CXCR7 is an active component of SDF-1 signalling in astrocytes and Schwann cells

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Summary

The alternative SDF-1 (stromal cell derived factor-1) receptor, CXCR7, has been suggested to act as either a scavenger of extracellular SDF-1 or a modulator of the primary SDF-1 receptor, CXCR4. CXCR7, however, also directly affects the function of various tumor-cell types. Here, we demonstrate that CXCR7 is an active component of SDF-1 signalling in astrocytes and Schwann cells. Cultured cortical astrocytes and peripheral nerve Schwann cells exhibit comparable total and cell-surface levels of expression of both SDF-1 receptors. Stimulation of astrocytes with SDF-1 resulted in the temporary activation of Erk1/2, Akt and PKCζ/λ, but not p38 and PKCα/β. Schwann cells showed SDF-1-induced activation of Erk1/2, Akt and p38, but not PKCα/β and PKCζ/λ. The respective signalling pattern remained fully inducible in astrocytes from CXCR4-deficient mice, but was abrogated following depletion of astrocytic CXCR7 by RNAi. In Schwann cells, RNAi-mediated depletion of either CXCR4 or CXCR7 silenced SDF-1 signalling. The findings of the astrocytic receptor-depletion experiments were reproduced by CXCR7 antagonist CCX754, but not by CXCR4 antagonist AMD3100, both of which abolished astrocytic SDF-1 signalling. Further underlining the functional importance of CXCR7 signalling in glial cells, we show that the mitogenic effects of SDF-1 on both glial cell types are impaired upon depleting CXCR7.

Key words: Chemokines, Astrocytes, Schwann cells, Cell proliferation, Erk, p38, PKC, Akt

Introduction

The CXC chemokine SDF-1 (stromal cell derived factor-1; also known as CXCL12) was originally isolated as a pre-B-cell growth-stimulating factor and was subsequently shown to regulate trafficking, transendothelial migration, proliferation and differentiation of hematopoietic cells (Broxmeyer, 2008). Outside the hematopoietic system, SDF-1 is essential for cardiogenesis, vascularization of the gastrointestinal tract, limb myogenesis, and morphogenesis of the central and peripheral nervous systems (CNS and PNS) (Ma et al., 1998; Zou et al., 1998; Tachibana et al., 1998; Li and Ransohoff, 2008; Ödemis et al., 2005). In the adult nervous system, expression of SDF-1 and its receptor, CXCR4, persists in astrocytes, microglial cells, Schwann cells, distinct neuronal populations and endothelial cells (Li and Ransohoff, 2008), modulating neurotransmitter release from neurons and glia as well as hormone secretion from neuroendocrine cells (Bezzi et al., 2001; Barbieri et al., 2007; Lazarini et al., 2003; Rostène et al., 2007). SDF-1 and CXCR4 are additionally involved in several brain pathologies, such as acute ischemic and traumatic brain injuries, chronic brain infections and autoimmune diseases (Cartier et al., 2005; Li and Ransohoff, 2008). Furthermore, CXCR4 binds the HIV-1 envelope protein gp120 and hence serves in virus co-entry and as a mediator of HIV-associated dementia (Kaul and Lipton, 2006). In addition, CXCR4 controls growth, migration and invasion of various tumours, including glioma and astrocytoma (Balkwill, 2004).

The long-held exclusivity of the SDF-1–CXCR4 interaction only recently ended with the identification of the previously orphan G protein CXCR7 (also known as RDC-1) as an alternative SDF-1 receptor (Balabanian et al., 2005). CXCR7 is expressed in the haematopoetic system, heart, vascular endothelial cells, bone, kidney and brain (Sierro et al., 2007; Schönemeier et al., 2008a; Gerrits et al., 2008; Infantino et al., 2006; Balabanian et al., 2005). CXCR7 exhibits a distinctly higher binding affinity for SDF-1 than CXCR4 (Balabanian et al., 2005; Crump et al., 1997) and has a second ligand in the form of interferon-inducible T cell α chemoattractant (I-TAC, also known as CXCL11) (Burns et al., 2006). In contrast to CXCR4, CXCR7 is a non-classical G-protein-coupled receptor (GPCR) that apparently fails to activate bound G proteins (Levoye et al., 2009), but instead can signal through β-arrestin-2 (Zabel et al., 2009; Kalatskaya et al., 2009). Studies on different (cell) systems have so far unravelled a broad range of CXCR7 functions. During development, CXCR7 seems to act as a SDF-1 scavenger, which directs CXCR4-induced migration of primordial cells by shaping the extracellular SDF-1 gradient (Boldajipour et al., 2008; Dambly-Chaudiere et al., 2007). CXCR7 further modulates CXCR4-dependent cell signalling by heterodimerizing with CXCR4 (Sierro et al., 2007; Levoye et al., 2009). Active CXCR7-dependent cell signalling seems to occur at least in tumor cells and is involved in the control of tumor cell growth, adhesion and invasion, but not tumor cell migration (Wang et al., 2008; Meijer et al., 2008; Burns et al., 2006; Miao et al., 2007; Jin et al., 2009; Yoshida et al., 2009).

Stimulated by this rather puzzling picture of the function of CXCR7, we now sought to elucidate the role of CXCR7 and CXCR4 in SDF-1 signalling in astrocytes and Schwann cells. We report that CXCR7 is an active SDF-1 signalling component in astrocytes. We further unravel that CXCR7, together with CXCR4, constitutes the active signalling unit in Schwann cells.

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Results

Analysis of CXCR4 and CXCR7 expression in primary astrocytes and Schwann cells

In accordance with previous reports (Tanabe et al., 1997; Ödemis et al., 2002; Küry et al., 2003), expression of the SDF-1 receptor CXCR4 was readily detectable by western blotting in cultures of rat cortical astrocytes and rat Schwann cells (Fig. 1A). Further corroborating recent findings by Schönemeier et al. (Schönemeier et al., 2008a), mRNA encoding CXCR7 could be amplified from rat astrocytes by real-time reverse transcriptase (RT)-PCR analysis (Fig. 1B). Likewise, CXCR7 transcripts were detectable in Schwann cells (Fig. 1B). Western blot analysis further demonstrated the presence of a CXCR7-immunoreactive protein band with a molecular weight of about 50 kDa in astrocytes and Schwann cells (Fig. 1C,D). The 50 kDa immunoreactive protein band was clearly reduced in astrocytes transfected with specific CXCR7 small interfering (si)RNA (Fig. 1C), hence confirming that the antibody specifically recognized CXCR7. Previous studies using fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) provided evidence that CXCR4 and CXCR7 exist not only as preformed homodimers, but also as preformed heterodimers (Wang et al., 2006; Sierro et al., 2007; Luker et al., 2009). To additionally determine whether CXCR4 and CXCR7 form heterodimers in the different glial cell types, we initially examined astrocytes for CXCR4 and CXCR7 coexpression using flow cytometric analysis. We found that 46% of all astrocytes in culture coexpress CXCR4 and CXCR7 (Fig. 1E,F). About 9% and 17% of the cultured astrocytes only expressed CXCR4 or CXCR7, respectively, whereas roughly one fourth of the cells expressed neither CXCR4 nor CXCR7. In a subsequent series of experiments, we then immunoprecipitated CXCR4 from astrocytes and Schwann cells, and analyzed the isolated protein for co-precipitated CXCR7 using western blotting. For control purposes, immunoprecipitation experiments were performed with astrocytes cultured from a CXCR4-deficient (CXCR4–/–) mouse line and age-matched wild-type (CXCR4+/+) E18 littermates. In both Schwann cells and CXCR4+/+ astrocytes, we detected large amounts of co-precipitated CXCR7, which remained undetectable in CXCR4–/– astrocytes (Fig. 1E). These findings indicate that CXCR4 and CXCR7 exist as heterodimers in glial cells.

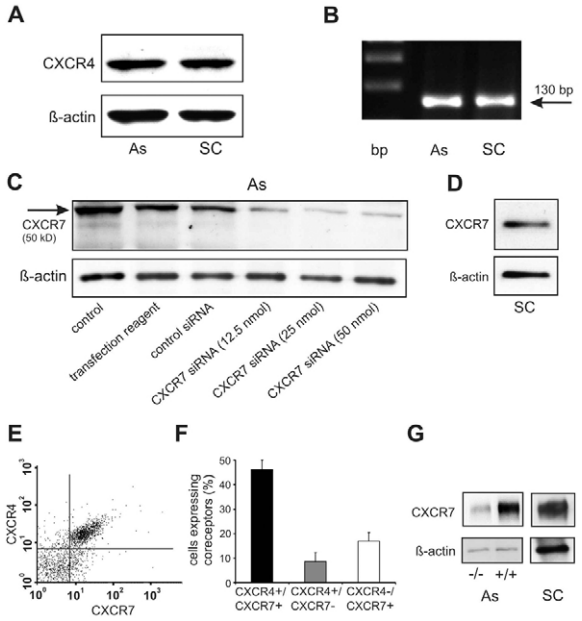

Fig. 1. Analysis of CXCR4 and CXCR7 expression in cortical astrocytes and Schwann cells. (**A**) Western blot analysis demonstrating that astrocytes and Schwann cells contain similar amounts of CXCR4 protein. β-actin served as a loading control. (**B**) RT-PCR allowed the amplification of mRNA encoding CXCR4 in astrocytes and Schwann cells. (**C**) Western blot analysis demonstrating the presence of a CXCR7 immunoreactive protein band in astrocytes, which decreased 48 hours after transfection of the cells with the indicated concentrations of selective CXCR7 siRNA. (**D**) The CXCR7 protein level in Schwann cells as determined by western blotting. (**E**) Representative flow cytometry plot demonstrating CXCR4 and CXCR7 coexpression in astrocytes. Astrocytes were double labelled with CXCR4 and CXCR7 antibodies, and analyzed by gating on forward and side scatter. (**F**) Flow cytometry data for astrocytic CXCR4 and CXCR7 expression compiled from three experiments. (**G**) Evidence for the existence of preformed CXCR4-CXCR7 heterodimers in primary glial cells. CXCR4 was immunoprecipitated from astrocytes derived from either E18 CXCR4 knockout animals (–/–) or age-matched wild-type littermates (+/+) as well as rat Schwann cells, and analyzed for coprecipitated CXCR7 by western blotting. As, astrocyte; bp, base pairs; SC, Schwann cell.

Because recent work demonstrated that CXCR7 is rarely expressed at the cell surface of T lymphocytes and $CD34^+$ progenitors, and is predominantly localized underneath the plasma membrane of these cells (Hartmann et al., 2008), we additionally stained cultured glial cells with CXCR7 antibodies and subjected them to flow cytometric analysis. We found that 72±8% and 66±5% of astrocytes and Schwann cells, respectively, express CXCR7 at their cell surface (Fig. 2A,C). Confirming the specificity of the staining, we further found that CXCR7 cell-surface expression decreased in astrocytes following transfection with CXCR7 siRNA (Fig. 2B). Collectively, these findings establish that astrocytes and Schwann cells express CXCR4 and CXCR7 at their cell surface. In addition, our findings reveal that, at least in part, CXCR4 and CXCR7 form heterodimers in glial cells.

Analysis of SDF-1-induced cell signalling in astrocytes and Schwann cells

To analyze SDF-1-induced signalling in the different glial cell types, we focused on p38, extracellular-signal-regulated kinases 1 and 2 (Erk1/2), Akt, the conventional protein kinase C (PKC) isoforms α and β (PKCα/β), and the atypical PKC isoforms ζ and λ (PKCζ/λ) – signalling molecules and pathways that are activated by SDF-1 (Wang et al., 2000; Bajetto et al., 2001; Ödemis et al., 2007; Li and Ransohoff, 2008). Stimulation of cultured astrocytes with SDF-1 (1-100 ng/ml; 10 minutes) resulted in the dose-dependent activation (phosphorylation) of Erk1/2, Akt and PKCζ/λ, but not p38 and PKCα/β (Fig. 3). Activation of Erk1/2 and Akt occurred with 1 ng/ml SDF-1; activation of PKCζ/λ required SDF-1 at 10 ng/ml (Fig. 3).

In Schwann cells, SDF-1 resulted in activation of p38, Erk1/2 and Akt, but not the conventional and atypical PKC isoforms (Fig. 4). Depending on the signalling molecule, activation required SDF-1 at 1 ng/ml to 10 ng/ml (Fig. 4). Analysis of the time-course of activation further revealed that, in astrocytes and Schwann cells, the respective signalling molecules are activated within 2-5 minutes of addition of SDF-1 and remained activated for up to 30 minutes (supplementary material Fig. S1). Together, these findings establish distinct differences in SDF-1 signalling between astrocytes and Schwann cells.

Contribution of CXCR4 and CXCR7 to SDF-1 signalling in astrocytes

To analyze the contribution of CXCR4 and CXCR7 to SDF-1-dependent signalling in astrocytes, we employed CXCR4 antagonist

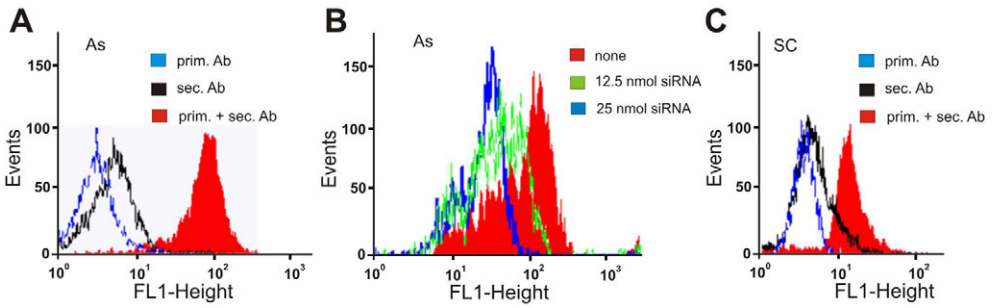

Fig. 2. Determination of CXCR7 cell-surface expression in glial cells by flow cytometry. Flow cytometry was performed on (**A**,**B**) cortical astrocytes (As) and (**C**) Schwann cells (SC) stained with CXCR7 antibodies in combination with secondary FITC-labelled antibodies (red) or with either the primary (blue) or secondary (black) antibodies alone. (B) CXCR7 cell-surface expression decreased in astrocytes 48 hours after transfection with the indicated concentrations of selective CXCR7 siRNA.

AMD3100 (1 μM) (Hatse et al., 2002) and CXCR7 antagonist CCX754, which selectively inhibits binding of SDF-1 to CXCR7 without affecting binding of SDF-1 to CXCR4 (Burns et al., 2006; Luker et al., 2009). CCX754 was applied at a concentration of 2 μM, which was previously shown to maximally displace SDF-1 binding from CXCR7 (Burns et al., 2006). Blocking astrocytic CXCR4 with AMD3100 completely abolished the SDF-1-induced activation of Erk, Akt and PKCζ/λ (Fig. 5). Moreover, SDF-1-induced signalling in astrocytes was likewise abrogated following blockage of CXCR7 with CCX754 (Fig. 5).

To verify our findings obtained with receptor antagonists, we studied SDF-1 signalling in astrocytes derived from CXCR4-null mice. Since homozygous CXCR4–/– mice die before birth (Zou et al., 1998), astrocytes were obtained from E18 animals. Fluorescence-activated cell sorting (FACS) analysis revealed that cell-surface expression levels of CXCR7 in CXCR4–/– astrocytes are similar to those of wild-type astrocytes (supplementary material Fig. S2). As with astrocytic cultures of the early postnatal rat cortex, treatment of astrocytes cultured from the cortex of prenatal wild-type mice with SDF-1 resulted in dose-dependent activation of Erk1/2 and Akt. However, in contrast to postnatal rat astrocytes, SDF-1 failed to activate PKCζ/λ in prenatal mouse astrocytes (Fig. 6A). The Erk1/2 and Akt responses were fully reproduced by astrocytes cultured from the cortex of age-matched homozygous CXCR4–/– mice (Fig. 6B). The signalling responses remained detectable in astrocytes in which CXCR4 expression was inhibited by RNAi (data not shown). CXCR4 depletion further remained without obvious effects on the time course of SDF-1-induced activation of Erk1/2 and Akt (supplementary material Fig. S3). The SDF-1-induced signalling response in CXCR4–/– astrocytes was, however, abrogated by CCX754 (supplementary material Fig. S4).

In a complementary series of experiments, we then determined SDF-1 signalling in astrocytes with depleted CXCR7 expression. Because CXCR7-null mice have not been at our disposal, we inhibited the expression of CXCR7 in cultured rat astrocytes by RNAi (see Fig. 1D). Treatment of CXCR7-depleted astrocytes with SDF-1 revealed attenuated activation of Erk, Akt and PKCζ/λ compared to controls (Fig. 7). Collectively, these findings identify CXCR7 as an active component of SDF-1 signalling in astrocytes. In addition, our findings point to the limited suitability of SDF-1 receptor antagonists for dissecting CXCR4- and CXCR7-dependent SDF-1 signalling.

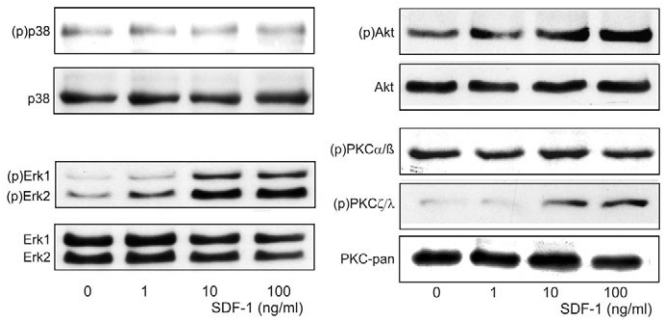

Fig. 3. SDF-1-dependent cell signalling in cortical astrocytes. Cultures of rat cortical astrocytes were stimulated for 15 minutes with the indicated concentrations of SDF-1. Levels of activated (phosphorylated) signalling proteins were subsequently determined by western blotting using phospho-specific (p) antibodies. As loading control, blots were additionally stained with antibodies recognizing the respective signalling molecule independent of its phosphorylation status. In the case of PKC, an antibody that does not distinguish the various protein isoforms (PKC-pan) served as loading control. SDF-1 resulted in dose-dependent activation of Erk, Akt and PKCζ/λ, but not of p38 and PKCα/β. One representative blot out of 3-5 independent experiments is shown.

SDF-1 signalling in CXCR4- and CXCR7-depleted Schwann cells

The contribution of CXCR4 and CXCR7 to SDF-1 signalling in Schwann cells was assessed by RNAi. Depletion of either CXCR4 or CXCR7 completely abrogated SDF-1-induced activation of p38,

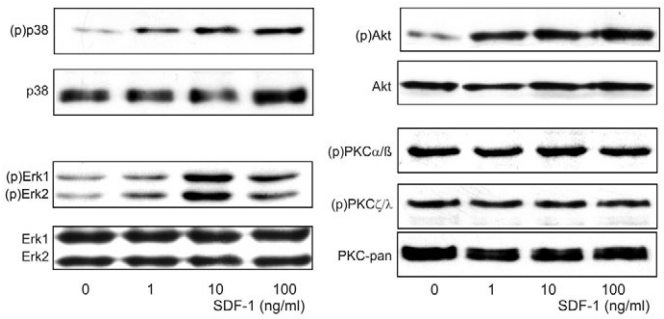

Fig. 4. SDF-1-dependent cell signalling in Schwann cells. Cultured Schwann cells were stimulated with the indicated concentrations of SDF-1 for 15 minutes and analyzed for activated signalling molecules as described in Fig. 3. In contrast with the findings from astrocytes, SDF-1 activates p38, Erk1/2 and Akt, but not PKCα/β and PKCζ/λ in Schwann cells. Experiments were replicated three times with similar results.

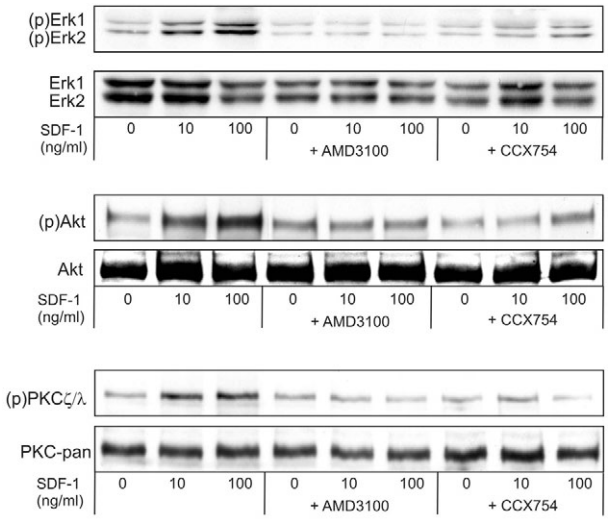

Fig. 5. Effects of selective CXCR4 and CXCR7 receptor antagonists on SDF-1-induced signalling in cortical astrocytes. Rat cortical astrocytes were stimulated for 15 minutes with varying concentrations of SDF-1 in the additional presence of either CXCR4 antagonist AMD3100 or CXCR7 antagonist CCX754. SDF-1-induced cell signalling was analyzed by western blotting as described in Fig. 3. SDF-1-dependent activation of Erk1/2, Akt and PKCζ/λ was likewise abolished by AMD3100 and CCX754. Results were reproduced in three independent experiments.

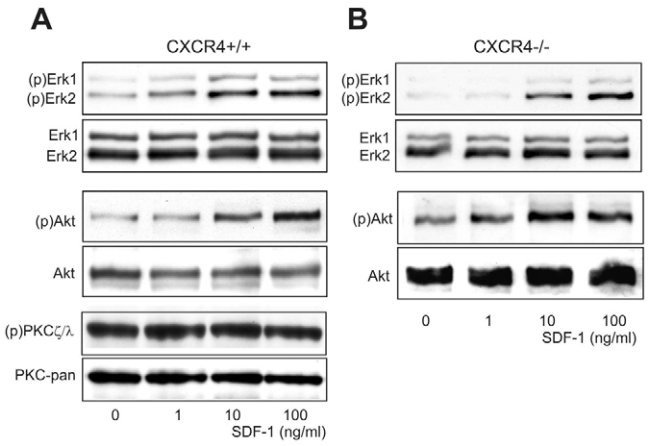

Fig. 6. CXCR4 deficiency does not impair SDF-1 signalling in astrocytes. Astrocytic cultures were established from the cortex of (A) E18 wild-type (CXCR4+/+) mice or (B) age-matched CXCR4 knockout (CXCR4–/–) mice. Activation of signalling molecules was determined by western blotting as described in Fig. 3, following stimulation with the indicated concentrations of SDF-1 for 15 minutes. In contrast with the findings from postnatal rat astrocytes, SDF-1 activated Erk1/2 and Akt, but not PKCζ/λ in astrocytes derived from E18 wild-type mice. CXCR4–/– astrocytes still responded to SDF-1 with activation of Erk1/2 and Akt. Findings were reproduced in three independent experiments.

Erk1/2 and Akt (Fig. 8). These findings imply that, in contrast to astrocytes, SDF-1 signalling in Schwann cells depends on the cooperation of CXCR7 and CXCR4.

Consequences of CXCR4 and CXCR7 deficiency for SDF-1-induced glial proliferation

In accordance with the recent demonstration of a mitogenic effect of SDF-1 on astrocytes (Bajetto et al., 2001), treatment of astrocytic cultures of E18 wild-type mice with SDF-1 (48 hours) resulted in a dose-dependent, maximum 2.5-fold increase in the number of cells expressing the proliferation marker Ki67 (Fig. 9A). This increase in the number of Ki67-expressing cells basically persisted in astrocytic cultures of age-matched CXCR4–/– littermates. However, CXCR4–/– astrocytes and wild-type astrocytes completely failed to respond to SDF-1 by means of cell proliferation in the additional presence of the CXCR7 antagonist CCX754 (Fig. 9A,C).

Treatment of cultured Schwann cells with SDF-1 at 10 or 100 ng/ml for 48 hours resulted in a similar 1.5-fold increase in the number of Ki67-expressing cells. Following depletion of CXCR4 by RNAi, SDF-1 at 10 ng/ml remained without effecting the number of Ki67-expressing cells, whereas Ki67 cell numbers increased with SDF-1 at 100 ng/ml (Fig. 9B). Independent of the applied concentration, SDF-1 failed to induce Ki67 expression in Schwann cells with RNAi-mediated depletion of CXCR7 (Fig. 9B).

Together, these findings point to a pivotal role for CXCR7 in the SDF-1-dependent proliferation of astrocytes. In addition, our findings suggest that CXCR4 and CXCR7 cooperatively control proliferation of Schwann cells.

Discussion

Previous work on the cellular function of the alternative SDF-1 receptor CXCR7 (RDC-1) has to date produced a rather puzzling picture. Some studies provided evidence that CXCR7 represents a silent receptor, which is responsible for either sequestering extracellular SDF-1 (Mahabaleshwar et al., 2008; Tiveron and Cremer, 2008) or modulating CXCR4 signalling by forming CXCR7-CXCR4 heterodimers (Sierro et al., 2007; Levoye et al., 2009). In contrast, other studies using various tumor cells demonstrated that CXCR7 can actively control cell growth and survival, as well as cell adhesion and transendothelial migration (Wang et al., 2008; Meijer et al., 2008; Burns et al., 2006; Miao et al., 2007; Balabanian et al., 2005; Zabel et al., 2009). We now identify CXCR7 as a key SDF-1 signalling component in astrocytes and Schwann cells.

Confirming and extending previous studies (Tanabe et al., 1997; Ödemis et al., 2002; Schönemeier et al., 2008a; Küry et al., 2003), we demonstrate that cultured astrocytes and Schwann cells express comparable levels of CXCR4 and CXCR7. Moreover, immunoprecipitation studies allowed us to demonstrate that, in both glial cell types, CXCR4 and CXCR7 are (partially) present as preformed heterodimers (Wang et al., 2006; Sierro et al., 2007; Luker et al., 2009). We further show that, despite the expression of both SDF-1 receptors, the chemokine activates distinct, nevertheless partially overlapping, arrays of signalling molecules in both glial cell types. This included Erk, Akt and PKCζ/λ, but not p38 and PKCα/β, in astrocytes, whereas Schwann cells showed SDF-1-dependent activation of p38, Erk and Akt, but not of PKCα/β and PKCζ/λ. These findings favour the assumption that differences in the signalling response to SDF-1 do not necessarily relate to the absence or presence of CXCR4 or CXCR7, but rather are generated downstream of these receptors, for example, by cell-type-specific expression and regulation of G proteins or activation of G-protein-independent signalling (Wettschureck and Offermanns, 2005; Sun et al., 2007; Xie and Palmer, 2007). Another observed difference

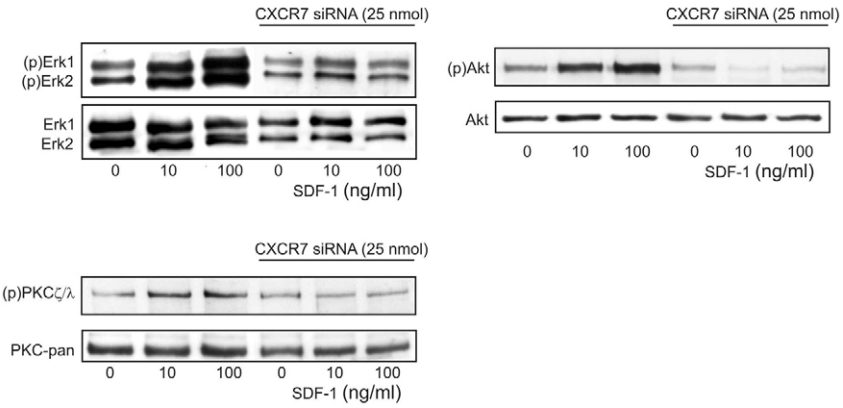

Fig. 7. Depletion of CXCR7 abrogates SDF-1 signalling in astrocytes. Rat cortical astrocytes were transfected with selective CXCR7 siRNA as described in Fig. 1. On day 2 post-transfection, cells were stimulated with the indicated concentrations of SDF-1 for 15 minutes and analyzed for activated signalling molecules by western blotting. Depletion of CXCR7 completely prevented SDF-1-induced activation of Erk1/2, Akt and PKCζ/λ. Experiments were replicated three times with similar results.

concerned SDF-1-induced cell signalling in cultured postnatal rat astrocytes and cultured late embryonic mouse astrocytes; the latter showed activation of only Erk and Akt, but not of PKCα/β and PKCζ/λ. Whether these differences relate to species and/or age remains to be established.

A subsequent analysis in which we attempted to dissect the contribution of CXCR4 and CXCR7 to SDF-1-induced cell signalling using CXCR4- or CXCR7-depleted glial cells led to the somewhat unexpected finding that, in astrocytes, all analyzed signalling events are entirely mediated by CXCR7. In fact, astrocytes cultured from CXCR4-deficient mice exhibited a signalling response to SDF-1 that was indistinguishable from that of astrocytes derived from age-matched wild-type littermates. However, astrocytic SDF-1 signalling was completely impaired upon depleting CXCR7 by RNAi. Because our studies only focused on a subset of signalling molecules and pathways activated by SDF-1 (Li and Ransohoff, 2008), the observed primary role of CXCR7 in these signalling events does not necessarily imply that CXCR4 would be completely silent in astrocytes. Because of the apparent failure of CXCR7 to induce Ca^{2+} mobilization (Burns et al., 2006; Sierro et al., 2007; Proost et al., 2007), CXCR4 could well mediate the increases in intracellular Ca^{2+} levels previously seen in primary astrocytes following exposure to SDF-1 (Tanabe et al., 1997; Bajetto et al., 1999). Our analysis further revealed that, in contrast to astrocytes, CXCR4 and CXCR7 are likewise required for SDF-1 signalling in Schwann cells. In this glial cell type, depletion of either CXCR4 or CXCR7 completely prevented SDF-1-induced activation of p38, Erk and Akt. These observations imply that, in Schwann cells, CXCR7 does not simply act as a modulator of CXCR4 signalling, as previously demonstrated for other cell types (Sierro et al., 2007), but instead represents an indispensable component of a CXCR7-CXCR4 signalling unit.

Interestingly, our findings on the function of CXCR4 and CXCR7 in astrocytes, as revealed by receptor depletion, were only partially reproduced by a pharmacological approach using established CXCR4 and CXCR7 receptor antagonists. Specifically, we found that astrocytic SDF-1 signalling is prevented not only by the selective CXCR7 antagonist CCX754 (Burns et al., 2006; Hartmann et al., 2008; Luker et al., 2009), but also by the selective CXCR4 antagonist AMD3100 (Khan et al., 2007). Because, as already mentioned above, CXCR4 and CXCR7 exist as preformed heterodimers in primary glial cells, a putative explanation for the false positive results obtained with AMD3100 could be that this bicyclam co-silences CXCR7 when bound to CXCR4. In this respect, it is also of note that the AMD3100 concentration used in our studies (1 μM) is distinctly lower than that recently shown to enable binding to CXCR7 homodimers (Kalatskaya et al., 2009). More generally, these observations emphasize that, unless characterized in more detail, the available receptor antagonists are of limited use for the reliable dissection of CXCR4- and CXCR7-induced cell signalling.

In the developing and injured brain, astrocytes show the rare expression of CXCR4, whereas throughout the brain a much larger subset of astrocytes seem to express CXCR7 (Stumm et al., 2002; Tissir et al., 2004; Schönemeier et al., 2008a; Schönemeier et al., 2008b). It is consequently feasible to assume that the predominant role of CXCR7 in astrocytic SDF-1 signalling, as established by our present in vitro studies, could well extend to the in vivo situation. This conclusion is also in line with our demonstration that SDF-1-dependent control of glial growth – a presumed function of SDF-1 in the developing and injured brain (Bonavia et al., 2003) – is entirely mediated by CXCR7. The contribution of CXCR7 to the recognized 'adult' functions of SDF-1 in astrocytes, such as

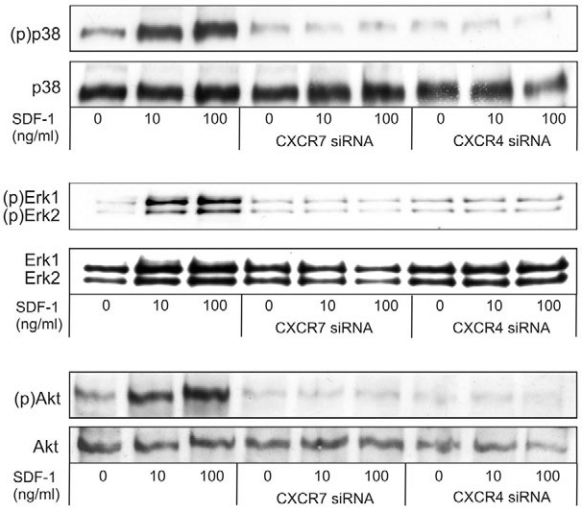

Fig. 8. Depletion of either CXCR4 or CXCR7 silences SDF-1 signalling in Schwann cells. Cultured Schwann cells were stimulated with SDF-1 (15 minutes) 48 h post-transfection, with either selective CXCR4 siRNA or selective CXCR7 siRNA. Subsequent western blotting demonstrated that SDF-1-induced activation of p38, Erk1/2 and Akt is abrogated following depletion of either CXCR7 or CXCR4. Findings were reproduced in three independent experiments.

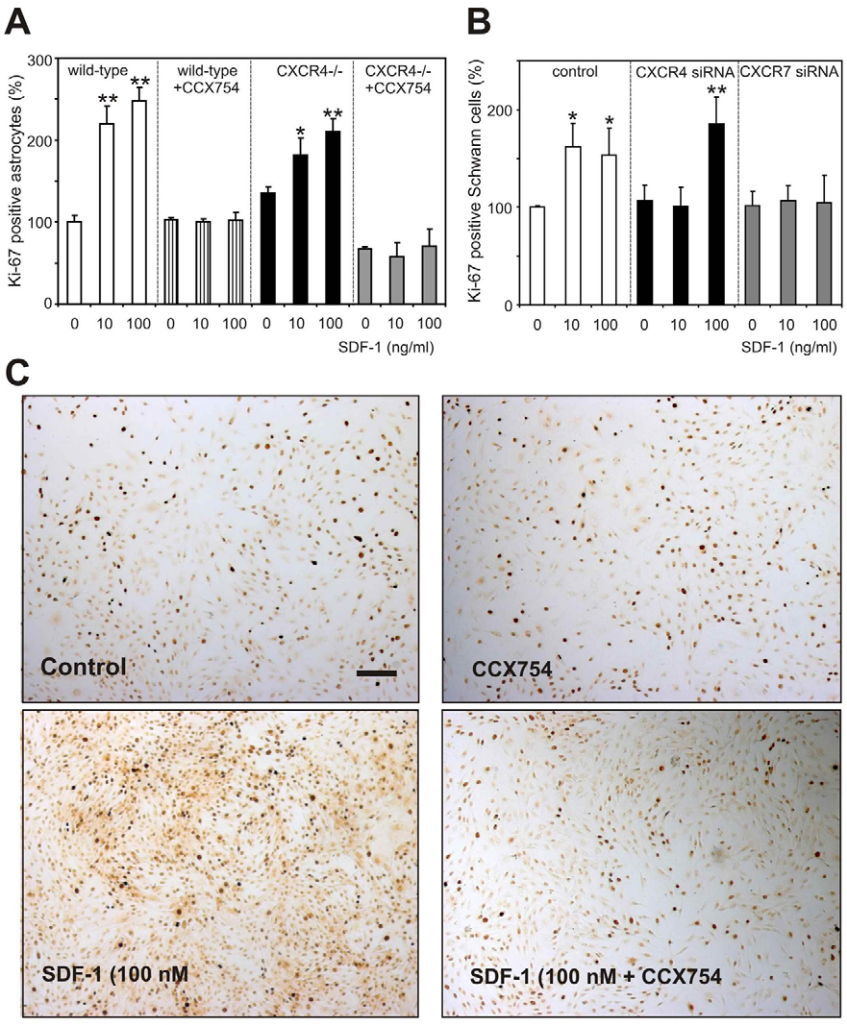

Fig. 9. Contribution of CXCR4 and CXCR7 to the mitogenic effects of SDF-1 on primary glia. (**A**) Astrocytes were cultured from E18 CXCR4–/– mice or age-matched littermates and maintained for 48 hours with the indicated concentrations of SDF-1 in the absence or presence of CXCR7 antagonist CCX754 (2 μM). Cells were subsequently stained with antibodies against the proliferation marker Ki67 and quantified by flow cytometry. The number of Ki67-positive cells present in controls was set at 100%. In both CXCR4+/+ and CXCR4–/– astrocytes, SDF-1 resulted in a statistically significant (n=3; *P<0.05, **P<0.001; SDF-1 treatment versus control; Tukey's test) increase in Ki67 expression. This increase was absent in wild-type and CXCR4–/– astrocytes additionally maintained with CCX754. (**B**) Cultured Schwann cells were transfected with either selective CXCR4 siRNA or selective CXCR7 siRNA, and subsequently maintained for 48 hours with the indicated concentrations of SDF-1. Ki67-expressing cells were identified by immunocytochemistry and quantified by flow cytometry. The number of Ki67-expressing Schwann cells present in untreated control cultures was set to 100%. SDF-1 increased Ki67 expression in non-transfectants. In CXCR4 transfectants, such increases were only detectable with high (100 ng/ml), but not with low (10 ng/ml), concentrations of SDF-1. CXCR7 transfectants completely failed to respond to SDF-1 with an increase in Ki67 expression (n=4; *P<0.05, **P<0.001; SDF-1 treatment versus control; Tukey's test). (**C**) Representative immunocytochemical staining of wild-type astrocytic cultures, maintained with SDF-1 and CCX754 alone or in combination, with antibodies against Ki67. Scale bar: 100 μm.

neuromodulation (Li and Ransohoff, 2008), remains to be established. At present, little information is available on the effects of SDF-1 on Schwann cells in the developing and injured nervous system. Our observation that SDF-1 increases expression of the proliferation marker Ki67 in cultured Schwann cells suggests a role for this chemokine in the control of Schwann-cell proliferation. It is of note that, again consistent with the suggested role of CXCR4 and CXCR7 as a functional receptor unit in Schwann cells, the chemokine required the presence of both CXCR4 and CXCR7 to induce optimal mitogenic responses in Schwann cells. CXCR4 expression persists in Schwann cells of the adult nerve (Küry et al., 2002; Bhangoo et al., 2007) and contributes to neuropathic pain (Bhangoo et al., 2007). Whether this effect likewise requires CXCR7 awaits clarification.

In summary, our studies have established the major involvement of CXCR7 in SDF-1 signalling in astrocytes and Schwann cells. This might be relevant to the development and regeneration of the nervous system, and could be additionally involved in the formation of brain tumours (Fulton, 2009).

Materials and Methods

Cell cultures

Enriched astroglial cultures, containing >90% glial fibrillary acidic protein (GFAP)-expressing cells, were established from the cerebral hemispheres of postnatal day 2-3 Sprague Dawley rats or embryonic day (E)18 CXCR4–/– mice, according to a previously established protocol (Figiel et al., 2003). A detailed description of the

breeding of CXCR4–/– mice and their subsequent genotyping is given elsewhere (Zou et al., 1998; Ödemis et al., 2005). Schwann-cell cultures were prepared from postnatal day 0-1 rats according to Brockes et al. (Brockes et al., 1979). For experiments, cells were seeded into either 12-well cluster plates (TPP, Trasadingen, Switzerland; for Western blot analysis, RNA interference, RT-PCR) or 6-well cell culture plates (TPP; for FACS), and were further maintained with serum-free N2 medium additionally supplemented with SDF-1 (1-100 ng/ml; Calbiochem, Gaitherburg, MD), AMD3100 (1 μM; Calbiochem) or CCX754 (2 μM; ChemoCentryx).

Western blot analysis

Cells were lysed by ultrasonication in 62.5 mM Tris-HCl containing 2% SDS and 10% sucrose. Proteins were denatured at 95°C for 5 minutes and further diluted in sample buffer (250 mM Tris-HCl pH 6.8 containing 4% SDS, 10% glycerol and 2% β-mercaptoethanol). For detection of phosphorylated proteins, sample buffer was additionally supplemented with sodium orthovanadate (10 mM). The protein content of cell lysates was determined using the BCA protein estimation kit (Pierce; Rockford, IL) with BSA as a standard. Proteins (15 μg/lane) were separated by SDS (8%, 10% or 12%) polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose by electroblotting. Upon blocking non-specific binding sites with 5% non-fat milk for 60 minutes, blots were incubated overnight at 4°C with one of the following antibodies: anti-CXCR4 (1:1000; ProSci, Poway, CA); anti-CXCR7 (1:1000; Abcam, Cambridge, MA); phosphospecific anti-Erk1/2 (1:3000; Biosource, Camarillo, CA); phosphospecific anti-p38 (1:1000; Cell Signaling Technology, Danvers, MA); phosphospecific anti-Akt (1:3000; Biosource); phosphospecific anti-PKCζ/λ (1:1000; Cell Signaling Technology); phosphospecific anti-PKCα/β (1:1000; Cell Signaling Technology); phosphospecific PKC-pan (1:3000; Cell Signaling Technology). Antibody labelling was detected by incubating cultures for 2 hours at room temperature with appropriate horseradish-peroxidase-labelled secondary antibodies (Dianova, Hamburg, Germany) and visualized with the enhanced chemiluminescence kit (Amersham Pharmacia, Freiburg, Germany). To control protein loading, blots were additionally stained with either anti-β-actin (1:6000; BD Transduction Laboratories, San Jose, CA) or antibodies against the following non-phosphorylated signalling molecules: p38 (1:1000; Cell Signalling); Erk1/2 (1:6000; Biosource); Akt (1:2000; Biosource). In the case of the PKC isoforms, protein loading was controlled by labelling blots with β-actin and antibodies recognizing all phosphorylated PKC isoforms (PKC pan). Integrated optical densities of immunoreactive protein bands were measured using the Image Master VDS software (Pharmacia).

RNA isolation and RT-PCR analysis

Total RNA was isolated from astrocytes and Schwann cells using the PeqGold isolation kit (PeqLab, Schwalbach, Germany) according to the manufacturer's instructions. Concentrations were measured by spectrophotometric absorbance at 260 nm. A total of 5 μg of RNA was reverse transcribed using 200 units/μl of moloney murine leukaemia virus reverse transcriptase (Sigma, St Louis, MI) and 2 μg of random hexamer primers (Thermo Hybaid, Ulm, Germany). Obtained templates were amplified in a final volume of 50 μl. mRNA levels were quantified by real-time PCR using the following primers: CXCR4 (accession number: NM_022205), sense (5′-AGT GGC TGA CCT CCT CTT TGT-3′) and antisense (5′-GCC CAC ATA GAC TGC CTT TTC-3′), product size 458 bp; CXCR7 (accession number: NM_053352), sense (5′-TGG TCA GTC TCG TGC AGC-3′) and antisense (5′-GCC AGC AGA CAA CGA AGA CC-3′), product size 130 bp. Amplification was carried out by 30 PCR cycles of 93°C for 60 seconds, 58°C for 45 seconds (CXCR4) or 54°C for 45 seconds (CXCR7), and 72°C for 120 seconds. Reaction products were separated on 1.5% (w/v) agarose gel and visualized with ethidium bromide.

Immunoprecipitation

For immunoprecipitation, cells were lysed in ice-cold NP-40 buffer [50 mM Tris-HCl pH 7.5 containing 0.5% (v/v) Igepal CA-630, 1 mM sodium orthovanadate, 50 mM NaCl, 0.5 mM EDTA]. After preclearing, lysates were incubated with precipitating antibodies at 4°C for 1 hour, followed by another 1 hour incubation period in the additional presence of protein A or protein G Sepharose beads (Calbiochem). Bound proteins were resuspended in sample buffer and separated (30 μg protein/lane) by 10% SDS-PAGE.

Proliferation assay

Proliferating cells were identified by Ki67 expression and quantified by flow cytometric analysis. Cells were fixed with paraformaldehyde (2% w/v) in PBS for 15 minutes, permeabilized with 0.05% saponin and incubated for 45 minutes at 4°C with anti-Ki67-antibodies (1:500; Dako) and for another 30 minutes with fluorescein isothiocyanate (FITC)-labelled secondary antibodies (1:500; Jackson Laboratories, West Grove, PA). Flow cytometric analysis was performed with a FACScan (Becton Dickinson). The forward narrow-angle light scatter was used to exclude dead and aggregated cells. For visual inspection, cultured cells were subsequently incubated with Ki67 antibodies for 24 hours and biotinylated secondary antibodies (1:400; Vector Laboratories, Burlingame, CA) for 2 hours. Labelling was detected using the ABC kit (Vector Laboratories) and 3,3′-diaminobenzidine (10 mg/ml in Tris-HCl, pH 7.6).

RNAi

Rat CXCR4 (Rn_Cxcr4_6), CXCR7 (Rn_Cmkor1_5) and non-homologous (nh) siRNAs were purchased from Qiagen (Hilden, Germany). One hour prior to transfection, culture medium was replaced by 400 μl DMEM medium containing 1% HS. Transfection was performed with the siPORT^{TM} amine transfection agent (Ambion, Huntingdon, UK), according to the manufacturer's recommendations. For transfection, cultures additionally received 100 μl of serum-free DMEM containing the indicated concentrations of CXCR4 siRNA, CXCR7 siRNA or nh siRNA and 3 μl of transfection reagent, and were incubated overnight. Transfected cultures were further maintained with DMEM containing 1% HS and SDF-1 at the indicated concentrations.

CXCR7 and CXCR4 cell-surface expression

Cell-surface expression of CXCR7 and CXCR4 was quantified by flow cytometric analysis. Cultured astrocytes or Schwann cells were suspended in PBS supplemented with 0.3% BSA and 0.1% sodium azide, and subsequently incubated for 20 minutes at 4°C with either anti-CXCR4 (1:500; ProSci, Poway, CA) or anti-CXCR7 antibodies (1:500; Abcam), and for another 20 minutes with FITC-labelled secondary antibodies (1:500; Jackson Laboratories, West Grove, PA). Flow cytometric analysis was performed with a FACScan (Becton Dickinson). The forward narrow-angle light scatter was used to exclude dead and aggregated cells. For coexpression analysis, CXCR4 and CXCR7 antibody labelling was detected using Cy5- (1:500; Jackson Laboratories, West Grove, PA) and FITC-labelled labelled secondary antibodies, respectively. Double-labelled cells were analyzed by gating on forward and side scatter.

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Supplementary material available online at
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