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Cxcr7 Controls Neuronal Migration by Regulating Chemokine Responsiveness

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SUMMARY

The chemokine Cxcl12 binds Cxcr4 and Cxcr7 receptors to control cell migration in multiple biological contexts, including brain development, leukocyte trafficking, and tumorigenesis. Both receptors are expressed in the CNS, but how they cooperate during migration has not been elucidated. Here, we used the migration of cortical interneurons as a model to study this process. We found that Cxcr4 and Cxcr7 are coexpressed in migrating interneurons, and that Cxcr7 is essential for chemokine signaling. Intriguingly, this process does not exclusively involve Cxcr7, but most critically the modulation of Cxcr4 function. Thus, Cxcr7 is necessary to regulate Cxcr4 protein levels, thereby adapting chemokine responsiveness in migrating cells. This demonstrates that a chemokine receptor modulates the function of another chemokine receptor by controlling the amount of protein that is made available for signaling at the cell surface.

INTRODUCTION

Chemokines are a large family of small proteins that are characterized by their ability to induce chemotaxis in responsive cells. Several chemokines and their receptors are expressed in the developing CNS, among which Cxcl12 (also known as Stromal cell-derived factor-1, SDF1) is the most studied. Cxcl12 has been shown to promote the migration of granule cells in the cerebellum and hippocampus (Bagri et al., 2002; Ma et al., 1998; Zhu et al., 2002; Zou et al., 1998), Cajal-Retzius cells (Borrell and Marín, 2006; Paredes et al., 2006), cortical interneurons (Li et al., 2008; López-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006) and, more recently, pontine neurons (Zhu et al., 2009).

The best characterized receptor for Cxcl12 is a member of the family of alpha-chemokine receptors, Cxcr4 (Bleul et al., 1996; Oberlin et al., 1996). Initially identified as a coreceptor for the human immunodeficiency virus, this G protein-coupled receptor (GPCR) is an essential mediator of the chemotactic responses induced by Cxcl12 in migrating cells. In the brain, loss of *Cxcr4* function leads to neuronal defects that are remarkably similar to those found in *Cxcl12* mutants (Stumm et al., 2003; Tiveron et al., 2006; Zou et al., 1998). These results, along with similar observations in other tissues, led to the notion that Cxcr4 was the only physiological receptor for Cxcl12.

This view was challenged with the discovery that the orphan receptor RDC1, now designated as Cxcr7, is also able to bind Cxcl12 (Balabanian et al., 2005a; Burns et al., 2006). The function of Cxcr7 in cell migration is under intense debate, as it seems to differ depending on the cellular context (Boldajipour et al., 2008; Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Thus, while some reports have suggested that Cxcl12 binding to Cxcr7 may induce cell chemotaxis and activate the characteristic intracellular responses triggered by GPCRs (Balabanian et al., 2005a; Wang et al., 2008), other studies indicate that this receptor does not signal per se through a classical GPCR pathway (Burns et al., 2006; Hartmann et al., 2008; Levoye et al., 2009; Rajagopal et al., 2010; Sierro et al., 2007). Moreover, recent work in zebrafish suggests that while Cxcr4 is expressed by migrating cells, Cxcr7 may function primarily by removing Cxcl12 from nontarget territories (Boldajipour et al., 2008; Cubedo et al., 2009; Sasado et al., 2008). Consistent with this hypothesis, migrating cells continue to respond to Cxcl12 in the absence of Cxcr7, but end up in undesirable locations because accumulations of Cxcl12 prevent directional migration (Boldajipour et al., 2008). Thus, the most plausible biological function for Cxcr7 reported so far is the regulation of chemokine gradients through a non-cell-autonomous mechanism.

The tangential migration of cortical interneurons has been previously used as a model to study the function of chemokines and their receptors in regulating neuronal migration (Li et al., 2008; López-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006). Most cortical interneurons derive from the medial ganglionic eminence (MGE, Batista-Brito and Fishell, 2009; Wonders and Anderson, 2006), a transient structure in the developing basal telencephalon, and migrate toward the cortex in response to a combination of chemoattractive and chemorepulsive cues (Marín et al., 2010; Métin et al., 2006). Once in the cortex, migrating interneurons actively avoid settling in the developing cortical plate (CP) and disperse tangentially through highly stereotypic routes in the marginal zone (MZ) and the

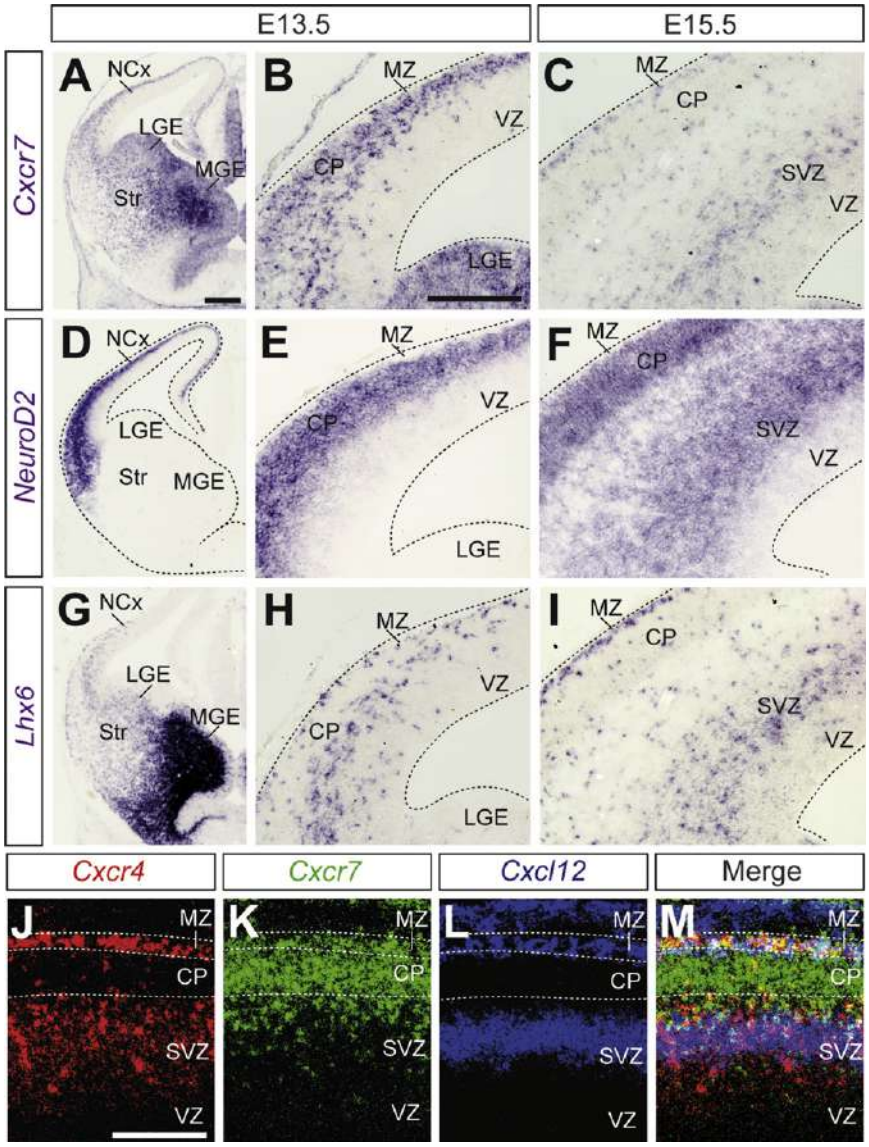


Figure 1. Cxcr7 Expression in the Developing Mouse Cortex

(A–I) Coronal sections through the telencephalon of E13.5 (A, B, D, E, G, and H) and E15.5 (C, F, and I) embryos showing mRNA expression for *Cxcr7* (A–C), *NeuroD2* (D–F), and *Lhx6* (G–I).

(J–M) Serial sagittal sections through the telencephalon of an E14.5 embryo showing mRNA expression for *Cxcr4* (J and M), *Cxcr7* (K and M), and *Cxcl12* (L and M). The pseudocolor triple in situ image (M) was compiled from adjacent sections (J, K, and L) using Photoshop software. CP, cortical plate; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, marginal zone; NCx, neocortex; Str, striatum; SVZ, subventricular zone; VZ, ventricular zone. Scale bars equal 300 μm (A, D, and G), 200 μm (B, C, E, F, H, and I), and 100 μm (J, K, L, and M).

subventricular zone (SVZ) (Hevner et al., 2004; Lavdas et al., 1999; López-Bendito et al., 2008; Pla et al., 2006). This process involves Cxcl12-induced chemotaxis via Cxcr4, because disruption of either Cxcl12 or Cxcr4 causes disorganization of this migratory pattern and premature CP entry (Li et al., 2008; López-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006).

Here we have investigated the function of the chemokine receptor Cxcr7 in neuronal migration by using cortical interneurons as a model system. We found that Cxcr7 is transiently expressed by cells of the cortex that are located in regions typically avoided by tangentially migrating interneurons, which is consistent with the previously suggested function of Cxcr7 as a scavenger receptor. However, we also found that most MGE-derived interneurons coexpress both Cxcl12 receptors, indicating that Cxcr7 may also regulate chemokine responsiveness in migrating neurons. Consistent with this hypothesis, we found that conditional deletion of Cxcr7 exclusively from migrating interneurons renders then insensitive to Cxcl12, which causes important defects in their migration. These alterations are caused by the loss of Cxcr4 protein in migrating neurons, which is degraded when migrating cells confront Cxcl12 in the absence of Cxcr7. In conclusion, our results demonstrate that Cxcr7 modulates chemokine responsiveness in migrating neurons by regulating the levels of Cxcr4 receptors that are available to bind Cxcl12, and that loss of Cxcr7 function results de facto in the generation of neurons that are functionally deficient for both chemokine receptors.

RESULTS

Cxcr7 Expression in the Developing Cortex

Previous studies have shown that numerous cells in the embryonic rat cortex express *Cxcr7* (Schonemeier et al., 2008). In particular, *Cxcr7* was found to be very abundant in neurons forming the CP during initial stages of corticogenesis. To verify that this expression pattern is conserved in mice, we analyzed the distribution of *Cxcr7* mRNA at different stages of mouse cortical development. Comparison of the expression patterns of *Cxcr7* and *NeuroD2*, a transcription factor that is strongly expressed in the developing CP, revealed that many cells in this region also express *Cxcr7* at embryonic day (E) 13.5 (Figures 1A, 1B, 1D, and 1E). Detailed analysis of adjacent sections using sensitive radioactive probes confirmed that *Cxcr7* transcripts are very abundant in the early CP, from where *Cxcr4*-expressing cells are largely absent at this stage (Figures 1J–1M). Interestingly, we observed that the expression of *Cxcr7* in the CP is very transient, because *Cxcr7* is virtually excluded from the CP already at E15.5 (Figures 1C and 1F).

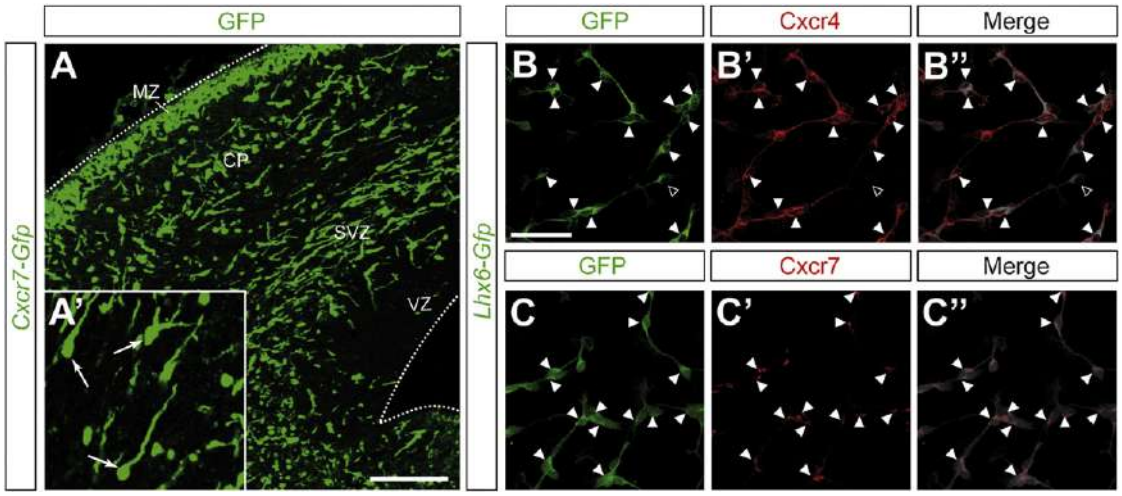


Figure 2. Cxcr7 Receptors Are Expressed in Migrating Cortical Interneurons

(A and A′) Immunohistochemistry for GFP in a coronal section through the telencephalon of an E13.5 *Cxcr7-Gfp* embryo. (A′) is a high magnification view of GFP-expressing cells in the SVZ that have the morphology of tangentially migrating interneurons. The arrows point to cells with the morphology of tangentially migrating interneurons.

(B–C″) Immunohistochemistry for GFP (B, B″, C, and C″), Cxcr4 (B′ and B″) and Cxcr7 (C′ and C″) in migrating cells derived from MGE explants obtained from E13.5 *Lhx6-Gfp* (*Lhx6-Cre;Rosa-EYFP*) embryos, after 24 hr in culture. Arrowheads point to GFP/Cxcr4 (B–B″) or GFP/Cxcr7 (C–C″) double-positive interneurons. Open arrowheads point to cells that do not seem to contain Cxcr4.

Scale bar equals 100 μm (A) and 40 μm (B–C″).

We also noticed that many cells outside the CP also express *Cxcr7* as early as E13.5 (Figures 1B, 1C, and 1K). Indeed, comparison of the distribution of cells expressing *Cxcr7* and *Lhx6*, a gene encoding for a transcription factor that is exclusively found in neurons derived from the MGE (Lavdas et al., 1999), suggests that this chemokine receptor may also be expressed by tangentially migrating interneurons (Figures 1A–1C and 1G–1I) (Schonemeier et al., 2008). Consistent with this idea, analysis of transgenic mice in which the gene encoding for the enhanced green fluorescent protein (EGFP) is expressed under the control of the *Cxcr7* promoter (*Cxcr7-EGFP*) revealed the existence of many cells with the morphology of tangentially migrating interneurons in the developing cortex (Figures 2A and 2A′). To quantify the expression of chemokine receptors in cortical interneurons, we cultured MGE explants obtained from *Lhx6-Cre;Rosa-EYFP* embryos on glass coverslips and stained migrating cells with antibodies against Cxcr4 and Cxcr7. We found that the large majority of MGE-derived interneurons express Cxcr4 (97.5% ± 1.0%, n = 879 cells; Figures 2B–2B″) and Cxcr7 (virtually all cells, n = 650 cells; Figures 2C–2C″). In summary, our analysis revealed that Cxcr7 is expressed in at least two populations of cortical neurons: one seems to correspond to pyramidal cells in the early CP, while the other consists of tangentially migrating interneurons that also contain Cxcr4 receptors. While the expression of Cxcr7 in the early CP is consistent with the previously reported function of this receptor as a "scavenger" removing Cxcl12 from undesirable locations (Boldajipour et al., 2008), coexpression of Cxcr4 and Cxcr7 in migrating interneurons suggests that the function of this latter receptor in neuronal migration might be more complex than previously anticipated.

Abnormal Migration of Cortical Interneurons in *Cxcr7* Null Mutants

To study the function of Cxcr7 in the migration of cortical interneurons, we first generated *Cxcr7*-deficient mice using a conditional approach (Sierro et al., 2007). In brief, $Cxcr7^{lox/+}$ mice were crossed to CMV-Cre transgenic mice (Schwenk et al., 1995) to produce germ-line deletion of *Cxcr7*. We then examined the distribution of MGE-derived cortical interneurons as identified by the expression of *Lhx6*. We found no significant differences in the routes of migration followed by *Lhx6*-expressing interneurons from the subpallium to the cortex in E16.5 control and *Cxcr7* null embryos (data not shown). However, analysis of the distribution of migrating cells within the cortex revealed important differences between both genotypes. Compared with controls, we found that many *Lhx6*-expressing interneurons deviate from their normal routes of migration within the MZ and SVZ and accumulate within the CP of *Cxcr7* null mutants (Figures 3A–3C). Thus, complete loss of Cxcr7 leads to abnormal intracortical migration of interneurons and premature invasion of the CP.

Cxcr7 Is Required in Migrating Interneurons for Intracortical Distribution

The previous analysis revealed that Cxcr7 function is required for the migration of cortical interneurons. The abnormal distribution of migrating interneurons in the developing cortex, which greatly resembled that previously reported for *Cxcr4* mutants (Li et al., 2008; López-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006), could be caused by the loss of Cxcr7 in the early CP, migrating interneurons, or both. To distinguish between these possibilities, we generated conditional mutants in which

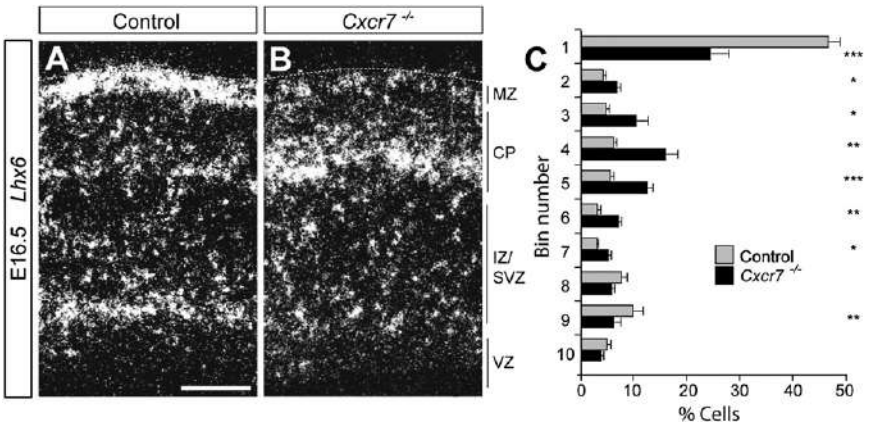


Figure 3. Complete Loss of Cxcr7 Leads to Abnormal Intracortical Distribution of GABAergic Interneurons during Embryonic Development

(A and B) Coronal sections through the telencephalon of E16.5 control (A) and *Cxcr7*$^{-/-}$ (B) embryos showing mRNA expression for *Lhx6*.

(C) Quantification of the distribution of *Lhx6*-expressing cells (putative MGE-derived interneurons) in control and *Cxcr7*$^{-/-}$ embryos. Numbers in ordinates identify bins for quantification, from the marginal zone (MZ, bin 1) to the ventricular zone (VZ, bin 10). n = 12 embryos, χ^2 test: **p < 0.01; ***p < 0.001. CP, cortical plate; IZ/SVZ, intermediate zone/subventricular zone. Histograms show average ± SEM.

Scale bar equals 100 μm.

Cxcr7 was specifically removed from cortical interneurons. To this end, we crossed *Cxcr7*$^{lox/+}$ mice with *Dlx5/6-Cre-IRES-Gfp* transgenic mice, in which Cre recombinase is expressed by most forebrain GABAergic neurons (Stenman et al., 2003). Compared to control embryos (*Dlx5/6-Cre-IRES-Gfp*;*Cxcr7*$^{lox/+}$ or *Dlx5/6-Cre-IRES-Gfp*;*Cxcr7*$^{+/+}$) analysis of interneuron-specific *Cxcr7* mutants (*Dlx5/6-Cre-IRES-Gfp*;*Cxcr7*$^{lox/lox}$, termed here *IN-Cxcr7* for brevity) revealed no significant differences in the number of interneurons that reach the embryonic cortex (control: 2563.4 ± 199.0 cells/mm^2, n = 4; *IN-Cxcr7*: 2368.4 ± 179.4 cells/mm^2, n = 6). However, we observed that many migrating interneurons fail to maintain their normal routes of migration through the MZ and SVZ in the absence of *Cxcr7* function (Figures 4A, 4B, 4E–4G, and S1, available online). Instead, many interneurons were found to be abnormally located within the CP (Figures 4A, 4B, 4E–4G, and S1).

In contrast to *Cxcr7* null mutant mice, which die soon after birth, *IN-Cxcr7* mutants survive into adulthood. This allowed us to study the postnatal consequences of the abnormal intracortical migration of embryonic interneurons. We found that the density of wild-type and mutant interneurons, identified in both cases by the expression of GFP from the *Dlx5/6-Cre-IRES-Gfp* transgene, was comparable in the motor, somatosensory, and visual cortices of control and *IN-Cxcr7* mutant mice at P21

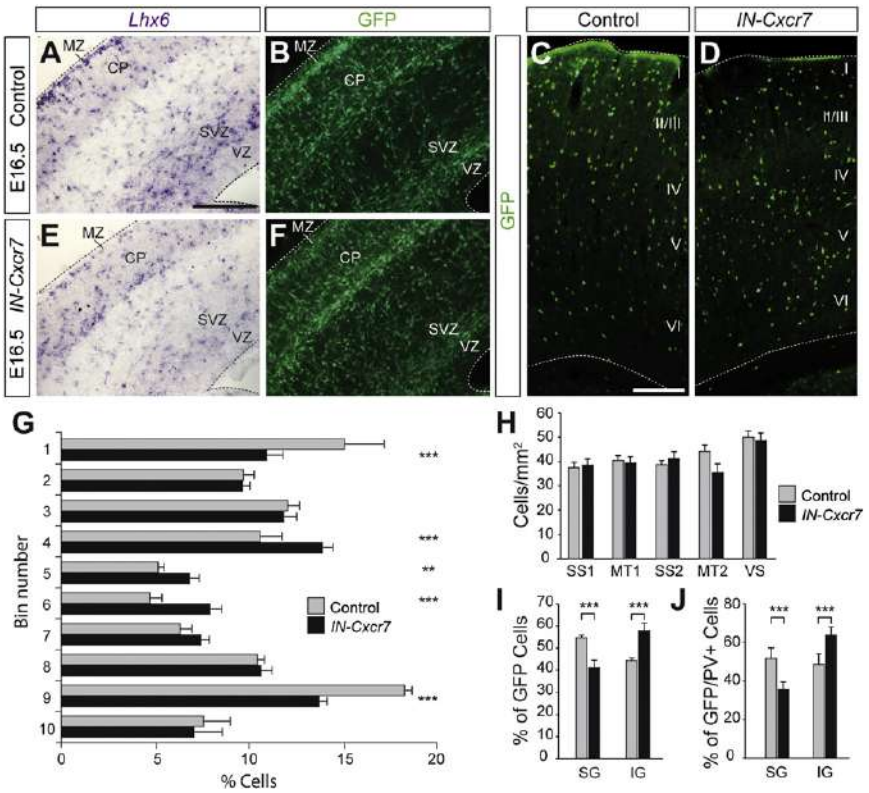


Figure 4. Loss of *Cxcr7* in GABAergic Interneurons Disrupts Intracortical Dispersion and Final Distribution

(A, B, E, and F) Coronal sections through the telencephalon of E16.5 control (A and B) and *IN-Cxcr7* (E and F) embryos showing mRNA expression for *Lhx6* (A and E) and immunohistochemistry for GFP (B and F). (C and D) Coronal sections through the somatosensory cortex of P21 control (C) and *IN-Cxcr7* mutant (D) mice showing immunohistochemistry for GFP. (G) Quantification of the distribution of GFP-expressing cells in control and *IN-Cxcr7* embryos. Numbers in ordinates identify bins for quantification, from the marginal zone (MZ, bin 1) to the ventricular zone (VZ, bin 10). Control: n = 4 embryos, *IN-Cxcr7*: n = 6. χ^2-test: **p < 0.01; ***p < 0.001. CP, cortical plate; SVZ, subventricular zone. (H) Quantification of the density of GFP-expressing cells in different cortical regions at P21. SS1 and SS2, rostral and caudal somatosensory cortex, respectively; MT1 and MT2, rostral and caudal motor cortex, respectively; VS, visual cortex. (I and J) Quantification of the distribution of GFP-expressing (I) and GFP/Parvalbumin-expressing interneurons in the somatosensory cortex of P21 control and *IN-Cxcr7* mutant mice. Control: n = 6 mice, *IN-Cxcr7*: n = 6. χ^2 test: **p < 0.01; ***p < 0.001. IG, infragranular layers; SG, supragranular layers (including layer IV). Histograms show average ± SEM. Scale bar equals 200 μm.

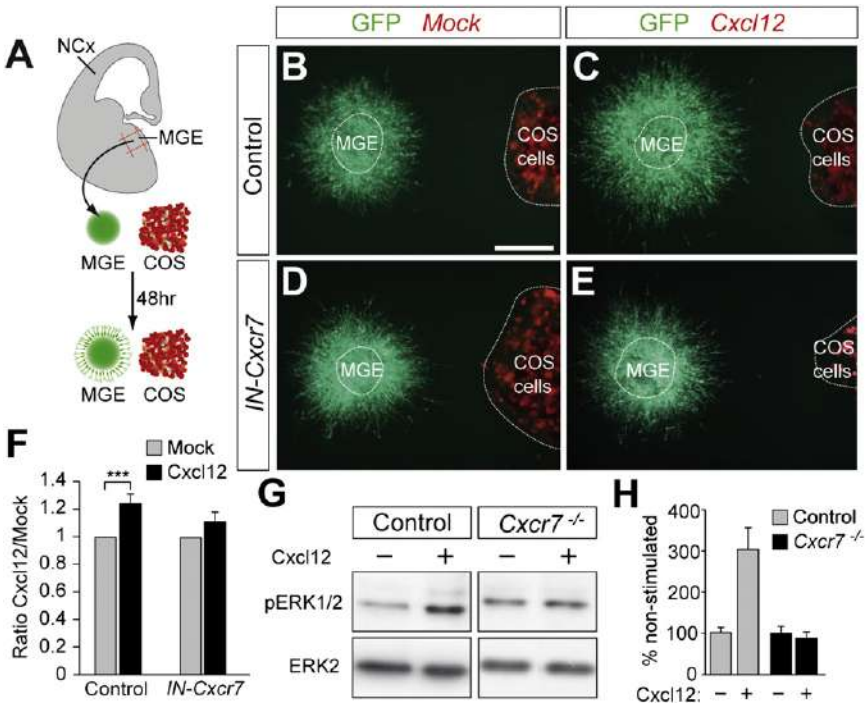


Figure 5. Cxcr7 Function in Interneurons Is Required for Cxcl12-Induced Chemotaxis

(A) Schematic of the experimental design.

(B–E) Migration of control (B and C) and *IN-Cxcr7* mutant (D and E) MGE-derived cells in response to mock-transfected (B and D) or *Cxcl12*-transfected (C and E) COS cell aggregates cultured in collagen matrices for 48 hr. Dotted lines indicate the limits of the explants and COS cell aggregates.

(F) Quantification of confrontation assays. Control versus mock, n = 48 explants; Control versus *Cxcl12*, *n* = 56; *IN-Cxcr7* versus mock, n = 33; *IN-Cxcr7* versus *Cxcl12*, n = 34, t test: ***p < 0.001.

(G) Immunoblots for pErk1/2 and Erk2 protein in telencephalic cultures obtained from control and $Cxcr7^{-/-}$ embryos, after 1 DIV. Cultures were treated (+) or not (–) with Cxcl12.

(H) Quantification of fold activation for pErk1/2 in control and $Cxcr7^{-/-}$ embryos. n = 6 cultures from two control and two $Cxcr7^{-/-}$ embryos, t test: ***p < 0.001. Histograms show average ± SEM.

Scale bar equals 200 μm.

(Figure 4H). However, we observed that the relative density of GFP-expressing interneurons within infragranular and supragranular layers of the somatosensory cortex was significantly different between control and *IN-Cxcr7* mutant mice. Specifically, interneurons were abnormally abundant in infragranular layers of *IN-Cxcr7* mutant mice compared with controls, whereas the reverse was found for supragranular layers (Figures 4C, 4D, and 4I). Similar results were obtained when we analyzed the distribution of cells expressing Parvalbumin, which identifies the largest population of MGE-derived cortical interneurons (Figure 4J). Thus, Cxcr7 function in interneurons is required for their normal distribution in the adult cortex.

Cxcr7-Deficient Interneurons Do Not Respond to Cxcl12

The previous experiments indicated that, independently of its possible function in the CP, *Cxcr7* is indispensable in cortical interneurons for their correct intracortical migration and final distribution. This suggests that Cxcr7 receptors might be required within cortical interneurons to respond to Cxcl12. To test this hypothesis, we cocultured E13.5 MGE explants obtained from control and *IN-Cxcr7* mutant embryos with aggregates of COS cells transfected with *Cxcl12* (Figure 5A). As described before (López-Bendito et al., 2008), we observed that Cxcl12 strongly promotes the migration of MGE-derived cells obtained from control embryos (Figures 5B, 5C, and 5F). In contrast, we found that MGE-derived cells obtained from *IN-Cxcr7* mutants fail to respond to Cxcl12 (Figures 5D–5F). Thus, Cxcr7 is necessary for the chemotaxis of cortical interneurons in response to Cxcl12.

The previous results were unexpected, since most MGE-derived cells express both Cxcr4 and Cxcr7 receptors (Figure 2) and Cxcr4 mediates the Cxcl12-dependent migration of these neurons (Li et al., 2008; López-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006). A possible explanation might be that both chemokine receptors cooperate in migrating interneurons and that one receptor alone is not sufficient to elicit a response to Cxcl12. Alternatively, Cxcr7 might be required for normal Cxcr4 function. To distinguish between both possibilities, we examined whether Cxcr4 signaling was impaired in the absence of Cxcr7. To this end, we prepared cultures from the ventral telencephalon of control and *Cxcr7* mutant embryos, and stimulated them with recombinant Cxcl12. As expected from previous reports on Cxcr4 signaling (Li and Ransohoff, 2008), stimulation with Cxcl12 strongly promoted the phosphorylation of the extracellular signal-regulated kinases 1 and 2 (Erk1/2) in control cells (Figures 5G and 5H). In contrast, Cxcl12 stimulation failed to elicit phosphorylation of Erk1/2 in cells obtained from *Cxcr7* mutants (Figures 5G and 5H).

Cxcr7 Is Required to Sustain Cxcr4 Protein Levels in Migrating Interneurons

The previous experiments reinforced the hypothesis that Cxcr4 function is compromised in the absence of Cxcr7. One possible mechanism could be that Cxcr7 is required for normal *Cxcr4* expression. To test this idea, we analyzed the distribution of *Cxcr4*-expressing cells in the cortex of control and *IN-Cxcr7* mutant embryos. We found that *Cxcr4* mRNA is normally expressed in the absence of Cxcr7. However, as predicted from the MGE coculture experiments, *Cxcr4*-expressing neurons were found to distribute abnormally in the cortex of *IN-Cxcr7* mutant embryos (Figures 6A and 6D). Indeed, the distribution of *Cxcr4*-expressing cells closely resembled that observed for *Lhx6*-expressing cells in *IN-Cxcr7* mutant embryos.

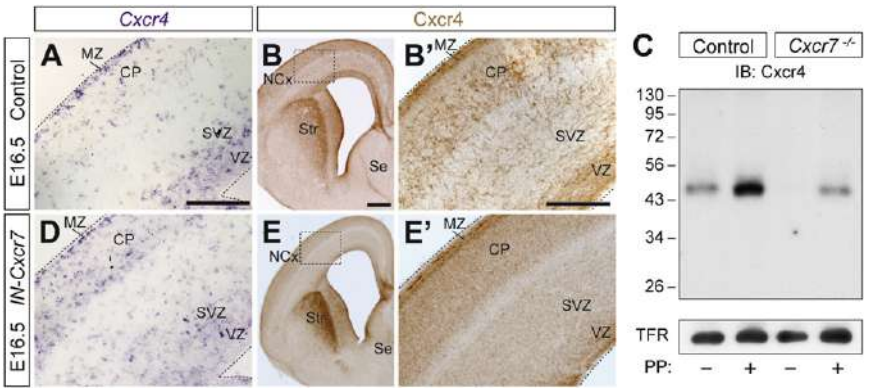


Figure 6. Cxcr7 Is Required to Sustain Cxcr4 Protein Levels in Migrating Interneurons

(A, B, B′, D, E, and E′) Coronal sections through the telencephalon of E16.5 control (A–B′) and *IN-Cxcr7* mutant (D–E′) embryos showing *Cxcr4* mRNA expression (A and D) and immunohistochemistry for Cxcr4 (B, B′, E, and E′). (B′) and (E′) are high-magnification images of the boxed areas in (B) and (E), respectively. (C) Immunoblots for Cxcr4 protein in the telencephalon of control and $Cxcr7^{-/-}$ embryos, with (+) or without (–) dephosphorylation of protein lysates with protein phosphatase lambda (PP) prior to blotting. Transferrin receptor (TFR) levels were used as charge control. CP, cortical plate; MZ, marginal zone; NCx, neocortex; Se, septum; Str, striatum; SVZ, subventricular zone; VZ, ventricular zone. Scale bar equals 200 μm (A, B′, D, and E′) and 300 μm (B and E).

We next wondered whether the levels of Cxcr4 protein were normal in *Cxcr7* mutant interneurons. We found that Cxcr4 immunoreactivity was reduced in the subpallium of *Cxcr7* mutant embryos compared with controls (Figures 6B and 6E). Most strikingly, Cxcr4 immunoreactivity was almost entirely absent from the cortex of *IN-Cxcr7* mutant embryos (Figures 6B, 6B′, 6E, and 6E′). These defects were also obvious in *Cxcr7* null mutants (Figures S2A–S2D). Because the antibody used to detect Cxcr4 in these experiments does not recognize the activated, phosphorylated form of Cxcr4 (Figures S2E and S2F), these results indicate that either all Cxcr4 present in *Cxcr7*-deficient interneurons has been phosphorylated, or that Cxcr4 is indeed absent from these cells. To distinguish between these two possibilities, we prepared lysates from the telencephalon of control and *Cxcr7* mutants and blotted for Cxcr4 with or without previous treatment with lambda phosphatase. We detected a moderate signal in lysates from wild-type embryos in which dephosphorylation was omitted (Figure 6C). The signal was approximately five times stronger after dephosphorylation, which indicated that roughly 80% of Cxcr4 receptors were present in the activated state. As expected from our histological observations, Cxcr4 was almost undetectable in lysates obtained from *Cxcr7* mutants that were not treated with phosphatase (Figure 6C). Treatment with phosphatase revealed a small fraction of Cxcr4 receptors in *Cxcr7* mutants, which was nevertheless much smaller than the total amount of Cxcr4 receptors found in controls (Figure 6C). Thus, the total amount of receptor is severely reduced in the telencephalon of *Cxcr7* mutants compared with controls, and the few receptors that are left in these embryos are present in a phosphorylated/activated form.

Interneurons Uptake Cxcl12 via Cxcr7 Receptors to Regulate Cxcr4 Levels

We next wondered about the mechanism through which Cxcr7 could regulate the expression of Cxcr4 receptors in migrating neurons. It is well established that persistent Cxcl12 stimulation causes Cxcr4 degradation in different cells (Figures S2G and S2H) (see, for example, Kolodziej et al., 2008), and so one possible explanation for the previous results is that Cxcr7 receptors are required in migrating neurons to adjust the concentration of Cxcl12 that these cells encounter as they move through the cortex. Indeed, Cxcr7 has been shown to be able to uptake and degrade Cxcl12 with great affinity in other cells (Balabanian et al., 2005a; Naumann et al., 2010), so we hypothesized that this receptor may play a similar role in migrating neurons. We reasoned that if this were the case, then Cxcr7 should be found at the plasma membrane of interneurons. Unexpectedly, we found that Cxcr7 is barely detectable in the membrane of permeabilized interneurons (i.e., those fixed and treated with Triton X-100), whereas it is relatively abundant in intracellular compartments (Figures 7A and 7A″). By contrast, Cxcr4 is clearly detectable in the plasma membrane of the same cells (Figures 7A′ and 7A″). This suggested that the fraction of Cxcr7 receptor that is normally present in the cell surface of interneurons is relatively small compared to that of Cxcr4. To confirm this, we performed surface labeling of living interneurons by incubating MGE explants with antibodies directed against the N terminus of Cxcr7 at 4°C to prevent receptor internalization. Using this approach, we unequivocally detected expression of endogenous Cxcr7 receptors in the membrane of migrating interneurons (Figures 7B–7C″). Interestingly, incubation of antibodies against Cxcr7 with living interneurons at 37°C revealed that Cxcr7 receptors are rapidly internalized in these cells, even in the absence of its ligand (Figures S3A–S3B″). We next analyzed the subcellular location of Cxcr7 receptors by performing double labeling immunohistochemistry with markers of different types of intracellular organelles. We found that Cxcr7 puncta largely overlap with the marker of recycling endosomes Rab4 (Figures S3A–S3B″; Cxcr7/Rab4 double-labeled puncta: 81.9% ± 4.52%, average ± SEM; n = 52 cells from two different cultures), but not with markers of other types of endosomes (data not shown). All together, our results indicate that Cxcr7 receptors are indeed present in the plasma membrane of migrating interneurons, but they typically recycle from the membrane to intracellular compartments, where the largest fraction of receptors is normally present.

We next wondered whether Cxcr7 is indeed used by interneurons to bind and uptake Cxcl12. To tackle this question, we cultured ventral telencephalic neurons and carried out

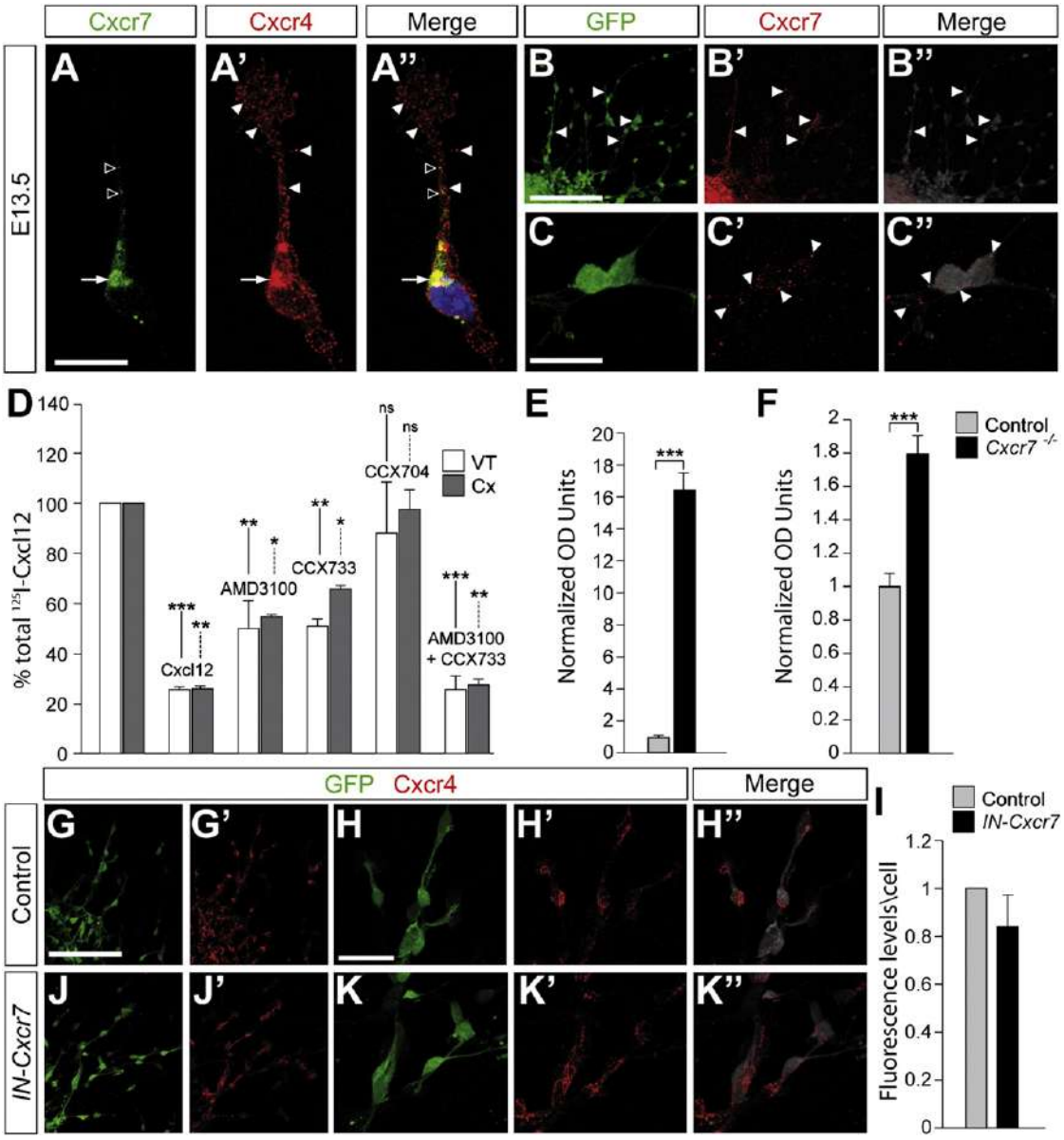


Figure 7. Interneurons Use Cxcr7 to Bind and Uptake Cxcl12

(A–A″) Double immunohistochemistry for Cxcr4 and Cxcr7 in an interneuron that has been fixed and permeabilized. Arrowheads and open arrowheads point to dots that are positive for Cxcr4 and Cxcr7, respectively. The arrow points to an intracellular accumulation of Cxcr4 and Cxcr7 signal, which in part overlaps with the Golgi apparatus (data not shown).

(B–C″) Low-magnification (B–B″) and high-magnification (C–C″) images showing immunohistochemistry for GFP (B, B″, C, and C″) and Cxcr7 (B′, B″, C′ and C″) in migrating cells derived from an E13.5 MGE explant obtained from a Gfp transgenic embryo. Cxcr7 antibodies were incubated with living cells at 4°C for 1 hr prior to fixation to detect endogenous Cxcr7 receptors present in these cells. Arrowheads point to dots that are positive for Cxcr7.

(D) Quantification of ^{125}I-Cxcl12 uptake in cell cultures prepared from the ventral telencephalon (VT, white bars) or cortex (Cx, gray bars) of E16 wild-type embryos. All values are normalized against control uptake at 1 hr. ANOVA test with post hoc Bonferroni was used for ventral telencephalic data, whereas Welch test with post hoc Games-Howell was applied for cortex data. All statistical analyses referred to control values. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Histograms show average ± SEM.

(E and F) ELISA measurements of the concentration of Cxcl12 present in the medium of cortical cultures prepared from wild-type and *Cxcr7*$^{-/-}$ embryos after 5 DIV (E) or from supernatants obtained from cortical homogenates of E14.5 wild-type and *Cxcr7*$^{-/-}$ embryos (F). t test, ***$p < 0.001$. Histograms show average ± SEM.

(G–H″ and J–K″) Immunohistochemistry for GFP (G, H, H″, J, K, and K″) and Cxcr4 (G′, H′, H″, J′, K′, and K″) in migrating cells derived from an MGE explant obtained from E13.5 control (G–H″) and *IN-Cxcr7* mutant (J–K″) embryos, after 24 hr in culture.

(I) Quantification of relative fluorescent levels for Cxcr7 in control and *IN-Cxcr7* mutant interneurons derived from MGE explants. Control, n = 35 cells and *IN-Cxcr7*, n = 29 cells.

Scale bar equals 10 μm (A–A″), 25 μm (C–C″, H–H″, and K–K″) and 100 μm (B–B′, G–G′, and J–J′).

radioligand binding assays in which neurons were exposed to Cxcl12 labeled with Iodine-125 (^{125}I) for different periods of time. We found that ventral telencephalic neurons bind and uptake increasing amounts of radiolabeled ligand with time (data not shown), reaching peak levels after 1 hr of incubation. The observed binding was specific for Cxcl12, as demonstrated by the ability of unlabeled Cxcl12 (40 nM) to effectively compete radiolabeled ligand binding (Figure 7D). We also observed that Cxcl12 uptake was partially blocked by a saturating concentration of the Cxcr4 antagonist AMD3100 (Figure 7D). This experiment suggested that neurons in the ventral telencephalon might also use Cxcr7 receptors to bind Cxcl12, because uptake was not completely abolished by the Cxcr4 antagonist. To unequivocally demonstrate this, we performed another series of experiments using CCX733, a small compound that has been shown to specifically compete with Cxcl12 for Cxcr7 binding (Luker et al., 2010; Rajagopal et al., 2010). We found that CCX733 (but not the closely related control molecule CCX704) severely reduces Cxcl12 uptake in ventral telencephalic neurons (Figure 7D), which reinforced the view that Cxcr7 receptors in migrating interneurons bind and uptake Cxcl12. Furthermore, incubation of interneurons with both AMD3100 and CCX733 reduces Cxcl12 binding to background levels (Figure 7D), which demonstrated that both receptors are functionally active in this population of neurons.

To verify that interneurons continue to bind and uptake Cxcl12 once they have arrived to the cortex, we repeated the previous experiments with cells obtained from the cortex of E16 embryos, a stage at which CP cells no longer express *Cxcr7* (Figure 1C). We found that cells in the cortex bind and uptake Cxcl12, and that this is in part mediated by Cxcr7 receptors (Figure 7D). Together with our immunocytochemical observations, these results strongly suggested that migrating interneurons bind and uptake Cxcl12 through both Cxcr4 and Cxcr7 receptors, although the latter receptor seems to follow a much more rapid dynamic of internalization than Cxcr4.

The previous results are consistent with our hypothesis that Cxcr7 receptors expressed in interneurons modulate the levels of Cxcl12 that these cells encounter during their migration, thereby preventing the rapid desensitization of Cxcr4 receptors. We reasoned that if this were the case, then Cxcl12 should accumulate in the absence of these receptors. To test this hypothesis, we prepared cortical cultures from control and *Cxcr7* null embryos and measured the concentration of Cxcl12 in the medium after 5 days in vitro (DIV). We found that Cxcl12 was ~15 times more abundant in cortical cultures obtained from *Cxcr7* null embryos compared with those from controls (Figure 7E). To extend these observations in vivo, we next prepared cortical homogenates from control and *Cxcr7* null embryos and measured the concentration of Cxcl12 present in the supernatants. We found that the concentration of Cxcl12 was significantly increased in *Cxcr7* mutants over that of controls (Figure 7F). Considering that the expression of *Cxcl12* mRNA is not altered in *Cxcr7* null or *IN-Cxcr7* mutants (Figures S3C–S3F), these experiments strongly suggested that Cxcr7 is required to titrate the amount of Cxcl12 available in the developing cortex.

Finally, if Cxcr4 levels depend on the concentration of Cxcl12 that they encounter, then Cxcr4 expression should not be altered in *Cxcr7* mutant interneurons cultured in the absence of Cxcl12. To test this hypothesis, we cultured MGE explants from control and *IN-Cxcr7* mutants in the absence of Cxcl12, which is not expressed in the MGE. In this context, quantification of Cxcr4 fluorescence after immunohistochemistry revealed no significant differences between control and *IN-Cxcr7* mutant interneurons (Figures 7G–7K″). Moreover, analysis of the expression of Cxcr4 in single confocal planes of cells stained with wheat germ agglutinin (WGA) lectin, which labels the plasma membrane, revealed a similar degree of colocalization in both controls and *IN-Cxcr7* mutant interneurons (Figures S3G–S3I). These results indicated that Cxcr7 is not essential for the synthesis or transport of Cxcr4 to the plasma membrane. All together, our experiments suggested that the function of Cxcr7 in migrating interneurons is to titrate the concentration of Cxcl12 available for these cells, thereby modulating the levels of Cxcr4 receptors. In the absence of Cxcr7, Cxcr4 becomes degraded, and interneurons fail to respond to Cxcl12.

Migrating Neurons Collectively Regulate Chemokine Responsiveness

Our analysis of *IN-Cxcr7* mutants clearly demonstrated that Cxcr7 is required in interneurons for normal intracortical migration. One remaining question, however, is whether Cxcr7 is required in each individual interneuron (i.e., whether Cxcr7-mediated Cxcl12 uptake in each individual interneuron prevents Cxcr4 degradation) or whether migrating interneurons collectively adjust Cxcl12 levels for the entire population (i.e., whether interneurons clean up excessive Cxcl12 for other interneurons). To answer this question, we tested whether the migratory phenotype observed for *Cxcr7* mutant interneurons could be rescued when transplanted in a wild-type environment in which many other interneurons migrate and uptake Cxcl12 from the extracellular space. We first performed microtransplantation assays in slices, as described before (López-Bendito et al., 2008) (Figure 8A). We previously showed that E13.5 wild-type MGE-derived interneurons isochronically transplanted into the cortex disperse tangentially and avoid entering the CP, as they normally do in vivo, whereas *Cxcr4* mutant interneurons prematurely invade the CP (López-Bendito et al., 2008). Unexpectedly, the migration of *Cxcr7* mutant interneurons transplanted into wild-type cortices was indistinguishable from that of wild-type cells (Figures 8B, 8D, and 8E). Furthermore, we observed that most *Cxcr7* mutant interneurons contained detectable levels of Cxcr4 while migrating into wild-type slices (Figures 8C and 8F), which demonstrated that the function of Cxcr7 in nearby interneurons is enough to sustain the levels of Cxcr4 receptors in *Cxcr7* mutant interneurons.

To confirm these observations, we next carried out similar transplantation experiments in vivo (Figure 8G). We previously showed that E15.5 wild-type interneurons transplanted isochronically and homotypically in utero end up primarily in superficial layers of the cortex (López-Bendito et al., 2008; Pla et al., 2006), as they normally do in vivo. In contrast, many E15.5 *Cxcr4* mutant interneurons end up in deep cortical layers, probably because they prematurely invade the CP (López-Bendito et al., 2008). As predicted by our organotypic cultures, E15.5

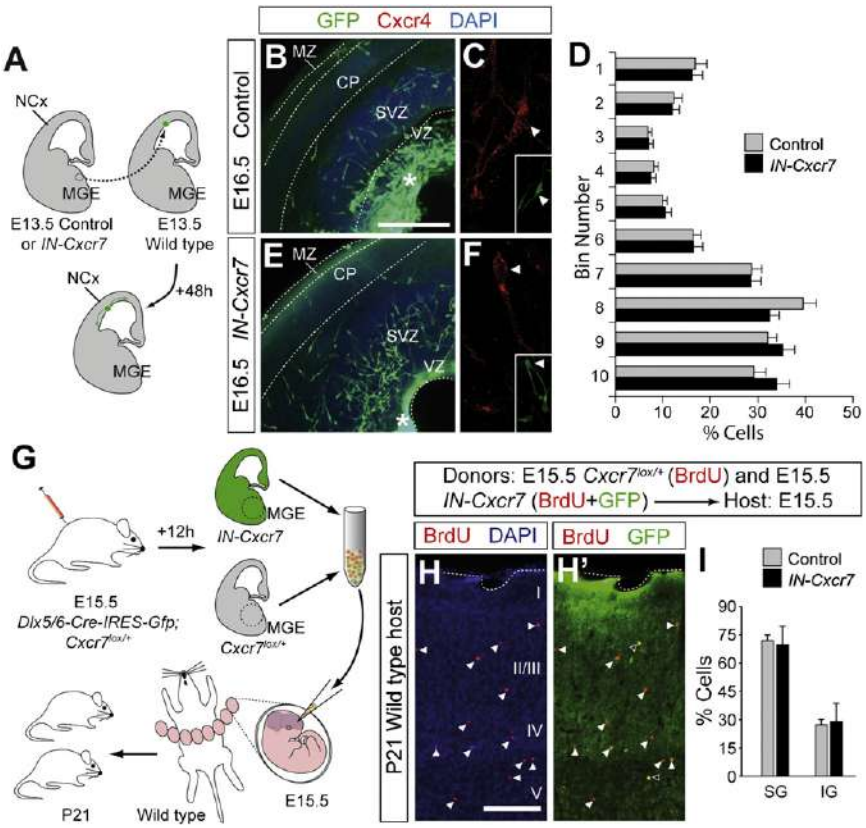


Figure 8. Population Effects of Cxcr7 Function on Intracortical Interneuron Migration

(A) Schematic of the experimental design. (B, C, E, and F) Immunohistochemistry against GFP in microtransplantation slice assays in which E13.5 control (B and C) or *IN-Cxcr7* mutant (E and F) MGE explants were transplanted into the cortical VZ of E13.5 wild-type slices and cultured for 48 hr. (C) and (F) show high-magnification images of migrating neurons stained against Cxcr4. The insets indicated that the labeled cells derived from the MGE transplants. (D) Quantification of the distribution of E13.5 MGE-derived cells from control or *IN-Cxcr7* mutant embryos. Numbers in ordinates identify bins for quantification, from the marginal zone (MZ, bin 1) to the ventricular zone (VZ, bin 10). Control, n = 39; *IN-Cxcr7*, n = 40. χ^2-test: **p < 0.01; ***p < 0.001. CP, cortical plate; SVZ, subventricular zone. (G) Schematic of the experimental design. *Dlx5/6-Cre-IRES-Gfp;Cxcr7*$^{lox/+}$ pregnant females (mated with *Cxcr7*$^{lox/lox}$ males) received a single pulse of BrdU at E15.5. Twelve hours later, the MGEs from *Cxcr7*$^{fl/+}$ and *IN-Cxcr7* embryos were collected, dissociated, and pooled together. Pooled donor MGE cells were injected into the MGE of E15.5 wild-type host embryos using ultrasound imaging. Host embryos were analyzed at P21. (H and H′) Coronal section through the somatosensory cortex of a transplanted P21 mouse showing the distribution of transplanted control (BrdU+) and *Cxcr7* mutant interneurons (BrdU+/GFP+). Solid and open arrowheads point to control and mutant cells, respectively. (I) Quantification of the distribution of control and Cxcr7 mutant interneurons in P21 somatosensory cortex. IG, infragranular layers; SG, supragranular layers (including layer IV). Histograms show average ± SEM. Scale bar equals 200 μm.

MGE-derived *Cxcr7* mutant interneurons transplanted into wild-type embryos adopt a laminar pattern that is indistinguishable from control interneurons born isochronically (Figures 8H and 8I). Altogether, our experiments demonstrated that Cxcr7 is not essential within each individual interneuron for their migration. Instead, these results revealed that Cxcr7 functions at the population level to regulate the migration of cortical interneurons.

DISCUSSION

In this study, we have used cortical interneurons as a model system to investigate the function of the atypical chemokine receptor Cxcr7 in neuronal migration. We have found that Cxcr7 is required in migrating interneurons to regulate the levels of Cxcr4 receptors expressed by these cells, through a process that requires the interaction of migrating cells with the chemokine Cxcl12. Interestingly, this function emerges as a property of the entire population of migrating interneurons, because the loss of Cxcr7 in an individual cell can be rescued by the function of Cxcr7 in other migrating interneurons. These results provide a clear demonstration that an atypical chemokine receptor can modulate the highly specialized function of a classical chemokine receptor by controlling the amount of receptor that is made available for signaling at the cell surface. Beyond its relevance in neuronal migration, we believe that these findings may have important implications for understanding the role of Cxcr7 in other biological contexts, such as tumorigenesis.

Cxcr7 Expression in the Early CP

Previous studies have suggested that the main function of Cxcr7 is to sequester Cxcl12 from undesirable locations, thereby contributing to dynamically shape the chemokine gradients for cell migration (Boldajipour et al., 2008). The expression of Cxcr7 in the early CP suggests that this receptor may play such a role in the developing cortex. Thus, Cxcr7 expression in the early CP may contribute to the organization of the routes of interneuron migration in the cortex, and the analysis of null mutants for *Cxcr7* is consistent with this possibility. However, the similarity of the defects found in *Cxcr7*$^{-/-}$ and *IN-Cxcr7* mutants suggest that the expression of Cxcr7 in migrating neurons is indispensable for normal migration. Thus, although the CP may play a role in buffering chemokines through the function of Cxcr7, our results demonstrate that this receptor is uniquely required for normal migration in cells that also express Cxcr4 receptors.

Coexpression of Cxcr4 and Cxcr7 in Migrating Neurons

Our results indicate that migrating cortical interneurons express both Cxcl12 receptors, Cxcr4 and Cxcr7. This is in contrast with many other systems, in which Cxcr7 is largely restricted to nonmigratory cells that modulate the migration of Cxcr4-expressing cells (Boldajipour et al., 2008), or is expressed by a set of migrating cells that do not express Cxcr4 (Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Previous experiments using simultaneous transfection of Cxcr4 and Cxcr7 in cell lines suggested that these receptors have the potential to dimerize, and that Cxcr7 may modulate signaling through Cxcr4 in response to Cxcl12 (Levoye et al., 2009; Sierro et al., 2007). In migrating interneurons, however, the different subcellular location of both receptors suggests that they may not interact extensively. Thus, while Cxcr4 is copiously found in the plasma membrane, Cxcr7 is more abundant in recycling endosomes. Our experiments nevertheless suggest that interneurons also uptake Cxcl12 through Cxcr7, although this receptor follows a very rapid dynamic of internalization. This is in agreement with recent experiments in other cell types in which Cxcr7 spontaneously internalizes even in the absence of ligand (Luker et al., 2010; Naumann et al., 2010), a behavior that is common to other atypical chemokine receptors such as D6, CCX-CKR, and DARC (Graham, 2009).

We cannot exclude the possibility that the antibodies used in our study only recognize a fraction of Cxcr7 receptors, and that therefore Cxcr4 and Cxcr7 may dimerize under some circumstances in migrating interneurons. It should be noted, however, that a recent study using different antibodies than those used here has described a very similar expression pattern for endogenous Cxcr7 receptors in T cells. As in interneurons, Cxcr7 is nearly exclusively confined to cytosolic compartments of leukocytes (Hartmann et al., 2008). In sum, our results suggest that a large fraction of Cxcr7 concentrates underneath the plasma membrane, and so modulation of Cxcr4 expression and function is unlikely to occur in these cells through the interaction of these receptors at the cell surface.

Regulation of Cxcr4 Protein Levels by Cxcr7 Function

We have shown that Cxcr7 is required in migrating interneurons to sustain normal levels of Cxcr4 receptors in response to Cxcl12. This defect does not seem to involve a transcriptional link between *Cxcr4* and *Cxcr7*, as proposed in other systems (Dambly-Chaudiere et al., 2007; Wang et al., 2008), because *Cxcr4* mRNA levels are normal in the absence of *Cxcr7*. Most notably, Cxcr4 receptors are found in the plasma membrane of *Cxcr7* mutant interneurons in the absence of Cxcl12, which indicates that Cxcr7 is not required for the normal trafficking of Cxcr4 to the membrane. This suggests that the defects observed in vivo might be linked to the regulation of receptor endocytosis. For GPCRs, endocytosis serves as a mechanism to regulate cell-surface receptor levels, thereby modulating chemokine responsiveness. Upon internalization, GPCRs can be recycled back to the plasma membrane or sorted to the lysosome for degradation, and the fraction of receptors that recycle back to the membrane depends on several factors (Marchese et al., 2003). In the case of Cxcr4 receptors, nearly 70% of the activated receptors are targeted for degradation following prolonged Cxcl12 stimulation (Kolodziej et al., 2008; Marchese and Benovic, 2001; Tarasova et al., 1998). This indicates that saturating signals of Cxcl12 render cells insensitive to the chemokine in a very short time. In this context, given its high affinity for Cxcl12 (Balabanian et al., 2005a), expression of Cxcr7 in migrating neurons may contribute to the regulation of Cxcl12 levels in the microenvironment of each cell to prevent the rapid desensitization of Cxcr4 receptors. In the absence of Cxcr7, synthesis of Cxcr4 in migrating neurons might not be enough to cope with the degradation rate imposed by the surplus of Cxcl12 that the cells encounter as they enter the cortex. In sum, our experiments suggest that Cxcr7 fine-tunes the response of Cxcr4 to changing concentrations of Cxcl12, thereby enabling directional migration.

It is conceivable that Cxcr7 might also be necessary for the proper internalization of Cxcr4 receptors, or that Cxcr7 may somehow protect a fraction of Cxcr4 receptors from degradation. This would imply strictly cell-autonomous functions of Cxcr7 in migrating interneurons, which we have not been able to test. However, our transplantation experiments strongly suggest that Cxcr4 expression can be rescued in *Cxcr7* mutant interneurons when they migrate in a wild-type environment, which indicates that if Cxcr7 plays a strictly cell-autonomous role in vivo, it may only have a relative impact in the migratory behavior of this population of cells.

Recent work suggests that a crosstalk between Cxcr4 and Cxcr7 exists in T cells, in which blocking Cxcr7 function perturbs Cxcr4-mediated adhesiveness induced by surface-bound Cxcl12 (Hartmann et al., 2008). In these cells, however, Cxcr7 inhibition does not prevent Cxcr4-mediated Erk1/2 activation or chemotaxis toward Cxcl12 (Hartmann et al., 2008). Moreover, while the disruption of Cxcr4-mediated adhesiveness in T cells might be related to the inability of a fraction of Cxcr4 receptors to target the membrane in the absence of Cxcr7, our results suggest that Cxcr4 receptors do indeed reach the membrane in *Cxcr7* mutant interneurons in the absence of Cxcl12. Thus, Cxcr7 seems to modulate chemotaxis in T lymphocytes by directly regulating trafficking, and not levels, of Cxcr4, which indicates that the interaction between these two receptors might be different in lymphocytes and neurons.

Our observations suggest that the regulation of cell surface levels of Cxcr4 by Cxcr7 depends on the concentration of Cxcl12, but it is presently unclear whether Cxcr7 merely functions as a decoy receptor, sequestering Cxcl12 from the surface of migrating cells, or if Cxcl12-induced Cxcr7 signaling also plays a role in neuronal migration. Our transplantation experiments suggest that a strictly cell-autonomous function of Cxcr7 is not required for migration, because *Cxcr7* mutant interneurons migrate normally when transplanted into a wild-type environment. Nevertheless, it is conceivable that Cxcl12 binding could elicit other cellular responses through Cxcr7 that may contribute to the regulation of neuronal migration. For example, Cxcr4 signaling and degradation requires interaction with β-arrestin2 (Fong et al., 2002; Sun et al., 2002), a protein that also seems to play a major role downstream of Cxcr7 signaling (Kalatskaya et al., 2009; Luker et al., 2009; Rajagopal et al., 2010; Zabel et al., 2009). Considering the high affinity of Cxcr7 for Cxcl12, activation of Cxcr7 receptors by its ligand may

sequester β-arrestin2 away from Cxcr4, thereby modulating the internalization rate of this receptor. Future experiments should aim at identifying to what extent Cxcr7 signaling may directly influence neuronal migration.

We believe that our findings may have important implications in other processes in which the chemokine Cxcl12 has been implicated, such as tumorigenesis. Cxcl12 has been involved in multiple steps of tumor progression and metastasis in more than 20 different cancers, including neuroectodermal tumors and breast cancer metastasis to the brain (Burger and Kipps, 2006; Murphy, 2001). In this context, recent studies have shown that Cxcr7 expression increases tumor formation and metastasis for some cancers (Miao et al., 2007; Raggo et al., 2005; Wang et al., 2008), which suggests that this receptor plays an important role in this process. If one important function of Cxcr7 is to prevent desensitization of Cxcr4, as reported here, then high expression of this receptor in tumor cells may contribute to excessive signaling through Cxcr4, a landmark of the physiopathology of WHIM syndrome that is also associated with tumor growth and metastasis formation (Balabanian et al., 2005b; Kawai and Malech, 2009). Consequently, our observations indicate that blocking Cxcr7 function may represent an effective therapy to treat the population of cancer cells in which both receptors are coexpressed.

Intracortical Migration and Postnatal Distribution of Interneurons

Our present results, along with previous observations (Li et al., 2008; López-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006), indicate that the chemokine Cxcl12 and its receptors play important roles in regulating the intracortical migration of interneurons. Disruption of the embryonic dispersion of interneurons influences their final distribution in the adult, which underlines the relevance of this process in the development of inhibitory circuitries in the cerebral cortex. It is worth mentioning that the postnatal defects found in *IN-Cxcr7* mutants (present study), as well as those reported for interneurons lacking *Cxcr4* (Li et al., 2008; López-Bendito et al., 2008), have a strong regional bias. In the case of *IN-Cxcr7* mutants, for example, the abnormal distribution of cortical interneurons affects the somatosensory cortex, but not the motor or visual cortices. This suggests that chemokine signaling might be particularly important to prevent the concentration of interneurons in the first region they encounter when they enter the cortex, the developing parietal cortex. Alternatively, other chemokines expressed in the developing brain may play additional roles in controlling the distribution of interneurons in other cortical regions.

EXPERIMENTAL PROCEDURES

Mouse Strains

Wild-type mice maintained in a CD1 background were used for expression analysis and tissue culture experiments. *Lhx6-Cre* (Fogarty et al., 2007), *Rosa-EYFP* (Srinivas et al., 2001), *Cxcr7^{lox}* (Sierro et al., 2007), and *Dlx5/6-Cre-IRES-Gfp* (Stenman et al., 2003) were maintained in a C57b/6 background. *Cxcr7-EGFP* BAC transgenic mice, maintained in a hybrid FVB/N-IcrTac/ICR background (Gong et al., 2003), were obtained from the Gene Expression Nervous System Atlas (GENSAT) Project, NINDS Contracts N01NS02331 and HHSN271200723701C to The Rockefeller University (New York, NY). *IN-Cxcr7* conditional mutants were obtained by crossing *Cxcr7$^{lox/lox}$* mice with *Dlx5/6-Cre-IRES-Gfp;Cxcr7^{lox}* mice. *Dlx5/6-Cre-IRES-Gfp;Cxcr7$^{lox/7+}$* and *Cxcr7$^{lox/lox}$* mice were indistinctively used as controls in our experiments, as no differences were observed between these genotypes. The day of vaginal plug was considered as E0.5. Animals were kept at the Instituto de Neurociencias de Alicante under Spanish, German, and EU regulation.

Binding Assays

^{125}I–SDF-1α (2200 Ci/mmol; 25 μCi/ml) was purified immediately before use with Micro Bio-Spin Columns (Bio-Rad) to exclude degradation products. Primary neurons (E16 ventral telencephalon or cortex) were seeded at a density of 500,000 cells/well in Neurobasal medium/B27-supplement (Invitrogen). Assays were performed after 24 hr in 400 μl fresh culture medium containing 25 pmol/l ^{125}I –SDF-1α, and terminated by washing twice with ice cold phosphate buffered saline (PBS) and subsequent hypotonic lysis in 400 μl 10 mM Tris buffer (pH 7.4). In some conditions, 1 μM AMD3100 (Sigma) or CCX733 (ChemoCentrix) were applied to block Cxcr4- or Cxcr7-dependent binding, respectively. CCX704 (ChemoCentrix), a compound related to CCX733 with no binding affinity for Cxcr7, was used as control. Uptake of ^{125}I –SDF-1α was considered to be the amount of ^{125}I recovered in the cell lysates and was expressed as percentage of the uptake observed in nontreated controls. The concentration of Cxcl12 present in the medium of cortical cultures was quantified using the mouse CXCL12/SDF-1 alpha Quantikine ELISA Kit (R&D Systems).

Cxcl12 Quantification

Cortical cultures were prepared from E15.5 control and *Cxcr7* mutant embryos and the supernatant was collected after 5 DIV. In other series of experiments, cortical homogenates were prepared from control and *Cxcr7* mutant embryos at E14.5. Cortical homogenates were then centrifuged and supernatants were collected. In both types of experiments, the concentration of Cxcl12 was quantified using the mouse CXCL12/SDF-1 alpha Quantikine ELISA Kit (R&D Systems).

MGE Explants Cultures

MGE explants were dissected out from organotypic slices and cultured on glass coverslips coated with poly-L-Lysine and laminin in Neurobasal medium containing 0.4% methylcellulose (Sigma). Alternatively, MGE explants were confronted with COS7 cell aggregates expressing *DsRed* or *DsRed* and *Cxcl12* and were cultured in collagen matrices (BD-Biosciences) as described previously (López-Bendito et al., 2008).

Cxcr4 Signaling

E16 telencephalic neurons were plated onto poly-L-lysine-coated 24-well plates (500,000 cells per well). Sixty minutes before Cxcl12-stimulation (20 nM) culture medium was replaced by BSS consisting of (in mmol/l) 143 Na, 5.5 K, 1.8 Ca_2, 1.8 Mg_2, 125 Cl, 26 HCO_3, 1 PO_4, 0.8 SO_4, and 4.5 g/l glucose (pH 7.4). Ten minutes after stimulation cultures, were lysed in 250 μl of boiling SDS sample buffer. Lysates were subjected to SDS-PAGE and electroblotting according to standard protocols. Phospho-p44/42 MAP kinase (Thr202/Tyr204) E10 monoclonal antibody (1:2000, Cell Signaling Technology) and Erk2 C-14 rabbit polyclonal IgG (1:10000, Sc-154, Santa Cruz Biotechnology) were detected with the ECL Western Blotting kit (GE Healthcare).

In Situ Hybridization and Immunohistochemistry

For in situ hybridization, brains were fixed overnight in 4% paraformaldehyde (PFA) in PBS. Twenty-micrometer frozen sections were hybridized with digoxigenin-labeled probes as described before (Flames et al., 2007). Alternatively, brains were fresh-frozen and in situ hybridization was performed using ^{35}S-labeled riboprobes as described before (Stumm et al., 2002). The following cDNA probes were used in this study: *Lhx6* (kindly provided by V. Pachnis, NIMR, London, UK); *Cxcr7* (kindly provided by E. Arenas, Karolinska Institutet, Sweden); *Cxcr4* (Invitrogen, BG174412), and *NeuroD2* (kindly provided by F. Guillemot, NIMR, London, UK). Immunohistochemistry was performed on vibratome, cryotome, or paraffin-embedded sections, as well as in MGE

explants, as described before (Flames et al., 2007; López-Bendito et al., 2008; Stumm et al., 2007). The following primary antibodies were used: rat anti-BrdU (1:100, Accurate), chicken anti-GFP (1:1000, Aves Labs), goat anti-GFP (1:1000, Abcam), rabbit anti-Cxcr4 (1:50, UMB-2 clone) (Fischer et al., 2008), mouse anti-Cxcr7 (1:250, 11G8 clone; kindly provided by Mark Penfold, ChemoCentryx, Mountain View, CA) (Burns et al., 2006), rabbit anti-PV (1:3000, Swant), and mouse anti-Rab4 (BD Biosciences). The specificity of the mouse anti-human Cxcr7 was tested in interneurons obtained from *Dlx5/6-Cre-IRES-Gfp;Cxcr7*$^{lox/lox}$ embryos (Figure S4). The following secondary antibodies were used: goat anti-chicken 488, donkey anti-rabbit 555, goat anti-rabbit 594, donkey anti-mouse 488 (Molecular Probes), donkey anti-rat Cy3 and donkey anti-mouse Fab Cy3 (Jackson Laboratories), donkey anti-rabbit Cy5 and donkey anti-mouse Cy3 (Chemicon), and goat anti-rabbit peroxidase (Pierce). The immunofluorescence detection of EYFP was performed using an anti-GFP antibody. DAPI (Sigma) was used for fluorescent nuclear counterstaining. The membrane labeling was performed using wheat germ agglutinin (WGA) lectin conjugated with Evans blue (Sigma).

Analysis of Cxcr4 Expression by Western Blot

For detection of Cxcr4 in the telencephalon of E16 mice, brain samples from two embryos were pooled in 1 ml RIPA buffer, sonicated for 5 s, and gently inverted for 1 hr at 4°C before centrifugation for 30 min at 23,000 × g at 4°C. The lysate was then divided in two aliquots. Glycoproteins were enriched using wheat germ lectin agarose beads as described (Stumm et al., 2002). Beads were washed in RIPA buffer and then gently inverted for 1 hr at 37°C in 170 μl 1× NEBuffer for Protein MetalloPhosphatases. One aliquot received 400 units lambda protein phosphatase (New England Biolabs, #P0753) for dephosphorylation of Cxcr4. Beads were washed with RIPA buffer before proteins were eluted for 15 min at 60°C with SDS sample buffer. Samples were then subjected to 10% SDS-polyacrylamide gel electrophoresis and immunoblotted onto nitrocellulose. Western blot analyses of Cxcr4 in human embryonic kidney cells (HEK293 cells) were done after transfection with a plasmid encoding for *Cxcr4* fused to a hemagglutinin (HA) epitope tag at the amino terminus.

Transplantation Experiments

Microtransplantation experiments in telencephalic slices were performed using the MGE of control and *IN-Cxcr7* embryos, as described before (López-Bendito et al., 2008). *Dlx5/6-Cre-IRES-Gfp;Cxcr7*$^{lox/+}$ and *Dlx5/6-Cre-IRES-Gfp* embryos were indistinctly used as controls in these experiments. In utero ultrasound-guided transplantation of MGE-derived cells was performed as previously described (Pla et al., 2006). Donor pregnant females were injected with BrdU 12 hr before dissection. *Cxcr7*$^{f/+}$ embryos were used as controls in these experiments.

Quantification

Quantification was performed using Canvas (ACD Systems) or Neurolucida Explorer (MBF Bioscience) software. For the quantification of migration in MGE explants, the distance migrated by the 40 furthest cells was measured. For the analysis of interneuron migration in vivo, the number of GFP-expressing cells was quantified in the same region located in the prospective somatosensory cortex for each brain. The area quantified was divided into 10 equal bins and the percentage of cells in each bin was calculated. For GFP and PV analysis at P21, the same region of the somatosensory, motor, and visual areas was quantified in control and mutant brains. Layers were drawn following nuclear staining. Layers I, II/III, and IV were grouped as supragranular layers, while layers V and VI were grouped as infragranular layers. Cxcr4 fluorescence levels and colocalization of Cxcr4 and WGA was measured using ImageJ software (NIH, http://rsb.info.nih.gov/ij/). In the first case, stacks of individual cells were taken using a Leica Confocal microscope (MCSII) every 1μm. Fluorescence intensity was measured in every stack of cell and the total fluorescence was calculated as the sum of the fluorescence of all stacks of the cell. For Cxcr4/WGA measurements, a single confocal plane was obtained per cell and the Mander's coefficient was used to calculate colocalization. For statistical analyses, normality and variance tests were first applied to all experimental data. When data followed a normal distribution, paired comparisons were analyzed with t test, while multiple comparisons were analyzed using either ANOVA with post hoc Bonferroni correction (equal variances) or the Welch test with post hoc Games-Howell (different variances). A χ^2 test was applied to analyze the distribution of cells in either bins or layers.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes four figures and can be found with this article online at doi:10.1016/j.neuron.2010.12.006.

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