possibly through altered endocannabinoid signaling) and toward excitation in the NRT and cortex have a pro-epileptic effect on SWDs. Unexpected net outcomes of pharmacological or genetic manipulation may be due to differential impact on different key nodes of the cortico-thalamo-cortical network and/or to astrocytic ability to both preserve and reverse the sign of the input signal.

The Classic Chicken and Egg Situation. Which Comes First: Astroglial Ca^{2+} Or Seizures?

Intracellular Ca^{2+} oscillations are one of the most studied indicators of astroglial activity and information coding

mechanism at the core of the astroglial signaling cascade resulting, among others, in gliotransmission (86). In the context of convulsive epilepsy, excitotoxic spilling of glutamate, GABA and ATP resulting from excessive network activity as well as dying cells induce perturbation in astroglial Ca^{2+} signals (224, 225). Conversely, spontaneous as well as induced Ca^{2+} oscillations lead to gliotransmission thus influencing neuronal synchrony and E/I balance (226–234). Notably, astroglial Ca^{2+} elevations precede temporally neuronal engagement and their attenuation results in reduction of the epileptic activity in an *in vivo* model of temporal lobe epilepsy (TLE) (235). Moreover, astroglial Ca^{2+} activity is associated with spreading depolarization-mediated seizure termination (236). However, current research is far from understanding astroglial Ca^{2+} contribution to seizure generation, propagation, severity, and termination both in mechanistic and logical (sufficiency and/or necessity) terms. In particular, research on the contribution of astroglial Ca^{2+} signaling in seizure phenotype has not yet provided causative links to the SWD pathophysiology. Nevertheless, in the following paragraph we include some observations that encourage further research on the topic.

Thalamic astroglial networks display multi-cellular Ca^{2+} oscillations in absence of neuronal input and induce glutamate release and NMDA-receptor mediated long lasting inward currents in thalamocortical neurons as studied in acute brain slice preparations (226, 237). Thalamic astrocytes segregate into two groups: a first group with mGluR5-dependent and no voltage-dependent Ca^{2+} oscillations in response to cortico-thalamic activation, and a second group with no mGluR5- but voltage-dependent Ca^{2+} responses (183). Moreover, thalamic astroglial Ca^{2+} responses were recorded after acute *ex vivo* application of the weak $GABA_B$ receptor agonist GHB (177), thus suggesting a putative role of astrocytes in the regulation of GABAergic signaling in the thalamus and possible in the phenomenology of SWDs. Notably, sustained $GABA_B$ receptor activation led to a decrease in glutamate release from astrocytes (177). In addition, Ca^{2+} signaling and GABA seem to be connected since artificial inhibition of Ca^{2+} oscillation in striatal astrocytes leads to GAT3 functional upregulation and increased GABA uptake (238). Further evidence suggesting an integrative role of thalamic astrocytes in cortico-thalamic interactions comes from the observation that astroglial glutamate- and NMDA receptor-mediated slow inward currents (SICs) in the thalamo-cortical neurons are largely resistant to afferent cortico-thalamic inputs in their emergence but not in their frequency upon sustained input (239, 240). Moreover, cortico-thalamic glutamatergic input induced disinhibition of thalamo-cortical neurons through astroglial mGluR2 activation, Ca^{2+}-dependent glutamate release and inhibition of presynaptic GABAergic projections from the NRT (241). In the NRT astrocytes also enhance $GABA_A$ receptor signaling (242). Astrocyte-induced glutamate-mediated SICs of thalamo-cortical neurons seem to be dependent on extracellular glutamate levels, since exogenous exposure to the glutamate-mimetic D-aspartate increased the frequency of SICs (243). Although it is still unclear if abnormal or hypersynchronous astroglial Ca^{2+} signals could promote epileptiform network activity by itself, this evidence further supports an astroglial contribution to the propagation and self-sustain of seizure-like activity (244, 245).

The role of astrocytic Ca^{2+} signaling in epilepsy, and particularly in SWD-displaying epilepsies, is far from being understood. Yet, association studies on CAE and other idiopathic epileptic forms displaying SWDs as well as the evaluation of the genetic etiology of rodent absence epilepsy models point to a plethora of genes involved in voltage-gated Ca^{2+} channel signaling and G protein-coupled receptor signaling that is worth further assessment (7).

Astroglia display spontaneous Ca^{2+} oscillations responsible for gliotransmission and homeostatic control of the E/I balance as well as network synchronicity. Moreover, astrocytes respond to physiological network activity and pathological neurotransmitter spilling and release from dying cells by Ca^{2+} elevations, typically further contributing to network priming, seizure initiation and progression. Conversely, Ca^{2+} signaling-induced gliotransmitter release and modulation of astroglial neurotransmitter receptors and transporters may underlie putative (or potential) anti-epileptic roles of Ca^{2+} signaling. Notably, astroglial Ca^{2+} signaling may also contribute to seizure suppression. To which extent this applies to SWDs is still unclear.

Reactive Astrogliosis and the Astrocyte-Derived Inflammatory Response May Contribute to the Pathology of SWDs

Astroglial proliferation and morphological, biochemical, and functional changes associated with epilepsy as well as with other neurodegenerative diseases are commonly referred to as reactive astrogliosis (246–248). The term is misleading since it implies that the pathological phenotype of astrocytes results from the epileptiform activity and oversees the possible causative role of astrocyte modifications in its genesis (249–251). In GAERS rats, cortical as well as thalamic astrocytes display enhanced expression of the glial fibrillary acidic protein (GFAP) even before the onset of absence seizures (193). Similarly, increased levels of GFAP expression can be found in adult WAG/Rij rats, though to a lesser extent than in GAERS rats (252). Astonishingly, the number of glial cells in the somatosensory cortex is significantly decreased (253). This suggests that biochemical and functional changes may contribute to a greater extent to the pathology of absence seizures than morphological alteration or that the latter involves qualitative astroglial reorganization, e.g., overlap of the astroglial processes, astroglial domain reorganization, structural and quantitative alteration of synaptic contacts or blood-brain barrier dysfunction. Notably, valproic acid diminishes the overlap of astroglial processes observed in correspondence of epileptic foci in several pathological models of convulsive seizures (254). Nevertheless, it is not clear if the same is happening in the pharmacodynamics of valproic acid in the context of SWDs. The same is true for the alterations of the blood-brain barrier (BBB) which have been associated with many pathological scenarios, including epilepsy (255), but whose role in SWDs has not been extensively addressed yet.

Pathological stimulation of astrocytes during convulsive epileptiform activity leads to astrocytic upregulation and release of proinflammatory cytokines, with IL-1β, Il-6, and TNFα as the most prominent ones. These factors, in turn, can induce astroglial dysfunction leading to, among others, increased glutamate release, decreased glutamate uptake, down-regulation of $K_{ir}4.1$, AQP4, connexins, and glutamine synthetase as well as upregulation of adenosine kinase (256–258). IL-1β is induced in reactive astroglia in the somatosensory cortex (and not in other regions of the cortex) in adult GAERS rats with mature SWDs and interestingly also in some young GAERS in association with immature forms of SWDs (259). Furthermore, inhibition of IL-1β biosynthesis in adult GAERS reduced both the number as well as the duration of SWDs. Conversely, IL-1β intraperitoneal administration in WAG/Rij rats induced a significant increase in SWDs and worsened the proepileptic effects of the GABA reuptake inhibitor tiagabine (260). TNFα administration also aggravates SWDs but with kinetics incompatible with a direct effect and therefore possibly through *de novo* production of IL-1β itself. Moreover, before the onset of SWDs, young WAG/Rij rats showed increased TNFα blood levels, which gradually decreased with age and returned to physiological levels in adult rats displaying mature SWDs, thus possibly suggesting a neuroprotective role of TNFα (260). The precise mechanism of TNFα action in this scenario is not clear, although it is known that TNFα reduces astroglial glutamate uptake and decreases neuronal $GABA_A$ receptor expression (261, 262). Notably, IL-1β-, TNFα-, and IL-6-inducing lipopolysaccharide (LPS) injection in WAG/Rij also promoted SWDs and the increase in the latter was prevented by blocking the inflammatory response with indomethacin (263, 264) as well as blockers of the mTOR pathway (265, 266). Similarly, LPS effects on SWDs were later confirmed in GAERS rats (267). Although IL-1β is believed to increase the levels of glutamate, co-administration of LPS and the NMDA receptor antagonist D-(-)-2-Amino-5-phosphonopentanoic acid (AP-5) did not counteract LPS effects as expected, but conversely prolonged them (264). Recently, it has been reported that IL-6 receptor (IL-6R) blockage *via* tocilizumab (a humanized monoclonal antibody against IL-6R) reduces SWDs in WAG/Rij rats and inhibits their LPS-induced worsening (268). In line with that, human CAE is known to be associated with detectable levels of IL-6 and IL-8 in the cerebrospinal fluid (269) and treatment with valproic acid reduces IL-6 serum levels in children with tonic-clonic generalized seizures (270).

Several lines of evidence support a role for a direct contribution of pro-inflammatory cytokines in the genesis and worsening of SWDs. Notably, astroglial alterations and cytokine release precede SWD onset, although it cannot be excluded that these cell responses may be due to subclinical epileptiform activities or genetic predispositions. IL-1β, IL-6 and TNFα may contribute to the pathology of SWDs, possibly through impaired K^+ clearance, glutamine-glutamate-GABA cycle, adenosine metabolism, gliotransmission, and neurotransmitter reuptake. Other morphological alterations, such as astroglial overlap, connectivity, and synaptic coverage, may play a role as well.

ABSENCE SEIZURES AND NREM SLEEP: TWO SIDES OF THE SAME COIN?

The cortico-thalamo-cortical network processes behaviourally relevant internal and external information and determines vigilance states as well as neuronal network oscillation during sleep (**Figure 4A**), thus playing a fundamental role in both physiology and pathology (25, 276–281). Several lines of evidence suggest that epilepsy and sleep are strongly related (282). Notably, various forms of epilepsy display different incidences across the 24 h sleep/wake cycle and among different sleep stages, possibly due to specific seizure susceptibility dependent on brain excitability and network engagement (283–285).

Till recently, SWDs were considered the pathological transformation of sleep spindles (also known also thalamocortical spindles) occurring during stage II NREM sleep (**Figure 4B**) (272, 286, 287). This concept was mainly supported by studies on the temporal coincidence of sleep spindles and SWDs (288, 289) and on the progressive transformation of sleep spindles into SWDs observed after intramuscular injection of penicillin in cats (24, 290). Indeed, to some extent both sleep spindles and SWDs share some anatomical, cellular and molecular mechanisms (291) and they are functionally correlated (292, 293). However, the identification of SWDs as pathological transitions from sleep spindles has been recently challenged (294–296), in favor of a predominant role of cortical slow (<1 Hz) oscillations alternating active (Up) and silent (Down) cortical activity and concomitantly occurring during NREM sleep (**Figures 4C,D**) (273, 274, 297–301). SWDs largely arise in a specific critical vigilance window in correspondence with passive wakefulness, transitions to NREM slow-wave sleep as well as during transitions between internal substages of NREM sleep (stage I to III; N1: light sleep or passive wakefulness, N2: light slow-wave sleep and N3: deep slow-wave sleep, respectively) (**Figure 4E**). Moreover, SWDs are disrupted by arousing stimuli and do not transition to REM sleep directly (38, 275, 302–308), thus suggesting that absence seizures prefer low and shifting-vigilance periods during superficial slow-wave NREM sleep (282). With respect to the incidence of seizures across the 24 h cycle, the distribution of generalized SWDs is still under debate. Seizures originating in the frontal lobe (as absence seizures are currently believed to be) are more frequent at night and in sleep (309, 310). Conversely though, dialeptic and atonic seizures occur more often during daytime (310). Generalized pediatric seizures, including absence seizures, were reported to occur predominantly during wakefulness (311, 312) but were restricted to NREM sleep stages I and II when occurring during the night and were almost absent during REM sleep (313, 314). Moreover, a study on idiopathic generalized epilepsies including CAE and other SWD-displaying epilepsies showed that interictal epileptic discharges are more frequent during NREM sleep and occur mainly at sleep onset (315). In WAG/Rij rats, SWDs are most

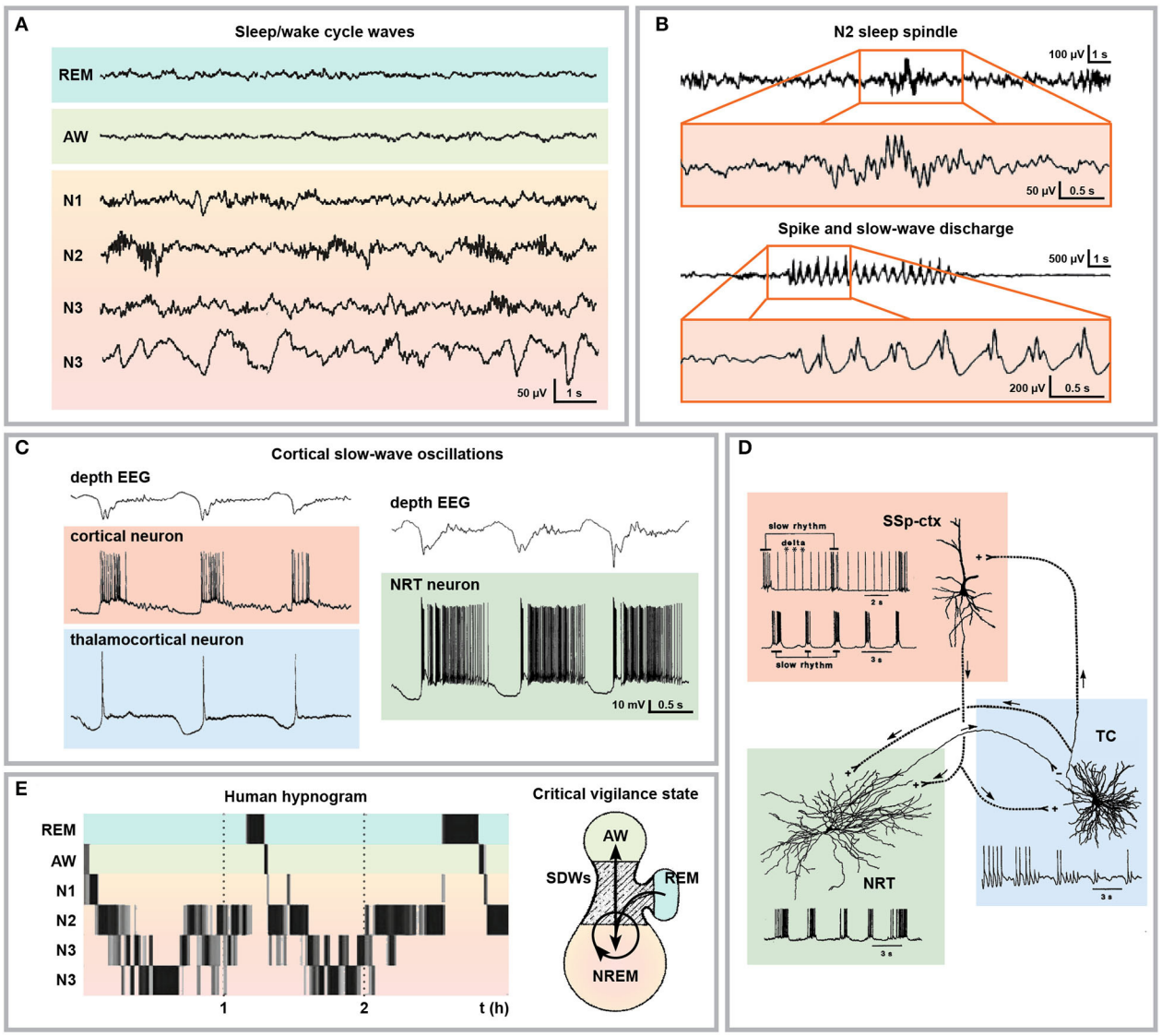


FIGURE 4 | Electrophysiological and cellular bases of sleep and SWD relationship. **(A)** Representative human electroencephalographical wave recordings during wakefulness (AW), REM sleep and different NREM sleep stages (N1, passive wakefulness or light sleep; N2, light slow-wave sleep; N3, deep slow-wave sleep). **(B)** Sleep spindle typically occurring during stage N2 of NREM sleep with respective magnification and comparison with SWDs. **(C)** Depth cortical EEG recording displaying cortical slow wave oscillations (upper traces) and time-matched intracellular recordings from cortical, thalamocortical, and NRT neurons with typical burst firing activity. **(D)** Schematic representation of the cellular and electrical components of cortico-thalamo-cortical oscillations. **(E)** Human hypnogram displaying two typical sleep cycles characterized by the succession of the NREM sleep stages followed by one episode of REM sleep (left) and schematic representation of the critical vigilance level (hatched area) promoting SWD occurrence during transitions between NREM and wakefulness, between NREM stages and from (but not to) REM sleep (right). Modified from **(A)**, (271); **(B)**, (272), **(C)**, (273); **(D)**, (274); **(E)**, (271, 275).

frequent in the beginning of the dark phase and are at their minimum frequency at the onset of the light phase (316, 317). If rats are artificially kept in dim light (thus disrupting the 12:12 light-dark cycle), SWDs still display 24 h cyclicity, proving its endogenous rhythmicity, but the cycle is desynchronized with respect to the rhythm of the general motor activity, thus suggesting that the mechanism governing SWDs and sleep/wake cycles are different (317, 318). Interestingly, after an artificial shifting in the light-dark cycle, SWDs resynchronized at the same speed of light slow-wave speed in comparison with both REM and deep slow-wave sleep (319), pointing at the existence of a common circadian mechanism governing SWDs and light slow-wave sleep. Taken together, it seems that conditions associated with highly desynchronized (active wakefulness and REM sleep) and highly synchronized (deep slow-wave sleep) cortical activity tend to inhibit SWDs. In line with this hypothesis, the anti-absence molecule uridine (320) impacts sleep architecture by fragmenting sleep, thus increasing the frequency of NREM-REM transitions and by inducing preferentially REM sleep (321).

With respect to the putative interdependency of SWDs and NREM sleep waves, it was reported that sleep deprivation has a proepileptic effect on both humans (322–325) and rodents (304, 305, 326, 327). On the other side, epilepsy is associated with sleep alterations, including sleep fragmentation, day-time drowsiness and difficulties in sleep initiation (328). To date, the field still lacks a systematic clinical study on the effect of absence epilepsy on sleep. Nevertheless, it was shown in WAG/Rij rats that SWDs disrupt NREM sleep and sleep architecture (329). Moreover, epilepsy-induced sleep alterations depend on the timing of the epileptiform activity. In a time-controlled kindling epileptic model in rats, seizure induction at the transition from light to dark (*zeitgeber* time (ZT) 0) and from dark to light (ZT13) altered both NREM and REM duration without affecting sleep/wake cycles and the sole seizure induction at ZT13 induced increased levels of IL-1 and increased NREM sleep specifically (330). Interestingly, both IL-1β and TNFα increase the amount of NREM sleep (331), which could contribute to the increase of SWD number after LPS injection (263, 264) by an increased state of passive awareness and slow-wave sleep. Indeed, *in silico* meta-analysis of differentially expressed proteins from the fronto-parietal cortex and thalamus of LPS-treated WAG/Rij rats supports this scenario, given the overrepresentation of proteins associated with sleep regulation (332). Moreover, the pathological activation of the mTOR pathway involved in LPS-induced increase in SWDs (265, 266) is responsible for the upregulation of the core clock gene product aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL), also known as brain and muscle ARNT-Like 1 (BMAL1), as observed in a model of tuberous sclerosis complex, a neurological disorder displaying epileptic activity (333). BMAL1 not only is a key component of both circadian and sleep/wake cycles (334) as well as susceptibility to seizures and epilepsy (335), but it is also at the basis of cell-autonomous circadian clock of astrocytes (336, 337).

SWD occurrence varies during the sleep/wake cycle with respect to both sleep and vigilance states. Notably, SWDs peak in correspondence of low and shifting-vigilance periods during superficial slow-wave NREM sleep and are underrepresented during active wakefulness and REM sleep. Resynchronization studies after shifting in the light-dark cycle suggest a common circadian mechanism governing SWDs and NREM sleep. Moreover, even though the nature and the causative link between the two are far from being clearly understood, SWDs and NREM sleep are similarly and consistently altered by a number of pathological and pharmacological alterations.

Both Sleep Architecture and Sleep/Wake Cycle Are Shaped by Astroglial Activity

Given the fact that both SWDs and NREM recruit the cortico-thalamo-cortical network, further insights into the role of astrocytes in the pathophysiology of SWDs may derive from evidence in their contribution to sleep (particularly sleep architecture and sleep/wake cycle) (**Figure 5**) as well as the circadian cycle. Astroglial impact on circadian clock mechanisms generated in deep structures may contribute to epilepsy in non-intuitive ways (335). In the hypothalamic primary timekeeping center, the suprachiasmatic nucleus (SCN), astrocyte-derived glutamate inhibits neuronal firing through presynaptic NMDA receptors specifically during the night (338). Moreover, astrocytes release adenosine in a CB1 receptor- and intracellular Ca^{2+} signaling-dependent manner and induce the disinhibition of SCN neurons (339). WAG/Rij rats are also characterized by astrogliosis and impaired GABAergic transmission in the thalamic intergeniculate leaflet, which coordinates inputs from the retina and outputs to the SCN (340). The research on the role of astrocytes on timekeeping is still at its early days, but several lines of evidence support astroglial contribution to sleep, namely the modulation of sleep homeostasis, sleep pressure, vigilance states and sleep-dependent cognitive function, brain energetics and network metabolic supply, network excitability and sleep-associated waste clearance (341, 342).

The sleep/wake cycle is associated with changes in interstitial fluid and of the ionic composition with increased extracellular space and decreased interstitial K^+ concentration during sleep (343–345), a process that involves norepinephrine-mediated inhibition of the astroglial Na^+/K^+ pump during wakefulness (346). This process is responsible for widespread neuronal hyperpolarization and decreased firing rate particularly during NREM sleep (347, 348). In parallel, it was recently reported that children with an autism-associated epilepsy phenotype carrying a gain-of-function mutation in the $K_{ir}4.1$ coding KCHJ10 gene display abnormal slow-wave sleep with a significantly longer slow-wave period (349). Norepinephrine induces astroglial process elongation and astroglial synaptic coverage during wakefulness (345, 350). Conversely, decreased levels of norepinephrine may be responsible for reduction of direct and indirect astroglial release of ATP/adenosine and D-serine, thus contributing to the overall decreased synaptic failure and increased release probability during sleep. Interestingly, norepinephrine level is particularly low during NREM sleep, due to suppression of noradrenergic neuronal firing (351).

Astroglial-dependent cerebrospinal fluid (CSF) flow is responsible for waste and interstitial fluid clearance during sleep (352) and inward flow of CSF through astroglial AQP4 occurs mainly during NREM sleep (343, 353). The CSF flow is under circadian control mediated by changes of AQP4 polarization (354). Recently, a haplotype of AQP4 carrying several single nucleotide polymorphisms (SNPs), among which some associated with reduced AQP4 expression, has been linked to altered slow-wave NREM sleep modulation (355). Moreover, astroglial gap junction coupling is likely to contribute to the regulation of the sleep/wake by means of modulation of both CSF flow and waste clearance. To date, most studies addressing GJ coupling in astrocytes are focused on the altered metabolite trafficking (namely glucose and lactate) resulting from GJ manipulation that impairs the fundamental role of astrocytes in synaptic energy support and brain energy metabolism (356). Astrocyte-specific conditional knock-out of Cx43 in mice resulted in enhanced sleepiness, fragmented wakefulness, and impaired neuromodulation of the sleep/wake cycle (357). Conversely,

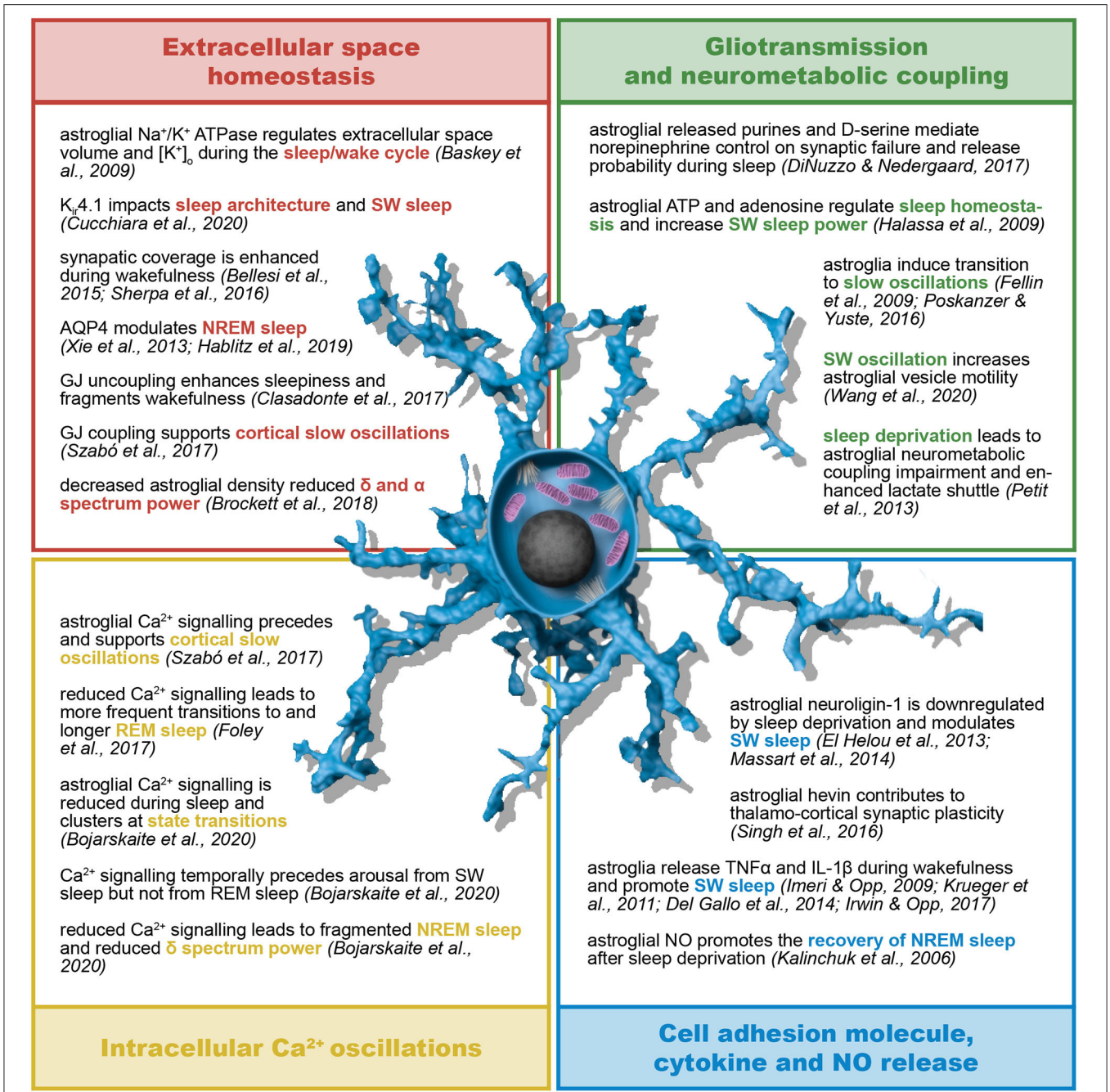


FIGURE 5 | Astroglial role in sleep/wake cycle and sleep architecture. Astrocytes contribute to sleep homeostasis in terms of both sleep/wake cycle regulation and sleep architecture and dynamics. The astroglial regulation of sleep relies on their control of extracellular K^+ concentration, interstitial fluid exchanges and spatial buffering through gap junctions. Moreover, astroglia support and influence neural activity by means of their neurometabolic coupling to neurons, intracellular Ca^{2+} oscillations, gliotransmission, and release of, among other, cell adhesion molecules, cytokines and nitric oxide. AQP4, aquaporin-4; ATP, adenosine triphosphate; GJ, gap junction; IL-1β, interleukin-1β; K_{ir}, inward rectifying K^+ channels; NO, nitric oxide; (N)REM, (non-) rapid eye movement; SW, slow wave; TNFα, tumor necrosis factor α.

astroglial neurometabolic coupling impairment results from sleep deprivation that leads to astroglial upregulation of the transporters GLUT1, GLT1, the Na^+/K^+ pump as well as other components of the astrocyte-neuron lactate shuttle (358). Sleep deprivation could therefore possibly contribute to increased network activity also through enhanced lactate delivery to neurons as suggested by the fact that the anticonvulsant stiripentol is a lactate dehydrogenase inhibitor (359) in addition to being a positive allosteric modulator of $GABA_A$ receptors (360).

Impairment of astroglial exocytosis and gliotransmission using cell-specific expression of dnSNARE results in reduced tonic A1R adenosinergic signaling, altered sleep homeostasis and reduced slow-wave power, and reduced sleep pressure in mice (361), as confirmed by previous evidence suggesting the role of A1 receptors in augmented sleep pressure (362–364). The same genetic manipulation suppressed the LPS-induced increase in slow-wave power during NREM sleep, proving the astroglial contribution to inflammatory-derived increased sleep pressure (365). Although the role of A2 receptor (A2R) activation by astroglial adenosine is still controversial, A2Rs may play a role in sleep homeostasis through activation of A2AR-expressing neurons in the nucleus accumbens core involved in the induction of slow-wave sleep (366). In the cortex, altered gliotransmission resulted in reduced neuronal NMDA receptor activity and reduced slow oscillations (367), whereas astroglial specific activation induced neuronal transition to slow oscillations (368). *In vivo* characterization of Ca^{2+} signaling in both rat cortical astrocytes and neurons revealed that astroglial synchronized activity reliably precedes neuronal oscillations and that both astrocyte uncoupling and intracellular Ca^{2+} chelation reduced the fraction of astrocytes and neurons involved in the cortical slow waves. Remarkably, neurons closer to active astrocytes were more likely involved in the oscillations (369). Recently, simultaneously recording of BOLD fMRI and astroglial Ca^{2+} signaling in anesthetized rats revealed that a fraction of intrinsic cortical Ca^{2+} signals were associated with reduced EEG power and negative fMRI signal throughout the cortex (correlated with decreased neuronal activity) and that increased activity in the thalamus specifically preceded these signals (370). Conversely, reduction in the density of cortical astrocytes in the medial prefrontal cortex (and therefore putatively in their connectivity) has been linked to a decrease in δ (0.5–4 Hz) and α (8–12 Hz) spectrum power (371). Moreover, mice overexpressing an astrocyte-specific inositol triphosphate (IP_3) phosphatase, and therefore displaying reduced IP_3-dependent Ca^{2+} activity, spent more time in REM sleep and revealed more transitions to REM sleep from passive wakefulness (372). In line with that, mice lacking the inositol 1,4,5-triphosphate receptor type 2 exhibit reduced Ca^{2+} signaling, a more fragmented and shorter NREM sleep associated with decreased δ spectrum power and more frequent microarousals (373). Remarkably, astroglial intracellular Ca^{2+} increases precede the transition from NREM sleep to wakefulness but follow arousal from REM sleep (373), thus suggesting that astroglia could mediate norepinephrine-induced arousal from NREM sleep (374). Furthermore, it has been recently shown that *in vitro* application of an oscillatory electric field specifically in the slow-wave range (and not at higher frequencies) increased astroglial synaptic vesicle mobility (375), thus suggesting a positive feedback mechanism for slow-wave state perpetuation. Taken together, these data suggest that astroglial connectivity, astroglial Ca^{2+} waves and gliotransmitter release may initiate and/or support the initiation of cortical slow wave oscillations or favor this transition over others (e.g., passive wakefulness-REM).

Another piece of evidence supporting the contribution of astroglial synaptic plasticity in the modulation of sleep derives from studies focused on the extracellular matrix components in the synaptic cleft. In the mouse forebrain, sleep pressure after sleep deprivation decreased the expression of astroglial neuroligin-1 (376), a cell adhesion molecule binding to presynaptic neurexins (377, 378). Conversely, neuroligin-1 knock-out mice have increased slow-wave sleep and enhanced synchrony during sleep (379). Astrocytes contribute to glutamatergic synaptic plasticity of thalamo-cortical synapses through secretion of hevin, a synaptogenic protein inducing the interaction between non-canonical synaptic partners including neuroligin-1 (380). Knock-out mice lacking another member of the neuroligin family, neuroligin-2, develop SWDs and behavioral arrests, a phenotype blocked by ethosuximide and attenuated by expression of neuroligin-2 selectively in the thalamic neurons or optogenetic activation of GABAergic projections from the NRT (381). This could be due to an interaction with $GABA_A$ receptors as suggested by previous studies on sleep-deprived mice (382). Mice with a missense mutation in neuroligin-3 exhibit an altered EEG power spectrum (383). Moreover, variations in the copy number of the gene encoding the postsynaptic scaffolding protein Shank3, which interacts with both neuroligin and glutamate receptors thus regulating synaptic plasticity (384, 385), have been linked to epileptiform activity specifically arising during slow-wave sleep (386). Shank3 loss-of-function mutations have been associated with several different epileptic forms but most commonly with atypical absence seizures (387).

Finally, as mentioned already above, inflammation and sleep are tightly intertwined. TNFα and IL-1β increase during wakefulness and decrease during sleep both at mRNA and protein level (388–390) and their systemic injection selectively increase slow-wave sleep (389, 391). Mice lacking IL-1β and TNFα receptors are characterized by less slow-wave and REM sleep (392, 393). Notably, agonists of both P2X- and P2Y-type purinergic receptors are known to activate the astroglial release of TNFα and IL-1β (394) and pannexin-1 knock-out mice with impaired ATP release display altered slow-wave sleep (395). Interestingly, unilateral cortical TNFα injection induces state-specific EEG asymmetries during NREM sleep (396) and ipsilateral increase in the number of IL-1β positive cells (mainly astrocytes) in the cortex, the NRT and in the thalamic nuclei (397). Along with TNFα and IL-1β, reactive astroglia also produce nitric oxide (NO) *via* inducible NO synthetase (iNOS) (398, 399) and NO has been linked to the pathology of epilepsy (400, 401). NO affects both NREM and REM sleep (402, 403) but inhibiting iNOS in sleep-deprived mice specifically impairs NREM recovery, whereas inhibiting neuronal NOS (nNOS) impairs the recovery of REM sleep (404), thus suggesting a specific role of astrocyte in NREM physiology.

Astroglial homeostatic control of the extracellular space and synaptic transmission through K^+ clearance mediates widespread neuronal hyperpolarization and decreased firing activity during sleep and impacts NREM sleep architecture. Astroglial regulation of the extracellular volume controls both waste and cerebrospinal

fluid clearance essential for correct functioning of the brain and relies on AQP4-mediated water influx and GJ coupling. Adenosine released by astrocytes governs sleep homeostasis, sleep pressure and slow-wave power and astroglial gliotransmission contributes to neuronal slow oscillations in the cortex. Moreover, artificial lowering of astroglial Ca^{2+} oscillations and connectivity leads to reduced slow-wave power and relative shortening and fragmentation of NREM sleep. Finally, astroglial TNFα, IL-1β, and NO are likely to impact sleep architecture by selective increase of slow-wave sleep. These findings support the fact that astroglia play a fundamental role in the physiology of NREM sleep and therefore represent a promising target to study pathophysiological alterations inducing or sustaining the abnormal recruitment of the cortico-thalamo-cortical network during SWDs.

CONCLUSION

From a clinical, social and human point of view, epilepsy is probably one of the most heterogeneous neurological diseases. This variability partially relies on the fact that epilepsy may originate from a plethora of different conditions, among others traumatic injury, stroke, CNS infections or inflammation, brain tumor, genetic predisposition, and drug or alcohol abuse. In addition, in six out of 10 epilepsy cases the pathological origin is unknown. Yet, from a scientific point of view, epilepsy can ultimately be reduced to a local imbalance of excitation and inhibition and altered synchrony and functioning of neural networks in the brain. The heterogeneity of pathological outcomes associated with epilepsy arises from the variety and complexity of functions carried out by the human brain and the multiple layers of fine-tuning that each of them requires for reliable physiological functioning of the electrical activity in neural circuits. Given the existence of common molecular and cellular mechanisms at the basis of epilepsy and their nature as pathological transitions of altered physiological processes, both epilepsy research and clinical treatment benefit from the understanding of the inner functioning of neural networks.

Since spike and slow-wave discharges (SWDs) share some key anatomical and functional physiological brain oscillations naturally occurring during slow-wave sleep, absence seizure research could advance our understanding of both epilepsy and healthy brain mechanisms. In this review, we collected evidence supporting the functional and mechanistic relationship between slow-wave sleep and SWDs, thus providing insights into network alterations that contribute to the pathology of SWDs. Moreover, proving and characterizing the interdependency between epilepsy, sleep architecture and sleep/wake cycles possess an undeniable therapeutic value, since sleep is a pre-existing condition affecting any treatment outcome and efficacy.

We focused our attention on the role of astrocytes in the physiology of sleep and in their putative pathophysiological contribution to SWDs. Astroglial control on extracellular homeostasis in terms of ionic composition, volume regulation and transmitter clearance, astroglial connectivity, Ca^{2+} signaling and gliotransmission as well as cytokine release are hallmarks of astroglial function for physiological brain performance and were addressed in the context of SWDs and sleep research. Please note that it is insufficient and underestimating of the system complexity to label the astrocytic contribution to neural homeostasis as exclusively anti- or pro-epileptic. Many astroglial mechanisms may be beneficial or detrimental with respect to different forms of epilepsy, not to mention different network connectivities and states. Current research on astroglial contribution to epileptic brain functioning mostly relies on studies focused on convulsive seizures, possibly due to their lower remission rate and their clinical symptoms which appear more obvious and life threatening. Nevertheless, some clues suggest the mechanisms governing network excitability and synchrony may have a role in SWDs, too. This work was not intended to be and is far from being comprehensive neither of the role of astroglia in epilepsy nor of their contribution to sleep homeostasis and architecture but provides with significant associations in the tripartite synapse engaging astroglia, epilepsy and sleep in the context of the pathophysiology of cortico-thalamo-cortical oscillations. Understanding how astroglia contribute to the mechanisms underlying slow-wave sleep and how these are altered in pathology could possibly shine light on new therapeutical targets for a plethora of epileptic forms displaying SWDs, among which absence epilepsy, a condition that still affects 50 million people worldwide and is pharmacoresistant in almost one third of those.

AUTHOR CONTRIBUTIONS

DG screened the literature, conceptualized the review focus, wrote the first draft, designed and realized the figures, and finalized the manuscript. AS and FK contributed to figure conceptualization, reviewed and finalized the manuscript. All authors approved on the final version of the manuscript.

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