Chapter 5:

Expression of CXCL4 in microglia *in vitro* and *in vivo* and its signaling through CXCR3

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Abstract
Signaling through chemokine receptor CXCR3 in the brain has been implicated in various brain diseases, since CXCR3 and its ligands are found under these conditions. Recently, a new chemokine ligand for CXCR3 was reported. In humans an alternatively spliced variant of CXCR3 expressed on microvascular endothelial cells, named CXCR3b, was shown to bind CXCL4. In the periphery, the cellular expression and functions of CXCL4 are well described but in the brain its expression and function are unknown. Here we show that brain microglia are a cellular source of CXCL4 in vitro and in vivo under neurodegenerating conditions. Microglial migration induced by CXCL4 is mediated by CXCR3 as demonstrated in CXCR3-deficient microglia. CXCL4 also attenuates LPS-induced microglial phagocytosis and nitric oxide production. We propose that locally released CXCL4 controls the microglial response and prevents overactivation.

Introduction
Chemokines belong to the cytokine family and induce cellular migration in immune cells. Chemokines are divided into four families (C, CC, CXC and CX3C) based on the positioning of one or two conserved cysteine residues in the N-terminal domain of the protein (Rossi and Zlotnik, 2000; Fernandez and Lolis, 2002). To date, nearly 50 chemokines and 25 chemokine receptors have been identified (Laing and Secombes, 2004; Murphy, 2002).

In the periphery, the role of chemokines in regulating migration of immune cells is widely recognized (Mackay, 2001; Moser et al., 2004; Rot and von Andrian, 2004). Also in the central nervous system, chemokines are now considered an important component of the inflammatory response following neural injury (Bajetto et al., 2002; Biber et al., 2006; Ransohoff and Tani, 1998).

In this respect, CXCR3 is an important chemokine receptor in the brain. CXCR3 has been associated with various neurodegenerative diseases and models of neural injury (Liu et al., 2006; Rappert et al., 2004; Sorensen et al., 1999; Wang et al., 2000). The ligands for CXCR3 are chemokines CXCL9, CXCL10, CXCL11 and CCL21 (Cole et al., 1998; Loetscher et al., 1996; Soto et al., 1998; Biber et al., 2001; Dijkstra et al., 2004b) all of which have been associated with various brain diseases (Sorensen et al., 1999; Simpson et al., 2000a; Poluektova et al., 2001; Sorensen et al., 2002; Fife et al., 2001; Wang et al., 1998; Hamilton et al., 2002; McColl et al., 2004; Biber et al., 2001; Biber et al., 2002b; de Jong et al., 2005). The importance of CXCR3 signaling has been further established in CXCR3-/- animals. In the entorhinal cortex lesion (ECL) model of neurodegeneration, impaired microglia activation and
increased neuronal survival after the lesion was reported in CXCR3-/- animals compared to wild type animals (Rappert et al., 2004). In the same study no effect was found in CXCR3-/- animals in response to facial nerve injury. In an animal model for multiple sclerosis, a recent study showed an increase in disease severity in the CXCR3-/- animals compared to wild types (Liu et al., 2006). These findings demonstrate an apparent different role for this receptor in the central nervous system under varying experimental disease conditions. The reason for these different effects of CXCR3 signaling are yet not understood, but may in part be explained by differential expression of CXCR3 ligands in the brain. Understanding the regulation and expression pattern of CXCR3 and its ligands will therefore provide important insight in the development of neuropathologies.

Recently, CXCL4 (previously platelet factor 4) was proposed to be a possible novel ligand for CXCR3 since it was shown to bind an alternatively spliced variant of CXCR3, named CXCR3b, which is expressed on human microvascular endothelial cells (Lasagni et al., 2003). In order to determine a possible role of CXCL4 in the brain we have determined its expression in cultured brain cells and in vivo under neurodegenerating conditions. It is presented here that CXCL4 is specifically expressed in microglia and not in neurons or astrocytes in vitro. Neurodegeneration induced CXCL4 expression in microglia in vivo and in organotypic brain slices. Wild type microglia but not CXCR3-deficient microglia migrated in response to CXCL4. Finally, it is described here that CXCL4 attenuates LPS-induced microglial activation. This is the first description of CXCL4 in endogenous brain cells and identifies CXCR3 as the receptor for CXCL4 in microglia.

Materials and Methods

Chemicals

Dulbecco’s Modified Eagle Medium (DMEM), Fetal Calf Serum, MEM Sodium Pyruvaat, L-glutamin, Penicillin, Streptomycin, Hank’s balanced salt solution (HBSS), Trypsin and PBS from GibcoBRL Life Technologies (Breda, The Netherlands); Poly-D/L-Lysin, Lipopolysacharide (LPS) from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands); DNAse I from Roche (Mannheim, Germany); Recombinant mCXCL4 from R&D; iQ SYBR Green Supermix (Biorad, Hercules CA); Yellow-Green Fluospheres from Invitrogen; recombinant mCXCL4 (R&D). Antibodies: Iba1 (Wako); GFAP (Dako); NeuN (Chemicon); fluorescent conjugated secondary antibodies (Jackson ImmunoResearch); Hoechst (Fluka).
Cell cultures

Cortical Neurons

Cultures of cortical neurons were established as described before (Biber et al., 2001). In brief, pregnant mice (NMRI) were anesthetized with isoflurane, sacrificed by cervical dislocation and ED 16 embryos were removed. Cortices were dissected in ice-cold HBSS supplemented with 30% glucose. After meninges were removed, cortices were placed in a 0.25% trypsin solution at 37 degrees Celsius for 20 minutes. Subsequently, tissue was gently dissociated by trituration and then filtered through a cell strainer (70 µm, Falcon). After one washing step (100x g for 10 min), cells were seeded out on poly-D-coated glass cover slides and maintained in Neurobasal/B27 medium for at least 7 days in a humidified atmosphere with 5% CO₂ at 37 degrees Celsius.

Astrocyte and microglia cultures

Mixed glial cell cultures were prepared by dissection of mouse cortex from newborn mouse pups (1-3 days old) on a chilled platform under sterile conditions. Tissue was collected in medium A (HBSS with 0.6% Glucose, 15 mM HEPES buffer and 1% Pen/Strep). The cortex was chopped and trypsinized for 20 minutes (0.25% trypsin in medium A and DNase I) at 37°C. Reaction was stopped with trypsin inhibition medium (medium A with 20% FCS, 1% DNase I and 1% trypsin inhibitor), and washed with wash medium (medium A with 10% FCS and 1% DNase). The cortices were triturated with fire polished glass pipettes, centrifuged at 800 RPM for 10 min in 25 ml of culture medium (DMEM with 4.5 g/l glucose, 2mM Glutamine, 1% pen/Strep and 1% Sodium Pyruvate) and resuspended in 1 ml culture medium. Suspended cells of 1.5 mouse brain were added per culture flask (75 cm²) with 10 ml culture medium. Cultures were maintained at 37 °C, in a humidified atmosphere (5% CO₂). Culture medium was replaced after 2 days and every 3 days thereafter. After 7-10 days in culture pure microglia were harvested by shaking the T75 flasks on 200 rpm for 2 h at 37 degrees and replated on poly-L-lysine coated coverslips. After 4-5 weeks in culture, astrocytes were trypsinized and replated 3-4 times in order to obtain pure astrocyte cultures.

Entorhinal cortex lesion model

C57Bl/6 and CXCR3-/- mice were kept under standardized conditions at constant temperature and controlled lighting and had ad libitum access to food and water. Unilateral entorhinal cortex lesion was performed as described before (Rappert et al., 2004). Briefly, the animals were anesthetized with a mixture consisting of 20% ketamine (CuraMED
GmbH) and 8% Rompun (Bayer, Wuppertal, Germany) dissolved in 0.9% NaCl. The animals were fixed in a stereotaxic apparatus (Kopf Instruments) and the following coordinates measured from λ were used for lesions: anteroposterior, 0.4 mm; lateral, 1.2 mm; and dorsoventral, down to the base of the skull. The monopolar blade of the surgical needle was lowered only once to the base of the skull.

**Chemotaxis assay**

Cell migration in response to neuronal supernatants or chemokines was assessed using a 48-well chemotaxis microchamber (NeuroProbe) as described (Dijkstra et al., 2004). Briefly, chemokine stock solutions were prepared in PBS and further diluted in culture medium for use in the assay. Culture medium without chemokines served as a control in the assay. 28µl of recombinant mCXCL4 solution or control medium was added to the lower wells, lower and upper well were separated by a polyvinylpyrrolidone-free polycarbonate filter (8 µm pore size), 1.5·10⁴ microglial cells were applied per well (50 µl) in the upper chamber. Determinations were done in hexaplicate. The chamber was incubated at 37°C, 5% CO₂ in a humidified atmosphere for 120 min. At the end of incubation the filter was washed, fixed in methanol and stained with toluidine blue. Migrated cells were counted with a scored eyepiece and data are presented as percentages of control-migration. In order to prevent bias, chemotaxis filters were occasionally counted by blinded investigators.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde for 30 min then rinsed twice with PBS. Cells were pre-incubated using PBS with 0.3% Triton X-100 and 5% fetal calf serum for 30 min. After rinsing twice with PBS, cells were incubated with primary antibody (CXCL4/Iba1) in PBS with 0.3% Triton X-100 overnight. After rinsing three times with PBS, appropriate secondary antibodies labeled with Fluorescein Isothiocyanate (FITC) or CY3 were incubated in PBS for 1 hour. A nuclear staining was performed using Hoechst at a concentration of 1 ug/ml for 15 min. After rinsing three times with PBS glass coverslips were mounted on object glasses. Analysis was done on an AOBS confocal image system (Leica) or on a Zeiss Axioskope 2 with Leica FFC300 FX camera and Leica software. Control experiments for specificity were done in absence of the primary antibody.
TCA precipitation
2 ml culture supernatant (w/o FCS) was mixed with trichloric acetic acid (TCA) up to a final concentration of 10% and incubated overnight at -20°C. The mixtures were centrifuged (10 min;15.000xg ), the supernatant discarded, and the pellet was washed twice with ice-cold acetone. Thereafter the pellet was air-dried, resuspended in sample buffer (0.175M TrisHCl pH 6.8, 4% SDS, 4M Urea, 0.2 M DTT, 20% glycerol, 0.1% bromophenolblue), and subjected to SDS-PAGE.

Western Blot Analysis
Supernatant extracts were loaded onto 12.5% SDS-polyacrylamide gels and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). The membranes were blocked with 10% nonfat dry milk in PBS and incubated with the primary antibody (CXCL4 1:1500, R&D) overnight, followed by a 2-hr incubation with the appropriate horseradish peroxidase-conjugated antibody (Amersham Biosciences). Specific bands were visualized by ECL™ Western blotting Detection System. 10 ng Recombinant mCXCL4 (R&D) was used as a positive control.

Real-time PCR analysis of CXCL4 mRNA
Brain tissue or cell cultures were lysed in guanidinium isothyocyanate/ mercaptoethanol buffer, total RNA was extracted and transcribed into cDNA as described previously (Biber et al., 2001). Primers were designed using Primer Designer version 3.0. CXCL4 primers sequences were: forward primer 5’-CAGTCCTGAGCTGCTGTTCT-3‘; reverse primer: 5’-TCCAGGCTGGTGATGTGCTTA-3‘. Hydroxymethylbilane synthase (HMBS) primer sequences were: forward 5’-CCGAGCCAAGGACCAGGATA-3‘; reverse primer 5’-CTCCTTCCAGGTCGCTCAGA-3‘. HMBS was used as normalizing gene because its expression remained stable under various experimental conditions. Experiments were conducted in 25 ul, using iQ SYBR Green Supermix with primers in a concentration of 250 nmol in 96 wells plates in an i-cycler (Biorad). Reaction conditions were 3 min at 95°C, followed by 50 cycles of 10 s at 95°C and 45 s at 58°C, followed by 1 min at 95°C and by 1 min at 55°C. To determine the amount of CXCL4 amplification at various time points after entorhinal cortex lesion or LPS stimulation, was normalized to the reference gene HMBS and related to a control sample, the comparative cycle threshold Ct method was used as described previously (Livak and Schmittgen, 2001).
Phagocytosis Assay
24h after seeding microglia on 12mm coverslips (10^5 cells/coverslip in 24 wells plates in 50% DMEM 10%FCS/ 50% glial conditioned medium), cells were stimulated with LPS (500 ng/ml o/n) or CXCL4 (10nM) or both in which case CXCL4 was applied 1h before LPS stimulation. After treatment, medium was collected for nitric oxide measurements (below) and replaced with culture medium containing 0,1% yellow-green fluospheres for 30 min. Thereafter, cells were rinsed 5 times in warm PBS to completely remove non-phagocytosed beads. Cells were subsequently fixated with 4% paraformaldehyde. After fixation, nuclei were stained using Hoechst and phagocytosis was measured using a method according to Koenigsknecht (Koenigsknecht and Landreth, 2004).

Nitric Oxide Assay
Culture media were obtained from control and stimulated microglia and nitrite levels were determined using the Griess reaction. Briefly, 50 ul of culture medium was mixed in a 96-wells plate with equal volume of 0,1% N-1-naphylethylenediaminedihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid. After colour development (10 min RT in the dark), samples were measured at 550 nm on a plate reader (Labsystems Multiskan).

Statistical analysis
To compare experimental conditions versus controls and to compare between groups, a one-way ANOVA was used. The significance levels in each experiment were calculated by dividing p= 0.05 by the number of experimental conditions.
Results

**CXCL4 expression is found in microglia and not in neurons and astrocytes**

In order to determine if neural cells express CXCL4, RT-PCR was performed in pure cell cultures. In figure 1 it is shown that at equal RNA levels, only microglia express CXCL4 mRNA.

![RT-PCR Image](image)

**Figure 1. Microglia specifically express CXCL4 in vitro**

(A) RT-PCR in pure neural cell cultures revealed CXCL4 mRNA expression in microglial cultures only. MM is molecular marker; N is neurons; A is astrocytes; MIC is microglia; NC is negative control; GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

To confirm this finding on the protein level, immunocytochemistry in microglia cultures was performed (Figure 2A-E). Figure 2A and 2B show control stainings to demonstrate the specificity of the antibodies for microglial marker Iba1 and CXCL4 respectively. Figure 2C shows that in numerous microglia a perinuclear staining of CXCL4 was observed. Co-stainings with Iba1 identified the CXCL4-expressing cells as microglia (Figure 2D-E). Figure 2D shows CXCL4 protein (red) expressed in Iba1-positive microglia (green). However, not all microglia in culture were positive for CXCL4 indicating heterogeneity in the microglial population (Figure2E; arrowhead indicates CXCL4-negative microglia). In these cultures CXCL4 immunoreactivity in Iba1-negative cells (<1%) was never observed.

Furthermore, trichloroacetic acid (TCA) precipitation and subsequent westernblot analyzes revealed the presence of CXCL4 protein in the supernatant of cultured microglia (Figure 2F).
Figure 2. Microglia express and release CXCL4 protein

(A-E) Immunostainings of cultured primary microglia demonstrating specific expression of CXCL4 in these cells. (A) Negative control for microglial marker Iba1. (B) Negative control for CXCL4. (C) Perinuclear staining of CXCL4 (in red) is clearly distinguishable (arrowhead). (D) Co-staining of CXCL4 and Iba1 showing that CXCL4 protein is expressed in microglia specifically. (E) Co-staining of CXCL4 and Iba1 demonstrating that not all microglia are positive for CXCL4. (F) Representative western blot showing that CXCL4 is detectable in the supernatants of cultured primary microglia (arrowhead). As a positive control recombinant CXCL4 was used.

Entorhinal cortex lesion induces CXCL4 mRNA and protein expression in microglia

In subsequent experiments CXCL4 expression was investigated in the mouse brain after ECL. In the ECL model, the cortex-hippocampus projection is transected resulting in microglial activation in the dentate gyrus (Bechmann and Nitsch, 2000). RNA was extracted 6h and 48h after ECL and analyzed for CXCL4 expression by realtime PCR (Figure 3A). Already 6h after ECL, an increase of CXCL4 mRNA was detected compared to control brains. This difference became significant 48h after ECL (Figure 3A).

To determine the CXCL4 expressing cell type, additional immunohistochemical studies were performed. In control brains CXCL4 immunoreactivity was found, but solely present specifically inside blood vessels in structures without a nucleus as demonstrated by using the
nuclear dye Hoechst (Figure 3C-D). In ECL brains, CXCL4 immunoreactivity was also observed outside blood vessels in nucleated cells. Co-stainings were performed to determine whether they were neurons (NeuN), astrocytes (GFAP) or microglia (Iba1). These cells were shown to be microglia as demonstrated by costaining with the microglial marker Iba1 (Figure 3E-F). Immunoreactivity of CXCL4 could not be colocalized with NeuN or GFAP (data not shown).

Recently Bechmann et al have shown that in the ECL model of neurodegeneration, within 48h, there is a significant influx of immune cells from the periphery (Bechmann et al., 2005). To exclude possible interference of infiltrating immune cells in the CXCL4 signal, an *ex vivo* hippocampal slice culture system was used. To induce neurodegeneration in this model, slice cultures were treated with 50 uM NMDA for 4h and examined after 4, 8, 24 and 48h after treatment for CXCL4 expression (Figure 3B). This figure shows that CXCL4 is rapidly upregulated in hippocampal slice cultures after NMDA treatment. From this it becomes clear that in absence of peripheral immune cells, CXCL4 is significantly upregulated in this model of neurodegeneration.
Figure 3. Upregulation of CXCL4 in microglia after entorhinal cortex lesion (ECL)
(A) Quantitative PCR showing the upregulation of CXCL4 mRNA in brain samples 6h and 48h after ECL. (B) Quantitative PCR showing the upregulation of CXCL4 mRNA in hippocampal slice cultures 0, 4, 8, 24, and 48h after 4h of 50 μM NMDA treatment. (C-D) Immunohistochemistry on control brains showing CXCL4 immunoreactivity in structures without nucleus inside blood vessels. (E-F) Immunohistochemistry on ECL brains showing CXCL4 immunoreactivity in cells that are co-stained with microglial marker Iba1.
CXCL4 attracts microglia through CXCR3
Since CXCR3 is discussed as a potential receptor for CXCL4 (Lasagni et al., 2003) and since microglia express CXCR3 (Biber et al., 2002; Dijkstra et al., 2004; Flynn et al., 2003; van der Meer et al., 2001) we here tested whether microglia would respond to CXCL4 stimulation. Microglia derived from wildtype animals migrated significantly when stimulated with different concentrations of CXCL4 (Fig 4). No effect of CXCL4 stimulation was observed in CXCR3-deficient microglia. Also astrocytes are known to express CXCR3 (Biber et al., 2002; Croitoru-Lamoury et al., 2003; Flynn et al., 2003; van der Meer et al., 2001) and respond to the appropriate ligands with intracellular calcium transients. In contrast to microglia, neither wildtype or CXCR3-deficient astrocytes responded to CXCL4 in calcium imaging experiments. Both wildtype and and CXCR3-deficient astrocytes responded to a positive control (ATP) but not to CXCL4 (N=112 wt/ N=126 CXCR3-/-; data not shown).

Figure 4. CXCR3 mediates CXCL4-induced microglial migration
Concentration dependent chemotaxis induced by CXCL4 was observed only in wildtype microglia, not CXCR3-deficient microglia. Experiments were performed in hexaplicate in three independent experiments.

CXCL4 attenuates microglial phagocytosis and nitric oxide production
Since chemokines are known to affect microglia activation, it was here investigated whether CXCL4 would potentiate or attenuate microglial activation in vitro. The microglial cell line
BV-2 or primary microglia were treated with 10 nM CXCL4, 500 ng/ml LPS or both in two experimental setups; measuring nitric oxide production or cellular phagocytosis. As shown in Figure 5A LPS (500ng/ml for 24h) induced phagocytosis in primary microglia (set at 100%). CXCL4 alone did not affect phagocytosis in microglia when compared to control conditions, but inhibited LPS induced phagocytosis significantly by around 25%. Similar findings have been observed for nitric oxide production in BV-2 cells. Nitric oxide production induced by LPS (500ng/ml for 24h) was significantly attenuated by CXCL4 co-stimulation, whereas CXCL4 alone did not affect nitric oxide production in BV-2 cells (Figure 5B).

![Figure 5. CXCL4 attenuation of microglial activation](image)

Primary microglia or BV-2 cells were seeded out on glass coverslips, stimulated with 10 nM CXCL4, 500 ng/ml lipopolysaccharide (LPS) for 24h or a combination of both in which case CXCL4 was administered 1h before LPS stimulation. (A) CXCL4 attenuates LPS induced phagocytosis in primary microglia. (B) CXCL4 attenuates LPS induced nitric oxide production in BV-2 cells. Each experiment was performed in triplicate and graph depicts results of three independent experiments.

**Discussion**

Here we provide evidence that microglia are the only neural cell type that produce the chemokine CXCL4 in vitro and in vivo. CXCL4 induces chemotaxis in microglia, a process mediated by chemokine receptor CXCR3. Furthermore, CXCL4 attenuates LPS induced microglial phagocytosis and nitric oxide production.

**Microglial production of CXCL4 in vitro and in vivo**

Although CXCL4 was sequenced more than 25 years ago and its functions in the periphery have been well documented, this is the first report showing that CXCL4 expression is localized specifically in microglia after brain injury.
Few previous papers have suggested that CXCL4 might be expressed in the brain. A recent paper showed that in experimental pneumococcal meningitis, CXCL4 protein could be detected in brain homogenates but the cellular source was not identified in this study (Klein et al., 2006). Another report on microglia microarray analysis showed that CXCL4 was one of the many factors that were induced in microglia cultures after herpes simplex virus infection (Aravalli et al., 2005).

In primary cell cultures we show that only microglia produce CXCL4. The basal level of CXCL4 expression is likely due to culturing conditions itself, leaving microglia in a mildly activated state. *In vivo*, CXCL4 expression was never colocalized with resting microglia in control brains. However, CXCL4 immunoreactivity was not completely absent in control brains. CXCL4 was found in structures without nuclei in the lumen of bloodvessels, most likely platelets, a well known source of CXCL4. They circulate in the bloodstream and become activated when blood coagulation and wound healing is required (reviewed in (Michelson, 2003; Ruggeri, 2002; Slungaard, 2005).

In mouse brains subjected to entorhinal cortex lesion (ECL) *in vivo*, CXCL4 was induced. The ECL model was used because it has a well characterized pattern of neurodegeneration and microglial activation in specific areas of the hippocampus (Bechmann and Nitsch, 2000). Upregulation of CXCL4 mRNA expression after ECL is probably not solely attributable to CXCL4 induction in microglia since the procedure of the ECL results in damage of the bloodvessels of the brain and will result in accumulation of platelets. However, immunoreactivity in the brain parenchyma was specific for microglia and indicates that primarily these cells induce CXCL4 under these conditions. Furthermore, we demonstrated that in hippocampal slice cultures, where interference of the peripheral immune system is eliminated, CXCL4 expression is still significantly increased after neuronal injury induced by NMDA treatment. Combined with immunohistochemistry and the observation that only microglia, not neurons or astrocytes produce CXCL4, these results show that specifically endogenous microglia express CXCL4.

**CXCR3 is the functional receptor for CXCL4 in microglia**

Chemotaxis assays showed significant migration of wildtype microglia towards CXCL4 which was not present in CXCR3-/- microglia indicating the involvement of CXCR3 in this process. In calcium imaging studies, neither wildtype or CXCR3-/- astrocytes were responsive to CXCL4. It is not understood why microglia do respond to CXCL4 and astrocytes do not since both cells are known to express CXCR3 (Biber et al., 2002a; Croitoru-Lamoury et al.,
Differential levels of expression of CXCR3 between cell types may be part of the explanation. Another hypothesis is the expression a different variant of CXCR3 on their surface. As described earlier, in humans two variants of CXCR3 exist of which only CXCR3b is receptive for CXCL4 (Lasagni et al., 2003). However, no evidence exists today to support this hypothesis since a murine alternative variant of CXCR3 has not yet been described.

The role of CXCL4 in the brain

Although CXCL4 has been detected in serum of patients suffering from stroke (Shah et al., 1985), these measures were related to platelet activity. The present data show that it is possible that platelet activity is not the only component that contributes to the rising levels of CXCL4 in the brain after injury, since microglia produce and release CXCL4 as well. It will be interesting to investigate whether CXCL4 is also upregulated in human microglia after brain injury. The function of microglial CXCL4 in brain injury is not yet understood. There is one report showing that exogenously applied CXCL4 inhibits facial nerve regeneration (Chen et al., 1999) and other experiments have shown that exogenously applied CXCL4 in the brain after stroke increases the severity of the disease (Wen et al., 1995). However, the mechanisms behind this latter observation are not clear. There is evidence from human monocytes that CXCL4 induces an increase in phagocytic activity in these cells (Pervushina et al., 2004; Scheuerer et al., 2000). Since microglia are of myeloid lineage and are considered the monocytes/macrophages of the brain, increased phagocytosis may be part of the mechanism that results in increased severity after infusion of CXCL4 in a stroke model.

Our data support a role for CXCL4 in attracting other microglia or immune cells to the site of injury but do not support the idea that CXCL4 is responsible for increased phagocytosis in microglia. We have demonstrated that CXCL4 in fact attenuates microglial activity as was shown by reduced phagocytosis and nitric oxide production. It should be noted that the report showing increased phagocytosis in monocytes used a thousand times higher concentrated CXCL4 solution than in our experimental setup (Pervushina et al., 2004; Scheuerer et al., 2000). Without dismissing the notion that at very high CXCL4 concentrations, increased phagocytosis may take place, we propose here that in the physiological range of chemokine levels in vivo, the opposite effect occurs in microglia.

Taken together, this is the first report showing CXCL4 expression in microglia in vitro and in vivo. Studies with CXCR3 deficient mice identified CXCR3 as responsible receptor for CXCL4 in microglia. Furthermore, CXCL4 stimulation inhibited LPS-induced phagocytosis
and nitric oxide production in microglia indicating that CXCL4 is a local and possible autocrine signal which downregulates detrimental microglial activity.
Reference List


