Published in final edited form as:

Nat Rev Neurol. 2022 December ; 18(12): 707–722. doi:10.1038/s41582-022-00727-5.

Astrocytes in the initiation and progression of epilepsy

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Abstract

Epilepsy is a neurological disease affecting about 65 million persons worldwide and characterized by an “enduring predisposition to generate epileptic seizures”. Although the etiology of epilepsy in many individuals is unknown, identified causes are either acquired or linked to genetic mutations. The first-line treatment for epilepsy consists of more than 20 anti-seizure medications, nonetheless, about one-third of patients fail to achieve seizure control. Anti-seizure medications act primarily on neurons and provide symptomatic control of seizures in drug-sensitive patients, but do not interfere with the onset and progression of epilepsy, and cause serious side effects. Thus, medications with new cellular and molecular targets and mechanisms of action are needed. Among the pivotal cellular components of the epileptogenic circuitry, astrocytes raised increasing interest for their contribution to epilepsy. Progress in understanding astrocyte function in health and disease led to new discoveries on how astroglia-mediated changes in brain metabolism and excitability play a role in epilepsy. This review reports our understanding of how dysregulation of key astrocyte-based functions, namely *gliotransmission, cell metabolism,* and *immune properties,* play a role in the development and expression of hyperexcitability in epilepsy. Strategies to mitigate astrocyte dysfunctions and methods to monitor cell activation states in vivo will be

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Annamaria Vezzani supervised the manuscript preparation, wrote introduction and conclusions and with Eleonora Aronica wrote the section related to the immune and inflammatory functions; Eleonora Aronica wrote Box 1; Peter Bedner and Christian Steinhäuser wrote the section related to gliotransmission; Detlev Boison wrote the section related to cell metabolism; Teresa Ravizza wrote the section related to *in vivo* monitoring of astrocyte reactivity, and prepared Box 2 and the reference list. All the authors contributed to prepare Box 3 and the figures, and equally contributed to revise and edit the final manuscript.

Competing interests

The authors declare no competing interests

discussed, with the overarching goal of defining new targets to prevent the initiation and progression of epilepsy.

Introduction

Astrocytes are specialized CNS cells of neuroepithelial origin that critically contribute to tissue homeostasis and neural signaling. There are mainly two types of astrocytes, protoplasmic and fibrous, which differ in morphologic appearance and location (gray and white matter, respectively). Recent studies further highlighted astrocyte diversity based on transcriptomic profiling, immunohistochemistry, and functional read-outs. Thus, astrocytes comprise a heterogeneous population of cells that display brain region- and disease-specific properties[1]. Among their various interrelated physiological functions, astrocytes play a key role in maintaining blood-brain barrier (BBB) structure and permeability properties[2] by directly interacting with endothelial cells and pericytes; they mediate neurovascular coupling[3], and are involved in blood flow regulation and energy metabolism[2,3], thus providing metabolic support to neurons. As part of the tripartite synapse[4], astrocytes regulate extracellular levels of neurotransmitters implicated in seizures (e.g., glutamate and GABA), as well as ions, water, pH, and they release gliotransmitters, thus contributing to synaptic transmission and neuronal excitability[5,6].

In healthy conditions, astrocytes are constantly activated in response to physiological stimuli whereas various types of CNS insults and pathologies shift their physiological state to an “activated state” through the process of “reactive astrogliosis”[7]. The latter involves complex and dynamic cell responses to diverse pathological conditions which involve a spectrum of genetic, epigenetic, molecular, metabolic, morphological and functional changes that are context-dependent and regulated by specific signaling events. Reactive astrogliosis is a hallmark of the epileptic focus both in human and experimental epilepsy[5,6] (Fig. 1). Glial fibrillary acidic protein (GFAP), a major constituent of intermediate filaments, has been mostly used as a cellular marker of reactive astrocytes in epilepsy[8], although other markers such as vimentin, connexin (Cx) 43, S100β, adenosine kinase (ADK), and glutamine synthase (GS) have also been assessed[9–12]. A recent study provided evidence for the presence of GFAP/S100β-positive immature astroglia in close apposition with the neurogenic niche in the dentate gyrus of the hippocampus in human mesial temporal lobe epilepsy (MTLE) but not in control tissue, suggesting it represents a pathological phenotype of this type of epilepsy with a functional role in modulating epileptic activity[13]. In addition, modulation of astrocyte activation in immature rodent brain improved memory outcomes after febrile seizures[14].

Comparative analyses suggested that there is substantial similarity between human and rodent astrocytes with regard to their functional properties and extensive conservation in astrocytic gene expression, which warrants employing mouse or rat models to investigate the involvement of astrocytes in the etiology of human CNS diseases[15,16]. Animal models of epilepsy mimicking human etiologies[17] allowed to characterize the complex and dynamic structural, cellular, molecular alterations occurring in the brain during epilepsy development. Functional and interventional studies showed that some alterations contribute to disease

onset and development (*epileptogenesis*), or to mechanisms of seizure generation and recurrence (*ictogenesis*). In this context, reactive astrocytes may critically contribute to epilepsy initiation and progression due to impairment of their homeostatic functions or gain of aberrant properties[9,11,18]. In particular, reactive astrocytes can either initiate the disease (as in the genetic gliopathy Alexander disease[19]; Box 1) or they might contribute to disease onset and progression, as suggested in acquired epilepsy[5,20]. For example, the induction of reactive astrocytes by knocking out the β1-integrin gene in transgenic mice, or delivering a specific adenoviral vector in the healthy mouse brain, elicits spontaneous seizures associated with impairment of GABAergic transmission[21,22]. However, the process of reactive astrogliosis is not uniformly negative since beneficial functions have been described in epilepsy such as defense from oxidative stress, production of anti-inflammatory mediators and neuroprotective molecules. These "resilient astrocytes"[7] often co-exist in the epileptic focus with maladaptive astrocytes[23–26], or the same cells may express both homeostatic/compensatory and pathologic markers. Overall, the evidence suggests that these compensatory states are likely to be insufficient in epilepsy for contrasting the detrimental consequences of dysfunctional astrocytes[27].

The following sections will present key pathophysiological features of astrocytes which are shared by CNS pathologies associated with neuronal network hyperexcitability and seizures. There is growing evidence and consensus that key mechanisms of the epileptogenic process, including the pathogenic role of astrocytes, are shared across a wide spectrum of acquired epilepsies[28]. Because astrocytes in culture may undergo dramatic changes and often acquire artificial functional properties, we have confined this review to data obtained *in situ*, after acute isolation or *in vivo* and explicitly indicated the few cases where culture work was cited.

Gliotransmission

Astrocytic processes are tightly associated with synapses, and it has been estimated that one astrocyte can oversee more than 100,000 synapses[29]. The close spatial and functional interactions of synapses and astrocytic perisynaptic processes gave rise to the concept of the 'tripartite synapse' according to which astrocytes detect and respond to synaptic signals through release of 'gliotransmitters' which influence neuronal activity[4]. Different forms of neuron–glia interaction across brain regions have since been described but are still controversially discussed[30,31]. However, increasing evidence does suggest that gliotransmission is an important component of normal brain function and that abnormalities in gliotransmission are involved in epilepsy.

Gliotransmission in the healthy brain

The first gliotransmitter shown to modify neuronal activity in cell culture was glutamate[32,33]. Subsequently, vesicular structures and Ca^{2+}-dependent astroglial glutamate release were identified *in situ*[34]. The presence of vesicular glutamate transporters in astrocytes has been questioned but these cells express many components required for vesicular release[35]. Astrocytic Ca^{2+} increase and glutamate release by astrocytes triggered by GABA, glutamate, endocannabinoids and ATP entail slow inward currents (SICs) in

neurons through extrasynaptic GluN receptors (GluNRs) (Fig. 2), which synchronizes neuronal activity[36,37] and increases synaptic strength[38,39]. Besides glutamate, astrocytes release the GluNR co-agonist D-serine and thereby control long-term potentiation[40]. Ca^{2+} elevations and D-serine release from astrocytes influence learning and memory[41,42]. ATP is also a gliotransmitter, which after its release activates purinergic P2 receptors or, after degradation by ectonucleotidases to adenosine, affects neurotransmitter release through presynaptic A1 receptors and regulates sleep homeostasis[43] (Fig. 2). Moreover, astrocytes release GABA, e.g., in the thalamus, where the gliotransmitter provokes outward currents in thalamocortical neurons, in the olfactory bulb, where astroglial GABA inhibits mitral and granule cells, in the hippocampus or in the cerebellum, where GABA release by astrocytes or Bergmann glia mediates tonic inhibition[44] (Fig. 2). In addition to Ca^{2+} dependent exocytosis, several other release mechanisms for gliotransmitters have been proposed, including through Cx43 hemichannels, pannexin-1 (Panx1) channels, volume regulated anion channels, bestrophin1 (Best1) anion channels, two-pore domain K^+ (K2P) channels, reversal of amino acid transporters, or through P2X7 receptors (P2X7Rs). Ultimately, however, it is still largely unclear in which brain regions astrocytes are equipped with which transmitter release machinery.

Since gliotransmitters modulate neuronal excitability, it is likely that disturbance of this astrocyte function is implicated in ictogenesis and epileptogenesis, as described in the following section.

Gliotransmission in the initiation and progression of epilepsy

Enhanced glutamate release and loss of astrocyte gap junction coupling

—The most intensively studied gliotransmitter in the context of epilepsy is glutamate (Fig. 2). Extracellular glutamate levels are elevated in the hippocampus in human and experimental TLE, particularly before (interictally) and during seizures[45,46]. First hints for an involvement of astrocytic glutamate release in epileptogenesis came from studies showing that Ca^{2+} oscillations in astrocytes evoke SICs through extrasynaptic GluNRs in neurons and promotes synchronization of neuronal discharges[36,37] (cf. above and Fig. 2). This led to the hypothesis that excessive release of the gliotransmitter underlies the hypersynchronous neuronal firing characterizing epileptic activity, and this was supported by the finding that astrocytic glutamate triggers neuronal paroxysmal depolarization shifts, which are the cellular correlates of interictal discharges[47,48]. However, other work suggested a modulatory rather than a causative role of the gliotransmitter[49], e.g. by lowering the threshold for ictal activity[50]. This idea was recently supported by showing that in mice injected intracortically with kainate, astrocytic Ca^{2+} increases precede seizures by several seconds[51]. That Ca^{2+}-dependent gliotransmission is crucially involved in epileptogenesis and neurodegeneration was also concluded from studies in rodents using pharmacological or genetic tools to inhibit astrocytic transmitter release[52,53]. In the sclerotic hippocampus of human and experimental TLE, astrocytes undergo dramatic morphological and functional alterations, which affects their sensitivity to glutamate and impairs their control of the extracellular glutamate concentration. Besides loss of gap junction coupling, glutamate transporters are replaced by ionotropic GluARs, particularly GluA1 flip leading to prolonged astroglial

depolarization[15,18,54]. This activates a vicious circle, in which extracellular glutamate increasingly accumulates, depolarizes glia, and generates neuronal hyperactivity[55].

ATP and purinergic receptors—Changes in extracellular ATP levels during seizures and epilepsy have been investigated in a number of studies with inconsistent results[56]. Among other reasons, this inconsistency is probably due to the difficulty of determining extracellular ATP levels because of its very short half-life and degradation to adenosine[56]. Nevertheless, using a luciferin-luciferase assay, Dossi and colleagues[57] demonstrated that extracellular ATP levels increase significantly during ictal discharges in cortical tissue samples from patients with drug-resistant epilepsy. ATP and adenosine play opposite roles in epilepsy. While it is well established that extracellular adenosine exerts anticonvulsive and neuroprotective effects by acting on pre- or postsynaptic A_1 receptors (A_1Rs), activation of purinergic receptors by elevated extracellular ATP levels promote seizures and epileptogenesis[43] (Fig. 2). In this context, the ionotropic P2X7R attracted much attention, as several studies indicate that its inhibition has anti-convulsive and neuroprotective effects[43,58]. As in human and rodent epileptic tissue P2X7Rs are mainly expressed by microglia and excitatory neurons (but not astrocytes) (Fig. 2), the protective effects of P2X7R inhibition are probably mediated by suppressing neuroinflammation and neuronal glutamate release[43]. However, the current data on the role of P2X7Rs in epilepsy are incomplete, partly contradictory and require further investigation. Among the metabotropic purinergic receptors, the Gq protein-coupled $P2Y_1$ subtype ($P2Y_1$R) has been implicated in epilepsy (Fig. 2). This receptor is activated by ADP and ATP, and is expressed by neurons, astrocytes and microglia[59]. Upregulation of $P2Y_1$R expression was shown in the hippocampus of human and experimental TLE[60]. Inhibition of $P2Y_1$R has anticonvulsant, antiepileptogenic and/or neuroprotective effects[59–63], possibly due to inhibition of $P2Y_1$R-mediated Ca^{2+}-dependent glutamate release from astrocytes[61–63]. $P2Y_1$Rs seem to be crucial in astrocyte-to-astrocyte propagation of Ca^{2+} waves and thus synchronisation of neuronal activity[63], and in regulating synaptic activity by autocrine astrocytic ATP signalling[61,62]. However, others have failed to detect $P2Y_1$Rs in astrocytes of epileptic tissue and suggested that the observed attenuation of inflammatory processes and antiepileptic effects were due to inhibition of microglial $P2Y_1$Rs[59,64]. Thus, although there are still several inconsistencies and unanswered questions about the role of astrocytic ATP in epilepsy, evidence is accumulating that this gliotransmitter is actively involved in genesis and/or progression of the disease.

GABA and extrasynaptic $GABA_A$ receptors—Loss of GABAergic interneurons and synaptic inhibition have been shown in the hippocampus of human and experimental TLE[65,66]. Despite this loss, several studies reported preserved or increased tonic (extrasynaptic) $GABA_A$ receptor ($GABA_A$R)-mediated currents in TLE, indicating high ambient GABA concentrations of non-neuronal origin[65–67]. Work from two groups suggested that in TLE mouse models, reactive astrocytes aberrantly overproduce GABA through *de novo* synthesis and/or decarboxylation of excess glutamate (Fig. 2). After its release into the extracellular space, possibly through Best1 channels, the gliotransmitter activates tonic $GABA_A$R-mediated currents in excitatory neurons and reduces seizure susceptibility (Fig. 2), thus apparently compensating for the loss of interneurons[66,67].

Summary—Overall, the above evidence underscores reactive astrocytes may release either excitatory or inhibitory gliotransmitters in epilepsy. A future challenge is to dissect out the involvement of each of these opposite functions, and which one might prevail, during epilepsy development. Additionally, although abnormal release of excitatory gliotransmitters seems to promote seizures and neurodegeneration, it might not itself be sufficient to trigger the pathology. It is conceivable, however, that in line with other astrocytic dysfunctions, it constitutes a main cause of some forms of epilepsy. For instance, loss of astrocytic gap junctional coupling and the consequential impairments in K^+ and glutamate homeostasis are critically involved in the initiation and progression of TLE[18]. Accordingly, enhanced gliotransmission in concert with impairment of homeostatic functions of astrocytes might drive neuronal networks into epileptiform activity.

Therapeutic potential of targeting gliotransmission

The need of alternative strategies for efficient therapies in patients with pharmacoresistant epilepsy provided a rational to determine whether glial dysfunctions, such as aberrant gliotransmission, represent promising therapeutic targets[43,53]. Work by Tian et al[47] showed that the antiseizure medications (ASMs) valproate, gabapentin, and phenytoin suppress astrocytic Ca^{2+} signaling during experimental seizures suggesting that these drugs exert part of their therapeutic effects by inhibiting gliotransmission. Several pharmacological agents targeting different sites of pathological gliotransmitter release affected epileptogenesis in experimental models[43,53]. In this context, the P2Y1R antagonists MRS2179 or MRS2500 were repeatedly shown to inhibit Ca^{2+}-mediated gliotransmission and also to exert antiseizure, antiepileptogenic and neuroprotective effects in experimental models, at least when administered after status epilepticus (SE)[59,61–63], a recurrent seizure event that triggers epilepsy. Dossi et al[57] reported that high K^+-induced ictal discharges in postoperative human epilepsy brain tissue cause ATP release through Panx1 channels, although it remained unclear in that study whether ATP was released by neurons or astrocytes. Inhibition of these channels with probenecid or mefloquine (approved drugs for the treatment of gout and malaria) efficiently blocked ictal discharges in human cortical brain tissue slices and displayed anticonvulsive effects in a mouse model of acquired epilepsy[57].

Deregulation of astrocytic metabotropic glutamate receptors (mGluR) has been described in epilepsy[68,69]. In particular, mGlu1R and mGlu5R antagonists (LY456236, LY367385, MPEP) were reported to decrease neuronal excitability in an in vitro model of focal seizures and in ex vivo brain slices from kindled rats, probably by attenuating glutamate release from both astrocytes and pre-synaptic terminals[50,61]. P2X7R antagonists, such as A438079[58] or JNJ-47965567[70], reduced SE severity and the consequent neurodegeneration or chronic seizures, respectively, in a kainate model of epilepsy, presumably by inhibiting neuroinflammation (see also Ref[71]). Drugs increasing GABA expression or release from astrocytes may also be of therapeutic interest in epilepsy, but to our knowledge such studies have not yet been conducted. Thus, although there is experimental evidence supporting the therapeutic potential of targeting gliotransmission in epilepsy, no compounds affecting this process have yet been investigated in clinical trials. This is likely because the mechanisms of action of the compounds showing anticonvulsive/antiepileptogenic effects in experimental models are often not well understood, or drugs are non-specific or have poor brain

penetration and side effects[57]. A deeper understanding of the mechanisms and impact of astrocytic gliotransmitter release in the normal and diseased brain is needed to allow the development of more specific drugs with clinical relevance for the treatment of epilepsy.

Cell metabolism

As early as the late 1970s it was already recognized that “the dynamic system that controls glucose and lipid metabolism, and thus electrolyte balance, is abnormal” in persons with epilepsy[72]. It is now well established that seizures cause profound changes in metabolism, and that maladaptive changes in biochemical pathways contribute to seizure generation and the development of epilepsy and its progression[73,74]. Astrocytes play a key role in the regulation of biochemical pathways related to adenosine, glucose, glutamate and GABA metabolism, and transmethylation (Fig. 3), which constitute a new frontier for therapy development with the prospect to not only treat seizures, but to treat epilepsy, its development, and its underlying comorbidities more comprehensively[75].

Dysregulation of adenosine metabolism

Adenosine is an evolutionary ancient master regulator of energy homeostasis[76]. Any energy crisis broadly leads to a drop in ATP and a resulting increase in adenosine, which acts as global inhibitor of metabolic and physiological activity. From an energetic standpoint, an epileptic seizure is a state of excessive energy consumption, which prompts the generation of adenosine acting as endogenous anticonvulsant and seizure terminator[77]. Adenosine-based inhibitory activity in the brain is largely mediated by activation of G_i protein coupled adenosine A_1 receptors, which can be blocked by the non-selective adenosine receptor antagonists caffeine and theophylline which therefore can have proconvulsant properties[78]. Maladaptive overexpression of the largely astrocyte-based adenosine metabolizing enzyme ADK has emerged as a pathological hallmark of experimental and human TLE[79]. Specifically, in multiple epilepsy models, reactive astrogliosis is associated with an overexpression of ADK and the resulting adenosine deficiency is not only sufficient to trigger seizures[80] but also contributes to the epileptogenic process[81]. Consequently, adenosine augmentation is an effective therapeutic strategy, not only for the suppression of epileptic seizures[82], but also to interrupt epileptogenesis[81]. Therefore, a derangement in adenosine metabolism affects the pathophysiology of epilepsy broadly and offers new metabolism-based therapeutic options, which directly interfere with the epileptogenic process[83].

Dysregulation of glutamate, glutamine, and GABA metabolism

An additional astrocyte based metabolic enzyme of importance for the pathophysiology of epilepsy is GS. This enzyme aminates glutamate into glutamine, which, after transport into inhibitory or excitatory neurons, can be converted into GABA or re-converted into glutamate, respectively. Therefore, GS plays an important role in the regulation of the glutamate and GABA balance, which is under metabolic control because GABAergic neurons are more sensitive to an hypoenergetic state[11,84]. In line with this mechanism, loss of GS activity in human TLE can cause seizures and supports the epileptogenic process by promoting *(i)* an increase in extracellular glutamate, regulated by astrocytes, and *(ii)*

a decrease in GABA production in neurons because of a decrease in glutamine supply[11]. Specifically, neuroinflammation through oxidative stress inhibits GS activity suggesting that these processes may affect GS in epilepsy[27]. Dysregulation of extracellular glutamate is further exacerbated by impaired astrocytic glutamate clearance through the glutamate transporter-1 (GLT-1), which is downregulated in the epileptic brain[18,54,85]. Indeed, the lack of GLT-1 in mice causes epilepsy and increased brain injury[86]. Together, the consequences of this metabolic derangement contribute to both ictogenesis and epileptogenesis.

Role of the astrocyte-neuron lactate shuttle

Epileptic neuronal networks have a high demand for energy. Excessive synaptic activity stimulates astrocytic glycolysis causing a rapid drop of glucose and a corresponding rise in lactate, which becomes an essential energy source for neurons. Increased ictal glycogen and glucose utilization lead to the formation of lactate from pyruvate through lactate dehydrogenase (LDH). Through this mechanism, astrocytes can potentially provide a significant supply of energy via the astrocyte-neuron lactate shuttle to sustain the energy demands of hyperactive neuronal networks[87]. The hypothesis that seizure-induced lactate formation enables epileptic activity is supported by findings that LDH inhibitors such as stiripentol provide robust anti-seizure effects[88]. In this scenario, LDH inhibition likely interferes with glycolysis by limiting the availability of NAD^+ and supporting the oxidative mitochondrial metabolism of pyruvate. However, a recent study demonstrated that both astrocytic glycolysis and the astrocyte-neuron lactate shuttle depend on the energy sensor astroglial AMP activated protein kinase (AMPK)[89]. The genetic deletion of AMPK in mice led to the depletion of lactate and a reduction in seizure threshold, whereas the deletion of AMPK in astrocytes, but not in neurons, triggered neuronal cell loss in mice and flies[89]. This apparent contradiction demonstrates the complexity of astrocyte metabolism in the regulation of lactate and brain energy homeostasis, which are of crucial importance for the balance of seizure thresholds.

Dysregulation of transmethylation reactions in epilepsy

The methylation hypothesis of epileptogenesis posits that maladaptive changes in DNA methylation drive and propagate the epileptogenic process[90]. Indeed, multiple studies show that epigenetic processes and in particular increased DNA methyltransferase activity and aberrant DNA methylation are intricately linked to the epileptogenic process[81,91]. In surgically resected hippocampal and neocortical specimens from patients with TLE and hippocampal sclerosis, progressive changes in DNA methylation were associated with prolonged epilepsy duration and induction of genes related to inflammatory processes, lending further support to the notion that aberrant DNA methylation is linked to the epileptogenic process[92]. Metabolically, all transmethylation reactions, including DNA methylation, are linked to adenosine metabolism through ADK[81,93]. The genetic disruption or pharmacological inhibition of ADK leads to an increase in adenosine, which shifts the thermodynamic equilibrium of the S-adenosylhomocysteine hydrolase reaction towards the formation of S-adenosylhomocysteine, a potent inhibitor of DNA methyltransferases. Conversely, pathological overexpression of ADK as part of the epileptogenic process, drives increased DNA methylation and thereby promotes epileptogenesis[81].

Metabolic therapies for epilepsy and its prevention

Because astrocytes play a key role as metabolic master regulator of brain activity, and because maladaptive changes in metabolism and basic biochemistry are linked to seizure generation and the epileptogenic process, astrocytes are attractive targets for metabolic therapies[75] (Fig. 3).

Ketones, fatty acids, and glucose restriction—The most widely used metabolic therapy is a high-fat, low-carbohydrate ketogenic diet, which forces the brain to use ketone bodies rather than glucose as the main energy source, and has been in clinical use for a century. Ketogenic diets enhance ketone body production in the liver, but astrocytes constitute a major site for fatty acid oxidation in the brain and they are the only cell types in the brain capable of producing ketone bodies. Through multiple mechanisms directly or indirectly linked to glucose restriction and elevated ketone bodies[94], ketogenic diets and related metabolic therapies suppress seizures in a wide spectrum of pharmacoresistant epilepsies[95]. Intriguingly, both in clinical studies, as well as in preclinical models, it was shown that ketogenic diet-based therapeutic interventions had lasting disease modifying, and possibly antiepileptogenic properties[83]. Of note, ketogenic diet therapy affects gene expression differentially in neurons and astrocytes, with a marked reduction of astrocytic gene expression related to pathways controlling glutamatergic signalling, endoplasmic reticulum processing, and insulin signalling. Because glucose restriction is one of the mechanisms of ketogenic diets, the disruption of glycolysis with the glycolytic inhibitor 2-deoxy-D-glucose (2DG) has been considered as an alternative to a strict dietary regimen[96]. In rodent models 2DG exerts acute antiseizure effects[97] with the involvement of a presynaptic mechanism[98] and delayed kinding development in rats[97]. Similarly, in a controlled cortical impact model of neurotrauma in mice, 2DG attenuated the development of epileptiform activities in *in vitro* cortical slice preparations and after one week of *in vivo* treatment immediately after injury, supporting disease modifying properties of metabolic therapies[99]. Together those findings support the notion that glucose restriction rather than ketone production provides key therapeutic benefits of metabolic therapies. This is important, because astrocytes take up glucose from the blood capillaries via the glucose transporter GLUT-1 and thereby assume a role as gatekeeper for energy homeostasis in the brain.

Adenosine for epilepsy prevention—An additional, ketone-independent mechanism of ketogenic diet therapy is an increase in adenosine, a multifunctional metabolite controlled by astrocytic metabolic clearance, in conjunction with downregulation of ADK, which is a likely explanation for the disease modifying properties of metabolic therapies[100,101]. Because maladaptive increases of ADK expression in astrocytes drive the epileptogenic process through increased DNA methylation, therapeutic adenosine augmentation is a rational approach for epilepsy prevention. Thus, adenosine delivered via cell-based brain implants suppressed both kindling epileptogenesis and the development of chronic seizures in kainate induced epilepsy models[80,102,103]. Adenosine delivered locally and transiently to the hippocampus of rats after the onset of epilepsy via silk-based implants prevented epilepsy progression[81], and a transient systemic dose of a small molecule ADK inhibitor attenuated the epileptogenic process in mice after an intrahippocampal injection of

kainate[104]. Because one isoform of ADK is specifically expressed in the cell nucleus (ADK-L) the opportunity exists to directly target nuclear adenosine metabolism in an effort to capitalize on the epigenetic mechanisms of adenosine, while minimizing excessive rise of adenosine in the extracellular space. Importantly, a transient treatment with adenosine (for ten days after the onset of epilepsy in rats) or an adenosine elevating drug given transiently during the latent period of epileptogenesis (for five days beginning with a delay of three days after an epilepsy triggering SE) was found to provide lasting suppression of epilepsy progression and development, respectively[81,104]. Early intervention would also mitigate additional risk factors associated with progression of epilepsy such as sudden unexpected death in epilepsy (SUDEP) and the development of comorbidities and ASM resistance. The epilepsy preventing properties of adenosine are in line with clinical findings showing that a single nucleotide polymorphism in the *Adk* gene constitutes a biomarker for increased risk of posttraumatic epileptogenesis after a traumatic brain injury[105]. Together, those studies demonstrate that transient metabolic treatments and strategies, which decrease astrocytic adenosine metabolism and thereby increase adenosine availability have the unique potential to disrupt the epileptogenic process via an epigenetic mechanism[75,83]. The transient use of preventative treatments would mitigate adverse effects associated with chronic treatment regimens in established epilepsy.

Immune and inflammatory functions

Astrocytes have been shown to initiate, regulate, and amplify immune-mediated mechanisms in different CNS diseases[5,26]. These cells represent a source of immunologically relevant cytokines and chemokines in both human and experimental epilepsy[5,26,27]. Reciprocally, an inflammatory phenotype in astrocytes can be induced by soluble molecules (cytokines, chemokines, danger signals) either released by surrounding parenchymal cells, like reactive microglia[106,107] or the pericytes and the microvasculature[108,109], or imported by blood leukocytes[110]. Spatio-temporal dynamics of glia activation in rodents after SE showed rapid microglia reactivity (within 24 h) leading to subsequent activation of astrocytes that exhibit IP3 R2-mediated Ca^{2+} hyperactivity[24], and express inflammatory molecules[25]. Notably, these reactive astrocytes are essential for promoting chronic enhanced seizure susceptibility after SE[24]. Increased neuronal activity is sufficient to elicit biosynthesis and release of pro-inflammatory molecules by astrocytes, and from other brain cells, a mechanism defined “neurogenic neuroinflammation”[111]. In support, activated neurons release danger signals such as HMGB1[112,113], ATP[114], prostaglandins[115], and complement factors[116,117] that activate their cognate receptors in astrocytes thereby inducing their inflammatory phenotype. Pattern-recognition receptors (PRRs) are innate immunity receptors activated by endogenous danger signals which are pivotal for the induction of the inflammatory cascade in glia and peripheral immune cells[118].

Immunohistochemical and large-scale transcriptomic studies in human brain tissue from TLE patients, and epileptogenic developmental pathologies, together with studies in animal models, showed induction of various inflammatory pathways in reactive astrocytes[5,26,27], some of which contribute to hyperexcitability and seizures, as discussed later.

Below we describe the astrocyte innate immunity properties relevant for seizure generation, cell loss and neurological comorbidities in epilepsy.

Astrocyte dysfunction linked to their inflammatory state

Here we focus on the inflammatory phenotype of astrocytes, considering both cell autocrine and paracrine responses to cytokines and danger signals released following epileptogenic lesions or during recurrent seizures. Astrocytes are key players to sustain the neuroinflammatory response in epilepsy as shown by their transcriptomic profiles and by histological co-localization of inflammatory markers with astrocyte-specific markers[27]. The key role of the crosstalk between astrocytes and neuronal and non-neuronal cells, in particular microglia and the microvasculature, in hyperexcitability and seizure generation has been confirmed in genetic and acquired epilepsies, and most recently in glioneuronal tumor-related epileptogenesis[5,26,119].

The following sections describe the neuromodulatory effects of inflammatory mediators expressed by astrocytes, and how inflammatory astrocytes lose their homeostatic functions[5,26,120], thus contributing to an extracellular *milieu* permissive to hyperexcitability and cell death.

Neuronal hyperexcitability driven by cytokines and chemokines—Cytokines and chemokines possess neuromodulatory properties evoked by their neuronal receptors [118,121]. In normal brain physiology, these immunity-related molecules play a role in synaptic transmission and plasticity[121], however, upon overproduction they can induce network hyperexcitability and reduce seizure threshold by modifying the expression and the function of voltage-gated and receptor-coupled ion channels[27,121]. Cytokines released after brain injury or hyperthermia may induce astrocyte dysfunctions, including block of gap junction coupling, which results in impaired ion and transmitter homeostasis and the generation of spontaneous recurrent seizures[18,122,123].

BBB dysfunction—Astrocytic endfeet ensheath blood vessels providing, together with endothelial cells and pericytes, structural barriers that promote vascular integrity and function[124]. Astrocytes contribute to the maintenance of tight-junctions between endothelial cells and regulate movements of water, soluble molecules, and immune cells across brain parenchyma. BBB permeability properties are altered in acquired epilepsies and this phenomenon occurs early on, and persists, after the epileptogenic injury in animal models[124], and is often associated with newly generated leaky vessels[125]. Perivascular astrocytes are pivotally involved in structural and functional changes of the microvasculature in epilepsy by releasing cytokines, such as vascular endothelial growth factor (VEGF), IL-1β, HMGB1 and TNF, chemokines and downstream effectors, which activate their receptors in pericytes and endothelial cells of microvessels[108,126]. As a consequence, BBB permeability is affected at multiple levels, including disruption of tight-junctions, increased transendothelial vesicular transport, and leukocyte trafficking. A converging consequence of BBB dysfunction in epilepsy is brain extravasation of serum albumin and the subsequent activation of transforming growth factor receptor 2 (TGFβRII)-SMAD signaling in perivascular astrocytes. This leads to transcriptional activation of inflammatory genes and

downregulation of Kir4.1, GLT-1, and AQP4 density as well as subcellular reorganization of Cx43 in astrocytic endfeet[124,127]. This chain of events reduces seizure thresholds and promotes epilepsy development in rodents. In accord, pharmacological blockade of TGFβRII-SMAD signaling for 3 weeks post-SE rescued some of the molecular changes in astrocytes and reduced epilepsy incidence and chronic seizure frequency[124] (but see Ref[128]). Mimicking BBB dysfunction in rodents is sufficient to induce epileptogenesis[124]. Albumin induces TGFβ1 and inhibits astrocyte gap junction coupling[129], thus reinforcing signaling activation, and elicits reactive astrogliosis through mitogen-activated protein kinase (MAPK) pathways[130].

The close contact of astrocytes with BBB constituents allows the sensing of immune molecules released by circulating leukocytes and facilitates astrocyte and peripheral immune cell interactions, which may shape the astrocyte activation state and function.

Extracellular matrix and synaptic remodeling—Inflammatory astrocytes in human TLE and related animal models express high levels of matrix metallopeptidase family proteins under the control of TGFβR[131] and IL-1β signalings[132,133], thereby inducing extracellular matrix remodeling and pathological synaptic plasticity. In particular, astrocytes mediate excitatory synaptogenesis *in vitro*, and *in vivo* before epilepsy develops, by activation of TGFβR signaling[124] and they are possibly involved in axonal sprouting[134], and are implicated in the degradation of perineuronal nets around fast-spiking inhibitory interneurons, as shown in brain tissue from TLE patients and rodent models of acquired epilepsy[124,135,136]. These modifications are associated with hyperexcitability and spontaneous seizure generation, and they are prevented by TGFβR signaling blockers which also inhibited epilepsy development in animals[124,136–138] (but see Ref[128]). Notably, endothelial cells activated *in vitro* by inflammatory stimuli can induce an extracellular matrix remodeling profile in astrocytic primary cultures[109].

Treatment with an MMP2/9 inhibitor reduced seizure severity in the rapid-kindling model and decreased the number of chronic spontaneous seizures and cognitive deficits in the kainate model during seven days treatment post-SE and up to seven weeks after drug withdrawal[133].

Oxidative stress—Neuroinflammation and oxidative stress (OS) are linked phenomena, and the “redox proteome” is intimately related to immune responses. There is evidence for the redox-based control of hyperexcitability and seizures in epilepsy[139], which mainly involves astrocytes. Astrocytes are major producers of glutathione (GSH) from glutamate, cysteine and glycine, and the astrocyte-neuronal GSH shuttle replenishes the neuronal GSH pool. This provides a major antioxidant defence mechanism in the brain by neutralising reactive oxygen species (ROS) and hydrogen peroxide[140]. Biomarkers of OS [e.g., inducible nitric oxide, cysteine transporter (xCT), transcriptional nuclear factor Nrf2] are increased in astrocytes of rodent brain during epileptogenesis and human and rodent chronic epileptic foci[141,142]. Astrocytic mitochondrial function seems to be maintained during seizures, when neuronal mitochondria depolarize and fail[143] suggesting that astrocytes provide sustained antioxidant defence. Moreover, increased neuronal firing activates astrocytic Nrf2, thus promoting antioxidant, anti-inflammatory and cytoprotective effects[144]. These homeostatic

functions of astrocytes co-exist with their inflammatory features, in agreement with the complex transcriptomic spectrum of astrocytes described during epileptogenesis in a SE mouse model[25], and in other CNS diseases[7,107]. To add to this complexity, a given molecular alteration may result in opposing functional outcomes. For example, elevated extracellular glutamate is a key contributor to cortical hyperexcitability, epileptiform activity, and excitotoxicity[145]. In addition to GLUT-1, extracellular glutamate is also controlled by the xCT antiporter system. Increased expression and activity of xCT in astrocytes not only promotes GSH biosynthesis but may also result in enhanced extracellular glutamate levels, which correlates with glioma-associated epilepsy[146]. Accordingly, sulfasalazine, the FDA-approved drug blocking xCT, reduces established seizures *in vivo*[147].

Preclinical studies demonstrate that the altered redox status in epilepsy is sensitive to therapeutic targeting by activation of the Nrf2 pathway with brain-penetrant small molecules or replenishing the GSH pool with N-acetylcysteine. These interventions decrease established chronic seizures, but also arrest disease progression when administered after SE and before epilepsy develops, also affording neuroprotection and improvement of cognitive deficits[27,141,142]. This evidence supports that antioxidant defense mechanisms are inefficient in epilepsy, therefore their implementation may offer new therapeutic opportunities.

Dysregulation of iron metabolism—Recent evidence from genetic epilepsies due to mTOR pathway dysregulation, indicates that OS and iron metabolism are tightly linked and may act synergistically to exacerbate cell dysfunction in epilepsy[148]. Iron is a key element for CNS development and proper function, and astrocytes are crucial players for maintaining iron homeostasis[149]. Accordingly, high concentrations of unbound Fe^{2+} enhance the generation of ROS, thereby promoting cell dysfunction or death[150]. Intracerebral hemorrhages after stroke or neurotrauma, and the consequent epilepsy, are characterized by brain iron deposition, and intracortical iron injection evokes seizures in experimental models[150]. Moreover, BBB dysfunction may contribute to brain iron overload by leakage of iron-rich blood components. A recent study addressed iron metabolism-based alterations in surgically resected brain specimens from patients with TLE or in autoptic tissue from patients who died after SE, and in a rat model of TLE[151]. The study showed that iron overload is likely due to BBB leakage and it accumulates in neurons with pycnotic morphology, in microglia and astrocytes, the latter cells showing also elevated ferritin levels. Human astrocyte cultures challenged with iron and ROS increased their antioxidant and iron-binding capacity, but simultaneously developed pro-inflammatory features upon chronic exposure[151]. Thus, reducing iron uptake into astrocytes should reduce their inflammatory state with potential therapeutic effects in epilepsy[149,150].

Mechanisms of drug-resistance—Release of glutamate and inflammatory mediators by perivascular astrocytes may contribute to up-regulation of multidrug transport proteins on BBB endothelial cells. These proteins, in particular p-glycoprotein (Pgp), are overexpressed at the luminal side of endothelial cells and astrocytic endfeet in TLE patients and in developmental glioneuronal lesions[152]. Pgp overexpression may limit access of several ASMs to their brain targets thus reducing their therapeutic effects and contributing to drug-resistance[152]. A strict association was found between astrocytic COX-2, prostaglandin

E2 and Pgp expression in microvessels in brain specimens from TLE patients and animal models[153,154]. Notably, COX-2 inhibitors prevented an increase in Pgp expression and function in rats with chronic seizures, and reversed ASM-resistance in these rats[154].

Antiinflammatory therapies

Interventional studies in rodent models of epilepsy helped to establish that the activation of specific inflammatory pathways in brain cells lowers neuronal excitability threshold thereby promoting seizures, and reciprocally the ensuing seizures perpetuate neuroinflammation[27]. In particular, specific antiinflammatory treatments, also including anti-oxidant drugs, were shown to decrease both acute and chronic seizures in animal models[27]. Additionally, such drugs, transiently administered after SE either before the clinical onset of epilepsy or right afterwards, could interfere with epileptogenesis thus mediating strong reduction in ensuing seizures, neuroprotection and in some instances also improving cognitive deficits[27,155]. Since the molecular targets of these treatments are the ictogenic inflammatory molecules induced in reactive astrocytes, this cell population is likely to be significantly involved in the observed therapeutic effects.

Some drugs tested in animals are already in medical use for autoinflammatory or autoimmune diseases and are well-tolerated, therefore, they may be considered for treating patients with drug-resistant epilepsy, or individuals at high risk of developing seizures after acute brain injuries[27,28]. One recent example of the therapeutic response is represented by the compassionate use of the antiinflammatory drug anakinra, the human recombinant IL-1 receptor antagonist, in patients with unremitting seizures due to new onset refractory SE[27,156], or the traditional use of immunosuppressive steroids in pediatric epileptic encephalopathies[27].

In vivo monitoring of astrocyte reactivity

The availability of minimally invasive tools to monitor astrocyte activation *in vivo* may both help to better understand the astrocyte involvement in the pathophysiology of epilepsy and to discover biomarkers for epilepsy outcomes. To meet these goals, two complementary approaches have been developed in animals and humans, namely *in vivo* imaging of reactive astrocytes and measurement of astrocyte-associated molecules in biofluids (Fig. 4). These two approaches, and related tools and applied methodologies, permit in principle to monitor astrocytic activation during epileptogenesis in the same animal, and possibly in humans, and to investigate whether therapeutic interventions have an impact on astrocyte reactivity. Additionally, they may provide epilepsy biomarkers as diagnostic tools to identify an epileptic focus, to predict the effect of new treatments, or to prospectively identify the risk of developing epilepsy and pharmacoresistance. However, the evidence for the use of astrocyte-based biomarkers of epilepsy is currently still weak. In particular, the majority of relevant studies did not include a receiver operating characteristic (ROC) analysis of the results, the sample size of the analyzed cohorts is generally small, and validation cohorts for candidate biomarkers have been used rarely.

Moreover, the specificity of potential biomarkers for epilepsy compared with other CNS diseases has not been validated. Whether these candidate markers are useful for

monitoring epileptogenesis in humans has not been tested as yet. Despite these limitations, neuroimaging and blood proteins have great potential as biomarkers due to minimal invasiveness, accessibility, and feasibility of quantification.

Imaging of reactive astrocytes

Human epilepsy.—Proton magnetic resonance spectroscopy (^{1}H-MSR) and positron emission tomography (PET) are imaging techniques used in epilepsy patients to help identify the epileptogenic zone. ^{1}H-MSR identifies various cell metabolites including myo-Inositol (mIns) which is considered an astrocyte marker[157]. ^{1}H-MSR coupled with GFAP immunohistochemistry showed that increased mIns levels are associated with reactive astrogliosis[157], supporting the view that mIns level mirrors alterations in astrocyte activation. mIns was increased in the epileptogenic zone of patients with structural epilepsies, namely TSC, Rasmussen's encephalitis and TLE[158–160]. However, hippocampal mIns variably changed in TLE patients compared to non-neurological controls[161] which may be due to the plethora of ASMs, the different ^{1}H-MSR protocols used in the studies, or differences in seizures between the cohorts analysed. This variability may hinder the usefulness of mIns measurement to help detecting the epileptic focus.

An alternative molecular target of reactive astrocytes that has been tested in epilepsy is monoamine-oxidase B (MAO-B), an enzyme induced in astrocytes in pathological conditions. The autoradiographic binding of [^{3}H]-L-deprenyl, an irreversible MAO-B inhibitor, was increased in surgical hippocampal tissue from TLE patients versus autopsy controls, and it correlated with histologically assessed reactive astrogliosis[162]. This study prompted PET development using [^{11}C]Deuterium-deprenyl as a diagnostic tool for localizing the epilepsy focus. [^{11}C]Deuterium-deprenyl signal was increased in the sclerotic hippocampus of TLE patients compared to non-neurological controls[163,164], and the identification of the epileptic focus was equivalent to PET with [^{18}F]FDG, the gold standard approach as determined in a subsequent validation study[163]. The diagnostic value of [^{11}C]Deuterium-deprenyl was confirmed by single-photon emission computed tomography using the MAO-B inhibitor [^{123}I]Ro43–0463[165]. The histopathological examination of the epileptogenic zone confirmed the presence of reactive astrogliosis. However, differently from seizures originating in the temporal lobe, the [^{11}C]Deuterium-deprenyl signal was unchanged in patients with neocortical epilepsy thus highlighting potential mechanistic differences in glial functions or in the type of reactive gliosis between the two clinical conditions[163]. Optimized MAO-B-based PET tracers were recently developed in patients with psychiatric or neurodegenerative conditions[166] fostering further application in epilepsy.

Additional astrocyte-based PET molecular targets were developed in chronic neurodegenerative diseases which may inform about their potential use in epilepsy. Examples are the imidazoline$_2$ binding sites (I_2BS) localized in the outer membrane of mitochondria in astrocytes[167] and which regulate GFAP expression[168], and adenosine A_{2A} receptors expressed by reactive astrocytes in the hippocampus of TLE patients[169]. Notably, I_2BS and A_{2A} PET tracers were used to map disease progression in multiple sclerosis, Alzheimer's disease and Parkinson disease[166,170–172].

Experimental models.—^{1}H-MSR was applied to experimental models of acquired epilepsy to measure mIns levels during epileptogenesis as an index of astrocytic reactivity to be correlated with pathological outcomes. In rat models of SE-induced epileptogenesis, hippocampal mIns increased within days after the acute injury[173] and before the onset of spontaneous seizures[174]. The latter study established a negative correlation between mIns level and the extent of neuronal cell loss in epileptic rats [174]. Morever, hippocampal mIns level predicted the onset of epilepsy in a SE rat model with 65% epilepsy incidence, and ROC analysis provided a measure of good predictive accuracy (AUC=0.83)[175]. This set of evidence suggests mIns is a potential prognostic biomarker of epileptogenesis which could be validated in humans exposed to epileptogenic injuries.

One study used [^{18}F]-deprenyl autoradiography in *ex vivo* brain slices from SE-exposed rats for investigating astrocytic activation during epileptogenesis[176]. It was found that the signal increased in the epileptic circuitry during disease progression supporting further PET-based approaches.

Whether *in vivo* astrocyte imaging may help to develop pharmacodynamic biomarkers of drug effect is still unexplored. In support, neural stem cell transplantation, which provides anti-inflammatory effects in a mouse model of Alzheimer's disease, was associated with both decreased astrocyte proliferation and mIns levels as assessed by ^{1}H-MRS[177].

Additional tools could be developed for monitoring astrocyte reactivity by exploiting existing imaging modalities that include astrocyte's contribution to the generation of MRI or PET signal (Box 2).

Measurements of astrocyte-associated molecules in biofluids

The majority of studies on circulating markers of astrocytes measured GFAP and S100β in blood or CSF with the intent of identifying potential biomarkers for epilepsy. The cerebral origin of these proteins was supported by studies showing increased CSF levels of GFAP in children with epilepsy of various aetiologies[178] or S100β in adult TLE patients[179] compared to respective controls. Since S100β is a releasable protein while GFAP is retained intracellularly in healthy astrocytes, their respective increase in biofluids is likely to reflect astrocyte reactivity or injury.

GFAP—Serum GFAP was higher in epilepsy patients with seizures of focal or generalized onset, although no correlation was found with seizure frequency or disease duration[180,181]. Notably, GFAP serum level was higher in patients with epileptic seizures compared with psychogenic seizures and controls[180] offering a potential diagnostic tool to differentiate between the two types of seizures (ROC analysis provided an AUC=0.68), thus reducing misdiagnosis and the need of video-EEG monitoring. Moreover, children with epilepsy and well controlled seizures also displayed increased GFAP serum level vs controls, suggesting this protein may be a marker of the underlying pathology, rather than purely reflecting cell damage due to recurrent seizures[181].

Recent studies reported anti-GFAP antibodies in blood -in the absence of neuronal antibodies- in ~5% of children and adult epilepsy patients[182]. This autoimmune response

may occur in predisposed individuals as consequence of GFAP elevation in peripheral blood. Whether this is a bystander effect of seizures or contributes to epileptogenesis needs further investigation.

S100β—A systematic review and meta-analysis showed that blood level of S100β was significantly higher in epilepsy patients compared to healthy controls[183]. In particular, serum S100β levels were more elevated in TLE patients with hippocampal sclerosis compared to those without hippocampal sclerosis[184], possibly reflecting the extent of reactive astrogliosis and the structural damage in the epileptic focus. Interpretation of these data, however, has to consider that S100β is also expressed by other cell types in the brain, including neurons and NG2 glia[185].

Conclusions

Rapidly growing evidence from both human epilepsy and animal models has linked astrocyte dysfunction to neuronal network perturbations leading to seizures and cell loss. Since astrocytes are broadly involved in brain homeostasis, and oversee large number of synapses, their functional alterations may lead to widespread and progressive changes in neuronal network activation, thus contributing to the development and aggravation of epilepsy. Most of our knowledge on the role of astrocytes in epilepsy is derived from animal models, which have the unique advantage to offer the possibility of monitoring the epileptogenic process longitudinally, and which allow for the identification of commonalities in the pathogenic and/or homeostatic processes across models. However, it is important to consider that any preclinical work may have implicit bias based on the experimental rigor of each individual study as it pertains to factors such as reproducibility, blinding, statistical power, age, genetic background, and sex[186]. Importantly, the pathological role of astrocytes was intensively studied in human drug-resistant focal epilepsies, thanks to the availability of resected brain tissue, which allowed the validation of animal model findings. In general, findings from animal studies are in agreement with validation studies from human resected epileptogenic tissue. However, information on the role of astrocytes in genetic forms of epilepsy, although plausible, remains still elusive (Box 3). Supportive evidence includes the effect of gliotransmission on thalamo-cortical circuitry that is involved in genetic generalized epilepsy[44], the evidence of reactive and inflammatory astrocytes in the somatosensory cortex of GAERS rats, an animal model of absence seizures[187], or dysfunctional and inflammatory astrocytes in mouse models of genetic epileptic encephalopathy[188], or Lafora progressive myoclonus epilepsy[189].

Although mechanistic investigations in epilepsy models highlight that several signaling pathways are modified in reactive astrocytes, there is still inconsistency and open questions (Box 3). This includes the role of ATP and purinergic receptors, or how the complexity of energy metabolism affects seizure threshold (cf. *related sections*), pointing out the need for further investigations. Moreover, the reactivity of astrocytes in epilepsy is accompanied by dynamic transcriptomic and molecular changes, reflecting complex combinations of compensatory and detrimental phenotypes during epileptogenesis. This is exemplified by inflammatory and OS mediators that may coexist with anti-inflammatory and detoxification molecules in astrocytes from the same brain area, and in some instances are co-expressed

by the same cells[27,190]. Another point of consideration refers to the context-specific role of some signaling pathways activated in astrocytes, such as the dual role of P2Y1 receptors or COX-2 and prostaglandins in exacerbating or reducing seizures depending on timing of activation in the epilepsy model[59,191]. Single cell transcriptomics and accompanying functional approaches may help to clarify these aspects that are crucial for better understanding the consequences of astrocyte reactivity in epilepsy, and to guide focused therapeutic interventions. In order to elucidate the dynamic role of astrocytes in the pathogenesis of epilepsy, innovative cell-specific approaches may be used, which include gene therapy to express light-sensitive opsins that allow to modulate cell activation[192], or gene editing with CRISPR-CAS technologies[193], or chemogenetic compounds that selectively target a genetically-modified G protein-coupled receptor (DREADD) or a chimeric ion channel (PSAM)[194]. These new approaches have been mostly used for modulating neuronal activity but they can be extended to glial cells, and possibly envisaged as therapeutic interventions, although their clinical application is still remote.

Notably, before drugs targeting astrocyte dysfunctions are developed and validated in the clinic, it is crucial to elucidate the proper time of treatment initiation and duration during epilepsy development and to select the most suitable patient population. Animal models can help determining the dynamics of astrocytic changes and their consequences for pathological outcomes, but there are limitations for translating these animal-based dynamics to the clinical context. The identification of imaging biomarkers of astrocyte activation/reactivity states, or biofluid signatures reflecting astrocyte dysfunctional state (as discussed above), may help for designing effective treatment schedules and for patient stratification in clinical studies. However, validation of such biomarkers is required in prospective studies in larger cohorts of well-phenotyped patients with differing epilepsy etiology.

In the near future, astrocyte related research may open new opportunities to develop a largely unexplored therapeutic niche with more selective drugs specifically targeting pathological mechanisms, with good BBB penetration, and limited side-effects (Box 2). These may include modulators of gliotransmission and gap junction coupling, metabolic therapies, anti-inflammatory/anti-oxidant treatments, and drugs mitigating BBB dysfunction. A resolution of those processes that chiefly involve astrocytes during pathogenesis may offer new therapeutic opportunities for patients with pharmacoresistant epilepsies, and more broadly in CNS diseases associated with astrocytes dysfunctions[195].

Acknowledgements

The authors gratefully acknowledge their sources of support Era-Net Neuron Ebio2, and American Epilepsy Society Seed Grant and NORSE Institute (AV), the Associazione Italiana Contro l'Epilessia (AICE-FIRE) (TR). DB is funded through the National Institutes of Health (NIH grants NS103740, NS065957, NS127846, the Office of the Assistant Secretary of Defense for Health Affairs through the Epilepsy Research Program under Award No. W81XWH2210638, and a Catalyst Award from CURE Epilepsy. CS acknowledges support by grants from the EU (H2020-MSCA-ITN project No 722053 EU-GliaPhD) and BMBF (16GW0182 CONNEXIN, 01DN20001 CONNEX). AE acknowledges support by grants from the EU (H2020-MSCA-ITN project No 722053 EU-GliaPhD; H2020-Twinning project EpiEpiNet (No 952455) and the Dutch Organization for Medical Sciences (ZonMw).

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BOX 1.

Gliopathy in genetic epilepsy

Epilepsy is a frequent neurological feature of neurodevelopmental disorders associated with gene mutations[196]. Astrocyte reactivity is commonly observed with a spectrum of complex morphological and functional changes[119,188]. Thanks to rapid advances in genetic technology, various studies described the astrogliopathology associated with specific genetic mutations, emphasizing the aberrant neuro-glia crosstalk, as well as interglial communication contributing to the clinical phenotype. Examples are provided below.

Tuberous sclerosis complex (TSC) represents the prototypic monogenic disorder of the mammalian target of rapamycin (mTOR) pathway dysregulation. TSC is typically characterized by benign tumors in diverse organs and neurological manifestations that include epilepsy, neurodevelopmental delay and neuropsychiatric disorders[197]. Astrocytes display a reactive phenotype associated with increased proliferation and enhanced expression of pro-inflammatory mediators in cortical tubers. Moreover, astrocytes are characterized by decreased homeostatic functions related to ion homeostasis and neurotransmitter metabolism[119]. Understanding mTOR-related astrocyte dysfunction may offer new insights into common neurobiological mechanisms underlying epilepsy as well as the cognitive and behavioral comorbidities that are characteristic of the spectrum of mTOR-associated neurodevelopmental disorders[119].

Alexander disease is a fatal disorder of the CNS typically characterized by macrocephaly, rapid neurological deterioration, seizures, spasticity and retarded psychomotor development. It represents a classical example of a primary astrogliopathy caused by a dominant mutation of the GFAP gene, resulting in various pathological features, including changes in cell morphology, and dysfunction[198] associated with the activation of cell stress and inflammatory pathways[19,198].

Aicardi-Goutieres syndrome is a rare genetically determined infantile encephalopathy characterized by progressive microcephaly, psychomotor retardation, and premature death. Astrocytes are key players in the phenotypic presentations through cell-autonomous and non-cell-autonomous mechanisms[199] contributing to the inflammatory response with increased interferon-α production[200].

The application of sophisticated research strategies (single nucleus RNA sequencing, spatial transcriptomics, human iPSC-derived astrocytes), represents an ideal approach towards a better identification of human astrocyte subpopulations and elucidation of the phenotypic spectrum of genetic epilepsies.

BOX 2.

Additional tools to monitor in vivo astrocyte reactivity

[^{18}F]FDG PET signal in human epilepsy foci is considered an index of neuronal glucose consumption during intense neuronal activity. Recent evidence re-evaluated the cell-specific contribution to [^{18}F]FDG PET signal by considering that astrocytes are critical for glucose uptake and for coupling neuronal activity to glucose utilization[201] (see above *astrocyte-neuron lactate shuttle*). This provides a new perspective for interpretation of [^{18}F]FDG PET signal changes in epilepsy.

Diffusion-weighted MRI (DW-MRI) allows to detect changes in water molecules trajectories in brain parenchyma consequent to alterations in cell volume and/or water circulation in the extracellular space. Since reactive astrocytes are often characterized by hypertrophic cell body, in principle changes in DW-MRI-associated measures may reflect astrogliosis. In this context, an advanced DW-MRI sequence was recently developed to image astrocytes and microglia reactivity to inflammatory stimuli in the gray matter of rats, by building a multicompartment tissue model based on glial cell morphology[202].

Functional MRI (fMRI) is based on the blood oxygenation level-dependent (BOLD) signal, which is considered a surrogate marker of neuronal activity. However, optogenetic astrocyte activation in the cortex of awake transgenic mice, whose cortical neurons or astrocytes express channel rhodopsin-2, evoked an fMRI BOLD response in the absence of changes in local neuronal activation[203]. This response was associated with oxidative glucose metabolism at the site of astrocyte, but not neuron, evoked signal, as assessed by mass spectrometry in brain sections. This evidence implies that astrocytes contribute to the generation of the BOLD signal, although the extent of this contribution in pathophysiological conditions awaits further investigation for a correct interpretation of data.

BOX 3.

Outstanding questions

- Heterogeneity of astrocyte properties and dysfunctions across brain regions is insufficiently understood
- Which mechanisms astrocytes use for gliotransmitter release, whether multiple gliotransmitters are co-released by individual astrocytes and under which conditions, is still largely unknown
- Pathophysiological effects of ATP release vs adenosine degradation, and their cognate receptors activation, need further elucidation: autocrine vs. paracrine effects, target cells, pre- vs post-synaptic effects
- Information on the role of astrocytes in genetic forms of epilepsy remains elusive
- Reactivity of astrocytes in epilepsy reflects complex combinations of compensatory and detrimental phenotypes, dynamics and consequences need better understanding
- Selective drugs specifically targeting pathological mechanisms activated in astrocytes, with good BBB penetration, and limited side-effects are still needed

BOX 4.

Literature search strategy

We searched PubMed for peer-reviewed publications published between January 1990 and April 2022 with the term “astrocytes”. We then refined our search terms to be “astrocytes” AND (as individual combinatory terms) “epilepsy”, “epileptogenesis”, “seizure(s)”, “(neuro)inflammation”, “imaging”, “biomarker(s)”, “neurobiology”, “metabolism”, “gliotransmission”, “treatment(s)”. Selection criteria from full-text outputs were novelty of study findings and their relevance to neurologists, with inclusion decided collectively by all authors. One relevant historical reference outside the search timeframe was also included.

Key points

- In healthy conditions, astrocytes play key functions in the CNS which are compromised in disease states such as epilepsy, thus contributing to pathologic changes in synaptic transmission and to hyperexcitability.
- The metabolic and biochemical underpinnings of epilepsy are tightly linked to astrocyte pathophysiology.
- Astrocyte-mediated inflammation can promote epileptogenesis and seizure recurrence, especially when the endogenous resolution mechanisms fail.
- Imaging of astrocyte activation states and related blood fingerprint of soluble molecules may represent prognostic and diagnostic biomarkers of epilepsy useful for patient stratification in clinical studies.
- A resolution of those processes that chiefly involve astrocytes during pathogenesis constitute a new frontier for therapy development.

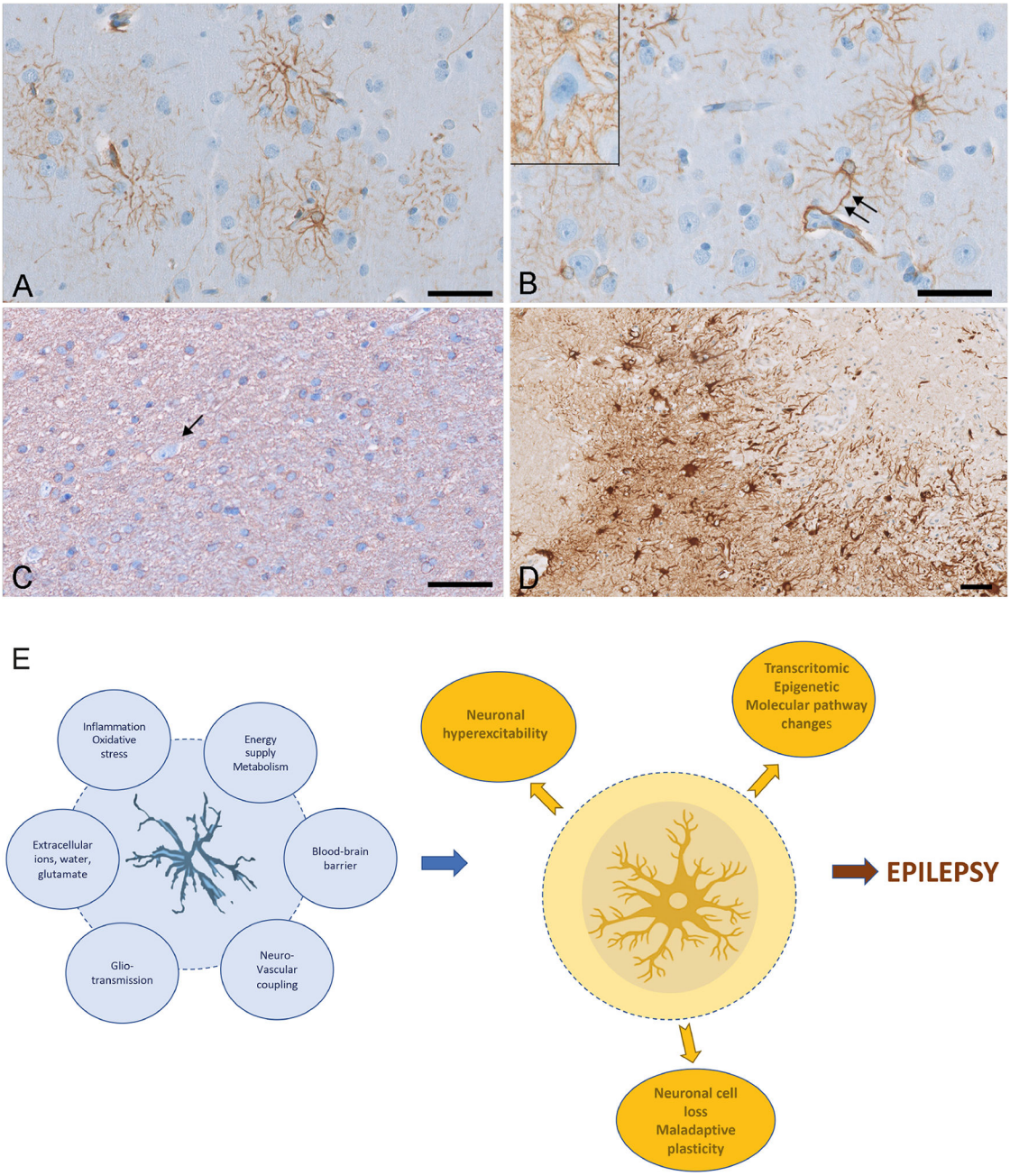

Figure 1. Astrocytes and their interactions with neurons and blood vessels

Photomicrographs depict GFAP-positive immunostaining in human astrocytes. **A,B**: Protoplasmic astrocytes morphology in normal human temporal cortex depicting highly branched bushy processes; cells are widely distributed between neurons. An astrocyte embracing a neuron is shown in B (magnification in inset). Arrows indicate a large astroglia process extending its foot along a blood vessel. **C,D**: Chronic dense fibrillary gliosis in human TLE sclerotic hippocampus. Arrow in C indicates a surviving CA1 neuron entrapped in dense glial meshwork. Scale bars: 50 μm.

There are different splice variants of GFAP (β, γ, δ, κ) including the predominant GFAPα form[8]. The minor isoforms are increased in subpopulations of astrocytes in focal lesions associated with epilepsy, however, their functional consequences for the epileptic network are unclear.

E: Schematic representation of the shift of astrocytic functional states (in healthy conditions, blue color) to reactive pathologic states (orange color). Reactive astrocytes, due to impairment of their homeostatic functions (blue circles), cause several alterations in surrounding brain tissue (orange shape) that contribute to epilepsy initiation and progression.

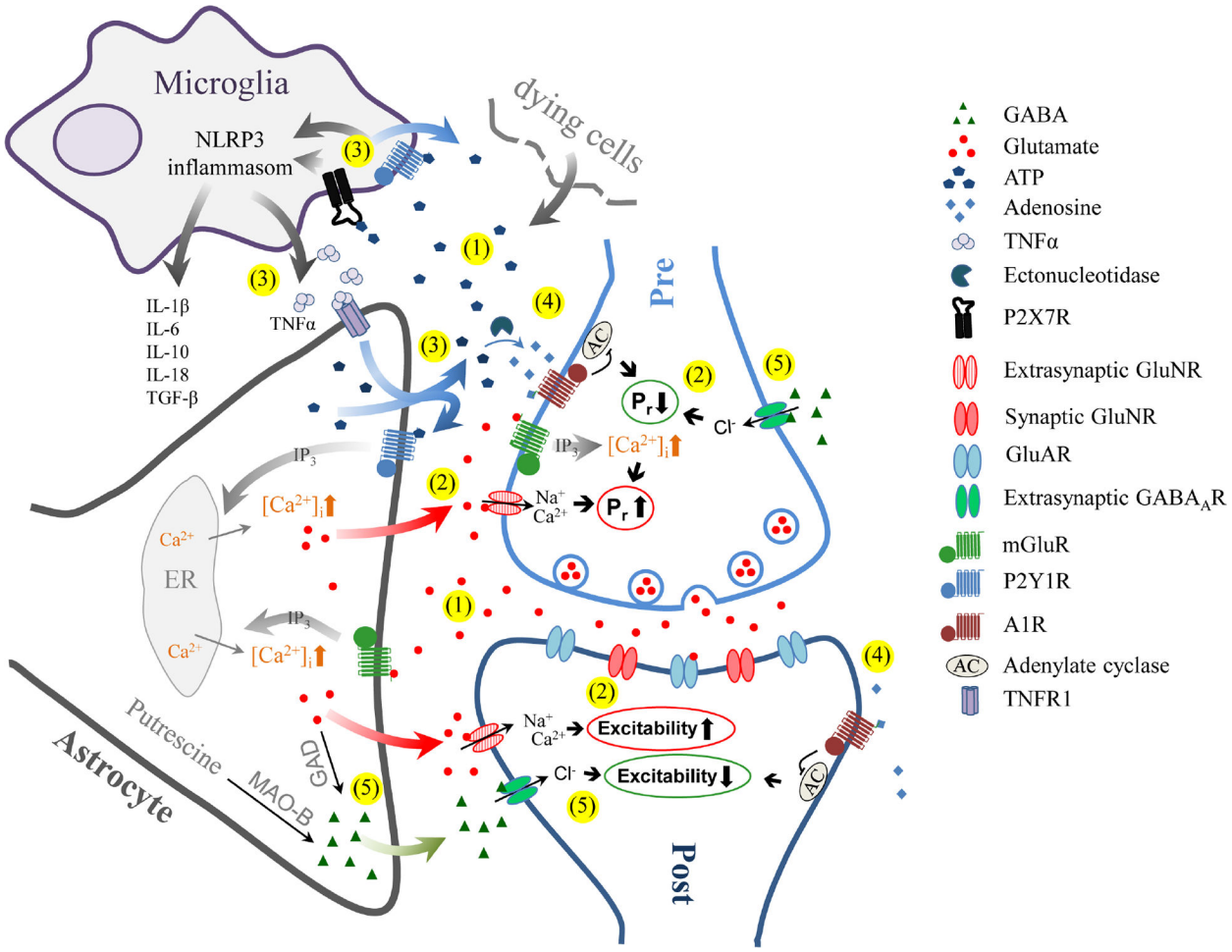

Figure 2. Gliotransmission in the epileptic brain

(1) Glutamate or ATP derived from neurons, astrocytes or microglia trigger an increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in astrocytes, which promotes the release of gliotransmitters. (2) Glutamate released by astrocytes activates extrasynaptic neuronal GluNRs and mGluRs, thereby increasing presynaptic release probability (Pr) and postsynaptic excitability. (3) Astrocytic ATP release evoked by elevated $[Ca^{2+}]_i$ levels or activation of cytokine receptors lead to autocrine or paracrine activation of P2YRs, resulting in potentiation and intercellular propagation of astrocytic Ca^{2+} signals. It also triggers microglial activation and release of pro-inflammatory cytokines through activation of microglial P2X7 and/or P2Y1Rs. (4) Extracellular ATP is rapidly hydrolysed by ectonucleotidases to adenosine, with the latter targeting pre- and postsynaptic A1Rs to decrease neuronal excitability. (5) Epileptic activity triggers astrocytic GABA production via decarboxylation of glutamate by glutamate decarboxylase (GAD) and degradation of putrescine by monoamine oxidase B (MAO-B). Upon release into the extracellular space, GABA activates extrasynaptic $GABA_A$Rs on excitatory neurons and elicits tonic inhibitory Cl^- currents, which attenuates neuronal excitability. Abbreviations: GluAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ER – endoplasmic reticulum; $GABA_A$R – γ-aminobutyric acid receptor; IL– interleukin; IP_3 – inositol 1,4,5-trisphosphate; NLRP3 – NLR family pyrin domain containing 3; GluNR – N-methyl-d-

aspartate receptor; TGF-β – transforming growth factor β; TNFα – tumor necrosis factor α; TNFR1 – tumor necrosis factor α receptor 1.

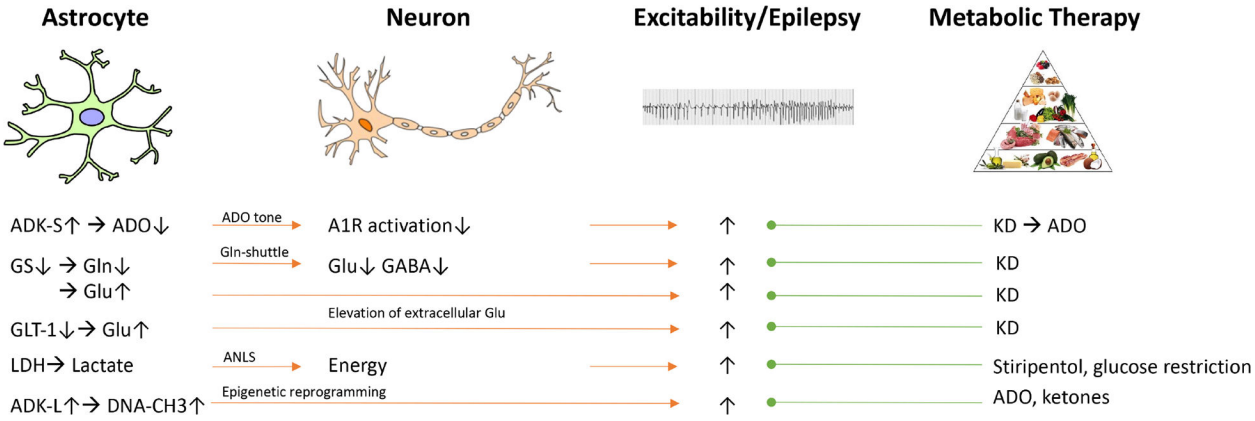

Figure 3. Key mechanisms through which metabolic dysfunction in astrocytes contributes to increased neuronal excitability

Changes in key metabolic enzymes or transporters lead to an imbalance in the key neurotransmitters and neuromodulators glutamate, glutamine, GABA, and adenosine. Glycolysis can lead to the formation of lactate, which is a key energy metabolite for neurons supplied by the astrocyte neuron lactate shuttle. Adenosine in the cell nucleus, regulated by ADK-L contributes to epigenetic reprogramming as driver of the epileptogenic process. Consequently, metabolic therapies, such as ketogenic diet, glucose restriction, adenosine, and ketones can reduce epileptic activity through multiple mechanisms. Abbreviations: ADO – adenosine; ADK-L – long isoform of adenosine kinase; ADK-S – short isoform of adenosine kinase; ANLS – astrocyte neuron lactate shuttle; A1R – adenosine A1 receptor; DNA-CH3 – DNA methylation; Gln – glutamine; GLT-1 – astroglial glutamate transporter 1; Glu – glutamate; GS – glutamine synthetase; KD – ketogenic diet; LDH – lactate dehydrogenase.

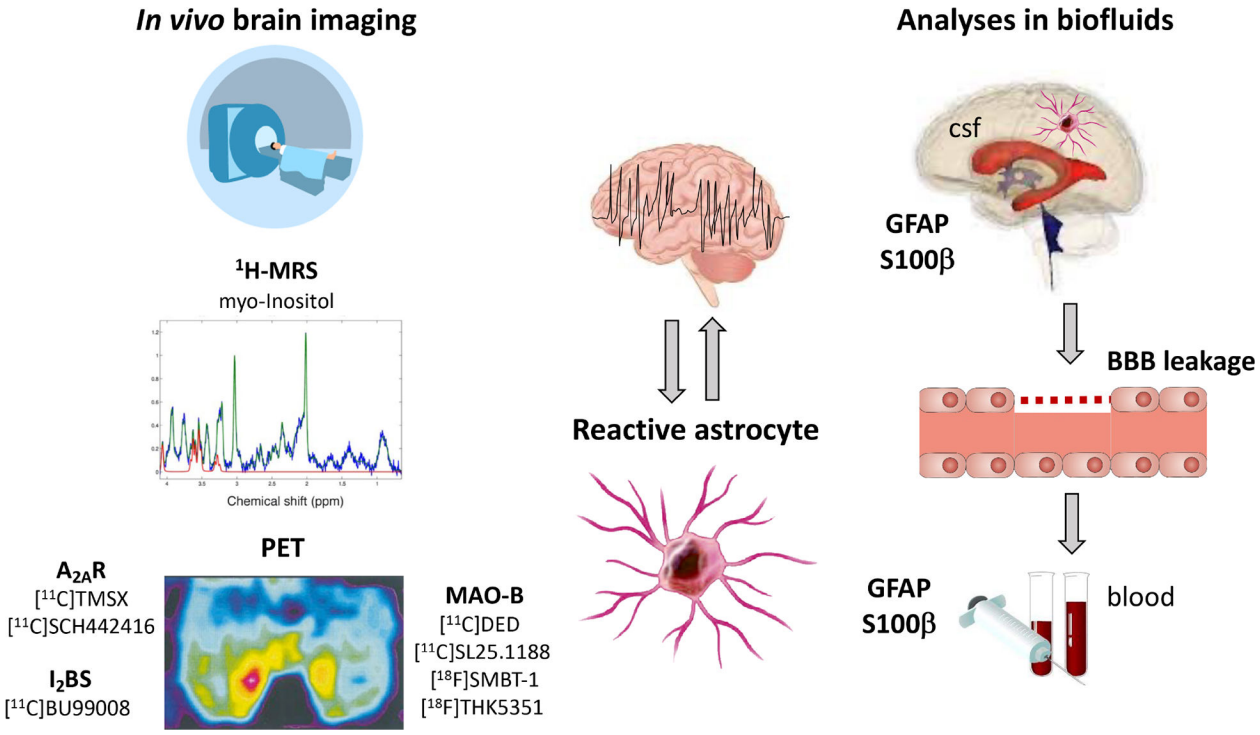

Figure 4. Imaging modalities and biofluids fingerprint of astrocyte reactivity

Brain imaging using proton magnetic resonance spectroscopy (^{1}H-MRS) and positron emission tomography (PET) are minimally invasive tools to monitor astrocyte activation *in vivo* by targeting molecular markers enriched in reactive astrocytes, namely myo-Inositol, adenosine 2A receptors ($A_{2A}R$), imidazoline$_2$ binding sites (I_2BS), monoamine-oxidase B (MAO-B). Additionally, analysis of circulating markers of astrocytes such as GFAP and S100β in blood or CSF may provide an index of astrocyte reactivity or injury. These astrocyte-related biomarkers may help to identify epileptic foci, to monitor epileptogenesis or the effect of therapies.