Inflammation and epilepsy: the contribution of astrocytes
Zurolo, E.

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DISCUSSION

Astrocytes are the most abundant glial cells in the brain and their role in physiology and pathology is still not completely understood. The discovery of gliotransmitters and the fact that astrocytes express receptors for neurotransmitters changed the classical view according to which astrocytes were the passive cells of the brain, only supporting the main players: the neurons. Indeed there is growing evidence of bidirectional communication between neurons and astrocytes and presently the term tripartite synapse is commonly used to indicate that communication within the brain not only involves pre- and post-synaptic neurons, but also the surrounding astrocytic processes (Perea, Navarrete et al. 2009).

The active communication between neurons and astrocytes points also to the latter cell type as pathological substrate for different CNS disorders. Moreover astrocytes have the potential to secrete various types of cytokines and chemokines which result in a long term communication. Indeed astrocytes are key players in inflammatory processes in the brain and they have been associated with different CNS disorders with an inflammatory component (reviewed in chapter 1.2). Increasing data suggest that inflammatory processes can influence neuronal excitability (Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010), seizure duration and intensity. Epilepsy for instance, especially in its pharmacoresistant forms, has been associated with increased expression of inflammatory markers and astrocytosis, which is a reactive process that astrocytes undergo in different pathological conditions. Moreover the fact that almost 30% of epilepsies are drug-resistant emphasizes the need to elucidate the mechanisms underlying the etiopathogenesis of this complex disease. This thesis aimed to investigate the role of astrocytes in epilepsy, focusing on molecular pathways involved in the etiopathogenesis of this disease, thus contributing to the identification of new molecular targets.

Inflammatory pathways in epilepsy

Epilepsy is a common neurological disorder characterized by recurrent seizures which have two main characteristics: hyper-excitability and synchronization of neurons. Astrocytes can influence both parameters releasing molecules that can increase the neuronal response or synchronize populations of neurons (Fellin, Pascual et al. 2006). Moreover astrocytes are an important cell type responsible for the amplification and propagation of the inflammatory stimuli in the brain. Increasing evidences support the concept of activation of innate immune response in experimental and human epilepsy (Vezzani, Balosso et al. 2010; Aronica and Crino 2011; Vezzani, Aronica et al. 2011). In chapter 2.1 of this thesis we showed that activation of plasminogen system occurs in refractory epilepsy. Upregulation of PAs and uPAR might contribute to the mechanisms underlying the epileptogenicity of focal lesions,
through direct modulation of neuronal activity or indirectly through regulation of inflammatory response and tissue remodeling. Indeed brain inflammation can contribute significantly in determining seizure threshold in susceptible brain regions, thus playing a role in seizure precipitation and their recurrence (Dube, Vezzani et al. 2005; Kulkarni and Dhir 2009; Riazi, Galic et al. 2010; Vezzani, Aronica et al. 2011; Vezzani, Maroso et al. 2011). Various in vitro and in vivo findings suggest that specific sets of inflammatory molecules and their cognate receptors, can contribute to the epilepsy associated molecular changes and synaptic plasticity. The IL-1/TLR signaling for example has been shown to influence seizure precipitation in different experimental paradigms (Viviani, Bantesaghi et al. 2003; Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010). In chapter 2.2 we investigated the inflammatory pathways activated in FCD I and II confirming the occurrence of complex inflammatory changes involving both innate and adaptive immune response. In particular in FCD II we found a greater activation of inflammation, as suggested by the presence of microglia, T lymphocytes and dendritic cells. These findings suggest that the activation of inflammation is not simply an effect of seizure activity (FCD I and II had a comparable history of seizure), but an active mechanism that involves different molecules and pathways. In chapter 2.3 we showed that HMGB1-TLR4 pathway is upregulated in human epileptic developmental lesions in particular in astrocytes confirming previous data in experimental models and human TLE (Maroso, Balosso et al. 2010). We also showed that there is translocation of HMGB1 from
the nucleus to the cytoplasm in astrocytes of patients with MCD, and that IL-1β can induce HMGB1 release in cultured astrocytes suggesting that astrocytes may play a key role in the amplification of the inflammatory response. HMGB1 represents a crucial regulator of chromatin conformation and inflammatory gene transcription. Upon its nuclear-to-cytoplasmatic translocation, which is induced by injury or specific biological stressors, and its subsequent release HMGB1 acquires the properties of a proinflammatory cytokine mediated by both TLRs and RAGE. Activation of RAGE by HMGB1 also induces differentiation of immature neurons and has a role in axonal and dendritic elongation (Rauvala and Rouhiainen 2010), an effect also shared by other endogenous ligands of the S100/calgranulin family (Ding and Keller 2005). S100b is a member of a multigenic Ca²⁺ regulated S100 proteins, and is abundant in CNS where is mainly expressed by astrocytes. It can be released by astrocytes and affects neurons in a paracrine manner. Although both HMGB1- and S100b-mediated activation of TLRs or RAGE subserves physiological and homeostatic functions, excessive release of these molecules and the concomitant overactivation of their receptors can result in neuropathology. These molecules have been implicated in disease progression in several CNS pathological conditions (Schmidt, Yan et al. 2000; Stern, Yan et al. 2002; Huttunen and Rauvala 2004; Rauvala and Rouhiainen 2010). For example, HMGB1 release from mature neurons is involved in the development of ischemic injury and blood-brain barrier (BBB) disruption induced by stroke, and both RAGE and TLRs may mediate these deleterious effects by activation of NF-kB and ERK-signaling, leading to the synthesis of key proinflammatory molecules (Qiu, Nishimura et al. 2008).

Previous studies have demonstrated a long-term increase in brain excitability in mice overexpressing cytokines in astroglia (Campbell, Abraham et al. 1993; Akassoglou, Probert et al. 1997; Stalder, Carson et al. 1998), or in rodent brain after the induction of an inflammatory challenge, particularly if this event occurs during the early post-natal life (Riazi et al., 2010). With this background we could envision that inflammation influences seizure susceptibility in two different ways, with an immediate effect, that influences directly neuronal excitability or a long term mechanisms resulting in long lasting modifications of the brain through the astrocytic network.

In chapter 2.4 we described an acute effect of two proinflammatory molecules in an ex vivo model of focal epilepsy: **HMGB1 and IL-1β could increase the focal ictal discharge (fID) in acute brain slices from mice.** These two molecules were already shown to be proconvulsant in a mouse epilepsy model (Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010) enhancing the total time spent in seizure activity. In our study, HMGB1 and IL-1β were found to be proictogenic, increasing the excitability of the neurons in response to NMDA stimulation. The increased excitability of the network corresponded to an increase in the calcium
signaling in particular in astrocytes, which have been previously shown to have a pivotal role in the generation of fID in enthorinal cortex slices (Gomez-Gonzalo, Losi et al. 2010; Losi, Cammarota et al. 2010). The proictogenic effect of HMGB1 and IL-1β was prevented by application of tetrodotoxin (TTX, that blocks the synaptic transmission by blocking voltage-gated Na⁺ channels) suggesting that there is an active communication between neurons and astrocytes which can be targeted to reduce excitability.

Astrocytes are also important in K⁺ homeostasis, in particular they express Kir 4.1, an inward rectifier potassium channel which is the main responsible for the control of potassium influx and resting potential (Kucheryavykh, Kucheryavykh et al. 2007; Inyushin, Kucheryavykh et al. 2010). To further investigate the role of inflammation in epilepsy, we analyzed the expression of IL-1β in a rat model for TLE (chapter 2.5) and in different tumors associated with epilepsy in relation to Kir4.1. In the present study, we observed that **IL-1β levels peaked in the temporal cortex at 1 day after SE, which corresponds to the time point of prominent reduction of Kir4.1 expression.** These observations suggest a role for IL-1β in the regulation of Kir4.1 mRNA expression, which was further investigated in vitro, using glial cells in culture. Further experiments on cultured astrocytes showed that **application of IL-1β could reduce the expression levels of Kir4.1 mRNA and protein in human astrocytes.** Previous studies showed that ablation of Kir4.1 protein results in altered astrocytes function, changed resting potential and impaired K⁺ buffering (Inyushin, Kucheryavykh et al. 2010) and that Kir 4.1 KO leads to a lethal phenotype characterized by seizures recurrence (Djukic, Casper et al. 2007). The reduction of Kir 4.1 after IL-1β could be an additional mechanism by which the inflammation contributes to hyperexcitability of the neurons in an indirect way: lack of potassium buffering could increase the concentration of K⁺ in the extracellular space, setting the resting potential of neurons to more depolarized values, closer to threshold of action potential.

**Regulation of inflammation in astrocytes: the role of miR-146a**

Micro RNAs are small (=20 nucleotides) regulatory molecules which act as post-translational suppressors of protein expression. Since their discovery in 1993 (Lee, Feinbaum et al. 1993), they have been the focus of attention of the scientific community for their ability to specifically suppress protein synthesis, by preventing the mRNA translation. Several miRNAs have been found in the human brain, and they are found to play a crucial role in a wide range of biological processes, including the regulation of the innate and adaptive immune response (Pedersen and David 2008; Sonkoly, Stahle et al. 2008; Pauley, Cha et al. 2009). In this thesis we showed that **miR-146a is upregulated in experimental models of epilepsy, as well as in human TLE (chapter 3.1).** In particular, in a rat model of TLE strong upregulation was detected in astrocytes 1 week after status epilepticus (during epileptogenesis) which corre-
Corresponds to the latent period and the time of maximal astroglial activation and upregulation of various genes involved in the immune response (Gorter, Van Vliet et al. 2006; Aronica and Gorter 2007). These observations are in line with other studies supporting the association between this specific miRNA and human inflammatory diseases (Quinn and O'Neill 2011; Rusca and Monticelli 2011). Cell specific upregulation suggests a key role of miRNA-146a in governing astrocyte activation and function. We further demonstrated that the pro-inflammatory cytokine IL-1β can increase the expression levels of mir146a on human cultured astrocytes (chapter 3.2) and we analyzed the two targets of mir146a: IRAK1 and TRAF6, in the pathway that leads to NF-kB activation. These results suggest that inflammation is a key component of epilepsy associated disease, involving astrocytes which in turn activate miR-146a pathway to reduce the inflammatory signaling. In the future this pathway could be targeted to reduce inflammation, for instance by overexpressing miR-146a: our results in vitro demonstrate that increased miR146a leads to downregulation of IRAK1 and TRAF6. The increase in miR-146a that occurs after IL-1β stimulation could be a regulatory mechanism to damper inflammation to limit tissue damage. Moreover miR-146a serves as a marker for inflammation and could be targeted to reduce the inflammatory response. To further investigate the effect of this microRNA more studies are needed, especially regarding in vivo overexpression of miR-146a in relation to epilepsy and inflammation. Recent data support the possibility of using miRs for in vivo therapy, with the result of silencing protein expression. AntagomiR-134 for example, was shown to suppress seizures in a mouse model of epilepsy when injected after KA administration and subsequent status epilepticus (Jimenez-Mateos, Engel et al. 2012).

However one of the main problems of application of miRNA is the so called off-target effect, which derives from the fact that each microRNA can have more that one target, in some cases hundreds of them. For instance miR-146a can also target complement factor H (CFH), which is an anti-inflammatory component of the complement system, resulting in increase of inflammation. For these reason artificial miRs, small molecules specifically designed to target only one sequence, have a better perspective of use in the therapy.

Astrocytes-mediated signaling and epilepsy

Astrocytes can send information to neighboring cells via release of gliotransmitters. One of the first to be recognized was glutamate, identified in 1994 (Parpura, Basarsky et al. 1994) and found to regulate synaptic transmission in cultured hippocampal cells. Nowadays we know that astrocytes may release different neuroactive molecules such as glutamate, D-serine, ATP, adenosine, GABA, TNFα, prostaglandins (Perea, Navarrete et al. 2009). Adenosine, for instance is an important modulator of synaptic strength and it is recognized as endog-
enous anticonvulsant and neuroprotectant (Ribeiro 2005; Stone, Ceruti et al. 2009; Boison 2010) mainly acting on $A_1$ receptors causing presynaptic inhibition (Fredholm, Chen et al. 2005). The levels of extracellular adenosine are dependent on the expression levels of the enzyme adenosine kinase (ADK) (for rev. see (Boison 2012)) which, in the adult brain is predominantly expressed in astrocytes (Studer, Fedele et al. 2006) and reduces the adenosine in the extrasynaptic cleft. Experimental up-regulation of ADK within astrocytes was shown to be sufficient to trigger spontaneous recurrent seizures in the absence of any other epileptogenic event, whereas ADK downregulation almost completely abolished spontaneous recurrent seizures (Theofilas, Brar et al. 2011). In the last part of the thesis we showed increased expression of the enzyme ADK in an experimental model as well as in patients with TLE (chapter 4.1) and also in human astrocytic tumors associated with epilepsy (chapter 4.2). These findings suggest that overexpression of ADK is a common pathological hallmark of medically intractable chronic epilepsy and could be an important target for treatment. More studies are needed, in particular to assess the functionality of the enzyme ADK in relation to the excitability of the neurons.

Another signaling system in which astrocytes play an important role is constituted by the endocannabinoids (ECBs) and their receptors. ECBs were classically considered inhibitory as they bind CB1 receptors in neurons inhibiting neurotransmitter release from presynaptic terminals (Chevaleyre, Takahashi et al. 2006; Hashimotodani, Ohno-Shosaku et al. 2007). The discovery that astrocytes express functional CB1R suggested a role for this cell type in ECB signaling. Indeed astrocytes were shown to modulate neuronal excitability upon activation of CB1R (Navarrete and Araque 2008) responding to ECBs with Ca$^{2+}$ elevations and subsequent release of glutamate that activates neurons more distant from the site of ECBs release (Navarrete and Araque 2010). The model that they propose is that ECBs can have either inhibitory effect acting directly on presynaptic neurons or an excitatory effect through activation of astrocytes that in turn activate neurons. In chapter 4.3 of this thesis we analyzed the expression pattern and distribution of CBRs in developing human cerebral cortex and showed an increase of their expression levels in focal malformation of cortical development (MCD) in particular in astrocytes that could contribute to the epileptogenicity of those lesions. In fact dysregulation of the ECB system, with abnormal expression of CB1, has been reported in both human and experimental temporal lobe epilepsy (TLE; (Falenski, Blair et al. 2007; Ludanyi, Eross et al. 2008)). Moreover in vitro studies show that the ECB system is necessary to stimulate astrocytes Ca$^{2+}$ rises in order to maintain the epileptiform activity in hippocampal cultured slices (Coiret, Ster et al. 2012). However, the complexity of the ECB system, the differential expression of their receptors, and the fact that ECB-mediated activation of astrocytes has an excitatory rather that inhibitory effect on neurons, emphasize the
need for further studies to clarify pathways that could be a target for development of new therapeutic strategies (Hofmann and Frazier 2011).

Clinical implications and future directions
During the past decade, detailed molecular characterization of astrocytes, in particular reactive astrocytes, demonstrates that these cells are active players in the development and progression of the immune/inflammatory response that takes place in epileptic brain tissue. Both human and experimental data suggest the activation of specific proinflammatory pathways in astrocytes, with consequent release of inflammatory molecules that can alter neuronal network excitability. The identification of “harmful” pro-inflammatory pathways contributing to seizure onset and recurrence highlights the possibility of developing a therapeutic strategy targeting the astrocyte-mediated inflammatory signaling. However, we need to wait for the outcome of clinical studies before we can consider whether this approach is the right strategy. If this is so, it may not only improve control of seizures, but may also act as disease-modifying therapy in patients with epilepsy resistant to conventional antiepileptic drugs.

Fig. 2. Summary of the results of this thesis. The color legend indicates the different processes in which inflammation and astrocytes can play a role in epilepsy.
REFERENCES


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