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CXCR7 Receptor Controls the Maintenance of Subpial Positioning of Cajal–Retzius Cells

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Cajal–Retzius (CR) cells are essential for cortical development and lamination. These pioneer neurons arise from distinct progenitor sources, including the cortical hem and the ventral pallium at pallium–subpallium boundary (PSB). CXCR4, the canonical receptor for the chemokine CXCL12, controls the superficial location of hem-derived CR cells. However, recent studies showed that CXCR7, a second CXCL12 receptor, is also expressed in CR cells at early developmental stages. We thus investigated the role of CXCR7 during CR cell development using multiple loss-of-function approaches. *Cxcr7* gene inactivation led to aberrant localization of Reelin-positive cells within the pallium. In addition, *Cxcr7*$^{-/-}$ mice were characterized by significant accumulation of ectopic CR cells in the lateral part of the dorsal pallium compared with *Cxcr4* knockout mice. Loss-of-function approaches, using either gene targeting or pharmacological receptor inhibition, reveal that CXCR7 and CXCR4 act both in CR positioning. Finally, conditional *Cxcr7* deletion in cells derived from *Dbx1*-expressing progenitors indicates an essential role of CXCR7 in controlling the positioning of a subpopulation of PSB-derived CR cells. Our data demonstrate that CXCR7 has a role in the positioning of hem and PSB-derived CR cells, CXCL12 regulating CR cell subpial localization through the combined action of CXCR4 and CXCR7.

Keywords: chemokine receptor, Dbx1, mouse, neocortical development, pallium–subpallium boundary, Reelin

Introduction

Cajal–Retzius (CR) cells play an essential role in establishing the laminar arrangement of cortical neurons in the mammalian cerebral cortex. CR cells are located in the marginal zone (MZ) and participate in the control of the radial migration of pyramidal neurons by secreting the extracellular protein Reelin (D'Arcangelo et al. 1995; Ogawa et al. 1995; Rice and Curran 2001; Tissir and Goffinet 2003). CR cells are generated in several regions of the cortical primordium, including the cortical hem, the septum, and the ventral pallium at the pallium–subpallium boundary (PSB) region, and migrate tangentially to populate the MZ all over the cortex (Takiguchi-Hayashi et al. 2004; Bielle et al. 2005; Yoshida et al. 2006; Griveau et al. 2010). However, each CR subtypes primarily populate specific regions of the early developing cortex, namely the rostromedial (CR cells from the septum), dorso-caudal (CR cells from the hem), and lateral (CR cells from the PSB) pallium by E12.5 (Bielle et al. 2005; Griveau et al. 2010). During their tangential migration within the MZ, CR cells remain closely apposed to the meninges which secrete the chemokine CXCL12 (Borrell and Marin 2006; Paredes et al. 2006; Ceci et al. 2010). CXCL12 interaction with its canonical receptor CXCR4 is required for retaining hem-derived CR cells within the MZ. Indeed, CXCR4 signaling defects do not affect tangential migration, but leads to the presence of ectopic CR cells in the deep cortical plate (CP) and intermediate zone (IZ) of the dorsal telencephalon at E16.5 (Borrell and Marin 2006; Paredes et al. 2006).

CXCL12 also binds to the CXCR7 (or RDC1) receptor (Burns et al. 2006; Sierro et al. 2007). CXCR7 is considered to act as a CXCL12 "scavenger" to control CXCL12 availability for interaction with CXCR4 (Balabanian et al. 2005; Luker et al. 2010). CXCR7 may also regulate CXCR4 signaling through receptor heterodimerization. Depending on the cell type, binding of CXCL12 to CXCR7-CXCR4 heterodimers affects CXCR4 signaling by altering CXCR4 binding to G protein, or enhancing CXCR4 chemotaxis (Sierro et al. 2007; Levoye et al. 2009). CXCR7 can also act on its own, for instance by influencing tumor growth and angiogenesis (Hernandez et al. 2011; Hattermann and Mentlein 2013). Moreover, during zebrafish development, CXCR7 is required to direct the migration of several cell types, including germ cells, motoneurons, and lateral line precursor cells (Dambly-Chaudiere et al. 2007; Valentin et al. 2007; Boldajipour et al. 2008; Cubedo et al. 2009). Using the zebrafish lateral line primordium as a model, 2 recent studies elegantly demonstrated that CXCR7–CXCL12 interaction is required to establish the CXCL12 gradient necessary for directional cell migration (Donà et al. 2013; Venkiteswaran et al. 2013).

In the rodent central nervous system, early CXCR7 expression is detected in progenitors of the ganglionic eminence and in preplate neurons. Preplate neurons include different transient subpopulations: CR cells that will remain in a subpial position, pioneer neurons positioned below the CR cells and the future subplate neurons (Espinosa et al. 2009). Expression of the 2 receptors, CXCR4 and CXCR7, is differentially regulated during cortical development. CXCR4 is first detected in the cortical hem at E11.5. Conversely, CXCR7 is present in all preplate cells as early as E11.5. Then, CXCR4 continues to be expressed in hem-derived CR cells and in migrating CR cells originating from the hem, whereas CXCR7 is down-regulated in CR cells from E13.5 onward (Schönemeier et al. 2008; Tiveron et al. 2010). Only a low percentage of CR cells seem to co-express CXCR4 and CXCR7, suggesting that most CXCR7-positive CR cells could be distinct from CXCR4-positive CR cells and therefore CXCL12 might act in different CR subtypes through distinct receptor combinations. The role of CXCR7 in CR cells using conditional inactivation in the Emx1 lineage reported

normal CR cell development, arguing that hem-derived CR cells are independent on CXCR7 for their localization (Wang et al. 2011). However, the specific role of CXCR7 and CXCR4 in the positioning of different CR subtypes remains unexplored.

Here, we show that constitutive deletion of *Cxcr7* leads to mislocalization of Reelin-positive cells in the deep nascent CP and IZ/subventricular zone (SVZ) of the lateral and dorsal part of the dorsal pallium. Using specific markers for CR cell subtypes, we show that the ectopic CR cells originate from the hem and PSB regions. Comparison of $Cxcr7^{-/-}$, $Cxcl12^{-/-}$, and $Cxcr4^{-/-}$ embryos indicates that deletion of *Cxcl12* or *Cxcr7* has a more severe effect on CR localization than *Cxcr4* knockout. We also show that, upon pharmacologically inhibition of CXCR4 in $Cxcr7^{-/-}$ embryos, CXCR4 signaling is not altered in the absence of *Cxcr7*. Finally, we addressed the specific function of CXCR7 by deleting *Cxcr7* in cells derived from *Dbx1*-positive progenitors and demonstrated the role of CXCR7 in the positioning of a subpopulation of PSB-derived CR cells.

In conclusion, our results demonstrate that CXCR7 regulates the positioning of CR cells originating from the cortical hem and the PSB, and hence CXCL12 controls the final location of CR cells via both CXCR4 and CXCR7 signaling.

Materials and Methods

Mice

All experimental procedures complied with the INSERM and Montpellier University animal welfare guidelines. $Cxcl12^{-/-}$ and $Cxcr4^{-/-}$ mice were described previously (Nagasawa et al. 1996). *Cxcr7* floxed mice were generated at the Mouse Clinical Institute (Illkirch, France). Exon 2, which contains the entire coding region, was flanked by loxP sites and the obtained *Cxcr7* floxed mice were then backcrossed with C57BL/6J animals (Iffa-Credo, France). The progeny was crossed with CMV-Cre deleter mice to obtain $Cxcr7^{+/-}$ males and females (Schwenk et al. 1995). The absence of *Cxcr7* mRNA expression in $Cxcr7^{-/-}$ embryos was confirmed by in situ hybridization on E14.5 sections. The following primers were used to detect the targeted and wild-type alleles:

p1: CCTGGTGCTGGCTTTGATACGCAGC, p2: CTGGTTGCTTGAGTGGTATGAAGAG,
p3: CCTTTGCAATATCCATCTGCCAACC, p4: GAGTCAATTGAGTGGGCAAGGAATG.

For lineage analysis, $Dbx1^{Cre}$ and $ROSA26^{tdTomato}$ transgenic mice were generated and genotyped as previously described (Bielle et al. 2005; Madisen et al. 2010; Teissier et al. 2010). *Cxcr7* conditional mutants were obtained by crossing $Cxcr7^{lox/lox}$ mice with double heterozygous $Dbx1^{Cre/+}/Cxcr7^{lox/+}$ mice. Cre-mediated recombination occurs at the *Cxcr7* locus only in cells derived from *Dbx1*-positive progenitors, leading to permanent and irreversible gene deletion.

In Utero Drug Administration

In vivo receptor inhibition was obtained by injecting 1–2 µL of 12.6 mM AMD3100 (CXCR4 antagonist; Sigma) or 10 µM CCX771 (CXCR7 antagonist; ChemoCentryx) in the telencephalic lateral ventricle of E12.5 $Cxcr7^{-/-}$ embryos and wild-type littermates (AMD3100) or C57BL/6J embryos (CCX771), as previously described (Borrell and Marin 2006). Vehicle solution (phosphate-buffered saline, PBS) and 10 µM CCX704 (close analog of CCX771 without binding affinity, ChemoCentryx) were used as negative controls for AMD3100 and CCX771, respectively. All injected solutions contain Oregon green 488 dextran (3 µm; Invitrogen) in order to label the injected area. Embryos were analyzed 48 h later (20 embryos obtained from 10 litters were analyzed), the quantifications were restricted to the dextran-positive region (see below). In addition, cell counts were conducted in a double-blind manner by 2 independent investigators.

Tissue Processing and RNA In Situ Hybridization

For in situ hybridization, vibratome (50 µm) or cryostat (12 µm) sections were processed as previously described (Daniel et al. 2005; Tiveron et al. 2006). *Cxcr7* probe was provided by ImaGene (clone# 4242244, BC015254). Double fluorescent in situ hybridization and immunohistochemistry experiments were performed with digoxigenin-labeled riboprobes and Fast Red (Roche Diagnostics GmbH) and/or the Tyramide amplification system (PerkinElmer) as substrates. The *Cxcr4*, *Gad1*, and *Lhx6* riboprobes have been described previously (Tiveron et al. 2006).

Immunohistochemistry

The following primary antibodies were used: mouse anti-Reelin (1 : 500, G10, Chemicon), rabbit anti-Tbr1 (1 : 500, #ab31940, Abcam), rabbit anti-Calretinin (1 : 500, Chemicon), goat anti-p73 (1 : 200, #SC-9651, SantaCruz), rabbit anti-Calbindin (1 : 1000, #CN-38a, Swant), rabbit anti-Gaba (1 : 2000 #A2052, Sigma), and rabbit anti-CXCR7 (1 : 100, #72100, Abcam). Secondary antibodies included donkey anti-mouse, anti-rabbit, and anti-goat antibodies conjugated to Alexa 488 (1 : 1000, Molecular Probes), Cy3 (1/500, Jackson Immunoresearch), or horseradish peroxidase.

Imaging and Cell Counting

Images were acquired using a Zeiss Axio Imager microscope and a Leica SPE confocal microscope. Coronal cryostat sections of 12 µm were used to quantify immunostaining and in situ hybridization. Regions were defined using morphological landmarks on cryostat and vibratome sections using Nomarsky optical sectioning for mutant analysis. Cell counting was carried within defined fields in the dorsal and lateral part of the dorsal pallium (one field = 500 × 500 µm) on 6–10 sections per embryo for mutant analysis and pharmacological studies. The number of embryo studied per genotype is indicated for each experiment. The total numbers of mutant embryos for quantification (ISH and immunohistochemistry) were, respectively: $Cxcr7^{-/-}$, $n = 14$; $Cxcr4^{-/-}$, $n = 3$; $Cxcl12^{-/-}$, $n = 6$; Cxcr7–TdTomato, $n = 3$; and $Dbx1^{Cre}/Cxcr7^{lox/lox}$, $n = 4$. For pharmacological experiments, we defined a restricted area labeled by Oregon Green dextran that was positioned at the medial level along the rostro-caudal axis, within the previous defined field (500 × 500 µm).

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed using the GraphPad Prism software. For multiple comparisons, different tests were used: non-parametric Kruskal–Wallis and Dunn's multiple comparison tests, and, one-way ANOVA with post hoc Bonferroni's multiple comparison test.

Results

Dynamic Cxcr7 Expression in CR Cells

Cxcr7 expression in the developing mouse cortex was investigated from E11.5 to E14.5 at 3 rostro-caudal levels. At E11.5 and E12.5, *Cxcr7* transcripts are localized in the subpallial ventricular zone (VZ) and in the emerging preplate in the pallium up to the medially located cortical hem (Fig. 1*A*, data not shown, Tiveron et al. 2010; Sánchez-Alcañiz et al. 2011; Wang et al. 2011). Comparison on adjacent sections of *Cxcr7* and *Reelin* expression in the preplate at E12.5 shows that *Cxcr7*-positive cells are present over the whole thickness of the preplate (Fig. 1*B*). Conversely, cells expressing *Reelin*, a pan-marker for CR cells (Meyer et al. 1999), are localized in the subpial area throughout the pallium (Fig. 1*C*). Combined

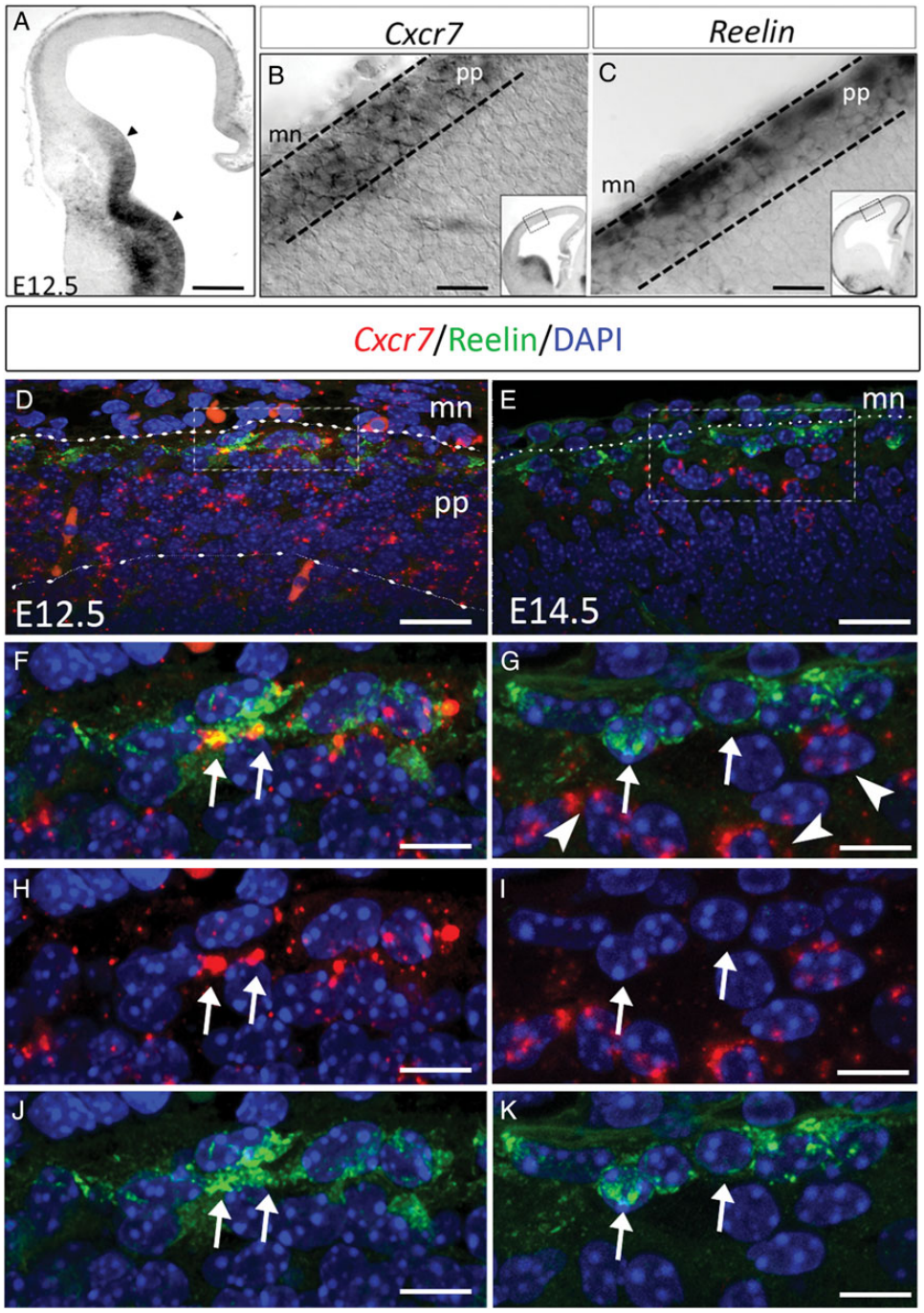

Figure 1. Dynamic regulation of *Cxcr7* expression in the developing pallium. (*A*) At E12.5, *Cxcr7* expression is observed in the preplate and ganglionic eminences (arrowheads). (*B* and *C*) Within the preplate, *Cxcr7* and *Reelin* mRNA expression profiles are overlapping. (*D*, *F*, *H*, and *J*) Double labeling with a *Cxcr7* riboprobe and anti-Reelin antibody show co-expression in CR cells at E12.5 (*F*, *H*, and J, arrows). (*E*, *G*, *I*, and *K*) At E14.5, *Cxcr7* expression (arrowheads) is confined in cells just below CR cells (arrows). mn, meninges (dotted lines); pp, preplate (dashed line). Scale bars: *A*, 160 μm; *B* and *C*, 70 μm; *D*, 25 μm; *E*, 20 μm; *F–H*, *G–I*, 8 μm.

Cxcr7 in situ hybridization and Reelin immunolabeling show that *Cxcr7* is expressed in Reelin-positive cells all over the pallium (Fig. 1*D*,*F*,*H*,*J*, arrows). In addition, double immunolabeling for CXCR7 and Reelin confirmed in situ hybridization data (Supplementary Fig. 1*A–C*). Quantitative analysis at E12.5 indicates that 81 ± 7.8% of Reelin-positive cells also expressed *Cxcr7*.

The expression patterns of *Cxcr7* and Reelin diverge at E13.5 and at E14.5 since 90 ± 9% of Reelin-positive cells are *Cxcr7*-negative (Fig. 1*E*,*G*,*I*,*K*, arrows), whereas the neurons localized just below express *Cxcr7* but not Reelin (arrowheads in Fig. 1*G* and Supplementary Movie 1).

In summary, at E12.5, *Cxcr7* is expressed in preplate neurons of the developing cortex, including CR cells. Then, *Cxcr7* is rapidly down-regulated and at E14.5, the most superficial cells are Reelin-positive and *Cxcr7*-negative (CR cells), whereas the immediately underlying cells are Reelin-negative and *Cxcr7*-positive.

CXCR7 Is Required for the Proper Localization of CR Cells in the Developing Cortex

The early expression of *Cxcr7* in CR cells suggests a possible role in CR migration and/or final localization. To address this question, a conditional (floxed) *Cxcr7* mutant mouse line was generated (Fig. 2*A*,*B*) and the entire *Cxcr7* coding region was deleted by crossing $Cxcr7^{Flox/Flox}$ mice with an ubiquitous CMV-Cre deleter mice (Schwenk et al. 1995). Immunolabeling at E12.5 and in situ hybridization at E14.5 show that CXCR7 protein as well as *Cxcr7* mRNA transcripts are absent in homozygous ($Cxcr7^{-/-}$) embryos after Cre recombination (Supplementary Fig. 1*D*–*F* and Fig. 2*C*,*D*). Homozygous mice die at E17.5 as previously reported (Sierro et al. 2007; Wang et al. 2011).

Reelin mRNA expression was used to determine the localization of CR cells in $Cxcr7^{-/-}$ and $Cxcr7^{+/+}$ brains at E12.5 and E14.5, respectively. At E12.5, *Reelin*-positive cells are found in the entire preplate with rare ectopic cells within the pallium (data not shown), suggesting that the initial tangential migration of CR cells does not rely on Cxcr7 signaling. At E14.5, all *Reelin*-positive cells are confined to the subpial zone in control animals (Fig. 2*E*,*F*,*I*,*J*). Conversely, in $Cxcr7^{-/-}$ mutants, many ectopic *Reelin*-positive cells are detected deep in the lateral (Fig. 2*G*,*K*) and dorsal (Fig. 2*H*,*L*) portions of the dorsal pallium, down to the prospective piriform cortex (Fig. 2*H*,*M*) at different rostro-caudal levels.

The CR Cell Phenotype Is More Severe in $Cxcl12^{-/-}$ Than in $Cxcr4^{-/-}$ or $Cxcr7^{-/-}$ Embryos

A previous comparison of CR cell position in $Cxcl12^{-/-}$ and $Cxcr4^{-/-}$ embryos showed no major difference (Stumm et al. 2003; Borrell and Marin 2006; Paredes et al. 2006), consistent

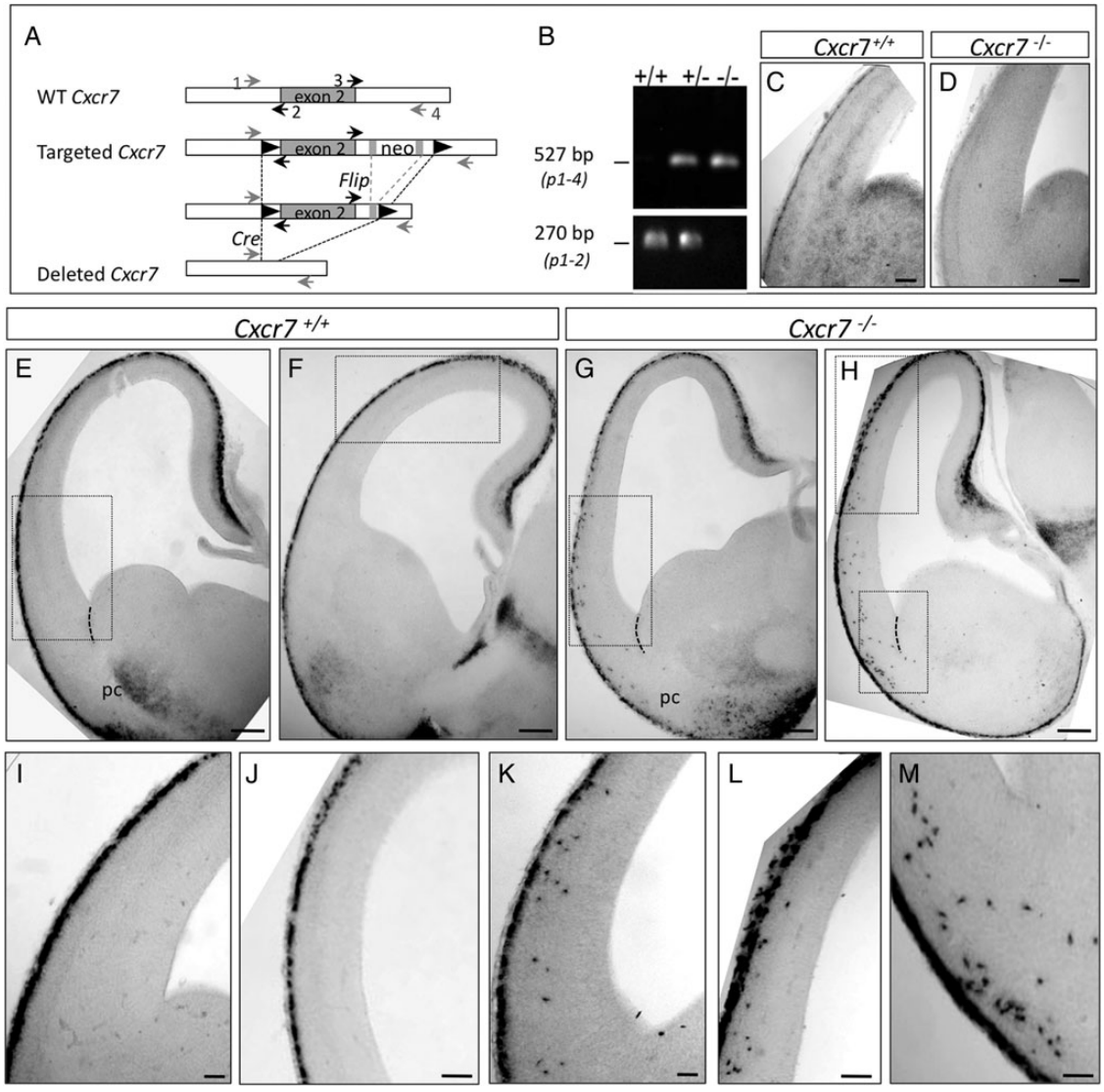

Figure 2. CXCR7 is necessary for the localization of a subpopulation of CR cells at E14.5. (*A*) Schematic representation of the wild-type (WT) *Cxcr7* locus, the targeted allele, and the deleted locus. The boxed region represents exon 2. The neomycin resistance cassette (neo) was excised by FRT/Flip recombination (gray rectangles). LoxP sites: black triangles; primers 1, 2, 3, and 4 used for genotyping are indicated by small arrows. (*B*) PCR analysis of genomic DNA from E14.5 WT (+/+), heterozygous (+/−), or homozygous (−/−) animals. (*C* and *D*) Absence of *Cxcr7* mRNA expression in $Cxcr7^{-/-}$ (*D*) compared with control brains (*C*). (*E*, *I*, *F*, and *J*) *Reelin*-positive cells are all localized in the MZ in control embryos. (*G*, *H*, and *K*–*M*) In $Cxcr7^{-/-}$ embryos, ectopic *Reelin*-positive cells are observed in the ventrolateral part (*G* and *K*) and dorsal part of the dorsal pallium (*H* and *L*) as well as in the piriform cortex (*H* and *M*) at different rostro-caudal levels (*G* and *H*). pc, piriform cortex, dotted line indicates the PSB region. Scale bars: *C* and *D*, 100 μm; *E*–*H*, 200 μm; *I*–*K*, 80 μm; *J*–*L*, 100 μm; *M*, 60 μm.

with the idea that CXCL12 acts exclusively through its canonical CXCR4 receptor. However, as this comparison was made at a relatively late developmental stage (E18.5, Stumm et al. 2003), we decided to analyze the *Cxcl12*$^{-/-}$ and *Cxcr4*$^{-/-}$ phenotypes at earlier stages in comparison with *Cxcr7* mutant (Fig. 3*A–D′*). We found ectopic CR cells scattered throughout the CP and SVZ/IZ at all levels along the rostro-caudal axis in *Cxcl12* mutants (Fig. 3*B*,*B′*) as well as in *Cxcr4* and *Cxcr7* mutants (Fig. 3*C*,*C′* and *D*,*D′*, respectively).

First, we quantified total *Reelin*-positive CR cells in the neocortex and found that *Cxcr4*$^{-/-}$ and *Cxcl12*$^{-/-}$ have fewer CR cells compared with control (36% and 37%, respectively, Fig. 3*E*). No significant decrease was observed in *Cxcr7* mutants.

Next, we compared the distribution of ectopic *Reelin*-positive cells at E14.5. We found a significant reduction in the number of *Reelin*-positive cells in the MZ for the 3 mutants ($P < 0.001$), associated with a mislocalization within the CP, and the IZ/SVZ areas (Fig. 3*F–H*, $P < 0.05$). A significant higher proportion of ectopic cells in the IZ/SVZ were found in *Cxcl12* mutants (22%) compared with *Cxcr7* (7.1%) and *Cxcr4* (5.8%) mutants (Fig. 3*H*). Thus, both receptors might be involved in CR cells positioning in the MZ. The proportion of ectopic cells represented, respectively, 15%, 25%, and 32% of total *Reelin*-positive cells in *Cxcr4*$^{-/-}$, *Cxcr7*$^{-/-}$, and *Cxcl12*$^{-/-}$ brains (Fig. 3*H*).

Then, we analyzed the repartition of ectopic cells along the dorso-ventral axis of the neocortex to determine the preferential

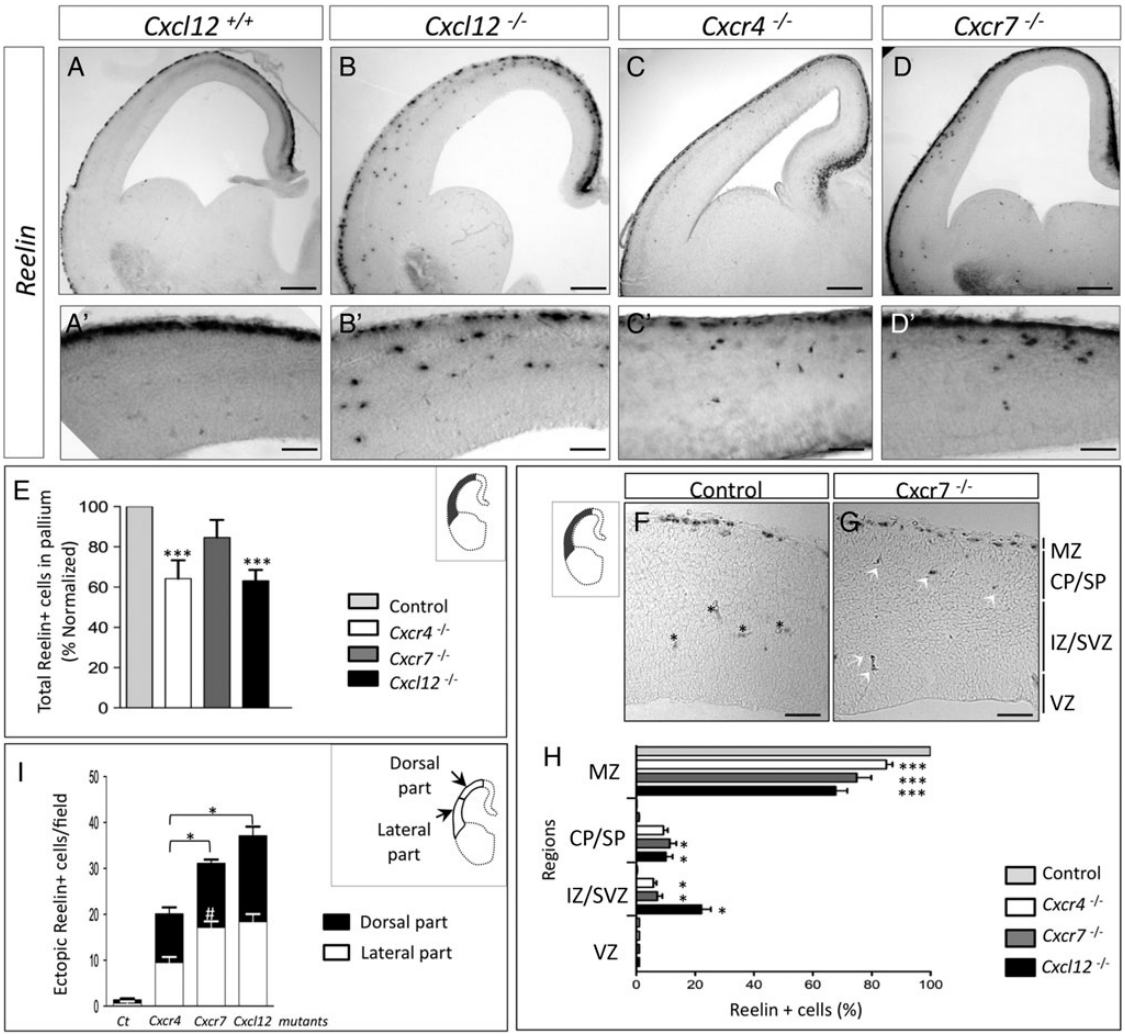

Figure 3. Comparison of abnormal localization of CR cells within *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$, and Cxcr7$^{-/-}$ mutants. (*A–C*) *Reelin* mRNA expression in E14.5 embryos. *Reelin*-positive cells are observed in the deep cortical layers of the dorsal and lateral part of neocortex in *Cxcl12*$^{-/-}$ (*B*), *Cxcr4*$^{-/-}$ (*C*), and *Cxcr7*$^{-/-}$ (*D*) brains. (*A′–D′*) Higher magnifications of the lateral part of dorsal pallium from (*A–D*) showing the distribution of ectopic cells. (*E*) Quantitative analysis of the total number of *Reelin*-positive cells in the mutant cortices. We found that both Cxcl12$^{-/-}$ and Cxcr4$^{-/-}$ mutants had ∼36% fewer *Reelin*-positive cells in the dorsal pallium when compared with controls and *Cxcr7*$^{-/-}$ ($n = 3$–4 brains per condition, 10 slices per brain; $^{***}P < 0.001$, one-way ANOVA). (*F* and *G*) Coronal cryostat sections of E14.5 Control (*F*) and *Cxcr7*$^{-/-}$ brains (*G*) from dorsal pallium (black area) after *Reelin* in situ hybridization. Asterisks: blood cells; arrowheads: ectopic cells. (*H*) Quantification of the distribution of *Reelin*-expressing cells in control and mutants in the thickness of the pallium, from the MZ to the ventricular zone VZ. The laminar distribution of ectopic cells shows localization in deeper layers: CP and IZ/SVZ with a large proportion for the *Cxcl12* mutant. No ectopic cells were observed in the VZ. Histograms show average ± SEM (*Cxcl12*$^{-/-}$: $n = 4$, *Cxcr4*$^{-/-}$: $n = 3$, and Cxcr7$^{-/-}$: $n = 8$). Non-parametric Kruskal–Wallis and Dunn's multiple comparison tests were performed with *P*-values denoted as follows: $^{*}P < 0.05$, $^{***}P < 0.001$. (*I*) Quantification of *Reelin*-ectopic cells in the lateral and dorsal part of the dorsal pallium in wild-type (control) and *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$, and *Cxcr7*$^{-/-}$ brains at E14.5. Significantly more ectopic *Reelin*-positive cells are observed in the lateral part of the dorsal pallium of *Cxcr7*$^{-/-}$ compared with *Cxcr4*$^{-/-}$ embryos ($^{\#}P < 0.05$). The total number (dorsolateral + ventrolateral parts) of ectopic *Reelin*-positive cells is significantly higher in *Cxcl12*$^{-/-}$ and *Cxcr7*$^{-/-}$ embryos compared with *Cxcr4*$^{-/-}$ animals. Non-parametric Kruskal–Wallis and Dunn's multiple comparison tests were performed with *P*-value, $^{*}P < 0.05$ (10 slices per brain). MZ, marginal zone; CP/SP: cortical plate/subplate; IZ/SVZ: intermediate zone/subventricular zone; VZ: ventricular zone. Scale bars: *A–D*, 150 μm; *A′* and *B′*, 70 μm; *C′* and *D′*, 90 μm; *F* and *G*, 120 μm.

localization site. Quantification analysis indicates that the chemokine ablation has a stronger effect on CR positioning than *Cxcr4* knockout (respectively, *Cxcl12*: 37.7 ± 3.2 ectopic *Reelin*-positive cells/field, $n = 4$; *Cxcr4*: 19.4 ± 1.6, $n = 3$, $P < 0.05$) (Fig. 3*I*). Conversely, comparison between *Cxcl12*$^{-/-}$ and *Cxcr7*$^{-/-}$ embryos reveals comparable distribution of ectopic *Reelin*-positive cells in the dorsal or the lateral regions (*Cxcl12*: 37.7 ± 3.2 ectopic *Reelin*-positive cells/field, $n = 4$; *Cxcr7*$^{-/-}$: 33.1 ± 1.4, $n = 8$, Fig. 3*I*). In addition, *Cxcr7* $^{-/-}$ displays a higher number of ectopic *Reelin*-positive cells compared with *Cxcr4*$^{-/-}$ (respectively, 25 ± 1.6, $n = 3$ and 33.1 ± 1.4, $n = 14$, $P < 0.05$, Fig. 3*I*): this difference was mainly due to an accumulation of ectopic *Reelin*-positive cells within the lateral part (L) (*Cxcr4*$^{-/-}$: 9.5 ± 1.1, $n = 3$ vs. *Cxcr7*$^{-/-}$: 18.2 ± 1.2, $n = 8$, $^{\#}P < 0.001$, Fig. 3*I*). Our data not only suggest that CXCR7 plays an important role (together with *Cxcr4*$^{-/-}$) in maintaining CR cells in the MZ, but also might acts particularly in the lateral region of the developing neocortex.

At E12.5, ectopic *Reelin*-positive cells are scattered deep within the parenchyma of *Cxcl12*$^{-/-}$ brains, but only in the lateral portion of the dorsal pallium (Supplementary Fig. 2). These cells are unlikely to be hem-derived CR cells, because at this developmental stage few cells have reached the lateral part of the dorsal pallium (Takiguchi-Hayashi et al. 2004; Borrell and Marin 2006; Yoshida et al. 2006; Tiveron et al. 2010), which is mostly populated by PSB-derived CR cells (Bielle et al. 2005; Griveau et al. 2010).

In conclusion, the phenotypes of *Cxcl12*, *Cxcr4*, and *Cxcr7* mutants indicate that both receptors are involved in CR cell positioning originated from the hem. In addition, preferentially mislocalized CR cells in the lateral part of the dorsal pallium in *Cxcr7* mutants is consistent with the hypothesis that CXCR7 plays a specific role in the superficial localization of CR cells in this region.

Characterization of Ectopic Reelin-Positive Cells

The difference in the phenotypes observed between *Cxcr7* and *Cxcr4* mutants is intriguing and raises the question of the nature and origin of ectopic cells in *Cxcr7* mutants.

The ectopic localization of *Reelin*-positive cells in *Cxcr7*$^{-/-}$ telencephalon could be the result of a premature *Reelin* expression in interneurons. During normal development, Reelin is expressed only after E15.5 in interneurons that migrate tangentially in the MZ and the IZ/SVZ (Alcántara et al. 1998). We combined double and triple immunolabeling for Reelin with specific interneuron markers (Calbindin, GABA, *Lhx6*, or *Gad1*) and we did not detect co-labeling with Reelin-positive ectopic cells in *Cxcr7*$^{-/-}$ embryos at E14.5 (Fig. 4*B–G* and Supplementary Fig. 3), thus excluding premature expression of Reelin in interneurons.

Moreover, co-expression of Reelin and Tbr1, a transcription factor expressed in glutamatergic pallial cells (Hevner et al. 2001), demonstrates that the ectopic Reelin-positive cells originate from the pallial region (88.3 ± 7.2%, Fig. 5*B,C* —arrows and *P*).

To further characterize the phenotype and origin of the ectopic Reelin-positive cells in *Cxcr7*$^{-/-}$ embryos, we explored the expression of Calretinin (normally detected in CR cells derived from the cortical hem and PSB) and p73 (a marker of CR cells derived from the cortical hem and the septum, but not from the PSB) (Takiguchi-Hayashi et al. 2004; Bielle et al. 2005; Hanashima et al. 2007; Griveau et al. 2010). At E14.5, p73 is co-expressed in most of Reelin-positive cells in the MZ both in control and *Cxcr7*$^{-/-}$ mice (Fig. 5*D–F*, arrows). However, only 56.5 ± 4.7% of ectopic Reelin-positive cells express p73 in *Cxcr7*$^{-/-}$ embryos (Fig. 5*P*). Note that p73-negative ectopic Reelin cells are also negative for the interneuron marker Calbindin (Fig.4*D–D′*, arrowhead). In addition, 69.4 ± 9.1% of ectopic Reelin-positive cells expressed also Calretinin in *Cxcr7*$^{-/-}$ embryos (Fig. 5*G–H′*—arrows and *P*).

Next, we analyzed *Cxcr4*, as a hem-derived CR cell marker, and found its expression in CR cells located in the MZ, comparable in *Cxcr7*$^{-/-}$ and control embryos (Fig. 5*I*—*O*, arrows). However, in ectopic positions (CP and IZ/SVZ), both *Cxcr4*-positive and *Cxcr4*-negative/Reelin-positive cells are detected in the dorsal and lateral territories of the developing cortex (Fig. 5*M–O*). Indeed, 59.9 ± 5.6% of ectopic Reelin-positive cells co-expressed *Cxcr4* (Fig. 5*P*), which is consistent with the percentage of p73-positive ectopic cells (56.5%) and likely reflects a cortical hem origin.

These findings clearly show that ectopic Reelin-positive cells are not interneurons, but CR cells as they express Tbr1 and Calretinin but do not express interneuron markers. We show that more than half (~60%) of Reelin-ectopic cells originate from the cortical hem (p73+ *Cxcr4*+), while the remaining (~40%) p73- and *Cxcr4*-negative Reelin-ectopic cells show a PSB molecular identity.

CXCR7 in the Dbx1 Lineage Is Involved in the Positioning of a Subpopulation of CR Cells

PSB-derived CR cells are produced by progenitors that express the *Dbx1* gene and predominately populate the ventrolateral part of the pallium (Bielle et al. 2005). We analyzed *Cxcr7* expression in Dbx1-derived cells using *Dbx1*^{cre}/*Rosa26*^{tdTomato} embryos. At E12.5 and E14.5, *Cxcr7*-tdTomato-positive cells are observed predominantly in the prospective piriform cortex and the MZ of the lateral part of the dorsal pallium (Fig. 6*A–C*, $n = 3$). At E14.5, they represent 63 ± 5.7% of all *tdTomato* labeled cells in the subpial position, consistent with a preferential localization of Dbx1-derived CR cells within the lateral pallium at earlier embryonic stages (Bielle et al. 2005; Griveau et al. 2010). To investigate the specific function of CXCR7 in the Dbx1 lineage, *Dbx1*^{cre} mice were crossed with *Cxcr7*$^{lox/lox}$ mice to inactivate *Cxcr7* specifically in Dbx1-derived cells. In *Dbx1*^{cre}/*Cxcr7*$^{lox/lox}$ mutant embryos, ectopic mRNA *Reelin*-positive cells are specifically observed in the lateral portion of the dorsal pallium, although they are less numerous (4.5 ± 0.5 ectopic cells/field, $n = 4$, $P < 0.05$, Fig. 6*D–F*) compared with *Cxcr7*$^{-/-}$ embryos (18.2 ± 1.2, $n = 14$, Fig. 3*I*). In addition, the nature of the ectopic cells was assessed using co-detection of Reelin/p73 and Reelin/Calbindin (Fig. 6*G,H*). None of ectopic cells expressed Calbindin, confirming that these cells were not interneurons. The absence of p73 expression demonstrates their PSB origin.

We conclude that CXCR7 activity in *Dbx1*-positive progenitors regulates the positioning of a subpopulation of CR cells derived from the PSB. Such regulation is likely independent of CXCR4, since no *Cxcr4* expression is detected in these cells (Tiveron et al. 2010).

CXCR4 Function in Cxcr7$^{-/-}$ *Embryos*

To further confirm CXCR7 role in CR positioning, CCX771 (a specific pharmacological inhibitor of CXCR7) or CCX704

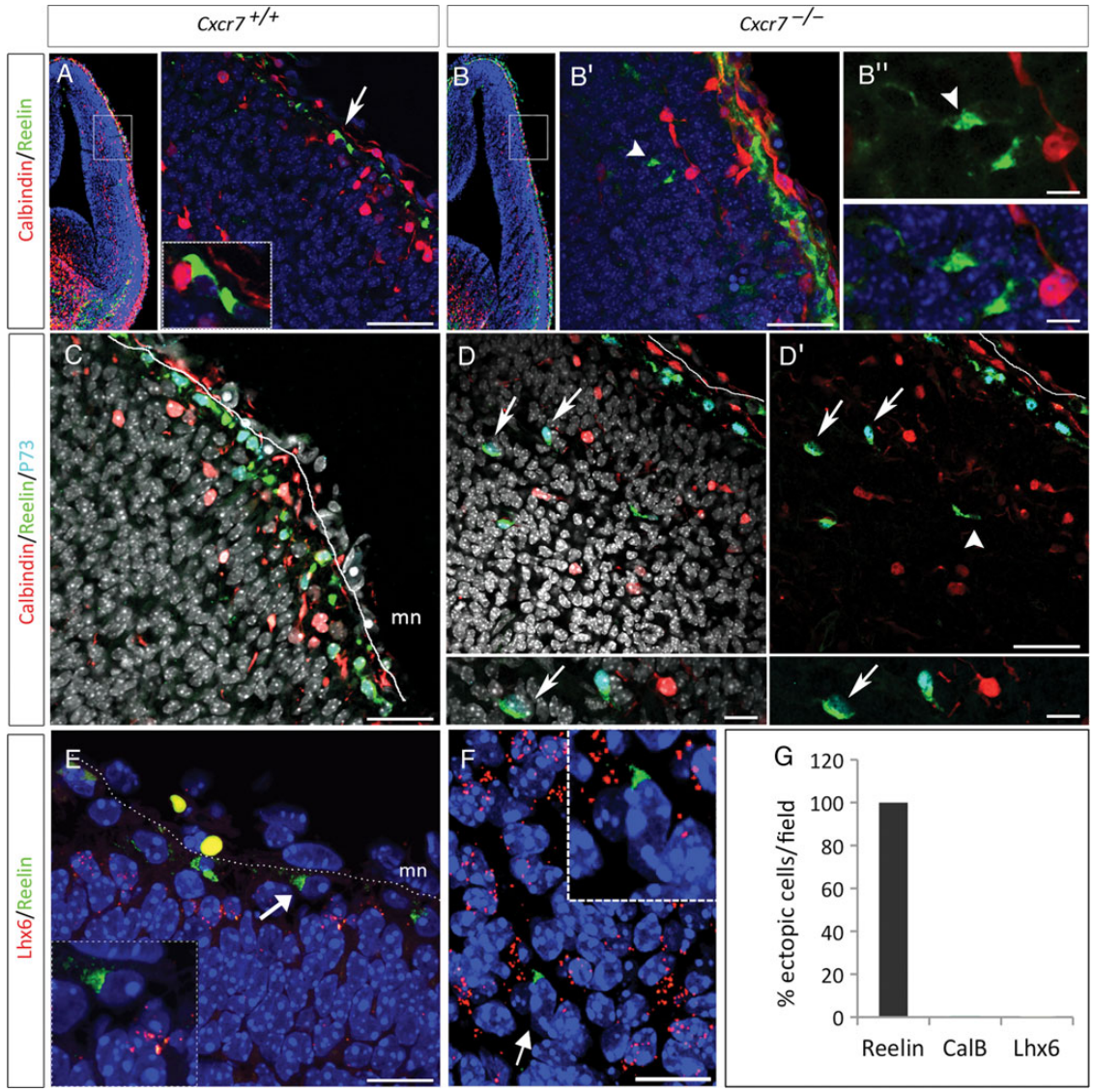

Figure 4. Characterization of ectopic Reelin-positive cells in the cortex of *Cxcr7*$^{-/-}$ E14.5 embryos. Immunohistochemistry for Reelin/Calbindin (*A* and *B″*) or Reelin/Calbindin/p73 (*C* and *D′*) on coronal sections of *Cxcr7*$^{-/-}$ mutant. (*A* and *B″*) Immunostaining for Calbindin, a marker of immature interneurons. (*A′* and *B′*) High magnifications of the dashed white boxes in *A* and *B*. In control, the CR cells are negative for Calbindin in the MZ (arrow and dashed box). (*B* and *B″*) In Cxcr7$^{-/-}$ embryos, the ectopic Reelin-positive cells do not express Calbindin (arrowhead). (*C* and *D′*) Immunostaining for Calbindin, p73, and Reelin shows that double-positive Reelin/p73 cells are not labeled with Calbindin (arrows, *G*). In addition, example of Reelin-positive/p73-negative cell that is not Calbindin positive (*D′*, arrowhead). (*E* and *F*) *Lhx6* and Reelin expression in *Cxcr7*$^{+/+}$ and *Cxcr7*$^{-/-}$ mutant. The absence of co-expression of *Lhx6* and Reelin demonstrates that ectopic cells are not interneurons. (*G*) Quantification of ectopic cells that co-express Reelin with Calbindin (CalB) or *Lhx6* in *Cxcr7*$^{-/-}$ mutant: neither *Lhx6* or CalB were co-expressed with Reelin. Scale bars: *A′*, 90 μm; *B′*, *C*, *D*, and *D′*, 60 μm; *B″*, 15 μm; *E*, 30 μm; *F*, 15 μm.

(a control compound unable to bind CXCR7) was injected in the lateral ventricle of E12.5 wild-type C57BL/6J embryos in utero and then the location of Reelin-positive cells was assessed and quantified in a defined area corresponding to the injection site (dextran labeled) after 2 days, by immunohistochemistry.

CCX771 injection is associated with the presence of ectopic Reelin-positive cells specifically in the lateral part of the dorsal pallium compared with CCX704 (6.5 ± 1.2 and 2.1 ± 0.3 ectopic cells/field, respectively; $P < 0.004$, $n = 3$/each group; Fig. 7*A*,*B*,*G*). We demonstrate that pharmacological inhibition of Cxcr7 signaling results in a mislocalization of CR cells as observed in *Cxcr7*$^{-/-}$ embryos (7.5 ± 0.5 ectopic cells/field, Fig. 7*G*).

To assess whether CXCR4 are functional in *Cxcr7* mutants, CXCR4 function was blocked by injecting the specific inhibitor AMD3100 in the lateral ventricle of E12.5 *Cxcr7*$^{-/-}$ and wild-type littermates. At E14.5, AMD3100-treated wild-type embryos show a disorganized MZ and ectopic Reelin-positive cells in the MZ and CP of the dorsal pallium (Fig. 7*C*,*E*), compared with *Cxcr7*$^{+/+}$ vehicle injected (data not shown). Quantification of Reelin-ectopic cells is significantly higher in AMD3100-injected *Cxcr7*$^{-/-}$ embryos compared with wild-type littermates (*Cxcr7*$^{-/-}$/AMD3100: 15.5 ± 1.3 and *Cxcr7*$^{+/+}$/AMD3100: 11.4 ± 1.1 of ectopic cells/field, $n = 3$/group, $^{*}P < 0.05$, Mann–Whitney test; Fig. 7*D*,*F*, *G*). The inhibition of CXCR4 in the *Cxcr7* mutant causes an increase of Reelin-ectopic cells when compared with *Cxcr7* mutant (*Cxcr7*$^{-/-}$: 7.65 ± 0.5, $^{***}P < 0.001$), reflecting that CXCR4 receptors remain functional in the absence of *Cxcr7*. Altogether, these data indicate that both receptors are required for proper CR cell localization.

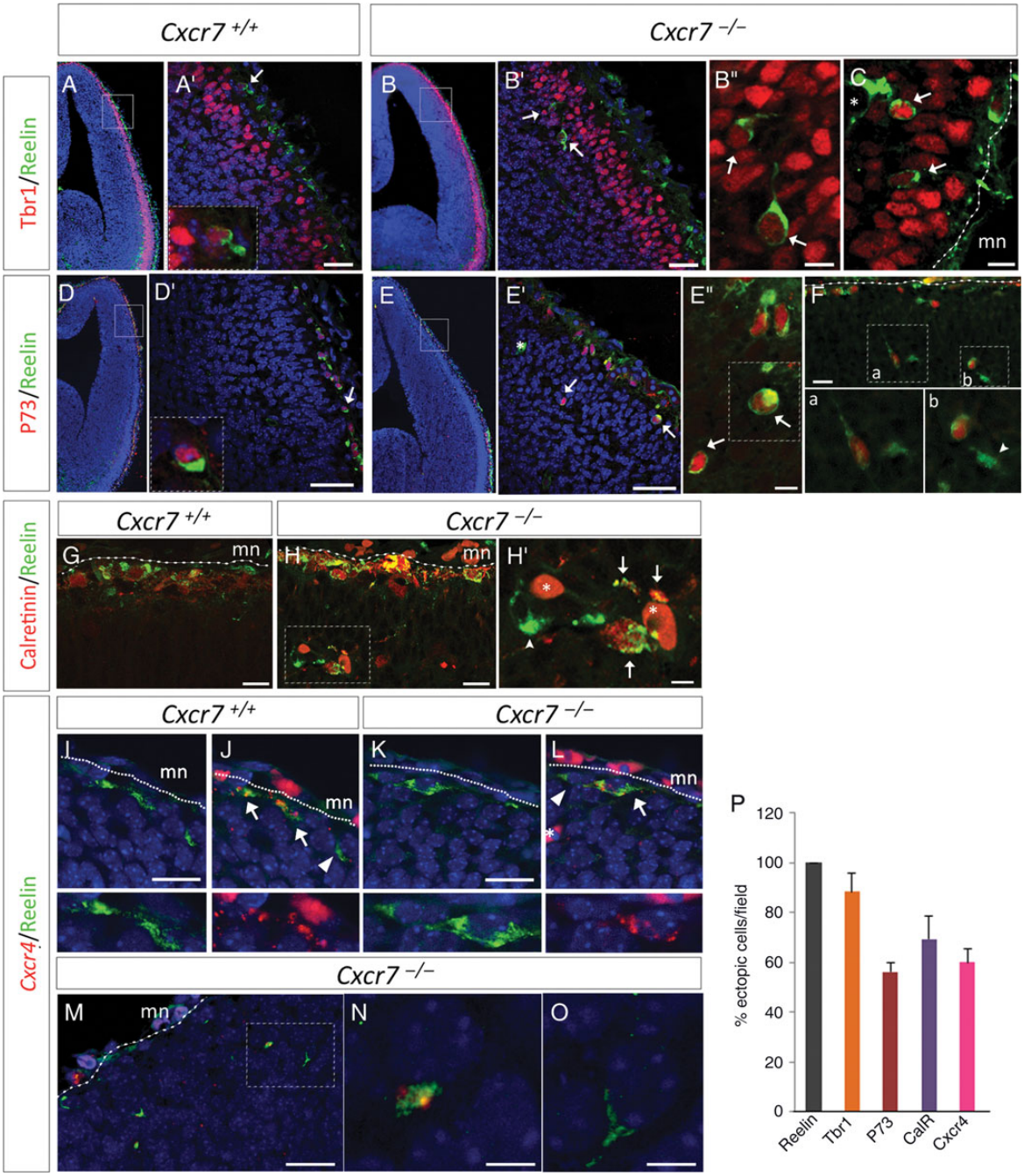

Figure 5. Pallial origin of ectopic Reelin-positive cells. Immunostaining for Tbr1 (*A*–*C*), p73 (*D*–*F*), Calretinin (*G* and *H*′), and *Cxcr4* (*I*–*O*). (*A*′, *B*′, *D*′, and *E*′) High magnifications of the dashed white boxes in *A*, *B*, *D*, and *E*. (*A* and *A*′) Reelin-positive cells express the pallial marker Tbr1 (arrow) in the MZ in control. (*B* and *C*) Ectopic Reelin-positive cells express Tbr1 in *Cxcr7*$^{-/-}$ mutant (arrows). (*B*″) High magnification of *B*′ illustrating double Reelin/Tbr1-positive cells. (*C*) Another example showing Tbr1 immunoreactivity in 2 ectopic Reelin-positive cells. (*D* and *D*′) In E14.5 *Cxcr7*$^{+/+}$ embryos, all Reelin-positive cells express p73 (marker of cortical hem- and septum-derived CR cells) in the MZ. (*E* and *E*″) In the dorsal pallium of E14.5 *Cxcr7*$^{-/-}$ embryos, ectopic Reelin-positive and p73-positive cells are observed (arrows, magnification in *E*″). (*F*) Another example showing an ectopic Reelin-positive/p73-negative (arrowhead). (*G*) In E14.5 *Cxcr7*$^{+/+}$ embryos, Reelin- and Calretinin-positive cells were observed in the MZ. (*H* and *H*′) In *Cxcr7*$^{-/-}$ embryos, ectopic Reelin-positive cells in the CP express Calretinin. (*H*′) Higher magnification of the boxed area in *H* shows a Reelin-positive/Calretinin-negative cell (arrowhead) among cells that co-express Reelin and Calretinin (arrows). (*I* and *O*) Coronal sections of control (*I* and *J*) and *Cxcr7*$^{-/-}$ mutant mice (*K* and *O*) at E14.5 analyzed by ISH *Cxcr4* combined to immunostaining for Reelin. (*I* and *J*) Reelin-positive cells that co-express *Cxcr4* mRNA (arrows) are intermingled with Reelin-positive/*Cxcr4*-negative cells (arrowhead) in the CR layer. (*M*–*O*) Analysis of *Cxcr4* and Reelin expression in the depth of the CP (*M*). Higher magnification of the boxed area in *M* showing an ectopic cell that co-expresses *Cxcr4* and Reelin (*N*) next to an ectopic Reelin-positive and *Cxcr4*-negative cell (*O*). (*P*) Quantification of ectopic cells that co-express Reelin and Tbr1 or p73 or Calretinin (CalR) or *Cxcr4* in *Cxcr7*$^{-/-}$ E14.5 embryos. mn, meninges (dotted lines); asterisks, blood cells. Scale bars: *A*′, *B*′, *D*′, and *E*′, 50 μm; *B*″, *C*, and *E*″, 10 μm; *F*–*H*, 30 μm; *H*′, 25 μm; *I*–*M*, 30 μm; *N* and *O*, 8 μm.

Discussion

CXCL12/CXCR4 signaling controls the position of hem-derived CR cells in the MZ. In the present study, we demonstrate that loss of *Cxcr7* is associated with ectopic location of Reelin-positive cells in the pallium. Comparison of *Cxcl12*$^{-/-}$, *Cxcr4*$^{-/-}$, *Cxcr7*$^{-/-}$ embryos indicates that both receptors are

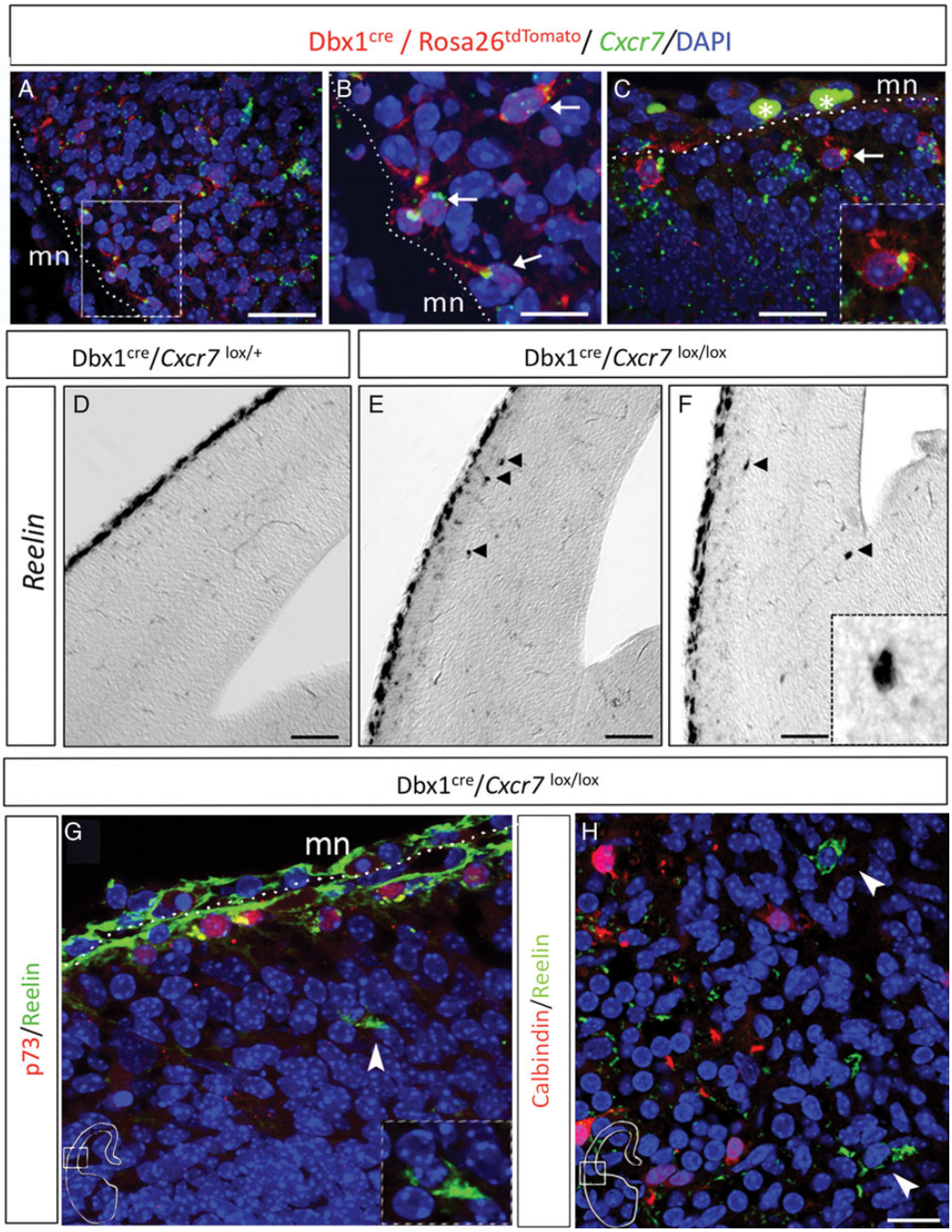

Figure 6. Distinct function of CXCR7 in CR cells derived from *Dbx1*-positive progenitors. (*A–C*) Expression of *Cxcr7* mRNA in *Dbx1*^{cre}/*Rosa26*^{tdTomato} embryos shows *Cxcr7*/tdTomato co-localization (arrows) in the lateral part of the dorsal pallium at E12.5 (*A* and *B*) and E14.5 (*C*). Asterisk indicates blood cells in the meninges (*D–F*) *Reelin* mRNA expression at E14.5 in Dbx1^{Cre}/Cxcr7$^{lox/lox}$ embryos shows ectopic *Reelin*-positive cells in the lateral pallium in different sections (*E* and *F*, arrowheads) compared with wild-type embryos (*D*). In (*F*), the inset represents a high magnification of an ectopic *Reelin*-positive cell. (*G* and *H*) Reelin-positive cells in Dbx1^{Cre}/Crcr7$^{lox/lox}$ do not express p73 or Calbindin in the lateral part of the dorsal pallium (arrowhead). mn, meninges (dotted lines). Scale bars: *A*, 40 μm; *B* and *C*, 15 μm; *D*, 30 μm; *E* and *F*, 50 μm; *G*, 30 μm; *H*, 25 μm.

involved in CXCL12 signaling with a major contribution by CXCR7. Moreover a decrease of total CR cells is retrieved in *Cxcl12* and *Cxcr4* mutants but not in *Cxcr7*, indicating a distinct function for both receptors.

Finally, the specific *Cxcr7* ablation in cells derived from *Dbx1*-positive progenitors shows that CXCR7 is required in the Dbx1 lineage to position PSB-derived CR cells. We conclude that CXCR7 regulates the positioning of both hem- and PSB-derived CR cells while CXCR4 controls only the positioning of hem-derived CR cells.

CXCR7 Is Involved in the Positioning of Hem-Derived CR Cells

Cxcr4 and *Cxcr7* receptor knockout mice display mislocalized Reelin-positive cells. Interestingly, in *Cxcl12* mutants, CR cell

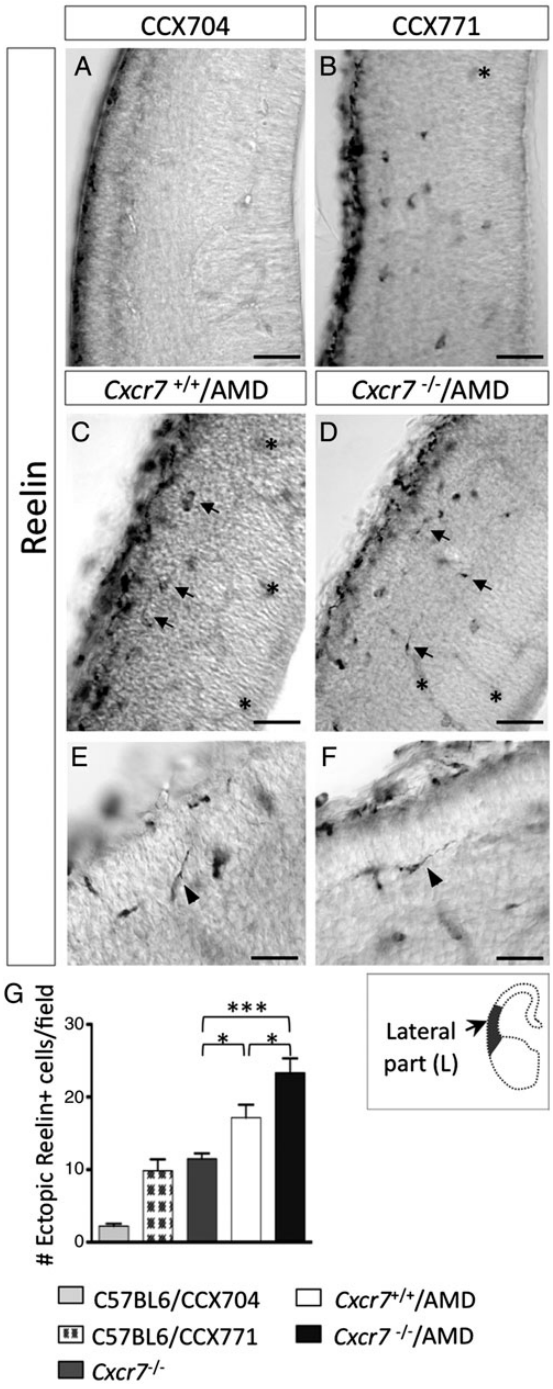

Figure 7. Pharmacological inhibition of CXCR7 or CXCR4. (*A–F*) Reelin immunolabeling at E14.5 after intraventricular injection of the CXCR7 or CXCR4 antagonist at E12.5: (*A*) control (C57BL/6J) embryos treated with CCX704, (*B*) C57BL/6J animals treated with the CXCR7 antagonist CCX771, (*C* and *E*) $Cxcr7^{+/+}$ animals treated with the CXCR4 antagonist AMD3100 (AMD), and (*D* and *F*) $Cxcr7^{-/-}$ animals treated with AMD3100. CR cells were confined to the MZ in control embryos, but after pharmacological blocking of CXCR7 with CCX771, some CR cells were scattered within the CP (compare *A* with *B*). (*C*) AMD3100-treated $Cxcr7^{+/+}$ embryos show ectopic Reelin-positive cells in the dorsal pallium (arrows). (*D*) The effect of AMD3100 treatment in $Cxcr7^{-/-}$ embryos is stronger in the lateral part of the dorsal pallium (arrows). (*E* and *F*) High magnification showing ectopic Reelin-positive cells (arrowheads). (*G*) Quantification of ectopic cells in a defined field (500 × 500 μm lateral area). One-way ANOVA test, with post hoc Bonferroni's multiple comparison test, was performed with *P*-values denoted as follows: $^{*}P < 0.05$, $^{***}P < 0.001$ ($n = 4$–5 brains per condition). Asterisks indicate blood vessels. Scale bars: *A–D*, 100 μm; *E* and *F*, 30 μm.

mispositioning is (1) more pronounced compared with *Cxcr4* mutants and (2) comparable to *Cxcr7* mutants. This finding suggests a redundancy in the function of both receptors in CR cell positioning. *Cxcr7* is expressed in 90% of CR cells at E12.5 when most CR cells have migrated tangentially from different sources, including hem, PSB, and septum, to cover the cortical surface. This present work shows that, in addition to CXCR4 signaling, CXCR7 is also required for proper location of hem-derived CR cells. First, in $Cxcr7^{-/-}$ embryos, the high proportion of ectopic CR cells that express the p73 marker (56%) or *Cxcr4* (59%) confirms that >50% of ectopic Reelin-positive cells originate from the hem.

The functional relationship between CXCR7 and CXCR4 in hem-derived CR cells is not completely elucidated, but our data strongly suggest that CXCR7 might have a non-cell autonomous effect through CXCR4.

Indeed, a down-regulation of CXCR4 expression has been shown after *Cxcr7* inactivation in interneurons that express both receptors (Schönemeier et al. 2008; Tiveron et al. 2010; Sánchez-Alcañiz et al. 2011; Abe et al. 2014). Indeed, in $Cxcr7^{-/-}$ mice, CXCR4 can still bind to an excess of CXCL12, thereby inducing excessive CXCR4 activation and internalization from the neuronal surface at E14.5 and E16.5 stages (Sánchez-Alcañiz et al. 2011; Abe et al. 2014). These data are consistent with the fact that deficiency of one chemokine receptor can lead to increase in the expression of its ligand, which would then promote internalization of the other receptor(s) for that ligand (Cardona et al. 2008). Our findings, in $Cxcr7^{-/-}$ embryos after CXCR4 antagonist, intraventricular injections at early developmental stages demonstrate an aggravation of $Cxcr7^{-/-}$ phenotype and indicate that CXCR4 might still be present in $Cxcr7^{-/-}$ embryos. In CR cells, CXCR4 down-regulation might not occurred at this early stage (E12.5), conversely as it is demonstrated at E14.5 and even more pronounced at E16.5 in interneurons (Sánchez-Alcañiz et al. 2011; Abe et al. 2014).

Many attempts to investigate the CXCR4 protein levels have not yielded conclusive results owing to nonspecific staining of the antibodies tested (personal observations and Fischer et al. 2008). Therefore, we cannot exclude non-cell autonomous effect of CXCR7 in hem-derived CR cells.

CXCR7 Plays a Role in a Subpopulation of CR Cells Originating from Dbx1 Progenitors

Defects in CR cells positioning in $Cxcr7^{-/-}$ and $Cxcl12^{-/-}$ mutants are more severe compared with $Cxcr4^{-/-}$ mutants, suggesting that the hem-derived CR cell subpopulation is not the only one affected.

Mapping studies indicate that CR cells originating from the PSB populate the lateral pallium and the piriform cortex, whereas hem- and pallial septum-derived CR cells invade preferentially the dorsal and rostral pallium, respectively (Bielle et al. 2005; Yoshida et al. 2006; Griveau et al. 2010).

The presence of ectopic Reelin-positive cells in the lateral part of the dorsal pallium of $Cxcr7^{-/-}$ embryos is therefore consistent with a role for CXCR7 in the subpial location of CR cells originating from the PSB, as also suggested by the fact that 40% of ectopic cells do not express p73 and *Cxcr4*. Moreover, conditional ablation of *Cxcr7* only in the *Dbx1* cell lineage impaired the localization of a small population of CR cells, preferentially in the lateral part of the pallium and almost

all ectopic Reelin cells do not express p73, a signature of PSB-derived CR cells (Hanashima et al. 2007; Griveau et al. 2010). The smaller number of ectopic CR cells in this mouse strain compared with *Cxcr7*$^{-/-}$ embryos in the same brain area might be explained by the fact that Cre recombination does not occur in the earliest cells derived from the *Dbx1* progenitors due to delayed Cre activity (Bielle et al. 2005). In addition, lineage analysis demonstrates that a large proportion of neurons derived from the *Dbx1* progenitors express *Cxcr7* at E12.5 and E14.5, and transcriptome analysis of CR cells derived from the *Dbx1* progenitors revealed strong expression of *Cxcr7* at early stages (Griveau et al. 2010; Ugo Borello and Alessandra Pierani, personal data). Taken together, these data strongly argue in favor of a cell autonomous effect of CXCR7 in positioning of PSB-derived CR cells.

The question of which cellular mechanisms are affected remains to be answered: in *Cxcr7*$^{-/-}$ mice, do CR cells fail to migrate or do they migrate, but are then unable to maintain their pial position (as in *Cxcr4*$^{-/-}$ mice)? The absence (or very low incidence) of ectopic cells in the dorsal pallium at E12.5, when migration is highly active and CR cells from different sources are invading the entire surface of the cortex, argues against the hypothesis of migration failure. However, *Cxcr7* deficiency might partially affect CR cell motility as reported for *Cxcr7*$^{-/-}$ interneurons, which develop shorter leading processes and are less motile, resulting in abnormal positions within the CP (Wang et al. 2011). On the other hand, receptor antagonist injection at E12.5 has an effect on CR cell positioning, suggesting that even after migration completion, CXCR7–CXCL12 interaction is required to retain CR cells in their subpial location. Based on our results, we propose that Cxcr7 signaling is not necessary for CR cell migration but is essential for maintaining CR cells in the MZ.

In addition, we propose that CXCR7 may also have a function of its own, independent of regulating CXCR4 signaling, by maintaining the subpial location of PSB-derived CR cells. CXCR7 and CXCR4 would then play complementary roles in, respectively, the lateral and dorsal portions of the dorsal pallium. CR cell streams originating from the hem and the PSB overlap only in the dorsolateral regions (Bielle et al. 2005; Yoshida et al. 2006) and appropriate cell density is obtained through cell interactions (Villar-Cerviño et al. 2013). Thus, a defect in either population may be partly compensated by the other one (Griveau et al. 2010).

Up to now, CXCL12 has been considered to influence only the migration of hem-derived CR cells (Borrell and Marin 2006; Paredes et al. 2006) and other CR populations were thought to be insensitive to CXCL12 signaling. Here, we demonstrate a novel role of CXCR7 in CR cells and complete the scheme of regulation that involved both receptors and include several CR populations.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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