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Abstract

A large series of clinical and experimental studies supports a link between inflammation and epilepsy, indicating that inflammatory processes within the brain are important contributors to seizure recurrence and precipitation. Systemic inflammation can precipitate seizures in children suffering from epileptic encephalopathies, and hallmarks of a chronic inflammatory state have been found in patients with temporal lobe epilepsy. Research performed on animal models of epilepsy further corroborates the idea that seizures upregulate inflammatory mediators, which in turn may enhance brain excitability and neuronal degeneration. Several inflammatory molecules and their signaling pathways have been implicated in epilepsy. Among these, the chemokine pathway has increasingly gained attention. Chemokines are small cytokines secreted by blood cells, which act as chemoattractants for leukocyte migration. Recent studies indicate that chemokines and their receptors are also produced by brain cells, and are involved in various neurological disorders including epilepsy. In this review, we will focus on a subset of pro-inflammatory chemokines (namely CCL2, CCL3, CCL5, CX3CL1) and their receptors, and their increasingly recognized role in seizure control.

Keywords	seizure, inflammation, CCL2, CCL3, CCL5, CX3CL1
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Corresponding Author	Yuri Bozzi
Corresponding Author's Institution	University of Trento
Order of Authors	Chiara Cerri, Matteo Caleo, Yuri Bozzi
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Center for Mind/Brain Sciences

Yuri Bozzi, Ph.D.
Professor of Physiology

Trento, July 13, 2017

To the kind attention of
David M. Treiman, M.D.
Editor-in-Chief, *Epilepsy Research*

Dear David,

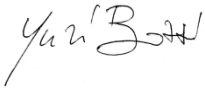
please find enclosed the newly revised version of our review manuscript entitled "Chemokines as new inflammatory players in the pathogenesis of epilepsy" by Chiara Cerri, Matteo Caleo and myself.

We would like to thank once again reviewer 2 for her/his patience and competence in editing our manuscript, and we apologize for the errors that were still present in our previous version. We have now addressed all her/his concerns, as detailed in our point-to-point response. All changes have been highlighted in red in the new version of the manuscript. We now hope the manuscript will be acceptable for publication.

Looking forward to your positive reply to this re-submission,

best regards,

Yuri



via Sommarive, 9 – 38123 Povo - Trento, Italy - tel. +39 0461 282961 e-mail: yuri.bozzi@unitn.it

EPIRES_2017_203-R2

“Chemokines as new inflammatory players in the pathogenesis of epilepsy” - Response to Reviewers’ comments.

We thank once again Reviewer 2 for her/his patience and competence in editing our manuscript, and we apologize for the errors that were still present in our previous version. We have now addressed all her/his concerns, as detailed below. All changes have been highlighted in red in the new version of the manuscript.

Page 3
- line 9 "patients appear" (not "appears") - done
- two lines before the end of 1. Introduction "then we will then focus": delete the first "then" - done
- last line "multiple sclerosis (MS) and Alzheimer's disease (AD)": add the acronyms here - done

Page 4
- line 8 "cellular responses can" (delete "to") - done
- line 10 "injury becoming" (delete "by") - done

Page 5
- lines 2-3: "and with a slower kinetic in the retina" (simplify!) - we simplified the sentence
- line 10, delete the comma after "PGs) - done
- lines 4 from bottom: "respectively, activate" (delete "and") - we rephrased and simplified the sentence

Page 6
- line 5 please rephrase as follows "It is important - no need for "Finally" - to mention that in the epileptic brain local inflammatory responses have been…" - done
- Section 2.2, lines 6,7, please rephrase as follows: "infection can trigger or sustain seizures.." - done

Page 7
- Line 6: delete the comma after "MTLE" - done
- Line 10 "seizures may" (not "might") - done
- Line 13, please rephrase as follows "such as the epileptic brain, can display an amplified" - done
- Line 14 delete the comma after "challenge" - done
- 2nd paragraph, lines 3,4 "targeting the …. pathway resulted in anti-convulsant effects…" - done
- 2nd paragraph, line 7 "epileptic patients" (and not "epilepsy patients" - done

Page 8
- Line 2: "… of leukocytes. These molecules are classified…" - done
- 2nd paragraph, line 7 change "they" into "chemokine receptors" - done

Page 9
- line 2: MS, AD (delete the name in extensor, since they should be abbreviated when first mentioned, as already indicated above) - done
- 2nd paragraph, line 3: CXC3CL1 (not "CxC3CL1") - done
- 3rd paragraph, line 2 "interestingly"; lines 5,6 "For instance, CCL2 plasma level could act" (simplify!) - we simplified the sentence. In the following line, delete "Importantly" (what is not important??): "It has also been proposed…" (just add "also") - done

Page 10
- Line 1 "could represent" - done
- Line 3 "in other neurological pathologies" (rather than "brain pathologies", since "neuropathic pain" is not really "brain" though the brain is involved!) - done

- Line 6 "cells to glioma in a rat model" (or "cells to a rat model of glioma", but not the current repetition) - done
- Line 5 from bottom "Chemokines can exert" - done

Page 11
- Lines 6,7: delete the commoas before and after "in hippocampal neurons" - done
- 2nd paragraph, lines 2-5 should become "after spatial learning and in hippocampal slices after LTP induction, and it has been proposed that CX3CL1 upregulation could modulate glutamate …… plasticity (Sheridan et al., 2014). Indeed CX3CL1 inhibits…. - done
- At the end of this paragraph, delete the comma after "epilepsy" - done
- Second line from page bottom "that the GABAergic" (add the article) - done

Page 12
- Section 5.2 Lines 8,9: "According to studies in humans, the majority of the results obtained in animal models": ??? - we rephrased the sentence
- Subsection 5.2.1 Line 2 "reportedly increased": where?? (brain, blood?) - we rephrased the sentence, indicating that the increase was observed in brain vasculature

Page 13
- Line 4 "related deleterious events - done
- 5.5.2 line 7 "focus and followed" (not "but") - done
- At the end of this paragraph, CCR2-positive needs to be hyphenated - done

Page 14
- Line 2 "see also subsection - not paragraph! - 2.2 above" - done
- Line 5 "seizure upregulation": what do you mean? ("seizure increase"? - seizures are not molecules…-) – we changed "seizure upregulation" to "seizure increase"
- 6. Conclusions, line 4 "in the last years, chemokines have been" - done

Highlights

- Chemokines signaling exerts multiple actions in the brain in both physiological and pathological conditions.
- CCL2, CCL3, CCL5 and CX3CL1 have a key role in epilepsy.
- CCL2, CCL2, CCL3, CCL5, CX3CL1 and their receptors may represent new therapeutic targets for seizure control.

Chemokines as new inflammatory players in the pathogenesis of epilepsy

Chiara Cerri[1,2], Matteo Caleo[1] and Yuri Bozzi[1,3*]

[1]CNR Neuroscience Institute, via G. Moruzzi 1, 56124, Pisa, Italy.

[2]Fondazione Umberto Veronesi, Piazza Velasca 5, 20122 Milano

[3]Neurodevelopmental Disorders Research Group, Centre for Mind/Brain Sciences, University of Trento, via Sommarive 9, 38123 Povo, Trento, Italy.

Email addresses of each author: chiara.cerri@in.cnr.it; caleo@in.cnr.it; yuri.bozzi@unitn.it

*Corresponding author:

Yuri Bozzi

Centre for Mind/Brain Sciences (CIMeC), University of Trento

via Sommarive 9, 38123 Povo, Trento, Italy

Phone: +39-0461-282961 Email: yuri.bozzi@unitn.it

Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; AEDs, antiepileptic drugs; BBB, blood-brain barrier; CNS, central nervous system; COX, cyclooxygenase; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; GABA, γ-aminobutyric acid; HAD, HIV associated dementia; HMGB1, high mobility group box 1; IL, interleukin; KA, kainic acid; LPS, lipopolysaccharide; MS, multiple sclerosis; MTLE, mesial temporal lobe epilepsy; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; PGs, prostaglandins; SE, status epilepticus; SRS, spontaneous recurrent seizures; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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[3]Neurodevelopmental Disorders Research Group, Centre for Mind/Brain Sciences, University of Trento, via Sommarive 9, 38123 Povo, Trento, Italy.

Email addresses of each author: chiara.cerri@in.cnr.it; caleo@in.cnr.it; yuri.bozzi@unitn.it

*Corresponding author:

Yuri Bozzi

Centre for Mind/Brain Sciences (CIMeC), University of Trento

via Sommarive 9, 38123 Povo, Trento, Italy

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Abstract

A large series of clinical and experimental studies supports a link between inflammation and epilepsy, indicating that inflammatory processes within the brain are important contributors to seizure recurrence and precipitation. Systemic inflammation can precipitate seizures in children suffering from epileptic encephalopathies, and hallmarks of a chronic inflammatory state have been found in patients with temporal lobe epilepsy. Research performed on animal models of epilepsy further corroborates the idea that seizures upregulate inflammatory mediators, which in turn may enhance brain excitability and neuronal degeneration. Several inflammatory molecules and their signaling pathways have been implicated in epilepsy. Among these, the chemokine pathway has increasingly gained attention. Chemokines are small cytokines secreted by blood cells, which act as chemoattractants for leukocyte migration. Recent studies indicate that chemokines and their receptors are also produced by brain cells, and are involved in various neurological disorders including epilepsy. In this review, we will focus on a subset of pro-inflammatory chemokines (namely CCL2, CCL3, CCL5, CX3CL1) and their receptors, and their increasingly recognized role in seizure control.

Keywords

seizure, inflammation, CCL2, CCL3, CCL5, CX3CL1

1. Introduction

Epilepsy is a chronic neurological disorder that affects approximately 65 million people of all ages in the world (Shakirullah et al., 2014). The hallmark of epilepsy is the repeated occurrence of two or more unprovoked seizures, whose clinical manifestation consists of sudden and transitory abnormal episodes of motor, sensory, autonomic, or psychic origin (Shakirullah et al., 2014). Seizure episodes are a result of excessive electrical discharges in a group of neurons in the brain and the behavioral outcome depends on the brain regions where synchronous firing of a neuronal cell group occurs. From a therapeutic point of view, conventional antiepileptic drugs (AEDs) are employed in epilepsy with the aim to reduce this abnormal neural activity. However, about 30% of epileptic patients appear to be resistant to current therapies (Scott Perry and Duchowny, 2013). Mesial temporal lobe epilepsy (MTLE) with hippocampal sclerosis is a typical example of drug-resistant epilepsy. For many MTLE patients, the surgical removal of the epileptic focus remains the only option to achieve an acceptable seizure control (Kwan et al., 2011; Lee, 2014). Thus, it is urgent to find alternative and less invasive approaches for drug-resistant epilepsy treatment. Understanding the functional, cellular and molecular mechanisms involved in the pathogenesis of epilepsy should favor the development of novel drugs that interfere with seizure generation.

A rapidly growing body of evidence indicates that inflammatory processes within the brain contribute to seizure recurrence and precipitation. In both epileptic patients and animal models, seizures upregulate or induce inflammatory mediators, which in turn may enhance brain excitability and neuronal degeneration (Vezzani et al., 2011). In this review we will first provide an overview of the involvement of inflammation in epilepsy, we will then focus on a subfamily of pro-inflammatory molecules, called chemokines, and their increasingly recognized role in seizure control.

2. Inflammation and epilepsy

It is now well accepted that inflammatory pathways are implicated in the pathogenesis of several neurodegenerative disorders, such as multiple sclerosis (MS) and Alzheimer disease (AD), and are known to be activated following neurologic infection, ischemic stroke, and traumatic brain injury

(Glass et al., 2010). The major players of the inflammatory response in the brain are resident cellular elements. Microglia and astrocytes are strongly activated in most neurodegenerative diseases and produce a variety of inflammatory mediators. In particular, in the diseased brain microglia rapidly reacts, changes morphology (a process referred to as “priming”) and can ultimately acquire a phagocytic function (Minghetti, 2005; Perry and Holmes, 2014). Whether the neuroinflammatory reaction is beneficial or detrimental for disease progression is still a matter of debate. Indeed, molecular and cellular responses can be either neuroprotective or neurotoxic depending on several parameters. The concept of "microglia polarization" provides a good example in this respect. According to the classical view, microglial cells respond to acute brain injury becoming activated and developing M1-like (proinflammatory) or M2-like (anti-inflammatory) phenotypes (Lan et al., 2017). It is, however, important to note that this classification has been recently questioned (Ransohoff, 2016).

In the last decade, clinical observations and experimental findings supported a link between inflammation and epilepsy. This link has been shown in human and experimental acquired epilepsies (de Vries et al., 2012; Devinsky et al., 2013; Fabene et al., 2008; Friedman and Dingledine, 2011; Pernot et al., 2011; Vezzani et al., 2013; Aronica et al., 2017), while evidence for a role of inflammation in genetic epilepsies is just beginning to emerge (Shandra et al., 2017). Complex febrile seizures in childhood have long been associated with the later development of temporal lobe epilepsy and febrile illnesses in people with epilepsy can trigger seizures. In surgically resected brain tissue from patients with drug-resistant epilepsy, all of the hallmarks of a chronic inflammatory state have been found, including reactive gliosis and overexpression of cytokines and chemokines. Research using experimental models further corroborates the idea that inflammatory processes have a crucial role in epilepsy. These investigations have also tried to elucidate some unresolved questions such as how inflammation is generated in the epileptic brain and whether inflammation exacerbates the epileptic phenotype.

2.1 Seizures upregulate inflammatory mediators

In rodent models of epilepsy, pharmacological or electrical stimulation of seizures triggers rapid induction of inflammatory mediators in the brain (Vezzani et al., 2013, 2011) and other areas of the central nervous system (CNS), such as the retina (Ahl et al., 2016). Pro-inflammatory cytokines including interleukin (IL) 1β, tumor necrosis factor (TNF) alpha, IL-6 and high mobility group box 1 (HMGB1) are rapidly released from glial cells (Vezzani et al., 2013, 2011) and cytokine receptor expression is induced or upregulated in neurons as well as in microglia and astrocytes (Balosso et al., 2005; Lehtimäki et al., 2003; Ravizza and Vezzani, 2006; Vezzani and Granata, 2005), following epileptic seizures. In addition to inflammatory cytokines, other inflammatory factors such as prostaglandins (PGs) markedly increase following seizures (Shimada et al., 2014). Consistent with this, the enzyme cyclooxygenase-2 (COX-2), responsible for PGs synthesis, is rapidly induced in the brain following seizures (Yoshikawa et al., 2006). A prominent activation of the classical complement pathway was also found in experimental and human temporal lobe epilepsy (Aronica et al., 2007). In addition to the molecules mentioned above, chemokines and their receptors are also produced during epileptic events (Cerri et al., 2016; Foresti et al., 2009; Manley et al., 2007; Xu et al., 2009).

It has been proposed that seizures induce inflammation first in brain endothelial cells (upregulating adhesion molecules and other factors; Fabene et al., 2008; Librizzi et al., 2007), then in perivascular glia, which produces and releases cytokines and PGs (Vezzani et al., 2011). Cytokines bind their receptors and activate signaling cascades that result in the synthesis of chemokines, cytokines, enzymes (e.g., COX-2) and receptors, which further sustain the inflammatory response. For example, IL-1β and HMGB1 respectively bind IL receptor 1 (IL-1R1) and Toll-like receptor 4 (TLR4); their binding activates intracellular pathways converging on the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB). The transcription factor NFkB is common to many pathways activated by inflammatory ligands, and modulates the expression of many genes involved in inflammation, cell death/survival, and synaptic plasticity (O'Neill and Kaltschmidt, 1997; Vezzani et al., 2011). Seizure-induced brain inflammation may trigger the recruitment of peripheral inflammatory cells. In particular, chemokines act as

chemoattractants for blood cells (Ley et al., 2007; Shi and Pamer, 2014) and are responsible for leucocyte recruitment in epileptic brains (Fabene et al., 2010, 2008; Ravizza et al., 2008; Zattoni et al., 2011; Varvel et al., 2016).

It is important to mention that in epileptic brain local inflammatory responses have been observed in conjunction with seizure-promoted blood-brain barrier (BBB) disruption (Fabene et al., 2008; Marchi et al., 2007; Zattoni et al., 2011).

2.2 Inflammation promotes seizures

Brain inflammatory pathways play a key role in seizure generation and exacerbation (Balosso et al., 2005; Maroso et al., 2010; Vezzani et al., 2000; Xiong et al., 2003). For example, IL-1β causes potent proconvulsant effects by mediating enhanced calcium influx through NMDA receptors (Vezzani et al., 2013). Peripheral inflammation can also impact on seizure propensity and this is already evident from the above outlined clinical observation that fever can cause seizures (Cross, 2012). Many experimental evidences further corroborate the notion that systemic infection can trigger or sustain seizures (de Vries et al., 2012; Friedman and Dingledine, 2011; Galic et al., 2008; Györffy et al., 2014; Marchi et al., 2014; Riazi et al., 2008; Sayyah et al., 2003; Zattoni et al., 2011). Systemic inflammation reduces the threshold for pharmacologically induced acute seizures in animals (Sayyah et al., 2003; Riazi et al., 2008), and this has been linked to upregulation of proinflammatory cytokines (Riazi et al., 2008). A systemic inflammatory challenge during a critical period in early development leaves a lasting impact on brain excitability and seizure susceptibility later in life (Galic et al., 2008). Interestingly, pilocarpine, one of the most widely used proconvulsant agents, does not require access to the brain to induce status epilepticus (SE, defined as a seizure lasting >30 min), apparently acting as a peripheral proinflammatory agent that triggers seizures (Marchi et al., 2014).

The proconvulsant effect of systemic inflammation is well described in several studies that used lipopolysaccharide (LPS) as inducer of peripheral infection (Cerri et al., 2016; Galic et al., 2008; Györffy et al., 2014; Sayyah et al., 2003). LPS administration produces convulsions in rats

treated with a subconvulsant dose of kainic acid (KA) (Heida et al., 2005). Systemic LPS also enhances baseline hippocampal excitability and increases progression of rapid kindling, an effect that is counteracted by neutralization of IL-1β (Auvin et al., 2010). Our recent work showed, for the first time, that LPS challenge is able to increase the frequency of spontaneous recurrent seizures (SRS) in a murine model of MTLE based on intrahippocampal injection of KA (Cerri et al., 2016). These data add a novel mechanistic insight since peripheral inflammation appears to target specifically the mechanisms responsible for seizure onset without affecting seizure termination. Indeed, LPS increased frequency of ictal events with no effect on seizure duration (Cerri et al., 2016). The effects of systemic infection on seizures may be explained considering that i) a peripheral inflammatory stimulus triggers a local brain inflammatory "mirror" reaction (i.e., cytokine and chemokine production) similar to the response elicited in the periphery (Perry and Holmes, 2014); ii) the diseased brain, such as the epileptic brain, can display an amplified, exaggerated response to a systemic inflammatory challenge as a result of microglia priming (Perry and Holmes, 2014).

Understanding the complex role of inflammation in the generation and exacerbation of epilepsy is crucial for the identification of new molecular targets for therapeutic intervention. Some strategies able to interfere with agents involved in inflammation have yielded positive outcomes in experimental models of epilepsy (Aronica et al., 2017). For example, targeting the IL-1β or HMGB1 pathway resulted in anti-convulsant effects in the KA model of MTLE (Maroso et al., 2011, 2010). Recent works have turned the spotlights on chemokines as new exploitable targets for controlling neuronal network hyperexcitability in epileptic patients (Bozzi and Caleo, 2016; Cerri et al., 2016; Fabene et al., 2010; Louboutin et al., 2011; Louboutin and Strayer, 2013; Roseti et al., 2015, 2013).

3. Chemokines: who are they and what do they do?

Chemokines are a family of small (8–14 kDa) secreted cytokines usually described as chemoattractants (hence their name) that guide directional migration of leukocytes. These molecules are classified into CXC, CC, CX3C or C chemokines based on the positioning of the conserved cysteine residues in their amino acid chain (Zlotnik and Yoshie, 2000). Based on their function, chemokines can be homeostatic or inflammatory. Homeostatic chemokines, for example CCL14 and CXCL13, are constitutively expressed and are involved in many physiological processes, such as embryonic development, immunity and angiogenesis. Inflammatory chemokines are induced by inflammatory stimuli in pathological conditions and actively participate in the inflammatory response attracting immune cells (monocytes/macrophages; T-lymphocytes, mast cells) to the site of inflammation. Examples are CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CX3CL1 (fractalkine) (Raman et al., 2011).

Chemokines interact with chemokine receptors that are G protein-coupled transmembrane proteins on the surfaces of their target cells. Receptors are divided into four families according to the type of chemokine they bind. For example, CCRs bind CC chemokines and CX3CR1 binds CX3CL1. Upon chemokine binding, they can undergo homo- or heterodimerization and activate many signaling cascades, including Rho-GTPases and MAP kinase pathways (Raman et al., 2011). Chemokine receptors are not restricted to leukocytes. In the diseased brain, in addition to infiltrated monocytes and T-cells, chemokine receptors are found in microglia, astrocytes, oligodendrocytes and neurons (for an overview of cellular localization of principal chemokines and their receptors in the brain, see Fig.1). Chemokines secreted by glia and their receptors are involved in neuronal migration and cell proliferation during brain development, in synaptic activity modulation and in the regulation of neuroendocrine functions (Banisadr et al., 2005; Callewaere et al., 2007; Cartier et al., 2005). On the other hand, it is well known that chemokine signaling is involved in various CNS pathologies, notably those with an inflammatory component.

4. Chemokines in CNS diseases

Chemokines and their receptors are involved in several neurodegenerative diseases, including MS, AD, HIV-associated dementia (HAD) and cerebral ischemia. Their role in the pathogenesis of these diseases remains controversial. Although most of the experimental studies on chemokines indicate that they contribute to the development of the diseases, some investigations suggest that they can also be neuroprotective (Azizi et al., 2014; Cartier et al., 2005). For example, evidence from studies of experimental autoimmune encephalomyelitis (EAE, a model of MS) induced in CCR2 knockout mice indicated that CCL2 and its receptor CCR2 are implicated in MS pathology (Gaupp et al., 2003; Mahad and Ransohoff, 2003). On the other hand, CCL2 signaling on monocytes seems to be neuroprotective in a model of cerebral ischemia (Chu et al., 2015).

CX3CL1 and its receptor CX3CR1 may be implicated in AD neurodegeneration since CX3CR1 deficiency prevented neuron loss in a mouse model of AD (Fuhrmann et al., 2010). However, due to its effect on amyloid β (Aβ) clearance (Merino et al., 2016), it is also proposed that CXC3CL1 signaling may have a protective role in AD (Chen et al., 2016). Disruption of CX3CR1 signaling might also contribute to neurodevelopmental and neuropsychiatric disorders, as suggested by the occurrence of social behavior deficits in mice lacking CX3CR1 (Zhan et al., 2014).

Elevated levels of chemokines have been found in post-mortem human brains in correspondence of MS lesions, Aβ plaques, and cerebral infarct. Interestingly, some chemokines are upregulated in the serum and cerebrospinal fluid (CSF) of MS, AD, HAD and stroke patients (Azizi et al., 2014; Bartosik-Psujek and Stelmasiak, 2005; Cartier et al., 2005). Thus, it has been suggested that chemokines may represent biomarkers for these diseases. For instance, CCL2 plasma level could act as a biomarker to monitor the inflammatory process in AD (Azizi et al., 2014). It has also been proposed that systemic levels of some chemokines might predict future stroke events. For example, in asymptomatic men, higher blood levels of CCL5 could represent a risk factor for stroke (Canouï-Poitrine et al., 2011). However, great caution should be used in judging the validity of plasma biomarkers for brain diseases.

Chemokines are implicated also in other neurological pathologies such as glioma and neuropathic pain. Glioma cells can produce chemokines that in turn control glioma functional behavior including tumor cell migration, invasion, and proliferation (Sciumè et al., 2010). For instance, CCL2 promotes tumor growth and recruits microglial cells to glioma in a rat model (Platten et al., 2003). In neuropathic pain, microglial cells expressing CCR2 play a well-established key role (Zhang et al., 2007). In addition to CCR2, CCR5 is also required for the development of neuropathic pain. Indeed, blocking CCR2- and CCR5-mediated monocyte chemotaxis leads to attenuation of rodent neuropathic pain (Padi et al., 2012).

5. Chemokines and epilepsy

Since epileptic seizures are characterized by an abnormal pattern of neural activity and evidence indicates that chemokines may modulate neural activity, it is important to first discuss the link between chemokines and activity modulation to better understand the role of chemokines in epilepsy.

5.1 Chemokines as modulators of neural excitability

Due to their wide expression in the CNS and putative role in the regulation of neural transmission (Ambrosini and Aloisi, 2004), chemokines and their receptors have been considered the third major transmitter system in the brain, acting in concert with neurotransmitters and neuropeptides (Adler et al., 2008). Chemokines can exert direct effects on neuronal excitability, most likely through their receptors, expressed on both pre and postsynaptic elements (Rostène et al., 2007). For example, CCL2 alters electrophysiological properties and Ca^{2+} signaling in cerebellar neurons (Van Gassen et al., 2005), reduces inhibitory responses in spinal cord neurons (Gosselin et al., 2005), and potentiates excitatory postsynaptic currents in the Schaffer collateral pathway of the hippocampus in vitro (Zhou et al., 2011). These latter effects are mediated by activation of the p38 MAP kinase pathway (Cho and Gruol, 2008).

In vitro studies have also demonstrated that CXCL12 induces the enhancement of spontaneous postsynaptic activity and a slow inward current in cerebellar neurons (Limatola et al., 2000) and modifies the activity of dopaminergic neurons acting on presynaptic γ-aminobutyric acid (GABA) and glutamate release (Guyon et al., 2006). Moreover, it has been shown that in hippocampal neurons CX3CL1 reduces spontaneous glutamate release and postsynaptic glutamate currents (Limatola et al., 2005; Meucci et al., 1998). The latter effect has been linked to increased intracellular calcium and dephosphorylation of the GluR1 glutamate receptor (Ragozzino et al., 2006).

Interestingly, a recent work demonstrated that CX3CL1 is upregulated *in vivo* in rat hippocampus after spatial learning and in hippocampal slices after LTP induction, and it has been proposed that CX3CL1 up-regulation could modulate glutamate neurotransmission during memory-associated synaptic plasticity (Sheridan et al., 2014). Indeed CX3CL1 inhibits LTP maintenance in the hippocampus in $GABA_A$ receptor-dependent manner and suppresses glutamate-mediated Ca^{2+} influx in hippocampal neurons (Sheridan et al., 2014). All the studies described above have examined the involvement of chemokines in neural activity modulation in physiological conditions. Uncovering how chemokines regulate neural activity in pathologies characterized by altered neural firing such as epilepsy is a crucial but still poorly explored issue.

Recently, Palma and co-workers have tackled this issue showing that CX3CL1 is responsible for a positive modulation of $GABA_A$ receptor function in human TLE brain tissue expressed in *Xenopus* oocytes. This effect was mediated by a reduction of $GABA_A$ receptor rundown current (Roseti et al., 2013) and was an intrinsic characteristic of the epileptogenic tissue since it was absent in non-epileptic control tissue. These data indicate that the GABAergic system is significantly modulated by CX3CL1 released in epileptic foci, thus opening a wide scenario of novel therapeutic opportunities for controlling neuronal network hyperexcitability in epileptic patients (Roseti et al., 2013).

5.2 Chemokines in human and experimental epilepsy

Data have indicated that levels of chemokines are upregulated in the brain of epileptic patients. In particular, CCL2 was found to be highly expressed in surgically resected brain tissues from patients with intractable epilepsy (Choi et al., 2009; Wu et al., 2007), and CCL3 and CCL4 have been reported to be up-regulated in patients with MTLE (Van Gassen et al., 2008). It is important to point out that data obtained from human autoptic tissues refer to the "end-point" of the inflammatory process, thus not allowing to draw significant conclusions on the causal role of chemokine expression in epileptogenesis. To date, the experimental studies that have analyzed the involvement of chemokines and their receptors in epilepsy are still limited. Results obtained in animal models point to a role for CCL2, CCL3, CCL4 and CCL5 signaling in epilepsy. Specifically, data indicate that these chemokines are not only produced and released in response to seizures but may also be causative for seizures.

5.2.1 CCR5

CCR5 is a member of the CC-chemokine receptor family that binds CCL3, CCL4, and CCL5. It is expressed in blood circulating cells as well as in microglia. CCR5 is reportedly increased in bran vasculature in animal model of epilepsy, as are its ligands in MTLE patients (Louboutin and Strayer, 2013; Van Gassen et al., 2008). How chemokine receptor expression is induced or upregulated during an ongoing seizure remains to be determined. In the KA model of MTLE, increased CCR5 expression by neuronal and glial cells could be due to a direct effect of KA on glutamate receptors or an indirect consequence of KA-induced excitotoxicity; CCR5 ligands could also be implicated in CCR5 induction/upregulation (Mennicken et al., 2002). Louboutin and colleagues (Louboutin et al., 2011; Louboutin and Strayer, 2013; Marusich et al., 2011) investigated the role of CCR5 in a rat model of seizures provoked by KA intraperitoneal administration. In particular, they showed that inhibition of CCR5 in cells circulating in the blood strongly protected rats from seizures, BBB leakage, CNS injury, and inflammation, and facilitated neurogenic repair. These results suggest that inhibition of CCR5 in circulating cells can decrease

their interaction with endothelial cells, thus reducing leukocyte migration across the BBB, and consequently neuroinflammation and related deleterious events.

5.2.2.CCL2

CCL2 and its receptor CCR2 have been reported to increase in the hippocampus following pilocarpine-induced seizures in rats (Foresti et al., 2009). CCL2 expression has been also found to be induced in hippocampal reactive astrocytes and blood vessel at late- time points following pilocarpine-induced SE in mice (Xu et al., 2009). Manley and co-workers (2007) described the temporal profile of CCL2 expression after KA-induced seizures in rat hippocampus. They found that CCL2 upregulation started 12 hours after KA infusion in the hippocampus. This upregulation was concurrent with immune cell recruitment at the epileptic focus and followed by the typical BBB permeability increase. Authors concluded that CCL2 upregulation and BBB permeability increase are two independent events and that CCL2 upregulation likely mediates the recruitment of immune cells, including circulating monocytes, at the epileptic focus (Manley et al., 2007). In keeping with this hypothesis, Varvel and co-workers (2016) showed that intraperitoneal KA-induced SE causes hippocampal recruitment of monocytes expressing the CCL2 receptor, CCR2. They also found higher levels of CCL2 expression in the hippocampus of KA-treated rats with respect to controls and identified perivascular macrophages and microglia as cellular sources of CCL2. Noteworthy, infiltrating CCR2-positive monocytes exacerbate seizure-induced neural damage. Indeed, CCR2 knockout mice displayed greatly reduced monocyte recruitment and attenuated neuronal damage after pilocarpine-induced SE (Varvel et al., 2016).

Recently, we showed a crucial role for CCL2 and its receptor CCR2 in seizure control (Cerri et al., 2016; Bozzi and Caleo, 2016). Mice with spontaneous recurrent seizures and neuropathology resembling MTLE were systemically injected with LPS to mimic a peripheral inflammatory challenge. LPS was found to increase seizure frequency and up-regulate the brain expression of many inflammatory proteins, including CCL2 (see also subsection 2.2 above). To test the potential role of CCL2 in seizure exacerbation, either a CCL2 transcription inhibitor

(bindarit) or a selective antagonist of the CCR2 receptor (RS102895) was administered systemically. Interference with CCL2 signaling potently suppressed LPS-induced seizure increase. Intracerebral administration of anti-CCL2 antibodies also abrogated LPS-mediated seizure enhancement in chronically epileptic animals (Cerri et al., 2016). These results reveal that CCL2 is a key mediator that link peripheral inflammation with neuronal hyperexcitability.

6. Conclusions

Chemokines are increasingly capturing neuroscientists' attention for their multiple actions in the brain in both physiological and pathological conditions. In particular, their involvement in several brain diseases makes chemokines a particularly interesting subject of investigation. Although their role in pathologies such as AD, MS, and stroke is still controversial, in the last years chemokines have been recognized as a "trademark" for these diseases. This may have important implications: i) chemokines could represent new targets for therapeutic intervention and ii) given their elevated levels in the serum of patients, circulating chemokines could be considered biomarkers for AD, MS and stroke. Despite the role of inflammation in the pathogenesis of epilepsy, to date a few but convincing works have analyzed the involvement of chemokines. Importantly, these studies have suggested that chemokines and their receptors (in particular CCL2, CX3CL1, CCR2 and CCR5) have a key role in epilepsy and may represent new therapeutic targets for seizure control. This is particularly important for drug- resistant epilepsies, such as MTLE, where the surgical removal of the epileptic focus is still today the only, not always resolutive, cure. Future efforts to deepen our knowledge on the link between chemokines and epilepsy are desirable. In particular, it would be clinically relevant to verify whether CCL2, CX3CL1, CCR2 and CCR5 may be considered biomarkers for epilepsy. In this view, further studies are needed to investigate whether plasma levels of these chemokines in epileptic patients could predict seizures and indicate a temporal window for their therapeutic control.

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Data reported in this review were identified by searches of PubMed (as of June 16, 2017). Abstracts and reports from meetings were not included, and only papers published in English were reviewed. However, due to the large amount of bibliographic material available on this subject, we apologize with those authors whose studies were not quoted. This work was supported by a Fondazione Veronesi [post-doctoral fellowship to C.C], AIRC [grant IG18925 to M.C], and University of Trento [start-up grant to Y.B.].

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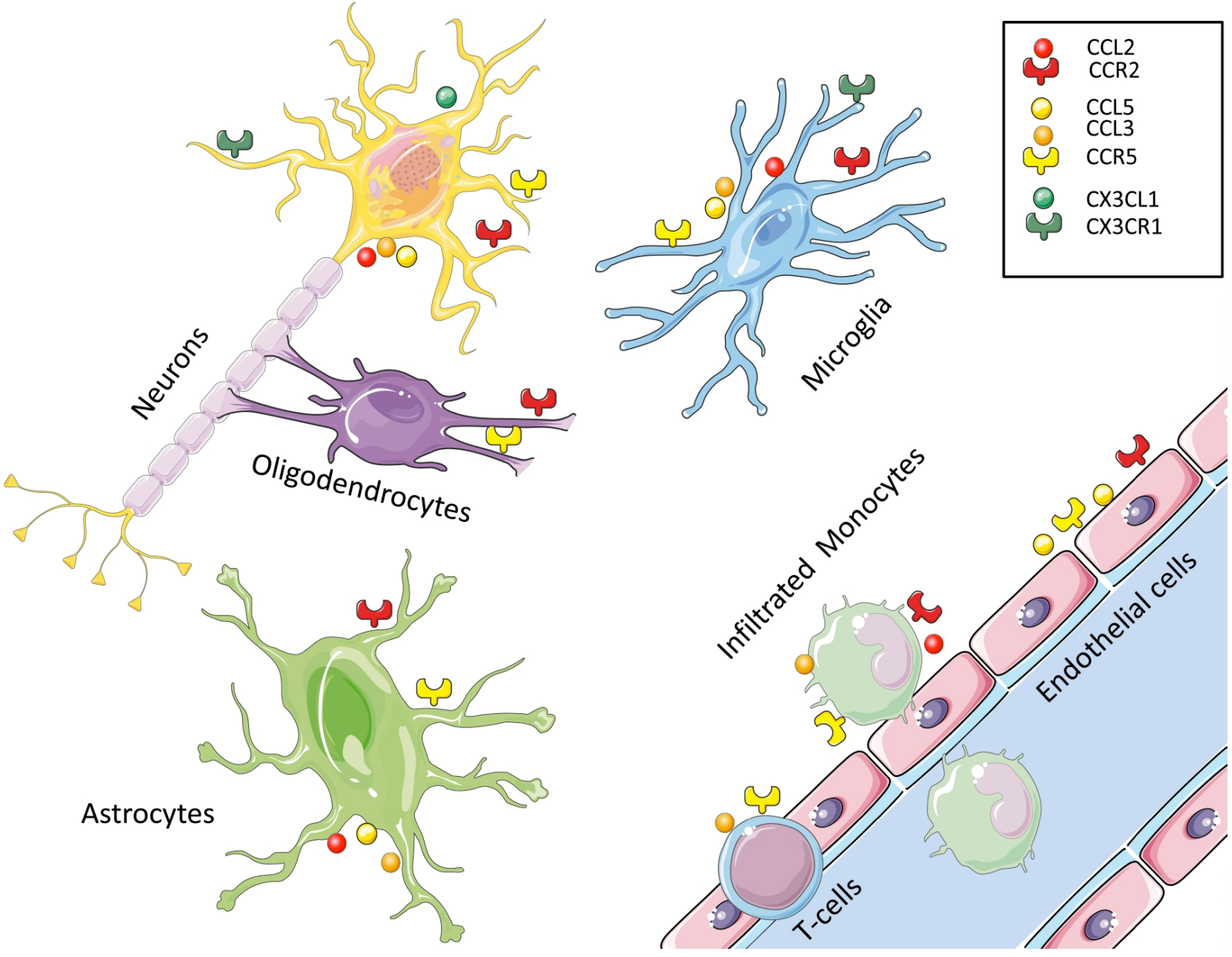
Figure 1. Cellular localization of chemokines and their receptors in the diseased brain.

In brain pathological conditions, neurons, microglia, astrocytes, and infiltrated monocytes can secrete CCL2 and express its receptor CCR2, which is also present on oligodendrocytes and endothelial cells. The CCR5 receptor is expressed by neurons, microglia, astrocytes, oligodendrocytes, infiltrated monocytes, T-cells, and endothelial cells. The cellular sources of its ligands are neurons, microglia, astrocytes, endothelial cells (CCL5) and infiltrated monocytes and T-cells (CCL3). CX3CL1 expression has been found in neurons while its receptor CX3CR1 is present on neurons and microglial cells (see Aronica et al., 2017; Azizi et al., 2014; Bozzi and Caleo, 2016; Ubogu et al., 2006, and references therein).


CCL2
CCR2
CCL5
CCL3
CCR5
CX3CL1
CX3CR1
Neurons
Oligodendrocytes
Microglia
Astrocytes
Infiltrated Monocytes
Endothelial cells
T-cells