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Glial Mechanisms of Inflammation during Seizures

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Abstract: It is now clearly established that microglia and astrocytes become reactive in brain regions experiencing seizures both in human and experimental epilepsies. The expression of these reactive phenotypes lead to the dysregulation of the physiological functions normally fulfilled by these glial cells and to the acquisition of inflammatory properties through which they influence the activity and the fate of brain cells, including neurons, glia and cells of the blood vessels. In this chapter, we review how dysregulation of astrocyte and microglia physiological functions and the emergence of specific reactive states impact epilepsy progression.

Keywords: astrocyte, microglia, temporal lobe epilepsy, cytokine, purinergic signaling, gliotransmission, inflammation

Introduction

Glia of the central nervous system (CNS) mainly comprises astrocytes, cells of the oligodendrocyte lineage and microglia. Beside their well-known supportive or metabolic functions for neurons, it is now clear that glial cells also dynamically interact with neurons to integrate neuronal activity and, in turn, directly influence this activity. In the case of astrocytes and microglia, this glial modulation of neuronal activity can operate through the release of mediators, either classical transmitters such as glutamate, GABA, D-serine and ATP or cytokines and growth factors. In physiological conditions, “metabolic” and “information processing” functions of glia operate on a time scale ranging from milliseconds to hours or even days, when considering brain development and hormonal cycles, and are fundamental for brain functions, including cognitive functions (Pellerin et al., 2007; Theodosis et al., 2008; Attwell et al., 2010; Araque et al., 2014; Khakh and Sofroniew, 2015; Dallérac and Rouach, 2016; Mosser et al., 2017; Salter and Stevens, 2017; Allen and Lyons, 2018; Thion et al., 2018; Santello et al., 2019; Vainchtein and Molofsky, 2020). In pathological conditions, microglia and astrocytes develop reactive phenotypes that result in i) an impairment of their homeostatic functions and ii) the up-regulation of “immune” functions through which microglia and astrocytes orchestrate the central inflammatory reaction. This remodelling of glial phenotype modifies their interactions with neurons but also with cells of the blood vessels (pericytes and endothelial cells), thereby leading to impaired neuronal activity and BBB functions (Halassa et al., 2007; Rossi and Volterra, 2009; Salter and Beggs, 2014; Gorter et al., 2015; Steinhäuser et al., 2016; Verkhratsky and Parpura, 2016; Wolf et al., 2017; Volk, 2017; Ising and Heneka, 2018; Cresto et al., 2019; Patel et al., 2019).

The relationships between inflammation, gliosis, seizure and epilepsy emerged at the beginning of this century from clinical and experimental observations. The best documented evidence for a direct causality between inflammation and human epilepsy comes from Rasmussen encephalitis in which brain inflammation of unknown origin triggers focal motor seizure (Varadkar and Cross, 2015). However, Rasmussen encephalitis remains an extreme condition which does not directly relate to other forms of epilepsy. 20 years later, there is a general agreement that brain inflammation represents a hallmark of seizure and epilepsy (Bauer et al., 2017; Terrone et al., 2017) but whether reducing inflammation has any clinical benefit for

patients remains an unanswered question. Another key question is whether inflammation and gliosis contribute to epileptogenesis and/or to the genesis of spontaneous recurrent seizures (ictogenesis). In other words, are these processes involving primarily non-neuronal cells a cause or a consequence of aberrant neuronal network activities? Answering these key questions is difficult for different reasons. First, experimental models of epileptogenesis leading to chronic seizures, even when considering only models of temporal lobe epilepsy (TLE), are diverse and often not strictly comparable, and the evolution of the immune response during the progression of the disease (acute versus chronic) remains poorly characterized. Second, investigating central immune response in human is mostly limited to brain resections obtained from patients undergoing surgical removal of epileptic foci since non-invasive approaches such as PET imaging of inflammatory markers remain rather crude.

In this chapter, we will first review the roles of astrocytes and microglia in homeostatic conditions before discussing the notion of gliosis or glial remodelling in non-homeostatic conditions, its link with inflammation, and how astrocytes and microglia influence the pathogenesis of epilepsies, focusing mostly on temporal lobe epilepsy (TLE).

1. Astrocytes and microglia in homeostatic conditions

1.1. Astrocytes

Homeostasis of K^+ and water

In physiological conditions astrocytes contribute to the maintenance of ion homeostasis and, in particular, regulate extracellular K^+ ($[K^+]_0$). The accumulation of K^+ in the extracellular space leads to sustained neuronal depolarization and hyperexcitability (Heinemann and Dieter Lux, 1977; Walz, 2000). Astrocytes control $[K^+]_0$ by two mechanisms: K^+ uptake and spatial buffering. The K^+ uptake is mediated through Na^+/K^+ pumps, $Na^+/K^+/Cl^-$ cotransporters and the large expression of inward-rectifier potassium (Kir) 4.1 channels; it also relies on the very negative resting potential of astrocytes (Ransom et al., 2000; D'Ambrosio et al., 2002; Kofuji and Newman, 2004; Seifert et al., 2009). Remarkably, mice lacking Kir4.1 channels on

astrocytes were impaired for K⁺ and glutamate uptake and were more susceptible to seizures (Djukic et al., 2007; Chever et al., 2010).

Control of ion homeostasis by astrocytes relies also on spatial buffering (Orkand et al., 1966), which is mediated by gap junctions (GJ), channels composed of connexin (Cx) 43 and Cx30 (Nagy and Rash, 2000). These channels allow the formation of a functional network between astrocytes (syncytium) and the diffusion of K⁺ following the electrochemical gradient: K⁺ is taken up by astrocytes where neuronal activity is high, diffuses through the syncytium and is released at remote sites with lower [K⁺]_o. In line with the spatial buffering concept, the physiological functioning of astroglial networks should have an anti-epileptic role, and indeed, a reduced threshold for generating epileptic activity was found in mice with coupling-deficient astrocytes (Wallraff et al., 2006; Pannasch et al., 2011).

Another important function in which astrocytes are involved is the regulation of extracellular space (ECS) volume and fluid osmolarity through aquaporin 4 (AQP4). This channel mediates transmembrane water movements according to osmotic gradients and that is expressed by astrocytes mostly at perisynaptic processes and perivascular end-feet (Schwartzkroin et al., 1998). Regulating water flux near synapses and blood vessels may be linked to K⁺ homeostasis (Papadopoulos and Verkman, 2013).

Neurotransmitter buffering

Another crucial role for astrocytes that directly impacts on neuronal excitability is neurotransmitter buffering. The removal of neurotransmitters, such as glutamate and GABA, from the synaptic cleft is crucial for maintaining a correct functioning of synapses and an appropriate balance between excitation and inhibition (E/I balance). Alterations in these buffering mechanisms could lead to neuronal hyper-excitability and excitotoxicity (Choi, 1988; Meldrum, 1994). The astrocytic buffering of neurotransmitters is mediated by uptake through transporters and their metabolism through the glutamine – glutamate – GABA cycle (Eid et al., 2016). Glutamate and GABA reuptake relies on specific transporters expressed by astrocytes: EAAT1 and EAAT2 (also known as GLAST and GLT1) for glutamate, and GAT1 and GAT3 for GABA. Once internalized in astrocytes, glutamate is converted into glutamine by glutamine synthetase (GS), while GABA enters the Krebs cycle which produces

alpha ketoglutarate, glutamate and glutamine. Glutamine produced in astrocytes is then released, taken up by neurons and used as a precursor for both glutamate and GABA synthesis.

Adenosine kinase (ADK) is another important astrocytic enzyme involved in E/I balance. By phosphorylating adenosine to adenosine-monophosphate (AMP) it exerts a key role in regulating extracellular levels of adenosine (Studer et al., 2006; Etherington et al., 2009), adenosine being known to dampen neuronal activity and its release to exert anti-convulsant effects (Lado and Moshé, 2008; Cunha, 2016).

Gliotransmission

The concept of the “tripartite synapse” emerged from experiments demonstrating that astrocytes are able to sense neurotransmitters released into the synaptic cleft by presynaptic neurons but also can release gliotransmitters that regulate neuronal and synaptic activities (Auld and Robitaille, 2003; Perea et al., 2009).

Several classical neurotransmitters have been also identified as gliotransmitters, among which glutamate, D-serine, ATP, adenosine and GABA have been the most studied for their modulation of neuronal excitability and of the activity of excitatory and inhibitory synapses (Angulo et al., 2008; Araque et al., 2014; Harada et al., 2016; Petrelli and Bezzi, 2016; Papouin et al., 2017). An increase in intracellular Ca^{2+} is often crucial for the release of gliotransmitters (Araque et al., 2014; Rusakov et al., 2014; Volterra et al., 2014; Khakh and Sofroniew, 2015; Bazargani and Attwell, 2016) that can be mediated through exocytotic or non-exocytotic pathways (Hamilton and Attwell, 2010; Verkhratsky et al., 2016). Yet, gliotransmitters can be released also through Ca^{2+} independent mechanisms involving connexin hemichannels or pannexin (PANX) channels (Abudara et al., 2018), exchangers such as the cysteine-glutamate antiporter, or reversal of neurotransmitter transporters (Malarkey and Parpura, 2008). It should be noted, however, that *in vivo* studies on gliotransmission are still sparse and that the mechanisms and the functional consequences of gliotransmission are still matters of debates and controversies. These issues have been discussed recently in several reviews (Araque et al., 2014; Rusakov et al., 2014; Khakh and Sofroniew, 2015; Bazargani and Attwell, 2016; Panatier and Robitaille, 2016;

Papouin et al., 2017; Dallérac et al., 2018; Fiacco and McCarthy, 2018; Savtchouk and Volterra, 2018).

Interactions with blood vessels

Another important aspect of astrocyte function that is affected by gliosis is their close interaction with brain vasculature. Astrocytes contact blood vessels with their endfeet and in physiological conditions contribute to the maintenance of the blood-brain barrier (BBB) structure by increasing the expression of tight junction proteins occludin and claudin-5 and by inhibiting the expression of chemokines and leukocyte adhesion molecules, which would favor BBB disruption (Andreone et al., 2015; Zhao et al., 2015).

Astrocytes have been also proposed to control cerebral blood flow according to neuronal activity and contribute to power neurons by supplying energy substrate needed to sustain synaptic activity (Giaume et al., 2010; Pellerin and Magistretti, 2012; Nortley and Attwell, 2017; Nippert et al., 2018).

1.2. Microglia

Microglial cells are the resident macrophages of the CNS and unlike the other mononuclear macrophages associated with the CNS (i.e. perivascular, meningeal and choroid plexus macrophages) they are in direct contact with other cells and with synapses in the CNS parenchyma (Tremblay et al., 2011; Kettenmann et al., 2013; Schafer et al., 2013; Wu et al., 2013; Nayak et al., 2014; Prinz and Priller, 2014). In line with their immune cell identity, microglial cells have been primarily studied in pathological conditions and, until recently, our knowledge on these cells was mostly derived from analyses of CNS pathologies. It is now clear, however, that these immune cells also interact with their neighboring partners of the CNS parenchyma and fulfill specific functions in homeostatic conditions.

Never resting microglia

During the last 15 years, the development of new tools for functional analysis and of new animal models has completely changed our view of microglia functions in physiological conditions. In 2005, two *in vivo* studies using two-photon microscopy in anesthetized knock-in CX3CR1-eGFP mice reported that microglial cells in

physiological conditions are not resting, as previously thought, but are permanently extending and retracting their thin processes (Davalos et al., 2005; Nimmerjahn et al., 2005). This motility of microglial processes may help to detect more rapidly danger signals appearing in the parenchyma. These two articles actually showed that in response to laser lesions or to local applications of ATP, which is considered as an alarm signal (Rodrigues et al., 2015), microglial cells rapidly extend their processes toward the lesion or the source of ATP, which acts here as a chemoattractant. Although other signaling pathways may control the oriented motility of microglia processes, purinergic P2Y₁₂ receptors and adenosine A_{2A} or P2Y₁₃ receptors are instrumental for rapid process extension and retraction, respectively, occurring right after an insult to the CNS parenchyma (Haynes et al., 2006; Orr et al., 2009; Milior et al., 2020).

Monitoring and modulating neuronal activity

Following these initial observations, other groups showed that during their surveillance of the parenchyma, which involves a two-pore domain K⁺ channel (Madry et al., 2018), microglial cell processes contact synapses. The occurrence and duration of these contacts are regulated by neuronal activity and can influence the activity and the fate of the synapses (Wake et al., 2009; Tremblay et al., 2010; Li et al., 2012; Dissing-Olesen et al., 2014; Eyo et al., 2014; Pfeiffer et al., 2016; Sipe et al., 2016). Interestingly, microglial cells promote the formation of new spines in the cortex of animals engaged in a motor learning task (Parkhurst et al., 2013). Parallel studies also showed that mobilization of microglia specific pathways could lead to the modulation of synaptic activity. For instance the fractalkine signaling pathway regulates excitatory synaptic transmission and plasticity in the hippocampus (Rogers et al., 2011; Scianni et al., 2013), microglial Toll Like Receptor (TLR) 4 signaling controls glutamate release probability of presynaptic fibers indirectly through the mobilization of astrocytes (Pascual et al., 2012) and microglial P2Y₁₂ receptors are necessary for synaptic plasticity during the critical period of ocular dominance (Sipe et al., 2016). A recent study also revealed the existence of contact between microglial processes and neuronal soma. These junctions, which can be rapidly induced by neuronal activation, are organized in specialized nanodomains enriched in Kv2.1 potassium channels, mitochondria and vesicular nucleotide transporter (Vnut) containing vesicles on the neuronal side and P2Y₁₂ on the microglial side (Cserép et

al., 2020). These junctions could therefore inform microglia on the excitability and metabolic state of the neuron. Curiously, these junctions, which are observed in a very large proportion (90%) of neurons in different regions of the CNS, had not been noticed before. Altogether, these influential studies have led to the notion that microglial cells monitor but also regulate synaptic and neuronal activity in the healthy brain.

Microglia diversity

Heterogeneity of microglia in physiological conditions was first evidenced during CNS development when their phenotype is constantly evolving from amoeboid cells, when entering the CNS parenchyma at embryonic stages, to fully ramified cells at juvenile stages. Remarkably, at a given stage of development, microglial cells with different morphological and functional phenotypes are differentially distributed in different areas of the CNS. This reflects the dynamics of microglia colonization of the brain and their exceptional ability to adapt to their environment, which explains the tight coupling between their own maturation and the maturation of other components of the CNS (Mosser et al., 2017). This variety of phenotypes underlies a variety of microglia functions during development that have been reviewed recently (Mosser et al., 2017; Paolicelli and Ferretti, 2017; Thion et al., 2018). More recent studies have also revealed the existence of different microglia populations in different regions of the CNS (Sierra et al., 2010; Baalman et al., 2015; De Biase et al., 2017; Stowell et al., 2018). Interestingly, microglia repopulating the CNS after a transient depletion in adult mice regain the specific phenotype that they normally express in a given brain area (De Biase et al., 2017), indicating that at any given time microglial cells retain the ability to adapt their phenotype to local environmental cues.

2. Astrocytes and microglia reactivity

The role of astrocytes and microglia in pathologies of the CNS is widely associated with reactive phenotypes developed by these cells in response to a rupture in CNS homeostasis. The acquisition of these reactive phenotypes, called gliosis, is specific of each glial cell type but is also dependent on the nature, duration and location of the pathological triggers. Because glial cells have homeostatic

functions in non-pathological conditions (see above), profound changes of their phenotype upon appearance of pathological conditions have two consequences: i) the loss of their homeostatic influence and ii) the emergence of new functions associated with the acquisition of reactive, inflammatory phenotype.

2.1. Astrogliosis in epilepsy

Astrogliosis is particularly evident in epileptic syndromes associated with hippocampal sclerosis (HS) that is characterized by a glial scar (Thom, 2014) and this reactivity of astrocytes is commonly revealed by the up-regulation of GFAP expression. Astrogliosis, however, involves many more changes at the molecular, cellular and functional levels (Dossi et al., 2018b), and these changes can precede the GFAP up-regulation (see below). The degree of alterations observed in reactive astrocytes varies according to the type of insult inducing the reaction and to the possibility of reversion of the damage (Sofroniew, 2009; Anderson et al., 2014). Transcriptomic analysis of reactive astrocytes has disclosed the occurrence of two broad phenotypes: a pro-inflammatory phenotype A1 having detrimental effects (Zamanian et al., 2012; Lian et al., 2015; Liddelw et al., 2017) and an immune-suppressive phenotype A2 with protective functions (Anderson et al., 2016; Liddelw and Barres, 2017). Interestingly, astrocyte A1 phenotype can be induced by the co-release of IL-1 α , TNF α and C1q from reactive microglia (Liddelw et al., 2017). Although the specific phenotype of astrocytes in different forms of epilepsy has not yet been firmly established, different studies rather point toward a deleterious A1 phenotype. For example, in epilepsy models, the release of Interleukin-1 β and High Mobility Group Box1 by astrocytes induces the activation of IL1R/TLR signaling pathways that favors the induction and maintenance of seizure activity (Maroso et al., 2010; Vezzani et al., 2011). On the other hand, mechanisms that oppose to this deleterious astrocyte phenotype have been described (Iyer et al., 2012; van Scheppingen et al., 2018; Korotkov et al., 2020) and transforming growth factor-beta (TGF- β) signaling, which is activated in astrocytes during epilepsy, reverts A1 phenotype *in vitro* (Liddelw et al., 2017). However, in most cases the population of astrocytes has been considered as a whole without taking into consideration a possible diversity of the astrocyte reactivity (e.g. lesion vs peri-lesional area).

A still debated question is whether astrocyte reactivity is a cause or a consequence of seizures. In favor of a causative role, astrocyte-specific deletion of genes (tuberous sclerosis complex 1 or Beta 1 integrin) or mutation of an astrocyte-specific gene (GFAP) induce astrogliosis and the occurrence of spontaneous seizures (Ortinski et al., 2010; Messing et al., 2012; Robel et al., 2015). On the other hand, there were no sign of astrogliosis in non-lesional epileptogenic cortical areas of patients with focal cortical dysplasia (Rossini et al., 2017). Moreover, in a guinea pig model of temporal lobe epilepsy with hippocampal sclerosis (HS-MTLE; unilateral, intra-hippocampal kainate), astrogliosis and cell damage were observed only in the injected, and not in the contralateral, hippocampus despite the occurrence of epileptiform activities in both hippocampi (Noè et al., 2019). The latter two examples suggest dissociation between the occurrence of seizure and astrogliosis, as defined with the classical marker GFAP. However, they do not exclude the contribution of astrocytes to the functional remodeling that occurs during epileptogenesis and to the generation of seizures, especially in view of the many functions of astrocytes by which they influence the excitability of neuronal networks.

Homeostasis of K⁺ and water

Different studies, both in humans and animal models, have highlighted a clear association between uncontrolled extracellular K⁺ increase and epilepsy (Steinhäuser et al., 2016). In particular, measurements of extracellular K⁺, patch-clamp recordings of Kir currents in astrocytes, single-cell RT-PCR and immunostaining of Kir proteins revealed a reduction in Kir-mediated current and a reduction of Kir4.1 expression in surgical specimen from patients suffering from MTLE as well as in rodent models of MTLE (Bordey and Sontheimer, 1998; Heinemann et al., 2000; Hinterkeuser et al., 2000; Kivi et al., 2000; Schröder et al., 2000; Jauch et al., 2002; Heuser et al., 2012). GJ communication within the astrocyte syncytium contributes to K⁺ homeostasis (see above) and is regulated by pro-inflammatory cytokines (IL-1 β & TNF α) (Meme et al., 2006). Evidence suggests that alterations in astrocyte coupling could be involved in the genesis and progression of epilepsy. In a mouse model of HS-MTLE (unilateral intracortical kainate injection), it was demonstrated that astrocyte coupling is impaired as early as 4 hours after the induction of status epilepticus (SE), a mechanism probably related to the release of IL-1 β and TNF α , and thus suggesting that impaired coupling represents a key event in epileptogenesis (Bedner et al., 2015). Moreover,

the authors showed a complete lack of GJ coupling in sclerotic hippocampi of HS-MTLE patients.

Mice lacking AQP4 have a mild ECS volume expansion, an elevated threshold but prolonged duration for electrographic seizures induced *in vivo* by hippocampal electrical stimulation (Binder and Steinhäuser, 2006; Binder et al., 2006). Subsequent analysis of these mice, however, indicated that they also displayed an enhanced gap junctional coupling and an improved spatial $[K^+]_o$ buffering (Strohschein et al., 2011). In human and in animal models of MTLE, there is a loss of astrocytic perivascular AQP4, which precedes the appearance of chronic seizures (Eid et al., 2005; Alvestad et al., 2013). This suggests that perturbed polarized expression of AQP4 during epileptogenesis, together with consequences on $[K^+]_o$ homeostasis, may be of pathophysiological relevance.

Neurotransmitter buffering

Alterations in neurotransmitter transporters are reported in different neurological diseases, including epilepsy (Coulter and Eid, 2012). The involvement of alterations in neurotransmitter homeostasis in epilepsy is demonstrated by an increase in extracellular glutamate levels in the sclerotic hippocampus of MTLE patients (Cavus et al., 2005, 2008). In experimental epilepsy, deletion of GLT1 in astrocytes enhanced responses to sub-convulsive doses of pentylentetrazol (Tanaka et al., 1997).

Alterations in GABA extracellular levels can also contribute to hyper-excitability and epilepsy. Depending on the epileptic syndrome, both decreased extracellular GABA levels and increased GABA receptor mediated activity can be detrimental for the maintenance of E/I balance. On the one hand, an increase in the expression levels of GAT3 astrocytic GABA transporter in TLE, leading to a reduction of extracellular GABA levels, has been reported just before seizure onset (During and Spencer, 1993; Lee et al., 2006). On the other hand, in absence epilepsy a dysfunction of the astrocytic GABA transporter GAT1 was responsible for the generation of non-convulsive seizures by determining an increase in GABA-A receptor mediated inhibition in thalamocortical neurons (Cope et al., 2009; Pirttimaki et al., 2013).

Reduction in GS function is involved in the pathogenesis of epilepsy by inducing extracellular glutamate accumulation, as reported in TLE patients (Eid et al., 2004, 2016; van der Hel et al., 2005). Evidence for GS involvement in the pathogenesis of epilepsy is reported also in animal models in which blocking of GS function with the inhibitor methionine sulfoximine was able to induce recurrent seizures, neuronal loss and hippocampal atrophy (Eid et al., 2008; Wang et al., 2009). Considering that glutamine is fundamental also for GABA synthesis in interneurons, GS dysfunction could be related also to impaired inhibitory transmission, as shown in an experimental model of TLE in which the downregulation of GS in astrocytes was responsible for a compromised inhibitory synaptic transmission (Ortinski et al., 2010).

Astrogliosis occurring in epilepsy is known to be associated with increased levels of ADK both in humans and in experimental models (Boison, 2016). The increase in ADK and the consequent reduction of extracellular adenosine levels are responsible for reducing seizure threshold (Boison, 2016).

Altogether these data clearly highlight how crucial is neurotransmitter buffering in order to maintain a functional synaptic activity and avoid the generation of a seizure-prone context.

Gliotransmission

In vitro models of seizure showed that an excitatory loop involving astrocyte Ca^{2+} signaling, glutamate and ATP signaling lowers seizure threshold for neurons (Gomez-Gonzalo et al., 2010). Moreover, Ca^{2+} signals in astrocytes facilitates spread of epileptiform activity *in vivo* (Heuser et al., 2018) and increased astrocyte Ca^{2+} signaling and glutamate release contribute to neuronal cell death in the neocortex *in vivo* after a pilocarpine induced SE (Ding et al., 2007). Astrocyte glutamate release occurs in different forms, one of which generates in neurons slow inward currents (SICs) resulting from the activation of extra-synaptic NMDA receptors and allowing the synchronization of small ensemble of neurons (Parri et al., 2001; Angulo et al., 2004; Fellin et al., 2004). It has been proposed that SICs generated by astrocytes could be at the origin of hypersynchronous excitation of neurons in models of epilepsy (Tian et al., 2005) but further pharmacological experiments clearly showed a dissociation between ictal events and SICs (Fellin et al., 2006).

ATP is an essential mediator of communication between astrocytes and other types brain cells (Butt, 2011). Expression of a dominant-negative SNARE that impairs the vesicular release of ATP from astrocytes (Pascual et al., 2005) delays the onset of recurrent seizures, decrease epileptiform activity and reduce hippocampal damage after a pilocarpine-induced SE (Clasadonte et al., 2013). Release of ATP through the other route constituted by pannexin 1 (PANX1) hemi-channels appears to contribute to ictal discharges in human epileptic brain tissue and in mouse models of MTLE (intra-hippocampal kainate) or SE (systemic kainate) (Santiago et al., 2011; Dossi et al., 2018a). In a rat model of TLE (rapid kindling), activation of P2Y1 receptors induces astrocyte Ca^{2+} hyperactivity that is responsible for an increase in glutamate release from astrocytes. This glutamate release activates neuronal presynaptic mGluR5, which increases the release probability at the CA3-CA1 synapses. Blocking astrocyte Ca^{2+} signalling or inhibiting P2Y1 or mGluR5 receptors relieved this abnormal enhancement of synaptic strength (Álvarez-Ferradas et al., 2015). Similarly, in a mouse model of TLE (unilateral intracortical kainate injection), enhanced astrocyte P2Y1 signalling increased astrocyte glutamate release. However, in the dentate gyrus this glutamate release activated neuronal presynaptic NMDA receptors that enhanced excitatory synaptic transmission in granule cells of the dentate gyrus (Nikolic et al., 2018). Remarkably, this enhanced P2Y1 signalling resulted from a constitutive activation of an autocrine loop involving astrocyte ATP release onto P2Y1 receptors (Shen et al., 2017). This autocrine loop, which can be triggered by $TNF\alpha$, was up-regulated before the initiation of the chronic stage of this TLE model (Nikolic et al., 2018). It should be remembered, however, that ATP released from astrocytes also activates P2Y1 expressed by inhibitory interneurons (Khakh et al., 2003; Tan et al., 2017) and that its degradation into adenosine inhibits the activity of excitatory pyramidal cells through A1 receptor activation (Shen et al., 2017; Tan et al., 2017).

Altogether, the majority of the above cited observations point toward the conclusion that the actions of astrocyte purinergic signalling determined by the combined effects of ATP, adenosine and glutamate can displace the equilibrium between excitation and inhibition and promote appearance of epileptiform activity. It is important to note that P2Y1 regulation of astrocyte Ca^{2+} signalling described in the context of epilepsy has been also reported in a mouse model of Alzheimer's disease (Delekate et al.,

2014; Reichenbach et al., 2018) and in a model of traumatic brain injury (Choo et al., 2013). In both cases, blocking this pathway had beneficial consequences on outcome of these models. Impaired astrocyte purinergic signalling could thus be a common signature of different CNS pathologies. Yet, additional *in vivo* experiments are needed to evaluate its weight on TLE progression.

Interactions with blood vessels

Modifications of astrocyte phenotype during astrogliosis can contribute to BBB impairment that occurs in epilepsy through disruption of tight-junction coupling, increased expression of adhesion molecules on endothelial cells determining an increase of pro-inflammatory cytokines and the extravasation of leukocytes in brain parenchyma (Marchi et al., 2014; van Vliet et al., 2015). In line with the idea that astrocytes contribute to perturbed BBB integrity is the observation of enhanced Ca^{2+} signaling in astrocyte endfeet during the latent phase of a mouse model of TLE (unilateral intra-cortical kainate; (Szokol et al., 2015)). This reactivity of astrocyte endfeet may be driven by the $TNF\alpha$ -induced endothelin-1 release that occurs after pilocarpine-triggered SE in rats which promotes the production of reactive oxygen species in astrocytes via endothelin receptor-B activation (Kim et al., 2013). Furthermore, BBB disruption in epilepsy leads to extravasation of serum albumin, which activates $TGF-\beta$ in astrocytes (Heinemann et al., 2012). $TGF-\beta$ signaling activation in astrocytes then leads to a series of modifications, including impaired K^+ , water and glutamate buffering capacity (Seiffert et al., 2004), increased excitatory synaptogenesis (Weissberg et al., 2015) and degradation of perineuronal net around inhibitory interneurons (Kim et al., 2017), which may promote hyperexcitability (Terrone et al., 2019). Interestingly, reactive astrocytes are associated with microglia-pericytes clusters along leaky capillaries in human and experimental epilepsies and may contribute to the formation of a perivascular scar at loci of impaired blood-brain barrier (Klement et al., 2018, 2019).

2.2. Microgliosis in epilepsy

Microglial cells are involved in a large number of CNS diseases and very often they are mobilized at early stages of these diseases. This mobilization leads to changes of their phenotype, which allows microglia to react and adapt in a specific manner to the new environment (Hanisch and Kettenmann, 2007; Ransohoff and

Perry, 2009; Shemer et al., 2015). Microglia reactivity was first described as a morphological change: from a small soma with long and thin ramified processes in physiological conditions, they adopt a less ramified morphology with fewer and thicker processes in pathological conditions, which can eventually lead to an amoeboid morphology, without process, in the most severe cases. We know now that these morphological changes are accompanied by important functional modifications including changes in gene expression and motility properties, increased proliferation, phagocytic activity and release of numerous pro- and anti-inflammatory mediators (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009; Kierdorf and Prinz, 2013). Yet, microglia activation is not an all-or-none process; it is progressive, depends on the actual pathological conditions and on the context in which the disease appears. There is therefore not a single reactive state but a diversity of phenotypes that are determined by a fine detection of environmental cues and that allows microglial cells to perform specific functions in different pathological conditions. Although microglial morphological alteration is a convenient mean to assess reactivity, it does not reflect the complexity of the functional remodeling of microglia associated to specific pathological context. This is best exemplified for P2Y₁₂ receptors whose expression is either up or down regulated in morphological similar reactive microglia but in different pathological context, status epilepticus or neuropathic pain, respectively (Haynes et al., 2006; Avignone et al., 2008; Tozaki-Saitoh et al., 2008). The diversity of functional remodeling of reactive microglia was further demonstrated by transcriptional analyzes performed in various pathological states. These studies firmly established that in a given pathology microglial reactivity constantly evolves, that there is a diversity of microglial reactive states at a given time of the disease, and that the transcriptional profile adopted by microglia is disease specific (Hirbec et al., 2019). Nevertheless, there is a core of genes that are commonly deregulated across different pathological states and may serve as unambiguous makers of microglial reactivity (Hirbec et al., 2018).

Microglial inflammatory signaling pathways in epilepsy

Recently, human microglial cells of patients suffering from MTLE and who underwent surgery were analyzed in depth, uncovering specific phenotypes in regions of sclerosis (CA1, CA3) versus areas with less neuronal damage (Morin-Bureau et al., 2018). These observations suggested that the repairing interleukin IL-

10 regulates the microglial phenotype in these sclerotic regions. Moreover, this study also provides evidence for an early and transient expression of several cytokines, including CXCL8, IL-1 β , after each spontaneous seizure (Morin-Brureau et al., 2018).

Increased expression and production of inflammatory cytokines in the hippocampus is well documented in experimental models of TLE and occurs rapidly following SE (Vezzani et al., 2019). These cytokines can be produced by different cell types, including neurons, astrocytes and microglia. However, in a mouse model of TLE induced by pilocarpine, microglial cells were shown to up-regulate transiently pro-inflammatory cytokines IL-1 β , IL-6 and TNF α mRNAs 3 days after SE (Benson et al., 2015). Interestingly, at this stage anti-inflammatory markers arginase-1, chitinase-3-like protein (Ym1), IL-4, but not IL-10, were also up-regulated by microglia (Benson et al., 2015). Because many of these cytokines are known to modulate neuronal excitability and astrocyte signaling, these observations suggests that microglial inflammatory mediators have a complex role during epileptogenesis. Surprisingly, none of these genes were found to be dysregulated in microglia 3 days post-SE in a model of intra-cerebroventricular injection of kainate (Bosco et al., 2018). These conflicting observations are likely due to differences between the two models and to methodological variations (RNASeq of purified microglia versus qPCR of hippocampal tissue).

Toll-like receptor (TLR) signaling could contribute to the production of cytokines and other mediators by microglial cells after SE. Activation of TLR3 and TLR4 receptors *in vitro* induces the production of inflammatory cytokines by microglia (Olson and Miller, 2004). TLR3 deficiency reduces microglia reactivity, the level of proinflammatory cytokines TNF α and interferon β as well as spontaneous recurrent seizures (Gross et al., 2017). Antagonizing TLR4 reduces acute and chronic seizure recurrence in a kainate model of TLE (Maroso et al., 2010). This effect of TLR4 could involve a signaling pathway including microglia, astrocytes and neurons. Pascual and co-workers showed that activation of microglial TLR4 induces the release of ATP by microglia, which activates astrocyte P2Y1 receptors, promoting the release of glutamate by astrocytes that in turn increases excitatory synaptic transmission in CA1 (Pascual et al., 2012).

Stromal cell-derived factor-1 (SDF-1 or CXCL12) and its receptor CXCR4 also seem to play a role in TLE. Rats treated with a CXCR4 antagonist decreased spontaneous seizures and aberrant adult neurogenesis in a kainate model (Song et al., 2016). Interestingly, CXCR4 is upregulated in hippocampal microglia 3 days post kainate-induced SE (Bosco et al., 2018). Again, some of these effects could involve both microglia and astrocytes as SDF-1 has been shown to trigger the release of TNF α by microglia that stimulates astrocyte glutamate release (Bezzi et al., 2001).

Fractalkine (CX3CL1) is a neuronal chemokine that exclusively signals to microglia, which are the only CNS cells expressing the fractalkine receptor CX3CR1. This signaling pathway is thought to act as an Off signal that opposes microglial reactivity (Cardona et al., 2006; Biber et al., 2007; Limatola and Ransohoff, 2014). Increased expression of fractalkine has been reported in the hippocampus and temporal neocortex of TLE patients and in a pilocarpine rat model (Xu et al., 2012). Fractalkine application was shown to restore inhibitory GABAergic currents in excitatory neurons in slices of TLE patients (Roseti et al., 2013). Moreover, fractalkine signaling favors microglial process convergence toward neuronal axons and dendrites that increases after pilocarpine or kainate induced SE, and CX3CR1 deficiency worsens seizure phenotype (Eyo et al., 2016). Interestingly, deficiency of P2Y12 receptors, which drive microglial process motility, also worsens kainate-induced seizure behaviors (Eyo et al., 2014).

Accumulating evidence favors a role of microglial purinergic receptors in experimental models of seizure. The transcripts of the 4 main microglial purinergic receptors, *i.e.* P2X4, P2X7, P2Y6 and P2Y12, are upregulated in the hippocampus in a mouse model of kainate-induced SE. The membrane currents induced by the activation of P2X7, P2Y6, P2Y12 and the microglial process motility toward an agonist of P2Y12 are increased after SE (Avignone et al., 2008). In the same model, P2X4 proteins are upregulated in reactive microglia and P2X4 deficiency reduces several aspects of microglial reactivity, the upregulation of several pro-inflammatory mediators and the neuronal death induced in the hippocampus by SE (Ulmann et al., 2013). However, while microglial P2X4 receptor has been involved in BDNF-mediated disinhibition and generation of local hyperexcitability in the spinal cord (Beggs et al., 2012), this receptor is also upregulated in neurons and it is still not

known whether P2X4 expressed by neurons, microglia or both contribute to inflammatory state post SE.

P2X7 receptors are prominently expressed in different immune cells, are one of the main trigger of IL1- β processing and contribute to TNF- α production. In the brain, P2X7 is expressed in homeostatic microglia but its expression increases after SE. For these reasons the potential involvement of P2X7 in epilepsy has been early investigated in different animal models using either pharmacological or genetic approaches to modulate the receptor activity. However, opposite results were reported depending on the way SE was triggered. In the model of pilocarpine-evoked SE, inhibition of P2X7 activity using either specific antagonists or P2X7-deficient mice increases seizure-susceptibility after pilocarpine injection, an effect that may be linked to PANX1 since siRNA-mediated down regulation of PANX1 also reduced the threshold of seizure apparition (Kim and Kang, 2011). On the contrary, in a model of intra-amygdala injection of KA, P2X7 activity seems to be pro-convulsive since P2X7-deficient mice show reduced electrographic seizure during SE and pre-treatment with P2X7 antagonists reduced behavioral seizures, neuronal cell death and IL1- β increase in the hippocampus. In this model, microRNA-22 repress the expression of P2X7 and thus has anti-convulsive effects (Jimenez-Mateos et al., 2015). Whether this regulation is also present in human is unknown. The reasons for these opposite results are not elucidated. In both studies the same pharmacological agents and the same strain of P2X7-deficient mice were used. However the two experimental models of SE present notable differences and it is possible that in the peripheral administration of pilocarpine might trigger mechanisms which are not present in the alternate model. Importantly, adhesion of circulating leukocytes, which express P2X7, on endothelial cerebral blood vessels cells is thought to be an initial and mandatory step of SE induction in the pilocarpine model (Fabene et al., 2008). This adhesion might depend on P2X7 activity and therefore impaired by P2X7 antagonists. Recent studies using P2X7 antagonist that crosses the BBB showed in rats that administration of this molecule three months after systemic kainate injections decreased the severity of spontaneous recurrent seizures (Amhaoul et al., 2016). Although the seizure frequency remained unaltered, this study further strength the idea that blocking P2X7 may have beneficial effects in regulating seizure during the acute but also the chronic phases of the disease.

While rodent studies support that P2X7 may represent a potential therapeutic target to reduce the burden of seizures, experimental data in human are still lacking. Particularly, all clinical trials testing the effectiveness of P2X7 antagonists in rheumatoid arthritis have failed to demonstrate any beneficial effect (Keystone et al., 2012), despite the clear demonstration of the involvement of this receptor in animal models of the disease. Nevertheless, in human specimen from patients suffering with Focal Cortical Dysplasia (FCD), P2X7 mRNA and protein expression was increased compared to control samples. This was accompanied with an increased immunoreactivity against IL1- β in microglia but also in dysmorphic neurons and balloon cells, which are characteristic cells of FCD (Wei et al., 2016). Other studies also reported an increase of P2X7 expression in the brain of epileptic patients, however because the specificity of the antibodies are questionable, these results have to be interpreted with caution.

Complex contribution of microglia in epilepsy

The above described studies indicate that targeting different signaling pathways dysregulated (mostly upregulated) in microglia can have either pro- or anti-convulsive effects. There are certainly concerns regarding the cell-type specificity of some of these manipulations but if we consider the most microglia-specific receptors that have been studied, *i.e.* CX3CR1 and P2Y12, it is interesting to note that microglial cells seem to have anti-convulsive actions. This is in agreement with different studies supporting the idea that, in pathological conditions, homeostatic interactions between microglia and neurons have rather an inhibiting effect on neuronal activities. This was initially described in the Zebrafish embryo where microglial processes are attracted by active neurons, in an ATP-dependent manner, and the resulting contacts lead to a reduction of both spontaneous and evoked neuronal activities (Li et al., 2012). Another study reports dynamic interactions of microglial processes with the axonal initial segment in conditions of neuronal hyperexcitability leading to a reversal of the depolarization (Kato et al., 2016). Interestingly, recent experiments of microglia depletion in mouse models of epilepsy also support such a calming effect of microglia on neuronal activity. Using 3 different genetic strategies to deplete microglia, Wu and collaborators showed that the absence of microglia aggravates both acute and chronic seizures in kainate mouse models of SE or TLE (Wu et al., 2020). In another study on seizure progression in a

mouse model of viral encephalitis, pharmacological depletion of microglia using the antagonist of the Csf1 receptor PLX5622, maximum seizure occurrence was reached more rapidly in microglia depleted mice but the overall seizure incidence was not affected (Waltl et al., 2018). Depletion of microglia with PLX5622 was also shown to induce seizures in a mouse model of low level picornavirus infection of the CNS (Sanchez et al., 2019). In contrast, the use of another Csf1 receptor antagonist, PLX3397, had anti-epileptic effects in mouse models of pilocarpine or intra-hippocampal kainate (Srivastava et al., 2018). However, that study used a much lower concentration of PLX3397 than the one required to deplete microglia (Elmore et al., 2014) and the authors indeed showed that microglia were not depleted (Srivastava et al., 2018). These observations suggest that inhibition of microglial Csf1 receptors have anti-seizure effects whereas microglial depletion worsens seizures.

The signaling pathway through which microglia cells inhibit active neurons in physiological or epileptic conditions remains to be identified. Converging evidence, however, indicates that P2Y₁₂ receptors are central in this calming effect. Indeed, pharmacological or genetic inhibition of P2Y₁₂ receptors result in aggravated neuronal death following transient ischemia (Cserép et al., 2020) or worsened seizure (Eyo et al., 2014; Mo et al., 2019). While P2Y₁₂ expression can be up regulated in specific reactive states associated with neuropathic pain (Inoue and Tsuda, 2012) or shortly after SE (Avignone et al., 2008), sustained microglia reactivity is often associated with total repression of P2Y₁₂ expression (Hirbec et al., 2019). Such a switch in the expression pattern of P2Y₁₂ may contribute to the opposite effects of microglia during seizure progression but many other pathways may also be involved. Interestingly, elevating mTOR signaling in mouse microglia induces a microglial reactive state without induction of pro-inflammatory cytokines and leads to the appearance of spontaneous recurrent seizures (Zhao et al., 2018). This study suggests that the loss of homeostatic functions of microglia may be more important than the acquisition of an inflammatory phenotype in promoting the appearance of seizures. However, the genetic strategy used in that study does not allow excluding the possibility that border-associated macrophages residing in the meninges, the choroid plexus, and the perivascular spaces or even some CX3CR1 expressing monocytes contribute to the observed effect.

Finally, the spatio-temporal dynamics of microglial reactivity within different parts of the epileptic hippocampus (e.g. sclerotic vs non-sclerotic regions) generate a diversity of microglial phenotypes that need to be characterized to understand the complex roles of these immune cells in regulating the activity and fate of neurons and other brain cells during epileptogenesis and at a chronic stage of the disease.

Concluding remarks

In the CNS glial cells have homeostatic functions that support and also finely tune neuronal and synaptic activities. Yet, in pathological conditions where homeostasis is durably lost, glial cells become reactive. These reactive states evolve along disease conditions and can be deleterious or protective. In the context of epilepsy, glial reactivity occurs only in brain areas experiencing seizures and increasing evidence indicates that this reactivity contributes to disease progression. Major challenges in the field aim at identifying the triggers, the time course and evolution of glial reactivity. However, better characterizing the consequences of the loss of glial homeostatic functions is certainly of equal importance. The urgent need of new therapeutic strategies will benefit from a better understanding of the signaling pathways through which glial cells regulate or dysregulate other brain cells and thereby contribute to the progression of different forms of epilepsy.

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