



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-glia 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).

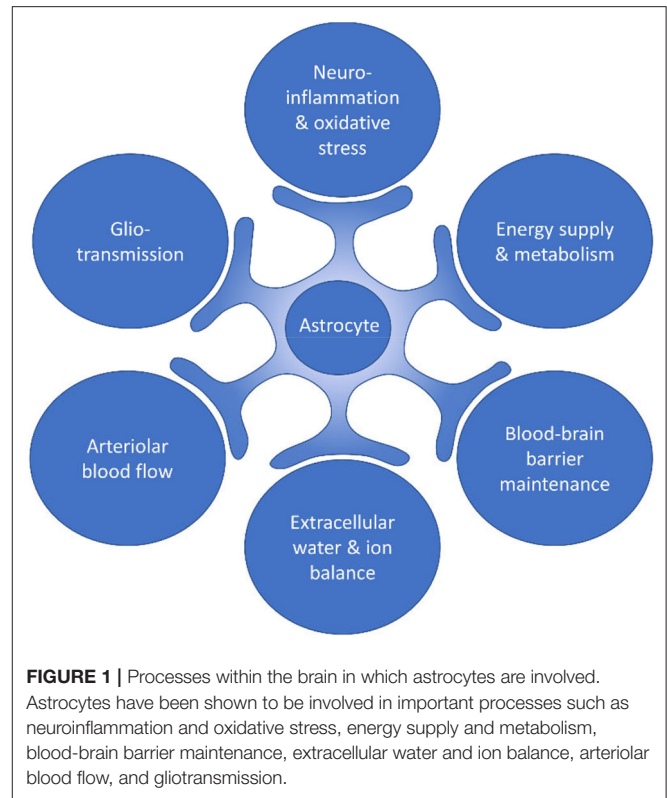
For many years, astrocytes were regarded as “glue” that bound neuronal elements together, providing mere structural support for the brain. In fact, astrocytes are playing a pivotal role in brain homeostasis. From recent transcriptome studies it became clear that different subtypes of astrocytes exist 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**).

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

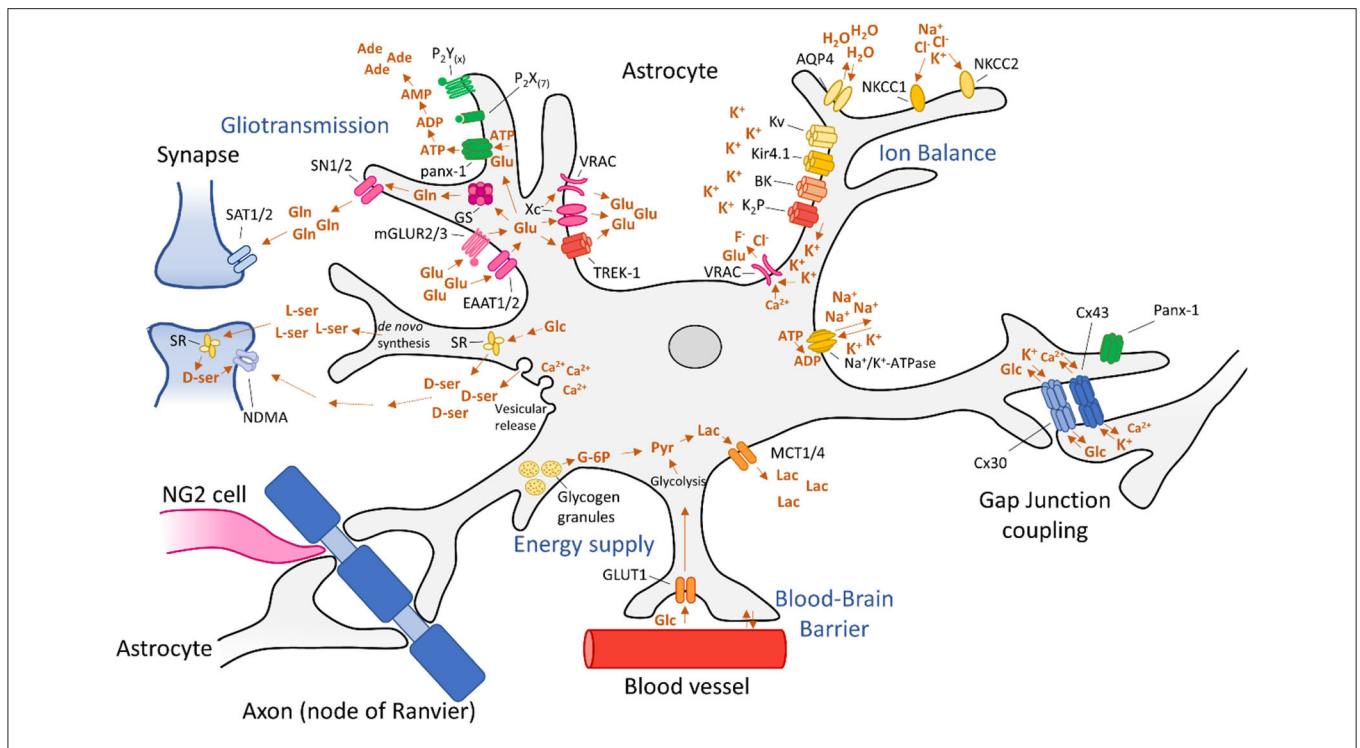


FIGURE 2 | Astrocytic processes involved in epileptogenesis. Dysregulation of astrocyte functions can lead to epileptogenesis via disturbed energy metabolism and gliotransmission, alterations in extracellular ion concentrations, as well as dysfunction of the blood-brain barrier and dysregulation of blood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

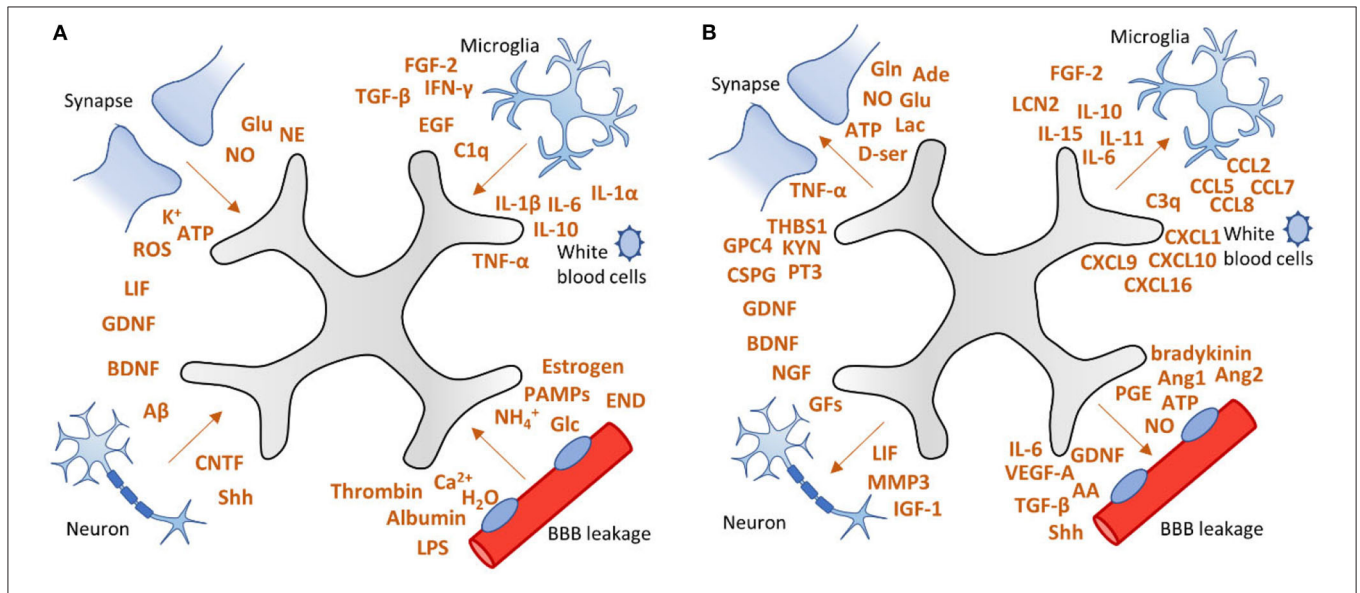


FIGURE 3 | Factors involved in astrogliosis. (A) After brain injury, astrocytes can receive “instructions” from their environment and respond to a plethora of signaling molecules. (B) In turn, astrocytes send “instructions” to their environment by releasing a variety of factors, including pro-inflammatory 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-1 β), 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 astrogliosis. 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-1 β , IL-6, and TNF- α in animals with (recurrent) seizures (53–59) and patients with epilepsy (60–63). IL-1 β 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 NMDA 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-1 β , 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 $\geq 50\%$ 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-1 β 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-1 α , 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 phosphoglucosyltransferase (PGM) and then into uridine triphosphate (UDP)-glucose by UDP-glucose pyrophosphatase (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 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 $\text{AQP4}^{-/-}$ 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 ($\text{P}_2\text{X}_7\text{R}$) [(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 $\text{P}_2\text{X}_7\text{R}$ (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 P₂X₇R 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 P₂X₇R 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 P₂X₇R presumably affects astrocytes and other glial cells indirectly as well, by blocking P₂X₇R-mediated excitotoxic IL-1 β release (255). P₂X₇R 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 administered 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 ($\text{A}_{2\text{A}}\text{R}$) (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 $\text{A}_{1\text{A}}\text{R}$ and $\text{A}_{2\text{A}}\text{R}$ are the most studied. Anti-epileptic effects are mainly mediated by $\text{A}_{1\text{A}}\text{R}$ 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 $\text{A}_{1\text{A}}$, $\text{A}_{2\text{A}}$, $\text{A}_{2\text{B}}$, 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 $\text{A}_{1\text{A}}\text{R}$ (310). However, elevated synaptic adenosine levels also activate the $\text{A}_{2\text{A}}\text{R}$, which signaling may in turn desensitize and downregulate the $\text{A}_{1\text{A}}\text{R}$ (312, 313). Indeed, it has been shown that in epileptogenic circuits, stimulation of $\text{A}_{2\text{A}}\text{R}$ downregulates $\text{A}_{1\text{A}}\text{R}$ (314, 315). Recently, it has been shown that a 3-fold induction of $\text{A}_{2\text{A}}\text{R}$ is present in astrocytes within the hippocampus of patients with TLE (316). Increased $\text{A}_{2\text{A}}\text{R}$ 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- κ B 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 Ca²⁺ 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⁺

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 ₂ O	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 ₂ P		↑ 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 ₂ X ₇		No change (253)
	PMCA	Transient upregulation	↑ Dentate gyrus (342)
	TRPA1		↑ (343)
	TRPCs		↑ (344–346)
	TRPV1		↑ Cortex, Hippocampus (347)
	Cl ⁻	NKCC1	Transient upregulation (333, 334)

↑: upregulation, ↓: downregulation.

ions, astrocytes express different types of K⁺-channels, including K_{ir} channels, Ca²⁺-sensitive potassium (BK) channels, voltage-gated potassium (K_v) channels, two-pore domain (K₂P) 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⁺/Ca²⁺-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).

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 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 $K_{ir4.1}$, 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 “ankyryn” 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).

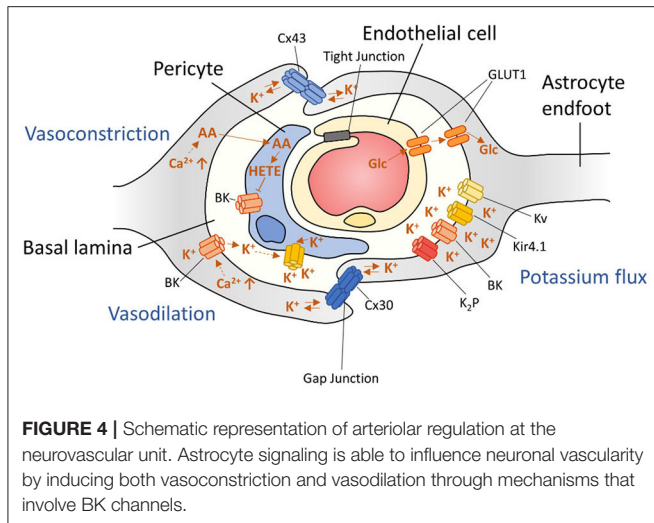
TRPCs are involved in store-operated Ca^{2+} entry and have been shown to contribute to Ca^{2+} -mediated glutamate release in astrocytes (413). On the other hand, glutamate can activate astrocytic NMDA receptors and thereby induce Ca^{2+} influx, although they are ~ 2 times less permeable than their 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 $K_{ir4.1}$



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- κ B) (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 (PLA₂) pathway and (2) BK channel mediated vascular control [(393, 479); **Figure 4**]. Increased PLA₂ 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 PLA₂ pathway, such as 20-hydroxyeicosatetraenoic acid (20-HETE), epoxy-eicosatetraenoic acids (EET)s, and prostaglandin E2 (PGE₂). 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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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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