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# Expression and function of CXCR7 in the mouse forebrain

Marie-Catherine Tiveron [a,*], Camille Boutin [a], Pascale Daou [a], Barbara Moepps [b], Harold Cremer [a]

[a] Institut de Biologie du Développement de Marseille Luminy, Unité Mixte de Recherche 6216, CNRS/Université de la Méditerrannée, Campus de Luminy, 13288 Marseile Cedex 09, France
[b] Department of Pharmacology and Toxicology, University of Ulm, 89081 Ulm, Germany

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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.

* Corresponding author. Tel.: +33 491 26 97 70; fax: +33 491 26 97 26.
*E-mail address:* tiveron@ibdml.univ-mrs.fr (M.-C. Tiveron).

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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