Embryonic cortical neural stem cells migrate ventrally and persist as postnatal striatal stem cells

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Introduction

A subpopulation of cortical neurons (γ-aminobutyric acid transmitting [GABAergic] interneurons) originates from an extracortical forebrain region during development, i.e. the ganglionic eminences that constitute the presumptive basal ganglia. Tracing experiments using thymidine and lipophilic dyes (De Carlos et al., 1996; Anderson et al., 1997a,b), as well as fate-mapping experiments in vitro (Anderson et al., 2001; Hitoshi et al., 2002) and in vivo (Wichterle et al., 2001), have provided direct evidence for the migration of these interneurons from the ventral developing striatum into the major dorsal region of the forebrain—the cortex. We provide evidence for another migratory stream traveling in the opposite direction between the developing cortex and striatum; i.e., the migration of Emx1-lineage embryonic cortical stem cells ventrally into the striatal germinal zone (GZ).

This study was prompted by the finding that neural stem cells have been isolated from the embryonic cerebral cortical GZ (Davis and Temple, 1994), but do not appear to persist in the adult cortex (Kornack and Rakic, 2001; Seaberg et al., 2005). In contrast, stem cells from the striatal GZ persist into adulthood and senescence (Tropepe et al., 1997) in the adult remnant of the striatal GZ, i.e., the subependyma that surrounds the forebrain lateral ventricles. Given that stem cells are defined by their long-term self-renewal capacity (Seaberg and van der Kooy, 2003), we sought to determine the fate of embryonic cortical stem cells by taking advantage of region-specific molecular marker expression in transgenic mice that express Cre recombinase under the control of the Emx1 gene (Fig. 1 A). Emx1 is a homeobox gene that is expressed in a dorsal-restricted pattern in the developing forebrain (Simeone et al., 1992) and persists in many adult cortical cells (Chan et al., 2001). Emx1IREScre mice have been shown to express Cre recombinase in a spatial and temporal pattern that recapitulates known endogenous Emx1 expression (Gorski et al., 2002). Emx1IREScre mice were mated to the Cre-dependent reporter strain LacZ/EGFP (Z/EG), which expresses EGFP upon Cre-mediated excision (Novak et al., 2000). In this way, we were able to indelibly mark and follow the fate of all cortical cells and their progeny that express Emx1 at any point during development.

Results

The early embryonic striatal GZ does not contain Emx1-lineage cells

At embryonic day (E) 15.5, the pattern of GFP expression in coronal forebrain sections from Emx1IREScre,Z/EG double-transgenic embryos recapitulated the pattern previously described for Emx1 mRNA (Simeone et al., 1992) and protein (Mallamaci et al., 1998); i.e., that GFP+ cells were confined to the developing cortex.
No GFP$^+$ cells were detected ventrally in the striatal GZ at this stage (Fig. 2, A–D), or in the “border” zone of the striatal GZ, which is defined by expression of Dbx1 and SFRP2 (Stenman et al., 2003a,b). A small number of GFP$^+$ cells were present in the postmitotic region of the striatum at E15.5, as has been reported previously (Gorski et al., 2002), but these cells were clearly absent from the striatal GZ at this stage (Fig. 2, C and D). RT-PCR analysis of E15.5 striatal GZ revealed that Emx1 is not expressed in this tissue at this age. To further confirm that the GFP$^+$ striatal GZ did not contain any GFP$^+$ neural stem cells, single cells from this region were cultured, and the resultant clonal neural sphere colonies were examined for GFP expression. The E15.5 striatal GZ yielded an average of 80.9 ± 2.7 colonies per 10,000 cells, and all were GFP$^-$. Cortical cell cultures from E15.5 Emx1$^{IRESCre;Z/EG}$ double-transgenic forebrains yielded an average of 34.1 ± 2.8 colonies per 10,000 cells, all of which were GFP$^+$ (Fig. 2 E). Each of these separate clonal cortical and striatal neural stem cell colonies were multipotent in that they generated neurons, astrocytes, and oligodendrocytes and were able to generate secondary colonies as shown previously (Davis and Temple, 1994; Tropepe et al., 1999).

**Emx1-lineage cortical neural stem cells migrate into the perinatal striatum**

Surprisingly, examination of coronal sections from postnatal day (PND) 1 Emx1$^{IRESCre;Z/EG}$ mice revealed that some GFP$^+$ cells were now scattered along the striatal and septal aspects of the lateral ventricle (Fig. 3 D). This is the first time that Emx1-lineage cells have been reported in the striatal GZ. GFP$^+$ cells were particularly numerous in the dorsolateral portion of the striatal GZ, which is an area well populated with colony-forming neural stem cells (Fig. 3 D; Morshead et al., 1994; Tropepe et al., 1997), and were also scattered less densely throughout the ventral extent of the striatal periventricular tissue. Note that in addition to the cortex, cortically derived corpus callosum and anterior commissure fibers, as well as internal capsule axonal fibers that traverse the striatum, were also GFP$^+$ (Fig. 3 D), as expected. As reported previously (Gorski et al., 2002), GFP$^+$ cells were also present in the rostral migratory stream and the olfactory bulbs.

To determine whether GFP$^+$ or GFP$^-$ cells in the striatal GZ were neural stem cells, single cells from dorsal and ventral regions of the striatal GZ were assayed for clonal colony formation. For all experiments at PND1 and adult, “dorsal” refers to the most dorsal 1/3 of the striatal GZ or subependyma for early postnatal and adult dissections, respectively. Similarly, “ventral” dissections included the most ventral 1/3. These regions are demarcated by red boxes. Note that an adult section is shown. Ctx, cortex; CC, corpus callosum; LV, lateral ventricle; Str, striatum; Sep, septum.
the PND1 striatal GZ (Fig. 5 D). Moreover, the finding that there were no GFP+ sphere colonies isolated from the cortical GZ suggests that the migration only occurs in one direction; striatal neural stem cells do not migrate dorsally into the cortex.

To further investigate whether Emx1-lineage cortical neural stem cells were indeed migrating ventrally into the striatal GZ, we performed an experiment to better characterize the time course over which these cells migrated. Thin (15-μm) cryosections of forebrains from E16.5, 17.5, and 18.5 mice were analyzed, and they demonstrated a progressive pattern of stem cell migration over this time period, with the majority of migration being completed between E16.5 and 18.5 (Fig. 3, A–C). In an effort to quantify the number of migratory cells, the number of GFP+ cells present in the striatal GZ at PND1 was determined using the optical dissector method. At the most dorsal level of the striatal GZ, Emx1-lineage GFP+ cells represent up to 19% of the total number of cells present. This proportion decreases in a dorsal-to-ventral manner along the striatal GZ, such that at the most ventral extent of the GZ only 1% of cells are Emx1-lineage GFP+ cells (Fig. 3 I).

To rule out the possibility that indigenous striatal GZ cells were up-regulating Emx1 over this time course, and to show more definitely that Emx1-lineage cortical stem cells were migrating, we used a technique that was previously effectively used to definitively demonstrate the ganglionic eminence origins of cortical interneurons (Anderson et al., 1997a,b). Live coronal slices were obtained from E15.5 Emx1IRES;Z/EG embryos, and one hemisphere of each slice was surgically transected along the presumptive cortical/striatal GZ boundary (Fig. 4 A). The other hemisphere was left intact throughout the period of in vitro culture. We predicted that if Emx1-lineage GFP+ cortical neural stem cells were indeed migrating ventrally into the striatal GZ, we should be able to isolate GFP+ stem cell colonies from the striatal GZ of intact cultured hemispheres, but not from the striatal GZ of hemispheres that had been transected at the beginning of the culture period. Indeed, GFP+ neurosphere colonies were isolated from 91% (n = 11) of intact hemispheres. In contrast, no GFP+ colonies were isolated from surgically transected hemispheres (n = 12). This difference cannot be attributed to cellular damage at the cortical/striatal boundary, by itself, because there was no difference in the number of GFP+ colonies generated from intact (35.9 ± 2.5) versus transected (32.2 ± 2.7) slices (P > 0.05), although the overall number of colonies obtained from these cultured slices was decreased compared with the number obtained from fresh slices, as might be expected (Fig. 4 B). Together, these data provide strong evidence that the GFP+ cortex is the source of migratory GFP+ neural stem cells in the perinatal striatal GZ.

To investigate the possibility that in cultured slices a critical signal from the adjacent cortex was responsible for up-regulating Emx1 in indigenous striatal GZ cells, a DiI labeling experiment was performed. Single DiI crystals were placed on the E15.5 cortical GZ in a position just dorsal to the dorsolateral aspect of the lateral ventricle, but carefully avoiding the striatal GZ (Fig. 4 C). After culture for 3 d (i.e., E15.5–18.5), analysis of these slices clearly revealed the migration of Emx1-lineage GFP+/DiI+ double-labeled cells from the cortical GZ into the striatal GZ (Fig. 4, D–G). Double-labeled cells demonstrated a typical bipolar morphology characteristic of migrating cells (Fig. 4, E–G). Importantly, near the migrating cells, other GFP+ cells were detected that did not display bipolar morphology and were not labeled with DiI (Fig. 4, E–G). This clearly demonstrates that DiI was not simply diffusing throughout the slice, but instead was specifically labeling cells migrating ventrally from the cortex.

To obtain direct evidence of cell migration, time-lapse video recordings of E16.5 coronal forebrain slices were performed. Analysis of the 4-d videos (E16.5–20.5) clearly showed the
dorsal-to-ventral migration of GFP\(^+\) cells originating from the immediately cortex-adjacent dorsal striatal GZ (Fig. 4 H).

The GFP\(^+\) cells migrated dorsally at an average speed of 9.8 ± 2.1 μm/h (n = 6), whereas adjacent GFP\(^-\) cells moved at an average speed of 1.5 ± 1.8 μm/h (n = 7).

To demonstrate that neural stem cell migration occurs in vivo and is not merely an artifact of slice culture, GFP\(^+\) cells from the cortical GZ of PND1 Emx1\(^{IRES-Cre}\) mice were homotopically transplanted into the cortical GZ of wild-type GFP\(^-\) PND1 mice. Mice in which cortical GZ cells were injected into the lateral ventricle were excluded from further analysis (n = 10). Of the remaining mice (n = 4), there were two independent cases in which the transplanted GFP\(^+\) cells integrated into the host cortical GZ and, after 5 d, were found within the striatal GZ (Fig. 4 I). In contrast, there were no examples of migration from control experiments in which GFP\(^+\) cells from the postmitotic cortical plate of PND1 Emx1\(^{IRES-Cre}\) mice were transplanted into host cortical GZ (n = 4). In summary, these data obtained from multiple experimental techniques demonstrate a novel dorsal-to-ventral migration pattern of neural stem cells in the perinatal forebrain.
Emx1-lineage migratory stem cells from the cortex acquire a striatal phenotype

Dlx2 is a homeobox gene that is characteristic of the developing striatum (Porteus et al., 1991), adult subependyma (Porteus et al., 1994), and striatal neural stem cells (Hitoshi et al., 2002). To determine whether migrating GFP+ Emx1-lineage cortical neural stem cells maintained Emx1 gene expression characteristic of their host tissue of origin or whether they acquired Dlx2 expression characteristic of their striatal neighbors, nested RT-PCR was performed on single GFP+ and GFP+ clonal colonies generated from dorsal and ventral aspects of the striatal GZ. GFP+ clonal neural stem cell colonies of both dorsal and ventral striatal origin expressed Dlx2, but not Emx1, as is expected of cells indigenous to the striatum. Interestingly, GFP+ Emx1-lineage...
clonal colonies from the ventral striatal GZ similarly did not express Emx1, but did express Dlx2. This result was confirmed by immunocytochemical analysis of forebrain slices; 70 ± 2% of GFP+ cells in the striatal GZ also express Dlx2. Most importantly, GFP+ clonal colonies of dorsal striatal GZ origin expressed both Emx1 and Dlx2 (Fig. 5, A and C), which is suggestive of a transitional gene-expression state. That these transitioning, migrating stem cells continue to express Emx1 was confirmed by the immunocytochemical detection of Emx1 in GFP+ cells in the PND1 striatal GZ (Fig. 5 D). The only other clonal colonies to express Emx1 were cortically derived GFP+ neural stem cell colonies. Real-time RT-PCR indicated that resident cortical neural stem cell colonies expressed a higher level of Emx1 than those that had migrated into the dorsal striatal GZ (Fig. 5 B). Although not detected by the nested RT-PCR performed on single clonal colonies (Fig. 5 A), minute levels of Emx1 were also found in pooled samples of dorsal striatal GFP− or ventral striatal GFP+ colonies (Fig. 5 B). A subpopulation of cortical neural stem cell colonies (6/20 or 30%) also expressed very low levels (<50% of the level expressed by striatal colonies in pooled samples) of Dlx2, as reported previously (Hack et al., 2004). Together, these data suggest that as Emx1-lineage cortical neural stem cells migrate ventrally they acquire the striatal characteristic of Dlx2 expression and, ultimately, down-regulate Emx1.

One of the criteria that differentiate neural stem cells from more restricted progenitor cells is that they persist throughout life (Tropepe et al., 1997; Seaberg and van der Kooy, 2003). Experiments were performed on adult animals to test if Emx1-lineage cortical neural stem cells do, indeed, persist throughout life in the adult remnant of the striatal GZ, the lateral ventricular subependyma. Examination of coronal sections from adult Emx1<sup>R<sub>ires</sub>,Z/EGR</sup> mice revealed that some GFP+ cells remained scattered along the striatal ventricular subependyma (Fig. 6, A and B). Cells were cultured from the striatal subependyma of adult Emx1<sup>R<sub>ires</sub>,Z/EGR</sup> double-transgenic animals at 6 wk and 8 mo of age. GFP+ Emx1-lineage neural stem cell colonies formed from striatal cultures at both ages (Fig. 6 C), indicating that Emx1-lineage cortical neural stem cells persist in the adult striatal subependyma. The data presented (Fig. 6 C) underestimate the total number of Emx1-lineage cortical neural stem cells surviving into adulthood, as ~33% of the GFP− neurons isolated from the adult striatal subependyma were β-galactosidase<sup>−</sup> by X-gal histochemistry, indicating some in vivo suppression of the GFP transgene over time. RT-PCR analysis demonstrated that Emx1 was no longer expressed by single GFP+ clonal adult colonies from dorsal or ventral striatal GZ (Fig. 6, D and E) and that all colonies expressed the striatal marker Dlx2. Adult cortical cultures did not yield any GFP− or GFP+ sphere colonies (Fig. 6 C). Interestingly, immunolabeling with a GFP antibody and GAD65/67 antibody (which labels GABAergic neurons) revealed the presence of double-labeled cells in the adult striatum, indicating that Emx1-lineage migratory neural stem cells generate GABAergic neurons in this region (Fig. 6 F). Cells that were GFP+/GAD65/67− were further analyzed by confocal microscopy; >90% of these single cells were confirmed to be double-labeled by this method.

**Discussion**

These data demonstrate that ventral migration is the postnatal fate of a subpopulation of cortical neural stem cells. However, it remains possible that another subpopulation of these cells may remain dormant in the adult cortex and be transformed into stemlike cells after long periods of culture (Palmer et al., 1999). Indeed, other subpopulations of adult progenitors may reside in the white matter; progenitors have been isolated directly from
adult human subcortical white matter (Nunes et al., 2003), postnatal mouse optic nerve (Seaberg et al., 2005), and rat postnatal optic nerve after “reprogramming” in vitro (Kondo and Raff, 2000). The dorsal-to-ventral migration of cortical stem cells could be the result of a passive mechanism whereby callosal cortical projection fibers separate the cortical plate from the cortical GZ and push cortical neural stem cells into the striatal GZ. Alternatively, stem cell migration may occur by an active push or pull mechanism involving Slit, Netrin, or Eph/ephrin signaling, as has been shown for migrating neuroblasts (Conover et al., 2000). Importantly, dorsal-to-ventral neural stem cell migration has not been previously described; this study represents not only the first study of in vivo neural stem cell migration but also the first study of a dorsal-to-ventral migration phenomenon of any cell type between the developing cortex and striatum. In addition to the well-established ventral-to-dorsal migration pathway of postmitotic neurons (De Carlos et al., 1996; Anderson et al., 1997a,b, 2001; Wichterle et al., 2001; Hitoshi et al., 2002), the other route described has been a lateral-to-medial route of “inward migration” of early and postmitotic preplate neurons from the piriform cortex to the striatum (Hamasaki et al., 2001). This migration takes place exclusively through the postmitotic mantle tissue, and does not involve the GZ. Interestingly, this mechanism of migration may explain the presence of the small number of previously described postmitotic Emx1

+ neurons identified in the mantle zone (Gorski et al., 2002), but never in the striatal GZ.

We previously demonstrated that neural stem cells from different embryonic brain regions maintain expression of molecular markers characteristic of their region of origin, even after passing in vitro (Hitoshi et al., 2002). Nevertheless, neural stem cells are able to alter their regional gene expression profile in response to their environment (Hitoshi et al., 2002; Gabay et al., 2003). This data demonstrate a dramatic in vivo example of neural stem cells altering their regional identity as they migrate from the cortical GZ to the striatal subependyma in situ. It is possible that this type of plasticity is a property specific to neural stem cells, as it has been demonstrated that migrating...
Dlx2-lineage GABAergic interneurons originating from the developing striatum never up-regulate Emx1, even though they persist in the cerebral cortex (Gorski et al., 2002). Interestingly, a subpopulation of Emx1-lineage cells in the adult striatum has been found to express markers of striatal neurons (Gorski et al., 2002). We demonstrate directly that a subpopulation of GABAergic neurons in the striatum are generated by Emx1-lineage neural stem cells that migrate perinatally from the cortex. Further, adult Emx1-lineage stem cells generate progeny that label with BrdU and migrate to the olfactory bulb; these GFP+/BrdU+ cells represent up to 20% of the BrdU+ population in both the adult subependyma and adult olfactory bulb (unpublished data). It is not known at this time whether Emx1 expression is required for the migration of cortical neural stem cells into the striatal GZ. Mutations of the Emx1 gene have been demonstrated to result in lack of corpus callosum formation (Qiu et al., 1996), although this may depend on the genetic background of the mice (Guo et al., 2000). However, in Emx1 mutants the cortex remains intact, according to histological and molecular analyses (Qiu et al., 1996; Guo et al., 2000), with only few subtle cortical defects reported in adult mice (Yoshida et al., 1997). A more severe cortical phenotype, featuring reduced cortical size, absence of the hippocampus and dentate gyrus, and olfactory bulb defects, is noted in double mutant when both Emx1 and Emx2 gene expression are disrupted (Bishop et al., 2003). Similar defects have been described in mice with mutations of the Gli3 gene, which is essential for Emx1 and Emx2 expression (Theil et al., 1999).

Defects in the striatum or striatal GZ have not been reported in Emx1 mutants. However, because migratory Emx1-lineage neural stem cells constitute a maximum of 19% of the cells at the dorsal limit of the striatal GZ at PND1, an analysis of early postnatal Emx1 mutants specifically investigating potential striatal GZ stem cell defects may be required to determine the possible requirement for Emx1 gene expression in this cortical neural stem cell migration phenomenon. It is of further interest to note that a related transcription factor, Emx2, has been shown to play a role in the symmetric division of both embryonic cortical neural stem cells (Heins et al., 2001) and adult striatal neural stem cells (Galli et al., 2002).

Intriguingly, cortical neurogenesis has been demonstrated after induced cell death in layer 6 pyramidal neurons (Magavi et al., 2000) and, more recently, in layer 5 corticospinal neurons (Chen et al., 2004). These studies suggested that the newly born neurons originated from the adult periventricular striatal subependyma and migrated into cortical regions (Magavi et al., 2000; Chen et al., 2004). In addition to subpopulations of striatal and olfactory bulb cells (Gorski et al., 2002), we hypothesize that the Emx1-lineage cortical neural stem cell pool that takes up residence in the adult periventricular striatal subependyma may be responsible for the generation of new adult cortical neurons. Although the role of Emx1-lineage migratory neural stem cells is not well understood at this point, what emerges from our studies is the identification of a novel dorsal-to-ventral perinatal neural stem cell migration phenomenon that differs in many ways from the well-established ventral-to-dorsal migration pathway of postmitotic neurons (De Carlos et al., 1996; Anderson et al., 1997a,b, 2001; Wichterle et al., 2001; Hitoshi et al., 2002). These differences include the time period of migration (postmitotic neurons are migratory by E12.5; stem cells not until E16.5), the direction of migration, and the identity of the migratory cells.

Materials and methods

Mice and tissue preparation

Emx1+/- mice were generated and genotyped as previously described (Gorski et al., 2002). Z/EG mice, which express lacZ before Cre excision and GFP after Cre excision, were a gift from C. Lobe (Sunnybrook and Women’s College Health Science Centre, Toronto, Ontario, Canada; Novak et al., 2000). Matings were timed such that noon of the day vaginal plugs appeared was considered E0.5. The day of birth was counted as PND0. Pups were killed by decapitation, and adult mice were killed by cervical dislocation. Brains were removed under a dissecting microscope (Carl Zeiss Microlmaging, Inc.) and sectioned using a Vibratome, as previously described (Seaberg and van der Kooy, 2002).

Cell culture and analysis

Live Vibratome sections were dissected, and the tissue fragments were mechanically dissociated into single cells and plated in defined serum-free media containing EGF and FGF2, as previously described (Tropepe et al., 1999; Seaberg and van der Kooy, 2002). Clonal stem cell neurosphere colonies have previously been demonstrated to arise from single cells, and not by cellular aggregation, under these conditions (Tropepe et al., 1999; Morshead et al., 2003). The number of GFP+ and GFP− sphere colonies was determined by visualizing fluorescence with an inverted microscope (Diaphot; Nikon) after 7 d in vitro. That Cre-mediated excision had not occurred in PND1 GFP− colonies was confirmed by the presence of β-galactosidase activity using X-gal histochemistry, as previously described (Tropepe et al., 2001). Individual clonal sphere colonies were picked for passaging or differentiation, as previously described (Seaberg and van der Kooy, 2002), or for single-colony RTPCR analysis (see RTPCR analysis). Colonies that were differentiated were immunostained after 5 d in vitro for the presence of neurons, astrocytes, and oligodendrocytes using antibodies against β3-tubulin, glial fibrillary acidic protein, and O4, respectively, as previously described (Seaberg and van der Kooy, 2002), as well as rabbit polyclonal anti-GABA (Sigma-Aldrich) used at 1:500.

For some experiments, primary PND1 striatal cells were isolated as described in the previous paragraph, and cells were sorted with an EPICS Elite Cell Sorter (Beckman-Coulter) based on GFP fluorescence. GFP+ and GFP− populations after FACs were determined to be 99.5% pure.

Slices culture and immunohistochemistry

Live coronal Vibratome sections (~200-μm thickness) of E15.5 forebrains were obtained and cultured essentially as previously described (Anderson et al., 1997a,b). One hemisphere of each forebrain slice was transected at the presumptive cortical/striatal border, and the other hemisphere was left intact. Slices were cultured for 5–10 d with media supplemented every 2 d.

For the direct labeling experiments, single Dil crystals (Invitrogen) were carefully placed on the cortical GZ of intact E15.5 Vibratome forebrain slices, just dorsal to the dorsolateral corner of the lateral ventricle. These slices were cultured for 3 d and analyzed for the presence of double-labeled Dil+/GFP+ migrating cells.

Time-lapse video microscopy was performed on live coronal Vibratome sections (~300-μm thickness) of E16.5 forebrains that were obtained and cultured essentially as previously described (Anderson et al., 1997a,b). The slices were maintained on the microscope stage in a humidified chamber at 37°C and 5% CO2. Video recording was initiated on the day of dissection. Slices were imaged at 10× magnification using an inverted microscope (Axiovert 200; Carl Zeiss Microlmaging, Inc.). Brightfield images were acquired at 2-min intervals to track the same fluorescent cell at each time point to correspond with the fluorescent images taken at 1-h intervals. Images were captured with a digital camera (KCD-SX900; Sony) using ImageJ software (National Institutes of Health).

For immunohistochemistry, brains were fixed, embedded, cryosectioned into 15-μm sections, and immunostained essentially as previously described (Chiaison et al., 1999) using rabbit polyclonal anti-GFP (Abcam) at 1:1,000, mouse monoclonal anti-GFAP (CHEMICON International, Inc.) at 1:300, rabbit polyclonal anti-GAD65/67 (CHEMICON International, Inc.) at 1:200, mouse monoclonal anti-HM3 (CHEMICON International, Inc.)
at 1:100, rabbit polyclonal anti-Dlx2 (a gift from D. Eisenstat, University of Manitoba, Winnipeg, Manitoba, Canada) at 1:100, rabbit polyclonal anti-Emx1 (Santa Cruz Biotechnology, Inc.) at 1:500, and anti-rabbit Alexa Fluor 488, anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 568 (all from Invitrogen) at 1:300 dilution. Cell nuclei were counterstained with DAPI. Fluorescent images were visualized using a motorized inverted research microscope (IX81; Olympus) or a confocal microscope (LSM 410; Carl Zeiss Microimaging, Inc.) and were captured using Microsuite Version 3.2 image analysis software (Olympus/Soft Imaging System Corp.).

Transplant experiments
To ensure correct placement of cells into the host cortical GZ, experiments were performed using Trypan blue as an injection site marker into PND1 CD1 mice (Charles River Laboratories). After 5 d, brains were fixed, embedded, cryosectioned into 15-μm sections, and analyzed for the presence of GFP+ cells, as described in the previous section.

RT-PCR analysis
Total RNA was extracted from individual sphere colonies using an extraction kit (RNeasy; QIAGEN). Samples were treated with DNase to avoid contamination with genomic DNA. Reverse transcription and the first round of PCR were performed using a RT-PCR kit (OneStep; QIAGEN) in a GeneAmp PCR System 9700 (Applied Biosystems) according to the manufacturer’s instructions. Initial PCR reactions were performed in 25 μl for 40 cycles because the relatively small amount of starting material involved in single colony RT-PCR. For Emx1 and Dlx2, 2 μl of a 1:10 dilution of the first product was further amplified with nested primers in 20 μl for 40 cycles. Controls run without RNA did not produce bands. Only single colony RNA isolates that were found to express β-actin were considered for further analysis. Stem cell colonies derived from the E15.5 spinal cord were used as a tissue-negative control.

Forward and reverse primers (5’ and 3’), expected product size, and annealing temperatures for RT-PCR were as follows: β-actin, ATC TGG TTT GAG ACC TTC AA and TCT GCG CAA GTT AGG TTT TGT C (825 bp; 56°C); Emx1, TGA GAA GAA TCA CTA CGT GG and AGG TGA CAT CAA TAT CCT CT (229 bp; 56°C); nested Emx1, AGC CGA GCG GAA GCA GC and TGG CCT GTG TGG CG (181 bp; 56°C); Dlx2, ACA CCG CCG GTG ACA CCT CCT A and CTT GCC GTT TTT CCA CAT CITT C (310 bp; 56°C); nested Dlx2, AGT TCG TCT CCG GTC AAC and CAT CTT CTT GAA CTT GGA TC (257 bp; 56°C).

RNA analysis by real-time RT-PCR
Levels of mRNA were quantified in pooled stem cell colony samples by real-time PCR using the sequence detection system (ABI/Prism 7000; Applied Biosystems). Reverse transcription was performed with random hexamers on DNase I-treated total RNA with Superscript II (Invitrogen). cDNA samples were amplified using specific primers for each target (Stratagene). Primer pairs were used at 100 nM and were validated for amplification efficiency against the HPRT or GAPDH internal control genes by running out a comparative standard curve analysis. Relative quantitation of transcript levels in unknown samples was performed by the [delta][delta]Ct method using HPRT as internal control. Amplification cycles comprised of 15 s at 95°C and 1 min at 59°C. All primers (5’ and 3’) were designed using the Primer Express software (Applied Biosystems) with melting temperatures as follows: Dlx2, CCA GTT CGT CCG TCG TCA AC (59°C) and TCG GAT TTC AGG CTC AAG GT (58°C); Emx1, GAA GAA GAA GGG TTC CCA CTT (59°C); and CCG TGG GCC TGT TTC GT (59°C).

Statistical analysis
All analyses used two-tailed tests. P < 0.05 was considered to be a significant difference between groups. The ‘n’ for each comparison and the statistical test used (either t test or single variable analysis of variance), is noted in the respective figure legend.

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References
Hitoshi, S., V. Tropepe, M. Ekker, and D. van der Kooy. 2002. Neural stem cell lineages are regionally specified, but not committed, within distinct compartment of the developing brain. Development. 129:233–244.


