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Astrocytes and Glutamine Synthetase in Epileptogenesis

Tore Eid[1,5], **Tih-Shih W. Lee**[2], **Peter Patrylo**[4], **Hitten P. Zaveri**[3]

[1]Department of Laboratory Medicine, Yale School of Medicine

[2]Department of Psychiatry, Yale School of Medicine

[3]Department of Neurology, Yale School of Medicine

[4]Department of Physiology, Southern Illinois University School of Medicine

[5]Department of Molecular Medicine, University of Oslo

Abstract

The cellular, molecular and metabolic mechanisms that underlie the development of mesial temporal lobe epilepsy are incompletely understood. Here we review the role of astrocytes in epilepsy development (a.k.a. epileptogenesis), particularly astrocyte pathologies related to: aquaporin 4, the inwardly rectifying potassium channel Kir4.1, monocarboxylate transporters MCT1 and MCT2, excitatory amino acid transporters EAAT1 and EAAT2, and glutamine synthetase. We propose that inhibition, dysfunction or loss of astrocytic glutamine synthetase is an important causative factor for some epilepsies, particularly mesial temporal lobe epilepsy and glioblastoma-associated epilepsy. We postulate that the regulatory mechanisms of glutamine synthetase as well as the downstream effects of glutamine synthetase dysfunction, represent attractive, new targets for antiepileptogenic interventions. Currently, no antiepileptogenic therapies are available for human use. The discovery of such interventions is important as it will fundamentally change the way we approach epilepsy by preventing the disease from ever becoming manifest after an epileptogenic insult to the brain.

Keywords

Ammonia; brain networks; epilepsy; excitotoxicity; glutamate; inflammation

1. Introduction

Approximately 1% of the general population suffers from epilepsy, which is a chronic disorder characterized by spontaneous recurrent seizures and an increased risk of comorbid conditions, particularly cognitive impairment, anxiety, depression and sudden unexpected death (Hauser et al. 1993; Tellez-Zenteno et al. 2007). Up to 40% of all patients with epilepsy continue to experience seizures and comorbid features despite optimal pharmacological treatment, and many antiepileptic drugs have significant side effects that

Correspondence: Tore Eid, MD, PhD, Associate Professor, Department of Laboratory Medicine, Yale School of Medicine, 330 Cedar Street, P.O. Box 208035, New Haven, CT 06520-8035, tore.eid@yale.edu, Fax: (203) 688-8597.

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limit their use (Berg 2008; Hu et al. 2018; Reynolds 1988; Rosati et al. 2018). More effective and specific antiepileptic interventions are therefore urgently needed. To date, all interventions for human use only relieve symptoms, particularly seizures, in patients with fully established disease, whereas no treatment can prevent epileptogenesis, which is the pathological process that underlies the development of epilepsy after an insult to the brain.

Discovering safe and effective antiepileptogenic interventions for human use has been challenging for several reasons. First, it can take months to years before epilepsy manifests after an insult to the brain, and many individuals subjected to such insults do not develop epilepsy. Thus, human studies of epileptogenesis are typically extensive, costly, time-consuming and associated with a significant drop-out rate. Second, such studies need to be low-risk – i.e. minimally invasive and observational. Highly invasive and informative approaches such as neuropathological evaluations of brain tissue, are only possible in subsets of patients with epilepsy, in conjunction with epilepsy surgery or analysis of tissue obtained at autopsy. Third, while the use of laboratory animals is cost-effective and allows for more extensive investigations of the epileptogenic process than human studies, animal models have limitations because they do not recapitulate all aspects of the human disease. Careful evaluations and comparisons of data from both humans and animals are therefore essential. For example, if a specific pathology is present in humans with established epilepsy and the same pathology drives epileptogenesis in animals, then the finding would be high on the list of candidate antiepileptogenic targets for human use.

While several investigations have focused on the role of neurons in epileptogenesis, less emphasis has been placed on other brain constituents such as the extracellular matrix, the neurovascular unit and glial cells. Expanding the search for pharmacological targets beyond neurons is important, because an effective antiepileptogenic therapy remains to be discovered.

Thus, the objective here is to review the role of nonneuronal constituents, specifically astrocytes, in epileptogenesis. We will focus on changes in the molecular phenotype and physiology of these cells in animal models of epileptogenesis, and where data are available we will correlate the changes with findings from human epilepsy. The astrocytic pathologies to be discussed involve: *(1) alterations in astrocyte transport – i.e. aquaporin 4 and the associated α-syntrophin-dystrophin complex; the inwardly rectifying potassium channel Kir4.1; the excitatory amino acid transporters EAAT1 and EAAT2; and the monocarboxylate transporters MCT1 and MCT2, and (2) perturbations in glutamine synthetase (GS).* Other astrocyte-related pathologies may also play important roles in epileptogenesis, and the reader is referred to several excellent papers on topics such as adenosine and adenosine kinase (Boison 2016), gap junctions and hemichannels (Bedner et al. 2015), inflammation (Vezzani 2015; Vezzani et al. 2013; Zhang et al. 2016), monoamines (Svob Strac et al. 2016), extracellular matrix components (Dzwonek and Wilczynski 2015; Kim et al. 2016) and the blood-brain barrier (Friedman et al. 2009).

The emphasis of this review will be on mesial temporal lobe epilepsy (MTLE), which is one of the most common forms of medication refractory focal epilepsies (Hauser et al. 1993). MTLE is characterized by spontaneous recurrent seizures that are thought to originate from

mesial temporal lobe structures, such as the hippocampus, amygdala and entorhinal cortex (Spencer 1994; Spencer and Spencer 1994). While surgical resection, ablation or disconnection of these structures can sometimes be used to treat refractory cases, the seizures will eventually recur in over 50% of surgically managed patients, suggesting that new (i.e. secondary) seizure foci (epileptogenic regions) develop elsewhere in the brain (McIntosh et al. 2012; Najm et al. 2013). Up to two-thirds of all patient with surgically treated MTLE exhibit a pathology known as classical hippocampal sclerosis, which is recognized by glial proliferation and preferential loss of neurons in CA1, CA3 and the polymorphic layer of the dentate gyrus of the hippocampal formation (de Lanerolle et al. 2003; Sommer 1880). Many of the pathological changes described in this review are from sclerotic hippocampi, and a key question is whether these changes are causes or consequences of seizures and epilepsy.

2. Astrocytic pathologies with potential relevance for epileptogenesis

2.1 Alterations in astrocyte transport

Aquaporins are transmembrane protein channels for movement of water and other molecules, such as ammonia, glycerol, CO_2 and urea across lipid bilayers (Benga 2012). Thirteen forms of aquaporins have been identified in mammals, with aquaporin 4 (AQP4) being the most abundant channel in the brain (King and Agre 1996). AQP4 is expressed on the plasma membrane of astrocytes and is highly enriched on the glial membrane surrounding brain capillaries (Neely et al. 2001). This enrichment is likely caused by anchoring of the channel via a cytoskeletal complex comprising several proteins, including α-syntrophin, α-dystrobrevin and dystrophin Dp71 (reviewed in (Amiry-Moghaddam et al. 2004)).

Using Western blotting, immunohistochemistry and quantitative immunogold electron microscopy, we found that the expression of AQP4 was increased in epileptogenic and sclerotic regions of the hippocampal formation in humans with long-standing, medically intractable mesial temporal lobe epilepsy (Lee et al. 2004). Moreover, the vascular enrichment of AQP4 was lost (Fig. 1) and accompanied by deficiencies in perivascular dystrophin Dp71 and α-syntrophin (Fig. 2) (Eid et al. 2005; Heuser et al. 2012).

Experimental studies of knockout mice have shown that clearance of extracellular K^+ is compromised by removal of the perivascular pool of AQP4, suggesting that such clearance depends on a concomitant water flux through the astrocyte membrane (Amiry-Moghaddam et al. 2003). Removal of the perivascular AQP4 pool is also associated with a higher intensity of hyperthermia-induced seizures (Amiry-Moghaddam et al. 2003), suggesting that the perturbations in AQP4 observed in human epilepsy may contribute to or worsen the seizures via a mechanism involving impaired potassium buffering. While the relationships among AQP4, potassium buffering, and seizures are not completely understood, it has been suggested that loss of perivascular AQP4 leads to less effective transport of potassium from the extracellular space of the brain to the blood with increased extracellular potassium levels and seizures as the result (Binder et al. 2012; Binder et al. 2006). It is also possible that the increased expression of AQP4 on the non-perivascular domain of astrocytes, increases the propensity of astrocyte swelling with resultant contraction of the extracellular space and

relatively increased potassium concentrations. Interestingly, downregulation of AQP4 has been reported in the hippocampal formation 1 day after status epilepticus in the intrahippocampal kainic acid model of mesial temporal lobe epilepsy with a gradual return to baseline levels and a significant increase in ipsilateral protein levels by 30 days after status epilepticus, suggesting a role of AQP4 in epileptogenesis (Hubbard et al. 2016). An earlier study, using immunogold electron microscopy, demonstrated changes in perivascular AQP4 in the intraperitoneal kainic acid model of MTLE, during the latent period, prior to the occurrence of epileptic seizures (Alvestad et al. 2013).

Single nucleotide polymorphisms in the gene for the inwardly rectifying potassium channel Kir4.1, which is preferentially localized to astrocytes, has been reported in cases of human temporal lobe epilepsy (Heuser et al. 2010). Moreover, mice with mutations in the human Kir4.1 gene are particularly susceptible to seizures (Djukic et al. 2007). Electrophysiological studies of human epilepsy brain tissue have demonstrated impaired extracellular potassium buffering via Kir4.1 (Bordey and Sontheimer 1998), and patients with mesial temporal lobe epilepsy have reduced levels of Kir4.1 on perivascular astrocyte processes in the sclerotic and epileptogenic hippocampal formation along with reductions in dystrophin Dp71 and α-syntrophin at the blood-brain-barrier interface (Heuser et al. 2012) (Fig. 3). The loss of Kir4.1 from glial end-feet membranes in human mesial temporal lobe epilepsy may be explained by a disruption in dystrophin and α-syntrophin anchoring of the channel to perivascular membrane domain, like that of aquaporin 4 (Amiry-Moghaddam et al. 2004).

Glutamate is one of the most abundant amino acids in mammals, being present in particularly high concentrations in brain tissue (5–15 mmol/kg wet weight) (Schousboe 1987). Glutamate is released preferentially from axon terminals into the synaptic cleft, where the transmitter binds to several types of glutamate receptors. To ensure high fidelity encoding of the synaptic glutamate response, glutamate is rapidly cleared from the synaptic cleft by diffusion into the surrounding extracellular space and by uptake via excitatory amino acid transporters (EAATs), which are located on the surface of several cell types, including astrocytes and neurons (Danbolt 2001). Furthermore, effective clearance of extracellular glutamate is important because sustained high levels of the transmitter can lead to seizures and neuronal death (Choi and Hartley 1993; Nadler et al. 1978; Olney et al. 1986).

The mammalian genome contains five genes encoding EAATs (for reviews, see (Danbolt 2001; Grewer et al. 2014; Vandenberg and Ryan 2013)): EAAT1 (GLAST; slc1a3), EAAT2 (GLT-1; slc1a2), EAAT3 (EAAC1; slc1a1), EAAT4 (slc1a6) and EAAT5 (slc1a7). While the cellular and subcellular localizations of EAATs are debated, there is general consensus that astrocytes express EAAT1 (Lehre et al. 1997; Lehre et al. 1995; Rauen et al. 1996; Regan et al. 2007) and EAAT2 (for a review, see (Danbolt et al. 2016)). Deletion of the EAAT2 gene in mice causes nearly complete (95%) loss of glutamate uptake activity and spontaneous recurrent (epileptic) seizures starting at about three weeks of age with sudden, unexpected death in half of the animals after 1–2 weeks (Otis and Kavanaugh 2000; Tanaka et al. 1997; Zhou et al. 2014). Moreover, there is an initial increase in the immunoreactivity for EAAT2 in the hippocampal formation in the intrahippocampal kainic acid model of epilepsy, followed by a marked downregulation at 4 and 7 days post status epilepticus, suggesting a

possible role for the transporter in the pathophysiology of epileptogenesis (Hubbard et al. 2016). Lack of EAAT1 does not lead to spontaneous seizures, but animals lacking the transporter have increased duration and severity of experimentally triggered seizures (Watanabe et al. 1999).

EAATs have also been studied in brain tissue samples from epilepsy patients. Using *in situ* hybridization and Western blotting, Tessler and colleagues found no changes in the levels of mRNA or protein for EAAT1 and EAAT2 in hippocampal specimens from patients with mesial temporal lobe epilepsy (Tessler et al. 1999). However, immunohistochemical staining of hippocampal tissue excised from patients with mesial temporal lobe epilepsy have shown variable results with respect to EAAT1 and EAAT2 expression (Bjornsen et al. 2007; Eid et al. 2004; Mathern et al. 1999; Proper et al. 2002). Factors such as ischemia-induced tissue proteolysis (Li et al. 2012), the area of the hippocampus being sampled (Bjornsen et al. 2007), and whether neuron loss and reactive gliosis (sclerosis) is present (Kim et al. 2016) possibly underlie this variability in the results.

Monocarboxylate transporters (MCTs) constitute a family of fourteen transmembrane protein channels (MCT1-14; SLC16A1-A14) that facilitate the movement of monocarboxylates, such as lactate, pyruvate, beta hydroxybutyrate and acetoacetate across cell membranes (Jones and Morris 2016). In the brain, expression of MCTs has been reported on astrocytes, neurons, endothelial cells and oligodendrocytes (Bergersen 2015). Using immunohistochemistry and quantitative immunogold electron microscopy we discovered that MCT1 and MCT2 were highly enriched along the perivascular astroglial plasma membrane in normal rat brain tissue and in, presumably, nonepileptogenic tissue from humans with mesial temporal lobe epilepsy (Lauritzen et al. 2011; Lauritzen et al. 2015; Lauritzen et al. 2012a; Lauritzen et al. 2012b). Intriguingly, the perivascular enrichment of MCT1 and MCT2 was lost whereas the remainder of the astrocytic labeling for the transporters was increased in the sclerotic and epileptogenic hippocampal formation from humans with mesial temporal lobe epilepsy (Lauritzen et al. 2011; Lauritzen et al. 2012a). Similar patterns of MCT1/MCT2 redistribution were seen in three rat models of mesial temporal lobe epilepsy: the intrahippocampal methionine sulfoximine model and two perforant path stimulation models (Lauritzen et al. 2010). The patterns of MCT1/MCT2 alterations resembled the changes in AQP4 and the α-syntrophin-dystrophin complex, suggesting a functional and anatomical relationship among these molecules. In fact, some studies indicate that water and monocarboxylates can be transported across cell membranes via the same transporter molecules (reviewed in (Rambow et al. 2014)).

While the role MCTs play in epilepsy and epileptogenesis is not understood, the change in transporter expression could indicate a potentially important role of small molecule metabolites and metabolic pathways in the causation of epilepsy (for a detailed review, see (Lauritzen et al. 2015)). Monocarboxylates, particularly ketone bodies, may be necessary to maintain normal mitochondrial function (Bough et al. 2006). This feature of monocarboxylates is important because mitochondrial dysfunction and oxidative stress may contribute to epileptogenesis (Kudin et al. 2009; Waldbaum and Patel 2010). Ketone bodies may have antiepileptogenic effects via several possible mechanisms. Beta-hydroxybutyrate may indirectly regulate excitatory neurotransmission through increasing the synthesis of

kynurenic acid, an endogenous antagonist of glutamatergic and α7-nicotinic receptors, which via interaction with presynaptic N-methyl-D-aspartate (NMDA) receptors may reduce the release of glutamate (Chmiel-Perzynska et al. 2011), in addition to reducing the postsynaptic NMDA receptor response. Acetoacetate modulates vesicular glutamate release and suppresses seizures evoked with 4-aminopyridine (Juge et al. 2010). Ketone bodies have also been reported to increase the levels and activity of mitochondrial uncoupling proteins, thus acting neuroprotective by decreasing the production of reactive oxygen species (Sullivan et al. 2004). Finally, ketone bodies have been reported to enhance GABAergic inhibition (Cantello et al. 2007), increase the brain levels of GABA through suppressing degradation (Suzuki et al. 2009) and increase GABA synthesis (Lund et al. 2011; Yudkoff et al. 2004), ultimately lowering the neural excitation within the cortex.

2.2 Glutamine synthetase

2.2.1 Glutamine synthetase is enriched in astrocytes and is critical for brain ammonia and neurotransmitter homeostasis—Glutamine ammonia ligase (GLUL), which encodes for the enzyme glutamine synthetase (GS; E.C. 6.3.1.2), is one of the phylogenetically oldest genes known to exist, dating more than 1,000 million years back in time, preceding the divergence of prokaryotes and eukaryotes (Kumada et al. 1993). GS catalyzes the formation of glutamine by combining ammonia with glutamate, and is the only known source of endogenous glutamine in mammals under physiological conditions (Eisenberg et al. 2000; Krebs 1935). GS is highly enriched in the mammalian brain and is preferentially found in the cytosol of astrocytes (Martinez-Hernandez et al. 1977). The enzyme is important for metabolism of ammonia, and a lack of astrocytic GS will likely lead to increased brain ammonia, which is neurotoxic, and if present in high concentrations can induce seizures (Navazio et al. 1961). Moreover, because glutamine is a precursor for the synthesis of neurotransmitters glutamate and GABA, astrocytic GS is believed to be key player in regulation of excitatory and inhibitory transmission in the brain (Eid et al. 2016; Rose et al. 2013).

2.2.2 Glutamine synthetase is deficient in astrocytes in the hippocampal formation and amygdala in human epilepsy and in tumor tissue from patients with malignant gliomas and concomitant epilepsy—We and others have observed that the activity and immunoreactivity of GS is dramatically reduced in astrocytes in several brain regions in patients with medically refractory epilepsies. In the sclerotic and epileptogenic hippocampal formation in humans with mesial temporal lobe epilepsy, GS is deficient in astrocytes, particularly in CA1, CA3 and the dentate hilus of the structure (Eid et al. 2004; van der Hel et al. 2005) (Fig. 4). In some patients with neocortical epilepsies, GS is deficient in the amygdala, whereas normal levels of the enzyme are present in the neocortex (Steffens et al. 2005). The lack of astroglial GS is also strongly correlated with risk for epilepsy in patients with malignant gliomas (Rosati et al. 2013). While it is possible that the loss of GS is an epiphenomenon with no significant causative role in epilepsy, several studies (see below) strongly support the idea that GS is a causative or contributing factor in some types of epilepsies.

2.2.3 Mutations in the gene for glutamine synthetase leads to severe epileptic encephalopathy in humans and increased susceptibility to seizures in knockout mice—Haberle and colleagues described the first known cases of homozygous congenital GLUL (GS gene) mutations in humans (Haberle et al. 2005; Haberle et al. 2006; Haberle et al. 2011; Jin et al. 2011). All three patients had severe brain malformations, and the two patients that survived beyond the first days of life exhibited epileptic seizures and several other clinical signs and symptoms, likely reflecting the effects of GS deficiency throughout the body [reviewed in (Spodenkiewicz et al. 2016)]. Attempts to create GLUL knockout mice have been met with limited success. Mice completely deficient in GLUL die at embryonic day E3 (He et al. 2007) and mice with deficiency limited to the CNS die approximately three days after birth (He et al. 2010), making studies of whole-body or whole brain knockouts difficult. However, mice with haploinsufficiency of GLUL in the brain survive and exhibit a greater susceptibility to experimental febrile seizures (van Gassen et al. 2009). Thus, while congenital deficiencies of GLUL have various manifestations depending on the type and cellular distribution of the mutation, epilepsy or increased susceptibility to seizures are common consequences in humans and animals that survive the genetic defect.

2.2.4 Inhibition of glutamine synthetase focally in mesial temporal structures of normal rats induces epileptogenesis and leads to recurrent seizures that become more severe over time—It is important to note that the loss of GS in adult patients with mesial temporal lobe, neocortical and glioblastoma-associated epilepsies are not generalized to the whole body or brain, but localized to specific regions, such as the hippocampal formation, the amygdala and the glioblastoma tissue (Eid et al. 2004; Rosati et al. 2009; Steffens et al. 2005; van der Hel et al. 2005). Thus, to assess the consequences of such localized deficiencies in brain GS, we inhibited the enzyme in different mesial temporal lobe structures in normal rats using chronic, brain-site specific infusions of methionine sulfoximine (MSO). This chemical binds irreversibly to the active site of GS, effectively blocking the normal catalytic conversion of glutamate and ammonia to glutamine (Eisenberg et al. 2000). While MSO is a potent inhibitor of GS, the chemical has other effects such as lowering tissue glutathione levels (Shaw and Bains 2002) and increasing astrocytic glycogen concentrations (Phelps 1975). Appropriate control experiments, such as measurement of tissue glutathione levels and the use of knockout approaches, were therefore used to validate the specificity of the MSO approach (Eid et al. 2008).

In the first study, MSO or phosphate buffered saline (control) was continuously infused into the right hippocampal formation of normal, adult (200–450 g) Sprague-Dawley rats. All animals were subjected to continuous video-intracranial electroencephalogram (EEG) recordings for several weeks followed by euthanasia and neuropathological investigations of the brain. Chronic infusion of MSO at a dose of 0.625 microg/h resulted in approximately 80% reduction in GS activity in the ipsilateral hippocampal formation, with minimal inhibition in the contralateral structure, as assessed by enzyme activity assay (Eid et al. 2008). The MSO-infused, but not the phosphate buffered saline infused rats, exhibited mostly low-grade (Racine scale ≤ 3), repetitive seizures that started a few hours after the onset of MSO infusion and lasted for approximately 24–48 hours (Fig. 5). The initial burst

of seizures was, in many rats followed by a seizure-free interval of approximately 7 days (range: 1.8 – 16.2 days), after which recurrent seizures commenced (Wang et al. 2009). This sequence of events, although contracted in time, is like that of human mesial temporal lobe epilepsy. Patients with this disease have often experienced an insult to the brain in early life, such as prolonged febrile seizures, which is followed by a clinically silent latent period of variable length before the first epileptic seizure occurs (French et al. 1993). Moreover, some of the MSO-infused rats exhibited proliferation of glial cells and patterned loss of neurons in the hippocampal formation, like human mesial temporal lobe epilepsy (Eid et al. 2008; Wang et al. 2009).

We next assessed whether the anatomical site of GS inhibition was a determining factor for induction of epileptogenesis and for the epileptic phenotype. MSO or phosphate buffered saline (PBS) were infused unilaterally into different mesial temporal lobe regions of adult rats, including (1) the angular bundle, (2) the deep entorhinal cortex (EC), (3) the stratum lacunosum-moleculare of CA1, (4) the molecular layer of the subiculum, (5) the hilus of the dentate gyrus, (6) the lateral ventricle, and (7) the central nucleus of the amygdala (Dhaher et al. 2015; Gruenbaum et al. 2015). All animals infused with MSO into the brain tissue exhibited recurrent seizures that were particularly frequent during the first 3 days of infusion and continued to recur for the entire 3-week EEG-recording period. Only a fraction of animals infused with MSO into the lateral ventricle had recurrent seizures, which occurred at a lower frequency compared with the other MSO infused group. Infusion of MSO into the hilus of the dentate gyrus and CA1 resulted in the highest total number of seizures observed over the 3-week recording period. Infusion of MSO into all brain regions studied, except for the lateral ventricle, led to a change in the composition of seizure severity over time. Low-grade (Racine stages 1–3) seizures were more prevalent early during infusion, while severe (stages 4–5) seizures were more prevalent later. Finally, infusion of MSO into the central nucleus of the amygdala also led to depressive-like behaviors, as evident by reduced sucrose consumption in the sucrose preference test (Gruenbaum et al. 2015). Thus, the site of GS inhibition within the brain appears to determine the pattern and temporal evolution of recurrent seizures as well as the expression of depressive-like features associated with epilepsy. The latter is of interest due to the high prevalence of depression and suicide in patients with epilepsy (Kalinin 2007; Mazza et al. 2004; Schmitz 2005; Verrotti et al. 2008).

2.2.5 Epileptogenesis induced by focal inhibition of glutamine synthetase is accompanied by activation of progressively larger neuronal networks over time—There is increasing evidence to suggest that the anatomical and electrophysiological substrates of focal epilepsies are not limited to a circumscribed brain region (i.e. a “focus”), as originally thought (Bartolomei et al. 2017; Dickten et al. 2016; Kramer and Cash 2012; Spencer et al. 2017; Spencer 2002). Clinical observations and EEG studies have reported the involvement of large scale aberrant “networks” in the generation and propagation of seizures. Evidence for spatially distributed alterations, or of aberrant network activity, including a change to the default mode network have also been reported during the interictal state, suggesting that epileptic networks are active and can be detected in the time between seizures (Bartolomei et al. 2017; Constable et al. 2013; Englot et al. 2016; Nissen et al. 2017; Sinha et al. 2017; Smith and Schevon 2016; Spencer et al. 2017; Tomlinson et al.

2017; Varotto et al. 2012; Zaveri et al. 2009). However, the development, physiological properties and anatomical substrates of such networks remain to be defined. For example, it is not known whether these are extant neuroanatomical networks which are "hijacked" during epileptogenesis, or if they are novel networks. It is also not known if these are static networks which form at the onset of epileptogenesis, or if these are time-varying networks which evolve during epileptogenesis. There has been speculation on the existence of a link between the networks underlying epilepsy and those underlying the neuropsychiatric comorbidities of epilepsy. This link, however, remains to be thoroughly demonstrated (Spencer et al. 2017).

To investigate the anatomical substrate of epileptic networks and the possible evolution of these networks, we infused MSO or PBS (control) into the right entorhinal-hippocampal region of normal rats. The brains were examined by intracranial EEG recordings and c-Fos immunohistochemistry (1 hour after a seizure, early and late in epileptogenesis (Albright et al. 2017). Congruent with previous studies, the seizures early in epileptogenesis were preferentially mild (Racine stage 1–2), whereas later in epileptogenesis theywere generally more severe (stages 4–5). During the early stage we observed activation (c-Fos staining) of neurons in the entorhinal-hippocampal area, the basolateral amygdala, the piriform cortex, the midline thalamus, and the anterior olfactory area (Albright et al. 2017) (Fig. 6). Late in epileptogenesis, we observed neuronal activation extending to the neocortex, the bed nucleus of the stria terminalis, the mediodorsal thalamus, and the central nucleus of the amygdala (Albright et al. 2017).

2.2.6 Focal glutamine synthetase inhibtion leads to widespread and progressive structural changes in the brain—We also performed whole brain diffusion tensor imaging (DTI) of MSO- and PBS-infused rats, early and late in epileptogenesis. There were significant changes in fractional anisotropy (FA) in numerous brain regions in rats undergoing epileptogenesis vs. controls (Wang et al. 2017) (Fig. 7 + 8). [FA is a measurement of the direction of water diffusion in a tissue. High FA values mean more constrained water diffusion, such as that of water within fiber bundles; whereas low values indicate diffusion in multiple directions, such as water within cell bodies or brain ventricles. FA measured by DTI is therefore used to visualize fiber pathways (high FA) and other less "directional" tissue components (e.g. cell bodies, glial tissue) in the whole brain, both *ex vivo* and *in vivo*]. Changes included decreases and increases in FA in, amongst other regions, the entorhinal-hippocampal area, amygdala, corpus callosum, thalamus, striatum, accumbens, and neocortex. The FA changes, when evaluated at the early time-point, significantly correlated with seizure load. The FA changes evolved over time as animals transitioned from early to late epileptogenesis. For example, some areas with significant decreases in FA early in epileptogenesis (Fig. 7) changed to significant increases late in epileptogenesis (Fig. 8). While some of the FA changes occurred in regions also stained by c-Fos, there were notable differences. These differences are likely due to the fact that DTI and c-Fos staining measure different parameters, i.e. water diffusivity and stress response, respectively.

Thus, inhibition of GS focally in the hippocampal formation triggers a process of epileptogenesis characterized by gradual worsening of seizure severity over a period of

several weeks (Dhaher et al. 2015). This gradual progress in epileptogenesis is accompanied by structural changes that evolve over time and include numerous brain areas both within and outside the GS-inhibited brain region (Albright et al. 2017; Wang et al. 2017). Several areas were preserved between the early and late stage of epileptogenesis, but new areas were also recruited. Further, in some of the persevered areas there was evidence of change. That is, while the network increased in size over time corresponding to an increase in the severity of seizures, there was evidence as well of reorganization within the developing network. These studies reveal the relatively large scale of the network and suggest a reorganization within the network during the epileptogenic process. It can be argued that the parts of the network responsible for seizure initiation, propagation and termination are the ones that are preserved while those responsible for increased seizure severity are the ones that are changed. It is possible though, that some of the new areas which are recruited into the network may be able to give rise to seizures through secondary epileptogenesis.

Our studies suggest two separate sets of future studies. First, the apparent correlation of increased seizure severity with an increase in the spatial extent of the network suggests that the network changes between the early and late stages of epileptogenesis support more severe seizures. That is, these network changes support the transmission of a seizure to areas of the brain which support expression of more severe seizure symptoms. In our studies we considered two time-points, early and late, and two broad categories of seizures, mild and severe. Further work is indicated to study spatial and temporal network changes through epileptogenesis to uncover in finer detail the time-points when changes in the developing network occur and the locations which are involved and to relate the increasingly more severe behavioral manifestations of seizures to network locations. Second, studies are indicated to probe the locations which are identified through the first set of studies, to better understand the mechanisms underlying ictogenesis and epileptogenesis in a network model of epilepsy. The second set of studies may also reveal if the areas which are recruited into the network can, in time, give rise to independent seizures. The two sets of studies may reveal temporal and spatial points of vulnerability when epileptogenesis can be arrested, modulated, or reversed.

2.2.7 The epileptogenic mechanism of the glutamine synthetase deficiency remains unknown—As discussed, there is increasing evidence to support the notion that loss of GS activity can initiate epileptogenesis and cause epilepsy. However, the mechanism by which the loss of GS induces epileptogenesis is incompletely understood and several possible scenarios have been proposed. One idea is that loss of GS leads to impaired clearance of extracellular glutamate through slowed metabolism of astrocytic glutamate, with backup of glutamate in the extracellular space (Eid et al. 2004). Sustained high levels of extracellular glutamate have been demonstrated in the epileptogenic and GS deficient hippocampal formation in humans with mesial temporal lobe epilepsy (Cavus et al. 2005), and the glutamate excess has been proposed to increase the likelihood of seizures and result in neuron loss due to the excitotoxic properties of glutamate and its analogues (Nadler et al. 1978; Olney 1978; Olney et al. 1986).

Another possibility is glutamate release from astrocytes. We have shown that GS-inhibited astrocytes have significantly increased concentrations of glutamate, raising the possibility

that the astrocytic glutamate excess might be released into the extracellular space, and thus contribute to seizures and neuron loss (Perez et al. 2010). Astrocytic glutamate release can occur *in vitro* via several possible mechanisms, particularly swelling of the cells (Kimelberg et al. 1990). Whether such release occurs in vivo, the mechanism of the glutamate release and its contribution to epileptogenesis, seizures and neuron loss are intensely debated (Hamilton and Attwell 2010), but have been proposed by some investigators (Tian et al. 2005).

Because the brain lacks a complete urea cycle, the main pathway for ammonia metabolism is the GS reaction (Benjamin and Quastel 1975; Cooper and Lai 1987). Inhibition or loss of astrocytic GS will therefore lead to increased brain ammonia, which may lead to seizures and brain injury. The convulsive and neurotoxic mechanisms of ammonia are not completely understood, but appear to involve factors such as brain edema (Blei 1991; Brusilow et al. 2010), mitochondrial dysfunction with free radical formation (Albrecht and Norenberg 2006; Gorg et al. 2010), impaired potassium buffering (Rangroo Thrane et al. 2013) and brain tissue inflammation (Butterworth 2016; Jayakumar et al. 2015).

With respect to the latter it is intriguing that blockade of astrocytic GS leads to microglial activation (Palmieri et al. 2017). Several studies in humans and animals have suggested that brain inflammation is involved in epileptogenesis and epilepsy (Vezzani et al. 2013) and it is plausible that the loss of astrocytic GS may, at least in part, be responsible for the inflammatory changes associated with epilepsy.

Finally, GS is the only enzyme known to produce significant quantities of glutamine in mammals. Because glutamine plays important roles in many other biological processes such as cell growth, energy metabolism, protein synthesis, immune metabolism, osmoregulation, pH regulation, epigenetic regulation and mTOR signaling, there are multiple possible mechanisms by which GS deficiency, and low glutamine levels, can impact epileptogenesis and epilepsy. The idea that low glutamine is implicated in epilepsy is supported by observations by Kanamori and Ross, who demonstrated markedly decreased levels of extracellular brain glutamine in the kainic acid model of epilepsy (Kanamori and Ross 2011; Kanamori and Ross 2013).

2.2.8 Several endogenous and environmental factors can lead to loss, inactivation or inhibition of astrocytic glutamine synthetase—Understanding the cause of astroglial GS deficiency in epilepsy is important because pharmacological targeting of upstream mechanisms could potentially be used to arrest, modulate, or reverse epileptogenesis. While the exact cause in most cases is not fully understood, several possibilities exist such as mutations of the GS gene (Haberle et al. 2005; Haberle et al. 2006; Haberle et al. 2011), nitrosative stress (Butterfield et al. 2006; Swamy et al. 2011), accumulation of beta amyloid (Boyd-Kimball et al. 2005), inflammation (Letournel-Boulland et al. 1994), and many environmental toxins derived from the oceanic ecosystem, terrestrial plants, fungi and herbicides (see (Eid et al. 2013) for a review of the topic). Upstream targeting of the GS deficiency could potentially involve the use of antioxidants, anti-inflammatory compounds and epigenetic modulators.

3. Summary and conclusions

Several changes in the phenotype and function of astrocytes occur during epileptogenesis in animal models of epilepsy, and some of these pathologies are also present in humans with fully established disease. GS, which is abundant in astrocytes, is of interest in this respect because inhibition, dysfunction or loss of the enzyme can initiate epileptogenesis and lead to neuron loss, spontaneous recurrent seizures, and comorbid features associated with epilepsy. Downregulation or inhibition of GS readily occurs in response to factors such as beta amyloid, nitrosative stress, tissue inflammation and several environmental agents. The upstream mechanisms controlling GS expression and activity, as well as the downstream effects of GS deficiency, are therefore attractive targets for future antiepileptogenic therapies.

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Significance Statement

Several types of injuries to the brain such as mechanical trauma, prolonged seizures, infection and stroke, can lead to epilepsy in susceptible individuals; however, the underlying mechanism remains unknown. Here we discuss the role of astrocytes in the causation of epilepsy, particularly the astrocyte-associated molecules aquaporin 4, the alpha-syntrophin-dystrophin complex, the inwardly rectifying potassium transporter Kir4.1, excitatory amino acid transporters EAAT1 and 2, monocarboxylate transporters MCT1 and 2, and glutamine synthetase. We postulate that inhibition, loss or dysfunction of astrocytic glutamine synthetase are strongly associated with epilepsy development and that glutamine synthetase represents an attractive target for novel antiepileptogenic therapies.

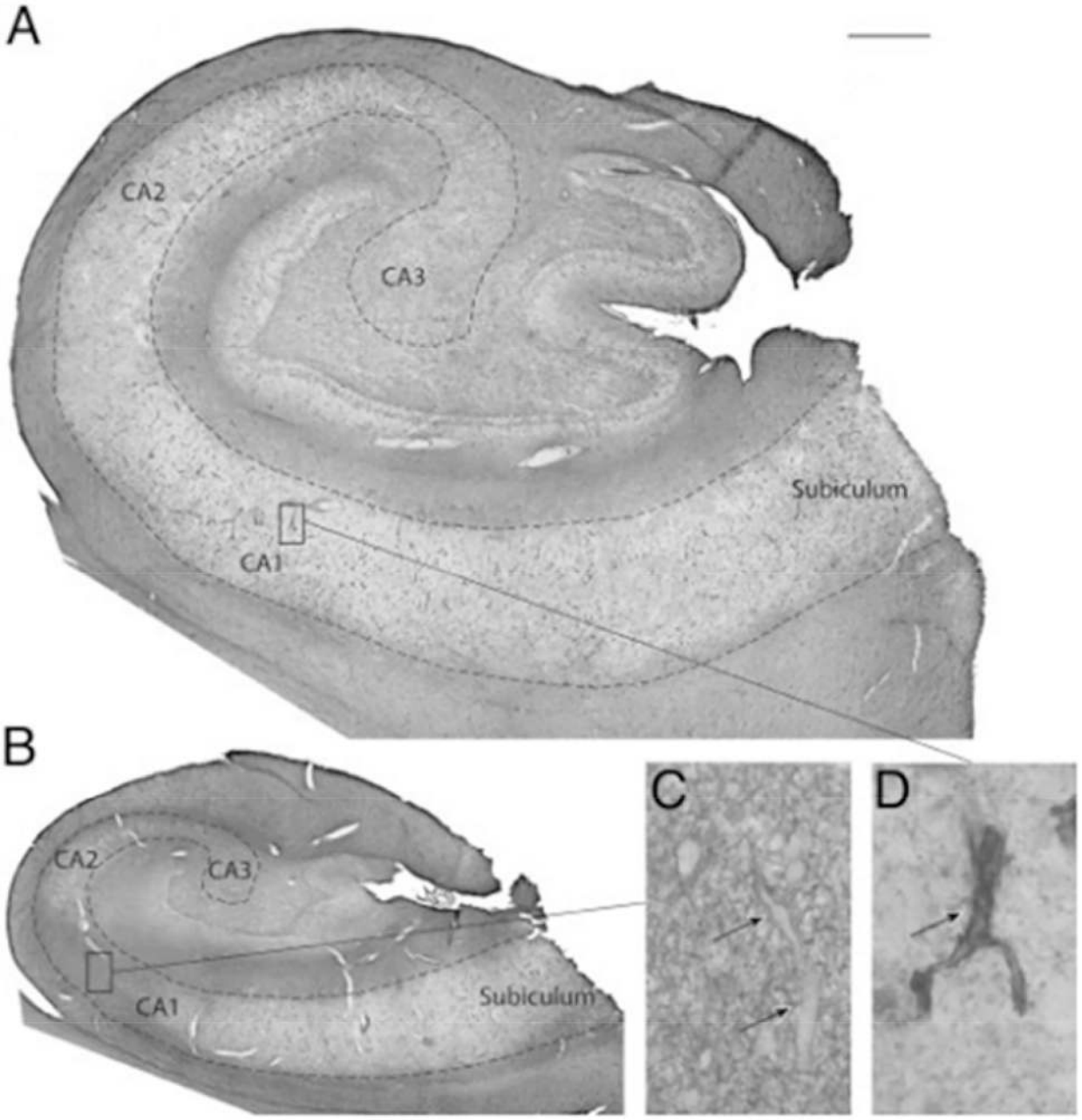

Figure 1.

Coronal sections of the hippocampus from two representative patients with mesial temporal lobe epilepsy without hippocampal sclerosis (A, D) and with hippocampal sclerosis (B, C). Sections were stained for aquaporin 4. Note dense staining for aquaporin 4 around blood vessels in CA1 of the nonsclerotic hippocampal formation, with less staining of the surrounding neuropil (arrow in D). In areas of hippocampal sclerosis, such as the CA1 in B, the enrichment of aquaporin 4 staining around blood vessels is lost (arrows in C). The overall protein expression of aquaporin 4 is increased in hippocampal formations with

sclerosis (data not shown). Scale bar = 1 mm. From (Eid et al. 2005). *Reproduced with permission from The National Academy of Sciences of the USA*.

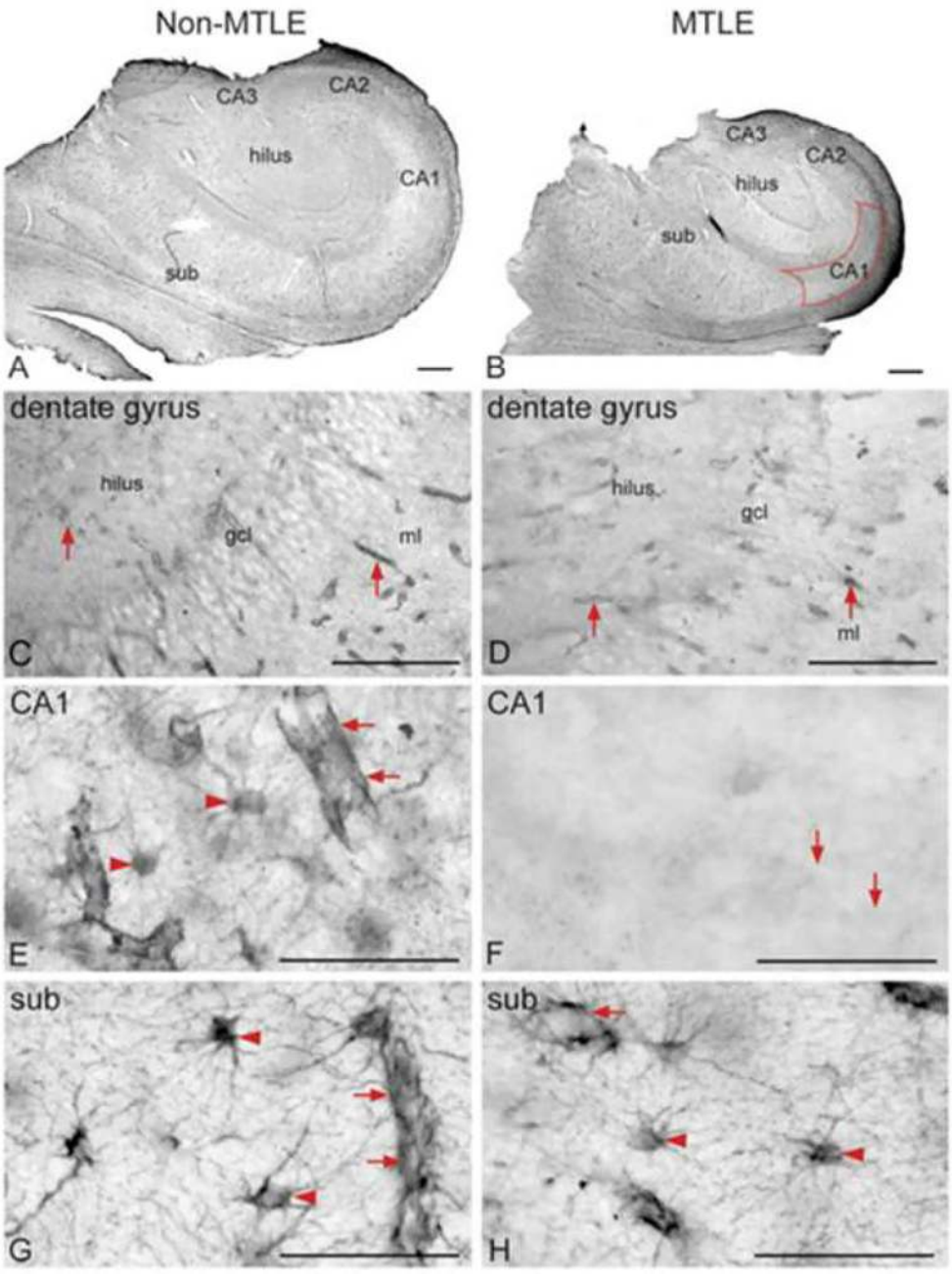

Figure 2.

Distribution of α-syntrophin immunoreactivity in coronal sections of the hippocampus in a patient with mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE) and a temporal lobe epilepsy patient without sclerosis (non-MTLE). (A-H) In non-MTLE patients (A, C, E, G), there is distinct perivascular α-syntrophin immunoreactivity (arrows) and somewhat weaker labeling of astrocytic somata (arrowheads) in all subfields of the hippocampal formation. In MTLE patients (B, D, F, H), perivascular α-syntrophin labeling is markedly reduced in sclerotic areas, particularly in CA1 (F), but also in the dentate hilus (D) and in CA3. In these subfields, the staining is very weak, without demarcation of vessels

or astrocytes. Subfields CA2 (B) and subiculum (sub) (H) display a similar labeling pattern to that in non-MTLE cases (A, G). The sclerotic area in CA1 in MTLE is marked with a dashed line. Scale bars = (A, B) 1 mm; (C, D) 500 μm; (E-H) 50 μm. From (Heuser et al. 2012). *Reproduced with permission from Oxford University Press.*

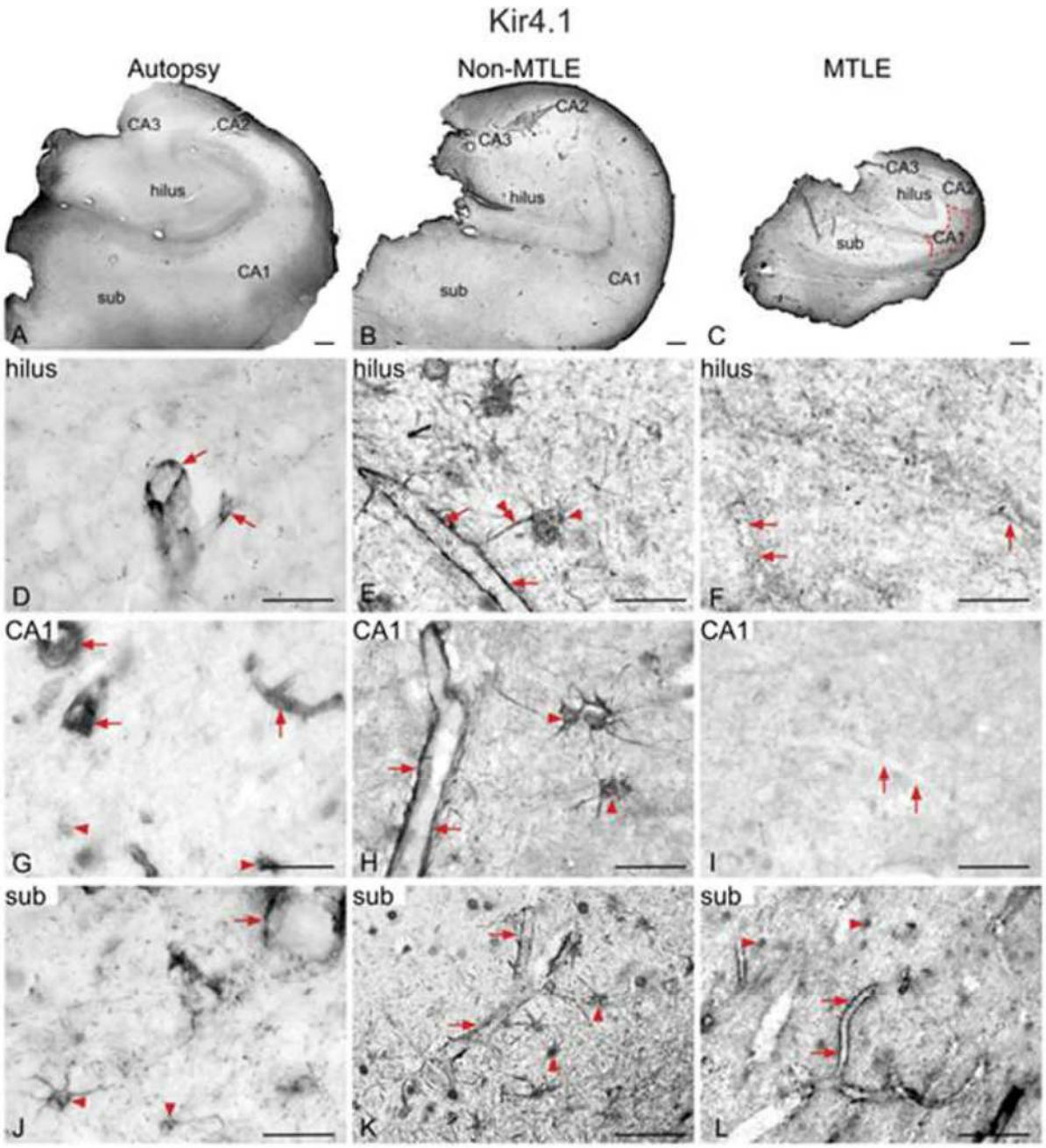

Figure 3.

Distribution of Kir4.1 immunoreactivity in coronal sections of hippocampi from nonepileptic autopsy controls and patients with temporal lobe epilepsy. (A–L) Kir4.1 labeling in subfields of the hippocampal formation from autopsy controls (A, D, G, J), patients without hippocampal sclerosis (non-MTLE) (B, E, H, K), and patients with hippocampal sclerosis (MTLE) (C, F, I, L). In autopsy controls and non-MTLE cases, Kir4.1 immunoreactivity resides in cells with morphology typical of astrocytes, including their somata (arrowheads), processes (double arrowhead in [E]), and perivascular end-feet (arrows). Hippocampi from patients with MTLE show a substantial loss of astrocytic Kir4.1

immunoreactivity in areas with neuronal loss and gliosis (F, I). Thus, the sclerotic CA1 are almost devoid of Kir4.1 labeling (I). The hilus shows changes that are somewhat less extensive than those in CA1 (F). Loss of perivascular Kir4.1 immunoreactivity is associated with reduced labeling of astrocytes. The subiculum (sub) in MTLE (L) displays a labeling pattern similar to that observed in autopsy controls (J) and non-MTLE (K). The sclerotic area in CA1 in MTLE is marked with a dashed line (C). Vertical panels (D, G, J), (E, H, K), and (F, I, L) are high-magnification fields of the respective areas in (A, B, C). Scale bars = (A–C) 1 mm; (D–I) 50 μm; (J–L) 100 μm. From (Heuser et al. 2012). *Reproduced with permission from Oxford University Press.*

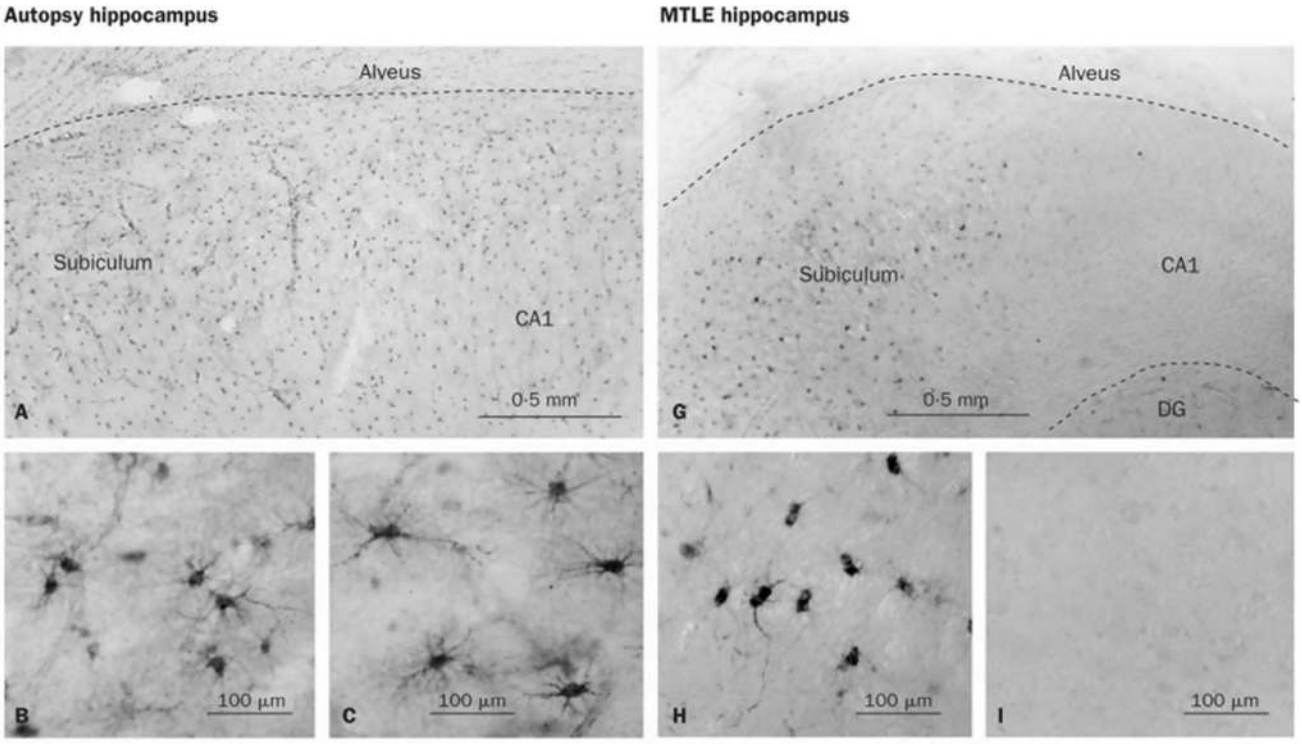

Figure 4.

Glutamine synthetase immunoreactivity of a representative hippocampus from a human subject without epilepsy (autopsy, A–C) and a subject with mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE, G–I). There is dense and even distribution of glutamine-synthetase-positive cells in the subiculum and area CA1 of the nonepilepsy hippocampus (A). High-power fields of the subiculum (B) and CA1 (C) in the nonepilepsy hippocampus show that staining is confined to astroglial cells. In the MTLE hippocampus (G), there are many glutamine-synthetase-positive cells in the subiculum, but area CA1 is severely deficient in staining for glutamine synthetase.. High-power field of the subiculum in (H) confirms presence of staining in astroglial cells, which have somewhat fewer processes than positive astrocytes in the corresponding area of the nonepilepsy hippocampus (B). High-power field of area CA1 (I) confirms lack of staining in this region. From (Eid et al. 2004). *Reproduced with permission from Elsevier.*

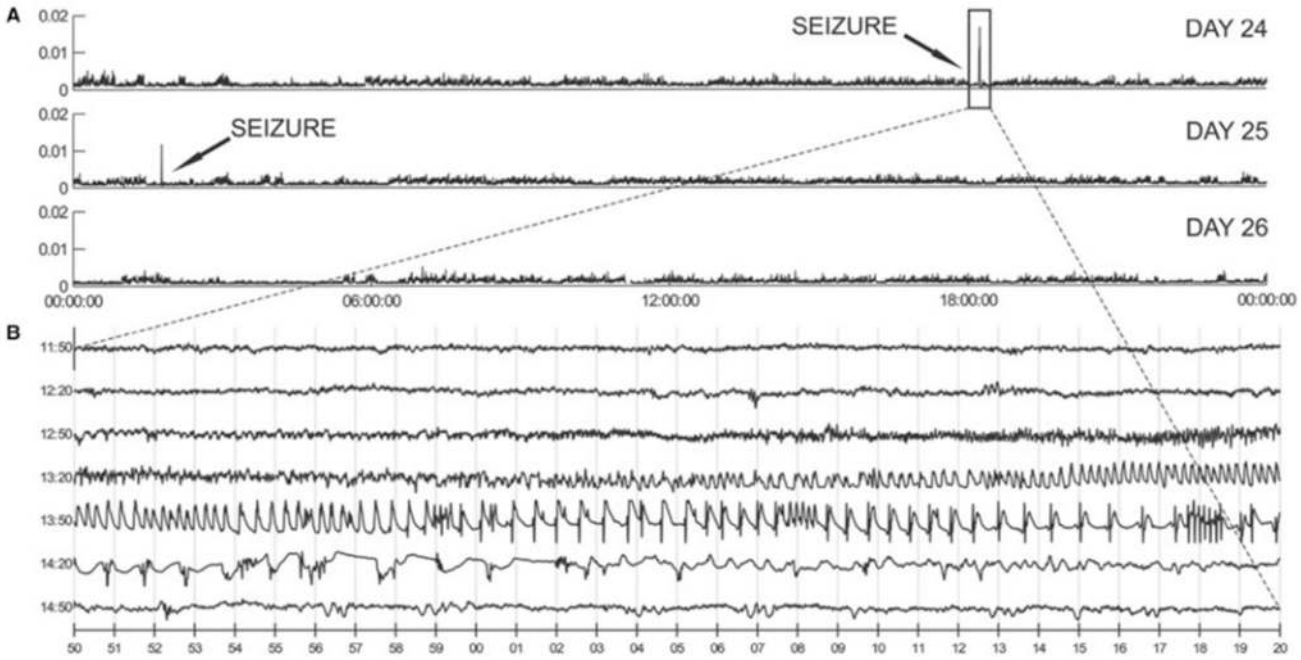

Figure 5.

Infusion of the glutamine synthetase inhibitor methionine sulfoximine (MSO) into the right hippocampal formation of normal rats precipitates recurrent seizures. (A) Continuous three-day-record (days 24 – 26 after surgery) of Teager energy of a single intracranial EEG time-series from a representative animal infused continuously with intrahippocampal MSO (0.625μg/h). Energy estimates were obtained at 1s resolution and averaged over a 5s running window (x-axis). A 24-h-period (midnight to midnight) is displayed in each trace. Two seizures were recorded during the 72-h-period. As in human epilepsy, seizures in the MSO model are readily identified by the difference in energy (y-axis) frombackground icEEG activity. (B) Continuous 3.5 min intracranial EEG (from day 24: 18: 11: 50 to 18: 15: 20) displaying the first seizure identified in A. From (Eid et al. 2008). *Reproduced with permission from Oxford University Press.*

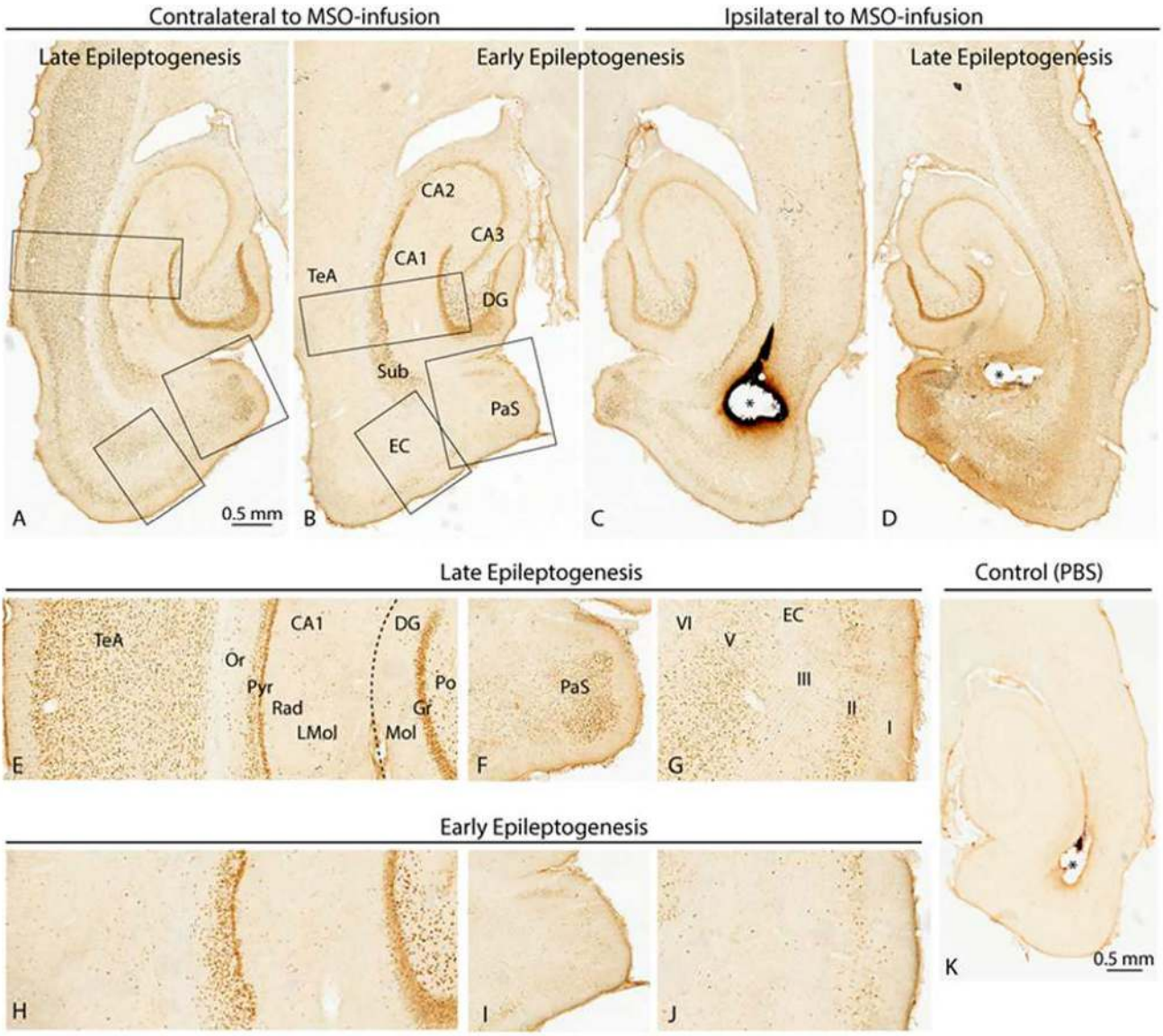

Figure 6.

Fig. 2. C-Fos staining in the entorhinal-hippocampal area of representative MSO-infused rats during early [B, C] and late [A,D] epileptogenesis. There is strong C-Fos staining of neurons in several regions ipsilateral to the MSO-infusion (asterisks) both early (C) and late (D) in epileptogenesis. Regions with particularly intense staining include granule cells (Gr) and polymorphic cells (Po) of the dentate gyrus (DG) as well as neurons in the CA1, subiculum (Sub), parasubiculum (PaS), and layers of the entorhinal cortex (EC) and temporal association cortex (TeA). In general, there is more extensive staining for c-Fos during late vs. early epileptogenesis ipsilateral to the MSO-infusion. Neurons in several areas contralateral to the MSO-infusion are stained as well with a marked increase in the number of stained cells late (A, E, F, G) vs. early (B,H, I, J) in epileptogenesis. In the contralateral hemisphere, numerous neurons are stained in the TeA, PaS, and layers V–VI of the EC during late epileptogenesis (E, F, G) whereas the corresponding areas show minimal if any

staining during early epileptogenesis (H, I, J). A nonepileptic (PBS-infused) rat is shown in K and is negative for c-Fos staining (K). Abbreviations: CA1–3, cornu ammonis subfields 1–3 of the hippocampus; LMol, stratum lacunosum-moleculare; Mol, molecular layer of the dentate gyrus; Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum. From (Albright et al. 2017). *Reproduced with permission from Elsevier.*

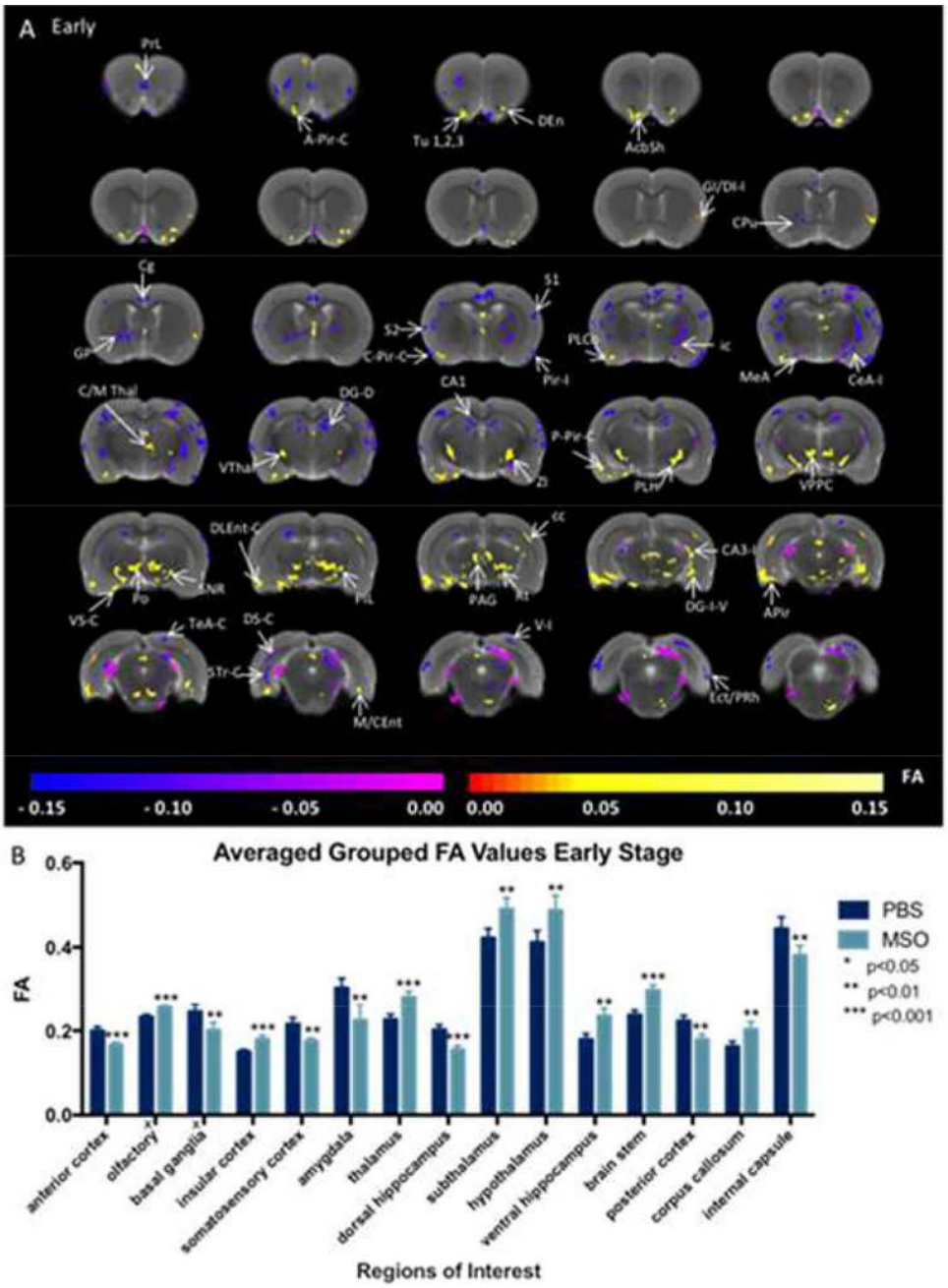

Figure 7.

Representative slices from anterior to posterior of fractional anisotropy (FA) differences ($p < 0.05$) between MSO-treated and PBS-treated animals at the *early* stage of epileptogenesis (top, A). Warm colors represent increases in FA, whereas cool colors represent decreases in FA in MSO-treated rats versus PBS-treated rats. Abbreviations: PrL, prelimbic cortex; A-Pir-C, anterior piriform cortex-contralateral; Tu 1,2,3, olfactory tubercles 1,2,3; DEn, dorsal endopiriform; AcbSh, accumbens shell; GI/DI-I, granular/dysgranular insular cortex-ipsilateral; CPu, caudate putamen; GP, globus pallidus; Cg, cingulate cortex; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; C-Pir-C, central piriform

cortex-contralateral; Pir-I, piriform cortex-ipsilateral; PLCo, posterolateral cortical amygdala; ic, internal capsule; MeA, medial amygdala; CeA-I, central amygdala-ipsilateral; C/M Thal, central/medial thalamus; VThal, ventral thalamus; DG-D, dentate gyrus-dorsal; CA1, CA1 of the hippocampus; ZI, zona incerta; P-Pir-C, posterior piriform cortex-contralateral; PLH, peduncular part of the lateral hypothalamus;VPPC, ventral posterior nucleus of the thalamus, parvicellular part; VS-C, ventral subiculum-contralateral; Po, posterior thalamus; SNR, substantia nigra; DLEnt-C, dorsolateral entorhinal cortex-contralateral; PIL, posterior intralaminar thalamic nucleus; PAG, periaqueductal gray; Rt, reticular formation; cc, corpus callosum; CA3-I, CA3 of the hippocampus-ipsilateral; DG-I-V, dentate gyrus-ipsilateral-ventral; APir, amygdalopiriform transition area; TeA-C, temporal association cortex-contralateral; STr-C, subiculum transition area-contralateral; DS-C, dorsal subiculum-contralateral; M/CEnt-medial/caudomedial entorhinal cortex; V-I, visual cortex-ipsilateral; Ect/Prh, ectorhinal/perirhinal cortex. Early Stage Grouped FA differences, arranged from anterior to posterior (gray matter) with white matter changes on the right (bottom, B). Regions with an×superscript consist of mixed FA increases and decreases. Statistical significance was determined using multiple t-tests with Holm-Sidak correction for multiple comparisons. Asterisks indicate levels of significance where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. From (Wang et al. 2017). *Reproduced with permission from The International League Against Epilepsy*.

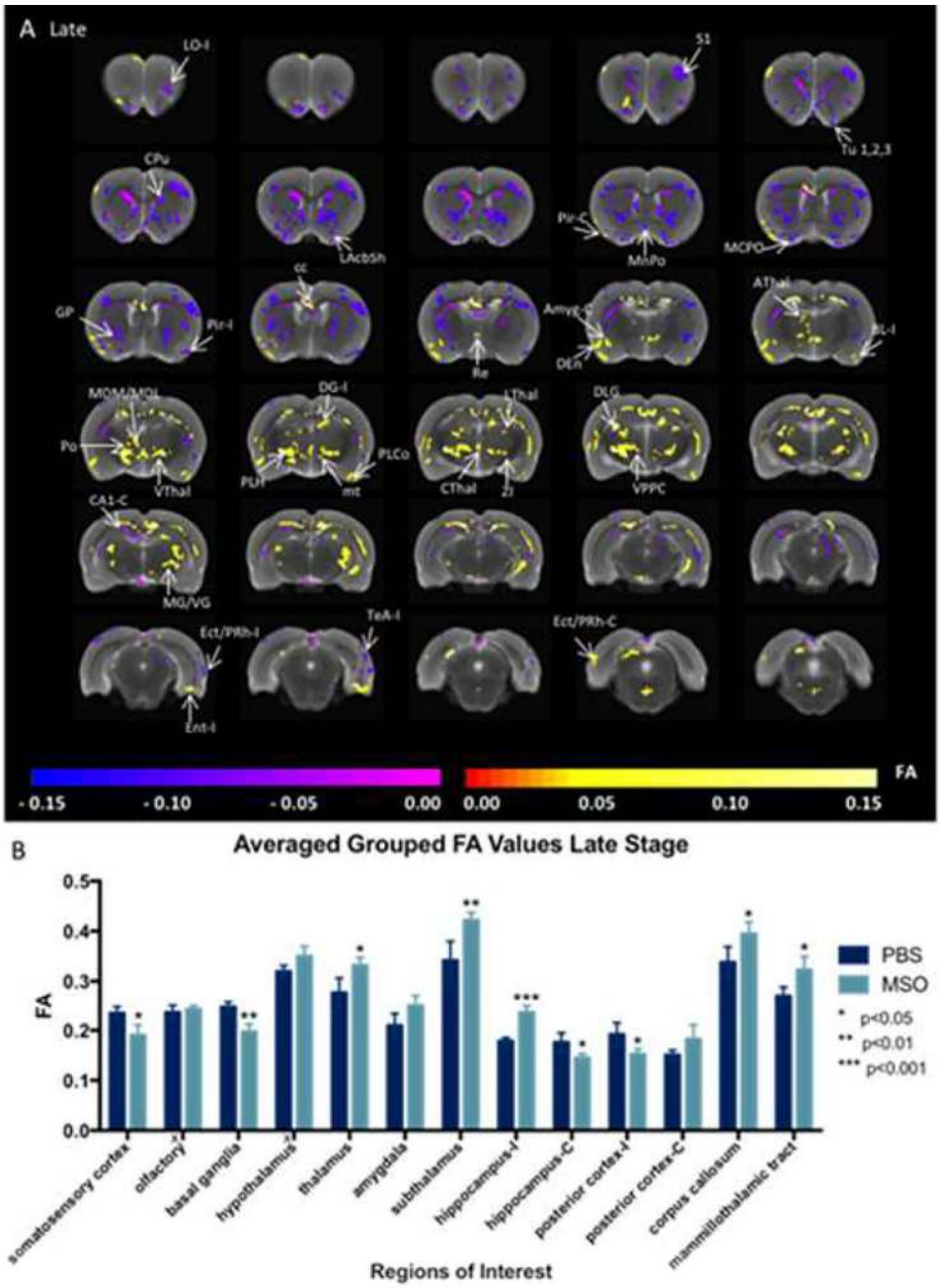

Figure 8.

Representative slices from anterior to posterior of FA differences ($p < 0.05$) between MSO-treated and PBS-treated rats at the *late* stage of epileptogenesis (top, A). Warm colors represent increases in FA, whereas cool colors represent decreases in FA. Abbreviations are as follows: LO-I, lateral orbital cortex; S1, primary somatosensory cortex; Tu 1,2,3, olfactory tubercles 1,2,3; CPu, caudate putamen; LAcbSh, lateral accumbens shell; Pir-C, piriform cortex-contralateral; MnPo, median preoptic nucleus; MCPO, magnocellular preoptic nucleus; GP, globus pallidus; Pir-I, piriform cortex-ipsilateral; cc, corpus callosum; Re, reuniens; Amyg-C, amygdala-contralateral; DEn, dorsal endopiriform; AThal, anterior

thalamus; BL-I, basolateral amygdala-ipsilateral; MDM/MDL, mediodorsal thalamus, medial/lateral; Po, posterior thalamus; VThal, ventral thalamus; PLH, peduncular part of the lateral hypothalamus; DG-I, dentate gyrus-ipsilateral; mt, mammillothalamic tract; PLCo, posterolateral cortical amygdala; CThal, central thalamus; LThal, lateral thalamus; ZI, zona incerta; DLG, dorsal lateral geniculate; VPPC, ventral posterior nucleus of the thalamus parvicellular part; CA1-C, CA1 of the hippocampus-contralateral; MG/VG, medial/ventral geniculate nucleus; Ent-I, entorhinal cortex-ipsilateral; Ect/PRh-I, ectorhinal/perirhinal cortex-ipsilateral; TeA-I, temporal association cortex-ipsilateral; Ect/PRh-C, ectorhinal/ perirhinal cortex-contralateral. Late Stage Grouped FA differences, arranged from anterior to posterior (gray matter) with white matter changes on the right (bottom, B). Regions with an×superscript consist of mixed FA increases and decreases. Statistical significance was determined using multiple t-tests with Holm-Sidak correction for multiple comparisons. Asterisks indicate levels of significance where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. From (Wang et al. 2017). *Reproduced with permission from The International League Against Epilepsy*.