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Meninges control tangential migration of hem-derived Cajal-Retzius cells via CXCL12/CXCR4 signaling

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Cajal-Retzius cells are critical in the development of the cerebral cortex, but little is known about the mechanisms controlling their development. Three focal sources of Cajal-Retzius cells have been identified in mice—the cortical hem, the ventral pallium and the septum—from where they migrate tangentially to populate the cortical surface. Using a variety of tissue culture assays and *in vivo* manipulations, we demonstrate that the tangential migration of cortical hem–derived Cajal-Retzius cells is controlled by the meninges. We show that the meningeal membranes are a necessary and sufficient substrate for the tangential migration of Cajal-Retzius cells. We also show that the chemokine CXCL12 secreted by the meninges enhances the dispersion of Cajal-Retzius cells along the cortical surface, while retaining them within the marginal zone in a CXCR4-dependent manner. Thus, the meningeal membranes are fundamental in the development of Cajal-Retzius cells and, hence, in the normal development of the cerebral cortex.

The cerebral cortex is critical for the integration and processing of information required for most animal behaviors, including those most characteristic of humans. The correct functioning of the cerebral cortex depends on the exquisitely precise laminar arrangement of neurons, axon collaterals and dendritic processes[1,2]. Such precise laminar organization arises during cortical development when newborn neurons migrate radially from the proliferative ventricular zone to the vicinity of the marginal zone (MZ), where successive generations of neurons coalesce into new cortical layers[3,4]. Cajal-Retzius (CR) cells, first identified in human infants by Gustav Retzius (1893) and Santiago Ramón y Cajal (1899), are a transient population of neurons located in the MZ of the developing cerebral cortex, apposed to the meningeal membranes[5–8]. From their strategic position in the MZ, and through the expression of the extracellular glycoprotein Reelin, CR cells fulfill the formidable task of orchestrating the radial migration and laminar arrangement of the myriads of neurons that constitute the adult cerebral cortex[4,9–14].

Cajal-Retzius cells have long been assumed to originate from cortical progenitors throughout the pallial ventricular zone and to translocate radially from there to the cortical surface[14–16]. However, recent studies have identified three focal points in the embryonic telencephalon as the major sources of CR cells in the mouse: the cortical hem[17,18], the septum and the ventral pallium[19]. These studies have also shown that CR cells do not migrate much radially, but rather spread tangentially for vast distances along the cortical surface until the entire cortical primordium is covered[17–19]. As the origins and basic developmental aspects of CR cells have only recently begun to be unraveled, virtually nothing is known about the cellular and molecular mechanisms involved in their tangential migration[17–19]. Nevertheless, in view of the newly discovered origin of CR cells, some clues about possible mechanisms influencing CR cell migration come from the reinterpretation of studies in which the meningeal membranes were physically or genetically disrupted. Notably, these manipulations consistently resulted in abnormal distributions of CR cells in the developing cortex[20–25], thus suggesting a role for the meninges in the migration of CR cells.

Chemokines are small secreted proteins that were first identified as important for the trafficking of leukocytes[26,27]. CXCL12, a member of the C-X-C subfamily of chemokines (also known as Stromal cell–derived factor-1, SDF-1) and its unique receptor CXCR4 (ref. 27) are critical for the normal development of the central nervous system, influencing the guidance of both growing axons and migrating neurons[28–36], among other functions. As *Cxcl12* is highly expressed by the cortical meninges throughout embryonic development and CR cells express *Cxcr4* during the same period[37–39], it is conceivable that CXCL12 and CXCR4 may have prominent roles in the development of CR cells.

Here we studied the mechanisms regulating the tangential migration of hem-derived CR cells. We provide evidence indicating that the meninges serve as a necessary and sufficient substrate for the tangential spread of hem-derived CR cells along the surface of the cortical primordium. Furthermore, the meninges exert a potent chemoattractive influence over migrating CR cells *in vitro*, a function served by CXCL12 via the CXCR4 receptor. In the context of the developing cortical primordium, the pharmacological or genetic disruption of CXCL12/CXCR4 signaling results in the derailment of migrating CR cells, which slide away from the cortical surface and invade the cortical plate. These results indicate that the meninges are key players in the recruitment of migrating CR cells to the marginal zone, a critical position for their function in cerebral cortical lamination.

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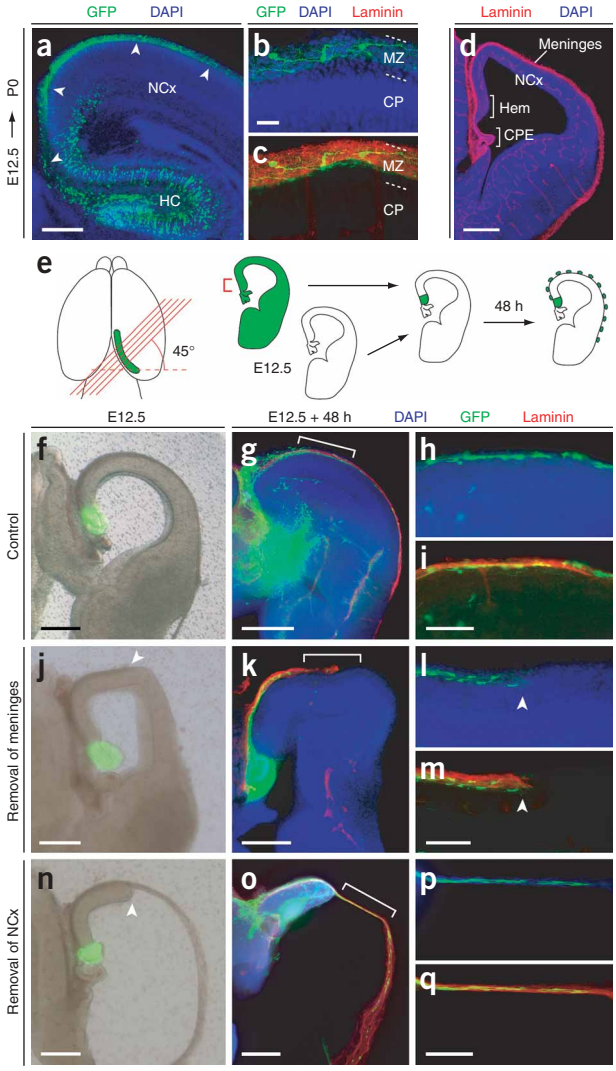

Figure 1 The meninges are necessary and sufficient for the migration of hem-derived CR cells. (**a**–**c**) CR cells in the marginal zone (MZ) of the neocortex (NCx) expressed GFP (green, arrowheads) after electroporation of the cortical hem. GFP+ CR cells overlapped with the laminin+ (red) meningeal membranes. Nuclear counterstain is shown in blue (DAPI). HC, hippocampus; CP, cortical plate. (**d**) Structures present in the dorsal telencephalon at E12.5: choroid plexus epithelium (CPE), hem, NCx and meninges (red). (**e**) Schematic of the experimental design. (**f**–**i**) Examples of control slices at the initial (**f**) and final (**g**–**i**) time points of the culture. GFP+ hem-derived cells were visible along the marginal zone (**h**,**i**) next to the meninges (**i**, red). (**j**–**m**) Effect of removing the lateral meninges, when these spanned only from the hem up to a random point (arrowhead). After 48 h (**k**–**m**), GFP+ cells were present along the cortical MZ containing meninges (red), but not beyond the edge of tissue removal (arrowhead). (**n**–**q**) Effect of removing the neocortical epithelium (NCx). The slice shown in **n** is from the initial time point; note that all ventrolateral telencephalic tissue was absent beyond an arbitrary dorsal point (arrowhead), whereas the surrounding meninges were left intact. (**o**–**q**) GFP+ cells were visible next to the meninges even in the absence of underlying neocortical epithelium (**p**,**q**). Scale bars: 300 μm in **a**,**d**,**f**,**g**,**j**,**k**,**n**,**o**; 30 μm in **b**,**c**; 100 μm in **h**,**i**,**l**,**m**,**p**,**q**.

RESULTS

The meninges are the substrate for CR cell migration

Two prominent features characterize the tangential migration of hem-derived CR cells. First, there is a marked apico-basal asymmetry in their migration, as CR cells systematically move in close apposition to the meninges, never invading the cortical epithelium or, at later stages of development, the cortical plate (**Fig. 1a–c**). Second, CR cells spread from the caudomedial wall to the rostrolateral domains of the telencephalon (refs. 17–19 and **Fig. 1a**), suggesting that their dispersion is a highly directional process. Due to the close relationship existing between CR cells and the meninges (**Fig. 1a–c**), we reasoned that the meninges may constitute a very favorable substrate for the migration of CR cells, which could thereby limit their dispersion through the most superficial part of the cortex (**Fig. 1d**). To address this question, we performed transplantation assays using oblique brain slices, as they represent an ideal system to manipulate hem-derived CR cell migration under relatively physiological conditions. In brief, the cortical hem of embryos expressing green fluorescent protein (GFP) was homotypically and isochronically transplanted into wild-type host slices, and the distribution of migrating cells was analyzed after 48 h in culture (**Fig. 1e**). In control slices, most GFP-positive (GFP+) cells exited the transplanted hem and migrated along the cortical surface, next to the meninges (**Fig. 1f–i**). To test for the requirement of the meninges as a necessary substrate for the migration of CR cells, we removed them from part of the neocortex while maintaining the underlying tissue intact (**Fig. 1j,k**). CR cell migration was completely normal until migrating cells reached the incised border of the meninges, where the advancement of CR cells was abruptly interrupted regardless of the presence of the underlying cortical neuroepithelium (**Fig. 1k–m** and **Supplementary Table 1** online). In complementary manipulations, part of the neocortical epithelium and the entire basal ganglia were removed from the slices, while leaving the meninges intact (**Fig. 1n,o**). Under these conditions, CR cells migrated along the cortical surface until they reached the cut border of the tissue, from where cells continued migrating along the naked meninges (**Fig. 1o–q** and **Supplementary Table 1**). Taken together, these experiments demonstrate that the meninges are a substrate both necessary and sufficient for CR cell migration along the surface of the developing neocortex.

The meninges are chemoattractive for hem-derived CR cells

The previous experiments demonstrated that the meninges constitute a favorable substrate for the migration of CR cells. However, it is likely that the dispersion of CR cells is also influenced by chemotropic signals present along their migratory route. Indeed, several components of the early embryonic telencephalon occupy strategic positions to influence the polarized migration of hem-derived CR cells (**Fig. 1d**). Thus, the meninges and the neocortical epithelium could influence the apico-basal distribution of migrating CR cells through the expression of attractants and repellents, respectively. In addition, the choroid plexus epithelium and the cortical hem could express chemorepellent molecules to instruct the caudomedial-to-rostrolateral direction of migration of CR cells, whereas rostrolateral regions of the cortex could contribute to this process through the expression of long-range attractants.

To test for the existence of these hypothetical cues influencing the migration of CR cells, we first evaluated the migration of hem-derived cells in three-dimensional Matrigel matrices. When cortical hem explants, obtained from telencephalic slices on embryonic days (E) 10.5 to E12.5, were cultured in Matrigel, hundreds of cells emigrated for long distances (**Fig. 2a**). These cells exhibited characteristic traits of CR cells, such as calretinin immunoreactivity (99.1%, $n = 1{,}593$ cells) and expression of *Reelin* (*Reln*) mRNA (98.7%, $n = 698$ cells; **Fig. 2b–d**). Hem-derived CR cells located outside the explant showed characteristic migratory morphologies, including an elaborate apical process with multiple branches, all tipped with growth cones (**Fig. 2b**). Migrating hem-derived CR cells were markedly longer and more elaborate than

other tangentially migrating cortical cells, such as medial ganglionic eminence (MGE)-derived GABAergic interneurons (**Fig. 2e**). Thus, early embryonic hem explant cultures constitute a relatively pure source of CR cells and therefore represent an ideal assay to search for factors influencing their migration.

We first searched for factors influencing the place of migration of CR cells in the apico-basal dimension. To this end, explants of E12.5 cortical hem were confronted with age-matched clumps of meningeal membranes or explants of neocortex. CR cells were markedly attracted toward the meninges, whereas they showed no response to neocortical explants (**Fig. 2f,g** and **Supplementary Table 2** online). This suggested that the migration of CR cells along the cortical surface might be driven by a chemoattractant secreted by the meninges, but not by a chemorepellent secreted by the cortical epithelium.

To identify biological activities driving the migration of CR cells away from the midline and toward the lateral cortex, explants of hem were confronted with explants of hem or the choroid plexus epithelium. Hem-derived CR cells showed no chemotactic responses to either the choroid plexus epithelium or the cortical hem (**Fig. 2h–k** and **Supplementary Table 2**), suggesting that diffusible factors from these tissues are not likely to determine the initial direction of migration for CR cells. Thus, the meninges seem to be the primary cellular structure influencing the migration of hem-derived CR cells in the embryonic telencephalon through a chemoattractive activity.

CR cell dispersion does not depend on long-range cues

One of the most notable features of hem-derived CR cell dispersion throughout the telencephalon is the apparent directionality of these migrations. As previous experiments showed that the meninges may be the only source of diffusible signals influencing the migration of hem-derived CR cells, we searched for the presence of distinct chemoattractive activities in different domains of the telencephalic meninges that could contribute to explaining the general caudomedial-to-rostrolateral direction of dispersion of hem-derived CR cells (**Fig. 3a**). When hem explants were confronted with either dorsal or ventral fragments of cortical meninges, CR cells had identical attractive responses, in both cases migrating about twice as far in the direction of the meninges as compared to the opposite direction (**Fig. 3b–e** and **Supplementary Table 2**). Similarly, no qualitative or quantitative differences were observed when hem explants were confronted with anterior or posterior fragments of cortical meninges (**Fig. 3f–i** and **Supplementary Table 2**). These results suggest that the meninges of the four telencephalic quadrants secret attractants with identical influence over CR cell migration. This possibility would suggest, in turn, that hem-derived CR cells are not bound to migrate exclusively in the caudomedial-to-rostrolateral direction but rather may have the potential to migrate in the opposite direction as well. We tested this hypothesis by transplanting GFP+ hems in the dorsal neocortex or at the pallial-subpallial boundary of E12.5 wild-type slices (**Fig. 3j**). In all experiments, GFP+ CR cells migrated massively along the neocortical surface both ventrolaterally, the normal direction of migration, and dorsomedially, the reverse direction of migration (**Fig. 3k,l** and **Supplementary Table 1**). These results indicate that hem-derived CR cells have the capacity to migrate in multiple and even opposite directions along the surface of the neocortex.

Our previous experiments suggested that the dispersion of hem-derived CR cells through the telencephalon does not depend on diffusible attractive or repulsive cues. An alternative mechanism that could contribute to the general dispersal of hem-derived CR cells from their origin to rostral and lateral regions of the cortex would be the existence of contact-inhibitory interactions between adjacent CR cells. According to this hypothesis, CR cells would tend to migrate from the region with the higher concentration of CR cells, the cortical hem, toward regions with a lower concentration of CR cells, which would effectively cause an overall caudomedial-to-rostrolateral dispersion of hem-derived CR cells. To test this hypothesis, hem explants were confronted by other hem explants on two-dimensional substrates, thereby forcing cell-cell interactions to occur between migrating CR cells. When explants were cocultured at approximately 500 μm from each other

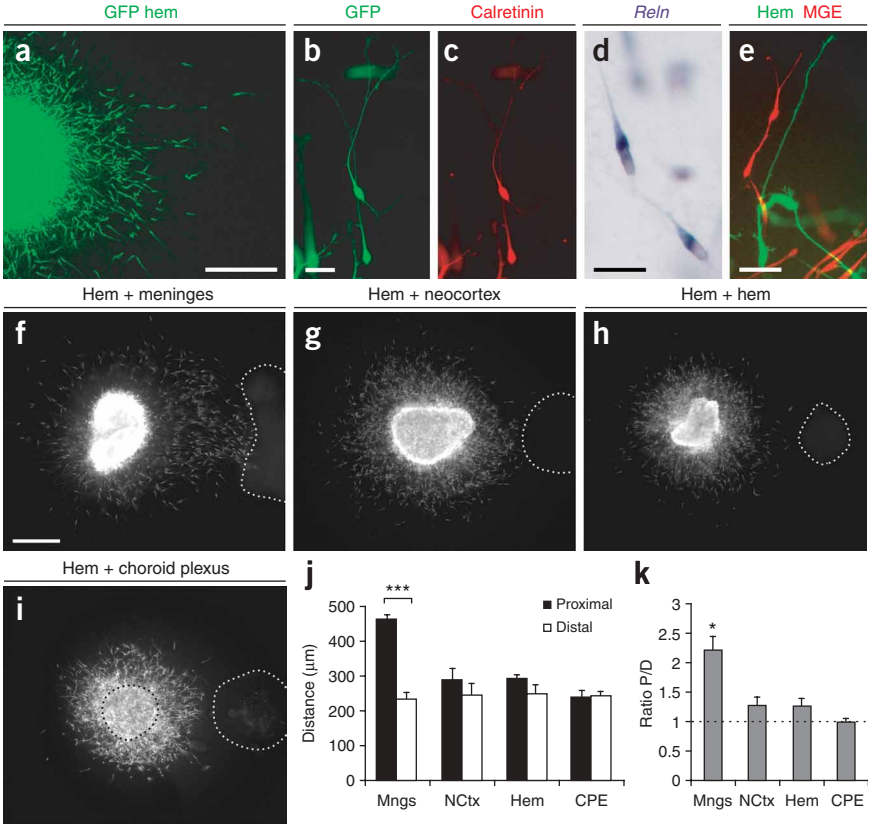

Figure 2 The meninges are chemoattractive for hem-derived CR cells. (**a**–**d**) Cells migrating out of hem explants in Matrigel cultures (**a**) were immunoreactive for calretinin (**b**,**c**) and expressed *Reln* mRNA (**d**). (**e**) Comparison of size and morphology between hem-derived and medial ganglionic eminence (MGE)-derived migrating cells. (**f**–**i**) Distribution of hem-derived cells in response to meninges (Mngs), neocortex, hem or choroid plexus epithelium, in Matrigel matrix. Dotted lines indicate the border of the explants. (**j**,**k**) Quantification of confrontation assays as in **f**–**i**. Plots show average + s.e.m. Hem explants were confronted with Mgns ($n = 11$), NCtx ($n = 8$), hem ($n = 9$) and CPE ($n = 10$). $^{***}P < 0.0005$, $^{*}P = 0.022$ comparing Mngs and NCtx, $^{*}P = 0.013$ comparing Mngs and hem, $^{*}P = 0.002$ comparing Mngs and CPE; *t*-test. Scale bars: 300 μm in **a**,**f**–**i**; 30 μm in **b**–**e**.

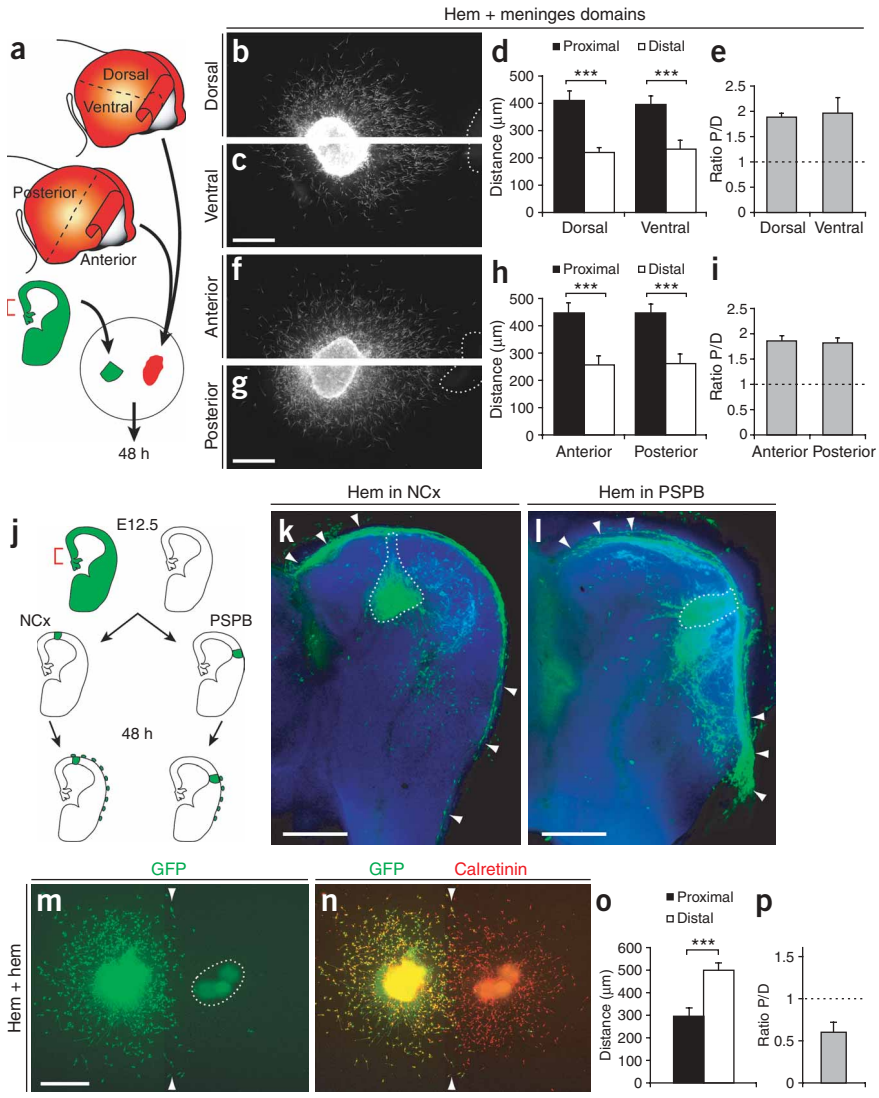

Figure 3 The directional dispersion of hem-derived CR cells does not depend on long-range cues. (**a**) Experiment schematic. Peeled meningeal membranes were split into two halves, and each was confronted with a GFP+ hem explant over 48 h. (**b**–**i**) Results from experiment depicted in **a**. Images show dispersion of hem-derived cells confronted with dorsal (**b**, $n = 10$ explants) versus ventral (**c**, $n = 8$) meninges, or anterior (**f**, $n = 9$) versus posterior (**g**, $n = 10$) meninges, in Matrigel matrix. Dotted lines indicate the border of the meningeal explants. Quantifications are shown in **d**,**e** and **h**,**i** as average + s.e.m. $^{***}P < 0.0005$, *t*-test. (**j**) GFP+ hem (red bracket) was transplanted into the dorsal neocortex (NCx) or the pallial-subpallial boundary (PSPB) of wild-type slices. (**k**,**l**) Results from experiment depicted in **j**. Images show distribution of hem-derived cells (green) along the cortical marginal zone. Dotted lines indicate the border of the hem explants. (**m**–**p**) Dispersion of GFP+ hem-derived cells (green) confronted with a second hem explant (**m**, dotted line; **n**, red) on a two-dimensional substrate. Aligned arrowheads indicate the intermediate distance between the two explants at which the dispersion of migrating cells was sharply reduced. Quantifications are shown in **o** and **p** as average + s.e.m., where $n = 10$ GFP+ explants. $^{***}P < 0.0005$, *t*-test. Scale bars: 300 μm in **b**,**c**,**f**,**g**; 500 μm in **k**,**l**,**m**,**n**.

(449.88 ± 45.52 μm, average ± s.e.m.; $n = 17$ explants, three independent experiments), CR cells migrated shorter distances toward the confronted explant than away from it (**Fig. 3m–p**; 14 of 17 explants). Thus, although CR cells densely populated the space between both explants, they did not approach the opposite hem beyond the halfway point (**Fig. 3m**,**n**). In contrast, when the confronted hem explants were separated by more than 800 μm (828.27 ± 36.89 μm; $n = 19$ explants, three independent experiments), CR cells migrated similar distances in all directions (17 of 19 explants; data not shown). These results suggest that CR cell migration is impaired by contact with neighboring CR cells, which is consistent with the notion that directional CR cell dispersion away from the hem and throughout the cortical surface may be mediated *in vivo* by contact-inhibitory interactions between CR cells.

The meninges express the CR cell attractant CXCL12

Once we had established the critical role of the meninges in the migration of hem-derived CR cells, both as a substrate and as a homogeneous source of a chemoattractive activity, we searched for suitable candidate signaling pathways. CXCL12, an extracellular molecule with well-characterized chemoattractive activity, is highly expressed by the meninges[37,39]. In agreement with previous findings, we observed high expression of *Cxcl12* mRNA in the meninges at E11.5 and E13.5 (**Fig. 4**), stages at which CR cells spread from the hem through the neocortex[17]. Complementarily, we observed that the cortical hem as well as cells in the marginal zone of the cortex express *Cxcr4*, the univocal receptor of CXCL12, at the same stages (**Fig. 4**). The distribution of *Cxcr4*+ cells was identical to that of *Reln*+ CR cells at E13.5, but not at E11.5, when *Cxcr4*+ cells extended only a few hundred microns laterally from the hem (**Fig. 4a–f**). Double staining with calretinin, a marker for mouse CR cells in the marginal zone[5], demonstrated that CR cells express *Cxcr4* mRNA and migrate next to the *Cxcl12*-expressing meninges (**Fig. 4g–i**).

To determine whether CXCL12 is chemoattractive for migrating CR cells, we confronted hem explants with aggregates of transfected COS cells (**Fig. 5a**,**b**). CR cells were markedly attracted toward *Cxcl12*-transfected COS cells, whereas they showed no response to control-transfected COS cells (**Fig. 5a–d** and **Supplementary Table 3** online). Next, we tested whether CR cells might prefer a CXCL12-reach surface as a migratory substrate (such as the meninges) to other extracellular components. To this end, we performed protein stripe assays in which hem explants were cultured on top of alternating CXCL12-containing and control lanes. CR cells strongly preferred to migrate on CXCL12-containing substrates (67.8%; $n = 5{,}585$ cells, three independent experiments) rather than on CXCL12-free substrates (**Fig. 5e**,**f**). Thus, our results suggest that CR cells migrate along the surface of the developing telencephalon owing to the attractive influence of CXCL12, which is secreted by the meningeal membranes overlying the cerebral cortex.

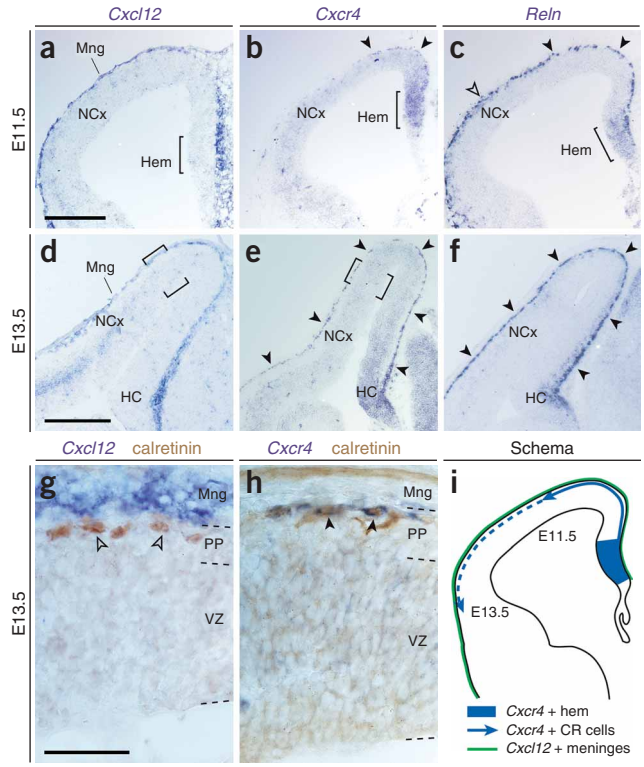

Figure 4 *Cxcl12* and *Cxcr4* are complementarily expressed in the meninges and in CR cells. (**a**–**f**) Pattern of expression of *Cxcl12*, *Cxcr4* and *Reln* mRNA in the dorsal telencephalon at E11.5 and E13.5. At E11.5, *Cxcl12* was expressed by the meninges (**a**; Mng), and *Cxcr4*-expressing cells (**b**) were found only in the hem and superficially in the medial-most region of the neocortex (NCx, solid arrowheads). *Reln*-expressing cells occupied the marginal zone of the entire telencephalic vesicle (**c**), even in lateral regions of the neocortex where *Cxcr4* expression was absent (open arrowhead). At E13.5 (**d**–**f**), *Cxcl12* was expressed by the meninges in the neocortex and hippocampus (HC); *Cxcr4*+ cells and *Reln*+ cells were found superficially throughout the neocortex and prospective hippocampus (arrowheads). (**g**,**h**) Double stains of areas bracketed in **d** and **e** showing calretinin immunoreactivity (brown) with mRNA expression of *Cxcl12* (**g**) and *Cxcr4* (**h**) (blue). Calretinin+ CR cells (arrowheads) expressed *Cxcr4* (**h**, blue; solid arrowheads) and were aligned right beneath the *Cxcl12*-expressing meninges (**g**, blue). PP, cortical preplate; VZ, ventricular zone. (**i**) Schematic illustrating the spatiotemporal relationship between *Cxcl12*- and *Cxcr4*-expressing cells in the early embryonic cerebral cortex. Scale bars: 500 μm in **a**–**c**,**d**–**f**; 50 μm in **g**,**h**.

CR cells are attracted by meningeal CXCL12 via CXCR4

We next investigated whether the attractive influence of CXCL12 over CR cells is mediated by CXCR4, the only identified receptor for CXCL12 (ref. 40). *Cxcr4* mRNA is expressed by CR cells in the embryonic neocortex (refs. 37,39 and **Fig. 4**), and most cells migrating from cortical hem explants were immunoreactive for CXCR4 (96.6%, $n = 1{,}260$ cells; data not shown). When hem explant cultures were confronted with *Cxcl12*-transfected COS cells in the presence of AMD3100, a highly specific CXCR4 antagonist[41–44], the attractive response of CR cells toward the source of CXCL12 was completely neutralized (**Fig. 6a–c** and **Supplementary Table 3**). These results suggest that CR cells express functional CXCR4 receptors, which mediate the attraction of these cells toward a source of CXCL12. Our data, however, did not demonstrate that the meningeal attractant for CR cells is CXCL12. This was next addressed by adding AMD3100 to explant cultures of cortical hem confronted with meninges. Whereas hem-derived CR cells showed their characteristic attractive response toward the meninges in the presence of vehicle solution, this response was neutralized in the presence of AMD3100 (**Fig. 6d–f** and **Supplementary Table 3**), suggesting that CR cells are attracted by meningeal CXCL12 and respond to it via the CXCR4 receptor. To confirm these findings, hem explants from *Cxcr4* mutant embryos[35] were confronted by control (GFP+) meninges. In most explants obtained from $Cxcr4^{+/+}$ or $Cxcr4^{+/-}$ embryos, CR cells were attracted toward the meninges (**Fig. 6g** and **Supplementary Table 3**). In contrast, most hem cultures derived from $Cxcr4^{-/-}$ embryos showed no attraction toward the meninges (**Fig. 6h,i** and **Supplementary Table 3**). These results demonstrate that the chemoattractive influence of meningeal CXCL12 over CR cells is signaled via the CXCR4 receptor and that CXCL12 is either the only or the predominant meningeal attractant for CR cells.

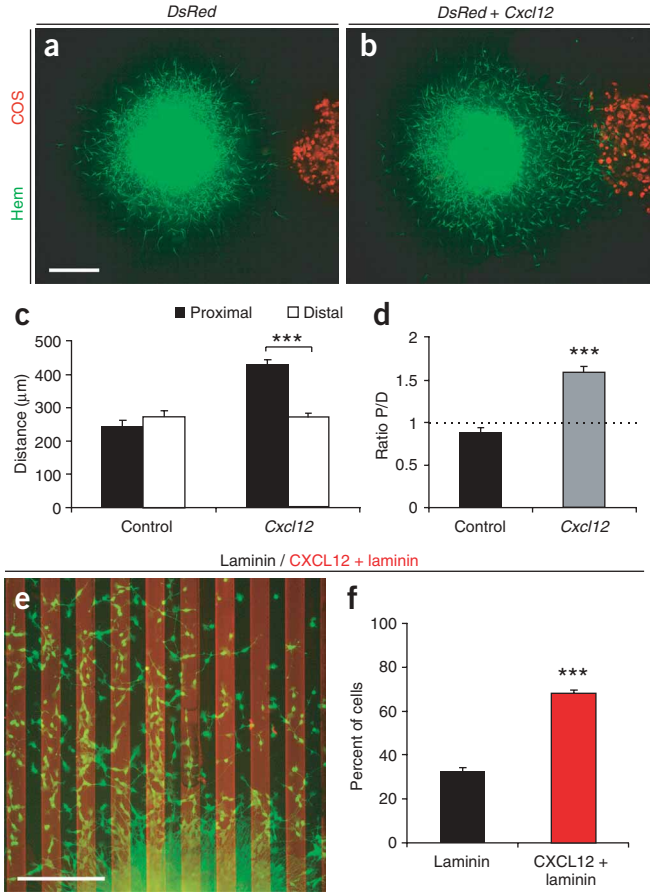

Figure 5 CXCL12 promotes the migration of hem-derived CR cells. (**a**,**b**) Dispersion of hem-derived CR cells (green) confronted with COS cell aggregates (red) expressing *DsRed* (**a**) or *DsRed* + *Cxcl12* (**b**) in Matrigel matrix. Quantifications are shown in **c** and **d**. $n = 13$ explants per condition. *** $P < 0.0005$, *t*-test. (**e**) Detail of a protein stripe assay in which CXCL12-containing stripes (red) alternate with control stripes (black). Hem-derived CR cells are in green, explant is to the bottom. (**f**) Quantification of the distribution of CR cells along CXCL12-containing stripes (red bar) versus control stripes (black bar; $n = 5{,}585$ cells, *** $P < 0.0005$, chi-squared test). Plots show average + s.e.m. Scale bars: 300 μm in **a**,**b**; 250 μm in **e**.

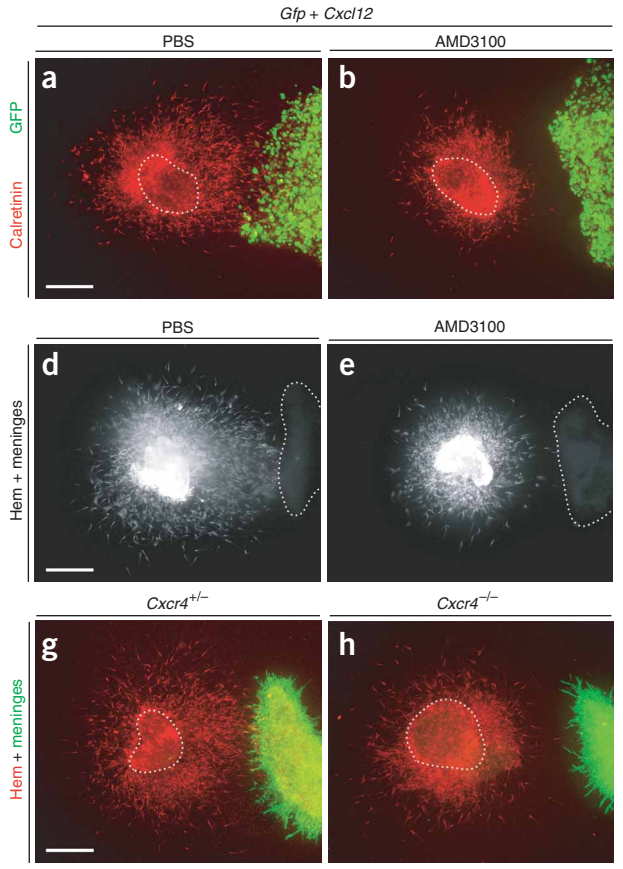

Figure 6 CR cells respond to meningeal CXCL12 via the CXCR4 receptor. (**a**–**c**) Dispersion of hem-derived calretinin-positive CR cells (red) confronted with COS cell aggregates transfected with *Gfp*+ *Cxcl12* (green) in the presence of AMD3100 (**b**) or vehicle solution (**a**). Dotted lines indicate the border of the hem explants. Quantifications are shown in **c**. $n = 10$ explants in the PBS group. $n = 20$ in the AMD3100 group. (**d**–**f**) Dispersion of hem-derived CR cells confronted with meninges (dotted lines) in the presence of AMD3100 (**e**) or vehicle solution (**d**). Quantifications are shown in **f**. $n = 10$ explants in the PBS group. $n = 19$ in the AMD3100 group. (**g**–**i**) Dispersion of hem-derived calretinin-positive CR cells (red) from a $Cxcr4^{+/-}$ embryo (**g**) or a $Cxcr4^{-/-}$ embryo (**h**) confronted with control meninges (green). Dotted lines indicate the border of the hem explants. Quantifications are shown in **i**. $n = 9$ $Cxcr4^{+/+}$ explants. $n = 10$ $Cxcr4^{+/-}$ explants. $n = 10$ $Cxcr4^{-/-}$ explants. Plots show average + s.e.m. $^{*}P = 0.042$, $^{**}P = 0.011$, $^{***}P < 0.0005$; *t*-test. Scale bar, 300 μm.

Meninges-associated migration of CR cells requires CXCR4

Of the two characteristic asymmetries described for the migration of hem-derived CR cells (superficial location next to the meninges and caudomedial-to-rostrolateral direction), the CXCL12/CXCR4-mediated chemoattraction is consistent with a role of the meninges in recruiting tangentially migrating CR cells in their vicinity. To better address this hypothesis, we blocked CXCR4 function in GFP+ hem-transplanted slice cultures (**Fig. 7a**). Treatment of slices with vehicle solution did not modify the normal behavior of migrating CR cells, as most of these cells followed a superficial route next to the overlying meninges (**Fig. 7b**). In AMD3100-treated slices, however, CR cells migrated following no particular route and with no apparent preference for the meninges over the neural epithelium (**Fig. 7c**), suggesting that CXCL12/CXCR4 signaling is fundamental in the recruitment of migrating CR cells within the vicinity of the meninges as these cells disperse tangentially through the cortex.

To determine the extent to which CXCL12/CXCR4 signaling is critical for the association of migrating CR cells with the meninges *in vivo*, CXCR4 function was blocked in developing wild-type embryos by injecting AMD3100 intraventricularly *in utero* at E12.5 and analyzing the distribution of CR cells 2 d after surgery (**Fig. 7d**). In control embryos, calretinin+ cells and *Reln*+ cells (putative CR cells) were exclusively located in the MZ of the cortex ($n = 11$ embryos, 3 litters; **Fig. 7e**,**f**). In contrast, in antagonist-injected embryos, calretinin+ cells were observed throughout the cortical plate, most frequently in the dorsolateral neocortex, in addition to those found in the MZ ($n = 21$ embryos, 3 litters; **Fig. 7g**). Analysis of *Reln* mRNA expression also showed important differences between AMD3100-treated and control embryos. Compared to controls, AMD3100-treated E14.5 embryos contained numerous ectopic *Reln*+ cells in the cortical plate and had occasional gaps between groups of *Reln*+ cells in the MZ, further suggestive of the derailment of CR cells from the MZ to the cortical plate (**Fig. 7g**,**h**).

To confirm the previous findings, we next analyzed the distribution of *Reln*+ and calretinin+ cells in *Cxcr4* mutant embryos. At E16.5, control embryos showed an almost continuous band of large and intensely labeled *Reln*+ and calretinin+ CR cells along the MZ (**Fig. 7i**,**j** and data not shown). Occasionally, weakly labeled *Reln*+ (calretinin–) cells were also found in the cortical plate and between intermediate zone (IZ) and subventricular zone (SVZ) (**Fig. 7i**,**j**). In the control hippocampus, large *Reln*+ CR cells were restricted to the vicinity of the hippocampal fissure (ref. 45 and **Fig. 7k**). In $Cxcr4^{-/-}$ embryos, the MZ was almost devoid of small *Reln*+ cells, and stretches of strongly labeled *Reln*+ and calretinin+ CR cells were frequently separated by large cell-free gaps (**Fig. 7l** and data not shown). CR cell-free gaps accounted for only 2.8 ± 0.4% of the MZ in control embryos, but 9.7 ± 2.0% in $Cxcr4^{-/-}$ embryos ($Cxcr4^{-/-}$, $n = 4$; $Cxcr4^{+/-}$, $n = 3$; $Cxcr4^{+/+}$, $n = 1$; two litters; $P < 0.0005$). In addition, large *Reln*+ and calretinin+ CR cells were frequently observed deep in the cortical plate and even in the IZ in $Cxcr4^{-/-}$ embryos, but they were only occasionally found in those locations in control embryos (cortical plate: 39 cells per brain in $Cxcr4^{-/-}$, 3.25 cells per brain in control embryos, $P < 0.0005$; IZ: 6.75 cells per brain in $Cxcr4^{-/-}$, 2.5 cells per brain in control embryos, $P = 0.087$). Small *Reln*+ cells were also very abundant throughout the depth of the cortical plate (**Fig. 7l**,**m** and data not shown). In the hippocampus of mutant embryos, large *Reln*+ CR cells were abundant not only in the vicinity of the hippocampal fissure but also away from the fissural meninges (70.5 cells per brain in $Cxcr4^{-/-}$, 4 cells per brain in control embryos; $P = 0.009$), in particular in the CA3 hippocampal subfield (**Fig. 7n**). Taken together, these observations demonstrate that CXCL12/CXCR4 signaling is critical for the recruitment of CR cells to the vicinity of the meninges during their tangential migration.

DISCUSSION

Our results show that the tangential migration of hem-derived CR cells along the surface of the cortical primordium depends on the meningeal

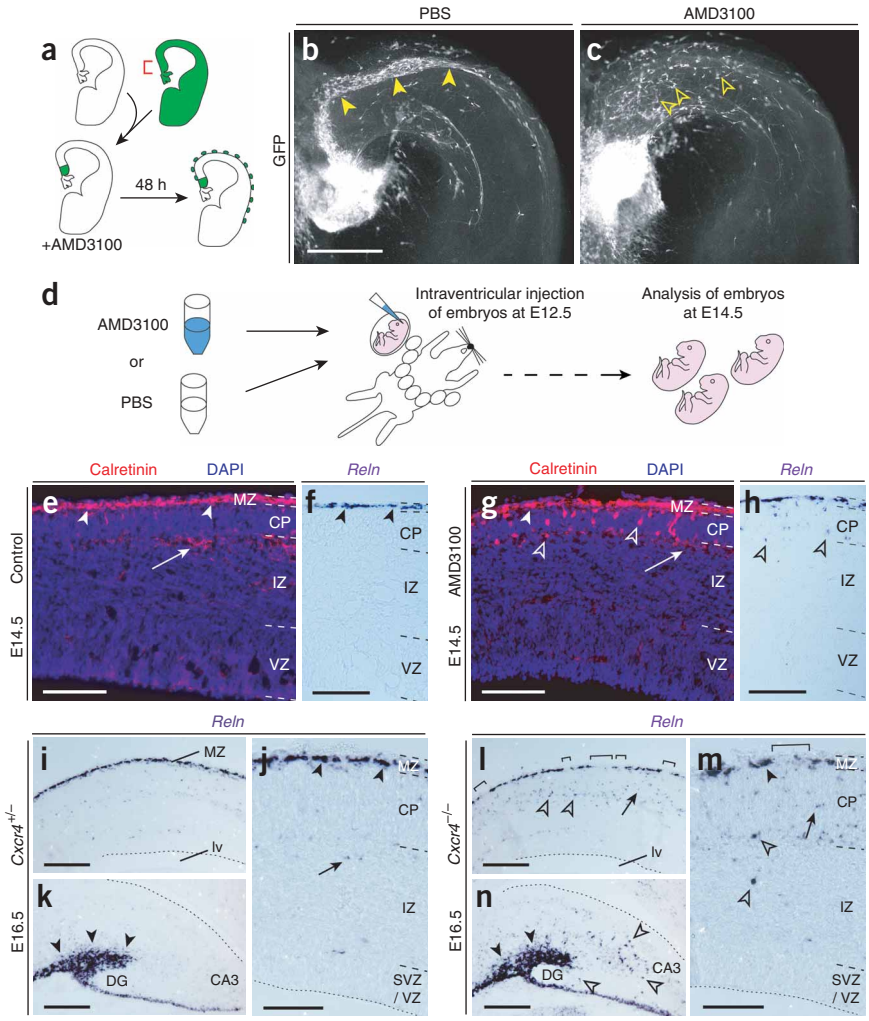

Figure 7 Detachment of CR cells from the marginal zone in the absence of CXCR4 function. (**a**) A GFP+ hem (red bracket) was transplanted in replacement of the original hem in a wild-type slice, which was then treated with AMD3100 or PBS (vehicle solution) over 48 h. (**b**,**c**) Results from experiment depicted in **a**. Images are representative examples of control and AMD3100-treated slices. In control slices, GFP+ hem-derived CR cells were restricted to the marginal zone (solid arrowheads), whereas in the presence of AMD3100, these were scattered throughout the depth of the developing neocortex (open arrowheads). (**d**) AMD3100 or vehicle solution was injected into the telencephalic lateral ventricle of E12.5 wild-type embryos *in utero*, and embryos were allowed to develop until E14.5. (**e**–**h**) Results from experiment depicted in **d**. Images show distribution of calretinin+ (**e**,**g**) and *Reln*+ (**f**,**h**) CR cells in the neocortex at E14.5 after injection of AMD3100 (**g**,**h**) or vehicle solution (**e**,**f**) at E12.5. CR cells were confined to the marginal zone (MZ) in control embryos (solid arrowheads), but in AMD3100-treated embryos, numerous CR cells were seen deep within the cortical plate (CP; open arrowheads). Arrows indicate calretinin+ thalamo-cortical afferents. (**i**–**n**) Distribution of *Reln*+ cells in the neocortex (**i**,**j**,**l**,**m**) and hippocampus (**k**,**n**) of E16.5 *Cxcr4*$^{+/+}$ (**i**–**k**) and *Cxcr4*$^{-/-}$ (**l**–**n**) embryos. In wild-type embryos (*Cxcr4*$^{+/+}$, $n = 3$; *Cxcr4*$^{+/-}$, $n = 2$), *Reln*+ cells were confined to the neocortical marginal zone (MZ; **i**,**j**) and hippocampal fissure (**k**) (solid arrowheads) with small *Reln*+ cells occasionally present in the intermediate zone (IZ) (arrow in **j**). In mutant embryos (*Cxcr4*$^{-/-}$, $n = 3$; **l**,**m**), the MZ had multiple gaps without *Reln*+ cells (brackets), whereas large (open arrowheads) and small (arrows) *Reln*+ cells were found in the cortical plate and IZ. In the mutant hippocampus (**n**), *Reln*+ cells were found in the hippocampal fissure (solid arrowheads) but also in deeper positions within the dentate gyrus (DG) and the CA3 subfield (open arrowheads). lv, lateral ventricle; SVZ/VZ, subventricular/ventricular zone. Scale bars: 300 μm in **b**,**c**; 100 μm in **e**–**h**,**j**,**k**,**m**,**n**; 200 μm in **i**,**l**.

membranes. The meninges are required in this process both as a physical substrate for migration along the cortical surface and as a source of the chemokine CXCL12, which promotes the migration of CR cells and maintains them at the cortical surface, away from the neural epithelium.

The meninges are essential for CR cell migration

Genetic manipulations and physical insults resulting in a disruption of the integrity of the meningeal membranes affect the normal distributions of CR cells in the cerebral cortex. These alterations include interruption of the continuous arrangement of CR cells along the MZ (refs. 20–22,24,25) or derailment of CR cells away from the MZ and into the cortical plate[23]. However, only recently have CR cells been shown to disperse tangentially through the cortex[17–19], and these studies have never addressed whether the integrity of the meninges is critical even for the initial spread of CR cells along the cortical surface. By using several embryonic slice culture manipulations, we demonstrated that the meningeal membranes are an essential, and sufficient, substrate for the tangential dispersion of CR cells from the hem and throughout the pallium.

We have also shown that the meninges, besides serving as substrate for migration, secrete diffusible factors that are chemoattractive for migrating CR cells *in vitro*. This finding also implicates the meninges in the directional migration of hem-derived CR cells, from caudomedial to rostrolateral domains of the telencephalon. Notably, meninges from different domains of the telencephalon are similarly chemoattractive for hem-derived CR cells, suggesting that the overall directional dispersion of these cells is not controlled by the meninges. Other components of the developing telencephalon that occupy strategic positions from whence to potentially exert this function, such as the choroid plexus or the neocortical primordium, have no chemotactic influences over these cells *in vitro*, suggesting they cannot exert long-range influences on migrating CR cells in the developing telencephalon.

So are there mechanisms responsible for directing the migration of hem-derived CR cells into appropriate areas of the cortex? Previous cell lineage–tracing studies showed that hem-derived CR cells have complementary distributions with respect to CR cells from other sources[18,19] and that the ablation of CR cells from the septum and ventral pallium is partly rescued by expansion of the territories occupied by hem-derived CR cells[19]. These results are in agreement with our present findings: according to our model, the meninges promote the tangential migration of CR cells in a nondirectional manner, and it is only the interaction between CR cells that mutually restricts their movement, thus determining their final patterns of distribution in the telencephalon.

Multiple roles of meningeal CXCL12 in CR cell migration

Chemokines are emerging as important regulators of the development of the mammalian central nervous system[27]. Several studies have shown that CXCL12/CXCR4 signaling has a critical role in the migration of neurons in various systems[27–29,32,37]. Here we showed that CR cells derived from the cortical hem are robustly chemoattracted by meningeal CXCL12 via the CXCR4 receptor. In the context of the cortical and hippocampal primordia, blockade or absence of CXCR4 function results in the derailment of CR cells from subpial positions down to the cortical plate, combined with an overall reduction in the abundance of CR cells in the cortical MZ (see also ref. 37).

Previous investigations of the role of CXCR4 in the migration of cortical neurons concluded that CXCR4 signaling is not involved in the migration of CR cells, as some large *Reln*+ cells (putative CR cells) were observed in the MZ of *Cxcr4*$^{-/-}$ embryos[37]. Unfortunately, these studies were performed before the identification of the origins and routes of migration of the various CR cell subtypes[17–19], and the results were interpreted assuming that CR cells were generated in the cortical VZ and migrated radially from there to the MZ (ref. 37). As described above, our experiments unequivocally demonstrate that CXCL12/CXCR4 signaling is essential for the development of CR cells derived from the cortical hem.

The mechanism through which the meninges control the tangential migration of CR cells shares some features with mechanisms in other regions of the central nervous system. In the cerebellum, for example, granule cell progenitors remain transiently in the external granule cell layer (EGL) before migrating radially into the internal granule layer (IGL). Several lines of evidence indicate that CXCL12 secreted by the cerebellar meninges acts as an anchoring mechanism that prevents premature inward migration of granule cells toward the IGL (refs. 31,46,47). In the cortical primordium, however, the chemoattractive activity of meningeal CXCL12 over CR cells cannot be interpreted as an anchoring system, as CXCL12-responsive hem-derived CR cells actually require the presence of the meninges in order to migrate tangentially over long distances. What is the role of CXCL12 in the migration of CR cells *in vivo*? We favor the interpretation that the chemoattractive influence of meningeal CXCL12 on the migration of hem-derived CR cells *in vitro* represents a stimulation of their movement along the MZ *in vivo*. Moreover, in addition to enhancing cell movement, meningeal CXCL12 is also involved in recruiting migrating CR cells to the MZ. In fact, the recruitment of migrating CR cells to the MZ may be a direct consequence of the chemoattractive influences of CXCL12, as the secretion of a motogenic factor from a polarized source (the meninges) seems to be sufficient to generate the polarized apico-basal distribution of migrating CR cells along the MZ (**Supplementary Fig. 1** online). In the absence of CXCL12/CXCR4 signaling, the positive influence of the meninges is no longer present or detectable, and many hem-derived CR cells disperse into deep layers of the developing cortex, whereas other CR cells may fail to migrate altogether (**Supplementary Fig. 1**). Many of these cells are probably left behind, stranded in the vicinity of the cortical hem; later, these cells probably correspond to the CR cells observed ectopically in the mutant hippocampus. Of note, the fact that many hem-derived CR cells migrate—albeit through abnormal paths—in the absence of CXCL12/CXCR4 signaling suggests that other factors stimulate the initial movement of hem-derived CR cells.

Emerging role of meninges in neuronal tangential migration

Our findings provide strong evidence that the meninges do not just have a structural or homeostatic role in the developing brain, but directly participate in the orchestration of this complex process. As shown in this study, CXCL12 secreted by the cortical meninges is critical for the tangential migration of hem-derived CR cells. *Cxcr4* does not seem to be expressed in CR cells derived from sources other than the cortical hem, as *Cxcr4* expression in the marginal zone at E11.5 does not extend to the lateral domains of the pallium, where, at this stage, mostly CR cells from the ventral pallium are found[17,19]. Because CR cells from all possible origins migrate along the pial surface, it is likely that the meninges express additional factors that influence the migration of CR cells from other sources in ways similar to how CXCL12 influences the migration of CXCR4+ hem-derived CR cells. This argument would also explain why many CR cells, probably derived from sources other than the cortical hem, remain correctly positioned in the MZ of *Cxcr4*$^{-/-}$ embryos.

In addition to CR cells, the marginal zone of the cortex contains other neuronal types that disperse tangentially through the cortical surface, namely the GABAergic interneurons and the so-called pioneer neurons[4,48]. Thus, it is conceivable that, as for CR cells, the meninges may be involved in the tangential migration of these cell populations. Notably, the distribution of cortical GABAergic interneurons in the cortex of mouse fetuses carrying loss-of-function alleles for *Cxcl12* or *Cxcr4* is abnormal[37], although the cellular mechanism underlying such defects remains unknown. In view of the role of CXCL12/CXCR4 signaling in the tangential migration of CR cells, it is tempting to speculate that the meninges may exert a similar effect on cortical GABAergic interneurons migrating through the marginal zone of the embryonic cortex. As it is likely that signaling systems other than CXCL12/CXCR4 are involved in this process, unraveling the molecular nature of such signal(s) will greatly contribute to our understanding of the mechanisms controlling tangential neuronal migration in the developing brain. In conclusion, our experiments point to the meninges as a key element in dispersing Cajal-Retzius cells throughout the surface of the developing cerebral cortex, a process essential for the subsequent functions of Cajal-Retzius cells in orchestrating the normal development and lamination of the cerebral cortex.

METHODS

Animals. Wild-type and GFP-expressing transgenic mice[49] were maintained in a CD1 background. Mice carrying a loss-of-function allele for *Cxcr4* (ref. 35) were maintained in a C57b/6 background. The day of vaginal plug was considered as embryonic day (E) 0.5. Mice were kept at the Instituto de Neurociencias de Alicante in accordance with Spanish and EU regulations.

***In utero* drug administration.** For *in vivo* blocking of CXCR4 receptors, surgery was performed as for *in utero* electroporation (**Supplementary Methods** online), and 1 μl of AMD3100 solution (Sigma; 1.26 mM or 12.6 mM) or vehicle solution (phosphate-buffered saline, PBS) was injected into the telencephalic lateral ventricle. Embryos were analyzed 48 h later.

Slice cultures. Brain slices were obtained from E12.5 embryos as described previously[17,50]. The cortical hem from host (wild-type) slices was discarded and substituted by the hem from donor slices (obtained from GFP mice). In a parallel series of experiments, the ventral, lateral and dorsolateral domains of the meninges were peeled from the telencephalon of E12.5 brains, which were then sliced as above. Alternatively, the lateral neocortex and the basal ganglia were mechanically separated from the overlying meninges and removed from the slice. Slices were maintained for 48 h *in vitro* before analysis.

Explant cultures. Slices obtained as above were dissected to obtain explants of the cortical hem, neocortex or choroid plexus epithelium. Clumps of meningeal membranes were obtained by mechanical peeling from intact wild-type or GFP brains. To obtain distinct domains of the meninges, whole peeled meningeal membranes were hemisected along the dorso-ventral or anterior-posterior axes. Explants were cultured in three-dimensional Matrigel matrix (Beckton-

Dickinson) for 48 h in Neurobasal medium (Invitrogen). For contact-inhibition assays, pairs of hem explants were cultured on two-dimensional glass coverslips coated with poly-lysine and laminin for 24–32 h in Neurobasal medium containing 0.4% methylcellulose (Sigma).

Quantification and statistics. For explant confrontation assays, the distance between each cell body and the edge of the explant was measured using ImageJ software. In matrigel cultures, the 50 cells farthest from the center of the explant were measured in the proximal and distal quadrants for each explant. In coated-glass cultures, the 30 cells farthest from each explant were measured in the proximal and distal hemiquadrants. The average value of these measures was taken as the distance of migration of each explant. The proportion between the average distances migrated in the proximal and distal quadrants defined the value of the proximal/distal ratio (P/D) for each explant. Individual cultures were additionally classified as indicated in **Supplementary Tables 1** and **3**.

To measure mispositioning of CR cells *in vivo*, changes in the presence of CR cells in the MZ of mutant versus control embryos were quantified by measuring the proportion of linear MZ devoid of CR cells, defined as large *Reln*+ cells. For each embryo, the added length of MZ segments devoid of CR cells was measured from five sections (20 μm thick) representative of the rostro-caudal extent of the telencephalon. Only neocortical MZ segments longer than 50 μm were included. For quantification of ectopic CR cells, large *Reln*+ cells observed in the neocortical cortical plate and IZ and in the hippocampus were counted from the same five sections per embryo as before.

Data were statistically analyzed with SPSS software using chi-squared tests, pair-wise *t*-test or independent samples *t*-test, as appropriate.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

V.B. and O.M. planned the experiments. V.B. performed the experiments and analyzed the data. O.M. provided reagents, materials and analysis tools. V.B. and O.M. discussed the results and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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