Expression of CXCL10 in cultured cortical neurons

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Chemokines are part of the larger cytokine family and induce cellular migration in cells expressing the appropriate chemokine receptors. Chemokines and their receptors have also been recognized as important mediators of cell-cell signaling in both the periphery (Rossi and Zlotnik 2000; Mackay 2001; Moser et al. 2004; Rot and von Andrian 2004) and in the central nervous system (for review see Bacon and Harrison 2000; Bajetto et al. 2002; Biber et al. 2002; Ambrosini and Aloisi 2004). Moreover, the induction of chemokine expression in the CNS has been associated with various neuronal insults and neurodegenerative diseases such as ischemia, epilepsy, Alzheimer’s disease, Amyotrophic Lateral Sclerosis, Multiple Sclerosis, and human immunodeficiency virus encephalitis (for review see Cartier et al. 2004). Although microglia and astrocytes are the principal source of chemokines in the CNS, recent findings have shown that neurons express and secrete chemokines as well (for review, see de Haas et al. 2007), suggesting that chemokines and their receptors play a role in local neuron-glia signaling (Hesselgesser and Horuk 1999; Ambrosini and Aloisi 2004; Biber et al. 2007).

Recently, we have shown that chemokine CCL21 is up-regulated in neurons in vivo and in vitro after injury. As CCL21 activates microglia through the chemokine receptor CXCR3, a role of CCL21-CXCR3 in neuron-microglia signaling has been proposed (Biber et al. 2001; Rappert et al. 2002; de Jong et al. 2005). CCL21 is not the only neuronal chemokine that binds and activates CXCR3 as CXCL10, a major CXCR3 ligand is found in neurons as well (Wang et al. 1998; Rappert et al. 2004; Klein et al. 2005). In order to investigate whether CXCL10 might also serve as a signal between endangered neurons and microglia, we have here examined the expression and regulation of CXCL10 in cultured neurons in more detail.

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Abbreviations used: Aβ, amyloid-beta peptide; DAB, 3,3¢-diaminobenzidine; DMEM, Dulbecco’s modified Eagle’s medium; EGFP, Enhanced green fluorescent protein; FACS, Fluorescence-activated cell sorting; FCS, Fetal Calf Serum; GFAP, Glial fibrillary acidic protein; HBSS, Hank’s balanced salt solution; HMBS, hydroxymethylbilane synthase; LPS, lipopolysaccharide; NG-108, neuroblastoma-glioma 108; NPY, Neuropeptide Y; PBS, phosphate-buffered saline; VAMP2, Vesicle-associated membrane protein 2.

Abstract
Chemokines expressed in neurons are important mediators in neuron-neuron and neuron-glia signaling. One of these chemokines is CCL21 that activates microglia via the chemokine receptor CXCR3. As neurons also express CXCL10, a main ligand for CXCR3, we have thus investigated in detail the expression pattern of CXCL10 in neurons. We show that CXCL10 is constitutively expressed by neurons, is stored in large dense-core vesicles and is not regulated by neuronal injury or stress. Neuronal CXCL10 release occurred constitutively at low level. In vivo CXCL10 expression was found in the developing brain at various embryonic stages and its peak expression correlates with the presence of CD11b- and GFAP-positive cells expressing CXCR3. These results suggest a possible role of neuronal CXCL10 in recruitment and homing of glial cells during embryogenesis. Keywords: chemokines, CXCL10, microglia, neuronal injury, neurons, vesicles.

Materials and methods

Chemicals
Culture media and supplements were all obtained from GIBCO® (Invitrogen, Breda, The Netherlands), unless mentioned otherwise. The amyloid-beta peptide 1–42 (Aβ1-42) used in this study was kindly provided by Prof. Dr. B. Penke from the Department of Medical Chemistry, University of Szeged, Hungary.

Cell cultures
Cortical neurons
Cultures of cortical neurons were prepared as described before (Biber et al. 2001). In brief, cortices from embryonic day 16 embryos were dissected in ice-cold Hank’s balanced salt solution (HBSS) supplemented with 30% glucose and were placed in a 0.25% trypsin solution at 37°C for 20 min. Subsequently, tissue was gently dissociated by trituration and then filtered through a 70 µm Falcon cell strainer (BD Biosciences, Erembodegem, Belgium). After one washing step (100 g for 10 min), cells were resuspended in complete neurobasal medium (2% B27-supplement, 0.5 mM glutamine, 1% pen/strep), seeded on poly-γ-lysine (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands)-coated glass coverslips and maintained at 37°C in a humidified atmosphere with 5% CO₂.

Mixed neuronal-glial co-cultures
Mouse cortex from newborn mouse pups (1–3 days old) were dissected in medium A (HBSS with 0.585% Glucose, 15 mM HEPES buffer and 1% pen/strep). Cortices were chopped and dissociated by trituration and then filtered through a 70 µm Falcon cell strainer. Cortical neurons were triturated with fire polished glass pipettes. Before centrifugation, 25 mL of culture medium [Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/L glucose, 2 mM l-glutamine, 1% pen/strep and 1% sodium pyruvate] was added. Cells were centrifuged (143 g for 10 min) and resuspended in complete Neurobasal medium and seeded in culture flask (75 cm²). Cultures were maintained at 37°C, in a humidified atmosphere (5% CO₂).

Neuroblastoma-glioma 108 cells
Neuroblastoma-glioma 108 (NG-108) cells were cultured in DMEM containing 10% Fetal Calf Serum (FCS), 1% pen/strept and 1% sodium pyruvate. Neuronal-like differentiation of NG-108 cells was done by transferring to differentiation medium (DMEM with 0.5% FCS, 1% pen/strept and 1% sodium pyruvate). Cortices were triturated with fire polished glass pipettes. Before centrifugation, 25 mL of culture medium [Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/L glucose, 2 mM l-glutamine, 1% pen/strep and 1% sodium pyruvate] was added. Cells were centrifuged (143 g for 10 min) and resuspended in complete Neurobasal medium and seeded in culture flask (75 cm²). Cultures were maintained at 37°C, in a humidified atmosphere (5% CO₂).

Neuronal insults
Excitotoxicity was induced by adding glutamate (100 µM) for 30 min to primary neurons medium as described previously (Biber et al. 2001) and mRNA/protein samples were taken 0, 2, 4, 6, 12 and 24 h after treatment. Cellular hypoxia was induced by treating 7 days in vitro primary neurons with 10 mM sodium azide (Sigma) for 5 min as described elsewhere (Grammatopoulos et al. 2004). The expression of CXCL10 mRNA and protein was evaluated 24 h after treatment. Beta-amyloid neurotoxicity was induced by incubating primary neurons with Aβ1-42 oligomers, which were prepared as follows: the solid Aβ1-42 peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma) at 1 mM and 1,1,1,3,3,3-hexafluoro-2-propanol was removed by evaporation in a SpeedVac (Savant Instruments, Holbrook, NY, USA). The Aβ1-42 films were dissolved in anhydrous dimethylsulfoxide (Sigma) at 5 mM and subsequently diluted in phenolred-free neurobasal medium to 100 µM (stock solution). The stock solution was incubated at 4°C for 24 h to enable Aβ1-42 oligomers formation. Primary neurons were incubated for 24 h with 50 µM of Aβ1-42 oligomers or phenolred-free neurobasal media as control. After the incubation with Aβ1-42 oligomers, the cell viability was determined and media samples were collected and snap frozen in liquid nitrogen for further analyses. To check if appropriate Aβ1-42 oligomers had formed, media were checked on western blot using the primary anti-Aβ antibody 6E10 (Signet Laboratories, Dedham, MA, USA). The last neurototoxic treatment applied was heat shock. Briefly, plates containing neurons were sealed with paraffin and were incubated in a waterbath at 42°C for 30 min. Neurons were then put back in the incubator and samples were taken after 24 h. Plates containing control neurons were only sealed with paraffin for 30 min.

Real-time PCR analysis of CXCL10 mRNA in cultured neurons
Cell lysis, RNA extraction and cDNA transcription were performed as described previously (Biber et al. 2001). The following primers for CXCL10 and hydroxymethylbilane synthase (HMBS) were designed using Primer Designer version 3.0: CXCL10F 5’-CCGGATTCA-CTCCTTCCAGGTGCCTCAGA-3’, CXCL10B 5’-ATAGAATTCAAAGGAGCCCTTTTAGAC-CTTTTTTGCTAAA, pCXCL10-5mRFP was kindly provided by Dr. R. Miller (Northwestern University, Chicago, USA) and pHluorin-N3 was kindly provided by Dr. R. Toonen (VUMC, Amsterdam, The Netherlands).

Transfection
Plasmids were transfected into differentiated NG-108 cells using transfection lipid nanofectamin (PAA Laboratories, Coïble, Germany) according to the manufacturer’s instructions. NG-108 cells were transfected in DMEM for 5 h and transferred back to differentiation medium.

Plasmids
The following primers have been used to amplify Neuropeptide Y-enhanced green fluorescent protein (NPY-EGFP) and Vesicle-associated membrane protein 2 (VAMP2)-EGFP for subcloning into Xhol-EcoRI sites of pEGFP-N2: NPYF 5’-ATAGAATTCAAAAGGAGCCCTTTTAGAC-CTTTTTTGCTAAA, pCXCL10-5mRFP was kindly provided by Dr. R. Miller (Northwestern University, Chicago, USA) and pHluorin-N3 was kindly provided by Dr. R. Toonen (VUMC, Amsterdam, The Netherlands).
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 72°C. Results were normalized to HMBS and the comparative cycle threshold Ct method was used as described previously (Livak and Schmitogen 2001).

**Immunoprecipitation**
Immunoprecipitation for CXCL10 was performed by adding 400 ng of rabbit anti-murine CXCL10 antibody (PeproTech, London, UK) to 1 mL supernatant of cultured neurons, NG-108 or HEK293 cells. Samples were incubated overnight at 4°C and Protein G-Sepharose 4 Fast Flow [GE Healthcare, Diegem, Belgium: 50 μL of 50% slurry in phosphate-buffered saline (PBS)] was added and incubated for another 4 h. Subsequently, samples were centrifuged at 125 g at 4°C and antibody-antigen-Protein G complexes were washed five times with PBS. Complexes were dissociated by adding 20 μL 2X sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer, heated for 5 min at 95°C and subjected to western blotting (see Fig. S1). As positive control, 80 ng of recombinant CXCL10 was added to 1 mL supernatant of cultured neurons, which was subjected to the same protocol.

**Immunocytochemistry**
Cells were fixed in 4% paraformaldehyde for 30 min, blocked 30 min in PBS containing 0.3% Triton X-100 and 5% normal goat serum and incubated O/N with rabbit anti-CXCL10 (PeproTech, 1 : 1000) and mouse anti-NeuN (Millipore, Amsterdam Zuidoost, The Netherlands, 1 : 1000) or mouse anti-Glial fibrillary acidic protein (GFAP, Millipore, 1 : 200) primary antibodies diluted in PBS with 0.3% Triton X-100. The next day, cells were incubated with FITC-goat anti-mouse (Millipore, 1 : 500) and CY3-goat anti-rabbit (Jackson Immuno-Research, Newmarket, Suffolk, UK, 1 : 500) secondary antibodies for 1 h. Glass coverslips were mounted on slides with Mowiol (Sigma). Control experiments for specificity were done in absence of the primary antibody. NG-108 cells transfected with CXCL10 mRFP were incubated O/N with rabbit anti-Chromogranin A and B (Novus Biologicals, Littleton, CO, USA, 1 : 100) and CY3 goat anti-rabbit was used as secondary antibody.

Mouse embryos were dissected, fixed overnight in 4% formaldehyde, embedded in paraffin and sectioned at 7 μm thickness. The sections were deparaffinized, hydrated in graded alcohols, heated for 10 min at 120°C, 1 kPa in 10 mM Na-citrate (pH 6.0) to retrieve antigens, blocked in Teng-T (10 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.25% (w/v) gelatin, 0.05% (v/v) Tween-20) and 10% FCS, and incubated overnight with the first antibody. Antibody-antigen reactions were detected using the biotin-streptavidin method and visualized with 3,3’-diaminobenzidine (DAB)/H₂O₂. The slides were rinsed, mounted with DEPEX and analyzed by light microscopy. Negative control experiments were performed with isotype-matched antibodies or by omitting primary antibodies.

**Electron microscopy**
Electron microscopy immunocytochemistry for the ultrastructural detection of CXCL10 was performed on cultured cortical neurons using a biotinylated secondary antibody and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) with DAB as chromogen. Intensification of the DAB reaction product was done using the gold-silver-substituted peroxidase method (Liem et al. 2001). Subsequently, neurons were fixed with 3% sodium thiosulfate, rinsed briefly in 0.1 M sodium acetate cacodylate buffer, pH 7.6 and were then osmicated in 1% OsO₄ (w/v) and 1.5% potassium hexacyanoferrate in the same buffer. Neurons were then dehydrated in a graded series of ethanol and embedded in Epon. One μm sections were then cut, counter-stained with uranyl acetate and lead citrate, and examined using a Philips CM100 transmission electron microscope (Aachen, Germany).

**Fluorescence-activated cell sorting (FACS) analysis**
Brains from embryonic day 14 embryos were dissected in ice-cold HBSS supplemented with 30% glucose and were placed in a 0.25% trypsin solution at 37°C for 20 min. Subsequently, tissue was gently dissociated by trituration and then filtered through a 70 μm Falcon cell strainer. After one washing step (100 g for 10 min), cells were resuspended in FACS buffer (PBS, supplemented with 0.5% bovine serum albumin and 0.01% sodium azide). Cells were incubated for 15 min with CD16/32 (eBioscience, Hatfield, UK, 1 : 100) to block Fc receptor binding. For the membrane-bound proteins CXCR3 (R&D Systems, Abingdon, Oxon, UK, 1 : 10), CD11b (BD Biosciences, 1 : 200) and isotype controls, fluorescent-conjugated antibodies were added and cells were incubated for 20 min on icle. For the detection of intracellular proteins in combination with membrane-bound markers, cells were fixated after membrane-antibody staining with freshly prepared 1% paraformaldehyde for 10 min. Subsequently, cells were washed twice with FACS buffer, supplemented with 0.1% saponin (FACS-SAP) and incubated for 30 min with the either rabbit anti-GFAP (Sigma, 1 : 200) or mouse anti-Microtubule-associated protein 2 (MAP2, Millipore, 1 : 200) diluted in FACS-SAP. After thoroughly washing with FACS-SAP, cells were incubated for 30 min with either goat anti-rabbit DyLight488 (JacksonImmunoresearch, 1 : 250) or goat anti-mouse Alexa488 (Invitrogen, 1 : 250), also diluted in FACS-SAP. After washing, unfixed and fixed cells were resuspended in FACS buffer and fluorescence was measured with a FACS Elite Flow Cytometer (Becton-Dickinson, Breda, The Netherlands).

**ELISA**
CXCL10 release in the supernatant of the cells was measured with an ELISA (R&D). Supernatants were spin down briefly to remove cells and then used in a mouse-CXCL10 specific ELISA as described in the manufacturer’s protocol. Recombinant murine CXCL10 served as the standard and detection limit was set at 15 pg/mL.

**Fluorescence and timelapse imaging**
Imaging of the immunofluorescent staining and the expression of the various fusion proteins was performed on a Leica AOB5_TCS SP2 confocal laser scanning microscope (Leica Microsystems, Rijswijk, The Netherlands). CXCL10-pHluorin was visualized in transfected NG-108 cells using a live-cell imaging set-up (Solamere Technology Group, Salt Lake City, UT, USA) equipped with an AR/Kr laser (Dynamic Lasers, Salt Lake City, UT, USA) and a CUS10 spinning Nipkow disk (Yokogawa, Amersfoort, The Netherlands). Coverslips
were placed in a motorized xyz stage of a DM IR2 Leica microscope which was placed in a custom made incubation chamber with an 37°C humidified atmosphere and Tyrode’s solution was used as buffer. A Stanford Photonics XR MEGA-10 Gen III iCCD camera (Stanford Photonics, Palo Alto, CA, USA) was used for acquisition. The setup was controlled by ‘InVivo’ software (Media Cybernetics, Bethesda, MD, USA). Images were acquired at 1 frame per second with 330 ms integration time per image using a 63× NA1.4 oil immersion objective +1.5 Optovar (Leica Microsystems).

Data analysis
Real time PCR results, Western and ELISA data were analyzed by one-way ANOVA followed by the Bonferroni correction. Values of $p < 0.05$ were considered statistically significant. FACS data were analyzed using WinList (Verity Software House, Topsham, ME, USA) for Windows. Confocal images and acquired time series were corrected for background noise and were deconvoluted using Huygens Pro Software (SVI, Hilversum, The Netherlands). Quantification of vesicle co-localization was performed using single channels of red-green confocal images saved separately. Images were then superimposed together and yellow, green and red puncta were counted.

Results
Expression of CXCL10 in cultured neurons
In order to study the distribution of CXCL10 in neurons, we performed fluorescent immunocytochemistry on E16 primary cortical neurons. Control experiments without primary antibody gave virtually no background staining (Fig. 1a). Our observations indicate that CXCL10 immunoreactivity was found abundantly throughout the whole cell as punctate signals and that most cells (> 95%) were positive for CXCL10 (red signal) (Fig. 1b). The majority (> 95%) of cells in culture that were positive for CXCL10 (red signal) were also NeuN positive (Fig. 1c). Few CXCL10-positive cells were not positive for NeuN (Fig. 1c, insert, arrowheads). GFAP immunocytochemistry revealed that these cells were CXCL10-positive astrocytes (Fig. 1d, arrowhead).

CXCL10 is stored in large dense-core vesicles
The punctate expression of CXCL10 in neurons (Fig. 1c) suggests the presence of CXCL10 in vesicle-like structures, similar to what has been reported for other cytokines/chemokines (Specht et al. 2003; Möller et al. 2006; Callewaere et al. 2008; de Jong et al. 2008; Jung et al. 2008; Tsakiri et al. 2008). Electron microscopic experiments confirmed the presence of CXCL10 in vesicle-like structures at the ultrastructural level. Moreover, CXCL10 was found in vesicles localized mainly at pre-synaptic sites as indicated for axo-somatic contacts (Fig. 2a), axo-axonal contacts (Fig. 2b) and axo-dendritic cell contacts (Fig. 2c).

To further characterize the type of vesicles in which CXCL10 is stored, we used differentiated NG-108 cells as a neuron-like cell model in which we transfected CXCL10-mRFP along with either NPY-EGFP or VAMP2-EGFP.
which are marker for large dense-core vesicles. 68 ± 5.9% of the CXCL10-containing vesicles were found to be co-localized with NPY (Fig. 3a). In the case of VAMP2-EGFP, only a partial co-localization (54 ± 6.6%) with CXCL10-mRFP was observed (Fig. 3b). We also processed differentiated NG-108 cells transfected only with CXCL10-mRFP for immunohistochemistry against Chromogranins A and B, another large dense-core vesicle marker, and observed a 34 ± 2.3% co-localization of CXCL10 vesicles with Chromogranins A and B (Fig. 3c).
Constitutive CXCL10 expression in cultured neurons is not changed after neurotoxic stimuli

Using quantitative real-time PCR, CXCL10 mRNA was detected in cultured neurons under control conditions, which was not affected by treatment with the NMDA-receptor antagonist MK801 (50 μM for 24 h) indicating that basal CXCL10 mRNA expression was not because of excitotoxic events in the culture (Fig. 4a). No significant differences in CXCL10 mRNA expression levels were observed after treatment with 100 μM glutamate for 0–24 h. These results were also confirmed at protein level (Fig. S1). Other neuronal insults such as treatment with amyloid beta Aβ_42 oligomers, chemical hypoxia induced with sodium azide and heat shock did not result in changes of CXCL10 mRNA expression 24 h after treatment (Fig. 4a). As CXCL10 is known to be expressed in glial cells, we also determined CXCL10 mRNA expression levels in mixed glia cell cultures. Compared to cultured neurons, a significant higher CXCL10 mRNA expression (10-folds increase) was found in mixed glia cells (Fig. 4b; \( p < 0.05 \)). The mRNA expression of CXCL10 in mixed glia cells was furthermore strongly stimulated by lipopolysaccharide (LPS, 100 ng/mL for 6 h) up to 170-times of control values (Fig. 4b). Twenty four hours after treatment with LPS, mixed glia cell cultures still expressed approximately 10-times more CXCL10 mRNA compared to controls (Fig. 4b).

Release of CXCL10

The vesicular presence of CXCL10 at pre-synaptic sites prompted us to investigate the release of CXCL10 from neuronal cultures. ELISA were performed with supernatants of primary cultured cortical neurons that were exposed to the various treatment mentioned above. Additionally, two conditions known to induce vesicular release (60 μM KCl for 6 h to induce depolarization and 1 μM ionomycin to induce calcium influx) were tested. None of these samples contained CXCL10 at a concentration that was significantly above the detection limit of our ELISA assay (15 pg/mL). This was in contrast with supernatants obtained from mixed glial cultures, containing astrocytes and microglia, in which CXCL10 was detected reliably (332.3 ± 8.1 pg/mL). Particularly after LPS treatment (100 ng/mL for 24 h), the concentration of CXCL10 was very high (71 168.0 ± 1341.8 pg/mL; Fig. 5a).

As no detectable amount of CXCL10 was found in the supernatants, we investigated if the vesicles containing CXCL10 were fusing with the membrane with the help of CXCL10-pHluorin. We therefore performed live imaging experiments in differentiated NG-108 cells that were transfected with CXCL10-pHluorin. We observed that CXCL10-filled vesicles are constitutively fusing with the cell membrane and suggesting that CXCL10 is indeed released. However, the number of fusion events was relatively low and treatment with 100 μM glutamate or...
50 mM KCl did not increase the frequency of release (data not shown). To confirm that CXCL10 is released from these vesicles, additional immunoprecipitation experiments were performed to detect CXCL10 in neuronal supernatants. Indeed, CXCL10 was found in neuronal supernatants under control conditions (Fig. 5c). Again, glutamate treatment (100 μM for 6 and 12 h) did not affect the neuronal release of CXCL10 (Fig. 5c). CXCL10 was also found in the supernatant of mixed glial cultures especially after treatment with 100 ng/mL LPS for 24 h. Data are given as mean ± SEM (n = 3). (b) Time lapse imaging of differentiated NG-108 cells transfected with CXCL10-pHluorin showed fluorescent signal at the beginning of the experiment indicative of vesicles fusing with the membrane (arrows, left panel). Several seconds later, these vesicles disappeared (arrows, right panel) and new fluorescent signal appeared elsewhere in the cells (arrowheads, right panel) indicating new fusion events. (c) Immunoprecipitation experiments revealed the presence of CXCL10 in supernatants of cultured neurons under control conditions (c) and after glutamate treatment (6 and 12 h) and of differentiated NG-108 cells (NG). HEK293 cells served as negative control (nc). As positive control, 80 ng of recombinant CXCL10 were added to neuronal supernatant and subjected to immunoprecipitation. Similar results have been obtained in four independent experiments for ELISA analysis and three independent experiments for immunoprecipitation.

CXCL10 is expressed in the developing mouse brain
In order to investigate whether neurons in vivo also express CXCL10, immunohistochemical studies have been performed. No neuronal CXCL10 was found in the brain of adult mice or neonatal animals (postnatal day 1–3), nor did we find CXCL10 immunoreactivity in neurons in hippocampal organotypic brain slice cultures (data not shown). In the developing brain, however, CXCL10 expression was found. In brain derived from embryonic day 10.5 CXCL10 was observed at the ventricular side of the developing neuropil (Fig. 6, arrows). At embryonic day 12.5 the whole developing brain was positive for CXCL10 (Fig. 6) and at embryonic day 17.5 CXCL10 expression was restricted to layers of the developing forebrain (Fig. 6, arrows).
The embryonic period in which neurons are expressing CXCL10 is characterized by the appearance of myeloid cells (future microglia) and astrocytes which are colonizing the CNS and proliferating (Alliot et al. 1999; Sauvageot and Stiles 2002). In order to identify CXCR3 receptor expressing cells in the developing brain, FACS analysis of unfixed E14 CNS cells were performed. Forward/side scatter analysis showed two main cell clusters (Fig. 7a), only one of which (region one) was positive for CXCR3 (Fig. 7b and c). Within the cells of region one, we detected two populations of CXCR3-expressing cells: one expressing high levels and one expressing low levels of CXCR3 (Fig. 7b). As 9% of the region one cells were CD11b positive (Fig. 7e), double staining experiments with CD11b and CXCR3 were performed. These experiments identified CD11b-positive cells as CXCR3 high expressors (Fig. 7f). In order to detect if, next to myeloid cells, also neurons and astrocytes expressed CXCR3, E14 CNS cells were fixed and double stained with MAP2/CXCR3 or GFAP/CXCR3. Cell fixation made the two populations less distinct on the forward/side scatter (Fig. 7g) but nevertheless CXCR3 was expressed mainly by cells of region one (Fig. 7h), although some cells of region two were also positive for CXCR3 (Fig. 7i), probably because of loss of cell granularity by fixation. Fixation also made the two CXCR3 population indistinguishable (Fig. 7h). MAP2-positive cells were found only in region two cells (Fig. 7j), confirming that neurons do not express CXCR3. Interestingly, GFAP-positive cells were found in both regions (Fig. 7k). Double staining experiments with CXCR3 and GFAP revealed that majority of astrocytes cells found in region 1 expressed CXCR3 (Fig. 7l). Again, because of fixation, some astrocytes belonging to region 1 were found in region 2 and expressed also CXCR3 (Fig. 7l).

**Discussion**

**Neuronal CXCL10 is constitutively expressed in vitro**

The present study shows that cultured cortical neurons constitutively express CXCL10, which is distributed throughout the whole cell including synaptic regions. Furthermore, CXCL10 mRNA and protein levels could not be induced by neuronal insults. Immunohistochemical studies in brain of mouse embryos detected CXCL10 expression at embryonic day 12.5 and 17.5. No neuronal CXCL10 expression was found in neonatal brains or organotypic hippocampal slices cultures derived from 3-day-old animals.
Fig. 7 CD11b and GFAP positive cells from E14 CNS express CXCR3 receptor. E14 CNS cells were processed for FACS analysis. (a) Two populations were identified in the forward/side scatter (region 1 and 2). CXCR3-positive cells were found in region 1 (b) but not in region 2 (c). Isotype control gave no signal (d). Staining with CD11b antibody showed expression only in region 1 (e). (f) Double labeling with CD11b and CXCR3 showed that all CD11b-positive cells expressed high levels of CXCR3, while isotype controls gave no signal. To identify, whether neurons and astrocytes expressed also CXCR3, E14 CNS were fixed and processed for FACS analysis. Although less distinct, the two population were observed on the forward/side scatter (g) and CXCR3 was present mainly in region 1 (h) but not in region 2 (i). MAP2 staining revealed that neurons are present only in region 2 (k), and thus do not express CXCR3. GFAP-positive cells were observed in both regions (k). Double labeling with GFAP and CXCR3 showed that part of the GFAP-positive cells of region 1 expressed also CXCR3 (l). Because of fixation, some GFAP-positive cells expressing CXCR3 were also observed in region 2 (l). Isotype controls gave no signals (l). FSC, forward scatter; SSC, side scatter.
In agreement with our results a constitutive expression of CXCL10 in cultured neurons (human fetal brain cells) has been described (Sui et al. 2004) whereas others did not find CXCL10 expression in untreated neurons (Patterson et al. 2003; Klein et al. 2005). The reason for these contradictory findings is not clear but might reflect differences in culture conditions. The first report describing a neuronal CXCL10 expression was by Wang et al. (1998) where they showed an induction of CXCL10 in neurons under ischemic conditions. However, they furthermore reported a low level of CXCL10 mRNA expression in sham-operated animals and also at the contralateral cortical side of treated animals, suggesting a basal level of neuronal CXCL10 mRNA expression. Thus, controversial results have been published concerning the expression of CXCL10 in unchallenged neurons, indicating that neuronal CXCL10 expression may depend on the developmental stage.

Release of neuronal CXCL10

CXCL10 was observed in punctuate structures that were identified as large dense-core vesicles using specific markers. These data are in accordance with other recent findings on the localization and release of neuronal cytokines or chemokines from vesicles (Specht et al. 2003; Möller et al. 2006; Callweaere et al. 2008; de Jong et al. 2008; Jung et al. 2008; Tsakiri et al. 2008). It further implies that neurons probably actively transport CXCL10 to various cell compartments as we previously demonstrated with CCL21 (de Jong et al. 2008).

The presence of CXCL10-vesicles in synaptic regions suggests that CXCL10 might be released from neurons. From our thorough analysis, we conclude that CXCL10 is released from cultured neurons, albeit at very low levels. This was in contrast with supernatants derived from mixed neuronal/glia cultures. There are three recent studies that investigated the release of CXCL10 from virus-infected neurons. Measles infection induced both CXCL10 and CCL5 in primary hippocampal neurons but interestingly, only CCL5 release was detectable by ELISA (Patterson et al. 2003). Using western blot analysis, Klein and colleagues showed high CXCL10 levels within West Nile virus infected cerebellar granule neurons but only low levels in the supernatant of these cells (Klein et al. 2005). Relatively high levels (around 1000 pg/mL) of CXCL10 were found in the supernatant of simian human immunodeficiency virus- or gp120-treated human fetal brain cultures (Sui et al. 2004). It should be noted, however, that the human fetal brain cultures used in this study contained approximately 30% astrocytes, whereas all other studies concerning CXCL10 expression in cultured neurons (including ours) used almost pure neuronal cultures (Patterson et al. 2003; Klein et al. 2005). It is thus apparent that CXCL10 in neurons can be reliably detected, whereas the presence of CXCL10 in neuronal supernatants is more difficult to show. Moreover, we show that the concentration of exogenously added CXCL10 into the supernatant of cultured neurons decreases, furthermore complicating the detection of CXCL10 in neuronal supernatants. Even though we show that CXCL10 is present in vesicles in synaptic areas, suggesting that CXCL10 is destined for release, we hardly detected its presence in the culture medium.

The function of neuronal CXCL10

The findings presented here concerning the expression of CXCL10 in embryonic cultured neurons and in the developing mouse brain suggest a function of this chemokine in brain development. Indeed, we show that myeloid cells (CD11b-positive) and some GFAP-positive cells express the receptor CXCR3. Myeloid cells, which will give rise to resident microglia, are known to colonize the CNS from E7 (Alliot et al. 1999), while astrocytes will start appearing at E12 (Sauvageot and Stiles 2002). As, this is the period when neuronal CXCL10 is expressed in the developing brain (between E12 and E17), a possible role for CXCL10 during embryogenesis can be envisaged. CXCL10 could function as a ‘homing’ molecule to attract CXCR3-positive cells. Indeed, several studies are showing a prominent role of chemokines in cellular migration also during development (Ragozzino 2002; Rezaie et al. 2002). However, as low amount of CXCL10 were detected in culture, it is more likely that CXCL10 provides a local signal. Thus, CXCL10 might be involved during apoptosis (which occurs abundantly during development) and cellular clearance from myeloid cells (Rice and Barone 2000) and/or could promote contact between glial cells and neurons, which is critical for synaptogenesis in later stages of development (Ullian et al. 2004). During embryogenesis, both radial glia and astrocytes express GFAP (Hartfuss et al. 2001) and the presence of these two cell types may explain why not all GFAP-positive cells expressed CXCR3. Moreover, we were not able to identify all the cells that expressed CXCR3. It is plausible that other cell types such as precursors cells and/or immature cells might express the receptor. In order to prove these hypotheses, however, further studies should be performed.

Earlier findings of our group suggested that the receptor for CXCL10, CXCR3, is instrumental in neuron-microglia signaling (Rappert et al. 2004). We have suggested CCL21 to provide the neuronal signal for this intercellular signaling. The data presented here do not support a function of CXCL10 in neuron-microglia signaling under excitotoxic conditions. However, this might be different in case of virus-infected neurons in which CXCL10 expression is induced.

In summary, it is shown here that CXCL10 is constitutively expressed in cultured cortical neurons and that its expression is not changed by endangering conditions. The function of neuronal CXCL10 is not yet understood,
however, the data presented here show that neuronal CXCL10 could play a role during embryogenesis.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Western blot analysis corroborated the presence of CXCL10 in cultured neurons.

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References


