During cerebral cortex development, different cell populations migrate tangentially through the preplate, traveling from their site of origin toward their final positions. One of the earliest populations formed, the Cajal–Retzius (C-R) cells, is mainly generated in different cortical hem (CH) domains, and they migrate along established and parallel routes to cover the whole cortical mantle. In this study, we present evidence that the phenotype of RETzius cells, as well as some of their migratory characteristics, is specified in the area where the cells are generated. Nevertheless, when implanted ectopically, these cells can follow new migratory routes, indicating that locally provided genetic cues along the migratory path nonautonomously influence the position of these cells emanating from different portions of the CH. This was witnessed by performing CH implants of tissue expressing fluorescent tracers in live whole embryos. In the same way, tracer injections into the hem of Small eye mutant mice were particularly informative since the lack of Pax6 affects some guidance factors in the migratory environment. As a result, in these animals, the C-R cell population is disorganized, and it forms 1 day late, showing certain differences in gene expression that might help explain these disruptions.

Keywords: culture, development, embryo, mouse, Pax6, tangential migration, tissue implants

Introduction

The correct development of the cerebral cortex largely depends on the appropriate migration of the cells generated during its development. These cells travel from their place of origin toward their final destination, where they will establish the necessary connections to integrate and process sensory impulses, thereby participating in higher intellectual functions, as well as providing the information required to develop complex patterns of behavior.

The Cajal–Retzius (C-R) cells are one of the first cell populations generated in the cortical neuroepithelium, although they subsequently coexist in the preplate with other cell populations such as subplate cells, subpial granular layer cells, interneurons of subpallial origin, and glial cells (Jiménez et al. 2003), as well as with several cell populations from different telencephalic origins that are in transit toward the olfactory cortex (García-Moreno et al. 2008). These cells were the first to be described in layer 1 (Cajal 1890; Retzius 1893), and they constitute an essential cell population for the development and establishment of the different layers of the cerebral cortex. During early cortical development (E10–E12 in mice), C-R cells are located at a subpial site where they secrete high levels of the glycoprotein Reelin (Rehn). From there, they are capable of organizing the radial migration of the cells generated in the germinative ventricular and subventricular zones, orchestrating the correct lamination of the cerebral cortex and favoring an inside-out gradient of generation (Caviness 1982; Ogawa et al. 1995; Schiffmann et al. 1997; Marín-Padilla 1998; Beffert et al. 2004; Soriano and Del Río 2005).

C-R cells have been described as a heterogeneous cell population based on the existence of multiple origin sites. Most of these cells are primarily originated in the cortical hem (CH; Takiguchi-Hayashi et al. 2004; Yoshida et al. 2006; García-Moreno et al. 2007; Gu et al. 2009), although other sites of generation such as the ventral pallium (VP) and the septum have been described (Gorski et al. 2002; Hevner et al. 2003; Bielle et al. 2005; Cabrera-Socorro et al. 2007; Abellán et al. 2009; Tisser et al. 2009).

Our previous studies (García-Moreno et al. 2007) revealed that the C-R cells generated in different domains of the CH (rostral [RCH], medial [MCH], and caudal [CCH]) migrate along defined parallel routes, maintaining a clear relationship between their site of origin and their final position.

Numerous genes appear to contribute to the specification and differentiation of this population of cortical interneurons, such as p73 (Meyer et al. 2002, 2004), p21 (Siegenthaler and Miller 2008) and Zic1-3 (Inoue et al. 2007) for the septal and CH-derived C-R cells and Ebf2 for the VP-derived cells (Hanashima et al. 2007). In fact, the expression of these genes among others was previously detected in E13.5-enriched mouse C-R cells by microarray analysis (Yamazaki et al. 2004). On the other hand, Foxg1 (forkhead box G1) and Lhx2 play a key role repressing the C-R phenotype, consequently limiting the generation of C-R cells to very specific areas (Tao and Lai 1992; Bulchand et al. 2001; Hanashima et al. 2004, 2007; Mangale et al. 2008). The pallial transcription factor Pax6 has been shown to be involved in many developmental processes, such as proliferation, migration and in specifying the adhesive properties of migrating cells, as well as in axonal growth and collateral branching (Caric et al. 1997; Valverde 2000; Estivill-Torrus et al. 2002; Tyas et al. 2003; Gopal and Golden 2008). Indeed, mutations in this gene can cause an increase in the number of Rehn” /Calretinin’ cells in the cortical marginal zone, although it is unknown whether this effect is caused through the re-specification of cells or due to alterations in the mechanisms that control cell migration (Stoykova et al. 2003).

To date, the relative contribution of the intrinsic and extrinsic influences that define how CH-derived C-R cells spread through the cerebral cortex remains unexplored. To determine whether these migratory routes are predicted by their spatial origins, we combined in vivo labeling with the implantation of genetically marked tissue. Here, we specifically address the importance of the signals coming from the site of origin and those signals in the microenvironment through which the cells move on the orientation of the different migratory routes taken by C-R cells. In addition, we evaluated the changes in migration of C-R cells in the Small eye (Sey) mutant mice, where Pax6 is not functional (Hill et al. 1991), by comparing their routes with wild-type (wt) mice.
gene expression in the mutant and wt mice, we hoped to identify potential guidance cues that might influence C-R cell migration. In this way, we provide evidence that the local environment has a significant influence on the migratory routes adopted by C-R cells and that altering genetic patterning affects the expression of guidance cues, altering the dispersion of these cells. Alternatively, we show that although neuroblasts originated in the CH can integrate into the host tissue when transferred to other locations, they are restricted to generate C-R cells.

Materials and Methods

Animals

In this study, we used wt mouse embryos (n = 111) obtained from 23 B6tgN pregnant mice (C57BL/6-TgN); embryos (n = 78) from 16 pregnant mice of a transgenic line that expresses an enhanced green fluorescent protein (GFP) under the control of the β-actin promoter (C57BL/6-TgN [ACTbEGFP] 10sb, JAXMice); and embryos from 5 pregnant heterozygote Sey mice carrying the Pax6 mutation (Pax6<sup>bmu</sup>–<sup>bmu</sup>, a gift from Jack Favor, GSF-Institut, Neuherberg, Germany). Animals were housed in an incubator, and they were maintained at 35 °C until needed. Total RNA was extracted using the RNeasy Micro Kit (AM1391; Ambion, Austin, TX) according to the manufacturer’s instructions.

Whole Embryo Culture

This method is used routinely in our laboratory, and it has already been described in detail elsewhere (De Carlos et al. 1996; Jiménez et al. 2002; García-Moreno et al. 2007, 2008). Briefly, embryos (E10–E12) were transferred to a small glass bottle containing 4 mL of culture medium. The medium used was heat-inactivated rat serum, obtained by centrifuging blood immediately after its extraction from donor rats (3 × 100 g, 6 min each), and it was supplemented with glucose (2 mg/mL rat serum) and penicillin-streptomycin (100 IU/mL; Gibco, Grand Island, NY) before it was filtered through a 0.45 μm filter (Sarstedt, Nümbrecht). The glass bottles were then used to attach an empty petri dish to a micropipette glued to a microperfusor (25 G 0.5 mm; BD Plastipak, Madrid, Spain) filled with an interface of Hanks balanced solution and mineral oil. Once the tissue was implanted, the embryo was introduced in a glass bottle to be cultured for 12–36 h.

Tracer Injections

Two different fluorescent tracers were injected into 2 adjacent CH domains in the same embryo: carboxy-fluorescein diacetate succinimidyl ester (CFDA: a 10-mM solution in dimethyl sulfoxide (DMSO); Molecular Probes, Inc., OR) and carboxyfluorescein diacetate succinimidyl ester (SNARF: a 10-mM solution in DMSO; Molecular Probes, Inc., OR).

Dissection and RNA Preparation

The RCH and CCH and the dorsal pallium were obtained from slices of E11 wt embryos. Three litters were used to obtain 3 different sets of samples. The entire dorsal pallium of wt (E11) and Pax6 (E12)-mutant embryos was recovered, avoiding both the pallial-subpallial boundary (PSB) and the CH area including the meningeal cells that have an active role in C-R cell migration. Each tissue sample was obtained from a minimum of 3 embryos, and we collected a minimum of 3 samples in each case. The tissues dissected were flash-frozen in dry ice and stored at −80 °C until needed. Total RNA was extracted using the RNeasy Micro Kit (AM1391; Ambion, Austin, TX) according to the manufacturer’s instructions.

RT–PCR Microarrays and Data Analysis

The cDNAs were synthesized from 500 ng of total mRNA. To quantify the expression of 95 genes implicated in cell adhesion and migration, we used the Custom Taqman low-density array (384-well microfluidic cards; Applied Biosystems, Foster City, CA; 4342259). Briefly, the cDNA sample was diluted in 100 μL of RNase-free water, and the solution was mixed with 100 μL of the Master Mix provided by Applied Biosystems. The microfluidic cards were charged with the solution mix, and the wells were filled by centrifugation. Amplification was recorded by real time (RT)-PCR using a 7900HT Fast Real-Time System, and the data were analyzed with the RQ Manager 1.2 software.

CII Implants

GFP-expressing transgenic and wt mouse embryos were used for the CII implants. The telencephalon was removed from 2 or 3 GFP embryos under a dissecting microscope (Nikon SMZ1500; Nikon Corp., Tokyo, Japan) using fine watchmaker’s forceps. The tissue to be implanted was obtained from the caudal portion of the CH, avoiding the menenchymal tissue. Using an oil-filled system, pieces of solid tissue from the caudal GFP-CH were removed and implanted into the desired site of the telencephalon of wt embryos. The injection system consisted of a micropipette glue to a microperforus (25 G 0.5 × 19 mm; Pic indolor, Como, Italy) that was connected to a 1 mL syringe (25 G 0.5 × 16 mm; BD Plastipak, Madrid, Spain) filled with an interface of Hanks balanced solution and mineral oil. Once the tissue was implanted, the embryo was introduced in a glass bottle to be cultured for 12–36 h.

Immunohistochemistry

Immunohistochemistry was carried out on 40-μm vibratome sections using the following antibodies and dilutions: mouse-anti-Reelin (MAB364 clone G10, 1:1000; Chemicon, Temecula, CA); rabbit-anti-Calbindin-D28K (CR, 1:10 000; Swant, Bellinzona, Switzerland); rabbit-anti-Calretinin (CR, 1:2000; Swant); mouse-anti-β tubulin type III (TuJ1, 1:1000; Chemicon); and rabbit-anti-Tbr1 (1:1000; Chemicon). The secondary antibodies were Alexa Fluor 568-conjugated goat-anti-mouse (1:2000; Molecular Probes, Inc.; A11004) or Alexa Fluor 568-conjugated goat-anti-rabbit (1:2000; Molecular Probes, Inc.; A11011). Sections were pretreated with 0.2% phosphate-buffered saline–Tween 20 (PBS-T), and they were blocked with 5% normal goat serum and 0.1% bovine serum albumin in 0.2% PBS-T. Primary antibodies were diluted in blocking solution and incubated with the tissue overnight at 4 °C. The sections were then washed with 0.2% PBS-T and incubated with the secondary antibody for 90 min at 4 °C. For Reelin immunohistochemistry, sections were treated with cold citrate buffer (pH 6) for 5 min and then for 1 min with citrate at 90 °C, and the sections were counterstained with bisbenzimide.

Image Acquisition

Fluorescent images were obtained using either a fluorescent dissecting microscope (Leica MZFL-III) to study whole brains, a fluorescent microscope (Nikon Eclipse E600) equipped with a digital camera (Nikon DMX 1200F) or a spectral confocal microscope (Leica TCS 4D).
Figure 1. Tangential migration of cell populations generated in adjacent domains of the CH in E11 mice embryos cultured in toto for 24 h. (A) Mouse head in which 2 different fluorescent tracers, SNARF (red) and CFDA (green), were injected into the RCH and MCH, respectively. Dorsal is to the left and rostral is below. (B) Site of CFDA injection into the MCH. Labeled cells migrate obliquely toward more rostral areas by dispersal in a discrete fan shape (bounded by dashed lines). A few cells leave their migratory pathway to reach the rostral domain (arrowheads). (C) Coronal section showing the injection site (asterisk) in the MCH. (D) Cell populations generated in the MCH run tangentially through the preplate following oblique caudorostral pathways (arrowheads). These cells do not transgress the PSB. (E) In coronal sections, at rostral levels, there is a mixture of cell populations generated in the MCH (green) and RCH (red). (F, G) There is no contamination between the 2 tracers. Thus, green cells (CFDA labeled) generated in the MCH cross the adjacent (rostral) injection site area (F, asterisk) without incorporating the second tracer (SNARF, red). (G) Shows the same field as that shown in (F) under the red filter. (H, I) In all cases examined, the migratory cells adopt a fusiform morphology and emit one or two opposite processes of variable length. (J) Schematic representation of the brain of an E11 mouse embryo as seen from the top. The shaded area corresponds to the extension of the CH delimited by the expression of wnt3a. The 3 black dots in the right hem indicate the divisions of the CH: RCH, MCH, and CCH. The parallel and oblique dotted lines indicate the plane of the section through each of these regions, corresponding to 45° sections (K–M). The drawings of each slice were made from real vibratome sections. (O–Q) In the sections obtained at an inclination of 45° that were used to follow the routes of migration, the overlapping routes of migration were always in rostral sections and at more distal (lateral) levels. Scale bars—(A–D): 200 μm; (O–Q): 100 μm; (E–G): 50 μm, and (H–I): 10 μm.
All photographs were adjusted for contrast and brightness using Adobe Photoshop CS3 (Adobe, San Jose, CA).

**Co-localization Analysis**
The co-localization of GFP-labeled cells with the different markers was quantified using the Cell Calculator plug-in (University of Sheffield) for the Image J software (Abramoff et al. 2004). Cell counts were obtained from serial confocal images taken every 2 μm from 40-μm thick vibratome sections, and only migratory fluorescent cells located beyond the implant area were considered in the quantitative analysis.

**Results**

**Discrete Overlapping of Cell Populations Generated in Adjacent Domains of the CH**
To determine whether any overlap existed between the parallel and adjacent routes taken by cells that originated in different CH domains, we performed dual tracer experiments. Accordingly, a green fluorescent tracer (CFDA) was injected exo utero into the MCH of E11 embryos (n = 7) and a red fluorescent tracer (SNARF) was injected into the RCH (Fig. 1A). After in toto culture for 24 h, green-labeled cells migrated tangentially through an oblique route toward the rostral lateral cortex, adopting a narrow fan-like dispersion (Fig. 1B). A similar behavior was observed in cells coming from the RCH (red cells) and CCH domains. Indeed, there was some intermingling between adjacent routes in all the injected embryos, with a few migratory cells coming from the MCH entering the rostral pathway (Fig. 1B, arrowheads and Supplementary Fig. 1A), although the cells never migrated in a pathway more caudal to the injection site. The analysis of coronal sections indicated that labeled cells reached the marginal zone or preplate by radial migration, and they moved tangentially from this location, maintaining a subpial position and halting at the PSB (Fig. 1C–E). In these experiments, double-labeled cells were never found, such that the migratory cells labeled with one tracer did not take up a second tracer, even though they might pass through the site of injection of this second tracer (Fig. 1F–G).

The cells coming from the RCH, MCH, and CCH had a similar morphology. These cells had a fusiform soma and one clearly visible rostral process (the leading process), which varied in length and usually ended in a growth cone that indicated the migratory direction of the cell (Fig. 1H). Occasionally, these migratory cells had 2 processes of similar length making it difficult to distinguish the leading from the trailing process (Fig. 1I). Since these cells migrated along oblique routes, they were also studied in sections oriented at 45° to the surface, following the direction of their migration (Fig. 1J–M). These oblique sections revealed an overlap in the most distal part of the adjacent routes (Fig. 1O–Q). However, despite this overlap, the majority of the cells emanating from the different CH domains always followed the correct route between their point of origin and their final position (see Supplementary Fig. 1A).

**Specifying the Direction of Cell Migration**
The idea that the progeny of each CH domain follows specific pathways could indicate certain conditioning in their origin. To evaluate whether the migratory pathway between the site of origin and the final position of the CH-derived cells was specified along the rostrocaudal extension of the CH, we dissected out the CCH tissue of GFP-transgenic mice and implanted it into different CH domains of wt embryos. We did not dissociate the nervous tissue removed to avoid the severe alterations that may affect the signals from the GFP-CH cell niche (Fig. 2A). We first confirmed that these cells were viable in the cultured embryos after the surgical procedure, as well as their capacity to integrate and migrate. Thus, part of the MCH from GFP embryos was implanted into the MCH of wt embryos of the same developmental age, and the recipient embryo was cultured in toto for 12–36 h. Selected embryos were then sectioned in the coronal plane and studied by fluorescence microscopy. In all cases (n = 3), GFP+ cells integrated into the host tissue and they moved tangentially toward the rostromedial cortex, always maintaining a subpial position and preserving their typical pattern of migration (Fig. 2B–D).

After verifying the feasibility and reproducibility of this approach, a series of experiments was carried out by implanting a CCH fragment from GFP-transgenic embryos into the RCH of wt embryos (Fig. 2E–G). In these experiments, the GFP+ cells moved rostromedially following the new rostral pathway. Significantly, in the converse rostral-to-caudal experiment, the implanted GFP cells now moved through the caudal routes (data not shown). This indicated that the migratory behavior of the cells was not conditioned by their original environment along the rostrocaudal level of the hem. To ensure this was the case, we compared the expression of several genes

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**Figure 2.** Four experiments involving the implantation of solid tissue from the CCH of GFP+ transgenic mice into different CH domains of wt mice. (A) Cartoon showing the experimental technique utilized. A tiny piece of the CCH was extracted and implanted exo utero (in petri dish) into the hem of another embryo of the same age, which was then cultured for 24 h in roller bottles as a whole embryo (whole embryo culture [WEC]). (B–D, first experiment): partial view of coronal sections (see insets) to illustrate the implants of GFP+CCH into the MCH of wt mice. Cells generated in the implants reach the marginal layer (preplate) and then migrate tangentially, aided by a leading process. (E, F, second experiment): an implant into the RCH domain that remains in the lateral ventricle, attached to the ventricular zone of the neuroepithelium (inset in F). Cells generated in the CCH reach the preplate by radial migration, and they move rostrally as if they were born in the rostral domain (E and inset). (G, third experiment): sometimes, the implants remained on the surface of the telencephalon, and they apparently did not break through the pial membrane. In those cases, the GFP+ cells generated did not enter the cortical neuroepithelium, and they migrated and remained on the leptomeninges, taking a wrong direction toward more caudal regions with respect to the implantation site. (H, I, fourth experiment): in this case, the implant was located in the dorsal telencephalon, close to the CH. In all these experiments (with the exception shown in G), the implanted cells adopted a new migratory route, which coincided with that of the site of implant, always moving toward more dorsal cortical areas. Although migration through the preplate is tangential from the initial medial location to another more lateral site (J, arrow), the implanted cells reach the upper strata by radial migration through a very narrow corridor (H, dashed lines). (J) Diagram showing the normal migratory behavior of the cells generated in the implants (green arrows), adopting new routes in a rostral lateral direction. Some cells lose contact with the neuroepithelium and therefore with their local molecular cues, migrating erroneously in a caudal direction (red arrow). (K) Diagram showing the 4 possibilities for the integration of implants into the host neuroepithelium shown in the earlier experiments. (1) A portion of the implant embeds into the host tissue by breaking the pial membrane while the ventricular zone (vz) is preserved. The cells generated in the implant reach the preplate without migrating into the deep layers. (2) The implant occupies the entire thickness of the neuroepithelium, which rarely occurred. (3) The implant is integrated with the ventricular zone (vz) at the site of injection and completely introduced into the ventricular cavity. (4) The implant lies above the neuroepithelium, and it is in contact with the leptomeninges, usually without breaking through the pial membrane. Cells generated in the unimplanted implants do not penetrate the neuroepithelium, and they migrate randomly and tangentially through the leptomeninges. Scale bars—(B, E, H, G): 50 μm; (D, L, I): 100 μm; and (J): 200 μm.
involved in cell adhesion and migration (Supplementary Table 1) between E11 RCH versus CCH by RT-PCR microarray. This analysis identified significant changes in the expression of a few genes (Table 1), and for example, *Fgfr3* and *Ccr11* were strongly enriched in the CCH. Indeed, these genes might influence the migratory capacity of CCH cells in relation to cells generated in the rostral domain, although they could also be implicated in other aspects of cell development.

To determine whether the implanted GFP<sup>+</sup> cells changed their migratory behavior in response to different signals coming from the new location, we implanted a piece of GFP-CCH into the dorsal pallium of wt embryos since this structure generates cells that migrate ventrally toward olfactory regions at early developmental stages (García-Moreno et al. 2008). As in previous experiments, cells generated from the heterotopically placed implant reached the preplate and they moved...
rostrolaterally, into the area where the cells were implanted. Nevertheless, these cells maintained the characteristic properties of migratory C-R cells, that is, a subpial tangential migration that ceased at the level of PSB, preventing their entry into the olfactory cortex.

**Dynamics of the GFP Cell Integration and Migration**

The migratory behavior of the implanted cells depended on the way the implant associated with the host tissue. When a piece of nerve tissue is implanted, it could be lost in the ventricular cavities or it may attach to the host tissue in 4 different ways (Fig. 2K). Thus, cells generated from an implant placed in the upper part of the host tissue, breaking the pial membrane and preserving the neuroepithelial ventricular zone (Fig. 2K, 1), reached the preplate without making contact with the ventricular zone and undertook an ascendant radial migration (Fig. 2C). Thus, these cells migrated tangentially in a caudorostral direction. When implants were introduced into the entire thickness of the pallial neuroepithelium (Fig. 2K, 2), migratory cells capable of correctly invading the host tissue were not evident (a situation only observed in one experiment). However, the most successful experiments were obtained when the implant was strongly associated with the ventricular zone and it introduced into the ventricular cavity (Fig. 2K, 3). The cells derived from such implants invaded the host tissue by migrating radially through a narrow corridor to reach the more superficial layers of the cortical neuroepithelium (Fig. 2F-H, insets). Finally, when the implant was placed on the neuroepithelium, above the pial membrane (Fig. 2K, 4), the GFP *+* cells migrated through the leptomeninges without making contact with the epithelium, thereby losing their sense of direction and moving randomly through the meninges, even in a caudal direction (Fig. 2G).

To ensure that migratory GFP *+* cells generated in the implants changed their migratory routes when heterotopically transplanted, we implanted a piece of CCH into the lateral ganglionceminence (LGE), an unrelated structure that is highly proliferative at this developmental stage (Fig. 3). After culturing the embryos, their whole brains were analyzed in the 3 dimensions of the telencephalon to observe the dispersion of GFP *+* cells (Fig. 3A-D). In these cases, GFP *+* neuroblasts moved along a new migratory pathway across the subpallium. In this new location, the cells did not migrate following their typical caudorostral orientation, but rather, they moved in an unrestricted manner throughout the subpallium in the opposite direction along the rostrocaudal axis. Even though migration was tangential and subpial, cells reached the preplate by radial migration (Fig. 3F, arrow). From there, the cells moved rostrally through the olfactory tubercle (Fig. 3G,H), reaching the telencephalic rostroventral tip where they lost their migratory morphology (fusiform and bipolar) and they acquired more ramifications. These processes were apparently dendritic (Fig. 3F, asterisk) since at the opposite side of the soma, it was possible to observe a thinner and shorter prolongation that seemed to be a growing axon (Fig. 3F, open arrowheads). However, other cells left their subpial position to settle in the olfactory tubercle, without entering the piriform cortex (PC; Fig. 3G,H). The cells that moved caudally reached the entorhinal cortex maintaining a fusiform and bipolar morphology typical of undifferentiated cells. Only the cells situated at deeper sites in the preplate, like the amygdaloid areas, had a more complex morphology (Fig. 3K). In the dorsoventral axis, migration was unrestricted and the majority of the cells did not cross the PSB, with only a few cells in more caudal positions reaching the lateral pallium (LP) (Fig. 3J). On the other hand, when the implant was situated into the VP, the GFP cells migrated to the outermost layer of the LP, expressing Reln, but they failed to invade the preplate of the dorsal pallium (Fig. 3L-N).

**Maintenance of the Phenotype of Implanted Cells**

Although the site of the CCH implant affects the final position of the GFP *+* cells, we analyzed the effect of this procedure on cell phenotype. Accordingly, we studied the co-localization of GFP with different markers following CH-LGE transplantation, including TuJ1, CR, Reln, Tbr1, and CB, and we compared these data with those obtained from CH-CH transplants. For the statistical analysis, we only considered migratory cells moving away from the implant, although we also found immunoreactivity inside the implant.

**Hem to LGE Implants**

The majority of migratory cells expressed typical C-R markers—Reln: 50 ± 7% (Fig. 3L); Tbr1: 75.5 ± 15% (Fig. 3G); and CR: 60.5 ± 25% (Fig. 3H). Indeed, TuJ1, a specific marker for postmitotic neurons, was expressed by most implanted cells (97.5%), although we did not find any GFP *+* cells expressing CB (Fig. 3K see the bar chart in Fig. 3L and the more detailed information in Supplementary Table 2).

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**Table 1**

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Note: A P value <0.05 is considered significant. ND, not detected. Genes with a 5-fold or greater superior change are shown in bold. Genes that have passed this criterion at a P value ≤ 0.01 are highlighted.

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Figure 3. Implant of CCH tissue from a GFP *+* mouse embryo (E11) into the LGE (G) of a wt mouse at the same developmental stage, which was then cultured for 24 h in toto. The images in this plate are from more rostral (E) toward more caudal (K) levels along the anteroposterior brain axis. (A, B): lateral view of the telencephalon with the GFP *+* implant. (C, D): ventrolateral view showing the anterior and posterior parts, taken by migratory cells generated in the implant throughout the ventral telencephalon. The boxes in (A) and (C) mark the exact site where the implant was deposited. Cells generated in the CCH implant migrate using new but established routes, in this case, through the subpallial structure into which they were integrated (f, arrow). These cells migrate radially to reach the marginal zone, where they change direction to move forward and backward subpially, some of them separating from the superficial layer to colonize other more caudal structures such as the olfactory tubercle (OT; G, H), the entorhinal cortex (EC; J), or the amygdaloid area (AA; K). These cells do not colonize the PC (G, H). At the rostral level (anterior telencephalic pole), the cells are more differentiated (F, asterisk) than those found at the caudal level, where they had a fusiform morphology and fewer branches (K). The implanted cells express the typical markers of C-R cells, including Reln (E), Tbr1 (G), and CR (F), and they were negative for CB (A). (L-M): implant of CCH tissue from a GFP *+* mouse embryo (E11) into the VP. The cells fail to reach the preplate of dorsal pallium and migrate to the most outer layer of LP. The strong expression of the C-R markers is represented in a bar chart (O). Scale bars: (A-D, I): 200 μm; (E, G, K): 50 μm; (F): 25 μm; (L, J, L-M): 100 μm.
Hem to Hem Implants

A similar expression of markers was observed to that seen when implants were introduced into the LGE—Reln: 63.7 ± 8% (Fig. 4A–F); TuJ1: 92 ± 10.4% (Fig. 4G–L); Tbr1: 64%; and CR: 46 ± 15% (Fig. 4M–O), and again, there was no immunoreactivity for CB (Fig. 4P). Since there was no difference in the expression of the different markers between the CH-CH or CH-LGE implants (Supplementary Table 2), we concluded that the phenotype of the implanted cells did not change in response to signals coming from the new location.
The mutation of the *Pax6* gene causes severe alterations in genetic patterning from very early stages of embryonic development that directly affect the migratory environment through which medial ganglionic eminence interneurons move toward the cerebral cortex (Gopal and Golden 2008). The same guidance system is employed by C-R cells (López-Bendito et al. 2008) and our data showed that a permissive or restricted environment seems to be sufficient to control the migratory behavior of the CH-derived C-R cells. Thus, we wondered how the migratory pathways from the RCH, MCH, and CCH would be established when the environment around these migrating cells is severely altered, as occurs in *Pax6* mutants. Hence, we characterized the migratory routes and the behavior of C-R cells when transplanted into the CH of Sey mutant embryos (*Pax6*−/−) that were in toto cultured for 12–36 h.

In Sey embryos, the C-R cells generated in the CH began to migrate on E11, reaching a peak of migration at E12. At the outset, the pathways followed by CH cells toward the lateral cortex appeared unclear, and they did not adopt their typical oblique orientation in migrating toward the rostrolateral territories. Furthermore, some labeled cells migrated caudally with regards the injection site (Fig. 5A–B), a phenomenon never seen in wt embryos. At E12, the migratory cells generated in the CH moved over long distances by tangential migration, showing a fan-like dispersion and occupying a large extent of

**Figure 4.** Immunohistochemical analysis of the cells generated by the implants. The bar chart (P) shows the percentage of GFP+ cells that express Reelin (Reln; B, C, E, F), β-tubulin-III (TuJ1; H, I, K, L), and CR (N, O). Arrowheads in (D–F) and (M–O) indicate individual cells co-expressing 2 different markers. Scale bars—(A–C, J–L): 50 μm; (D–F): 10 μm; (G–I, M–O): 20 μm.
Figure 5. Dual injection of fluorescent tracers (CFDA/SNARF) into the RCH and MCH of Sey mutant mice at E11 (A–C, G–J) and E12 (D–F, K–N). The dark yellow area defined in (C) and (F) represents the Wnt3a expression that labels the entire extension of the CH. In these mutant mice, the cells generated in the RCH and MCH migrate along strictly lateral routes rather than along their normal migratory pathways from the caudal to rostral areas. Furthermore, cells from different adjacent domains may intermingle during the first 24 h in culture (A–C). In (A and B), rostral is to the left and dorsal is toward the top. However, 1 day later (at E12), massive migration occurs from the CH, firstly in a very limited manner (D) and some hours later, cells spread throughout the rostral and caudal areas in a fan-like fashion (E, F). (D) and (E) is a view from the top. The asterisks in (G, H, K, and M) mark the injection site with the selected tracers in coronal sections: SNARF, red and CFDA, green. Labeled cells generated in the most caudal domain of the CH invade the adjacent anterior domain and migrate through the preplate, extending beyond the PSB. This is more evident at E12 (I, M, N; open arrowheads). In the mutants, migrating cells that do not lie in a subpial position are commonly observed, and they appear as debris from the preplate (J, arrowhead) or even in the deepest neuroepithelial strata (K, L; solid arrowheads). Scale bars—(A): 250 μm; (B, G–J, M, N): 100 μm; (D, E): 500 μm; (K, L): 50 μm.
the pallial tissue, both rostrally and caudally (Fig. 5D-F and Supplementary Fig. 1B). This migratory behavior was also observed in cells originating in the RCH and CCH.

A study of coronal sections taken from labeled brains of Sey mutant mice showed that C-R cells move tangentially through the preplate, although these cells transgressed the PSB and reached deep ventral areas (Fig. 5). This phenomenon was most evident at E12 (Fig. 5N). Along the entire migratory route, it was possible to observe a mixture of migratory cells coming from regions adjacent to the CH (Fig. 5M). Finally, in sections close to the injection site, some labeled cells abandoned their subpial position and occupied different strata in the neuroepithelium, some of which reached the ventricular area. The orientation of these cells was variable, and they could direct their leading processes tangentially, shortening them in the ventricular direction or toward the preplate (Fig. 5J-L, arrowheads).

Given the differences found in the migration of C-R cells in the telencephalon of wt and Sey mutant mice, we compared the expression of several genes involved in migration and cell adhesion in the mutant dorsal pallium with that in the same structure of wt animals by RT-PCR microarrays. In this way, we hoped to identify candidate genes that might be implicated in the aberrant migration of the C-R cell population. This analysis demonstrated differences in the expression of 29 genes (Table 2), although only 14 (represented in bold) were considered “of interest” owing to a change in expression that was greater than or equal to 5. The most interesting candidate genes were Ccl5, Cxcl12, Ilt8rb (Cxcr2), Ngrf, and Nrp2 since their change in expression was statistically significant (P ≤ 0.001, highlighted in Table 2). Surprisingly, the expression of most of these genes augmented in the cortex of the Pax6 mutant, whereas only Nrp2 expression diminished.

Discussion
We show here that C-R cells from the CH migrate along stereotypic routes defined by the permissiveness of the environmental signals to which they are exposed. These cells have an extraordinary capacity to become incorporated into different regions of the developing telencephalon, changing their normal migratory routes when they are implanted ectopically, while maintaining their phenotype and certain migratory features. Furthermore, we describe for the first time, the alterations experienced by the cells that originate in the CH of Sey mutant (Pax6<sup>−/−</sup>) mice where the environment through which C-R cells move is modified. These studies are strongly validated by the use of whole embryo culture, which preserves the 3D structure of the brain, as well as the integrity of molecular signals from different environments. In this way, cell behavior is not artificially conditioned as occurs in experiments with slices or in explants cultures.

Environmental Versus Cell Autonomous Influences on C-R Cell Migration
To define how cells spread along the entire cortical preplate, we injected 2 different tracers into adjacent domains of the CH. These double-labeling experiments showed that the C-R cells arising from the RCH, MCH, and CCH migrate simultaneously along parallel routes from the caudomedial toward the rostro-lateral cortex. However, certain cross talk exists between adjacent routes since some migrating cells may invade the adjacent rostral route; although migration caudal to the injection site was never found. At present, it is unclear whether certain molecular cues instruct the CH cells to migrate toward specific locations. The interplay between signals from the extracellular environment and intracellular regulatory cues appears to affect the behavior of cortical progenitors (Edlund and Jessell 1999). Hence, we evaluated the influence of the intrinsic versus extrinsic stimuli on the pathways followed by cells born at different levels within the CH and explored whether these migratory routes could be predicted by the site of cell origin. The fact that each cell population follows specific migratory pathways could indicate different conditioning in their origin. Thus, we wondered whether a different background along the rostrocaudal axis of the CH could be responsible for the migratory particularities of C-R cells. We addressed this question by performing CH implants into whole embryos. The implantation of labeled tissue into whole embryos shows that neuronal precursors generated in parts of the CCH implanted into a more rostral position can respond to local signals and mimic the migratory behavior of their new neighboring cells. Certain differences between cell populations generated in diverse CH domains must be recognized here because all of them respond to the specific environmental cues that change their migratory behavior. These differences are reflected by the variation between the RCH and CCH domains in the expression of some genes in the microarrays (Ccr1, Egfr3, etc.). In addition, we also found that the expression of Wnt3a gene, which delineates the full extent of the CH, is disrupted in Lhx5-mutant mice, where the expression of Wnt3a in the CCH disappears. This effect is concurrent with

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<th>Gene symbol</th>
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A P value <0.05 is considered significant. Genes with a 5-fold or greater superior change are shown in bold. Genes that have passed this criterion at a P value ≤0.01 are highlighted. Tel Sey, Telencephalon Sey; Tel wt, Telencephalon wild type.
a dramatic decrease of Reln$^+$ cells in the caudal third of the cortical mantle (Miquelajüregui A, Varela-Echavarria A, Ceci M.L, García-Moreno F, Ricaño I, Hoang K, Chowdhury T.G, Portera-Cailliau C, Tamariz E, De Carlos JA, Westphal H, Zhao Y, unpublished data). All these data might suggest the existence of some molecular heterogeneity between C-R cell populations generated in different CH domains, which could imply a role in cortical regionalization.

The same behavior was seen when pieces of CH tissue were implanted into subpallial structures (LGE). Indeed, most cells moved along a subpial route close to the meninges as they do in their natural environment. The fact that these cells retain many of their typical properties, even in ectopic areas, correlates with previous reports showing that central nervous system (CNS) progenitor cells grown in vitro can retain the memory of extrinsic signals that promote region-specific phenotypic properties through one or more cell division after the removal of such signals (Eagleson et al. 1997; Lillien 1998). Moreover, the fact that their markers are not only expressed in migratory cells but also in the cells that remain trapped in the implant shows that migration is not necessary for the expression of these characteristic proteins. Together, these and previous findings show that the fate of certain progenitor cells may be restricted by their exit from mitosis (Alvarado-Mallart et al. 1990; Martínez et al. 1991; Cohen-Tannoudji et al. 1994; Edlund and Jessell 1999; Valcanis and Tan 2003).

**Effect of the Pax6 Mutation on C-R Cell Migration**

*Pax6* is a transcription factor expressed in the pallium along a high rostrotemporal to low caudomedial gradient. Its expression is highly regulated during the early development of the nervous system, and it is opposite to the migration of CH-derived C-R cells (Stoykova et al. 1997; Osumi 2001). This factor has been correlated with numerous fundamental processes like proliferation, migration, cell adhesive properties, neuronal differentiation, lamination and regionalization of the cortex, and patterning events (Caric et al. 1997; Bishop et al. 2000, 2002; Estivill-Torrus et al. 2002; Muzio et al. 2002; Simpson and Price 2002; Tyas et al. 2003; Gopal and Golden 2008). In addition, the absence of *Pax6* causes an increase in the number of Reln$^+/\$ CR$^+$ cells in the marginal zone (MZ), which could produce defects including altered migration (Matsuo et al. 1993; Stoykova et al. 2003). Since our results pointed to environmental cues as playing a fundamental role in controlling the dispersion of C-R cells, we analyzed the migratory behavior of CH-derived C-R cells in these mutants. Our data revealed that in Sey mice, the onset of migration starts on E11 and it extends to E12/E12.5, close to when the preplate splits. On the other hand, more cells that arise from the CH of mutant embryos seem to migrate by unclear and expanded routes than in wild-type embryos (Supplementary Fig. 1B). These data correlate with a recent report showing that the disruption of Pax6 at the onset of neurogenesis in an *Emx1-Cre* line resulted in premature cell cycle exit of early progenitors leading to the expansion of specific neuronal MZ lineages (Tuo et al. 2009). The influence of *Pax6* on the cell cycle and differentiation was previously seen in *Pax6$^{-/-}$/Pax6$^{-/-}$ (Quinn et al. 2007) and Sey mice (Estivill-Torrus et al. 2002). By contrast, overexpression of Pax6 in early neurogenesis promotes apoptosis of radial glia progenitors (Berger et al. 2007).

In addition, these cells erroneously cross the PSB reaching the subpallial region, like olfactory neurons from the dorsal pallium of these mutants (Nomura et al. 2006). Furthermore, a large number of cells lose their subpial position and appear at different levels of the neuroepithelial thickness, also reaching the ventricular zone, demonstrating that the absence of *Pax6* has an effect on C-R cell migration.

In order to define the guidance cues potentially altered in *Pax6*-mutant mice, which might be responsible for the aberrant C-R cell migration, we analyzed the expression of different molecules using RT-PCR microarrays, and we found some interesting candidates, most belonging to the chemokine superfamily.

**Role of Chemokines in C-R Cell Migration**

In the CNS, specific chemokine receptors have been detected on microglia, astrocytes, oligodendrocytes, neurons, and in the brain microvasculature (Cardona and Ran光滑 ff 2007; Ran光滑 ff et al. 2007; Cardona et al. 2008). Indeed, there is evidence that chemokine signaling is involved in precursor cell proliferation and migration (Krathwohl and Kaiser 2004; Ni et al. 2004; Tran et al. 2005). In *Pax6* mice, where C-R cell migration is severely altered, we found that the expression of several chemokines and chemokine receptors is upregulated, including that of *Ccl5, Cxcl12, and Il18rb*, also known as *Ccxr2*. The Ccl5 receptor has been related to the migration of primary dorsal root ganglia neurons (Bolin et al. 2001), while Ccxr2 controls the migration of oligodendrocyte precursors (Tsai et al. 2002). However, the chemokine best recognized to participate in nervous tissue development is Cxcl12. In particular, C-R cells express Ccxr4, the receptor for the chemokine stromal cell-derived factor (SDF)-1, also known as Cxcl12 (Bagri et al. 2002; Lu et al. 2002; Tissir et al. 2004; Yamazaki et al. 2004). It is thought that the leptomeninges control the recruitment and dispersion of C-R cells in vitro, via the chemotaxtractant SDF-1 (Cxcl12) and its receptor Ccxr4 (Borrell and Marín 2006). Curiously, the same guidance system is employed by cortical γ-aminobutyric acid-ergic neurons (López-Bendito et al. 2008). However, the effect of Cxcl12 on C-R cells is less clear since in *Sdf1$^{-/-}$ or Ccxr4$^{-/-}$ mice show severe disruption of interneuron placement and proliferation, while the submeningeal position of C-R cells remains unaffected (Stumm et al. 2003; Paredes et al. 2006). Chemokines are not only chemokattactants but they are also mitogenic (Krathwohl and Kaiser 2004; Borrell and Marín 2006; Tiveron and Cremer 2008). Thus, the larger number of migrating cells derived from the CH in *Pax6* mutants could in part be explained by the overexpression of Cxcl12 and Ccl5. However, our concept of Cxcl12 activity must be revised following the identification of Ccxr7 (also known as CmKor1), a second receptor for this chemokine (Balabanian et al. 2005; Burns et al. 2006; Thelen M and Thelen S 2008; Raz and Mahabaleshwar 2009). The orderly migration and direction followed by CH-derived C-R cells cannot be explained by the expression of Ccxr4 alone, particularly since Cxcl12 is not polarized along this pathway. Ccxr7 is thought to act as an atypical receptor, binding and metabolizing Cxcl12 without evoking prototypical cell responses like chemotaxis but rather generating a local gradient responses like chemotaxis but rather generating a local gradient.
1997; Hansell et al. 2006; Cardona et al. 2008), and significantly, it is increased in Pax6-deficient pallium where C-R cell dispersion is altered.

In summary, our study highlights the importance of environmental signals on the migration of CH-derived cells as opposed to the prior conditioning at their site of origin, as well as the importance of niche signals on the phenotype of these cells. In addition, by analyzing the environmental factors that are altered in Pax6-deficient mice and their repercussion on C-R cell migration, we propose new candidate genes that might be implicated in C-R cell migration. It will now be of interest to confirm the role of these molecules in direct C-R cell migration.

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

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