Functional characteristics of chemokine-receptor interactions in oligodendrocytes

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   Neuroreport 2006, 17(11):1187-90
3. **Effects of interferon-beta on oligodendroglial cells**
Sandra Heine*, Jens Ebnet*, Samaneh Maysami, Martin Stangel
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**Poster presentations at scientific congresses:**

1. **Functional expression of chemokine receptors CXCR2, CXCR4, and CCR3 on rat oligodendrocyte progenitor cells.** Neuronal Systems Biology (Symbionic course; 31st Aug. - 3rd Sep. 2005; Trieste, Italy)
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2. **Characterization of oligodendrocyte responses towards chemokines CCL11 and CXCL2.** German Neuroscience Meeting (Goettingen, Germany, 17-20th Feb. 2005)
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- **Caroline Pitz,** student of human medicine: (Jan 2004-Jan 2006)
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- **PhD students of ZSN: Oct. 2004**
  Practical course on ‘Cellular Biology’

- **PhD students of ZSN: Nov. 2005**
  Practical course on ‘Cellular Biology’
Whether our efforts are, or not, favored by life, let us be able to say, when we come near the great goal, "I have done what I could”

(Louis Pasteur 1822-1895)
Dedicated

to all, who used to spare me a thought.
In fond rememberance

of all, who were dear to my heart and will always be.
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<th>Description</th>
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<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µM</td>
<td>Micromole</td>
</tr>
<tr>
<td>A2B5</td>
<td>A surface marker for oligodendrocyte progenitor cells</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer disease</td>
</tr>
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<td>App</td>
<td>Appendix</td>
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<td>APS</td>
<td>Ammoniumpersulfate</td>
</tr>
<tr>
<td>B104</td>
<td>A neuroblastoma cell line</td>
</tr>
<tr>
<td>B104-CM</td>
<td>B104 neuroblastoma cell line conditioned medium</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-Chloro-3-Indolyl phosphate</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCL11</td>
<td>(EOT), one of the ligands of CCR3 chemokine receptor</td>
</tr>
<tr>
<td>CCR3</td>
<td>A CC chemokine receptor</td>
</tr>
<tr>
<td>CG4</td>
<td>Rat oligodendrogial cell line</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Formerly Fractalkine, the only ligand of the chemokine receptor CX3CR1</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>(V28), the only CX3C chemokine receptor known so far</td>
</tr>
<tr>
<td>CXCL1</td>
<td>(KC/GRO-alpha), one of the ligands of the chemokine receptor CXCR2</td>
</tr>
<tr>
<td>CXCL12</td>
<td>(SDF), ligand of the chemokine receptor CXCR4</td>
</tr>
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<td>CXCL2</td>
<td>(MIP-2), one of the ligands of the chemokine receptor CXCR2</td>
</tr>
<tr>
<td>CXCL5</td>
<td>(LIX), one of the ligands of the chemokine receptor CXCR2</td>
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<tr>
<td>CXCR2</td>
<td>A CXC chemokine receptor with high sequence homology to IL-8 RB</td>
</tr>
<tr>
<td>CXCR4</td>
<td>A CXC chemokine receptor</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-Diazabicyclo[2.2.2]octane</td>
</tr>
<tr>
<td>DAPI</td>
<td>4´,6-diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy ribo nucleic acids</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>DNase I</td>
<td>Deoxyribonuclease I, which is an endonuclease</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>g</td>
<td>G force, which is pulling objects toward the earth by gravity</td>
</tr>
<tr>
<td>GalC</td>
<td>Galactocerebroside, surface marker for mature oligodendrocytes</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein, a marker for astrocytes</td>
</tr>
<tr>
<td>GPA</td>
<td>G-protein antagonist</td>
</tr>
<tr>
<td>H2SO4</td>
<td>Hydrogen sulfate or sulfuric acid</td>
</tr>
<tr>
<td>H³</td>
<td>Hydrogen-3 or tritium is a hydrogen isotope</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Symplex Virus</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ITS</td>
<td>ITS™ Tremix (Insulin, Transferrin, Selenous Acid)</td>
</tr>
<tr>
<td>M</td>
<td>Mole, the number of particles (6.02\times10^{23}) in a given amount of matter</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimole</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>N2B3</td>
<td>(SATO), a medium containing essentials for oligodendrocytes maturation</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide (Natriumhidroxyde)</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium chloride</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomole</td>
</tr>
<tr>
<td>O4</td>
<td>Surface marker for oligodendrocytes, which expressed later than A2B5</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte progenitor cells</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin-Streptomycin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF-aa</td>
<td>Platelet derived growth factor-aa</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>PSA</td>
<td>Poly sialic acid</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>T3</td>
<td>3,3',5-Triiodo-L-thyronine sodium salt</td>
</tr>
<tr>
<td>T4</td>
<td>3,3',5,5''-Tetraiodo-L-thyronine</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N’, N’- tetramethylenediamine</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-Amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>δ</td>
<td>Delta</td>
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I. Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) with an incidence of seven in 100,000 per year and a prevalence of approximately one in 1000 in the western world (Compston and Coles, 2002). This neuroinflammatory/demyelinating disease is characterized by inflammation, demyelination, gliosis, and axonal loss (Lassmann, 1998). There is a local infiltration of immune cells, activation of astrocytes and microglia, and consequently degeneration of myelin and/or axons. The exact etiology and pathogenesis of MS are still unknown. However, heterogeneities within the lesions were reported with respect to the extent and type of oligodendrocyte death, composition of the inflammatory infiltrates, and incidence of remyelination (Lucchinetti et al., 2000). Moreover, primary oligodendrocyte degeneration has been suggested as a most common histopathological pattern in progressive form of MS disease (Brück, 2005). Therefore, it seems that oligodendrocytes are the primary targets of the immune attack in this demyelinating disease.

Mature myelin-producing oligodendrocytes are able to synthesize and maintain myelin sheaths in the CNS (Fig.1). Many factors are known to be involved in the regulation of oligodendroglial functions including, growth factors, and chemokines (Baumann and Pham-Dinh, 2001, Robinson et al., 1998; Tsai et al., 2001). Chemokines or chemotactant cytokines, which interact with their 7-transmembrane G protein-coupled receptors (Rossi and Zlotnik, 2000), were primarily described to facilitate recruitment of leukocytes to the site of inflammation (Baggiolini, 1998). The expression of chemokines and chemokine receptors have already been described in the CNS (Bajetto et al., 2002). Several studies show that rodent oligodendrocytes express different chemokine receptors (Dziembowska et al., 2005; Nguyan and Stangel, 2001; Robinson et al., 1998; Tsai et al., 2001). However, very recent in vivo studies also revealed the expression of certain chemokine receptors on human oligodendrocytes (Filipovic et al, 2003; Omari et al., 2005; Omari et al., 2006) in health and disease. Neuronal cells also express chemokines (Maciejewski-Lenoir et al., 1999) as well as chemokine receptors (Boutet et al., 2001b; Coughlan et al., 2000; Hesselgesser et al., 1997; Horuk, 1997; Lavi et al., 1997;). Moreover, other glial cells such as astrocytes (Tanabe et al., 1997; Dorf et al., 2000; Tomita et al., 2005) and microglia (Tanabe et al., 1997; Aravalli et al., 2005; Dorf et al., 2000; Takanohashi et al., 2005) express a number of chemokine receptors and they are known as vivid sources of chemokines. This expression pattern of chemokines and their receptors on resident cells of the CNS shows that chemokines might play an important role in signalling between neurons and glia, which may influence the induction of
oligodendrocyte behaviors and thereby myelination of axons during development and diseases of CNS. Therefore, it is of great importance to investigate the characteristics of the chemokine receptor-ligand interaction on oligodendrocytes in order to obtain knowledge on the effect of this interaction on oligodendroglial cell biological activities. Ultimately, the outcome of this study might help us in better understanding of CNS developmental processes as well as progression of various demyelinating diseases including MS.
II. Literature review

II.1. Role of oligodendrocytes in health and demyelinating disease of CNS

As illustrated in figure 1 (Fig.1), precursor cells have the potential to differentiate into progenitors and then pre-oligodendrocytes. Consequently, these cells differentiate into immature and thereafter mature oligodendrocytes, which are producing myelin sheaths around axons. Mature myelin-forming oligodendrocytes produce myelin in a spiral structure from their plasma membrane extensions in the central nervous system (Baumann and Pham-Dinh, 2001). Along a myelinated fiber, flanking internodes are usually separated by the nodes of Ranvier, wherein the axolemma is exposed to the extracellular environment (Fig.1) (Baumann and Pham-Dinh, 2001). These nodes play a major role in the neural fast saltatory conduction. Breakdown of myelin sheaths (degeneration) and eventually transaction of axons occurs as these myelin-producing cells are damaged or destroyed. Most importantly, many resident cells of the CNS such as microglia and astrocytes as well as non-resident cells of the CNS like immune cells may play important roles in influencing the process of oligodendrocyte maturation via para- or exocrine secretion of different modulatory molecules (including growth factors and chemokines) during development and disease (Fig.1). From a pathological point of view, a demyelinating disease like multiple sclerosis manifests as acute focal demyelination and axonal loss with limited repair, which frequently ends in the chronic multifocal sclerosis plaques. Different extent of repair, which is observed in the demyelinating plaques, can be due to the presence of a variety of reactive inflammatory cells as well as apoptotic or inefficient oligodendrocytes in the vicinity of lesions. In brief, there are four modes suggested for etiological and pathological heterogeneity observed in pathogenesis of multiple sclerosis including, immune-cell mediated demyelination, antibody-complement mediated demyelination, distal oligodendroglial apoptosis, and primary oligodendrocyte degeneration (Lucchinetti et al., 2000). Interestingly, studies indicate that oligodendrocytes do have heightened vulnerability to undergo primary degeneration in the majority of patients with progressive MS (Brück, 2005), which implies the importance of the myelin producing cells in progression of this neurodegenerative / demyelinating disease.
**Fig.1: Differentiation of oligodendrocyte progenitor cells.** The diagram shows the process of maturation of OPC and the possible effect of the reactive cells during development or even in the progression (or repair) of the inflammatory-demyelinating diseases of the CNS. Note the maturation of oligodendrocytes from precursors towards the myelinating cells, surrounding the axon. A) Precursor cells, B) Progenitor cells, C) Pre-oligodendrocytes, D) Immature oligodendrocytes, E) Non-myelinating mature oligodendrocytes, F) Myelinating mature oligodendrocytes surrounding axons in the CNS and producing myelin sheaths. N: Node of Ranvier, gaps between myelin sheaths (Modified from Baumann and Pham-Dinh, 2001)
II.2. Chemokines

Chemokines are a family of low molecular weight (8-12kDa) proteins, which are primarily described as key mediators controlling infiltration of leukocytes in the vicinity of inflammation. Depending on the arrangement of the highly conserved cysteine motives, chemokines are classified into four major families CXC (α), CC (β), C (γ), and CX3C (δ) (Baggiolini et al., 1997). Although chemokines are known as signalling molecules at the site of inflammation, their importance was also shown as regulators of lymphopoesis (Nagasawa et al., 1996) and hemopoiesis during development (Horuk and Peiper, 1996; Nagasawa et al., 1996). Chemokines normally transduce their signals through the heptahelical receptor family which couple to trimeric (α, β, and γ) guanine-nucleotide binding proteins (7-G protein). Studies showed that an acidic residue in the N-terminal of the chemokine receptor (Hebert et al., 1993; Holmes et al., 1991; LaRosa et al., 1992) interacts with a certain basic residue of the ligand (Hebert et al., 1991; Hebert et al., 1993). This interaction leads to a transient mobilization of calcium in the cell (Holmes et al., 1991).

II.3. Chemokines and chemokine receptors in the CNS

II.3.a. Expression in CNS cells

The expression of chemokine receptors (CXCR4 and CCR3) was primarily shown in neuronal cells of human and non-human primates (Lavi et al., 1997; Hesselgesser et al., 1997; Hesselgesser et al., 1998; Zhang et al. 1998). Later on, studies revealed that human fetal neurons do not only express several chemokine receptors (CXCR2 and CXCR4) (Boutet et al., 2001b; Coughlan et al., 2000), but these cells are capable of producing and secreting certain chemokines as well (Coughlan et al., 2000).

Human (CXCR2, CXCR4 and CCR3) (Filipovic et al., 2003; Flynn et al., 2003) and rodent (CXCR4 and CX3CR1) (Jiang et al., 1998) glial cells (astrocytes and microglia) have also been shown to express chemokine receptors. In addition, previous in vitro studies revealed the expression of certain chemokine receptors including, CXCR2, CXCR4, CCR3, and CX3CR1 by rodent oligodendrocytes (Maysami et al., 2006a; Maysami et al., 2006b; Nguyen and Stangel, 2001; Stangel unpublished data - personal communication), which led us to investigate the characteristics of ligand-receptor interaction in modulation of oligodendrocyte biological activities.
II.3.b. Role in CNS development

The expression of chemokines (CXCL1 and CXCL2) or chemokine receptors (CXCR2) has already been observed in rodent CNS embryos (Filipovic et al., 2003; Luan et al., 2001). A number of studies has already shown the functional importance of some chemokines and/or chemokine receptors during development of the central nervous system. For instance, CXCR2 double knockout rodent embryos, which have the life span similar to their wild type littermates, show anomalous patterns of glial distribution in the CNS (Tsai et al., 2002). In contrast, the overexpression of CXCL1 (ligand of CXCR2) in transgenic animals, associates with decrease in life span, appearance of neurological syndromes, and increase in reactive microglia, astrocytes, and infiltrated neutrophiles throughout the CNS (Tani et al., 1996). In addition, the CXCL1 overexpression can cause anomalies in the myelin structures of neurons in the peri-ventricular area where immune cells are highly infiltrated (Tani et al., 1996).

CXCR4 chemokine receptor also plays an important role in the development of the central nervous system as well as other organs. Knockout embryos of CXCR4 chemokine receptor (Ma et al., 1998) or its ligand, CXCL12 (Nagasawa et al., 1996; Zou et al., 1998), are born with a cluster of anomalies in lymphopoiesis, bone marrow myelopoiesis, cardio-vascular system, and CNS. The abnormal structures of the granular cell layer of cerebellum observed in mice lacking CXCR4\(^{-}\) or CXCL12\(^{-}\) confirms the functional importance of this CXC chemokine and its receptor during development of the nervous system (Ma et al., 1998; Zou et al., 1998). Unfortunately, so far, no study evaluated the effect of CXCR4 (or CXCL12) overexpression on development of the CNS. Additionally, no report is available on the structural pattern of CNS and myelogenesis in knockout or transgenic animals of CX3CR1, CCR3 and/or their ligands.

II.3.c. Expression pattern in CNS disease

II.3.c.i. General overview

To date, several studies demonstrated the expression or upregulation of different chemokines and chemokine receptors in the neuroinflammatory / degenerative diseases of the central nervous system in human and rodents (Brouwer et al., 2004; Filipovic et al., 2003; Horuk et al., 1997; Miller et al., 2005; Omari et al., 2005; Ransohof et al., 1993; Sorensen et al., 1999; Sorensen et al., 2001; Spanaus et al., 1997). It is important to bear in mind, that not only
infiltrating cells of the immune system, but activated resident cells of the CNS including, type-I astrocytes, microglia, or even endothelial cells can express a wide range of chemokines or chemokine receptors (Cole et al., 1998; Salmaggi et al., 2002, Rezaie et al., 2002; Filipovic et al., 2003; Omari et al., 2005; Takanohashi et al., 2005; Tomita et al., 2005; Aravalli et al., 2005; Dorf et al., 2000). The upregulation of chemokines (Ransohoff et al., 1993) and chemokine receptors (Jiang et al., 1998;) was primarily shown in experimental autoimmune encephalomyelitis (EAE) animals. However, later on other studies revealed the expression of chemokines by activated astrocytes in the vicinity of MS lesions as well (Balashov et al., 1999; Omari et al., 2005; Sorensen et al., 1999). Very recent studies also showed the upregulation of chemokine receptors (CXCR2) in CNPase positive oligodendrocytes (Omari et al., 2005) and activated microglia (Filipovic et al., 2003) flanked by the demyelinating plaques in MS lesions. In addition, data showed that activated microglia can be a vivid source of chemokines (CXCL1) at the border of MS plaques as well (Filipovic et al., 2003).

An elevated level of certain chemokines was observed in the cerebrospinal fluid (CSF) of patients suffering from multiple sclerosis (Krumbholz et al., 2006) or other inflammatory diseases like acute bacterial meningitis (Spanaus et al., 1997). Studies showed that chemokines (CXCL12) and chemokine receptors (CXCR4) play important roles in neuronal apoptosis induced by viral (human immunodeficiency virus, HIV) infections too (Hesselgesser et al., 1998). Viral infections (herpes simplex virus, HSV) can activate glial cells (microglia) in a way that they highly express different chemokines (CXCL1 and CXCL2) (Aravalli et al., 2005). In addition, the upregulation of chemokine receptors (CXCR1 and CXCR2) in reactive glial cells (astrocytes), derived from HIV patients (Cota et al., 2000) suggest the contribution of these modulatory molecules toward the establishment of chronic inflammatory states in the central nervous system.

The upregulation of certain chemokine receptor (CXCR2) has also been observed in neurodegenerative disorders like Alzheimer’s disease (AD) (Horuk et al., 1997). Additionally, elevated levels of the expressed chemokines or chemokine receptors are also shown in other CNS diseases including, hypoxic ischemic injuries (Miller et al., 2005) and glioblastoma (Kouno et al., 2004).

Above all, the absence of chemokine(s) or chemokine receptor(s) usually reveals the functional importance of these modulatory molecules in differential vulnerability of animals to CNS disease. To date, data showed that a deletion mutation of certain chemokine (CCL2, ligand of CCR2) in mice can prevent these animals to develop severe form of EAE (Huang et al., 2001). The same outcome observed when EAE induced in the CCR2 deficient mice, these
animals either develop a mild form of EAE (Gaup et al., 2003) or they will be completely insusceptible (Fife et al., 2000).

Nevertheless, in contrast, chemokines (CX3CL1) might even have protective effects and prevent neurons (Meucci et al., 2000) or microglia (Boehme et al., 2000) from apoptotic stimuli. To date, no studies investigated the vulnerability of the knockout or transgenic animals of CXCR2, CXCR4, CCR3, and CX3CR1 (or their ligands) to neuroinflammatory / degenerative diseases of the nervous system. Therefore, the exact characteristics of these modulatory molecules and their receptors, in the time course of animal models of CNS disease, have to be investigated.

II.3.c.ii. CXCR2 chemokine receptor and its ligands, CXCL1, CXCL2, and CXCL5

The expression pattern of CXCR2 on human embryonal glial cells, observed in the ventricular zone (VZ) and subventricular zone (SVZ) (Filipovic et al., 2003), as well as the abnormal distribution and recruitment of oligodendrocyte progenitor cells shown in CXCR2 knockout embryos (Tsai et al., 2002), verify the importance of this receptor in CNS development. Data showed that the interaction of CXCL1 with the 7 G-protein coupled receptor, CXCR2, affects OPC proliferation and migration in vitro as well as in vivo (Robinson et al., 1998; Tsai et al., 2002). In addition, the upregulation of CXCR2 chemokine receptor, shown in activated glial cells at the border of MS plaques (Omari et al., 2005; Filipovic et al., 2003), suggests that this chemokine receptor may have an active role in CNS pathologies as well.

CXCL1

Primarily, in vivo data showed that leukocytes and astrocytes highly express CXCL1 (GRO-α) in EAE animals (Luo et al., 2000). In addition, very recent studies demonstrated the upregulation of this chemokine by hypertrophic astrocytes in active MS lesions adjacent to the CXCR2 expressing-oligodendrocytes (Omari et al., 2005 and 2006). Moreover, an elevated level of CXCL1, observed in the CSF of patients with acute bacterial meningitis (Spanaus et al., 1997), suggests the importance of this chemokine in other inflammatory conditions as well. It can be hypothesized that secretion of different modulatory-molecules such as cytokines (e.g.: interleukin) during the inflammatory states might be able to modulate the expression pattern of chemokines in glial or neuronal cells. There are two studies, which
support the effect of cytokines on increasing the secretion rate of different chemokines including CXCL1, CXCL2, and CX3CL1 in astrocytes (Omari et al., 2005 and 2006).

**CXCL2**

CXCL2 (MIP-2) was primarily described as a secreting molecule by stimulated macrophages (Wolpe et al., 1989). This chemokine shows 62.5% and 68.7% sequence homology with human and hamster CXCL1 respectively (Wolpe et al., 1989). It is shown that not only immune cells produce CXCL2, but activated microglia (Aravalli et al., 2005; Takanohashi et al., 2005) or astrocytes (Tomita et al., 2005) are also able to express this chemokine (Takanohashi et al., 2005). An increase in the expression of CXCL2 observed in both astrocytes and leukocytes in EAE mice in vivo (Luo et al., 2000) suggests a possible functional role for this ligand in an animal model of multiple sclerosis.

**CXCL5**

CXCL5 (LIX), another ligand of the CXCR2 chemokine receptor, is detectable in high levels in the cerebrospinal fluid (CSF) at the onset of acute bacterial meningitis compared to healthy controls (Spanaus et al., 1997). Additionally, in vitro studies showed the increase in the expression of all ligands of CXCR2 chemokine receptor, including CXCL5, in virally activated microglia (Aravalli et al., 2005). Furthermore, a very recent (in vitro) microarray analysis showed up-regulation of the CXCL5 gene expression in (cytokine and growth factor mediated) activated mixed glial cultures (Albright and Gonzalez-Scarano, 2004).

This suggests the potential role of CXCL1, CXCL2, and CXCL5 in the pathogenesis of various neuroinflammatory / degenerative diseases of the central nervous system.

### II.3.c.iii. CXCR4 chemokine receptor and its ligand, CXCL12

The expression of CXCR4 in the brain has already been shown in a variety of cell types including astrocytes (Bajetto et al., 1999; Boutet et al., 2001a; Tanabe et al, 1997) microglia (Boutet et al., 2001a; Lavi et al., 1997), cerebellar granule cells (Bajetto et al., 1999), neurons (Bajetto et al., 1999; Lavi et al., 1997; Meucci et al., 1998. Stumm et al., 2002), and oligodendrocytes (Dziembowska et al., 2005). However, it was initially shown in neuronal
cells that there is a high-affinity binding of CXCL12 (SDF) to the CXCR4 chemokine receptor (Hesselgesser et al., 1997). Moreover, data showed that the expression of CXCR4 on glial cells (astrocytes and microglia) and neurons is functional, since receptor-ligand interaction can transiently elevate the cytosolic Ca\(^{2+}\) in these cells (Bajetto et al., 1999; Tanabe et al., 1997).

In addition, neurons, meningeal, and endothelial cells (Stumm et al., 2002; Klein and Rubin et al., 2004) as well as oligodendrocytes (Gleichmann et al., 2000) are known as main resident producers of CXCL12 (ligand of CXCR4) in the brain. As mentioned before, double knockout CXCL12 or CXCR4 animals show obvious abnormalities in myelogenesis, and development of the CNS as well as other organs (e.g: lung and kidney), which consequently causes embryonic or perinatal death (Ma et al., 1998; Nagasawa et al., 1996; Zou et al., 1998). Furthermore, embryos lacking CXCR4 chemokine receptor do not only show decrease in the total number of PDGFR\(\alpha\) positive oligodendrocytes, but the remaining number of these myelin producing cells are mainly displaced in these knockout animals compared to the wild type control (Dziembowska et al., 2005).

Increase in the expression of CXCR4 chemokine receptor was primarily observed in EAE animals (Jiang et al., 1998). However, a very recent study showed that not only high levels of CXCL12 are detectable in the CSF of MS patients, but glial cells (astrocytes) also show upregulation of this chemokine in the vicinity of MS lesions (Krumbholz et al., 2006). Taken together, data suggest the functional importance of the CXCR4 receptor and its ligand, CXCL12, in formation of myelin around axons during development and possibly in demyelinating diseases like MS.

II.3.c.iv. CCR3 chemokine receptor and its ligand, CCL11

The expression of CCR3 chemokine receptor was initially shown on neuronal cells (Zheng et al., 1999) as well as astrocytes and microglia (Boutet et al., 2001a) in the CNS. Several ligands can interact with the CCR3 receptor; however, it is only CCL11 (Eotaxin), which can interact with this chemokine receptor and exert its chemoattractant effect to glial cells (astrocytes) (Dorf et al., 2000; Baggiolini et al., 1997).

CCR3 plays an essential role in eosinophil recruitment and allergic reactions (Humbles et al., 2002; Ma et al., 2002). However, unlike CXCL12 or CXCR4, the knockout CCR3 mice are usually born normal (Rothenberg et al., 1997). These mutants are fertile and they can grow as their wild type littermates, if they are kept under pathogen-free conditions (Humbles et al.,
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2002). In addition, CCL11\textsuperscript{−/−} animals did not show any detectable abnormalities in different organs, but data confirmed the involvement of this chemokine (similar to its receptor, CCR3) in eosinophil recruitment (Rothenberg et al., 1997 and 1999). To date, no in vivo study investigated the CNS structure, myelination pattern, or oligodendrocyte recruitment in these knockout mutants.

The upregulation of CCR3 chemokine receptor has been described primarily in the neurodegenerative disorder, Alzheimer’s disease (AD) (Xia et al., 1998). Later, increase in the expression of CCR3 was shown in EAE mice, which was mainly co-localized with lymphocytes (Fischer et al., 2000). Although, (unlike CXCL12) the increase of CCL11 in CSF of MS patients was not confirmed by several studies (Correale et al., 2004; Narikawa et al., 2004), the upregulation of CCR3 was observed in astrocytes and microglia in both plaques and normal white matter of at least one-third of MS patients (Simpson et al., 2000). However, it is still controversial, if immune cells such as lymphocytes do (Simpson et al., 2000) or do not (Balashov et al., 1999) express CCR3 chemokine receptor in the vicinity of MS plaques. The upregulation of CCR3 by (growth factor mediated) activated-microglia was also shown in a very recent in vitro study (Takanohashi et al., 2005). Taken together, the activation of this CC chemokine receptor might play a role in the pathogenesis and prognosis of inflammatory / degenerative diseases of the CNS.

II.3.c.v. CX3CR1 chemokine receptor and its ligand, CX3CL1

The CX3CR1 receptor (V28) was first identified in human and rodent neuronal cells (Meucci et al., 1998; Raport et al., 1995). Soon after, the functional expression of this chemokine receptor was shown in microglia (Boddeke et al., 1999; Hatori et al., 2002; Nishiyori et al., 1998; Tarozzo et al., 2002) and astrocytes (Maciejewski-Lenoir et al., 1999) of human and rodent origin.

Although former studies could not demonstrate any changes in the expression of this chemokine receptor in EAE models (Schwaeble et al., 1998), latest in vivo studies showed the elevated level of CX3CR1 mRNA over the time course of EAE (Jiang et al., 1998; Sunnemark et al., 2005). Moreover, studies on MS patients confirmed that CX3CR1 is highly expressed in reactive microglia / macrophages in the vicinity of MS lesions compared to controls (Hulshof et al., 2003). Although astrocytes and neurons express strongly CX3CR1 both in vitro and in vivo, no changes in the pattern of expression were observed in these cells in MS patients (Hulshof et al., 2003). In addition to the neuroinflammatory states, the
upregulation of CX3CR1 was also observed after induction of ischemic injuries (Tarozzo et al., 2002) or even on activated glial cells (microglia and astrocytes) in an animal model of degenerative disease of the CNS (prion) (Hughes et al., 2002).

The specific ligand of CX3CR1, CX3CL1 (fractalkine), belongs to the new class of membrane bound chemokine and has a completely different cysteine fingerprint (Bazan et al., 1997). This chemokine shows a high degree of homology within different species (Pan et al., 1997), associates with the cell membrane, but has one or two soluble versions that demonstrate chemotactic activity on leukocytes (Pan et al., 1997). CX3CL1 expression (unlike most other chemokines) was initially shown in different non-hematopoietic tissues including brain (Nishiyori et al., 1998; Rossi et al., 1998). This chemokine was mainly identified in neuronal cells of human, rodents, and other mammalian origin (Harrison et al., 1998; Hughes et al., 2002; Meucci et al., 1998; Nishiyori et al., 1998; Schwaeble et al., 1998; Tarozzo et al., 2003). Glial cells, including astrocytes (Hatori et al., 2002; Hughes et al., 2002) and microglia (Hughes et al., 2002; Nishiyori et al., 1998) also express this chemokine. Moreover, applying CX3CL1 to microglia (Boddeke et al., 1999; Maciejewski-Lenoir et al., 1999), astrocytes (Maciejewski-Lenoir et al., 1999) or even CX3CR1-transfected cells (Combadiere et al., 1998b; Imai et al., 1997) can transiently mobilize calcium in these cells, which suggests the functional importance of CX3CR1 receptor-ligand interaction. Although the expression of CX3CL1 is even shown at the embryonic level (Hatori et al., 2002), which might have implications for development of the central nervous system, to date, no study showed the expression pattern and recruitment of oligodendrocytes or other glial and neuronal cells in transgenic animals of CX3CL1 or its receptor.

Preliminary studies showed that the level of mRNA encoding CX3CL1 remained unchanged by EAE in vivo (Schwaebel et al., 1998). However, a very recent study in EAE animals confirmed more specifically the elevated level of CX3CL1 mRNA in astrocytes within the lesions (Sunnemark et al., 2005). In contrast, unlike CX3CR1 receptor, no significant differences were observed between the expression level of CX3CL1 in the brain of MS patients and control groups (Hulshof et al., 2003). Finally, the increase in the expression level of CX3CL1 was also reported after injuries due to ischemic insults (Tarozzo et al., 2002) or traumatically induced axotomy (Harrison et al., 1998). This overview suggests the functional importance of CX3CR1 receptor and its ligand during the course of different CNS injuries, including the inflammatory / demyelinating insults like MS.
III. **Aim of the study**

To date, several studies showed that chemokine-receptor interaction plays an important role in oligodendroglial cell development. Oligodendrocyte progenitor cells highly proliferate in the presence of certain chemokine(s) (Robinson et al., 1998), but may migrate less (Tsai et al., 2002) or sometimes more (Dziembowska et al., 2005) toward a specific gradient of chemokine(s). Moreover, previous and ongoing studies demonstrate the transcript expression of chemokine receptors CXCR4, CCR3 and CX3CR1 as well as CXCR2 in rat oligodendrocyte progenitor cells (Dziembowska et al., 2005; Kadi et al., 2006; Nguyen and Stangel, 2001; Maysami et al., 2006a; Maysami et al., 2006b; Stangel unpublished data). Here, we aimed to characterize whether the expression of these chemokine receptors on the myelin producing cells of the CNS is functional. We investigated how chemokine receptor-ligand interaction can influence the biological reactions of oligodendrocytes by evaluating the rate of migration, proliferation and differentiation of these cells toward specific ligand(s) of the expressed receptors. In addition, using a G-protein antagonist, helped us to examine whether modulatory effects of these chemokines are in fact due to interaction with the seven G-protein coupled receptors. Finally, our intention was to compare the response of the primary oligodendrocyte progenitor cells with a cell line to conclude whether precursor cells can be replaced by a cell line for in vitro studies. Results of this study may help us gain a better understanding of the role of chemokine receptor-ligand interaction on oligodendrocyte biological activities during CNS development and the course of demyelination / remyelination in diseases such as MS.
IV. Materials and methods

IV.1. Materials

IV.1.a. Cells

- A2B5 hybridoma cells (clone 105, ATCC CRL-1520, HB-29) used as a source of anti-A2B5 monoclonal mouse IgM.
- GalC hybridoma cells (EC-07, ECACC) used as a source of anti-galactocerebroside monoclonal mouse IgG3.
- B104 neuroblastoma cell line (Salk Institute, La Jolla, CA) used to make a conditioned medium that keeps oligodendrocytes in proliferating states.
- CG4 oligodendroglial cell line used from rat origin.
- Oligodendrocyte progenitor cells were used from newborn Sprague dawley rats.
- Microglial cells were used from Sprague dawley rats.

IV.1.b. Chemical reagents

- Acrylamide/Bis solution (Bio-Rad, 161-0146, Munich, Germany) used for making poly acrylamide gel.
- Ammonium hydroxide, (Merck, 105432, Darmstadt, Germany) used for reconstitution of reagents.
- APS, ammoniumpersulfate (Roth, 9592, Munich, Germany) is a catalyzator for acrylamid-polymerization, which used for protein electrophoresis.
- BCIP, 5-Bromo-4-chloro-3-indolyl phosphate (Sigma, B6274, Steinheim, Germany) dissolved in dimethyl-formamide and used for immunoblotting.
- Biotinylated protein ladder (Cell Signalling, 7727, Danvers, MA, USA) used as a protein marker in western blot (WB) experiments to detect the molecular weight of the target protein.
- Boric acid (Sigma, B6768, Steinheim, Germany) applied to decrease the acidity of solution in BrdU staining.
- BrdU (colorimetric) cell proliferation ELISA (Roche, 11647229, Indianapolis, IN, USA) used to measure proliferation indices of CG4 cells.
Materials

- BrdU, 5-Bromo-2'-Deoxy-Uridine (Roche, 280879, Indianapolis, IN, USA) used to measure proliferation indices of OPC.
- DAB, Diaminobenzidine (DakoCytomation, Glostrup, Denmark) is a chromogene substrate and used in peroxidase-based immunoenzymatic staining systems.
- DABCO, 1, 4-Diazabicyclo [2.2.2] octane (Sigma, D2522, Steinheim, Germany) is an anti fade reagent that is used to retard photo-bleaching of fluorescein and other fluorescent dyes.
- DAPI, 4’, 6-diamidino-2-phenylindole, dihydrochloride (Molecular Probes, D-1306, Leiden, Netherlands) is a nuclear and chromosome counterstain.
- Diff-Quik, a rapid staining set (DADE Behring, 130832, Deerfield, IL, USA), which contains fast green in methanol to fix cells, Thiazine dye in phosphate buffer, and Eosin G in phosphate buffer.
- DMSO, Dimethyl Sulfoxide (Sigma, D5879, Steinheim, Germany) used to protect living cells during cold storage. It is also used as a solvent for chemical reagents.
- DNase I, Deoxyribonuclease I (Sigma, D5025, Steinheim, Germany) is an endonuclease which hydrolysis the double stranded or single-stranded DNA. DNase I used to remove the DNA from the mixed glial preparation samples.
- EtOH, ethanol (J.T.Baker, 8006, Deventer, Netherlands) used for reconstitution of reagents.
- Glycerol (Fluka, 49770, Steinheim, Germany) used for sample preparation and gel formation in polyacrylamide gel electrophoresis.
- Glycine (Roth, 3908, Munich, Germany) applied for protein electrophoresis experiments.
- Methanol (J.T.Baker, 8045, Deventer, Netherlands) used for tissue fixation.
- N, N-Dimethylformamide (Sigma, 319937, Steinheim, Germany) used as a solvent for many hydrophobic organic compounds.
- NaCl, sodium chloride (Braun, Melsung, Germany) used to dilute protein samples before concentration determination.
- NaOH, sodium hydroxide (Merck, 109137, Darmstadt, Germany) used for reconstitution of certain chemical reagents.
- NBT, nitroblue tetrazolium chloride (Roche, 11087479001, Indianapolis, IN, USA) dissolved in dimethyl-formamide and used for immunoblotting.
• P/S, penicillin-streptomycin solution (Sigma, P0781, Steinheim, Germany) 100 units/ml penicillin and 100 µg/ml streptomycin applied for any cell culture medium used.
• PBS, phosphate buffered saline (Biochrom, L182-05, Berlin, Germany) used commonly in many tissue culture techniques.
• PFA, paraformaldehyde (Sigma, P6148, Steinheim, Germany) used for tissue fixation.
• PLL, poly-L-lysine hydrobromide (Sigma, P1274, Steinheim, Germany) used to promote cell adhesion to solid substrates.
• Quick start Brad ford dye reagent (Bio-Rad, 500-0205, Munich, Germany) used for protein concentration determination.
• SDS or sodium dodecyl sulfate (Roth, 2326, Munich, Germany) used for electrophoresis.
• Streptavidin-alkaline phosphatase [AP] conjugated (Roche, 11089161001, Indianapolis, IN, USA) used for immunoblotting. Alkaline phosphatase is a marker enzyme for immunoblotting making a stable and water insoluble product using X-phosphate/NBT.
• TEMED, N, N, N’, N’- tetramethylenediamine (Sigma, T9281, Steinheim, Germany) is a catalyzator for acrylamid-polymerization.
• Titriplex (Merck, 8418, Darmstadt, Germany) used in cell culture techniques.
• TRIS, 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Roth, 4855, Munich, Germany) used for electrophoresis.
• Triton X-100 (Serva, 37238.01, Heidelberg, Germany) used for immunocytochemistry.
• Trypan Blue (Sigma, T8154, Steinheim, Germany) is a stain used in dye exclusion procedures for viable cell counting.
• Trypsin inhibitor glycine max (Sigma, T6414, Steinheim, Germany) used (1mg/ml) for inactivating trypsin (v/v) in cell culture assays.
• Trypsin-EDTA (Biochrom, L2123, Berlin, Germany) used in several tissue culture techniques.
• Tween® 20 (Roth, 9127, Munich, Germany) is a detergent which is used for electrophoresis.
IV.1.c. Growth factors and cell culture reagents

- Biotin (Sigma, B4639, Steinheim, Germany) was reconstituted in ammonium hydroxide and used in differentiating or proliferating medium.
- FGF, recombinant human fibroblast growth factor basic (R&D systems, 233-FB, Nordenstadt Germany) is a mitogenic protein which stimulates proliferation of many cells of neuroectodermal origin. FGF (10 ng/ml final concentration) used in cultured medium of OPC in certain experiments.
- GPA, G protein antagonist (Sigma, G9541, Steinheim, Germany) reconstituted in pure DMSO and used in inhibition assays at final concentration of 2.5 µg/ml. G protein antagonist inhibits the activation of the G\(\alpha_i\) or G\(\alpha_o\).
- ITS, ITS\(^\text{TM}\) Tremain (BD Biosciences 354352) contains 12.5 mg Insulin, 12.5 mg Transferring, 12.5 µg Selenite, 2.5 g Bovine Serum Albumin (BSA), and 10.7 mg Lenoleic acid. This supplement diluted 1/100 for final working concentration. Reagents present in the ITS supplement are essential for maintenance of oligodendrocyte survival.
- PDGF-\(\text{AA}\), recombinant human platelet derived growth factor-\(\text{AA}\) (R&D systems, Nordenstadt Germany) is a potent mitogen for many cells. PDGF-\(\text{AA}\) (10 ng/ml) used in cultured medium of OPC in certain experiments.
- Progesterone (Sigma, P8783, Steinheim, Germany) is a steroid hormone produced by the corpus luteum and is one of the essential factors for oligodendrocyte continued existence. Progesterone was reconstituted in pure ethanol.
- PTX, pertussis toxin (Sigma, P7208, Steinheim, Germany) is a protein isolated from the gram negative Bordetella pertussis which can catalyze ADP-ribosylation of the α subunit of the guanine nucleotide regulatory proteins such as G\(i\), G\(o\), and G\(l\). PTX prevents the interaction of the G-protein heteromers from interacting with receptors proteins in the cell membrane. It has a great effect on cAMP accumulation since the adenylyl cyclase cannot be inactivated. The lyophilized powder were reconstituted in PBS and 100 or 200 ng/ml applied for inhibition assays.
- Putrescine dihydrochloride (Sigma, P5780, Steinheim, Germany) is reconstituted in H\(\text{2}\)O. It is used as one of the essential factors for oligodendrocyte continued existence.
- T3 or 3,3′,5-triiodo-L-thyronine sodium salt (Sigma, T6397, Steinheim, Germany) contains iodine and produced from thyroglobulin in the thyroid follicular cells. T3 is reconstituted in NaOH. This product is necessary for oligodendrocyte maturation.
• T4 or 3,3',5,5''-tetraiodo-L-thyronine (Sigma, T1775, Steinheim, Germany) contains iodine and is produced from thyroglobulin in the thyroid follicular cells. T4 is reconstituted in NaOH. Similar to T3, this product is also necessary for oligodendrocyte maturation.

• Trituating solution consists of 1% BSA, 0,5 mg/ml Trypsin inhibitor, and 0,03 mg/ml DNase in PBS. This solution is used in preparation of MGP.

IV.1.d. Media and sera

• B104 conditioned medium, B104-CM, was produced from B104 rat CNS neuroblastoma cell line (original source: Salk Institute, La Jolla, CA) by culturing 1x10^6 of these cells per 72cm^2 culture flask in the absence of FBS for 48 hours. The supernatant was then filtered using 0.2 µm pore sized filter (Nalgen) to get rid of debris and sterile filtration.

• B104 medium (formerly named O3 medium; Bottenstein et al., 1988) consists of DMEM plus 30% B104-CM (Bottenstein et al., 1988), 10 ng/ml biotin, 74 ng/ml progesterone, 16 µg/ml putrescin, 1% P/S. Other essentials including, 5 µg/ml Bovine insulin, 50 µg/ml human transferrin, and 30 nM sodium selenite were added by applying 1% ITS supplement. B104 medium keep oligodendrocytes in proliferating state (Asakura et al., 1997; Bottenstein et al., 1988).

• DMEM or Dulbecco’s modified eagle’s medium (Invitrogen 41965-039), which contains L-glutamin, and D-glucose without sodium pyruvat used for cell cultures.

• FBS, fetal bovine serum (Biochrom, S 0115, Berlin, Germany) used in making standard glial medium. The final concentration used was 10% of heat inactivated serum (56°C, 30 min.) in DMEM.

• HBSS, Hanks balanced salt solution (HBSS) (Sigma, H9269, Steinheim, Germany) with sodium bicarbonate used in mixed glial cell preparations.

• HEPES buffered medium, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma, H0887, Steinheim, Germany) is resistant to rapid pH changes and addition of HEPES raises the theoretical buffering range closer to optimal physiological pH. Applications of HEPES in this study were tissue homogenization and resuspension. The applied working concentration was 10mM in HBSS.

• N2B3 medium is made of 10 ng/ml biotin, 74 ng/ml progesterone, 16 µg/ml putrescin, 1% P/S, 1% ITS supplement in DMEM, plus T3 and T4 400 ng/ml each. N2B3
medium helps OPC to differentiate into mature oligodendrocytes (Omari et al., 2005; Wilson et al., 2003; Nicolas et al., 2001)

- Modified N2B3 medium is made of all the material used to make N2B3 medium except T3 and T4. Modified N2B3 medium used only for maintenance of oligodendrocytes, while in the absence of thyroid hormones OPC will transform slower into the differentiated oligodendrocytes.
- Normal glial medium is made of DMEM (plus 2 mM glutamine ) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin that was used in many cell culture techniques including mixed glial cell preparation (MGP).
- Normal Goat serum (Vector laboratories, S-1000, Burlingame, CA, USA) used for blocking non-specific binding sites in immunostaining.

IV.1.e. Antibodies

IV.1.e.i. Primary antibodies

- A2B5 hybridoma cells (LGC Promochem ATCC CRL-1520, HB-29) produce a monoclonal antibody that reacts with a glycolipid antigen localized on the cell bodies of chick retinal neurons as well as progenitor oligodendrocytes.
- Anti CX3CR1 (Torrey Pine Biolabs Inc. TP-501P) is a purified rabbit polyclonal antibody raised against the amino terminus of CX3CR1 of rat origin.
- Anti GalC (clone IC-07 ECACC) is a mouse anti-galactocerebroside monoclonal IgG3. The antibody reacts only with galactocerebroside antigen localized on the cell bodies of oligodendrocytes.
- Anti GFAP, glial fibrillary acidic protein (DakoCytometion, Z0334, Glostrup, Denmark) is a polyclonal rabbit which reacts with the cytoplasmic antigen of astrocytes.
- Anti O4, (Chemicon, MAB345, Temecula, CA, USA) is a mouse monoclonal IgM anti oligodendrocyte marker O4. This antibody has a strong reactivity with several species including mouse and rat.
- Anti OX42 (Cedarlane, CL042B, Hornby, Ontario, CANADA) is a mouse IgG2, which is reacting with the antigen localized on the cell bodies of microglial cells.
- Anti-bromodeoxyuridin (Roche, 1170376, Indianapolis, IN, USA) is a mouse monoclonal IgG1 and applied to detect incorporated BrdU in proliferating cells.
Materials

• Horseradish peroxidase (HRP) linked anti-biotin antibody (Cell Signalling, 7727, Danvers, MA, USA) used to bind biotinylated protein ladder in western blot experiments.

IV.1.e.ii.  Secondary antibodies

• Biotinylated goat anti rabbit IgG (Vector laboratories, BA-1000, Burlingame, CA, USA) applied for immuno-cytochemistry (ICC), immunohistochemistry (IHC), and WB.

• Cy2-conjugated goat anti rabbit IgG (Jakson, 111-225-144, West Grove, PA, USA) applied for ICC or IHC, reconstituted as suggested by the manufacturer and used at 7.5 µg/ml final concentration.

• Cy2-conjugated rabbit anti mouse IgG (Jakson, 315-225-008, West Grove, PA, USA) applied for ICC or IHC, reconstituted as suggested by the manufacturer and used at 6 µg/ml final concentration.

• Cy3- conjugated goat anti mouse IgM (Dianova, 115-165-020, Hamburg, Germany) applied for ICC or IHC, reconstituted as suggested by the manufacturer and used at 7.5 µg/ml final concentration.

• Cy3-conjugated rabbit anti mouse IgM, (Jackson, 315-165-020, West Grove, PA, USA) applied for ICC or IHC, reconstituted as suggested by the manufacturer and used at 7.5 µg/ml final concentration.

• Cy3-conjugated goat anti rabbit IgG (Dianova, 111-165-003, Hamburg, Germany) applied for ICC or IHC, reconstituted as suggested by the manufacturer and used at 7.5 µg/ml final concentration.

• MicroBeads-conjugated anti-mouse-IgM (MACS, 130-047-301, Auburn, CA, USA) is an IgG1 antibody of rat origin and used for immunisolation of OPC.

IV.1.f.  Chemokines

All chemokines were reconstituted in phosphate buffer saline (PBS) containing 0.1% BSA.

• CCL11 or recombinant mouse Eotaxin (R&D systems, 420-E, Nordenstadt Germany)

• CX3CL1 or recombinant rat Fractalkine (R&D systems, 537-FT, Nordenstadt Germany)

• CXCL1 or recombinant rat GRO/KC (Peprotech, 400-10, London, UK)
Materials

- CXCL12 or recombinant murine SDF-1α (Peprotech, 250-20A, London, UK)
- CXCL2 or recombinant rat GROβ/MIP-2 (Peprotech, 400-11, London, UK)
- CXCL5 or recombinant rat LIX, (R&D systems, 543-RL, Nordenstadt Germany)

IV.1.g. Instruments

- BioMagnet separator (Bioclone Inc, MS-02, San Diego, CA, USA) used for positive selection of OPC.
- Cryo-tubes (Greiner Bio-One, T-3800-1, Frickenhausen, Germany) used for cell storage in liquid nitrogen.
- Disposable semi-micro cuvettes (Brand, PMMA-759115, Wertheim, Germany) used for protein concentration determination.
- EagleSight software (Stratagene, version 3.0, La Jolla, CA 92037 USA) based on Microsoft windows program manager (version 3.11), used to measure the density of migrated CG4 oligodendroglial cells.
- Glass coverslips (Roth, P231, Munich, Germany) used for cell cultures.
- Heraeus tissue culture incubators (Heraeus Instruments, BB6060, Mannheim, Germany) used as standard environment in cell culture techniques. Temperature was kept at 37°C with constant flow of 5% CO2 in the incubators.
- Leica Cryostat (Leica microsystem, CM 3050S, Houston, TX, USA) used for cryosectioning fixed and snap-frozen tissues.
- Leica DMLB microscope, Leica DC 300 camera and Leica software-IM1000 (Leica microsystem AG, version 1.20, Houston, TX, USA) used for visualizing, taking pictures, and counting cells.
- Megafuge Heraeus 2.0R (Heraeus Instruments, Mannheim, Germany) was used at either 210g or 1800g for cell culture assays.
- Micro chemotaxis chamber (Neuro Probe, AP48, Gaithersburg, MD, USA) contains 48-wells and used for migration analysis of OPC and CG4 cells.
- Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, 170-3930, Munich, Germany) used for blotting.
- Mini-PROTEAN II System (Bio-Rad, Munich, Germany) used for protein electrophoresis
- Multidishes, polystyrene sterilized 96 well plates (Nunc, 167008, Wiesbaden, Germany) used for BrdU ELISA assay of CG4 cells.
Materials

- Multidishes, polystyrene sterilized four well plates (Nunc, 176740, Wiesbaden, Germany) used for routine OPC culture and ICC.
- Nalgene MF75 SFCA (Nalgene, 290-4520, Rochester, NY, USA) with a cellulose acetate membrane (pore size of 0.45 µm or 0.2 µm respectively) used for either filter-sterilization of chemical reagents or conditioned medium (B104).
- Neubauer Hemocytometer chamber (Brand, 149, Wertheim, Germany) used for cell counting.
- Nitrocellulose membrane, Hybond™-ECL (Amersham Biosciences, RPN68D, Freiburg, Germany) used for WB.
- Orbital shaker-incubator (Johanna Otto GmbH, Edmund Bühler KS-15 control and TH15) set at 180 rpm or 150 rpm to mechanically separate microglial cells or OPC, respectively. Temperature was kept at 37°C and CO2 preserved in the culture flasks during the shaking period.
- Polycarbonate membrane filter (NeuroProbe, PFB8, Gaithersburg, MD, USA), 25x80mm standard polycarbonate membrane with 8µm-sized pores used in migration assays of OPC and CG4 cells.
- Rocking Shaker (Heto, RS-PL 28-10, Allerod, Denmark) used for washing nitrocellulose membrane and applying antibodies or colorimetric reagents after WB.
- SLT (Tecan) Spectra microplate-reader (LabX, SE1433, Midland, ON, Canada) accompanied with TECAN software [version 2.01-3.91 based on MS-DOS (version 5.00)] detect wavelength range from 340 to 750nm and used for BrdU ELISA assay of CG4 cells.
- Spectrophotometer Du-70 (Beckman instrument, Fullerton, USA) used for protein concentration determination.
- Tissue culture polystyrene flasks with ventilation cap 75 cm² (Sarstedt, 83.1813.002, Nuembrecht, Germany) and 25 cm² (Sarstedt, 83.1810.002, Nuembrecht, Germany) used for cell culture assays.
IV.2. Methods

IV.2.a. Cell culture

IV.2.a.i. CG4 oligodendroglial cell line

CG4 oligodendroglial cell line (3 x 10⁶, in a frozen cryo-tube) was thawed in a water-bath, washed once, and then transferred into a PLL-coated medium-sized (75 cm²) tissue culture flask filled with B104 medium. Every two or three days medium was refreshed and cells were used or split when they were 80 - 90% confluent.

IV.2.a.ii. Preparation of primary oligodendrocytes

Primary cultures of mixed glial cells (MGP) were prepared from newborn Sprague-Dawley rat cerebra. Brains were freed from meninges, chopped in HBSS containing 10 mM HEPES and cells were chemically dissociated using 0.1% trypsin for 20 minutes at 37°C. After both mechanical and chemical dissociations, cells were centrifuged at 3000 rpm (1800 g) for 5 minutes and the supernatant as well as the meningeal layer on the top of pellet were discarded. Afterwards, cells were resuspended in 5 ml DNase (0.001%) and thereafter in 1 ml tritutating solution. Finally, by pipetting up and down, using glass-pasteur pipets (with different outlet diameters), a fine cell suspension was made and cells were plated into a PLL-coated medium sized culture flasks (1 - 2 brains per 75 cm²) and grown in normal glial medium. Medium was changed after 24 hours and then 72 hours after preparation. On day 7th of preparation, microglial cells were isolated after 30 - 45 minutes shaking at 200 rpm, the medium replaced, and then oligodendrocytes were mechanically dissociated overnight on an orbital shaker-incubator at 150 rpm. Each MGP cell culture flask were used two (max. three) times for mechanical dissociation of progenitor cells. In order to minimize the contaminating microglia from the isolated OPC, the cell suspension was incubated for 15 to 30 minutes in a non-coated medium-sized tissue culture flask in a standard incubator. Since microglia can adhere to the plastic, but OPC (non-adherent cells) can hardly attach to the culture flask, this cell suspension usually contains more than 80 to 85% OPC. However, there is always a small proportion of contaminating astrocytes (1-5%) and microglia (10-15%), as judged by applying immunostaining for GFAP and OX42, the astrocyte and microglial marker, respectively.
IV.2.a.iii. Storage of cells

All cells, including the CG4 oligodendroglial cell line, A2B5 and GalC hybridoma were stored in liquid nitrogen. Each cytotube filled with $3 \times 10^6$ / ml CG4 or hybridoma cells, was resuspended in either B104 medium or FCS plus 5% DMSO, respectively. In order to freeze cells, viability of more than 90% was considered. Trypan blue (50%) was applied to estimate the viability of cells. The ratio of viable cells was measured by dividing the number of living cells (excluding dead cells) to the total number of cells using a hemocytometer chamber.

IV.2.b. Positive immunoselection of OPC and cell-expansion

After isolation of OPC using an orbital shaker-incubator, $2 \times 10^6$ cells were labelled with the primary mouse IgM antibody, A2B5 hybridoma supernatant, for 30 minutes at 4°C on a rotating platform. Subsequently, cells were spun down at 600 g, washed twice in PBS, and magnetically labelled with the rat anti-mouse-IgM Microbeads (100 µl) for about 30 minutes at 4°C. The beads-conjugated cells were isolated with a magnet and transferred into a PLL-coated small-sized ($25 \text{ cm}^2$) tissue culture flask. Medium (B104) was exchanged the day after, and then every two or three days. Ultimately, cells were transferred into a medium-sized culture flask ($75 \text{ cm}^2$) when they were more than 75% confluent. The purified progenitor oligodendrocytes were subsequently used for further experimental analysis.

IV.2.c. Protein extraction and protein concentration determination of cultured cells

Medium was decanted from the tissue culture flask containing confluent immunoselected OPC ($10^7$) or purified microglial cells. Cells were washed once with PBS (RT) and the excess of PBS was aspirated. 1-2 ml boiling lysis buffer (SDS in TRIS) was added into the flask, cells were scraped from the dish, transferred into a microcentrifuge tube and boiled for additional 5 minutes. Then, the sample was passed several times through a 26-gauge needle to reduce the viscosity and centrifuged for another 5 minutes to pellet the insoluble materials. Afterwards, the protein concentration of the sample was measured using a spectrophotometer (Beckman). In brief, samples were resuspended in 1ml quick start Bradford dye reagent (Bio-Rad) after diluting (1:10) in 0.9% NaCl. The protein concentration was detected in a disposable cuvette (Brand) using a Beckman spectrophotometer (wave length 595 nm). Finally, 20 µg of this tissue lysis plus 1% sample buffer was loaded per well.
Methods

IV.2.d. Preparation of frozen sections

Rat brains (Sprague Dawley - P1), were carefully removed from the skull, after sacrificing the animals, and subjected to the pre-chilled 2-Methylbutan. The Coronal sections of 7.0 µm were cut from 3.1 to 4.5 mm Interaural using a Leica Cryostat (Leica CM) and brain slices were kept at -80°C before applying to IHC.

IV.2.e. Production of antibodies from hybridoma cells

IV.2.e.i. A2B5 and GalC monoclonal antibody

The A2B5 and GalC lymphocyte hybrid cells were thawed in a 37°C water bath, cells were washed once, transferred into a culture flask containing medium (DMEM plus 10% FCS), and kept in an standard incubator at 37°C plus 5% CO₂ and humidity. Medium was refreshed every 2 or 3 days, the supernatant containing the antibody was harvested and filter-sterilized (Nalgene). Supernatants kept at -20°C and both antibodies diluted 1:2 before used for the surface staining of OPC and mature oligodendrocytes, respectively.

IV.2.f. Staining methods

IV.2.f.i. Diff-Quik staining

Rapid staining set contains fast green in methanol for cell fixation, Thiazone dye in phosphate buffer (pH 6.6; 1.1 g/l), and Eosin G in phosphate buffer (pH 6.6; 1.22 g/l). This method was applied according to the manufacturer to stain the migrated cells on the polycarbonate membrane after wiping the non-migrated cells off.

IV.2.f.ii. Immunocytochemistry

Oligodendrocyte surface staining (A2B5 and GalC)

Purified OPC were plated on a PLL coated glass coverslip, incubated for 24 - 48 hours in B104 (in proliferation assays) or N2B3 medium (in proliferation and differentiation assays), and then the non-specific antigens were blocked using 5% FBS. Afterwards, A2B5 and/or
Methods

GalC primary antibodies (hybridoma supernatant) were applied 1:2 dilution and cells kept at 37°C for 1 hour. Following several washing steps, the secondary antibodies (Cy2-conjugated anti mouse IgG and Cy3-conjugated anti mouse IgM) were applied for 30 - 45 minutes (RT). Cells were fixed with 4% PFA, mounted in DABCO, and examined by fluorescence microscopy. In addition, DAPI was used for nuclear visualization. Primary antibodies (A2B5 or GalC) were omitted in control stainings.

BrdU staining

In order to label proliferating cells, 10 µM bromodeoxyuridine (BrdU) was applied overnight on cells. The day after cells were fixed on coverslips using pure chilled methanol at -20°C, histones were extracted by resuspending cells in 0.1 M HCl for 1 hour at 37°C and acid was diluted afterwards, applying several washing steps in PBS and borate buffer. Subsequently, 1-2 µg/ml anti-bromodeoxyuridin antibody was applied to bind to the incorporated BrdU for one hour (RT) and after washing the unbound primary antibody, the Cy2 conjugated secondary antibody was applied for 30 - 60 minutes (RT). Cells were mounted in DABCO and stainings were examined by a Leica fluorescence microscopy.

OX42 staining

In order to identify how many percentages of isolated OPC are contaminated with microglial cells, OX42 staining was applied. Purified cells were plated on glass coverslips and after 24 to 48 hours of incubation in B104 or N2B3 medium were subjected to surface staining. Cells were fixed using prechilled methanol, primary antibody (mouse IgG2, Cedarlane) applied for one hour at RT after blocking nonspecific binding sites using 5% FBS. Subsequent to several washing steps, the secondary antibody (Cy2-conjugated rabbit anti-mouse IgG, Jakson) applied for 30 to 45 minutes (RT). In addition, double staining for A2B5 was applied as explained before. Finally, DAPI applied for nuclear staining, cells were mounted in DABCO and stainings were examined by fluorescence microscopy (Leica). Cells were counted (100-200 cells / coverslips) in at least five different fields and the percentage of microglia (OX42 positive) and OPC (A2B5 positive) cells was calculated in total counted cells (DAPI positive) per coverslip.
GFAP staining

To identify the number of possible contaminating astrocytes in isolated OPC cultures, GFAP staining was applied. Purified cells were plated on glass coverslips and after 24 to 48 hours growing in B104 or N2B3 medium subjected to cytoplasmic staining. Cells were fixed using prechilled methanol and permeabilized with Triton X-100 (Serva), primary antibody (polyclonal rabbit, DakoCytomation) applied for one hour at RT, after blocking with 5% FBS. Subsequent to several washing steps, the secondary antibody (Cy2-conjugated goat anti rabbit IgG, Jakson) was applied for 30 to 45 minutes (RT). Moreover, the A2B5 surface staining was applied as explained before. Ultimately, DAPI applied for nuclear staining, cells were mounted in DABCO and stainings were examined by fluorescence microscopy (Leica). Cells were counted (100 - 200 cells / coverslips) in at least five different fields and the percentage of contaminated astrocytes (GFAP positive) and OPC (A2B5 positive) was calculated in total number of counted cells (DAPI positive) per coverslip.

IV.2.f.iii. Immunohistochemistry

The snap-frozen sections were rehydrated in PBS and fixed with methanol either before or after applying primary antibodies. The primary antibodies (anti O4, anti A2B5, and anti CX3CR1) applied at 4°C in a humidified chamber overnight, following blocking with 5% FBS (or the normal serum of the animal which the secondary antibodies were made). Subsequent to several washing steps, secondary antibodies (Cy2-conjugated goat anti rabbit IgG and Cy3-conjugated goat anti mouse IgM) were applied for 30 - 45 minutes (RT). Finally, the slices were mounted with DABCO (± DAPI for nuclear staining) and stainings were examined by fluorescence microscopy (Leica). The spleen frozen sections of newborn Sprague Dawley rat were used as a positive control for CX3CR1 staining. Negative controls were performed following the same procedure, but omitting the primary antibodies.
IV.2.g. Migration assay

Polycarbonate membrane (Neuroprobe) with 8µm pore size was coated with PLL for 1 to 2 hours (RT). First, the lower chamber wells were filled with medium (modified N2B3 or B104) with (0.1 - 100 ng/ml) or without (negative control) a specific chemokine (see materials section). After placing the precoated polycarbonate membrane between the lower and upper chamber, 5 x 10^4 CG4 cells or isolated OPC in modified N2B3 medium were applied into each well of the upper compartment (Fig.2). Subsequently, the micro-chemotaxis chamber was kept in the incubator at 37°C plus 5% CO2 and humidity overnight. After 16 hours, the non-migrated cells were wiped off from the upper side of the membrane, migrated oligodendrocytes were fixed and stained using Diff-Quik (Fig.2). Migrated OPC were then counted in at least five different fields using a Leica microscope (objective x 40). Density of migrated CG4 cells were measured using EagleSight software and the density-ratio of CG4 cells or the number of migrated OPC in the presence of a chemokine was normalized to the respective control without the chemokine.

IV.2.h. Proliferation assay – OPC

OPC were harvested after overnight shaking and applied (5 x 10^4 cells) per PLL-coated coverslip. In order to label proliferating cells, 10 µM bromodeoxyuridine was applied in N2B3 or B104 medium and cells were incubated overnight in presence (0.1 - 100 ng/ml) or absence (negative control) of a certain chemokine. The next day cells were subjected to A2B5 surface staining and BrdU nuclear staining as described before. Stainings were examined by fluorescence microscopy (Leica), cells were counted in at least five fields, and the ratio of proliferating (BrdU and A2B5 double positive) cells was compared to the total amount of A2B5 positive precursors (Fig.3). The proliferation indices were calculated by taking the ratio of proliferated OPC in the presence of a chemokine and the respective control culture.
Fig. 2: Migration assay: Boyden chamber. A) Lower chamber wells were filled with certain chemokine in triplets in modified N2B3 or B104 medium. B) PLL-coated polycarbonate membrane was laid between lower and upper part of the chamber, and cells (C) applied in the upper chamber wells. D) Finally, migrated cells were fixed and stained (Objective: x 40).
Methods

A) B)

Fig.3: Proliferation assay: Immunocytochemistry (ICC). OPC cultured in A) B104 or B) N2B3 medium plus 10 µM BrdU. After 24 hours incubation in the presence or absence of certain chemokine, cells were stained for BrdU (Cy2) and A2B5 (Cy3) (Objective x 40).

IV.2.i. Proliferation assay – CG4

BrdU proliferation ELISA (colorimetric immunoassay, Roche) was used to quantify cell proliferation based on the measurement of BrdU incorporation during DNA synthesis (Fig.4). CG4 cells (5 x 10⁴ per well) were plated in a PLL coated 96-well-plate and incubated in modified N2B3 or B104 medium in presence (0.1 - 100 ng/ml) or absence (negative control) of chemokines overnight. The next day 10 µM BrdU was added to the conditions above and cells were incubated for an additional 4 hours at 37°C in the standard incubator. Afterwards, the supernatant was removed and the cells were air-dried. In the next step, cells were fixed and DNA was denatured, using FixDenat (supplied by the manufacturer). Subsequently, anti-BrdU-POD was applied for 90 minutes (RT) and then cells were washed with the washing solution (supplied by the manufacturer). Ultimately, the final substrate was applied for 2 to 3 minutes and the reaction was stopped using 1 M H₂SO₄. Reactions were measured using a SLT (Tecan) Spectra microplate-reader (450 and 620 nm filters) and the rate of proliferating cells in presence of the chemokine was normalized to the respective negative control culture.
Methods

**Fig.4: Proliferation assay: ELISA.** BrdU proliferation ELISA (Roche) on CG4 oligodendroglial cells. This method is a colorimetric immunoassay, used to quantify cell proliferation based on the measurement of BrdU incorporation during DNA synthesis.

**IV.2.j. Differentiation assay**

Differentiation of OPC was examined by double-staining of OPC for the progenitor marker A2B5 and the oligodendrocyte marker GalC (Fig.5). OPC (5 x 10^4) were plated on PLL pre-coated glass cover slips in N2B3 medium in the absence (negative control) or presence (0.1 – 100 ng/ml) of a chemokine. Cells were stained for A2B5 and GalC after 48 hours of incubation as described in methods. The number of A2B5 and GalC positive cells (100 - 200 cells per coverslip) was counted in at least five visual fields (Leica, objective x 40). Differentiation index was calculated by taking the ratio of differentiated oligodendrocytes (GalC positive) to progenitor cells (A2B5 positive) normalized to the daily negative control.
Methods

**Fig.5: Differentiation assay: ICC.** Progenitor cells were cultured in N2B3 medium and after 48 hours of incubation cells were stained for (A) A2B5 precursor marker (Cy3) and (B) GalC oligodendrocyte marker (Cy2). OPC differentiation indices measured in the absence or presence of certain chemokine after 48 hours of incubation. C) Shows the phase contrast (objective x 40).

**IV.2.k. Inhibition of G-proteins from activation**

In order to confirm that modulatory effects of chemokines are indeed due to the interaction of these molecules with seven G protein coupled receptors, Pertussis toxin (PTX) or a G-protein antagonist was applied.

**IV.2.k.i. Inhibition of G-proteins in differentiation assays**

Cells were primarily pre-incubated in either GPA (2.5 µg/ml) or PTX (100 ng/ml) for 1 or 4 hours, respectively, before addition of the chemokine. Differentiation was then allowed for an additional 48 hours as described above, but this time in the absence (control) or presence of the blocker. Finally, the differentiation indices were determined as explained before. Note that only the concentration of 10 ng/ml of chemokines was applied in these inhibition assays that was able to modulate the differentiation index of OPC in previous experiments.
IV.2.k.ii. Inhibition of G-proteins in migration assays

In order to inhibit the modulatory effect of chemokines in migration assays, GPA used in the presence or absence of certain chemokines and experiments were run as explained before. GPA (2.5 µg/ml) was added directly to the lower chamber. Note that only one concentration of the applied chemokines was used, which had a modulatory effect in previous observations.

IV.2.l. SDS PAGE and Western blot

IV.2.l.i. 10% SDS polyacrylamide gel

For polyacrylamide gel, 1 mg/ml SDS, 8.66% acrylamide, 0.5 mg/ml APS, and 0.04% TEMED were used for the stacking gels. 1 mg/ml SDS, 10% acrylamide, 0.5 mg/ml APS, and 0.025% TEMED were applied for separating gels. All the solutions were prepared in the Tris buffer.

IV.2.l.ii. Gel electrophoresis and blotting

20 µg protein was loaded per well on 10% SDS gels. Samples were running on the gel using a Bio-Rad protein electrophoresis apparatus. A Biotinylated protein ladder (Cell Signalling) was also run on each gel. Protein bands were separated by gel electrophoresis and then blotted in the presence of a buffer containing 10 mg/ml Tris, 50 mg/ml Glycin. Protein samples transferred onto a nitrocellulose membrane for one hour using a Bio-Rad blotting apparatus. Subsequently, primary antibody (anti CX3CR1) was applied overnight at 4°C. The next day, the secondary antibody (Biotinilated-goat anti rabbit IgG) was applied for 1 hour (RT) and afterwards, membrane was incubated in streptavidin conjugated alkaline phosphatase (Streptavidin-AP, Roche, 1:1000). Finally, 0.38 mM X-phosphate / 0.41 mM NBT was dissolved in Tris buffer according to the manufacturer (Roche) and used to make an insoluble product band at the binding site of the Streptavidin-AP conjugated antibodies. 1.39 mM DAB / 0.01% H₂O₂ was made in Tris buffer according to the manufacturer (Cell signalling) and used to visualize protein ladder after applying horseradish peroxidase (HRP) conjugated antibody (1:1000). All incubation procedures accompanied with several washing steps in TBST with 5% blocker on a rocking shaker (Heto).
V. Statistical analysis

Two-tailed Student’s t test (type one) used for comparison between two unpaired groups of applied chemokine and control in migration, proliferation, and differentiation assays. The one way ANOVA (Neuman-Keul) test was used to compare more than two unpaired groups (Inhibition assays with GPA and PTX). The statistical analysis, and graphs, were made using SigmaStat (SPSS Inc. version 2.03), SigmaPlot (SPSS Inc. version 7.0), and Prism (GraphPad software Inc. version 3.00). Minimal statistical significance for each test used was fixed at $P < 0.05$. 
VI. Results

VI.1. Modulation of OPC migration

In general, migration assays were set in two different media including modified N2B3 and B104 medium (A modified-N2B3 medium plus 10 ng/ml FGF and 10 ng/ml PDGF applied in assays that CX3CL1 used). Data achieved from the migrated cells toward the gradients of chemokines were normalized to the respective negative control. Therefore, the random migration of these myelin producing cells in each culture medium is excluded. results show that OPC migrate 1.46 ± 0.01 SEM folds more in B104 than in modified N2B3 medium (Fig.6). In addition, in order to inhibit the effect of chemokines on OPC migration, the G-protein antagonist was applied directly into the lower chamber in the absence or presence of chemokines. Data shown as mean ± SEM of at least three independent experiments (Fig.7 to 11).

![Migration assay (Boyden chamber)](image)

Fig.6: Migration assay (Boyden chamber). OPC migrate more toward the B104 medium compared to modified N2B3. Mean of six independent experiments ± SEM. The asterisk shows significance of the difference (P<0.05).
VI.1.a.  CXCR2 chemokine receptor and its ligands, CXCL1, CXCL2, and CXCL5

The modulation observed in the migration rate of CG4 cells in the presence of all three ligands of CXCR2 is mainly in contrast to the observed assays on OPC. CG4 oligodendroglial cells show mainly a mild increase (concentration dependently) in the migration rate toward all three ligands (CXCL1, CXCL2, and CXCL5) of CXCR2 in B104 medium (Fig.7.A). In contrast, in modified N2B3 medium, CXCL1 and CXCL2 show dual effects which stimulate the migration of CG4 cells at very low (less than 1 ng/ml) and very high (50 – 100 ng/ml) concentrations, but both ligands (as well as CXCL5) have a chemorepellant effect at intermediate (10 - 25 ng/ml) concentrations.

In contrast, reduction in the migration rate of OPC toward different gradients of these chemokines was observed in the B104 medium (Fig.7.B). As mentioned before, inhibition of rat OPC migration toward the gradient of one of the ligands of CXCR2 chemokine receptor has already been shown in previously published data. Our data in OPC migration (not CG4 cell line) show a significant inhibition in migration of rat progenitor cells in the presence of CXCL1, CXCL2, and CXCL5 in conditioned medium (Fig.7.B), which is in accordance with the former studies (Tsai et al., 2002). In modified N2B3 medium, migration of OPC slightly increased toward a very low concentration of CXCL1 (0.1 ng/ml) and CXCL5 (0.5 ng/ml). However, this stimulatory effect was not robust in all ligands of CXCR2, for instance CXCL2 with 0.1 ng/ml value of the concentration or even CXCL1 at 1ng/ml had inhibitory effect on OPC migration in modified N2B3 medium (Fig.7.B).
Fig.7: Migration assay: CXCR2. CG4 cells (A) and OPC (B) migration was observed toward different concentration gradients of CXCR2 ligands, CXCL1, CXCL2 and CXCL5 in modified N2B3 (black) or B104 (gray). Statistical analysis was applied on three independent experiments. Asterisks show significant results ($P<0.05$).
VI.1.b. CXCR4 chemokine receptor and its ligand, CXCL12

Similar to the effect of CXCR2 ligands, an inhibition in OPC migration was observed when a gradient of 0.5 ng/ml CXCL12 was applied in B104 medium (Fig. 8.B), wherein this chemokine was mainly chemorepellent for progenitor cells. However, CG4 oligodendroglial cells show an opposite reaction toward this chemokine (Fig. 8.A). No statistically significant changes were observed for OPC or CG4 cell migration indices toward different gradients of CXCL12 in modified N2B3 medium (Fig. 8.A & B).

**Fig. 8: Migration assay: CXCR4.** CG4 cells (A) and OPC (B) migration was observed toward different gradients of CXCL12 in modified N2B3 (black) or B104 (gray). Statistical analysis was applied on three independent experiments. Asterisks show significant results ($P<0.05$).
VI.1.c. **CCR3 chemokine receptor and its ligand, CCL11**

Data show that CCL11, ligand of the CCR3 chemokine receptor, can act as a strong chemoattractant for CG4 oligodendroglial cells and increase the migration rate of these cells concentration dependently up to 40% in B104 medium compared to the control (Fig.9.A). However, in modified N2B3 medium (similar to ligands of CXCR2 receptor), this CC chemokine shows dual effects, which increases the migration index of CG4 cells at concentrations lower than 1 ng/ml or higher than 50 ng/ml, but has an inhibitory effect at 10 – 25 ng/ml (Fig.9.A).

In contrast to the cell line, CCL11 is mainly chemorepellant for OPC. These progenitor cells migrate (up to 25%) less toward 0.1 - 10ng/ml gradient range of this chemokine in B104 medium compared to the control (Fig.9.B). This CC chemokine (0.5 ng/ml) also shows a significant chemorepellent effect on OPC in modified N2B3 medium (Fig.9.B).

**Fig.9: Migration assay: CCR3.** CG4 cells (A) and OPC (B) migration was observed toward different concentrations of CCL11 in modified N2B3 (black) or B104 medium (gray). Statistical analysis was applied on three independent experiments. Asterisks show significant results ($P<0.05$).
VI.1.d.  CX3CR1 chemokine receptor and its ligand, CX3CL1

The CG4 cell line shows hardly dramatic changes in their migration rate toward CX3CL1 in both media (Fig.10.A). However, 10 - 25 ng/ml of this ligand can slightly increase (up to 5%) or mildly decrease the migration of these cells in B104 or modified N2B3 medium, respectively.

In contrast, CX3CL1 could inhibit (up to 50% compared to the control) the OPC migration at very low (<1 ng/ml) concentrations in medium containing 10 ng/ml FGF and PDGF (Fig.10.B). 100 ng/ml CX3CL1 could also mildly inhibit OPC migration in the presence of growth factors (Fig.10.B). In modified N2B3 medium, the migration rate of precursor cells toward different gradients of CX3CL1 is generally higher than the respective control culture (Fig.10.B). Furthermore, this chemokine showed a great potential as a strong chemoattractant (up to 150%) for progenitor cells at specific concentration gradients (10 - 25 ng/ml) in medium containing growth factors (FGF and PDGF) as well (Fig.10.B).

**Fig.10: Migration assay: CX3CR1.** CG4 cells (A) and OPC (B) migration was observed toward different concentration gradients of CCL11 in modified N2B3 (black) or modified N2B3 medium with 10 ng/ml FGF and 10 ng/ml PDGF (gray). Statistical analysis was applied on three independent experiments. Asterisks show significant results ($P<0.05$).
Inhibition of the effect of chemokines on OPC migration

In order to inhibit the modulatory effect of ligand-receptor interaction on OPC migration a G-protein antagonist (GPA) was applied. Data showed that the effect of receptor-ligand interaction on migrating OPC was completely and significantly blocked (Fig.11.A - C), when 2.5 µg/ml GPA was applied only into the lower chamber and the migration experiments were proceeded as described before. In inhibition assays on migration of OPC, GPA was applied in the presence of either 10 ng/ml CXCL1 (Fig.11.A), 0.5 ng/ml CXCL12 (Fig.11.B), or 10 ng/ml CX3CL1 (Fig.11.C).

**Fig.11: Inhibition of migration assay on OPC.** The GPA (Sigma) was applied in the lower chamber and OPC were let to migrate as described before. A) inhibition of OPC migration toward a gradient of 10 ng/ml CXCL1 (A), 0.5 ng/ml CXCL12 (B), or 10 ng/ml CX3CL1 (C) in the presence of growth factors. One way ANOVA (Neuman-Keul) used for statistical analysis on at least three independent experiments.
VI.2. Modulation of OPC proliferation

Proliferation of the CG4 oligodendroglial cell line was assayed using BrdU-ELISA assay (Fig.4). Experiments were set up (in triplets) in the presence (0.1 - 100 ng/ml) or absence of chemokines in B104 and N2B3 medium. However, due to the presence of contaminating astrocyte and microglia in mechanically isolated OPC and not having a homogenous culture, immunocytochemistry assays were used to evaluate the proliferation rate of these cells (Fig.3). The results of proliferating cells in the presence of chemokine (in both experiments ICC and ELISA) were normalized to the respective control medium. Therefore, the expected proliferation rate of these myelin producing cells in each culture condition was excluded. Results show that OPC proliferate 1.61 ± 0.22 SEM folds more in B104 compared to N2B3 medium (Fig.12). The proliferation data on OPC and CG4 cells are shown as mean ± SEM of three to five independent experiments (Fig.13 to 16).

Fig.12: Proliferation assay on OPC. OPC proliferate more in the medium containing growth factors (B104) compared to the N2B3 medium. Results are represented as mean of six independent experiments. The asterisk shows significance of the difference (P<0.05).
Fig.13: Proliferation assay: CXCR2. Proliferation of CG4 cells (A) and OPC (B) were observed in the presence or absence of CXCL1, CXCL2, and CXCL5 in two different medium N2B3 (black) and B104 medium (gray). Data are represented the mean value ± SEM of at least three independent experiments. Asterisks show significant results ($P<0.05$).
VI.2.a.  **CXCR2 chemokine receptor and its ligands, CXCL1, CXCL2, and CXCL5**

CXCL1 and CXCL2 could mildly increase the proliferation index of rat oligodendrogial cell line in B104 medium, which was dissimilar to the effect of CXCL5 (Fig. 13.A). In addition, in N2B3 medium, a significant increase in proliferation was observed in the presence of CXCL1 (25 ng/ml) and CXCL2 (0.5 ng/ml), but not CXCL5.

The effect of these ligands on proliferating OPC (similar to the migration assays, Fig. 7) was mainly inhibitory in all concentrations in B104 medium (Fig. 13.B). However, all three ligands of CXCR2 could increase the proliferation of OPC at very low (0.1 - 1 ng/ml) and very high (50 - 100 ng/ml) concentrations in N2B3 medium. This increase in OPC proliferation in N2B3 medium in the presence CXCR2 ligands represents the potential of these chemokines in stimulating the proliferation of progenitor cell in the absence of growth factors (Fig. 13.B).

VI.2.b.  **CXCR4 chemokine receptor and its ligand, CXCL12**

Applying CXCL12, ligand of the chemokine receptor CXCR4, showed that receptor-ligand interaction could hardly have any effect on CG4 cell proliferation (Fig. 14.A). In contrast, this chemokine causes a significant reduction (20%) in OPC proliferation in B104 medium; whereas, 50 ng/ml of this chemokine has a stimulatory effect on OPC proliferation in N2B3 medium (Fig. 14.B).

VI.2.c.  **CCR3 chemokine receptor and its ligand, CCL11**

Addition of the chemokine CCL11 in both culture media showed minimal inhibitory effects on the proliferation rate of CG4 cells (Fig. 15.A). In contrast, this ligand increased the proliferation rate of OPC up to 1.5 folds at very low (0.1 - 1 ng/ml) and very high (50 - 100 ng/ml) concentrations in N2B3 medium (Fig. 15.B). No significant changes were observed after applying 10 - 25 ng/ml of this chemokine. A slight inhibition of proliferation was observed when 0.1 and 10 ng/ml CCL11 applied in B104 medium. (Fig. 15.B)
Fig. 14: Proliferation assay: CXCR4. The proliferation of CG4 cells (A) and OPC (B) was observed in the presence or absence of CXCL12 in two different medium N2B3 (black) and B104 medium (gray). Data are represented as mean value ± SEM of at least three independent experiments. Asterisks show significant results ($P<0.05$).

Fig. 15: Proliferation assay: CCR3. Proliferation of CG4 cells (A) and OPC (B) was observed in the presence or absence of CCL11 in two different medium N2B3 (black) and B104 medium (gray). Data show the mean value ± SEM of at least three independent experiments. Asterisks show significant results ($P<0.05$).
VI.2.d. CX3CR1 chemokine receptor and its ligand, CX3CL1

CX3CL1 showed only insignificant modulations in the CG4 proliferation rate (Fig.16.A). In contrast, this chemokine inhibited the proliferation rate of progenitor cells concentration dependently in B104 medium (Fig.16.B). However, an opposite effect observed in N2B3 medium, where this modulatory molecule showed its potential in increasing the number of proliferating OPC in the absence of growth factors (Fig.16.B). This chemokine (like CCL11, and ligands of CXCR2) showed a dual stimulatory effect on proliferation of progenitor cells at a very low (0.5 - 1 ng/ml) and very high (50 - 100ng/ml) concentrations applied in N2B3 medium.

Fig.16: Proliferation assay: CX3CR1. Proliferation of CG4 cells (A) and OPC (B) was observed in the presence or absence of CX3CL1 in N2B3 (black) and B104 medium (gray). Data represent the mean value ± SEM of at least three independent experiments. Asterisks show significant results ($P<0.05$).

VI.3. Modulation of OPC differentiation

Two markers, GalC and A2B5, were used to observe the maturation rate of progenitor cells in N2B3 medium. GalC is mainly expressed by mature oligodendrocytes, whereas A2B5 is a
Results

marker for oligodendrocytes progenitors (OPC) (Fig.5). The maturation rate of OPC was calculated by division of the total number of mature oligodendrocytes (GalC positive) on the number of progenitor cells (A2B5 positive) in the presence or absence of chemokines. Differentiation indices were then normalized to the ratio observed in control cultures and data represented as mean ± SEM of three to five independent experiments (Fig.18 – 21).

![Differentiation assay on OPC](image)

**Fig.17: Differentiation assay on OPC.** After 48 hours incubation in N2B3 medium, 39.03% ± 0.26 SEM of OPC differentiated into mature oligodendrocytes and expressed GalC marker. Data show the mean value of six independent experiments.

**VI.3.a. CXCR2 chemokine receptor and its ligands**

Our data showed that the maturation rate (GalC+ / A2B5+) of OPC in control cultures of N2B3 was around 39.03 ± 0.26 SEM (Fig.17). However, addition of chemokines could significantly increase OPC differentiation at a specific concentration of 10 ng/ml of all chemokines used (Fig.18.A-C). Among the applied chemokines, the effect of CXCL1 was slightly prominent compared to CXCL5 and CXCL2 (Fig.18.A-C).
Fig.18: Differentiation assay on OPC: CXCR2. Differentiation of OPC was observed in the presence of the ligand of CXCR2 chemokine receptor in N2B3 medium. 10 ng/ml CXCL1 (A), CXCL2 (B), and CXCL5 (C) ligands of CXCR2 receptor could significantly increase the differentiation of OPC. Data are represented as mean value ± SEM of at least three independent experiments. Asterisks show significant results ($P<0.05$).
VI.3.b. CXCR4 and CCR3 chemokine receptors and their ligand

CXCL12 and CCL11 were also able to increase the differentiation index of oligodendrocyte progenitor cells at a concentration of 10 ng/ml (Fig.19.A and B).

**Fig.19: Differentiation assay on OPC: CXCR4 and CCR3.** Differentiation of OPC was observed in the presence of ligand of CXCR4 or CCR3 receptor in N2B3 medium. 10 ng/ml CXCL12 ligand of CXCR4 (A) and CCL11 ligand of CCR3 (B) could significantly increase the differentiation of OPC. Data show the mean value ± SEM of at least three independent experiments. Asterisks show significant results ($P<0.05$).
VI.3.c. CX3CR1 chemokine receptor and its ligand

CX3CL1 (10 ng/ml) could increase the OPC differentiation in N2B3 medium as well (Fig.20). However, applying 50ng/ml of this chemokine showed an inhibitory effect (20% inhibition of differentiation compared to the control).

**Fig.20: Differentiation assay on OPC: CX3CR1.** OPC differentiation was observed in the presence of CX3CL1 ligand of CX3CR1 receptor in N2B3 medium. 10 ng/ml CX3CL1 could significantly increase the differentiation of OPC. Data are represented as mean value ± SEM of at least three independent experiments. Asterisks show significant results ($P<0.05$).

VI.3.d. Inhibition of the effect of chemokines on OPC differentiation

G-protein antagonist (2.5 µg/ml) was applied to inhibit the modulatory effect of receptor-ligand interaction (Fig.21 and 22). In these inhibitory assays, progenitor cells were primarily pre-incubated for no less than one hour using the same concentration of antagonist, then experiments were carried on in the presence or absence of the chemokine. GPA could completely inhibit the modulatory effect of chemokines on differentiating OPC. However, using PTX (100 ng/ml) could not suppress the increased indices of differentiated cells in the presence of CXCR4 and CXCL2 (Fig.23.A & B).
Results

Fig. 21: Inhibition of differentiation assay on OPC. The increase in the differentiation indices of OPC in the presence of 10 ng/ml CXCL1 (A), CXCL2 (B), or CXCL5 (C) was completely blocked after applying 2.5 µg/ml GPA. Data show the mean value of at least three independent experiments. Asterisks show significance of the data ($P<0.05$).

VI.4. Expression of CX3CR1 chemokine receptor: in vitro

Expression of the chemokine receptor CX3CR1 was shown on immunoisolated OPC at the mRNA level (Stangel unpublished data - personal communication). In order to show the expression of CX3CR1 protein in these cells, the protein extract of the immunoisolated progenitor cells (Sprague Dawley rat, $P_1$) was subjected to western blot analysis. The process of OPC immunopanning will usually end up in having 99% pure cultured oligodendrocyte progenitor cells. The purity of these cultures were proven not only by morphological studies but by applying immunocytochemistry using specific marker for astrocytes (GFAP) and microglia (OX42), where no contaminating microglial cells were observed in immunisolated OPC culture. However, 0.1 - 0.5% GFAP positive astrocytes were present in these cultured cells. Applying 20 µg protein extract of the immunoisolated OPC showed the expression of the
Fig. 22: Inhibition of differentiation assay on OPC. The increase in the differentiation indices of OPC in the presence of 10 ng/ml CXCL12 (A), CCL11 (B), and CX3CL1 (C) was completely blocked after applying 2.5 µg/ml GPA. Data show the mean value of at least three independent experiments. Asterisks show significance of the data (P<0.05).

Fig. 23: Inhibition of differentiation assay on OPC. Pertussis toxin (100 ng/ml ) could not block the modulatory effect of CXCL12 (A) and CXCL2 (B). Data show the mean value of at least three independent experiments. Asterisks show significant results (P<0.05).
Results

CX3CR1 receptor (Fig.24, Line 1-3). The protein extract of microglial cells was applied as a positive control (Fig.24, Line 4). The CX3CR1 protein expression on OPC was shown in at least three different immunisolated OPC samples.

**Fig.24: Western blot (WB) analysis:** WB applied to evaluate the expression of CX3CR1 protein on immunisolated (A2B5 positive) OPC (O and line 1, 2 & 3). Microglial cell lysis used as a control (M and line 4).

**Fig.25: Immunocytochemistry on OPC.** OPC were cultured in B104 medium for 24 hours. Then ICC applied to show the expression of CX3CR1 (A) on A2B5 positive progenitor cells (B). Few A2B5 negative OPC also expressed CX3CR1 receptor (arrow-heads). C) shows DAPI nuclear staining (Objective x 40).
In addition, we showed the expression of CX3CR1 protein on isolated OPC using ICC as well. Progenitor cells were stained for A2B5 and CX3CR1 after 24 hours growing in B104 medium.

Fig. 26: Immunohistochemistry (IHC) on newborn rat brain slices. IHC applied on Sprague dawley rat (P1) to show the expression of CX3CR1 chemokine receptor on OPC. Immunohistochemistry: A) VZ, A2B5 (Cy3) C) SVZ, O4 (Cy3) E) VZ, CX3CR1 (Cy3) demonstrate staining on brain snap frozen sections (rat P1). G) CX3CR1 (Cy3) shows staining on spleen cryosections of Sprague Dawley rat (P1). B), D), F), and H) show DAPI nuclear staining. (Bar: 100µm)
Results

Data showed that A2B5 positive OPC express CX3CR1 (Fig.25.A-C). However, some A2B5 negative progenitor cells express this chemokine receptor as well (Fig.25.A – C, arrow-heads).

VI.5. Expression of CX3CR1 chemokine receptor: in vivo

Demonstrating the expression of CX3CR1 protein in purified OPC led us to investigate the possible pattern of expression of this chemokine receptor on oligodendrocytes in vivo. Snap frozen sections of newborn Sprague Dawley rat (P1) were subjected to IHC for A2B5, O4 and CX3CR1 staining to show the expression of this chemokine on precursor and/or mature oligodendrocytes in vivo. Data show the expression of A2B5 and O4 in new born rat brain SVZ and VZ (Fig.26.A - D). Single staining in serial sections shows that oligodendrocytes (A2B5 or O4 positive cells) do not express CX3CR1 in control barins (noninflammatory condition) (Fig.26.E & F). The spleen snap frozen sections of newborn Sprague Dawley rat were used as positive controls (Fig.26.G & H).
VII. Discussion

Myelination of axons is a crucial process in development and diseases of the nervous system. Sufficient numbers of OPC have to be recruited to the axons to be myelinated with subsequent differentiation into myelinating mature oligodendrocytes. Among many different signalling molecules, the chemokine receptor-ligand interaction has also been considered to play important modulatory effects on OPC functions. To date, the importance of CXCR2 (Nguyen and Stangel, 2001; Omari et al., 2006; Robinson et al., 1998; Tsai et al., 2002) and CXCR4 chemokine receptor (Dziembowska et al., 2005, Ma et al., 1998; Nagasawa et al., 1996; Zou et al., 1998) has been shown in CNS development and oligodendrocyte distribution. We investigated further characteristics and functional importance of these two receptors as well as CCR3 and CX3CR1 chemokine receptors on both rat precursor cells and an oligodendroglial cell line (CG4). Our in vitro data may provide a better understanding of chemokine-receptor effects on OPC responses during development and diseases of the CNS. Nevertheless, in vivo studies are required to accomplish these data.

VII.1. CXCR2 chemokine receptor and its ligands, CXCL1, CXCL2, and CXCL5

Previous studies showed that the CXCR2 chemokine receptor controls OPC positioning in the developing CNS (Tsai et al., 2002) and the overexpression of this chemokine receptor in transgenic animals causes a number of neurological symptoms and disruption of myelin (Tani et al., 1996). Moreover, the expression of this receptor on CNPase positive oligodendrocytes flanking MS plaques (Omari et al., 2005) suggests some importance of CXCR2 in demyelinating disease of the nervous system as well. In addition, the increase of CXCL1 expression by activated microglia at the border of MS lesions (Filipovic et al., 2003) and over expression of CXCL1 and CXCL2 by activated astrocytes in EAE animals (Luo et al., 2000), show the presence of CXCR2 ligands during inflammatory diseases of the CNS.

As described before, signalling of CXCR2 after interaction with CXCL1 could abolish the effect of the strong chemoattractant growth factor, PDGF, (Armstrong et al., 1990) on OPC in vitro (Tsai et al., 2002). This migrational arrest was rapid, concentration dependent, and reversible. Here, it is shown that all three ligands of the CXCR2 chemokine receptor, (CXCL1, CXCL2, and CXCL5) could inhibit OPC migration in conditioned medium, which was in accordance with the previously published data (Tsai et al., 2002). However, in N2B3 medium, CXCL1 and CXCL5 increased migration of OPC at 0.1 and 0.5 ng/ml respectively,
while CXCL2 showed an opposite effect. Despite minor differences observed in OPC migration (in N2B3), these three ligands showed yet again similar effects in modulation of OPC proliferation in vitro, where they had mainly inhibitory effects in conditioned medium, but showed a stimulatory effect on OPC proliferation in the absence of growth factors in N2B3 medium. Previous studies showed the stimulatory effect of CXCR2 ligands (CXCL2) on proliferation of immune cells (PMN) at very low (10 ng/ml) and very high concentrations (100 ng/ml) (Wolpe et al., 1989). Our observations also demonstrate a similar stimulatory pattern in all three ligands used, suggesting the importance of these modulatory molecules in proliferation of myelin producing cells at two different ranges of concentrations. The highest concentrations might be observed at the site of inflammation while the lowest concentrations could play a role in the absence of inflammatory stimuli (for instance during development). Interestingly, no changes were observed in OPC proliferation, when 10 - 25 ng/ml of these chemokines were applied, whereas 10 ng/ml of CXCL1, CXCL2, and CXCL5 could significantly stimulate OPC differentiation in N2B3 medium. Nevertheless, at 10 ng/ml of CXCL1 and CXCL5 no dramatic changes were observed in OPC migration. In addition, applying GPA could completely block the effect of CXCR2 receptor-ligand interaction (like previously published data; Tsai et al., 2002), which shows that these chemokines exert their effects via interacting with the 7-G-protein coupled receptor. Taking all together, data show that the secretion of CXCR2 ligands must be precisely tuned to have the optimal response in OPC proliferation and differentiation. In contrast to the precursor cells, CXCL1, CXCL2, and CXCL5 showed mainly opposite effects on modulation of CG4 cell biological activities. This disadvantage could limit the benefit of applying cell line in in vitro studies, and more importantly, the results of studies on cell lines should be interpreted cautiously.

**VII.2. CXCR4 chemokine receptor and its ligand, CXCL12**

A very recent study showed the functional expression of CXCR4 on OPC (Dziembowska et al., 2005; Maysami et al., 2006b). In addition, published data on knockout animals of CXCR4 or its ligand, CXCL12, revealed the importance of this chemokine receptor in organogenesis, development of the CNS (Ma et al., 1998; Nagasawa et al., 1996; Zou et al., 1998), and more specifically, in recruitment and distribution of OPC (Dziembowska et al., 2005). Moreover, increase in the expression of this chemokine receptor on glial cells (astrocytes and microglia) in EAE models (Jiang et al., 1998) or the increased expression of CXCL12 in the CSF and
Discussion

brain lesions of MS patients (Krumbholz et al., 2006) suggests the possible role of this chemokine and its receptor in demyelinating disease as well. Here we showed that CXCL12 inhibits migration of OPC at low concentration range (0.1 - 10 ng/ml). Although previous studies showed that CXCL12 is a potent chemoattractant for mouse oligodendrocytes (Dziembowska et al., 2005) as well as other resident cells in the CNS (Bleul et al., 1996; Hesselgesser et al., 1997), this chemoattractive effect was only observed when CXCL12 applied at high concentrations (20 - 1000 ng/ml). The differences observed between our results and the previously published data lies on the use of oligodendrocytes of different origin (rat instead of mouse) and using lower concentrations of this chemokine in our study (up to 10 ng/ml). CXCL12 can inhibit OPC proliferation in B104 medium as well. However, in N2B3 medium (in the absence of growth factors) this chemokine showed its stimulatory potential on proliferation of progenitor cells at a relatively high concentration (50 ng/ml), which is in accordance with the recently published data (Kadi et al., 2006). The stimulatory effect of CXCL12 on astrocyte proliferation in the absence of growthfactors has also been described in previous studies (Bajetto et al., 2001). In addition, increase in the differentiation index of OPC after applying 10 ng/ml of CXCL12 reveals the importance of this chemokine in maturation of OPC and production of myelin, which is again in accordance with the recently in print data (Kadi et al., 2006). Like CXCR2 ligands, the modulatory effects of CXCL12 on OPC migration and differentiation were completely blocked after applying GPA, which inhibits the activation of G-proteins subunits. Taken together, the secretion of CXCR4 ligand must be a finely tuned process to have the optimal response in myelin producing cells and this must be adjusted so that optimal response in OPC recruitment observes before oligodendrocytes go under arrest due to early maturation.

In contrast to the rat OPC, CXCL12 had contradictory effects on CG4 migration in B104 medium. This chemokine stimulated slightly CG4 cell migration in conditioned medium, but could not alter the migration index in the absence of growth factors. Nevertheless, CXCL12 could not significantly modulate CG4 cell proliferation in both culture media. This would again limit the advantage of using these cells in observational studies.

As mentioned above, the CXCR4 or CXCL12 knockout mutation is lethal. However, examining the susceptibility of CXCR4 and/or CXCL12 conditioned-knockout animals to develop EAE might reveal more information on the functional importance of this chemokine receptor and/or its ligand in animal models of demyelinating disease.
VII.3. CCR3 chemokine receptor and its ligand, CCL11

Although previously published data showed CCR3 expression on brain endothelial cells (Berger, 1999), glial (astrocytes and microglia) (Boutet et al., 2001a), and neurons (Zheng, 1999; Boutet et al., 2001a), the expression of CCR3 on isolated OPC is a novel report (Maysami et al., 2006a), which led us to investigate the characteristics of this receptor in myelin producing cells in vitro. Moreover, expression of CCR3 on microglia in demyelinating (Simpson et al., 2000) and degenerative disorders (Xia et al., 1998) may suggest a possible role for this chemokine receptor in different types of CNS diseases.

Previously published studies showed that CCL11 among several other ligands of CCR3 had a chemoattractive effect on astrocytes (Dorf, 2000; Baggiolini et al., 1997) and eosinophyl cells (Rothenberg et al., 1999). However, in our study, CCL11 was a chemorepellant for OPC, as a significant decrease was observed in OPC migration toward very low concentration gradients of this chemokine in B104 and N2B3 medium. The proliferation rate of OPC was also suppressed in the presence of low concentrations of this CC chemokine in B104 medium, whereas, (like other observed chemokines) CCL11 showed its potential in increasing OPC proliferation in the absence of growth factors in N2B3 medium at very low and very high concentrations. Although no significant changes were observed in OPC proliferation or migration in the presence of 10 ng/ml of this chemokine (in N2B3), differentiation was significantly upregulated at this concentration. Additionally, our data showed that the stimulatory effect of CCL11 on OPC differentiation was via activation of G-proteins.

CG4 cell migration was mainly in contrast to the progenitor cells in both medium and was particularly (concentration dependently) upregulated in the presence of CCL11 in B104 medium. Proliferation of these oligodendroglial cell line decreased in N2B3 culture medium, which is again in contrast to the result observed on OPC. Therefore, differential responses of CG4 cells compared to progenitors toward the CCL11 ligand would again limit the usage of this cell line.

To conclude, the functional expression of CCR3 and the modulatory effect of its ligand on OPC migration, proliferation and differentiation are described here for the first time (Maysami et al., 2006a). This may suggest a potential role of CCL11 in development and diseases of the nervous system. However, further in vivo studies are required to evaluate the recruitment and distribution of OPC or even myelogenesis in CCR3 and/or CCL11 transgenic animals. In addition, the susceptibility of these knockout or transgenic animals to demyelinating / degenerative diseases could be a new topic to take into consideration.
**VII.4. CX3CR1 chemokine receptor and its ligand, CX3CL1**

The functional expression of CX3CR1 has already been shown on microglial cells and astrocytes (Boddeke et al., 1999; Hulshof et al., 2003; Sunnemark et al., 2005; Tarozzo et al., 2002) as well as neurons (Meucci et al., 1998; Raport et al., 1995). Moreover, neurons (Harrison et al., 1998; Hatori et al., 2002; Hughes et al., 2002; Meucci et al., 1998) and astrocytes (Hatori et al., 2002; Hughes et al., 2002; Hulshof et al., 2003) express CX3CL1 as well. Although, to date, no study has evaluated the exact function of this chemokine and/or receptor during development of the nervous system, few studies showed the importance of these molecules in CNS disease. The upregulation of CX3CR1, observed in neuroinflammatory states in human (MS) (Hulshof et al., 2003) or rodents (EAE) (Sunnemark et al., 2005), and the increase in the expression of CX3CL1 in EAE lesions (Sunnemark et al., 2005), suggest a possible role for this receptor and its ligand in demyelinating diseases of the CNS.

Here we showed for the first time that OPC express a functional CX3CR1 chemokine receptor. Previously published data demonstrated that CX3CL1 increased leukocyte (Pan et al., 1997) and astrocyte (Maciejewski-Lenoir et al., 1999) migration. Our data also showed the stimulatory effect of this chemokine on OPC migration in N2B3 medium. However, inhibition of migration was observed when this chemokine was applied at low (<1 ng/ml) or high (>50 ng/ml) concentrations in conditioned medium. Moreover, unlike other chemokines used, after applying 10 - 25 ng/ml CX3CL1 in medium containing growth factors (FGF and PDGF) OPC migration increased significantly. Nevertheless, CX3CL1 could increase the differentiation of OPC at 10 ng/ml, but in a less effective manner (compared to other chemokines used). It is controversial from previous studies, whether CX3CL1 can (Hatori et al., 2002) or cannot (Maciejewski-Lenoir et al., 1999) induce proliferation of glial cells (microglia and astrocytes respectively). Here we observed the stimulatory effect of this chemokine on OPC proliferation in N2B3 medium. However, in conditioned medium, this chemokine inhibits the proliferation of OPC up to 40%.

The results of CG4 cells were not comparable to the precursor cells, where migration of these cells was only slightly changed in the presence of CX3CL1, and no statistically significant changes were observed in proliferation rate of CG4 cells in the presence of this chemokine.

We conclude that the results of the CG4 cell line with this chemokine and other applied chemokines must be interpreted cautiously. In addition, data suggest the potential stimulatory effect of CX3CL1 chemokine (like other chemokines used) in proliferation, and
differentiation of OPC in N2B3 medium. The OPC responses to this chemokine (as well as other CC and CXC ligands) observed in different assays, suggest that the timecourse of expression, secreted concentration, and the conditioned environment in which a chemokine is released must be finely tuned. Otherwise, optimal relocation, recruitment, and maturation of OPC will not be observed and consequently the impairment in myelogenesis and repair will occur. Furthermore, our data suggest that the modulations observed in migration, proliferation, and differentiation of oligodendrocytes are differentially regulated.
VIII. Future works

Here we showed that CC, CXC and CX3C chemokines have more or less similar modulatory effects on myelin producing cell functions. Differential responses observed on OPC proliferation, differentiation and migration suggest that these biological activities of precursor cells are differentially regulated possibly via activation or inactivation of different signalling pathways. Moreover, the upregulation of different signalling molecules after applying chemokines on neuronal or glial cells has already been investigated (Baggiolini, 1997 and 1998; Bajetto et al., 2001; Bajetto et al., 2002;; Maciejewski-Lenoir et al., 1999; Meucci et al., 1998; Shahabuddin et al., 2006; Xia et al., 2002). Therefore, further examinations on up- or down-regulation of signalling molecules, including activated Erk1/2, PI3K, and Akt are suggested, which could reveal how chemokines exert their modulatory effects on OPC functions at molecular level.

In this study, the functional expression of CCR3 and CX3CR1 was shown on rodent OPC for the first time. However, further in vivo investigations on knockout or transgenic animals of these chemokine receptors or their ligand reveal the importance of these molecules in CNS development, myelogenesis, distribution pattern and recruitment of OPC. These animals can also be subjected to inflammatory demyelinating diseases like EAE or Theiler's murine encephalomyelitis virus, to observe the susceptibility of them to develop a neuroinflammatory disease of the central nervous system.

In inhibition assay studies, the effect of chemokines was completely blocked after applying GPA, whereas PTX did not show such an effect. It is controversial between studies, whether PTX can (Heesen et al., 1996) or cannot (Tanabe et al., 1997; Wong et al., 2001) completely block modulatory effects of chemokines. Moreover, the effective PTX concentration and the method of application vary between different studies, where sometimes a very high concentration (µg/ml) of PTX and preincubation applications (Heesen et al., 1996) were needed to get an optimal blocking effect. Nevertheless, applying the specific receptor-antagonist of the chemokine receptor will surely demonstrate the specificity and selectivity of the receptor-ligand interaction to see how redundant these chemokines are.

In addition, our results suggest that CG4 cell line is not a good model to be used instead of progenitor cells for in vitro studies. Finally, further investigations on other oligodendrogial cells (derived from other species) are required for better interpretation of these data.
IX. Conclusion

The expression pattern of chemokines and their receptor on neuronal and glial cells suggests the functional importance of these modulatory molecules in glial/glial or neuron/glial interactions in health and disease. The finely controlled OPC responses toward different gradients of chemokines in different conditions (observed in our study as well as previously published data), suggest the requirement of the well-regulated dynamics during myelogenesis and repair.

Taken together, this study shows that the modulatory effects of applied chemokines (CC, CXC, and CX3C) were alike in many aspects, which implies a redundancy in chemokine system in the CNS. Moreover, the differences observed in the applied functional assays (proliferation, differentiation, and migration) suggest that OPC reactions toward chemokines are independently and differentially regulated.

Here we showed the functional importance of CCR3 and CX3CR1 on rodent OPC for the first time. However, additional in vivo investigations on knockout or transgenic animals of these chemokine receptors or their ligand reveal the exact characteristics of these molecules in development and diseases of the nervous system.

Moreover, it is also shown in here that chemokines exert their modulatory effects on OPC via interacting with the G-protein coupled receptors, while using a G-protein antagonist (which inhibits G-protein activation) could completely block the observed effects.

In addition, our data showed that the responses observed in rat cell line were mainly in contrast to the one observed in progenitor cells, which limit the usage of this cell line for in vivo studies.

Ultimately, results of these observations may help us in better understanding of developmental processes as well as progression of pathogenesis of various demyelinating diseases of the CNS including MS, and eventually, this may lead us to improve regenerative therapies.
X. Summary

The myelin-producing oligodendrocytes synthesize and maintain myelin sheaths in the CNS and many factors (including chemokines) are known to be involved in the regulation of their functions. To date, several studies showed that neuronal and glial cells (oligodendrocytes, astrocytes, and microglia) express different chemokine receptors in health and disease. This expression pattern suggests the possible functional importance of chemokines and their receptor(s) in neuronal/glial signalling, which may influence the induction and progression of oligodendrocyte recruitment, differentiation and thereafter myelination of axons during development or progression of CNS disease.

Previous studies demonstrated the expression of chemokine receptors CXCR4, CCR3 and CX3CR1 as well as CXCR2 on rat oligodendrocyte progenitor cells. However, we aimed to characterize the functional characteristics of these receptors on myelin producing cells (CG4 oligodendrocyte cell line and primary progenitor cells (OPC) of the CNS. Therefore, oligodendrocytes were subjected to migration (Boyden chemotaxis chamber), proliferation (BrdU incorporation ELISA or ICC), and differentiation (ICC) assays in the presence of different (CC, CXC, CX3C) chemokines.

Data showed that not only all three ligands (CXCL1, CXCL2, and CXCL5) of CXCR2 chemokine receptor had inhibitory effects on migration of OPC in conditioned medium containing growth factors, but CXCL12 (ligand of CXCR4), CCL11 (ligand of CCR3), and CX3CL1 (ligand of CX3CR1) showed almost similar inhibitory effects. This effect of chemokines was mainly observed at very low concentrations (<1 ng/ml). However, CX3CL1 (in contrast to other chemokines) could stimulate OPC migration in the presence of growth factors, when an intermediate concentration (10 ng/ml) of this chemokine was applied. All the applied chemokines could inhibit proliferation of OPC in conditioned medium (B104 medium) in a wide range of concentrations (0.1 - 100 ng/ml), while they showed their potential in stimulating OPC proliferation in the absence of growth factors. In addition, the differentiation of OPC was augmented in the presence of all CC, CXC, CX3C chemokines used (10 ng/ml), although the intensity varied between them.

Our data showed that chemokines exert their effect via activating the G-protein coupled receptors, while the G-protein antagonist could completely block the modulatory effects of these chemokines in OPC migration and differentiation. Ultimately, in contrast to progenitor cells, the results observed in migration and proliferation of rat oligodendroglial cell line were either completely opposite compared to the one obtained with progenitor cells or no
significant changes were observed, which urge us to interpret the obtained results from observations on cell lines more cautiously.

These data show that the production and secretion of chemkines must be finely tuned, so the optimal response in OPC proliferation or differentiation could be achieved, while a slight change in concentration can lead to premature differentiation of myelin producing cells and impairment of repair.
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…. Mache du nach Kräften kund, was Art du bist, wie auch, welches deine Fertigkeiten sind, welches dein Benehmen ist. Halte nicht das für das Beste, was man dir ins Ohr flüstert; auf das höre vielmehr, was einer offen redet!

…. halte nicht das für vortrefflich, was der Mächtige tut; was der Schwache leistet, das beachte vielmehr!

Darieos, d. Gr. (522-486 v. Chr)

Erklärung

(Die Erklärung muss unterschrieben und als vorletztes Blatt in die Gutachterversion der These eingebunden werden).

Hiermit erkläre ich, dass ich die Dissertation (Angabe des genauen Titels) selbständig verfasst habe. Bei der Anfertigung wurden folgende Hilfen Dritter in Anspruch genommen:

Functional characteristics of chemokine-receptor interactions in oligodendrocytes.


Ich habe die Dissertation an folgenden Institutionen angefertigt:

Department of Neurology, Medical University of Hannover and
Center for Systems Neuroscience, University of Veterinary Medicine Hannover

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

(Ist die Dissertation nicht an der Titelseite angefertigt worden, muss eine Erklärung des Leiters der Einrichtung beigefügt werden, dass er/sie mit der Einreichung der Arbeit als PhD-Arbeit an der Tierärztlichen Hochschule Hannover einverstanden ist.)

Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Unterschrift