Telencephalic progenitors maintain anteroposterior identities cell autonomously

Erqian Na*†, Maria McCarthy*†, Christine Neyt*, Eseng Lai‡ and Gord Fishell*

Grafting experiments have demonstrated that determination of anteroposterior (AP) identity is an early step in neural patterning that precedes dorsoventral (DV) specification [1,2]. These studies used pieces of tissue, however, rather than individual cells to address this question. It thus remains unclear whether the maintenance of AP identity is a cell-autonomous property or a result of signaling between cells within the grafted tissue. Previously, we and others [3–5] have used transplants of dissociated brain cells to show that individual telencephalic precursor cells can adopt host-specific DV identities when they integrate within novel regions of the telencephalon. We have now undertaken a set of transplantations during the same mid-neurogenic period used in the previous studies to assess the ability of telencephalic progenitors to integrate and differentiate into more posterior regions of the neuraxis. We observed that telencephalic progenitors were capable of integrating and migrating within different AP levels of the central nervous system (CNS). Despite this, we found that telencephalic progenitors that integrated within the diencephalon and the mesencephalon continued to express a telencephalic marker until adulthood. We speculate that during neurogenesis individual progenitors are determined in terms of their AP but not their DV identity. Hence, AP identity is maintained cell autonomously within individual progenitors.

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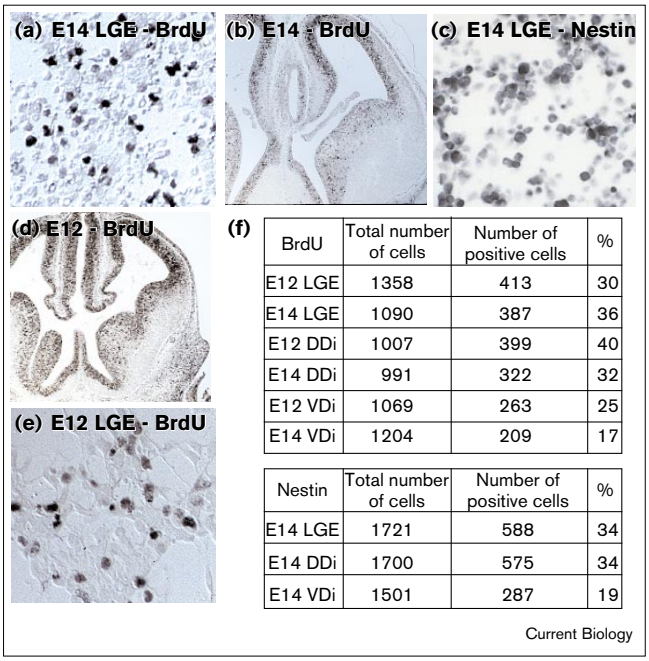
Results and discussion

To address whether the AP character of forebrain progenitors is intrinsically encoded, we grafted progenitors from mice embryos into the lumen of the forebrain ventricles. Our transplantation method is distinct from more conventional methods of grafting [6,7], in that individual progenitors are allowed to integrate of their own accord rather than being placed directly within the host tissue. Hence, progenitors are in essence given a choice of integrating homotopically, heterotopically or both. By evaluating the resulting patterns of integration and differentiation, as well as the expression of region-specific genes, the intrinsic character of different groups of progenitors can be inferred. As a donor population we examined progenitors from the lateral ganglionic eminence (LGE), a telencephalic region that gives rise to the striatum [8]. In

Figure 1



BrdU	Total number of cells	Number of positive cells	%
E12 LGE	1358	413	30
E14 LGE	1090	387	36
E12 DDi	1007	399	40
E14 DDi	991	322	32
E12 VDi	1069	263	25
E14 VDi	1204	209	17

Nestin	Total number of cells	Number of positive cells	%
E14 LGE	1721	588	34
E14 DDi	1700	575	34
E14 VDi	1501	287	19

Current Biology

Progenitor populations used for transplantation in this study were examined for their proliferative potential by assaying their ability to incorporate BrdU and their expression of the progenitor marker nestin. Timed-pregnant CD1 mice carrying E11 or E13 embryos were injected with BrdU (50 mg/kg) three times over a 12 h period. At 2 h after the last injection, the LGEs from E12 or E14 CD1 mouse embryos were dissected and incubated with trypsin–EDTA. Tissue was dissociated to a single cell suspension by trituration with DNase. **(a)** BrdU incorporation in E14 LGE progenitors after labeling *in vivo*. **(b)** BrdU incorporation in an intact E14 brain from embryos of the same litter used for the isolation of the progenitors shown in (a). **(c)** Nestin-positive cells in the E14 progenitors. **(d)** BrdU incorporation in an intact E12 brain from embryos of the same litter used for the isolation of the progenitors shown in (e). **(e)** BrdU incorporation in E12 LGE progenitors after labeling *in vivo*. **(f)** Quantification of the numbers of BrdU-positive and nestin-positive cells observed in the grafted E12 and E14 populations used in this study. DDi indicates dorsal diencephalon; VDi, ventral diencephalon.

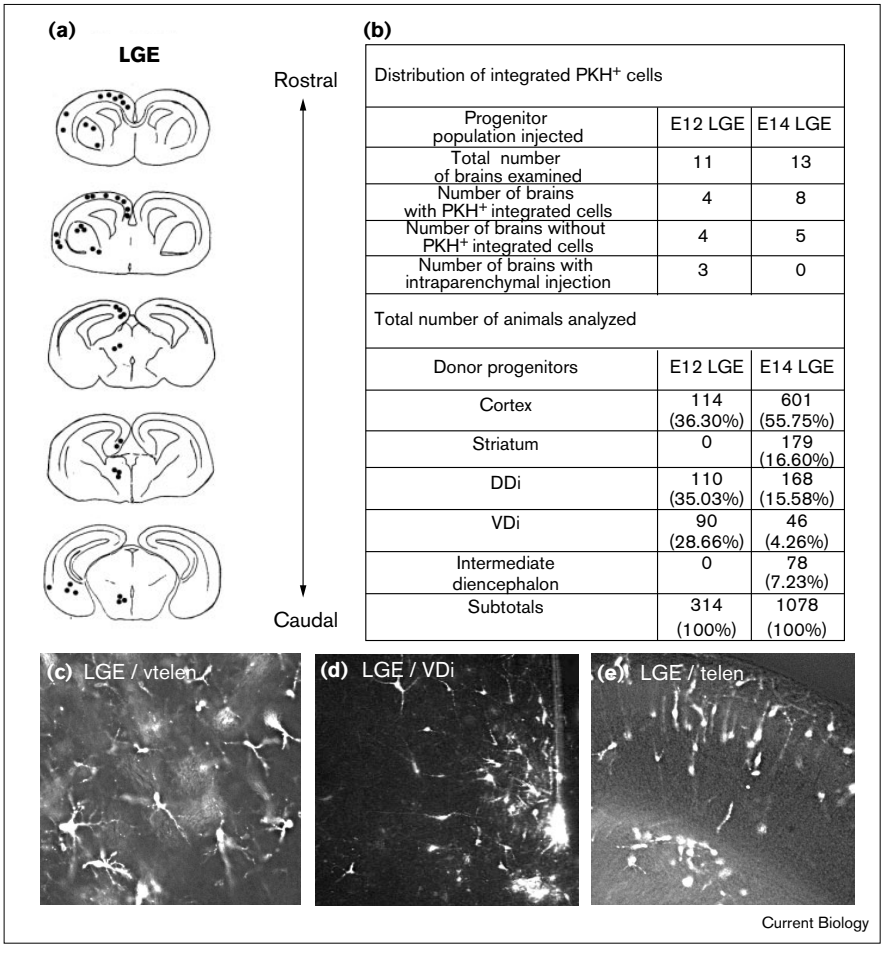
previous studies, we showed that this population of progenitor cells adopts the DV identity of the host region when it integrates elsewhere within the telencephalon [5]. We now ask if these progenitors can also change their AP identity if they integrate into more posterior CNS levels.

To take into account the neurogenic gradient that proceeds from caudal to rostral as forebrain development proceeds [9], tissue was isolated from two different stages of development, embryonic day 12 (E12) and E14. The donor progenitor populations were characterized by examining the proliferative state of the cells isolated for grafting. We found that approximately 30% of donor cells could incorporate bromodeoxyuridine (BrdU) or were positive for the neural progenitor marker nestin [10] (Figure 1). Hence, a substantial number of the donor progenitor cells were actively proliferating when they were isolated. It has been demonstrated that cells undergoing little or no proliferation within host animals are incapable of integrating into host brains after intraventricular grafting [11]. This suggests that it is the actively proliferating population that is able to integrate into the host tissues.

As expected from previous studies [3–5], progenitors from the ventral telencephalon were able to integrate into a wide number of host tissues, including the cortex, the diencephalon and the mesencephalon (Figure 2). Within the telencephalon the morphologies of grafted progenitors appear to be appropriate to the host region in which they reside [5]. Based on shape alone, however, it is difficult to discern whether the cells that integrated into other divisions of the CNS, such as the diencephalon and the mesencephalon have maintained or altered their telencephalic character.

To test whether progenitors maintain their AP identity after integrating in more posterior CNS regions, we examined whether they maintain expression of the telencephalic transcription factor brain factor 1 (BF-1) [12]. BF-1 expression

Figure 2



Distribution of integrated PKH+ cells		
Progenitor population injected	E12 LGE	E14 LGE
Total number of brains examined	11	13
Number of brains with PKH+ integrated cells	4	8
Number of brains without PKH+ integrated cells	4	5
Number of brains with intraparenchymal injection	3	0
Total number of animals analyzed		
Donor progenitors	E12 LGE	E14 LGE
Cortex	114 (36.30%)	601 (55.75%)
Striatum	0	179 (16.60%)
DDi	110 (35.03%)	168 (15.58%)
VDi	90 (28.66%)	46 (4.26%)
Intermediate diencephalon	0	78 (7.23%)
Subtotals	314 (100%)	1078 (100%)

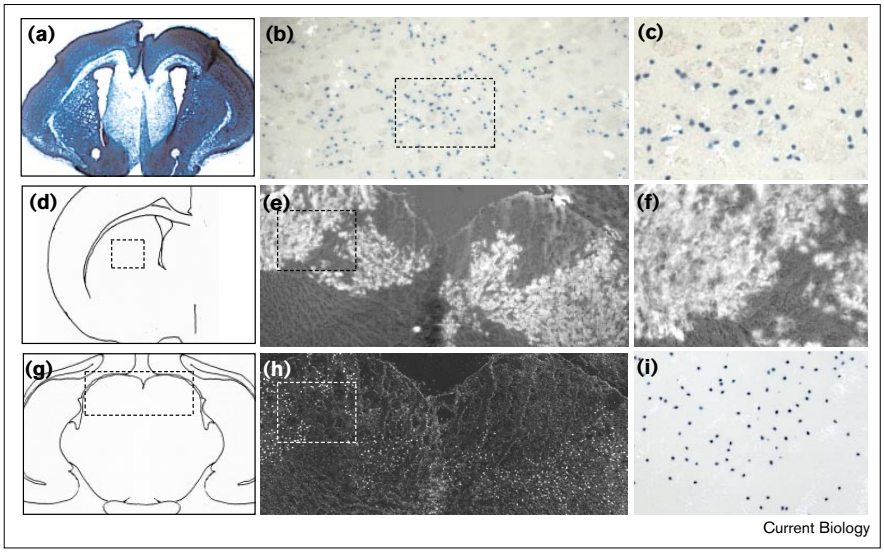
Quantitative and qualitative analysis of the patterns of homotopic and heterotopic integration of the grafted LGE progenitors. LGE progenitors were prepared as for Figure 1, labeled with the dye PKH-26 (Sigma) and 50,000 cells injected into the lateral ventricles of E14 mouse embryos as described [5]. **(a)** A summary of the integration patterns of E14 LGE grafts; mouse-to-mouse transplants were injected at E14 and sacrificed at birth. Each dot represents approximately 2% of the total labeling in a given region. As the integration patterns of transplanted cells were scored, the schematic drawings of the positions of the cells were assembled. Cells were only considered to have integrated successfully when they had migrated away from the proliferative zones and developed a differentiated morphology. **(b)** The pattern of integration of E12 or E14 forebrain progenitors transplanted into E14 hosts and sacrificed at birth. The total numbers of animals examined and the percent distribution of the cells integrated (PKH^+) according to position are tabulated. Due to the ambiguity of morphological landmarks at these later stages and the need to assign integrated cells to one of the five regions, the schematic in (a) provides a more accurate assessment of the DV distribution of donor cells than this table does. **(c–e)** Representative examples of homotopically and heterotopically integrated LGE cells after transplantation into the lateral ventricles. Note that the morphologies of LGE progenitors are appropriate to their region of integration. Hence, whereas LGE cells that integrated homotopically into the striatum took on the morphology of striatal medium spiny neurons, similar cells that integrated into the cortex appeared pyramidal in morphology. DDi indicates dorsal diencephalon; VDi, ventral diencephalon; telen, telencephalon; vtelen, ventral telencephalon.

appears just before the period when the telencephalon becomes morphologically distinguishable and is expressed strongly and ubiquitously within the telencephalon until adulthood (Figure 3a). BF-1 is also essential for telencephalic development [13]. Whereas BF-1 heterozygotes are phenotypically normal, in mice homozygous null for BF-1 the telencephalon is grossly deformed and only a fraction of its normal size. As a mouse line has been generated in which the *lacZ* reporter gene was targeted to the *BF-1* loci, transgenic heterozygotes can be used to detect BF-1 expression in grafted cells. We performed a series of intraventricular grafts utilizing LGE cells from *BF-1–lacZ* heterozygous embryos. By transplanting these mouse cells into rats we could follow both the general pattern of integration of grafted cells (using the mouse-specific M6 marker [14]) and simultaneously monitor whether the cells continue to express BF-1 (using the *lacZ* reporter). Strikingly, the majority of LGE progenitors that integrated into the diencephalon or the mesencephalon maintained BF-1 expression until adulthood (Figure 3h,i).

We also wished to evaluate whether mature markers are expressed in the basal telencephalic progenitors that integrate into other regions of the CNS. To do this we examined whether the striatal marker DARPP-32 becomes expressed in LGE progenitors that have integrated into the diencephalon or the mesencephalon. DARPP-32 is first expressed around birth, and becomes nearly ubiquitous in the striatum by post-natal day 15 (PD15) [15]. In no case did we see evidence that the LGE progenitors that integrated into the diencephalon or the mesencephalon expressed DARPP32, even if the recipient animals were examined as late as PD30 (data not shown). This is in marked contrast with numerous studies in which both isochronic and heterochronic transplants of these same progenitors were performed directly into the tissue of specific brain regions (that is, intraparenchymally). In these studies DARPP-32 is always expressed in the donor cells [16,17], even if the transplant is directed towards ectopic regions of the nervous system [18].

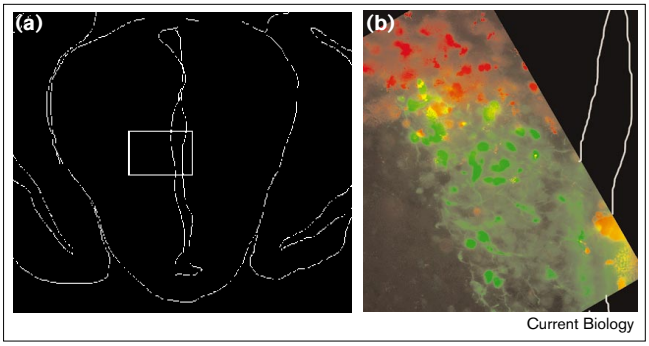
Perhaps progenitors are still able to express host regional markers in response to DV cues in the host environment while they maintain the telencephalic-specific marker BF-1. To investigate this question, we examined populations of telencephalic progenitors that integrated within the mid-ventral diencephalon to see if they expressed markers endogenous to the host regions. Specifically, we examined expression of the early regional marker Nkx2.1 [19] (sacrificing at E17 to E19) and the three more mature markers neurophysin, oxytocin and vasopressin [20] (sacrificing at PD30). In all cases, clear endogenous staining was observed. To the extent that negative data can be compelling, we found no evidence that any of these markers were induced in the telencephalic donor cells. A particularly striking example of this was found in sections double-labeled for vasopressin (Figure 4). Vasopressin-expressing cells were frequently found in close proximity with telencephalic cells

Figure 3



Telencephalic progenitors maintain region-specific markers after integration into heterotopic regions of the CNS. Cell suspensions from *BF-1–lacZ* heterozygous mice were prepared as for Figure 1. LGE progenitors were injected into the lateral ventricle of embryos from E16 timed-pregnant Sprague-Dawley rats. Brains were harvested at postnatal day 30 (PD30), cryo-preserved, cut in the coronal plane and stained for β-galactosidase or M6. **(a)** The normal expression pattern of BF-1 in mature telencephalon, in a heterozygous animal where BF-1 is directing the expression of *lacZ*. **(b)** A low power view and **(c)** a high power view of telencephalic striatum into which LGE progenitors have homotopically integrated. **(d)** A schematic of the region of the striatum shown in (b,c). **(e,f)** M6 expression within a recipient PD30 host, in a section taken adjacent to the section in (h,i). The M6 marker allows the extent of grafted tissue within host animals to be evaluated. (f) A high power photograph of the area shown in the square demarcated in (e). **(g)** A low power schematic of the region of mesencephalon shown in (e,f) and (h,i). **(h,i)** BF-1 expression in telencephalic progenitors that integrated into the mesencephalon. (h) A reverse contrast photograph showing β-galactosidase-positive cells (indicated as white dots) within the dorsal mesencephalon. (i) A high power photograph (without reverse contrast) of the area shown in the square demarcated in (h). The BF-1 staining pattern closely matches the M6 staining pattern in the adjacent section suggesting that over 90% of the grafted progenitors continue to express BF-1. In all regions examined the M6 staining was complemented exactly by BF-1 staining.

Figure 4



Telencephalic progenitors that integrate into the diencephalon do not express host-specific phenotypes. The telencephalic progenitors that integrated into the diencephalon were examined for the expression of a number of markers appropriate to their diencephalic host region, including Nkx2.1, oxytocin, neurophysin and vasopressin. **(a)** A low power schematic of the region of the diencephalon shown in (b). **(b)** The grafted cells (in red) are seen intermixed with endogenous vasopressin-expressing cells (in green). The small yellow areas, in the region of overlap of integrated cells and vasopressin-positive cells, are superimposed cells rather than transplanted cells expressing vasopressin.

that had integrated into the diencephalon. Despite the fact that the two populations were often intermixed, no double-labeled cells were ever observed.

Patterning during neurogenesis appears to require both cell intrinsic and extrinsic information [21]. The expression of BF-1, an early marker of telencephalic identity, is maintained in progenitors that integrate heterotopically. In contrast, a mature marker of regional differentiation, DARPP-32, fails to appear. Together, these results suggest that intimate contact between progenitors with the same regional character is essential for expression of more mature regional phenotypes. Gurdon [22] coined the term 'community effects' to describe the observation that synergy between populations of progenitors is needed to promote the differentiation of animal cells into mesoderm. Similarly, we speculate that although forebrain progenitors have intrinsic regional restrictions, they still require dynamic interactions with cells possessing similar regional character in order to differentiate appropriately during neurogenesis.

Whether individual neural progenitors are regionally specified also has clinical relevance. Numerous groups are presently exploring the possibility of using cell replacement strategies as a means of ameliorating neurological disorders [23]. If neural progenitors maintain regional character cell autonomously, it will be necessary to take this into account when using grafting as a therapy.

Supplementary material

Additional methodological details are published with this paper on the internet.

Acknowledgements

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Supplementary material

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Preparation of cell suspension

Donor cells were obtained from timed-pregnant CD1 mice (Charles River) at either E12 or E14. For the long survival animals LGE transplant experiments donor cells were derived from E14 *BF-1–lacZ* mice [13] (plug date was defined as day 1).

The LGE was dissected from the brains of both CD1 and *BF-1–lacZ* mice. Single cell suspensions were prepared by trypsinization (0.25% trypsin in a Ca^{2+}/Mg^{2+}-free PBS solution containing 0.02% EDTA, incubated for 30 min at 37°C) and trituration in DMEM containing 10% FBS and 0.05% DNase. Dissociated cells were labeled with a 1:500 solution of the lipophilic dye PKH-26-GL for 4 min (Sigma).

To get tissue from *BF-1–lacZ* transgenics, mice heterozygous for *BF-1–lacZ* (which carried a *lacZ* reporter driven off of the endogenous BF-1 promoter) were mated with wild-type females. Regions of interest were dissected from these animals as reported above. Heterozygous embryos were selected by X-gal staining the portion of the telencephalon not collected for transplantation. Tissues from heterozygous BF-1–lacZ animals were pooled and used for transplants. Notably, 100% of cells isolated from the LGE of these animals stained for *lacZ* activity after being cultured overnight *in vitro*.

Transplantation

E16 timed-pregnant Sprague-Dawley rats (Taconic Farms) and E14 CD1 mice were used as recipients (E16 rats and E14 mice are roughly comparable stages of development). The animals were anesthetized with sodium pentobarbital (50 mg/kg body weight for animals; for mouse, the anesthetic was diluted at 1:5 in 25 mg/ml $MgSO_4$). To gain access to the uterus a midline laparotomy was performed. After the uterine horns were exposed, each embryo was oriented in the uterine sac, by transillumination, such that the cerebral vesicles were identified using the calvarian sutures as a landmark. Approximately, 5×10^4 cells in 0.5 µl of medium of LGE suspensions were injected into the cerebral ventricle using a stoeling injection apparatus equipped with a glass micropipette. After transplantation, the uterine horns were placed back into the abdomen, and the mothers were sutured and left to recover. Since the dye used to mark cells is diluted to below detectable levels within three cellular divisions, the analysis of integrated dye-labeled cells was performed 3–8 days after the transplantation. Longer term survival relied on mouse to rat transplants, where the M6 marker can be used to trace the mouse donor population.

Tissue preparation

E17 mouse to PD2 rat embryos (3–8 days after transplantation) or PD30 rat pups were sacrificed and transcardially perfused with a PBS solution containing 2% paraformaldehyde. Brains were removed and post-fixed overnight in a PBS solution containing 2% paraformaldehyde and 30% sucrose. E17 mouse brains and E19 rat brains were embedded in 3% agarose and sectioned coronally using a vibrotome (Leica). Each brain was sectioned serially into 30–35 sections, each of which was 80 µm in thickness. Sections were collected sequentially from the olfactory bulb through to the midbrain. PKH-26 labeled donor cells were visualized in floating sections using a Zeiss Axiovert microscope. PD30 rat brains were embedded in Histo Prep media (Fisher Scientific). Sections at 25 µm thickness were cut using a cryosat and collected on probe-on plus (Fisher Scientific) microscope slides and stored at –20°C.

BrdU injection

E12 and E14 pregnant donor CD1 mice were injected with BrdU (50 mg/kg body weight, stock solution is 20 mg/ml BrdU and 2 mg/ml 5-fluoro-deoxyuridine; Sigma) three times over a 12 h period (at 0, 6 and 12 h) and sacrificed 2 h after the last injection. The brains of embryos were dissected out in ice-cold Leibovitz's L-15 medium (GIBCO/BRL). Some were fixed immediately overnight in 70% ethanol and processed for paraffin embedding. Paraffin sections, cut at 7 µm on a microtome, were used for antibody staining. Other brains were used for preparation of LGE cell suspensions.

Immunohistochemistry

1. BrdU stain

This stain was performed either on cell suspensions of LGE, DDi, and VDi or paraffin sections of E12 and E14 mouse brains with Amersham cell proliferation kit. Cell suspensions (10^6 cells/ml) were incubated on poly-D-lysine coated (100 mg/ml) tissue culture chambers for 6 h with serum-free culture medium (DMEM/F12 supplemented with 1% N2 and 2% B27 from Gibco, 0.1% mitoC, 1% glutamine of 200 mM stock and 1% penicillin and streptomycin), then fixed with acid–alcohol (90% ethanol :5% acetic acid : 5% water) at room temperature for 30 min. In cases using paraffin sections, the tissue was thoroughly de-waxed and rehydrated. Reconstituted nuclease and anti-BrdU was added to cell chambers and section slides and incubated for 1 h at room temperature. After a wash with PBS solution, peroxidase anti-mouse IgG2a was add to cover specimen for 30 min at room temperature and the immunohistochemical product was visualized using 3′,3′-diaminobenzidine (DAB) and nickel as a chromogen.

2. β-Galactosidase staining

Cryosections were incubated overnight in X-gal stain solution (0.87 g/liter NaCl, 0.1 M Na2HPO4, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide) and 1 mg/ml X-gal at room temperature. The sections were washed with PBS and analyzed using light microscopy.

3. Nestin staining

Freshly dissociated cell suspensions (10^6 cells/ml) were allowed to adhere to a poly-D-lysine coated tissue culture chambers for 1–2 h and fixed with 4% paraformaldehyde for 10 min. After a wash with PBS solution, cells were preincubated in PBS containing 10% normal goat serum and 0.1% triton for 1 h at room temperature. The specimens were incubated in mouse monoclonal anti-nestin supernatant (The Developmental Studies hybridoma bank) 1:5 at 4°C overnight. The immunohistochemical product was detected by using a biotinylated goat anti-mouse antibody (Jackson) and the ABC method (Vector Labs) and visualized by DAB enhanced with nickel.

4. M6 stain

Cryosections were preincubated in PBS containing 10% normal goat serum for 1 h at room temperature, and incubated with rat monoclonal antibody to M6 (gift of C. Lagenaur) at 1:30 overnight in 10% normal goat serum, at 4°C. After a wash with PBS solution, the sections were incubated with Cy3-conjugated goat anti-rat IgG for 1 h at room temperature.

Quantitation

For the quantitation of either BrdU incorporation or nestin staining five representative regions were counted for the numbers of positive or negative cells, within a 200 μm^2 region.

The data shown in Figure 2 schematically summarizes the data from the mouse to mouse transplants, injected at E14 and sacrificed at E17. The schematic is devised such that each dot represents approximately

2% of the total labeling. The table in Figure 2b gives the percentwise distribution of integration for each of the donor populations for both E12 and E14 transplants. Note that AP distribution data is only shown quantitatively in Figure 2a. The total numbers of animals analyzed are also shown. Intraparenchymal injections were noted but not quantified. For animals in which the graft was judged to have been placed intraventricularly, all cells that had integrated and differentiated within the host tissue were quantitated. Cells that remained in the ventricles failed to integrate and differentiate or resulted from intraparenchymal rather than intraventricular injections were not included in the schematic in Figure 2a or the quantitation of cell integration in Figure 2b. As the integration patterns of transplanted cells were scored, schematic drawings of the positions of the cells were assembled. The dots in Figure 2a are positioned according to a summary of these cumulative schematics and as noted above each dot represents a quantitatively accurate percentage (~2%) of the numbers of cells found in a given region.