

Review Article

Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks

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Chemokines and their receptors have crucial roles in the trafficking of leukocytes, and are of particular interest in the context of the unique immune responses elicited in the central nervous system (CNS). The chemokine system CC ligand 2 (CCL2) with its receptor CC receptor 2 (CCR2), as well as the receptor CXCR2 and its multiple ligands CXCL1, CXCL2 and CXCL8, have been implicated in a wide range of neuropathologies, including trauma, ischemic injury and multiple sclerosis. This review aims to overview the current understanding of chemokines as mediators of leukocyte migration into the CNS under neuroinflammatory conditions. We will specifically focus on the involvement of two chemokine networks, namely CCL2/CCR2 and CXCL8/CXCR2, in promoting macrophage and neutrophil infiltration, respectively, into the lesioned parenchyma after focal traumatic brain injury. The constitutive brain expression of these chemokines and their receptors, including their recently identified roles in the modulation of neuroprotection, neurogenesis, and neurotransmission, will be discussed. In conclusion, the value of evidence obtained from the use of *Ccl2*- and *Cxcr2*-deficient mice will be reported, in the context of potential therapeutics inhibiting chemokine activity which are currently in clinical trial for various inflammatory diseases.

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Introduction

Chemokines, or chemotactic cytokines, are classically defined by their ability to induce directional migration and activation of leukocyte subsets into inflammatory sites. Since the identification of the first human chemokines nearly two decades ago, extensive research has accumulated showing the significant contribution of these small, peptide mediators to inflammatory conditions. In the central nervous system (CNS), chemokines such as CC ligand 2 (CCL2) and its receptor CC receptor 2 (CCR2) have been implicated in neuropathologies

ranging from traumatic brain injury (TBI) to auto-immune diseases.

Chemokines are classified on the basis of structural features, which in turn, give rise to their functional specificity. The two main categories recognized are CXC or α -chemokines and CC or β -chemokines; CXC chemokines have one amino-acid residue separating two conserved cysteines and are primarily chemotactic for neutrophils, whereas CC chemokines that contain two adjacent cysteines are attractants for monocytic cells and lymphocytes (Zlotnik and Yoshie, 2000). There is considerable overlap and interaction between related chemokines and their receptors, whereby one chemokine can bind to various receptors, resulting in redundancy within the signaling network.

This review aims at providing an outline of the CCL2/CCR2 and CXCL8/CXCR2 chemokine networks in the brain, their functions, and contribution to pathologic conditions. Constitutive expression of chemokines in the CNS and the proposed roles of these mediators in neurogenesis, neuroprotection, and neurotransmission will also be discussed. Finally, we will report the current progress and issues associated with drug development aimed at therapeutically targeting

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chemokines in the CNS and systemic diseases. Multiple database searches were conducted from 2006 to 2009 to identify relevant references, and retrieved documents were hand-searched for additional publications. References included in this review were those deemed significant to the development of a solid understanding of the chemokine networks discussed.

The macrophage chemoattractant CCL2

There are five known members of the monocyte chemoattractant protein (MCP) family, designated as CCL2, CCL8, CCL7, CCL13, and CCL12 (MCP-1-5, respectively). Each family member attracts a different subset of leukocytes after binding with different affinities to several receptors (Gouwy *et al*, 2004). Although CCL2, CCL7, and CCL8 are able to signal through the CCR2 receptor, CCL2 is the most potent at activating signal transduction pathways leading to monocyte transmigration (Sozzani *et al*, 1994).

CCL2, also known as MCP-1/JE, was the first human chemokine to be characterized (Rollins, 1996; Yoshimura *et al*, 1989). Its ability to activate and attract cells of the monocyte lineage including macrophages, monocytes, and microglia has been repeatedly shown by *in vitro* assays (Rollins, 1991; Yoshimura and Leonard, 1992). Targeted *Ccl2* overexpression in the thymus and CNS of transgenic mice resulted in the accumulation of macrophages in these organs, which is further evidence of chemotactic function (Fuentes *et al*, 1995; Gunn *et al*, 1997; Wang *et al*, 2002). Conversely, mice deficient in *Ccl2* are reportedly unable to effectively recruit monocytes in response to an inflammatory stimulus, despite the presence of normal circulating leukocyte numbers (Lu *et al*, 1998).

In the brain, a positive correlation has been found between the level of *Ccl2* expression and the number of infiltrated macrophages after a cortical aspiration lesion (Hausmann *et al*, 1998). Concomitantly, recombinant CCL2 injected into the murine hippocampus induced leukocyte accumulation, with greater potency than did other monocyte chemokines, such as CCL5 (Bell *et al*, 1996). Besides monocytic cells, CCL2 is reportedly also a chemoattractant for T lymphocytes, basophils, natural killer cells, and astrocytes *in vitro* (Ge and Pachter, 2004; Rollins, 1996; Woldemar Carr *et al*, 1994). However, the physiologic impact of CCL2-mediated recruitment of these cells *in vivo* needs to be determined.

CCR2 receptor structure and intracellular signaling in the central nervous system

CCL2 binds primarily to the G-protein-coupled receptor CCR2, an interaction that is responsible for the initial phase of monocyte recruitment (Dzenko *et al*, 2001;

Dzenko *et al*, 2005; Kuziel *et al*, 1997). Dimerized CCL2 binds the receptor, which is then internalized and removed from the cell surface. This process has been proposed to regulate extracellular CCL2 levels (Mahad *et al*, 2006; Tylaska *et al*, 2002; Zhang and Rollins, 1995). Downstream targets of CCR2 signaling include phosphatidylinositol-3 kinase, mitogen-activated protein kinases, and protein kinase C, indicating that a wide range of intracellular pathways may be involved in cellular responses elicited by CCL2 (Stamatovic *et al*, 2005; Wain *et al*, 2002).

In the CNS, CCR2 expression has been reported on various cell types, including neurons, astrocytes, microglia, neural progenitor cells, and microvascular endothelial cells (Banisadr *et al*, 2002, 2005; Coughlan *et al*, 2000; Gourmala *et al*, 1997; Stamatovic *et al*, 2005). During normal conditions, expression seems to be at consistently low levels. Astrocyte and microglial CCR2 expression seems to be quite heterogeneous and subject to significant upregulation during an inflammatory response (Andjelkovic *et al*, 2002; Croitoru-Lamoury *et al*, 2003; White *et al*, 2005).

Consistent with studies using *Ccl2* knockout mice, deficiency of the *Ccr2* gene results in impaired macrophage recruitment in several injury and disease models (Göser *et al*, 2005). Decreased leukocyte adhesion and monocyte infiltration have been shown in *Ccr2*^{-/-} mice after an immune challenge with intraperitoneal thioglycollate injections (Kurihara *et al*, 1997), whereas delayed monocyte extravasation was observed after spinal cord contusion injuries in *Ccr2*^{-/-} mice (Ma *et al*, 2002). Emerging evidence indicates that CCR2 expression distinguishes the two subsets of blood monocytes in mice: the Ly-6C^{high} (Gr-1^{high}) CCR2⁺CX₃CR1^{low} phenotype is found on phagocytic monocyte cells which readily infiltrate into inflammatory sites, whereas Ly-6C^{low}(Gr-1^{low})-CCR2⁻CX₃CR1^{high} cells are less effective at transmigration and are believed to give rise to resident tissue macrophages (Geissmann *et al*, 2003). These subsets correspond, respectively, to the CCR2⁺CD16⁻ and CCR2⁻CD16⁺ monocyte populations in humans.

The CCL2/CCR2 network in the central nervous system

In the brain, CCL2 is predominantly produced by astrocytes and resident microglia, and to a lesser extent, by endothelial cells (Barna *et al*, 1994; Berman *et al*, 1996; Glabinski *et al*, 1996; Hanisch, 2002; Harkness *et al*, 2003). CCL2 is also released by infiltrating macrophages upon their migration into the brain parenchyma, implying the presence of autocrine regulation that perpetuates cell recruitment and activation (Clavo *et al*, 1996; Gourmala *et al*, 1997; Gunn *et al*, 1997; Peterson *et al*, 1997). Neurons are yet another source, producing detectable levels of CCL2 after brain ischemia (Che *et al*, 2001; Gourmala *et al*, 1997), transection of facial or

hypoglossal nerves (Flugel *et al*, 2001), and lipopolysaccharide administration (Gourmal *et al*, 1997).

A wide range of stimuli can trigger CCL2 production and release during an inflammatory response. Treatment with lipopolysaccharide, interferon, interleukin-1 beta (IL-1 β), colony-stimulating factor-1, transforming growth factor- β , and tumor necrosis factor- α (TNF α) can induce CCL2 expression in different cell types either *in vivo* or *in vitro* (Clavo *et al*, 1996; Harkness *et al*, 2003; Huang *et al*, 2000; Hurwitz *et al*, 1995; Thibeault *et al*, 2001). In contrast, retinoic acid, glucocorticoids, and estrogen reportedly inhibit CCL2 production (Melgarejo *et al*, 2009). A more comprehensive understanding of these mechanisms may provide fundamental evidence, whereby pathological neuroinflammation may be therapeutically manipulated.

The CCL2/CCR2 network in brain development and neurotransmission

A distinct pattern of CCL2 and CCR2 expression has been identified at different embryonic stages in relation to the cytoarchitectural organization of the CNS, implying a role for this chemokine network during brain development (Meng *et al*, 1999; Rezaie *et al*, 2002). Treatment of rat embryonic cultures with CCL2 and CCL7 increased the differentiation of cells toward a dopaminergic phenotype (Edman *et al*, 2008). Complementing these data, the application of CCL2 to dopaminergic neurons *in vivo* increased cell excitability, dopamine release, and locomotor activity in rats (Guyon *et al*, 2009). These novel findings show a previously unknown role for this chemokine in mediating dopaminergic neuron signaling and development.

CCL2 as a modulator of blood–brain barrier permeability

The highly selective blood–brain barrier (BBB) is largely impervious to circulating leukocytes. As CCL2 production in the brain is primarily intraparenchymal, it is still unclear how this hydrophilic protein communicates with the periphery to attract blood-borne monocytes. It is conceivable that CCL2 is released directly into the bloodstream by astrocytes and brain microvascular endothelial cells, which comprise the BBB. Alternatively, CCL2 may be transported transcellularly across the BBB, possibly by interaction with specific carrier molecules, such as caveolin-1 (Ge and Pachter, 2004).

In addition to its chemotactic properties, recent evidence indicates that CCL2 has direct effects on BBB permeability (Dzenko *et al*, 2005; Song and Pachter, 2004). Stamatovic *et al* (2005) showed that exposure of astrocytes and brain microvascular

endothelial cells to CCL2 *in vitro* induced changes in actin cytoskeletal structure and redistribution of tight junction protein expression, thereby rendering the BBB more porous and facilitating the transendothelial migration of blood-borne leukocytes into the brain. These effects were attenuated in CCL2-treated animals that had been previously depleted of peripheral macrophages, indicating that this chemokine acts directly on endothelial cells of the BBB, and indirectly, by the recruitment of macrophages and subsequent changes in BBB permeability (Stamatovic *et al*, 2005). In a separate study by the same group, a reduction in BBB permeability resulted from the use of antisense oligonucleotides or neutralizing antibodies blocking CCL2 in an *in vitro* model of ischemia–reperfusion injury (Dimitrijevic *et al*, 2006). According to this model, CCL2 modulation of BBB permeability appears to be mediated specifically by CCR2 expressed on endothelial cells, as tight junction protein distribution and BBB permeability were not altered by CCL2 treatment in *Ccr2*^{−/−} mice (Stamatovic *et al*, 2003).

The CCL2/CCR2 network in brain pathologies

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by extensive demyelination and inflammation, leading to a severe and progressive neurologic impairment. Inflammation has a critical role in MS, predominantly mediated by auto-reactive T cells infiltrating the brain parenchyma (Mahad and Ransohoff, 2003). The presence of CCL2 has been shown in autopsy tissue obtained from patients with both active and chronic MS, correlating with regions of hypertrophic astrocytes and macrophage infiltration, thus implicating CCL2 in this pathology (McManus *et al*, 1998; Simpson *et al*, 1998; Van Der Voorn *et al*, 1999). Interestingly however, CCL2 levels measured in the cerebrospinal fluid (CSF) of patients with MS was consistently attenuated compared with healthy individuals, perhaps as a result of its binding to circulating monocytes, which then downregulate CCR2 as they cross the BBB (Franciotta *et al*, 2001; Sindern *et al*, 2001).

Multiple sclerosis can be modeled in rodents by inducing experimental autoimmune encephalomyelitis (EAE), after inoculation of a myelin component, such as myelin basic protein. Elevated astrocytic *Ccl2* mRNA has been shown in conjunction with EAE relapses (Ransohoff *et al*, 1993), although subsequent to the entry of leukocytes into the brain, suggesting that this chemokine has an amplifying effect rather than an initiating role in MS (Glabinski *et al*, 1995). Both *Ccl2*^{−/−} and *Ccr2*^{−/−} mice show impaired macrophage recruitment during the course of EAE, and show less severe clinical symptoms compared with wild-type animals (Fife *et al*, 2000; Huang *et al*, 2001). Neutralizing antibodies or DNA

vaccination against CCL2 before EAE induction similarly alleviate the disease in mice (Kennedy *et al*, 1998; Youssef *et al*, 1999).

Ischemic Brain Injury

After cerebral stroke in patients, elevated CCL2 has been detected in both serum and CSF (Arakelyan *et al*, 2005; Losy and Zaremba, 2001). Transient occlusion of the middle cerebral artery (MCAO), an experimental model of ischemic stroke, similarly triggers CCL2 production in the rodent brain (Che *et al*, 2001; Gourmala *et al*, 1997). In transgenic *Ccl2* mice, MCAO induced enhanced recruitment of inflammatory cells and exacerbation of infarct volumes (Chen *et al*, 2003). In contrast, introduction of a nonfunctional *Ccl2* gene into rats by the adenoviral vector significantly reduced infarct volume and macrophage infiltration compared with control animals (Kumai *et al*, 2004). Similarly, Hughes *et al* (2002) showed that *Ccl2* deficiency is neuroprotective, as gene knockout mice exhibited smaller infarct volumes after MCAO as compared with wild-type mice, accompanied by a reduction in macrophage accumulation at 2 weeks (Hughes *et al*, 2002). A recent study has investigated the underlying mechanisms of CCL2's role in ischemic damage, by using green fluorescent protein transgenic bone marrow chimeras to differentiate between resident CNS microglia and blood-borne macrophages. Despite similar microglial activation early after MCAO, by 7 days, attenuated macrophage infiltration was apparent in both *Ccl2*- and *Ccr2*-deficient mice (Schilling *et al*, 2009a,b). These data corroborate the unequivocal function of *Ccl2* for the recruitment of blood-borne macrophages but not of microglia, and supports an association between macrophage accumulation and tissue damage.

Traumatic Brain Injury

Although implicated in TBI, the precise function of CCL2 in the time course of delayed brain damage after trauma still needs to be fully elucidated. A unique role for this chemokine in TBI is substantiated by the observation that *Ccl2* mRNA and protein levels are increased acutely in several models of mechanical injury to the brain. *Ccl2* expression was markedly increased by 3 h after stab wound injury, followed by an increase in protein levels at 12 h (Glabinski *et al*, 1996), whereas enhanced CCL2 has been detected as early as 2 h after aspiration cortical damage (Hausmann *et al*, 1998; Muessel *et al*, 2000). Increased CCL2 has also been reported after diffuse axonal injury (Babcock *et al*, 2003; Rancan *et al*, 2001), spinal cord contusion (Ma *et al*, 2002; McTigue *et al*, 1998), facial nerve injury (Flugel *et al*, 2001), and cryogenic cerebral trauma (Grzybicki *et al*, 1998). Our laboratory has shown that CCL2 that was upregulated after diffuse traumatic axonal injury

parallels the increase in sICAM (soluble intracellular adhesion molecule-1), which mediates leukocyte adhesion (Rancan *et al*, 2001). The involvement of sICAM-1 after TBI is supported by human studies, whereby increased sICAM-1 levels in the CSF correlate with brain contusion size and BBB dysfunction in patients with TBI (Pleines *et al*, 1998).

Most studies investigating the production of CCL2 in TBI have used noncontusional models, with some involving surgical penetration of the cortex (Babcock *et al*, 2003; Glabinski *et al*, 1996) or resection of the brain tissue (Hausmann *et al*, 1998; Muessel *et al*, 2000). Lateral fluid percussion and closed head injury models should be considered superior for examining inflammation associated with mechanisms of cerebral contusion formation and thus, most accurately reproducing human TBI. A recent study using a lateral fluid percussion model showed a transient increase in CCL2 peaking at 8 to 12 h in the injured cortex (Rhodes *et al*, 2009). In our laboratory, CCL2 protein levels peaked between 4 and 12 h after focal closed head injury in the mouse (Semple *et al*, manuscript in preparation). As this upregulation preceded the accumulation of mononuclear phagocytes in the brain peaking at 3 to 5 days after injury, we hypothesize that CCL2 expression is an early, intrinsic response to TBI, of primary importance in immune cell recruitment. The underlying mechanisms for this time discrepancy, between the early peak in CCL2 production and the delayed arrival of macrophages in the parenchyma several days later, are still unknown.

Although most chemokines target more than one receptor and most chemokine receptors bind to multiple ligands *in vivo* (Kurihara *et al*, 1997; Lu *et al*, 1998), additional evidence needs to be acquired to support the nonredundant role of CCL2 in recruiting macrophages in TBI pathology. One group using the aspiration cortical lesion model in *Ccl2*^{-/-} mice reported attenuated microglial activation correlating with transiently improved survival of thalamic neurons (Muessel *et al*, 2002). A recently completed study in our laboratory showed that *Ccl2*^{-/-} mice subjected to closed head injury developed smaller cortical lesions with reduced neuronal loss, diminished macrophage accumulation and astrocyte activation at 4 weeks after injury (Semple *et al*, manuscript in preparation, Figure 1). A parallel improvement in the neurological outcome of *Ccl2*^{-/-} mice supports a primarily deleterious role of CCL2 after focal TBI.

Neuroprotective and neurotrophic properties of CCL2-mediated signaling

Despite robust experimental evidence indicating that elevated CCL2 and subsequent recruitment of macrophages into the brain is detrimental, awareness of the fact that chemokines also possess pleiotropic and beneficial properties, beyond chemotaxis, is increasing. A potential role in tissue repair has been

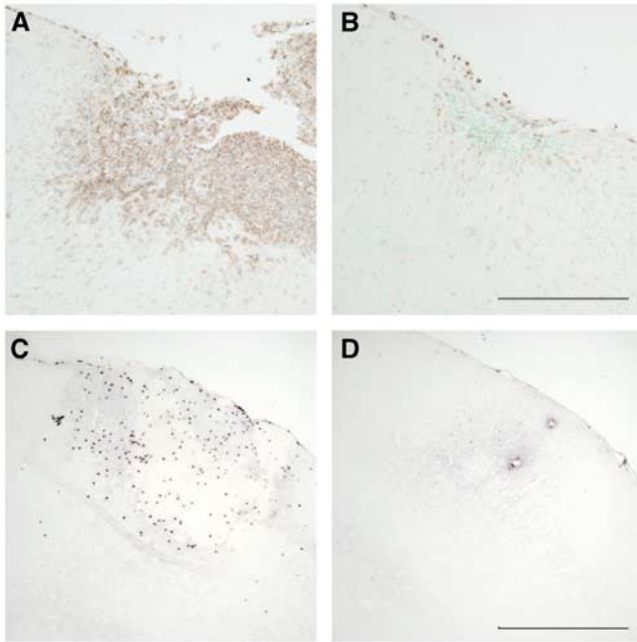


Figure 1 Effect of chemokine ligand or receptor deficiency on leukocyte infiltration after experimental focal traumatic brain injury. Upper microscope images illustrate the accumulation of macrophages and activated microglia in the injured cortex at 4 weeks after closed head injury, which was considerably reduced in *Ccl2*^{-/-} mice (panel B) compared with wild-type controls (panel A). Lower images show the influx of neutrophils peaking at 12 h after injury in wild-type mice (panel C). In contrast, few neutrophils are able to infiltrate the brains of *Cxcr2*^{-/-} mice (panel D). Stainings were performed with monoclonal anti-mouse F4/80 and NIMP-R14 antibodies (Serotec, Raleigh, NC, USA), for macrophages/microglia and neutrophils, respectively. Scale bar (panels A and B) = 200 μm; (panels C and D) = 500 μm. Unpublished data are obtained from the study by BD. Semple.

identified in the context of a skin wound model, with *Ccl2*^{-/-} mice exhibiting delayed wound reepithelization, angiogenesis, and impaired collagen synthesis compared with wild-type animals (Low *et al*, 2001). The apparent paradox of CCL2 function indicated by such data may be attributed to the heterogeneity of the macrophage response. After myocardial infarction, e.g., infiltration of Ly-6C^{high}CCR2⁺ macrophages dominates early, with cells exhibiting a phagocytic and inflammatory phenotype. Ly-6C^{low}CCR2⁻ cells that enter the tissue later display attenuated inflammatory properties and may be associated with tissue repair and regeneration (Nahrendorf *et al*, 2007). Neuroprotective effects associated with a reduction in CCL2-mediated macrophage infiltration after stroke or TBI may relate not only to the overall number of leukocytes present but also to their altered phenotypic state. Furthermore, any attempts to inhibit CCL2 production or to reduce CCR2 expression for therapeutic purposes under pathological conditions should be weighed carefully against its role in health maintenance and repair (Figure 2).

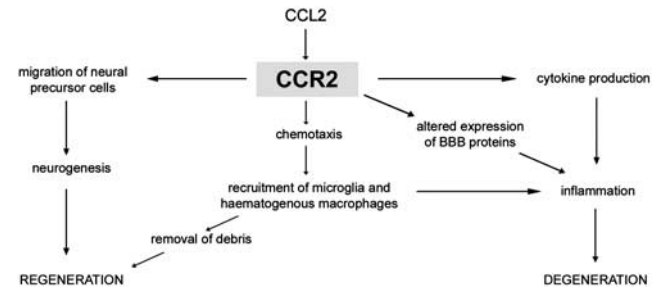


Figure 2 The roles of CCL2/CCR2 in brain inflammation and injury. CCL2 induces the recruitment of macrophages, production of cytokines, and direct alteration of the expression of endothelial cell tight-junction proteins to increase blood–brain barrier (BBB) permeability, which contributes to inflammation in the brain, potentially exacerbating neuronal loss. CCL2-mediated macrophage accumulation may also be beneficial, as these phagocytic cells remove myelin debris, which otherwise inhibits regeneration. Furthermore, CCL2 is chemotactic for neural precursor cells and thus, may influence repair after injury by enhancing neurogenesis.

In the brain, speculation that CCL2 may be important in neurogenesis has been gaining momentum over the past decade, as recombinant CCL2 was first shown to promote glial cell proliferation and growth *in vitro* (Rezaie *et al*, 2002). The ability of CCL2 to induce stem-cell migration into sites of damage in the adult injured brain has since been illustrated using hippocampal brain slices from ligand and receptor knockout mice (Belmadani *et al*, 2006; Wiedera *et al*, 2004). Cultured neural precursor cells migrate in response to CCL2 *in vitro*, whilst they move toward sites of recombinant CCL2 infused into the brain *in vivo* (Magge *et al*, 2009). Most recently, the first evidence that CCL2 may also direct differentiation of precursor cells into neurons, astrocytes, and oligodendrocytes has been published (Chintawar *et al*, 2009).

To date, the role of CCL2 in adult neurogenesis after brain injury has been investigated most thoroughly in models of stroke. Lui *et al* (2007) showed that MCAO induced a strong upregulation of CCL2 within the neurogenic subventricular zone. CCL2 reportedly promoted motility of adult neural progenitor cells and enhanced their differentiation into neurons, whereas the neutralization of the chemokine abolished these effects (Lui *et al*, 2007). Another group has also identified CCL2/CCR2-dependent migration of neuroblasts within the brain to sites of ischemic damage (Yan *et al*, 2007).

Current strategies for the therapeutic targeting of CCL2/CCR2

Accumulating preclinical data indicate that the CCL2/CCR2 network is a promising target to therapeutically reduce inflammatory cell infiltrates and tissue damage. Thus far, the pharmaceutical industry

has primarily focused on the use of CCR2 antagonists, resulting in several compounds entering the early phases of clinical trials for application in peripheral inflammatory conditions, including rheumatoid arthritis (small molecule antagonist INCB3284, Incyte, Wilmington, DE, USA), atherosclerotic cardiovascular disease (neutralizing antibody MLN1202, Millenium Pharmaceuticals, Cambridge, MA, USA), and IgA nephropathy (CCL2-LPM, Osprey Pharmaceuticals, Saint-Laurent, QC, Canada) (Horuk, 2009).

Information on the progress of clinical trials with CCR2 antagonists is scarce, with many companies reporting either no or only slight developments in their research. This lack of progress may reflect the complexity and redundancy of the chemokine network and its interactions, thus our limited understanding of this signaling network in disease (Horuk, 2009). Application of innovative technologies may assist in effectively targeting the CCL2/CCR2 network in neuroinflammation, e.g., with the use of interference RNA to silence specific genes, RNA oligonucleotides, or dominant negative mutants of chemokine ligands to eliminate receptor function.

The question also remains as to whether CCR2 antagonists would be able to effectively cross the BBB for treatment of CNS conditions, such as TBI, MS, or stroke. In the context of TBI, we know that the BBB has increased permeability for a short period of time after injury, which allows the infiltration of serum proteins and circulating leukocytes into the CNS (Habgood *et al*, 2007). This time course of BBB dysfunction may provide a therapeutic window for the administration of drugs which cannot normally cross the intact BBB.

The neutrophil chemoattractants CXCL1, CXCL2, and CXCL8

Just as CCL2 is considered to be the prototypical monocyte-attracting CC chemokine, CXCL8 (also known as IL-8) is the most intensely studied CXC chemokine, first identified as a powerful mediator able to induce morphologic changes and degranulation of neutrophils (Baggiolini *et al*, 1989). Since then, evidence for the role of CXCL8 as a key player in neutrophil transmigration has been shown both *in vivo* (Bell *et al*, 1996) and *in vitro* (Huber *et al*, 1991). Human CXCL8 can bind two receptors: CXCR2, which has been shown to be the most important receptor in chemotaxis, and CXCR1, which is believed to mediate activation of the neutrophil respiratory burst and release of myeloperoxidase (Rose *et al*, 2004). The CXCR2 receptor is 75% identical in humans and mice; however, no direct homology of the human ligand CXCL8 has been found in rodents to date. In addition, mice lack a corresponding homolog of CXCR1 (Lee *et al*, 1995).

In rodents, two main ligands perform the same functions as human CXCL8, namely CXCL1 (also known as keratinocyte-derived chemokine, KC) and CXCL2 (macrophage inflammatory protein, MIP-2). These chemokines share 78% sequence homology, and have been recognized as the most critical CXCR2 ligands mediating the neutrophil influx characteristic of inflammatory disorders, including psoriasis, rheumatoid arthritis, atherosclerosis, and irritable bowel disease. Compounding this nomenclature confusion further, CXCL1 and CXCL2 have also been identified in humans as growth-regulated gene- α (GRO α) and GRO β , respectively.

CXCR2 receptor structure and intracellular signaling in the central nervous system

As is the case for all known chemokine receptors, CXCR2 is a seven transmembrane G-protein-coupled receptor. Chemokines that are able to bind CXCR2 and mediate neutrophil chemotaxis contain the glutamic acid–leucine–arginine (ELR) tripeptide motif in their N-terminal domain, as opposed to non-ELR CXC chemokines (such as CXCL12), which lack this motif and generally attract lymphocytes. Upon ligand binding, CXCR2 activates G-protein-mediated phosphoinositide hydrolysis to generate diacylglycerol and inositol 1,4,5-trisphosphate, which then activate protein kinase C allowing the mobilization of calcium to initiate cellular responses (Wu *et al*, 1993). The ligand–receptor complex is phosphorylated and endocytosed after signaling through clathrin-dependent pathways, and once internalized, CXCR2 may be either degraded or transported back to the cell membrane for reexpression (Rose *et al*, 2004). Receptor endocytosis is believed to be essential for the regulation of chemotactic migration, although the evidence for this is still controversial (Rose *et al*, 2004; Yang *et al*, 1999). A recent study using receptor knockout mice has elegantly shown that functional CXCR2 is essential for the removal and thus, for the regulation of chemokines in the circulation and brain, identifying a novel scavenging role of these receptors in chemokine homeostasis (Cardona *et al*, 2008).

Cxcr2^{−/−} mice generated by homologous recombination have been widely used to show the crucial role of CXCR2 in neutrophil recruitment (Cacalano *et al*, 1994). Despite increased circulating neutrophils, the number of neutrophils recruited in *Cxcr2*^{−/−} mice in response to peritoneum thioglycollate injections was one-fifth that of wild-type mice (Cacalano *et al*, 1994; Luan *et al*, 2001). Neither human CXCL8 nor murine CXCL1 or CXCL2 can induce neutrophil accumulation in *Cxcr2*^{−/−} mice, confirming that CXCR2 is the primary receptor for these ligands. However, it is worth noting that neutrophils from *Cxcr2*^{−/−} mice can still respond

to other chemotactic molecules, such as leukotriene B₄, a lipid mediator which acts through a different receptor, indicating normal neutrophil locomotor activity (Mihara *et al*, 2005).

The CXCL8/CXCR2 network in the central nervous system

As with the CCL2/CCR2 network, ligands of the CXCR2 receptor appear to be expressed at low basal levels in the brain and upregulated in pathology. Reported sources of CXCL1, CXCL2, and CXCL8 include activated microglia, astrocytes, and endothelial cells; in addition, infiltrated neutrophils themselves are a major source of CXC chemokines, potentially amplifying leukocyte recruitment (Lu *et al*, 2005; Valles *et al*, 2006).

Peripherally, the receptor CXCR2 is most highly and uniformly expressed by neutrophils, although it has also been shown on eosinophils, mast cells, and on a small subset of effector T cells (Lippert *et al*, 2004). In the CNS, widespread neuronal CXCR2 has been detected by immunohistochemistry and *in situ* hybridization within several regions of the brain and spinal cord of nondiseased autopsied human tissue (Horuk *et al*, 1997). In the normal rat CNS, CXCR2 immunoreactivity has been reported within neurons of the cortex and striatum, as well as in several thalamic, hypothalamic, mesencephalic, and pontine nuclei (Luan *et al*, 2001; Valles *et al*, 2006). In contrast, astrocytes and microglia seem to express the CXCR2 receptor only once activated, e.g., *in vitro* by proinflammatory cytokines such as IL-1 β and TNF α , or *in vivo* after TBI or demyelination (Aloisi *et al*, 1992; Lindner *et al*, 2008; Otto *et al*, 2000; Saas *et al*, 2002). As with the CCR2 receptor and several other transmembrane receptors, the lack of specificity of commercially available antibodies continues to confound a clear understanding of expression localization (Semple *et al*, unpublished observations).

The CXCL8/CXCR2 network in brain development and neurotransmission

Recent data substantiating the involvement of CXCR2 signaling in neuronal electrical activity, neurotransmitter release, and synaptic plasticity in the CNS have contributed to the emerging concept of the 'chemokinergic' system as a new class of neurotransmitters (Parsadaniantz and Rostene, 2008). Giovannelli *et al* (1998) found that Purkinje neurons in mouse cerebellar slices respond to CXCL8 and CXCL1 treatment with a transient increase in calcium, neurotransmitter release, and impaired long-term depression. A separate study showed that CXCR2, when coexpressed on cerebellar Purkinje neurons with the Glut1 subunit of AMPA (α -amino-3-

hydroxyl-5-methyl-4-isoxazole-propionate) receptors, can alter the functional profile of these ion channel receptors by increasing channel opening frequency and thus, enhancing glutaminergic activity (Lax *et al*, 2002). The ability of CXCL8 to modulate calcium channel excitability through CXCR2 on rat septal neurons has also been shown (Puma *et al*, 2001). These findings corroborate a function for CXCR2 signaling beyond neutrophil chemoattraction, which is supported by widespread constitutive CXCR2 expression throughout the adult brain.

A role for this chemokine system in brain maturation has also been suggested, as the expression of both CXCL2 and CXCR2 is widely distributed during early developmental stages in the mouse forebrain, hippocampus, thalamus, and floor plate (Luan *et al*, 2001). Considering the chemotactic properties attributed to this chemokine family, it is conceivable that signaling through CXCR2 may contribute to the trafficking of neuronal processes to form appropriate synapses during brain development.

Furthermore, constitutive expression of CXCR2 by human oligodendrocytes has been shown both *in vitro* and *in vivo* (Omari *et al*, 2006). CXCR2-mediated signaling is reportedly crucial for oligodendrocyte development, as *Cxcr2*^{-/-} mice exhibit reduced oligodendrocyte precursors which are abnormally localized within the spinal cord, in conjunction with altered myelination distribution (Padovani-Claudio *et al*, 2006). By signaling through CXCR2, the ligand CXCL1 was shown to inhibit precursor cell migration, thus negatively regulating this developmental process (Tsai *et al*, 2002).

The CXCR2 network in brain pathologies

Multiple Sclerosis

The CXCL1/CXCR2 system seems to be involved in the pathology of MS. First, elevated CXCL8 was detected in the CSF of MS patients compared with controls, a phenomenon which is attenuated in patients receiving interferon β -1a therapy (Lund *et al*, 2004). Expression of CXCR2 has been described in normal and proliferating oligodendrocytes within active MS lesions (Omari *et al*, 2006), as well as on activated microglia bordering the lesion (Filipovic *et al*, 2003). Proximal reactive astrocytes reportedly secrete the ligand CXCL1, the production of which can also be induced *in vitro* with the proinflammatory cytokine IL-1 β , leading the authors to propose that this chemokine network may be involved in recruiting oligodendrocytes to mediate repair (Omari *et al*, 2006). A recent study showing that blockage of CXCR2 resulted in BBB compromise and heightened clinical manifestations in EAE mice, supports a role for this chemokine network in tissue repair of this autoimmune disease (Carlson *et al*, 2008).

Ischemic Brain Injury

A substantial number of studies have shown pronounced neutrophil infiltration in ischemic brain tissue (Emerich *et al*, 2002). Attenuation of neutrophil transmigration by an anti-CXCL8 antibody resulted in a 60% reduction in infarct volume after transient focal ischemia in rabbits (Matsumoto *et al*, 1997). The chemokine-mediated infiltration of neutrophils appears to contribute to reperfusion injury rather than to the formation of the initial infarct, as blockage of CXCR2 signaling by the noncompetitive allosteric inhibitor reparixin, was able to significantly reduce tissue damage after transient, but not permanent, MCAO in rats (Garau *et al*, 2005). Although reparixin binds with higher affinity to CXCR1 than to CXCR2, this compound has been shown to reduce neutrophil infiltration by 40–50%, reduce infarct volume, inhibit long-term inflammation, and improve recovery of sensorimotor function after experimental stroke (Garau *et al*, 2005; Villa *et al*, 2007). Another compound which was generated as a dual inhibitor of CXCR1 and CXCR2, DF2156A, showed similar neuroprotective effects (Garau *et al*, 2006).

Interestingly, several studies have detected the elevation of CXCL1 and CXCL8 in the periphery after ischemic brain injury, indicating the occurrence of systemic inflammatory events resulting from damage to the CNS. Circulating levels of CXCL8 were reportedly elevated in patients after stroke (Kostulas *et al*, 1998). CXCL1 was found to be increased in the plasma, liver, and lungs of mice after experimental MCAO at times preceding the peak of chemokine expression in the injured cortex and striatum (Chapman *et al*, 2009). These data illustrate the fact that chemokine expression in the body is both spatial and temporal, indicating that local production of chemokines within the brain parenchyma may be only one of the mechanisms contributing to the chemotactic gradients which regulate leukocyte migration.

Traumatic Brain Injury

In the clinic, we along with others have reported that CXCL8 elevated in the CSF of patients after severe TBI in both adults and children correlated with severe BBB dysfunction (Kossmann *et al*, 1997) and increased mortality (Whalen *et al*, 2000), suggesting that measurement of CSF CXCL8 may be an indicator of poor prognosis. Levels of CXCL8 were significantly higher in the CSF than in the serum, supporting intrathecal production as the prominent source. Our laboratory has previously shown that the adhesion molecule sICAM-1, the concentration of which in the CSF of TBI patients correlated with the severity of BBB breakdown (Pleines *et al*, 1998), is able to induce CXCL2 production in cultured mouse astrocytes and brain microvascular endothelial cells (Otto *et al*, 2002).

A prominent influx of neutrophils into the damaged brain parenchyma within the early hours after experimental TBI has been repeatedly shown by our group (Bye *et al*, 2007; Stahel *et al*, 2000). However, little work has been carried out to investigate the mechanisms underlying the contribution of CXCR2-mediated signaling to secondary tissue damage after TBI. We have previously shown that CXCL2 and CXCR2 are acutely increased in the ipsilateral hemisphere after closed head injury in mice, peaking between 4 and 8 h after injury (Otto *et al*, 2001). In a separate study, using a controlled cortical impact model in rats, Valles *et al* (2006) showed that production of the ligands CXCL1 and CXCL2 was increased as early as 2 h after trauma, remaining elevated for more than 24 h. Levels of the CXCR2 receptor were elevated in the ipsilateral cortex by 8 h after injury (Valles *et al*, 2006). Another study detected upregulated CXCR2 expression in the contused hemisphere by 4 h after lateral fluid percussion injury in mice (Rhodes *et al*, 2009). Recently completed work from our laboratory showing reduced accumulation of neutrophils in *Cxcr2*^{-/-} mice by ~80%, compared with wild-type controls after closed head injury (Semple *et al*, manuscript in preparation, Figure 1), emphasizes the essential role of CXCR2 in mediating neutrophil infiltration into the injured brain.

Interestingly, neither CXCL2 elevation nor neutrophil infiltration was observed by our group in a rat model of diffuse traumatic axonal injury (Rancan *et al*, 2001), suggesting that these key features of focal brain injury are not as relevant for patterns of diffuse brain damage. Clearly, more research into the specific role of CXCR2 in the context of different forms of TBI is warranted.

Neuroprotective and neurotrophic properties of CXCR2-mediated signaling

A robust neutrophilic infiltration is often the first cellular response to acute infection or injury. Rather than contributing to tissue damage, neutrophils in infectious diseases seem to have a vital role in the containment and neutralization of bacteria to minimize tissue degradation. For example, after inoculation with *Staphylococcus aureus*, mice depleted of neutrophils exhibit more severe brain abscesses and higher bacterial burdens than do controls (Kielian *et al*, 2001). The involvement of the CXCL8/CXCR2 network was shown by blockage of CXCR2, which increased bacterial load by up to 100-fold in animals with several pulmonary infections, resulting in higher mortality (Khan *et al*, 2007; Reutershan, 2006).

Independent of their chemotactic properties, additional neuroprotective and neurotrophic functions of the ligands CXCL8, CXCL1, and CXCL2 are emerging (Figure 3). First, it has been shown that CXCR2

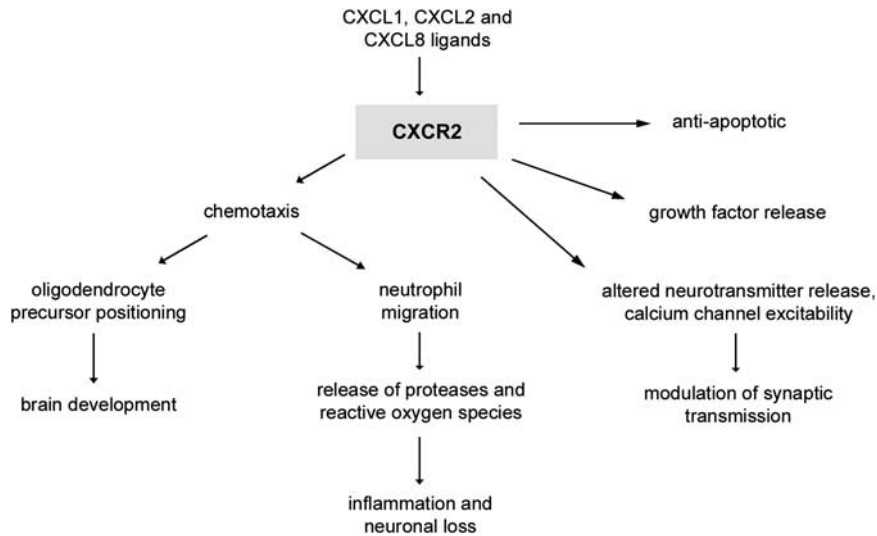


Figure 3 The multiple functions mediated by CXCR2 signaling in the CNS. CXCR2 is the main receptor involved in neutrophil chemotaxis, leading to cell migration into the brain during injury, infection or disease. Neutrophils perpetuate the neuroinflammatory response by the release of enzymes such as proteases, contributing to neuronal degeneration. Independent of this role, CXCR2 signaling is involved in chemotaxis of oligodendrocyte precursors during development, the release of growth factors, mediating self-defense mechanisms against Fas-initiated apoptotic cell death, and modulating synaptic transmission through altering calcium channel excitability and neurotransmitter release.

signaling is involved in the resistance of astrocytes and neurons to cell death *in vitro*. CXCL8 released by astrocytes signals in an autocrine manner, binding to CXCR2 expressed on their own cell surface to provide protection against Fas-mediated apoptosis in a self-defense mechanism (Saas *et al*, 2002). CXCL8 treatment of cerebellar granule neurons enhanced cell survival, an effect which was abolished by the inhibition of phosphatidylinositol-3 kinase signaling (Limatola *et al*, 2002). In hippocampal neurons, the ligand CXCL2 was shown to protect against amyloid-induced cell death by the activation of mitogen-activated protein kinase and phosphatidylinositol-3 kinase pathways (Watson and Fan, 2005). Conversely, a separate study has shown that CXCL2 can induce dose-dependent neurotoxicity of cultured primary motor neurons, illustrating the fact that consequences of CXCR2 signaling may depend on the ligand, concentration, cell type, and stimulus involved (De Paola *et al*, 2007).

Second, neurotrophic properties have been attributed to ligands signaling through CXCR2 *in vitro*. One group has shown that CXCL1 treatment of primary mouse cortical neurons stimulated mitogen-activated protein kinase and phosphatidylinositol-3 kinase-mediated growth factor signaling in a more potent manner as compared with known mediators, such as nerve growth factor and fibroblast growth factor (Xia and Hyman, 2002). Our laboratory has previously shown that CXCL8 stimulates nerve growth factor production by cultured astrocytes, when applied either as a recombinant peptide or as an endogenous chemokine collected from human CSF after TBI (Kossmann *et al*, 1997).

Both CXCL2 and CXCL8 have also been shown to be neurotrophic for cerebellar granule neurons, as treatment with these chemokines *in vitro* prevented potassium-depletion-induced cell death, an effect which appeared to be mediated by an association with AMPA-type ionotropic glutamate receptors (Limatola *et al*, 2000). Furthermore, exposure to CXCL8 increased the survival of cultured hippocampal neurons, likely by enhancing the proliferation of cocultured glial cells (Araujo and Cotman, 1993). Further investigation is required to examine whether these chemokines actually function *in vivo* as growth factors, with a potential impact in health or disease states.

Current strategies for the therapeutic targeting of CXCR2

Previous attempts to reduce neutrophil infiltration by the administration of neutralizing antibodies to individual chemokines have produced mixed results. This variability may depend on the model, the chemokine, and the organ being targeted. Experimentally, neutralizing antibodies against the receptor CXCR2 almost completely inhibited neutrophil infiltration in an intraperitoneal inflammation murine model, whereas blocking either CXCL1 or CXCL2 ligands alone produced only a partial attenuation (Tanimoto *et al*, 2007). Such studies emphasize the advantage of blocking receptors to combat the considerable redundancy within chemokine networks, as neutralization of an individual chemokine

may be compensated by another chemokine binding the same receptor. Any issues caused by currently unknown differences between human CXCL8 and its murine homolog ligands may also be avoided by targeting the joint receptor, CXCR2.

The alternative approach to study the role of CXCR2 is by modeling inflammatory diseases in mice overexpressing or genetically deficient for the specific gene. It is important to remember that, although they are invaluable for defining the roles of particular molecules *in vivo*, there are numerous drawbacks when using gene knockout and transgenic animal models. For example, although the *Cxcr2*^{-/-} phenotype is viable, these mice respond poorly to environmental stress, reproduce at low rates, and have smaller body weights. Upon autopsy of adult mice, abnormalities including splenomegaly, lymph node enlargement, and increased white bone marrow have been observed (Cacalano *et al*, 1994). A marked increase in total blood neutrophil counts and a considerable increase in myelopoiesis in *Cxcr2*^{-/-} mice suggests that this chemokine network has a vital role in inhibiting the differentiation of immune cells and in maintaining a healthy balance of all blood cell types (Broxmeyer *et al*, 1996; Coughlan *et al*, 2000; Luan *et al*, 2001).

Although these abnormalities provide important information on the role of CXCR2 in development and physiology, they can interfere with experimental studies investigating the role of this chemokine network in disease paradigms. Abnormalities present in knockout mice provide clues as to the possible side effects that may result from CXCR2 antagonism, indicating that patients who receive CXCR2-targeted therapeutics should be monitored carefully during clinical trials.

Conclusions

Chemokines are multifunctional mediators in the brain, both in health and pathology. The best characterized chemokine-receptor networks involving CCL2/CCR2 and CXCL8/CXCR2 have nonredundant functions not only in regulating immune cell infiltration into the CNS during neuropathology but also in physiologic processes, including neurogenesis, neuroprotection, and neurotransmission. The use of gene knockout and transgenic mice has been invaluable to increase our understanding of the different mechanisms regulated by chemokines. For conditions in which neuroinflammation is a key pathologic event such as MS, stroke, and TBI, therapeutic targeting of chemokine networks to reduce the inflammatory infiltrate and its consequences may have considerable potential benefit. Ultimately, targeting chemokines in heterogeneous conditions such as TBI with the aim of improving patient outcomes will most likely require the admission of multiple drug agents targeting several deleterious pathways, such as excitotoxicity and

oxidative stress, all of which contribute to downstream neuronal degeneration and subsequent neurologic impairment.

Conflict of interest

The authors declare no conflict of interest.

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