CXCL12/CXCR4 signaling is critical for cortical interneuron migration and their final laminar distribution. No information is yet available on CXCR7, a newly defined CXCL12 receptor. Here we demonstrated that CXCR7 regulated interneuron migration autonomously, as well as nonautonomously through its expression in immature projection neurons. Migrating cortical interneurons coexpressed CXCR4 and CXCR7, and CXCR7−/− and CXCR4−/− mutants had similar defects in interneuron positioning. Ectopic CXCL12 expression and pharmacological blockade of CXCR4 in CXCR7−/− mutants showed that both receptors were essential for responding to CXCL12 during interneuron migration. Furthermore, live imaging revealed that CXCR4−/− and CXCR7−/− mutants had opposite defects in interneuron motility and leading process morphology. In vivo inhibition of Gz (i/o) signaling in migrating interneurons phenocopied the interneuron lamination defects of CXCR4−/− and CXCR7−/− mutants. On the other hand, CXCL12 stimulation of CXCR7, but not CXCR4, promoted MAP kinase signaling. Thus, we suggest that CXCR4 and CXCR7 have distinct roles and signal transduction in regulating interneuron movement and laminar positioning.

INTRODUCTION

Cortical GABAergic interneurons are generated in multiple progenitor zones of the subpallial (subcortical) telencephalon, including the lateral, medial, and caudal ganglionic eminences (LGE, MGE, and CGE) (Anderson et al., 1997a; Butt et al., 2005; Fogarty et al., 2007; Marin and Rubenstein, 2003; Pleasure et al., 2000; Sussel et al., 1999; Wonders and Anderson, 2006). The specification, differentiation, and migration of these cells are regulated by multiple transcription factors, including the Dlx1,2,5,6 and Lhx6 homeobox genes. The Dlx genes are critical for interneuron migration and differentiation. For example, mice lacking Dlx1/2 show a block in the migration of most cortical and hippocampal interneurons (Anderson et al., 1997a; Pleasure et al., 2000). Mice lacking Dlx1 show defects in dendrite-innervating interneurons (Cobos et al., 2005), whereas mice lacking either Dlx5 or Dlx5/6 have defects in somal-innervating (parvalbumin+; PV+) interneurons (Wang et al., 2010).

Studies on transcriptional alterations in the Dlx1/2−/− mutants have begun to elucidate the molecular pathways that regulate interneuron development and function (Long et al., 2009a, 2009b). We have discovered that the Dlx genes promote the expression of two chemokine receptors, CXCR4 and CXCR7 (RDC1; CMKOR1) (Long et al., 2009a, 2009b; Wang et al., 2010). Furthermore, these receptors are also positively regulated by the Lhx6 transcription factor (Zhao et al., 2008) that is essential for the differentiation of PV+ and somatostatin+ (SS+) interneurons (Liodis et al., 2007; Zhao et al., 2008).

CXCR4 and CXCR7 are seven-transmembrane receptors that bind CXCL12, a chemokine also known as Stromal-derived factor 1 (SDF1) (Balabanian et al., 2005; Libert et al., 1991). CXCL12 binding to CXCR4 triggers Gαi protein-dependent signaling, whereas CXCL12 binding to CXCR7 does not activate Gαi signaling (Levoye et al., 2009; Sierro et al., 2007).

On the other hand, many lines of evidence indicate that CXCR7 has an important role in regulating cell signaling in culture and in vivo.

In developing zebrafish, CXCR4 and CXCR7 are both implicated in regulating migration of primordial germ cells (PGMs) and the posterior lateral line primordium, in part through their differential expression patterns (Boldajipour et al., 2008; Dambly-Chaudiere et al., 2007; Valentin et al., 2007). For instance, while CXCR4 is expressed in the germ cells, CXCR7 is expressed in adjacent cells. It has been proposed that CXCR7 functions as a “decoy” receptor to generate a gradient of CXCL12, through ligand sequestration; the CXCL12 gradient would impact CXCR4 signaling through controlling ligand availability (Boldajipour et al., 2008).

Another mode for CXCR7 function has been proposed based on experiments in which transiently transfected cells ectopically express both CXCR4 and CXCR7 (Levoye et al., 2009; Sierro et al., 2007). These studies showed that CXCR7 forms
heterodimers with CXCR4. In this context, CXCR7 dampened CXCR4 signaling.

More recently, transient transfection studies have provided evidence that CXCR7 is a signaling receptor. Unlike traditional seven-transmembrane receptors, which signal through both G proteins and β-arrestin, CXCR7 may only signal through β-arrestin (Rajagopal et al., 2010). β-arrestin activation then leads to stimulation of the MAP kinase cascade (Rajagopal et al., 2010; Xiao et al., 2010).

CXCL12 and CXCR4 cellular functions were first studied in leukocyte chemotaxis (D’Apuzzo et al., 1997; Valenzuela-Fernandez et al., 2002). However, their wider roles in cell migration are now appreciated, particularly in CNS development. Mice deficient in either CXCL12 or CXCR4 exhibit abnormal neuronal migration in the cerebellum, dentate gyrus, and dorsal root ganglia (Bagri et al., 2002; Belmadani et al., 2005; Ma et al., 1998). Meningeal expression of CXCL12 controls positioning and migration of Cajal-Retzius cells via CXCR4 signaling (Borrell and Marin, 2006; Paredes et al., 2006). Furthermore, CXCL12/CXCR4 signaling controls cortical interneuron migration by focusing the cells within migratory streams and controlling their position within the cortical plate (Li et al., 2008; Lopez-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006).

Analysis of CXCR7 function in mice is limited to studies that demonstrate its function in heart valve and septum development (Gerrits et al., 2008; Sierro et al., 2007). Here, using both constitutive and conditional null mouse mutants, we report that CXCR7 is essential for the migratory properties of mouse cortical interneurons. We demonstrated that Cxcr4 and Cxcr7 were coexpressed in migrating cortical interneurons. Each receptor was essential for interneuron migration based on several lines of evidence. First, Cxcr7−/− and Cxcr4−/− null mutants had remarkably similar histological phenotypes. Second, ectopic expression of CXCL12 controls positioning and migration of Cajal-Retzius cells via CXCR4 signaling (Borrell and Marin, 2006; Paredes et al., 2006). Furthermore, CXCL12/CXCR4 signaling controls cortical interneuron migration by focusing the cells within migratory streams and controlling their position within the cortical plate (Li et al., 2008; Lopez-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006).

Abnormal Interneuron Distribution in Cxcr7+/− Mutants

To analyze Cxcr7 function, we generated conditional null mutants in which exon 2 was flanked by LoxP sites; the entire coding region is included within exon 2 (Cxcr7fllox allele). By breeding these mice to deleter transgenic mice and then outcrossing to wild-type B6 mice, we established a stably transmitting mouse line with deletion of Cxcr7 exon 2 (Figures 2A–2C). To examine the cellular localization of CXCR7, we performed Cxcr7 antibody staining on the E13.5 MGE cells and 2 DIV. While Cxcr7+/− mutants showed no staining (Figure 2E), wild-type cells showed robust CXCR7 expression that appeared as intracellular aggregates in the close proximity to the nucleus (Figure 2D). The majority of Lhx6-GFP+ MGE cells expressed Cxcr7 protein (Figure 2F), consistent with our fluorescent in situ hybridization results (Figure 1Y).

We began our analysis with the constitutive null Cxcr7+/− mutants. To examine whether a loss of Cxcr7 function affected interneuron migration, we assessed expression of Lhx6-GFP+ cells by immunohistochemical staining. Lhx6-GFP is expressed in interneurons derived from the medial ganglionic eminence (Cobos et al., 2007). We followed the progression of phenotype over different developmental stages. At E13.5, in Cxcr7+/− brains, Lhx6-GFP+ cells formed the MZ and SVZ migratory streams; most SVZ cells had elongated processes pointing toward the dorsal cortex (Figure 2G). In contrast, Lhx6-GFP+ cells in the Cxcr7−/− cortex did not advance as far dorsally (dotted line in Figure 2H). Furthermore, mutant cells in the MZ and SVZ appeared to be intermingled, and their processes were less polarized along the dorsal (tangential) dimension (Figure 2H). In the E14.5 Cxcr7+/− cortex, Lhx6-GFP+ cells were mainly found in the MZ and SVZ/IZ (Figure 2I), whereas in the
Cxcr7−/− cortex, there were fewer Lhx6-GFP+ cells in the MZ and SVZ and more cells in the cortical plate (Figure 2J). At stages of E16.5 and E18.5, the mutants showed further depletion of Lhx6-GFP+ cells in the cortical MZ and SVZ, and the Lhx6-GFP+ cells continued to accumulate in the CP (Figures 2L, 2N, 2P, and 2R). The remaining Lhx6-GFP+ cells in the SVZ were chaotically oriented and displayed rudimentary processes (Figures 2N and 2R). The distribution of interneurons expressing Lhx6 and Dlx1 RNA showed the same phenotype as the Lhx6-GFP+ cells in Cxcr7−/− mutants (Figure S2).

Because Lhx6-GFP+ cells represent MGE-derived interneurons, but not interneurons derived from the dorsal CGE (dCGE; Zhao et al., 2008), we investigated whether the Cxcr7 mutation affected Lhx6-GFP− migrating interneurons by using double immunofluorescence labeling with anti-DLX2 and anti-GFP antibodies. In the E14.5 control cortex, ~55.5% of DLX2+ cells expressed Lhx6-GFP whereas ~99% of Lhx6-GFP+ cells expressed DLX2 (Figures 2S and 2T). Therefore, DLX2+/Lhx6-GFP+ cells (yellow) represented MGE-derived interneurons, whereas DLX2−/Lhx6-GFP− cells (red) represented dCGE-derived interneurons (Figure 2U). TBR1 antibody staining was used to mark developing cortical projection neurons. We analyzed the number of DLX2+/Lhx6-GFP+ cells and DLX2−/Lhx6-GFP− cells within each layer at E14.5 and E18.5. In Cxcr7−/− mutants at E14.5, Lhx6-GFP+ cells were reduced in the MZ and SVZ and accumulated in the CP; in contrast,
Neuron

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Lhx6-GFP+ cells had a normal distribution (Figures 2V–2W and 2Z). In Cxcrx7+/− mutants at E18.5, both Lhx6-GFP+ and Lhx6-GFP− cells displayed lamination defects in the MZ and SVZ and in the CP (Figures 2X–2Y and 2Z). Therefore, our data indicated that the Cxcrx7 mutation preferentially affected Lhx6-GFP+ interneurons at early stages, whereas it affected both Lhx6-GFP+ and Lhx6-GFP− interneurons at later stages.

Interneuron Phenotypes of Cxcrx7+/− and Cxcrx4+/− Mutants

Next, we asked whether Cxcrx4 and Cxcrx7 mutations affected interneuron migration in the same manner. We first studied Lhx6 mRNA expression in Cxcrx4+/− and Cxcrx7+/− mutants at E15.5 and quantified the total number of Lhx6+ cells in the lateral neocortex (Figures 3A–3C and 3A’–3C’). We found that both Cxcrx7+/− and Cxcrx4+/− mutants had ~25% fewer Lhx6+ cells in lateral neocortex as compared to controls (Figure 3D). Next, we compared the laminar distribution of Lhx6+ cells in both mutants and we found that they had very similar defects: reduced numbers of Lhx6+ cells in the MZ and SVZ, and an increase in the number of Lhx6+ cells in the CP, especially in the lower CP (Figures 3A’–3C’ and 3E). Thus, both Cxcrx7 and Cxcrx4 had similar functions in maintaining interneurons within the MZ and SVZ migratory streams and in controlling the timing for interneuron invasion into the cortical plate.

While Cxcrx4+/− and Cxcrx7+/− mutants share very similar interneuron laminar positioning phenotypes in the cortical plate, these deficits may arise from distinct alterations in migration dynamics. To further explore the migration behaviors of Cxcrx4+/− and Cxcrx7+/− interneurons in vivo, we performed real-time imaging of Lhx6-GFP+ cortices from control (Cxcrx4+/− or Cxcrx7+/−), Cxcrx4+/−, and Cxcrx7+/− embryos at E15.5. We first examined the transition from tangential to radial migration as interneurons migrated from either the MZ or the SVZ into the CP. In the MZ, Cxcrx4+/− and Cxcrx7+/− mutants demonstrated a 3-fold and 2.4-fold increase in the number of Lhx6-GFP+ interneurons switching from tangential to radial migration, respectively (Figures 4A–4C and 4G; Movies S1–S3). In the SVZ, Cxcrx4+/− mutants displayed a 2-fold increase in the number of Lhx6-GFP+ interneurons switching from tangential to radial migration and no effect was detected in the Cxcrx7+/− mutants (Figures 4D–4F and 4H; Movies S4–S6). Therefore, the laminar deficits from both mutants were mainly due to an increased number of Lhx6-GFP+ cells moving from either the MZ or the SVZ into the cortical plate.

Next, we studied the tangential migration rate of Lhx6-GFP+ cells that were maintained in the MZ and SVZ during the 20-hour live-imaging session. Compared to controls, Cxcrx7+/− mutants exhibited a substantial decrease in tangential migration rate in the MZ and SVZ; and Cxcrx4+/− mutants displayed a modest decrease in migration rate in the SVZ (Figure 4I). Thus, the decreased migration rate may underlie the reduced extent of interneuron migration into the dorsal cortex observed in both mutants during early embryonic stages.

Finally, we explored the migration behaviors of Lhx6-GFP+ cells after they entered into the cortical plate. Cxcrx4+/− and Cxcrx7+/− mutants displayed major differences in interneuron motility and leading process morphology. Cxcrx4+/− mutants exhibited a significant increase in the number of motile cells and in the leading process length of tangentially oriented cells, while Cxcrx7+/− mutants showed a significant decrease in the number of motile cells and in the leading process length of both radially and tangentially oriented cells (Figure 5 and Movies S7–S9). Together, these results indicated that CXCR4 and CXCR7 conferred interneurons with distinctive migratory properties after they entered the cortical plate.

Figure 2. Distribution and Cellular Morphology of Lhx6-GFP+ Cells in Cxcrx7+/− Mutants
(A) Schematic representation of the wild-type Cxcrx7 locus, the targeted allele, and the deleted locus. Shaded regions represent exon 2 (Ex2). The fragment includes Ex2 and about 100 bp up- and downstream of Ex2. The 5’ homologous arm was cloned from DNA immediately upstream of Ex2 (5’ arm). The 3’ homologous arm was cloned from DNA immediately downstream of Ex2 (3’ arm). LoxP sites are represented as black triangles and primer sites used for genotyping are represented as small arrowheads. E, EcoRV, neo, neomycin-resistance gene cassette.

(B) Southern blot analysis of embryonic stem cell clones showing non-targeted (“”) and targeted (“”) clones. The 9.4 kb wild-type and 7.1 kb targeted allele EcoRV-digested fragments were identified by an upstream probe, as shown in (A).

(C) PCR analysis of genomic DNA from E16.5 wild-type (“”) embryos or embryos heterozygous (“/”) or homozygous (“/”) for Cre-mediated deletion of the targeted locus. The 3.9 kb wild-type and 1.9 kb deleted products were from a single set of primers outside of Ex2, as shown in (A).

(D and E) Confocal images of Cxcrx7+/− mutant and control neurons from E13.5 MGE cultures stained with CXCR7 antibody after 2 DIV.

(F) Confocal image of control neurons from E13.5 Lhx6-GFP+ MGE cultures stained with CXCR7 and GFP antibodies after 2 DIV.

(G and H) Lhx6-GFP expression in the lateral cortices of Cxcrx7+/− and Cxcrx4+/− embryos carrying Lhx6-GFP transgene at E13.5. (I and J) Lhx6-GFP expression in the lateral cortices of Cxcrx7+/− and Cxcrx7+/− embryos at E14.5. Note that there are fewer Lhx6-GFP+ cells in the MZ and the SVZ (arrows) and more cells accumulate in the cortical plate in Cxcrx7+/− brains (asterisks). (K–P) Lhx6-GFP expression in the lateral cortices of Cxcrx7+/− and Cxcrx7+/− embryos at E16.5 and E18.5. Note that interneuron migration defects progress at these stages.

(S) Confocal image showing normal Lhx6-GFP (green) and DLX2 (red) expression in E14.5 lateral cortex. TBR1 (blue) labels deep cortical plate.

(T) Quantification of the percentage of DLX2+ cells expressing Lhx6-GFP and the percentage of Lhx6-GFP+ cells expressing DLX2 (n = 3).

(U) Graphic illustration of the percentage contribution of Lhx6-GFP+ and Lhx6-GFP− cells to DLX2+ cells.

(V–Y) Confocal images showing Lhx6-GFP+ (yellow) and Lhx6-GFP− cells (red) from Cxcrx7+/− and Cxcrx7+/− cortices at E14.5 and E18.5. Number-denoted areas are shown at higher magnification.

(Z) Quantification of the percentage of Lhx6-GFP+ and Lhx6-GFP− cells (n = 5; *p < 0.01, *p < 0.05, x2 test).

MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bars represent 15 μm (D–F), 100 μm (G–P, V1–V1, and V2–Y2), and 500 μm (V–Y). See also Figure S2.
is possible that CXCR7 also confers interneuron responsiveness to CXCL12 and that this chemoattraction is mediated by both CXCR4 and CXCR7. To determine whether cellular responsiveness to CXCL12 is mediated by both CXCR4 and CXCR7, we used in utero electroporation to ectopically express 
$\text{Cxcl12}$ and 
$\text{DsRed2}$ in the ventricular zone of the lateral neocortex of 
$\text{Cxcr7}^{+/+}$ and 
$\text{Cxcr4}^{+/+}$ E13.5 embryos. Electroporation with 
$\text{DsRed2}$ alone into wild-type cortices was used as a control (Figure S3). After ~48 hours, we used in situ hybridization to examine the location and extent of ectopic 
$\text{Cxcl12}$ expression (Figures 6B, 6D, and 6F) and the distribution of 
$\text{Lhx6}^+$ interneurons (Figures 6A, 6C and 6E).

Overexpression of 
$\text{Cxl12}$ in controls induced an accumulation of 
$\text{Lhx6}^+$ interneurons in the deep cortical plate (dCP) and intermediate zone (IZ) as well as a depletion of 
$\text{Lhx6}^+$ interneurons in the marginal zone (MZ) above the electroporation site (Figures 6A–6A2 and 6H; $n = 4$). In contrast, neither the 
$\text{Cxcr4}^{-/-}$ nor the 
$\text{Cxr7}^{-/-}$ mutants exhibited a measurable accumulation of 
$\text{Lhx6}^+$ interneurons around the site of ectopic 
$\text{Cxl12}$ expression (Figures 6C–C2, 6E–6E2, 6G, and 6H; $n = 4$). Thus, these results suggested that interneuron attraction to 
$\text{Cxl12}$ relied on the integrated function of both 
$\text{Cxcr4}$ and 
$\text{Cxcr7}$.

To further assess the role of CXCR7 in CXCL12-mediated chemoattraction, we studied the migration of E13.5 MGE cells by using a pharmacological inhibitor of CXCR7 (CCX771) in a 72-hour transwell migration assay. Control experiments showed a ~5-fold stimulation of migration by adding CXCL12 (100 nM) to the bottom chamber. On the other hand, treatment with the CXCR7 antagonist CCX771 (1 $\mu$M) reduced CXCL12-mediated cell migration (Figure S4). Similar inhibition was observed with the CXCR4 antagonist (AMD3100 [5 $\mu$M]) (Figure S4). Treatment with both CCX771 and AMD3100 did not lead to further reduction in migration (Figure S4).

As 
$\text{Cxr7}$ and 
$\text{Cxcr4}$ were coexpressed within interneurons (Figure 1K) and were both essential in regulating interneuron migration, we investigated whether simultaneous disruption of their function would lead to an enhanced phenotype. Because these genes are closely linked on mouse chromosome 1 (1.2 Mb apart), we could not generate a double mutant. We circumvented this problem by pharmacologically inhibiting 
$\text{Cxcr4}$ with a specific inhibitor, AMD3100 (Donzella et al., 1998; Lazarini et al., 2000) in 
$\text{Cxr7}^{-/-}$ mutants. We injected AMD3100 (1 $\mu$l, 12.6 mM in PBS) or PBS (control) into the lateral ventricle of E14.5 embryos carrying the 
$\text{Lhx6}$-GFP allele (some of which were 
$\text{Cxr7}^{-/-}$ mutants). We analyzed the location of GFP$^+$ cells after 15 hours. As reported previously (Lopez-Bendito et al., 2008), AMD3100-treated wild-type embryos showed interneuron defects that closely resembled those found in 
$\text{Cxcr4}^{-/-}$ mutants: fewer cells populated the MZ and SVZ, whereas
CXCR7 Regulates Cortical Interneuron Migration

CXcr7 mRNAs and CXcr7-GFP expression provided evidence that CXcr7 was expressed in the immature projection neurons of the cortical plate (Figure S1). Furthermore, we examined CXcr7 expression in E15.5 Dlx1/2−/− mutants. We found that CXcr7 expression was greatly reduced in the subpallium and was eliminated in most of the cortex, except for a band of cortical plate expression extending in a dorsoventral gradient in the dorsomedial pallium (Figures 7B and 7B′; Figure S5E). These CXcr7+ pallial cells are almost certainly not interneurons, as very few interneurons that reach the cortex in the Dlx1/2−/− mutants are present in a scattered pattern in the SVZ and intermediate zone (Cobos et al., 2006). Furthermore, E13.5 cortical cultures (2 DIV) showed that CXCR7 was detectable in ~10% of TBR1+ or CTIP2+ cortical projection neurons (Figure 7C and 7D). Therefore, in addition to its expression in migrating interneurons, CXcr7 is expressed in immature excitatory neurons of the cortical plate, especially of the dorsomedial cortical plate. Given this, we investigated CXcr7’s specific functions in immature GABAergic and glutamatergic neurons by using conditional mutants.

We selectively deleted CXcr7 gene in telencephalic GABAergic lineages (including cortical interneurons) and cortical glutamatergic lineages by using the Dlx1/2b-Cre allele (Pottet et al., 2009) and the Emx1-Cre allele (Guo et al., 2000), respectively. We compared the distribution of Lhx6+ cortical interneurons in the dorsomedial pallium of Emx1Cre−/− CXcr7f/f, Emx1Cre−/− CXcr7fl/fl, Dlx1/2bCre−/− CXcr7fl/fl, Dlx1/2bCre−/− CXcr7f/f, and Dlx1/2bCre−/− CXcr7f/f embryos at E15.5 and E18.5. At E15.5, both the Dlx1/2bCre and Emx1Cre CXcr7 conditional knockouts had reduced Lhx6+ cell numbers in the M2 and SVZ and excess number of cells in the CP (Figures 7E–7I and Figures S5F–S5J). These phenotypes persisted at E18.5 (Figures 7J–7M). We assessed whether the interneuron laminar defects in Emx1Cre−/−, CXcr7fl/fl were due to the abnormal lamination of Cajal–Retzius cells or cortical projection neurons. We did not detect defects in the laminar organization of Cajal–Retzius cells (based on Reelin and CR expression) and the cortical plate (based in Cux2, SATB2, CTIP2, and TBR1 expression) at E15.5 and E18.5 (Figure S6). Thus, we propose that CXcr7 functions in the dorsomedial cortical plate nonautonomously regulate interneuron migration without having a detectable effect on the position of cortical projection neurons.

To further determine whether the deletion of CXcr7 in interneurons affects their laminar postnatally, we examined interneuron phenotypes in the adult neocortex of Dlx1/2bCre−/− CXcr7f/f mutants by using a Cre-inducible R26R-EYFP reporter (Srinivas et al., 2001). We found that recombined (EYFP+) interneurons expressing CR, PV, or SS were more frequently present in the mutant’s deep cortical layers compared to control (Figure S7). Thus, interneuron-specific deletion of CXcr7 alters the laminar distribution of cortical interneurons pre- and postnatally.

CXCR4 and CXCR7 Signal through Different Molecular Mechanisms in Developing Cortical Interneurons

CXCR4 signals through Gαi, whereas CXCR7 is not believed to signal through heterotrimeric G proteins (Boldajipour et al., 2008; Levey et al., 2009; Sierro et al., 2007). On the other hand, recently, CXCR7 has been shown to signal through β-arrestin and thereby to activate the MAP kinase cascade in transiently transfected cells (Rajagopal et al., 2010; Regard et al., 2007; Xiao et al., 2010). To gain insights into the signaling mechanisms used by CXCR4 and CXCR7 in developing interneurons, we used in vivo and primary culture assays that employed CXCR4 and CXCR7 mutant mice, inhibition of Gαi/o with pertussis toxin (PTX), and CXCR4&7 inhibition with small molecule antagonists. We assessed the effects of these molecular perturbations on interneuron migration and MAP kinase signaling.

First, we inhibited Gαi/o in migrating interneurons in vivo by using PTX to probe the function of seven-transmembrane receptors (including CXCR4) that couple with this class of heteromeric G proteins. We used mice carrying an allele in which the S1 subunit of PTX was introduced into the Rosa26 locus (Regard et al., 2007). Expression of the S1 subunit of PTX in developing interneurons required removal of a floxed polyadenylation signal by using Cre recombinase; for this purpose, we used Dlx5/6 Cre (Kohwi et al., 2007). We analyzed the location of Lhx6+ migrating interneurons by using in situ hybridization in the Dlx5/6 Cre−/− mice, compared with PTX+/+ controls at E18.5 (Figures 8A–8C). We found that PTX-expressing embryos phenocopied the interneuron positioning defects observed in the cortical plate of CXcr4−/− mutants (compare Figure 8B and Figure S8B). Therefore, these results show that PTX-sensitive Gαi/o signaling regulates interneuron positioning. Given that CXCR4, and not CXCR7, is known to signal in this manner, these results provide strong evidence for the mechanism of CXCR4 signaling in migrating interneurons. They also suggest that there are no other receptors signaling through Gαi/o that have a major role in regulating early interneuron development.

We next tested whether CXCL12 promotes MAP kinase signaling through CXCR4 and/or CXCR7 in MGE primary cultures. In wild-type cells, CXCL12 increased phospho-p44/42 MAP Kinase (pErk1/2) expression (Figure 8G). pErk1/2 expression was asymmetrically localized in the cell, typically at the base of the primary process growing from the cell, and in that process (arrows in Figure 8G). In addition, pErk1/2 often colocalized with CXCR7 (arrowheads in Figure 8G).

Finally, we examined whether CXCL12 increased pErk1/2 expression in MGE cells lacking CXCR4 or CXCR7 or exposed to AMD3100 (CXCR4 antagonist), CXcr4−/− cells or AMD3100-treated cells showed no change in the CXCL12-mediated increase in pErk1/2 (Figures 8H, 8J, and 8L). On the other hand, CXcr7−/− mutant cells failed to show the CXCL12-mediated increase in pErk1/2 (Figures 8I and 8L). Thus, CXCR7, and not CXCR4, strongly activates the MAP kinase pathway in MGE cells.
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<th>Tangential to radial transition (MZ to CP)</th>
<th>Tangential to radial transition (SVZ to CP)</th>
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<td>Control</td>
<td>Cxcr4/-</td>
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![Images showing neuronal migration](image)

**G**

- % neurons undergoing tangential to radial transitions (MZ to CP)
- % neurons undergoing tangential to radial transitions (SVZ to CP)

**H**

- Rate of tangential migration (mm/h)

**I**

- Comparison of migration rates between Control, Cxcr4/-, and Cxcr7/- in MZ and SVZ.
DISCUSSION

CXCL12/CXCR4 signaling in interneurons controls their migration and final position in the cortical plate (Li et al., 2008; Lopez-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006). Here we demonstrated that CXCR7 regulated these processes through its distinct roles in immature interneurons and projection neurons. We showed that migrating immature cortical interneurons coexpressed CXCR4 and CXCR7 and that CXcr7–/– and CXcr4−/− single-mutant mice had similar defects in interneuron position. Both receptors were essential for responding to CXCL12 and for regulating interneuron migration, based on experiments that studied the effect of (1) ectopic CXCL12 expression and (2) pharmacological blockade of CXCR4 in Cxcr7−/− mutants. Furthermore, live imaging revealed that CXcr4−/− and CXcr7−/− mutants had opposite defects in interneuron motility and leading process morphology. We provided evidence that CXCR4 and CXCR7 signal through distinct mechanisms in immature interneurons. In vivo inhibition of Gαi/o signaling (the means through which CXCR4 is known to function) in differentiating interneurons phenocopied the interneuron laminar positioning defects observed in the prenatal cortex of the CXcr4−/− mutant. Finally, in MGE primary cultures Cxcr7, but not CXCR4, promoted CXCL12-mediated MAP kinase signaling. As such, we conclude that in migrating interneurons CXCR4 and CXCR7 perform essential but distinct functions in regulating interneuron movement and laminar positioning.

Cxc7 Regulates Interneuron Migration and Laminar Positioning

We found that Cxc7−/− constitutive null mutants and Dlx12bCre Cxcr7 conditional mutants (loss of expression in forebrain GABAergic neurons) had interneuron laminar positioning defects that were comparable to CXcr4−/− and Cxcl12−/− mutants (Figures 3 and 7; Li et al., 2008; Lopez-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006).

Live-imaging analysis provided insights into why CXCR4−/− and Cxcr7−/− mutants had laminar deficits of Lhx6+ interneurons on fixed brain sections. Both mutants showed an increased number of tangentially migrating mutant interneurons that switched from tangential migration (in the MZ and SVZ) to radial migration into the cortical plate. This is consistent with the idea that mutant interneurons have reduced responsiveness to CXCL12 in the SVZ and meninges. Furthermore, live imaging of both mutants detected a reduced rate of interneuron tangential migration in the SVZ; this may underlie why the mutant interneurons fail to advance as far dorsally as wild-type interneurons (arrows in Figure 3).

Cxc7 expression waned between E15.5 and E18.5 (Figure 1 and Figure S1). This interval correlates with the period when a large flux of interneurons invade the cortical plate (Lopez-Bendito et al., 2008). Perhaps downregulation of Cxc7 results in reduced interneuron responsiveness to CXCL12, thereby enabling their entry into the cortical plate. This is consistent with our electroporation experiments showing that interneurons require both CXCR4 and CXCR7 receptors to respond to CXCL12-mediated chemotraction.

CXCR4 and CXCR7 Perform Essential but Distinct Functions in Regulating Interneuron Migration

Several lines of evidence suggest that both CXCR4 and CXCR7 regulate CXCL12 signaling in migrating interneurons. CXCR4−/− and CXCR7−/− mutants had nearly equivalent interneuron positioning phenotypes. In addition, both CXcr4 and Cxcr7 were required for attraction to ectopic CXCL12. Thirdly, the CXCR4 blockage did not exacerbate the phenotype observed in the CXcr7−/− mutants. These experiments provide evidence that signaling through both CXCR4 and CXCR7 are essential in guiding migrating interneurons.

After entering the cortical plate, movements of Lhx6-GFP+ cells from the CXcr4−/− and CXcr7−/− mutants exhibited opposite phenotypes: CXCR4-deficient neurons were highly motile and elaborated a longer leading process, whereas CXCR7-deficient neurons were much less motile and had a shorter leading process. These results suggest that signaling downstream of CXCR4 and CXCR7 have distinct effects on cytoskeletal organization as previous studies have shown that actin and microtubule dynamics are the main cytoskeletal contribution responsible for cell motility (Baudoin et al., 2008; Etienne-Manneville, 2004; Reiner and Sapir, 2009). Our result also implies that both receptors are required for the interneurons to coordinate their movements in response to guidance cues in the cortex and eventually situate themselves in their appropriate cortical positions. Indeed, there are interneuron lamination defects in the adult cortex of interneuron-specific CXCR4 and Cxcr7 conditional mutants (Figure S7; Li et al. 2008).

Our data demonstrated that blocking Gαi/o signaling in vivo, using PTX, phenocopied the CXCR4 mutant (Figure 8B and Figure S8B), consistent with CXCR4’s known ability to signal through Gαi/o (Albert and Robillard, 2002; Hamm, 1998; Hessegser et al., 1998; Ma et al., 1998; Patrussi and Baldari, 2008; Teicher and Fricker, 2010). On the other hand, because Cxc7 does not appear to signal through heteromeric G

Figure 4. Real-Time Assessment of Interneuron Migration in the Cortical Wall Reveals Abnormal Migration in Cxcr4−/− and Cxcr7−/− Mutant Embryos

(A–C) Live imaging of E15.5 Lhx6-GFP+ cortices showing interneurons transitioning from tangential to radial migration along the MZ in Cxcr4−/− or Cxcr7−/− controls (A, cells in red), Cxcr4−/− (B, cells in blue), and Cxcr7−/− (C, cells in purple) mutants. (D–F) Intermembranes transitioning from tangential to radial migration along the SVZ in Cxcr4−/− or Cxcr7−/− controls (D, cells in red), Cxcr4−/− (E, cells in blue), and Cxcr7−/− (F, cells in purple) mutants. (G–H) Quantification of the percentage of interneurons transitioning from tangential to radial migration along the MZ and SVZ.

(i) Quantification of rates of tangential migration through the MZ and SVZ. Time elapsed since the onset of observations is indicated in minutes. n = 5; *p < 0.01 one-way ANOVA and Tukey-Kramer post-hoc test. MZ, marginal zone; CP, cortical plate; SVZ, subventricular zone. Scale bar represents 50 μm. See also Movies S1–S6.
proteins, the mechanism underlying its function has been controversial.

CXCR7 has been reported to regulate responses to CXCL12 in tissue culture cells, where CXCR4 and CXCR7 form heterodimers (Levoye et al., 2009; Sierro et al., 2007). CXCL12 stimulation of HEK293 cells results in stronger calcium flux and more robust phosphorylation of MAPKp42/44 in cells that coexpress CXCR4 and CXCR7 than in cells that express only CXCR4 (Sierro et al., 2007). This opens the possibility that CXCR4 and CXCR7 form heterodimers in migrating interneurons and that the balance of CXCR dimers and monomers modulates how the interneurons respond to CXCL12.

Recently, CXCR7 has been shown to signal through β-arrestin to activate MAP kinases in transiently transfected cells (Rajagopal et al., 2010; Regard et al., 2007; Xiao et al., 2010). Our data using cultures of MGE cells support these findings. We found that Cxcr7−/− mutant cells failed to show a CXCL12-mediated increase in pErk1/2, whereas loss of CXCR4 function did not alter this process (Figures 8H–8L). Thus, in immature MGE interneurons CXCR7, but not CXCR4, strongly promotes MAP kinase signaling.

Therefore, our data provide evidence that CXCR4 and CXCR7 signal through different pathways in the developing interneurons. Future studies are needed to test whether these signaling differences underlie the opposite defects in interneuron motility and leading process morphology of Cxcr4−/− and Cxcr7−/− mutants (Figure 5).

In the cultured MGE cells and cortical cells, CXCR7 is predominantly expressed inside the cell in perinuclear aggregates that may be internal membranous vesicles (Figures 2D, 2F, 7C, and 7D). CXCL12-induced pErk1/2 partially colocalized with CXCR7 (Figure 8G). There is evidence that internalized seven-transmembrane receptors continue to signal by G-protein-independent mechanisms, such as through β-arrestins to activate the MAP kinase cascade in signalsomes (Cottrell et al., 2009; Luttrell et al., 1999; Tohgo et al., 2002). Thus, perhaps CXCR7 activates pErk in endosomes, through β-arrestins. Currently we are uncertain of the location of the CXCR4, as all of the antibodies we tested continue to stain the surface of Cxcr4−/− cells. Thus, future studies are needed to fully elucidate how CXCR4 and CXCR7 differentially regulate signaling, morphology, and motility of developing interneurons.
CXCR7 Function in the Cortical Plate Regulates Interneuron Positioning

We showed that Cxcr7 mRNA and Cxcr7-GFP were expressed in the immature projection neurons of the cortical plate (Figure S1). In addition, the remaining Cxcr7 expression in the dorsomedial pallium of Dlx1/2−/− mutants also supports this idea, as Dlx1/2−/− mutants have very few cortical interneurons. On the other hand, we did not detect Cxcr4 expression in the neocortical plate (Figure S5B). Thus, we hypothesize that CXCR7 functions as homodimers in immature cortical projection neurons. Deletion of Cxcr7 in cortical plate cells with Emx1-Cre revealed that Cxcr7 non-cell-autonomously regulates interneuron migration, especially in the dorsomedial pallium. Furthermore, this regulation was not secondary to changes in the laminar position of Cajal-Retzius cells and projection neurons (Figure S6).

CXCR7 cortical plate expression followed a dorsoventral gradient (Figure S5E), which was opposite to the gradient of Cxcl12 SVZ expression (Figure S5A). The inverse gradients of the ligand and its receptor in the developing cortex are intriguing, because CXCR7 has been reported to act as a scavenger receptor that can reduce the concentration of CXCL12 available for signaling (Boldajipour et al., 2008). Possibly, CXCR7 in the cortical plate may lower the concentration of CXCL12 and generate a gradient from either MZ or SVZ to the cortical plate, thereby regulating the cortical invasion of migrating interneurons. In our study, elimination of Cxcr7 in the cortical plate may
Figure 7. Cxcr7 Has Cell-Autonomous and Non-Cell-Autonomous Functions in Regulating Interneuron Laminar Distribution

(A and B) In situ hybridization showing expression of Cxcr7 in Dlx1/2+/+ and Dlx1/2−/− brain sections. Boxed areas in (A) and (B) are shown in higher magnification.

(C and D) Confocal images of wild-type neurons from E13.5 cortical cultures stained with CXCR7 and TBR1 or CTIP2 antibodies after 2 DIV.

(E–H) Coronal sections through the telencephalons of Emx1cre+/; Cxcr7f/+ (E) and Emx1cre+/; Cxcr7f/− (F), Dlx12bCre+/; Cxcr7f/+ (G), and Dlx12bCre+/; Cxcr7f/− (H) showing Lhx6 expression at E15.5. Note that both conditional mutants exhibit laminar distribution defects of Lhx6+ interneurons.

(I) Quantitative analysis of the distribution of Lhx6+ cells/each layer at E15.5 (n = 3; *p < 0.01, ANOVA).

(J–M) E18.5 Lhx6 expression in the dorsomedial pallium of Emx1cre+/; Cxcr7f/+ (J) and Emx1cre+/; Cxcr7f/− (K), Dlx12bCre+/; Cxcr7f/+ (L), and Dlx12bCre+/; Cxcr7f/− (M) embryos (M).

MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bars represent 1 mm (A and B), 500 μm (A′–B′ and E–H), and 50 μm (C and D). See also Figures S5–S7.
disrupt this gradient and thus results in a premature interneuron entry.

**Transcription Factor Hierarchy Regulating Interneuron Development**

Several transcription factors have been demonstrated to regulate the migration of interneurons generated from the ganglionic eminences; these include Arx, Dlx1, Dlx2, Dlx5, Lhx6, Nkx2.1, and Sox6 (Alifragis et al., 2004; Anderson et al., 1997a, 1997b; Azim et al., 2009; Batista-Brito et al., 2009; Casarosa et al., 1999; Colasante et al., 2008; Liodis et al., 2007; Marin et al., 2000; Nobrega-Pereira et al., 2008; Pleasure et al., 2000; Sussel et al., 1999; Zhao et al., 2008). Effectors downstream genes that are implicated in regulating interneuron migration such as...
CXCR4 and CXCR7 are beginning to be identified (Long et al., 2007, 2009a, 2009b; Zhao et al., 2008).

In Dlx1/2−/− double mutants, expression of CXCR4 and CXCR7 is greatly reduced in cortical interneurons and the ganglionic eminences (Figure S5; Long et al., 2009a, 2009b). Our data provide evidence that CXCR4 and CXCR7 function is not required for interneurons to migrate from the ganglionic eminences to the cortex (Figure 3), whereas in Dlx1/2−/− mutants the migration block is nearly complete (Anderson et al., 1997a; Long et al., 2007). Thus, other factors must contribute to the failure of interneuron migration in the Dlx1/2−/− mutants such as overexpression of Pak3 (Cobos et al., 2007).

On the other hand, Lhx6, Cxcr4, and Cxcr7 mutants share similar defects in interneuron migration rate and loss from the cortical MZ (Zhao et al., 2008). Lhx6 mutants have greatly reduced Cxcr4 and Cxcr7 expression (Zhao et al., 2008). Thus, it is appealing to conclude that failure to express these receptors is a principle mechanism contributing to the interneuron migration defects in Lhx6 mutants.

EXPERIMENTAL PROCEDURES

Mice
All experimental procedures were approved by the Committees on Animal Health and Care at the University of California, San Francisco (UCSF). Mouse colonies were maintained at UCSF in accordance with National Institutes of Health and UCSF guidelines. Cxcr4 null mice were previously described (Ma et al., 1998; Stumm et al., 2003). The Dlx5/6Cre and Dlx12bCre lines were previously described (Kohwi et al., 2007; Potter et al., 2009), and the Lhx6-GFP/Cxcr7-GFP BAC lines were purchased from The Gene Expression Nervous System Atlas Project (GENSAT) at The Rockefeller University (New York, NY). The Rosa26-pertussis toxin (PTX) mice were obtained from Shaun Coughlin (Regard et al., 2007). See the Supplemental Data for details on the generation of Cxcr7flhox+ mice.

Histology
See the Supplemental Data for details on immunohistochemistry and in situ hybridization.

In Utero Electroporation and Drug Administration
In utero electroporation with pCAGGS-Cxcl12 and pCAGGS-DesRed2 or pCAGGS alone was performed as described (Li et al., 2008). For blocking of CXCR4 receptors, 1 μl of AMD3100 solution (12.6 mM; Sigma) or PBS was injected into the lateral ventricle at E14.5.

Real-Time Imaging
Coronal slices (200 μm) of E15.5 Cxcr4+/− or Cxcr7−/− control and littermate Cxcr4−/− or Cxcr7−/− mutant brains were placed onto nuclepore membrane filters over 35 mm glass-bottom dishes containing Minimum Essential Medium (GIBCO) and 10% FBS. Mounted slices were immediately transferred to a 37°C/5% CO2 live tissue incubation chamber attached to a Zeiss inverted microscope and a PASCAL confocal laser scanning system and imaged repeatedly every 12 min for up to 20 hr. Real-time interneuronal migration patterns were quantified by using Zeiss LSM Image Browser software.

Primary Cell Cultures and CXCR7 Immunohistochemistry
See the Supplemental Data for details on the primary cultures and the transwell assay. For CXCR7 staining, the cells were fixed in 2% formaldehyde for 30 min at RT and then permeabilized with permeabilization solution (PBS/0.1% BSA/0.2% saponin) for 10 min at RT. Cells were incubated in blocking solution (PBS/1% BSA/0.1% saponin/1% goat serum) for 30 min at RT. Cells were then incubated with CXCR7 antibody (11G8, 1:400; ChemogenCtryx) in blocking solution for 30 min on ice. For double labeling, cells were first incubated with CXCR7 antibody and then incubated with the other primary antibodies for 2 hr at RT.

Statistical Analysis
Statistical analysis was performed with Student’s t test, x2 test, or the one-way ANOVA and Tukey-Kramer post-hoc test using Prism4 (GraphPad Software) and XLSTAT (Addinsoft) software. p <0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes eight figures, nine movies, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2010.12.005.

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