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Expression and function of CXCR7 in the mouse forebrain

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ABSTRACT

The chemokine CXCL12/CXCR4 signaling system is important for the regulation of neuron migration in the developing forebrain. In particular it is crucial for correct distribution of Cajal–Retzius cells and migration of cortical interneurons. Here we investigated the expression of *CXCR7*, the second receptor for CXCL12, in comparison to *CXCR4*. We found that shifts in the expression of both receptors in the above cited cell populations coincide with major changes in their migratory behavior. Furthermore, we demonstrated that postnatally generated olfactory interneuron precursors express *CXCR7* but not *CXCR4* and that their distribution in the rostral migratory stream is affected by *CXCR7* downregulation. This suggests an involvement of CXCR7 in neuronal cell migration and indicates a possible action of CXCR7 independently of CXCR4 as a mediator of CXCL12 signaling.

1. Introduction

Neuronal migration is a key process in brain development and the slightest alterations can lead to major changes in brain architecture, connectivity and function. For example in the forebrain, Cajal–Reztius neurons are largely generated in the periphery of the developing cortex and are distributed in the marginal zone by tangential migration. Cortical projection neurons are produced in the ventricular zone of the dorsal telencephalon and migrate radially to form the cortical plate, while cortical interneurons are generated in the ganglionic eminences of the ventral telencephalon, and perform tangential migration into the cortex. Within the cortex, interneurons use two principal migratory routes localized in the subventricular zone (SVZ) and in the marginal zone (MZ) (Marin and Rubenstein, 2001). In the postnatal and adult brain, interneuron generation continues in the olfactory system. Here, neural stem cells in the periventricular region permanently generate neuronal precursors that migrate rostrally into the olfactory bulb (OB), thereby following a specific tangential migratory pathway, the rostral migratory stream (RMS) (Lois and Alvarez-Buylla, 1994).

Over the past decade, several signaling systems regulating the migration of the different neuronal populations have been identified, including semaphorins, netrins, slits and GDNF (Flames et al., 2004; Marin et al., 2001; Pozas and Ibanez, 2005; Stanco et al., 2009). Among these systems, the chemokine CXCL12 (or Stromal Derived Factor 1, SDF1) and its receptor CXCR4 appear to play key roles in the control of neuronal migration. Originally identified in the regulation of leukocyte trafficking, CXCL12/CXCR4 signaling has been shown to regulate the migration of neural cells as diverse as cerebellar granule neurons, Cajal–Retzius cells or cortical interneurons (Borrell and Marin, 2006; Li et al., 2008; Lopez-Bendito et al., 2008; Paredes et al., 2006; Stumm et al., 2003; Tiveron et al., 1996; Zhu et al., 2009; Zhu et al., 2002; Zou et al., 1998).

For many years, CXCR4 was accepted as the unique receptor of CXCL12, presenting an exception from the generally promiscuous binding properties of other chemokines. However, it has been shown recently that the chemokine receptor Cmkor/RDC1, now named CXCR7, is able to interact with CXCL12 (Balabanian et al., 2005), thereby regulating cell migration in different experimental systems. For example, in zebrafish, the migration of primordial germ cells (PGC) to the gonads and the formation of the posterior lateral line (PLL) depend on CXCR7. In both situations, it appears likely that CXCR7 acts as a non-signaling receptor, sequestering the ligand to regulate the CXCL12/CXCR4 guided migration (Boldajipour et al., 2008; Dambly-Chaudiere et al., 2007). In mammals, such a "scavenger" function was also determined in the mouse heart valve and in the human umbilical vein endothelial cells (Naumann et al., 2010). However evidence for an alternative function as an active signaling receptor has been presented (Odemis et al., 2010; Valentin et al., 2007).

Like CXCR4, CXCR7 is expressed in the vertebrate brain (Stumm and Hollt, 2007; Tiveron and Cremer, 2008) suggesting that it might be involved in the regulation of neuronal cell migration. Here we present a detailed analysis of *CXCR7* expression at the population and single cell level in the developing mouse forebrain. Furthermore, we combined *in vivo* electroporation and the use of shRNA to down-regulate *CXCR7* expression in olfactory interneuron precursors and address functional implications on their migration to the olfactory bulb.

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2. Materials and methods

2.1. Animals

All animals were treated according to protocols approved by the French Ethical Committee. CD1 mice were used. The day of appearance of the vaginal plug was considered as 0.5 day of gestation (E0.5).

2.2. In situ hybridization and immunohistochemistry

Mouse antisense RNA probes for *CXCR4*, *CXCR7* (kind gifts of B. Moepps, Ulm, Germany), *Lhx6* (Lavdas et al., 1999), *Reelin* (Schiffmann et al., 1997), *Tbr1* (Bulfone et al., 1995) and *CXCL12/SDF1* (Daniel et al., 2005) were labeled with either digoxigenin (DIG)-dUTP or FITC-dUTP (Roche, UK). Tissue preparation and *in situ* hybridization (ISH) were described previously (Tiveron et al., 1996). For double fluorescent ISH (DFISH), sections were co-hybridized with FITC-labeled *CXCR7* probe along with either DIG-labeled *Reelin* probe or DIG-labeled *Tbr1* probe, or with DIG-labeled *CXCR7* probe along with either FITC-labeled *Lhx6* probe or FITC-labeled *CXCR4* probe and processed as described by Dufour et al. (2006). For immunohistochemistry, CXCR4 polyclonal Goat antibody (1:400; Santa Cruz, sc-6190) and CXCR7/RDC1 polyclonal Rabbit antibody (1:400; Geneway) were used. Fifty micrometer sections obtained from brains fixed with 4% paraformaldehyde (PFA) were collected on Superfrost slides (Manzel-Gläser). Sections were pretreated according to the manufacturer's protocol for Tyramide Amplification System (TSA, Perkin Elmer). Furthermore, to detect CXCR7 antigen, sections were pretreated in boiling 10 mM citric acid, pH 6 for 60 s. The sections were incubated overnight at 4 °C with the primary antibody, then sequentially with the corresponding biotinylated secondary antibody (1:500; Jackson Laboratory) and with HRP coupled Streptavidin (1:100; Jackson laboratory) followed by a FITC-TSA (Perkin Elmer) reaction. Quantitative analysis was performed as described in Tiveron et al. (2006). Cells were counted on 4 independent sections in DFISH for *CXCR7* and *Lhx6* ($n=363$) and on 5 independent sections in DFISH for *CXCR7* and *CXCR4* ($n=448$).

2.3. Expression vectors

shRNA plasmids targeting *CXCR7* (sh660 and sh679) or off-target control shRNA (shcontrol) were obtained from SIGMA. The expression vector, pCXCR7-d1EGFP, coding for CXCR7 protein fused to a destabilized form of EGFP was obtained by subcloning a PCR fragment containing a *CXCR7* coding sequence in pd1EGFP (BD Biosciences Clontech). pCX-CXCR7, pCX-EGFP-N1 and pCX-tdTomato are expression vectors in which *CXCR7* or fluorescent reporter genes *GFP* (green) and *tdTomato* (red) were cloned in a pCAGGS derived vector, pCXmcs2 (Morin et al., 2007). In order to create a vector in which a reporter gene and a shRNA would be expressed from the same plasmid, we constructed pGFP-U6 as follows: a fragment containing the U6 promoter sequence was obtained after PCR amplification from pSilencer 1.0-U6 (Ambion) and subsequently subcloned into the enhanced GFP expressing vector, pCAAGS-AFP (Momose et al., 1999), downstream to the GFP expression unit. For cloning purposes EcoR1 and HindIII sites were inserted 3′ to the U6 promoter sequence. These sites were used to introduce the sh679 DNA sequence obtained by annealing two complementary oligonucleotides creating pGFP-U6_sh679.

2.4. In vitro shRNA validation

HEK293T cells were co-transfected with pCXCR7-d1GFP, pCX-tdTomato and either pBluescript (pBS) or the different shRNAs at a 1:1:1 ratio using Promofectin reagent (Promokine, Germany). Twenty-four hours later, 4 independent fields were photographed with an AxioCam camera on a Zeiss Observer-Z1 microscope and analyzed by ImageJ. The ratio of the green pixels (i.e. the green fluorescence emanating from the CXCR7-dGFP construct) on the red pixels (i.e. the red fluorescence emanating from the transfected cells) was determined and reported in a histogram. For shRNA validation with FACS analysis, COS cells were co-transfected with pCX-CXCR7 and the different shRNAs at a 1:2 ratio. After 24 h in culture, the cells were harvested, fixed in Cytofix-Cytoperm (BD Biosciences), labeled with a mouse IgG1 anti-CXCR7 (1:100; clone 9E11, gift of Chemocentryx) followed by anti-mouse-PE (1:1000; Coulter IMO855) labeling and then analyzed by FACS.

2.5. In vivo electroporation and quantitative analysis

In vivo electroporation procedures were carried out in P1 pups as described in Boutin et al. (2008). In the first set of experiments, pCX-EGFP alone or pCX-EGFP with shRNA at a ratio of 1:2 was used. In a second set of experiments pCX-EGFP-U6 or pCX-EGFP-U6_sh679 was injected in P1 pups. Four days after electroporation pups were perfused with 4% PFA, brains dissected out, and coronal sections mounted on Superfrost slides. GFP^+ cells were counted on sections at different rostrocaudal levels. Kruskal–Wallis and Mann–Whitney tests were used to assess differences between data groups. Differences were considered statistically significant when $P<0.05$.

3. Results

3.1. Comparison of expression pattern of the CXCL12 receptors, CXCR7 and CXCR4, in mouse embryonic forebrain

We established the expression pattern of *CXCR7* in comparison to *CXCR4* in the developing telencephalon. *CXCR7* mRNA expression was first observed at E11.5. In the ventral telencephalon, a hybridization signal was detected in the proliferative area of the medial ganglionic eminence (MGE) comprising the ventricular zone (VZ) and subventricular zone (SVZ), as well as in the ventral part of the lateral ganglionic eminence (LGE) (Fig. 1A, short arrowhead). At this time point, the dorsal telencephalon was devoid of staining except for a small number of cells localized in the cortical hem (Fig. 1A, long arrowhead), in a position similar to that of *CXCR4* positive cells, previously identified as Cajal–Retzius neurons (Fig. 1A,F; Borrell and Marin, 2006). At this stage *CXCR4* was expressed at low levels in the entire telencephalic VZ with slightly increased levels in the cortex (Fig. 1F).

At E12.5, numerous individual *CXCR7* expressing cells were found in the subpallium forming two continuums. One was located between the ganglionic eminence and the ventral pial surface (arrow; Fig. 1B; see also E11.5, arrow in Fig. 1A). Considering their location and the fact that the olfactory tubercle (OT) is labeled postnatally (Fig. 1E), these cells are likely to be the olfactory tubercle neuron precursors migrating from the LGE (Wichterle et al., 2001). The second continuum of *CXCR7* positive cells was positioned between the MGE and the ventral cortex, suggestive of cortical interneurons generated in the MGE and migrating towards the cortex (Fig. 1B; arrowheads). Furthermore, *CXCR7* expression appeared in the superficial aspect of the pallium in an area basal to the *CXCR4* expressing VZ (Fig. 1B,B' and G,G'). At E13.5, such a complementary expression of *CXCR7* and *CXCR4* was also observed in the subpallium where the caudate putamen expressed exclusively *CXCR4* and the germinal zone of the MGE expressed mainly *CXCR7* (Fig. 1C,H). In the dorsomedial cortex, expression of both genes was comparably exclusive while in the lateral cortex, overlapping expression appeared in cell populations in the marginal zone (MZ) and in the cortical SVZ (Fig. 1C,H; arrows). At E15.5, the overlapping expression of both receptors in the cortex was generalized. This was particularly obvious in two dense layers of cells in the MZ and in the SVZ, corresponding to the major migratory routes used by invading cortical interneurons (Marin and Rubenstein, 2001). At postnatal stages, *CXCR7* expression was observed in the striatal SVZ (Fig. 1E, arrow and E') while *CXCR4* labeling was restricted to cells

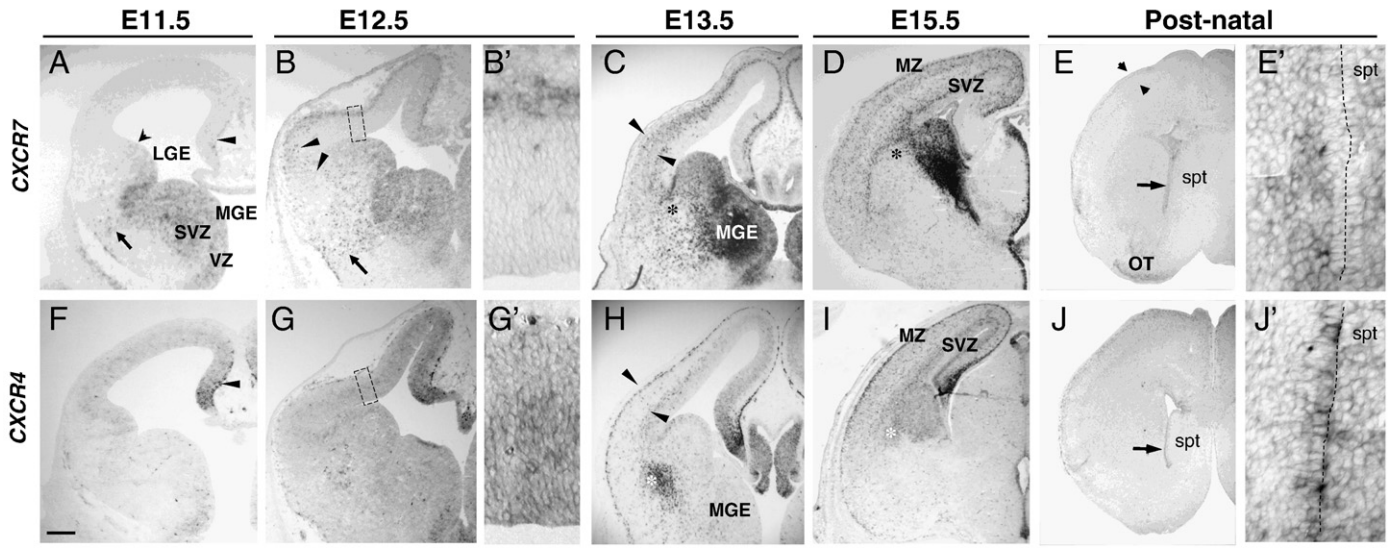

Fig. 1. Expression of the CXCL12 receptor genes, *CXCR7* and *CXCR4* in the developing telencephalon. *In situ* hybridization (ISH) for *CXCR7* (A–E') and *CXCR4* (F–J') on mouse brain coronal sections at E11.5 (A, F), E12.5 (B, B', G, G'), E13.5 (C, H), E15.5 (D, I) and P2 (E, J). (A) At E11.5 *CXCR7* ISH labels the ventral telencephalon in the proliferative zones (VZ and SVZ) of the MGE and ventral LGE (short arrowhead), in cells scattered between the LGE and the ventral pial surface (arrow), and some cells in a caudomedial region of the cortex, the hem region (long arrowhead), at a similar position to the *CXCR4* labeled Cajal–Retzius cells (arrowhead in F). At this stage, *CXCR4* but not *CXCR7* is expressed in the cortex (F). (B, G) At E12.5, two continuums of *CXCR7* labeled cells can be distinguished: one positioned between the GE and the ventral cortex (arrowheads) and one positioned between the GE and the pial surface, in an area corresponding to the future olfactory tubercle (OT) (arrow). As in postnatal stage *CXCR7* is expressed in OT (E), it is likely that these cells are the OT neuron precursors generated in the LGE. (B', G') High magnification of the area boxed in B and G respectively. *CXCR7* expression appears in the cortex in a superficial layer of cells that do not express *CXCR4*. (C, H) At E13.5, expression of *CXCR7* (C) and *CXCR4* (H) expressions overlap in one superficial and one deep stream of cells in the cortex (arrowheads). In ventral telencephalon, the caudate putamen (asterisk in C and H) expresses only *CXCR4* and the proliferative zone of the MGE shows a strong expression of *CXCR7*. (D, I) At E15.5, the *CXCR7* expression pattern in the cortex resembles that of *CXCR4*. The two lines of *CXCR4* labeled cells in the MZ and in the SVZ correspond to the tangentially migrating cortical interneurons. The asterisks underline the position of the developing caudate putamen that expresses *CXCR4* (I) but not *CXCR7* (D). (E, J) At P2, *CXCR7* ISH (E) and *CXCR4* ISH (J) show labeling in the periventricular zone (arrow). Moreover, *CXCR7* (E) labels the OT and two independent cortical layers, whereas *CXCR4* (J) labels cells throughout the cortex with concentrated signal in the superficial layer. (E', J') High magnification of areas boxed in E and J respectively. The dashed line marks the ventricle. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, marginal zone; OT, Olfactory tubercle; spt: septum; SVZ, subventricular zone. Scale bar: 200 μm (A, B, F, G); 40 μm (B', G'); 250 μm (C, H); 400 μm (D, I); 500 μm (E, J); 20 μ (E', J').

directly lining the ventricle (Fig. 1J, arrow and 1J'). Furthermore, *CXCR7* expressing cells were found concentrated in a deep and a superficial layer of the postnatal cortical plate (arrowheads in Fig. 1E).

In conclusion, the two CXCL12 receptors showed a largely exclusive expression pattern during early phases of forebrain development. At later stages their expression overlapped progressively.

3.2. Identification of CXCR7 expressing cells in the cortex

In order to identify the different *CXCR7* positive cell populations at cellular resolution, we used double fluorescent *in situ* hybridization (DFISH). At E12.5, the cortical preplate is formed above the VZ. This structure is composed of two populations of cells, the first postmitotic neurons generated from the cortical VZ and the Cajal–Retzius cells (CR) which are mainly generated in the caudomedial cortex, defined as the hem, and migrate tangentially to invade the entire cortical surface (Takiguchi-Hayashi et al., 2004). As cortical development proceeds, the two cell populations that form the preplate are split by the new born cortical pyramidal neurons, leading to the appearance of the MZ, populated mainly by CR cells and the subplate that underlies the newly formed cortical plate (Marin-Padilla, 1998).

We aimed at identifying the *CXCR7* positive cell populations in the superficial aspect of the E12.5 cortex (Fig. 1B). A BrdU incorporation experiment with a 2 h pulse showed that all *CXCR7* positive cells were postmitotic (Fig. 2C). Next, we performed DFISH for *CXCR7* and *Reelin*, a specific marker of CR cells. We found that *Reelin* expressing CR cells co-expressed *CXCR7* at E12.5 (Fig. 2A,A',A") in accordance to findings in the rat (Schonemeier et al., 2008). Surprisingly, one day later, at E13.5, *CXCR7* was strongly downregulated in CR cells overlying the cortex except for a small population of cells located in the medial part (Fig. 2B, B',B"). This was in contrast to the expression of *CXCR4* which persists in all hem-derived CR cells (Borrell and Marin, 2006; Tissir et al., 2004). Interestingly, loss of *CXCR7* coincided with the end of tangential migration of CR cells, suggesting a possible contribution of *CXCR7* to the control of CR cell dispersion.

The *CXCR7* expression domain in the cortex at E12.5 was too wide to be restricted solely to CR cells. Comparison of *CXCR7* expression with that of *Tbr1*, a marker for preplate neurons suggested that the subplate neurons expressed the receptor (Fig. 2D,E). This was confirmed at the cellular level by DFISH (Fig. 2F). Expression of *CXCR7* was downregulated coincidently with preplate splitting since no labeling was observed after the formation of a distinguishable cortical plate.

Thus, *CXCR7* was expressed transitorily by all preplate neurons. For CR cells as well as subplate neurons downregulation of the receptor was correlated with major changes in their migratory behavior.

Cortical interneurons are mainly generated in the MGE and traverse the ventral telencephalon to invade the cortex via two main migratory routes along the MZ and the SVZ (Marin and Rubenstein, 2001). It has been shown that the migrating cortical interneurons expressed *CXCR4* (Stumm et al., 2003; Tiveron et al., 2006) and that their tangential migration in the MZ and the SVZ depended on CXCL12/CXCR4 signaling (Li et al., 2008; Lopez-Bendito et al., 2008; Tiveron et al., 2006). *CXCR7* expression pattern dynamics in the developing forebrain in comparison with *CXCR4* suggested that one *CXCR7* positive cell population consisted of the immigrating cortical interneurons (Fig. 1). DFISH for *CXCR7* and *Lhx6*, coding for a transcription factor specifically expressed in MGE derived cortical interneurons, confirmed this hypothesis (Fig. 3A). Quantification demonstrated that 73.51% ± 4.72 of *Lhx6* positive in the MZ and 50.84% ± 5.67 in the SVZ co-expressed *CXCR7*. Thus *CXCR7* was expressed in the majority of interneurons migrating in the MZ and in about 50% in the SVZ.

As mentioned before, *CXCR4* is expressed and functions in migrating cortical interneurons (Stumm et al., 2003; Tiveron et al., 2006). In order to determine how *CXCR7* was distributed relative to

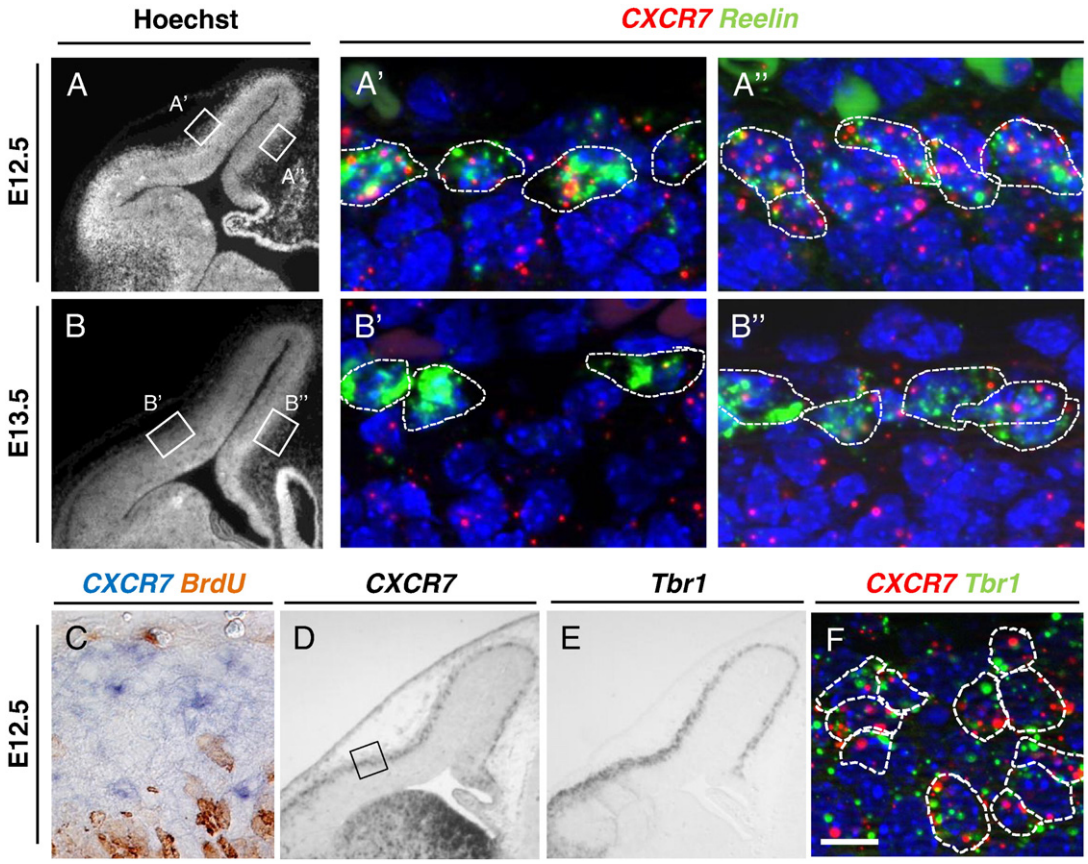

Fig. 2. *CXCR7* expression in preplate neurons. Coronal brain sections at E12.5 (A–A") and E13.5 (B–B") counterstained with Hoechst dye that reveals cell nuclei (A and B) or treated for double fluorescent *in situ* hybridization (DFISH) for *CXCR7* (red) and *Reelin* (green), a specific marker of Cajal–Retzius cells (A', A" and B', B"). The areas presented in A', A" and B', B" are high magnifications of cortical areas boxed in A and B respectively. Examples of labeled cells are lined in white. At E12.5 the *Reelin*$^+$ Cajal–Retzius cells express *CXCR7* all over the cortex (A' and A") while at E13.5 the double labeled cells are restricted to the medial cortex (B"). (C) Combined ISH for *CXCR7* (blue) with immunohistochemistry against BrdU (brown) on E12.5 coronal brain section at high magnification. The photographed field is equivalent to the boxed area in D. *CXCR7* expressing cells are not positive for BrdU indicating that they are postmitotic. (D, E) Single ISH on E12.5 coronal brain section for *CXCR7* (D) and for *Tbr1* (E), a marker for preplate neurons. (F) High magnification of DFISH for *CXCR7* (red) and *Tbr1* (green) shows double labeled cells (lined in white). Scale bar: 250 μm (A, B); 10 μm (A', A", B', B" and F); 20 μm (C); 220 μm (D, E).

CXCR4 in this population, we performed DFISH for both receptors at E15.5, a stage at which interneurons start to leave their tangential migrating routes in the MZ and SVZ to invade the cortical plate (CP).

In the MZ, more than 75% of all interneurons co-expressed both receptors while 17% expressed only *CXCR7* and less than 10% exclusively *CXCR4* (MZ: *CXCR4*$^+$ only, 6.8%±3.0; *CXCR7*$^+$;*CXCR4*$^+$, 75.78%±7.54; *CXCR7*$^+$ only, 17.49%±4.91; Fig. 3D). CR neurons were excluded from this analysis after their identification based on size and positioning. As in the MZ, the majority of interneurons in the SVZ/IZ expressed both receptor genes (*CXCR7*$^+$;*CXCR4*$^+$, 57.67%±8.17), while about 20% were single positive (*CXCR4*$^+$ only, 20.45%±5.18; *CXCR7*$^+$ only, 21.87%±6.15). Interestingly, the vast majority of interneurons invading the CP expressed either both receptors or exclusively *CXCR7* (*CXCR7*$^+$;*CXCR4*$^+$, 47.50%±2.11; *CXCR7*$^+$ only, 45.17%±3.93) while the *CXCR4*$^+$ only population was reduced to less than 10% (7.34%±2.55). Thus, as for preplate neurons, changes in the expression of the two CXCL12 receptors coincided with important alterations in their migration pattern. Here the expression of *CXCR7* appeared to be augmented when interneurons change from tangential to radial migration in the CP.

3.3. CXCR7 in the postnatal mouse forebrain

Neuronal migration continues in the postnatal and adult olfactory system, where large amounts of interneurons are generated in the periventricular region and invade the olfactory bulb via the rostral migratory stream.

Using Serial Analysis of Gene Expression (SAGE) on purified migratory interneuron precursors, we found that *CXCR7* was 5 times overexpressed compared to whole brain while *CXCR4* was not detected (Pennartz et al., 2004). These data corroborate the data presented in the Gene Expression Nervous System Atlas Project (GENESAT Project; Gong et al., 2003) in which transgenic mice carrying GFP under the control of the *CXCR7* locus (CXCR7-GFP mice) show GFP expression in the SVZ and RMS whereas CXCR4-GFP mice did not (www.gensat.org). We confirmed this expression data by *in situ* hybridization for *CXCR7* and *CXCR4* on postnatal brain sections. As expected, the SVZ and the RMS were labeled by *CXCR7* (Fig. 4A) but not by *CXCR4* (Fig. 4B). Furthermore, *CXCL12* expressing cells were positioned in the vicinity of the RMS (Fig. 4C) in the Accessory Olfactory Nucleus (AON; arrowheads) as well as in the OB meninges (arrow), thus compatible with a function in the guidance of olfactory interneuron migration. The above gene expression analyses were completed by immunohistochemistry for CXCR7 and CXCR4 protein on neonate brain (P0) coronal sections. At this stage the OB is immature and the rostral extension of the lateral ventricle is visible in the center of the proximal OB, with the SVZ surrounding the VZ. Immunoreactivity (IR) for CXCR7 but not for CXCR4 could be detected in the SVZ (arrows, Fig. 4D,E). Thus, OB interneurons represent a migratory cell population in the forebrain that expresses exclusively CXCR7, suggesting a CXCR4 independent function of the receptor in this system.

In order to unravel such a function for *CXCR7*, we performed a loss-of-function approach based on RNAi. Different shRNAs, targeting several

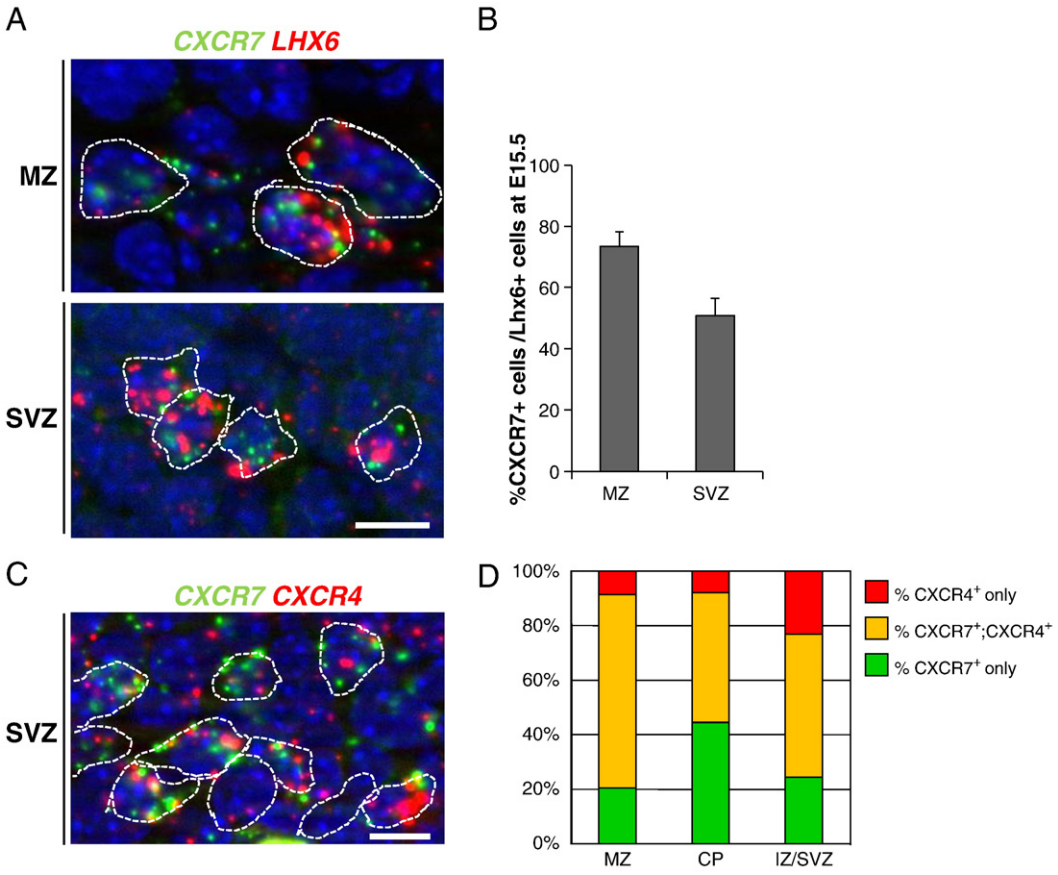

Fig. 3. *CXCR7* expression in cortical interneurons. (A) DFISH for *CXCR7* (green) and *Lhx6* (red) on E15.5 brain sections. Examples of double labeled cells in the MZ and in the SVZ are lined in white. (B) Quantification of *Lhx6* expressing cortical interneurons co-labeled with *CXCR7* in the MZ and in the SVZ. MZ: 73%±4.72 ($n=4$); SVZ: 50.84%±5.67 ($n=4$). (C) DFISH for *CXCR7* (green) and *CXCR4* (red) on E15.5 brain sections. Co-labeled cells are lined in white. (D) Distribution of cells labeled by either one SDF1 receptor or by both in three different cortical regions, the MZ, the cortical plate (CP) and the SVZ. MZ: $CXCR4^+$ only, 6.8%±3.0; $CXCR7^+CXCR4^+$, 75.78%±7.54; $CXCR7^+$ only, 17.49%±4.91 ($n=5$). CP: $CXCR4^+$ only, 7.34%±2.55; $CXCR7^+CXCR4^+$, 47.50%±2.11; $CXCR7^+$ only, 45.17%±3.93 ($n=5$). SVZ: $CXCR4^+$ only, 20.45%±5.18; $CXCR7^+CXCR4^+$, 57.67%±8.17; $CXCR7^+$ only, 21.87%±6.15 ($n=5$). Error bars indicate SEM. Scale bars: 10 µm.

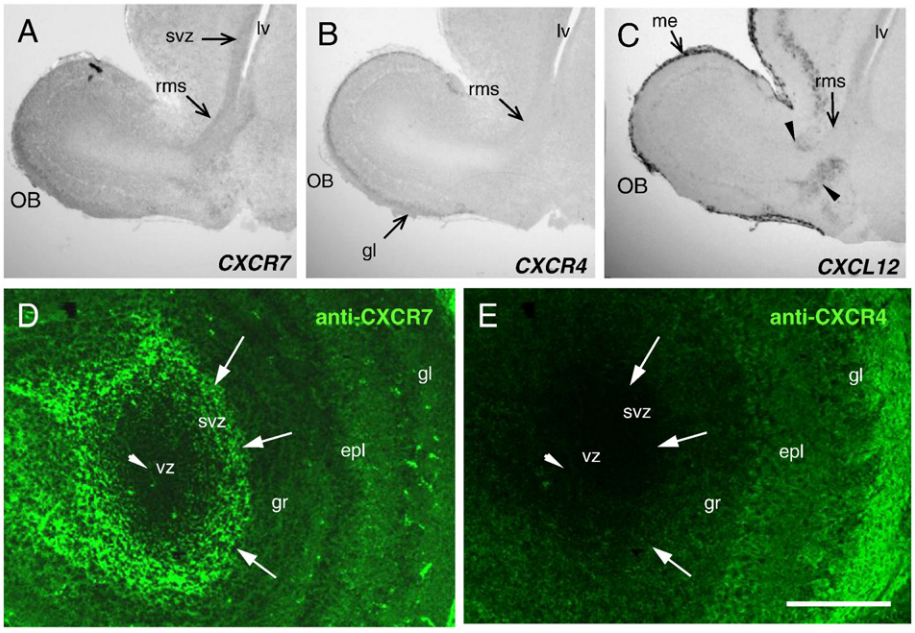

Fig. 4. Expression of *CXCR7*, *CXCR4* and their ligand CXCL12 in postnatal mouse forebrain. (A–C) ISH for *CXCR7* (A), *CXCR4* (B) and *CXCL12* (C) on P2 brain sagital sections. (D, E) Immunohistochemistry using anti-CXCR7 (D) or anti-CXCR4 (E) antibody on coronal sections of P1 mouse at a proximal level of the olfactory bulb. The olfactory interneuron precursors that are born in the subventricular zone (svz) of the lateral ventricle and migrate tangentially via the rostral migratory stream (rms) to the olfactory bulb (OB), express CXCR7 (A and D) but not CXCR4 (B and E). (C) The ligand CXCL12 is expressed in the accessory olfactory nucleus (arrowheads) at the vicinity of the $CXCR7^+$ rms. epl: external plexiform layer; gl: glomerular layer; gr: granular layer; lv: lateral ventricle; me, meninges; OB: olfactory bulb; rms: rostral migratory stream; svz: subventricular zone; vz: ventricular zone. Scale bar: 1 mm (A–C); 100 µm (D, E).

locations in the coding sequence, were tested for their ability to downregulate *CXCR7* expression *in vitro*. First, we used an expression construct producing CXCR7 protein fused to a destabilized form of the Green Fluorescent Protein (CXCR7-d1GFP). HEK293 cells were co-transfected with this vector and different *CXCR7*-specific and off-target control shRNAs as well as a Red Fluorescent Protein expression vector as transfection controls. One day after transfection, no significant difference in CXCR7-d1EGFP expression could be observed when either control shRNA (shcont) or sh660, one of the CXCR7 specific shRNA, was used. However, there was a striking reduction of GFP fluorescence intensity when sh679, another CXCR7 specific shRNA, was co-transfected, indicating that this shRNA was able to efficiently down-regulate *CXCR7* expression (Fig. 5A). These results were confirmed with a second method, in which expression of CXCR7 protein was analyzed in transfected cells by Fluorescent Activated Cell Sorting using an anti-CXCR7 antibody (Fig. 5B). Again sh679 appeared to strongly down-regulate *CXCR7* expression. Taken together these data validated sh679 as an efficient shRNA for inactivating *CXCR7*.

Then, *in vivo* electroporation of the postnatal olfactory system was performed (Boutin et al., 2008). Briefly, the different shRNAs together with a GFP reporter plasmid were injected in the lateral ventricle of 1 day old (P1) mouse pups and introduced in the progenitor cells lining the ventricle by electroporation. Four days after electroporation, the distribution of migratory neuronal precursors was analyzed in coronal brain sections. A schematic representation of the experimental protocol is presented in Fig. 5C. No difference in GFP^+ cell distribution was observed when the animals were electroporated with the GFP reporter alone or along with either shcont or sh660 (Fig. 5D). However, when sh679 was used, the rostrocaudal GFP^+ cell distribution was significantly different compared to controls: more cells accumulated close to

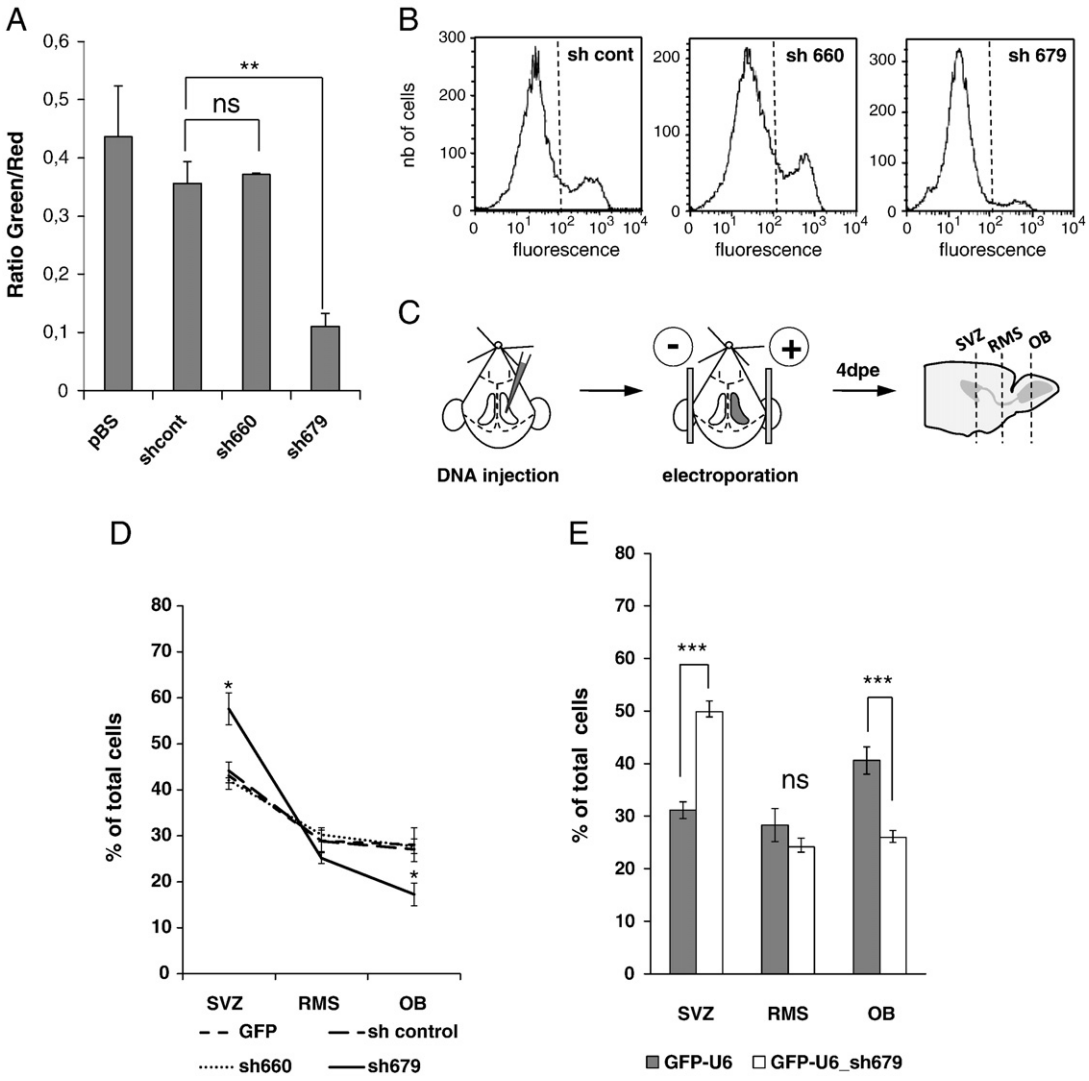

Fig. 5. Downregulation of *CXCR7* by specific shRNA. (A–B) *In vitro* validation of CXCR7 knock-down by shRNA. (A) Analysis of downregulation of CXCR7-d1GFP expressed in HEK293T cells in combination with pTomato (encoding a red fluorescent protein-RFP) and either pBS, control shRNA (shcont) or two *CXCR7* targeting shRNA, sh660 or sh679. Histograms represent the ratio of green signal among the red signal present in the transfected cells. pBS: 0.44 ± 0.09; shcont: 0.36 ± 0.09; sh660: 0.37 ± 0.02; sh679: 0.11 ± 0.04 (Statistics: Mann–Whitney). sh679 downregulates efficiently *CXCR7* (B) FACS analysis of *CXCR7* expression in COS7 cells co-transfected with pCX-CXCR7 and either shcont, sh660 or sh679. The peak on the left of the dashed line corresponds to the non-transfected cells. (C) Schematic representation of the experimental paradigm use for *in vivo* electroporation. Four days after electroporation (4 dpe), distribution of GFP+ cells was determined at 3 levels: SVZ, RMS and OB. (D, E) *In vivo* effect of *CXCR7* dowregulation by shRNA. (D) Caudorostral distribution of GFP^+ cells when GFP alone or GFP along with shcont, sh660 or sh679 was electroporated. In the GFP;sh679 condition, the GFP^+ cell distribution is significantly altered compared to the other conditions. More olfactory interneuron precursors remained in the SVZ and fewer cells have reached the OB. GFP: SVZ, 43.07% ± 2.94; RMS, 28.89% ± 2.34; OB, 28.04% ± 3.66 ($n = 5$). shcont: SVZ, 44.14% ± 3.92; RMS, 28.83% ± 1.06; OB, 27.03% ± 4.16 ($n = 5$). sh660: SVZ, 42.06% ± 0.52; RMS, 30.24% ± 1.53; OB, 27.71% ± 1.58 ($n = 4$). sh679: SVZ, 57.61% ± 3.50; RMS, 25.17% ± 1.16; OB, 17.23% ± 2.46 ($n = 5$). (Statistics: Kruskal–Wallis). (E) Caudorostral distribution of GFP^+ cells in brains electroporated with either pGFP-U6 or pGFP-U6_sh679. Altered cell distribution in presence of sh679 is confirmed. GFP-U6: SVZ, 31.12% ± 1.59; RMS, 28.30% ± 3.14; OB, 40.58% ± 2.59 ($n = 5$). GFP-U6_sh679: SVZ, 49.86% ± 2.04; RMS, 24.15% ± 1.65; OB, 25.99% ± 1.29 ($n = 8$). (Statistics: Mann–Whitney). ns: non-significant; $^*P<0.05$; $^{**}P<0.01$ $^{***}P<0.005$. Error bars indicate SEM.

the ventricle (level SVZ) while significantly fewer had reached the OB (level OB; Fig. 5D; $P<0.05$). In this experiment the GFP expressing plasmid and the shRNA were co-electroporated, raising the possibility that a subfraction of GFP^+ cells had not received the shRNA. To circumvent this problem, we constructed an expression vector that carried both the GFP and the sh679 sequence, denominated pGFP-U6-sh679, and repeated the same *in vivo* electroporation experiment. In these conditions, 49.86% ± 2.04 of the GFP^+;sh679 electroporated cells remained in the SVZ, representing a 60% increase when compared to the distribution of control cells electroporated with an empty GFP vector (31.12% ± 1.59). In the mean time, only 25.99% ± 1.29 of the GFP^+; sh679 had reached the OB compared to 40.58% ± 2.59 of GFP^+ control cells (Fig. 5D) ($P<0.005$). In agreement with the previous experiment, these results confirmed that the downregulation of *CXCR7* in the migrating interneuron precursors had affected the cell distribution along the caudorostral axis.

4. Discussion

In this work we investigated the expression of the alternative CXCL12 receptor CXCR7 in relation to CXCR4 in the forebrain. We identify the positive cell populations and show that expression of both receptors is highly dynamic and partially overlapping. At the cellular level, individual cells often show a co-expression of both receptors; however, cell populations that express exclusively *CXCR7* or *CXCR4* exist. Strikingly, changes in *CXCR7* expression by a given cell type coincided with changes in migratory behavior. Finally, we show that in postnatally generated interneurons of the olfactory bulb, CXCR7 is the unique CXCL12 receptor and that interference with its expression induces alterations in precursor distribution in the RMS.

CR cells are present in the MZ of the developing cerebral cortex and thus in close vicinity to the meningeal membrane, which expresses CXCL12 (Daniel et al., 2005; McGrath et al., 1999). They originate from three different locations of the telencephalon, the cortical hem, the septum and the ventral pallium, and migrate tangentially in the MZ to populate the entire cortical surface (Bielle et al., 2005; Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006). It was demonstrated that CXCL12 secreted by the meninges controls the positioning of the *CXCR4* expressing hem-derived CR cells by inhibiting their inward migration and retaining them within the MZ (Borrell and Marin, 2006; Paredes et al., 2006). We found that these CR cells express *CXCR7* during their tangential migration, but that this expression is lost when they stop migrating at E13.5. One possibility to explain this observation is that CXCR7 functions to reduce the action of CXCL12 on CXCR4 expressing CR cells thereby allowing their tangential translocation in the "sticky" MZ. Once in the right place, CXCR7 is downregulated and CR cells become fully responsive to meningeal CXCL12, thus maintaining them in their peripheral position. This interpretation is also in agreement with the observation that *CXCR7* is expressed in the CR cells that remain in the medial cortex at the level of the hippocampal anlage and that are also dependent on CXCL12/CXCR4 signaling for their positioning in the MZ. As development of the hippocampus is delayed compared to the neocortex, local tangential distribution of CR cells, possibly dependent on expression of *CXCR7*, like in the cortex, is therefore expected at a later time point.

In addition to controlling the migration of CR cells, CXCL12/CXCR4 signaling has been shown to be important for correct tangential migration of cortical interneurons by maintaining them on their principal migration routes in the SVZ and the MZ (Lopez-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006). In mutants for *CXCR4*, the majority of cortical interneurons leave the MZ and the SVZ routes to enter prematurely into the CP (Lopez-Bendito et al., 2008; Tiveron et al., 2006). Interestingly, neurons that leave the principal pathways do not downregulate *CXCR4*, suggesting that either CXCL12/CXCR4 signaling is inhibited by other means, or that so far unknown dominant mechanisms deroute interneurons from their main tracks. We found that a higher amount of interneurons presented *CXCR7* alone or co-expressed *CXCR4*/*CXCR7*, once these cells exit from the SVZ or MZ into the CP. This suggests that induction of *CXCR7* expression modifies the responsiveness of cortical interneurons to CXCL12 present in the SVZ and the MZ, thereby allowing their exit and terminal radial migration in the CP.

In conclusion, it is conceivable that in the cortex CXCR7 acts as a regulator of CXCL12/CXCR4 function, thereby fine tuning the balance between the reaction of a cell to stick to a CXCL12 source and it needs to either move in its presence or deviate and invade a ligand negative environment. For CR cells CXCR7 could weaken CXCL12/CXCR4 signaling thereby allowing their tangential translocation as long as necessary. For interneurons, regulation of the CXCL12/CXCR4 signal by CXCR7 might mediate the equilibrium between maintenance in the principal pathways versus exit into the cortical layers. It has been demonstrated in zebrafish that CXCR7 can bind CXCL12 and thereby function as a decoy receptor, reducing ligand concentrations locally (Boldajipour et al., 2008). Furthermore, in cultured mammalian cells CXCR7 acts as a specific receptor for CXCL12, mediating effective ligand internalization and degradation (Naumann et al., 2010). Such CXCL12 sequestration by CXCR7 appears to be active in mouse heart valves and human umbilical vein endothelium (Naumann et al., 2010). It appears well possible that such a scavenger function is also the basis for the altered responsiveness of the different cortical cell populations to CXCL12.

However, our findings in postnatally generated interneurons and destined for the OB are at odds with such a scenario. Three different approaches, SAGE gene expression analysis, *in situ* hybridization and immunohistochemistry demonstrated that these cells do express exclusively CXCR7 but never CXCR4 during their journey from the periventricular region into the OB. Interestingly, shRNA induced knock-down of *CXCR7* in this system leads to a phenotype that is suggestive of altered migration, altogether arguing for a CXCR4 independent, and therefore active, function of CXCR7 in this cell population. Such independent and active role has been recently reported in glial cells (Odemis et al., 2010). However, we are aware that the functional data we presented here are fragmentary and further analyses, for example based on the analysis of CXCR7 deficient mice will be necessary to formally make such a case.

Altogether, the present data are in agreement with a possible role of CXCR7 in controlling cell migration by adding another level of regulation to CXCL12/CXCR4 signaling. Whether this regulation is achieved via a ligand "sequestering" function, as suggested in the cortex, or via an active function of this receptor, as we can deduce from its role in the olfactory system, or both remains to be demonstrated.

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