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Host-Guided Migration Allows Targeted Introduction of Neurons into the Embryonic Brain

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Summary

The stereotyped positions occupied by individual classes of neurons are a fundamental characteristic of CNS cytoarchitecture. To study the regulation of neuronal positioning, we injected genetically labeled neural precursors derived from dorsal and ventral mouse forebrain into the telencephalic vesicles of embryonic rats. Cells from both areas were found to participate in the generation of telencephalic, diencephalic, and mesencephalic brain regions. Donor-derived neurons populated the host brain in distinct patterns and acquired phenotypic features appropriate for their final location. These observations indicate that neuronal migration and differentiation are predominantly regulated by non-cell-autonomous signals. Exploiting this phenomenon, intrauterine transplantation allows generation of controlled chimerism in the mammalian brain.

Introduction

During development, different regions of the CNS acquire characteristic patterns of cell organization. The molecular mechanisms involved in the generation of this cellular and regional diversity have become a major focus in modern neurobiology. It is commonly accepted that the initial patterning of the neural tube is established at the time of gastrulation (Doniach, 1993; Ruiz i Altaba, 1993). The relative contribution of cell-autonomous programs and environmental factors to this regional determination is unclear (McKay, 1989). Is an individual precursor cell itself restricted to a local fate, or is it the microenvironment that restricts the developmental potential of this cell? Transplantation experiments offer an opportunity to address these questions in vivo. Heterotopic transplantation of tissue fragments in the embryonic avian and neonatal rodent brain suggests that regional determination of neuronal progenitors is indeed an early developmental event (Alvarado-Mallart et al., 1990; Barbe and Levitt, 1991; Martinez et al., 1991; Cohen-Tannoudji et al., 1994). However, several recent grafting experiments involving transplantation of single-cell suspensions have shown that neural precursors heterotopically injected into selected developing brain regions migrate and differentiate in a way appropriate for their target site (McConnell, 1988; Renfranz et al., 1991; Fishell, 1995; Vicario-Abejón et al., 1995). The introduction of isolated cells into developing brain regions offers an exciting opportunity to "read out" developmental signals: heterotopic displacement can demonstrate whether single cells from distinct brain regions are able to respond correctly to their new environment and differentiate into a local phenotype, and heterochronic transplantation might determine whether developmental potentials are restricted to certain time periods. With few exceptions, neurogenesis in mammals is a prenatal event. Thus, to cover the aspect of precursor cell plasticity comprehensively, these cells have to be transplanted in utero. A reliable set of genetic markers is required to identify single donor cells and their offspring in a highly proliferative foreign environment. In this study, we transplanted precursor cells derived from the developing ventral and dorsal mouse telencephalon into the embryonic rat brain. To target a broad spectrum of brain regions, cells were not injected into the tissue but merely deposited in the telencephalic vesicles, thereby allowing them access to large areas of the host neuroepithelium. The transplanted cells were traced with two independent genetic labeling methods, in situ hybridization with a probe to mouse satellite DNA and X-Gal histochemistry following implantation of donor cells expressing the Escherichia coli *lacZ* gene. We show that, independently of their origin, cells from the dorsal and ventral telencephalon migrate similarly and in reproducible patterns into a variety of host brain regions, where they adopt the morphological and antigenic properties of their endogenous neighbors.

Results

Neuronal Integration Following Intrauterine Transplantation

Single-cell suspensions prepared from embryonic day 14 (E14) mouse cortex or ganglionic eminences were deposited in the telencephalic vesicles of E16–E18 rat fetuses. The recipient animals were sacrificed at different survival times, ranging from the day of birth to 5 weeks of age. Donor cells derived from wild-type and TgR(ROSA26) mice were visualized using DNA in situ hybridization and X-Gal histochemistry, respectively. TgR(ROSA26) represents a promoter trap strain exhibiting strong lacZ expression in all developing tissues (Friedrich and Soriano, 1991). The implanted cells were able to leave the ventricular system and to penetrate the host ventricular zone. From there, grafted precursors derived from either cortex or the ganglionic eminences incorporated with high efficiency into telencephalic, diencephalic, and mesenecephalic brain areas (see Figures 1, 2, and 5). Within these areas, they settled preferentially in regions undergoing neurogenesis at or after the time of implantation. Residual donor cells formed aggregates at various sites of the host ventricular system (data not shown). The donor-derived neurons appeared to migrate along endogenous pathways. In-

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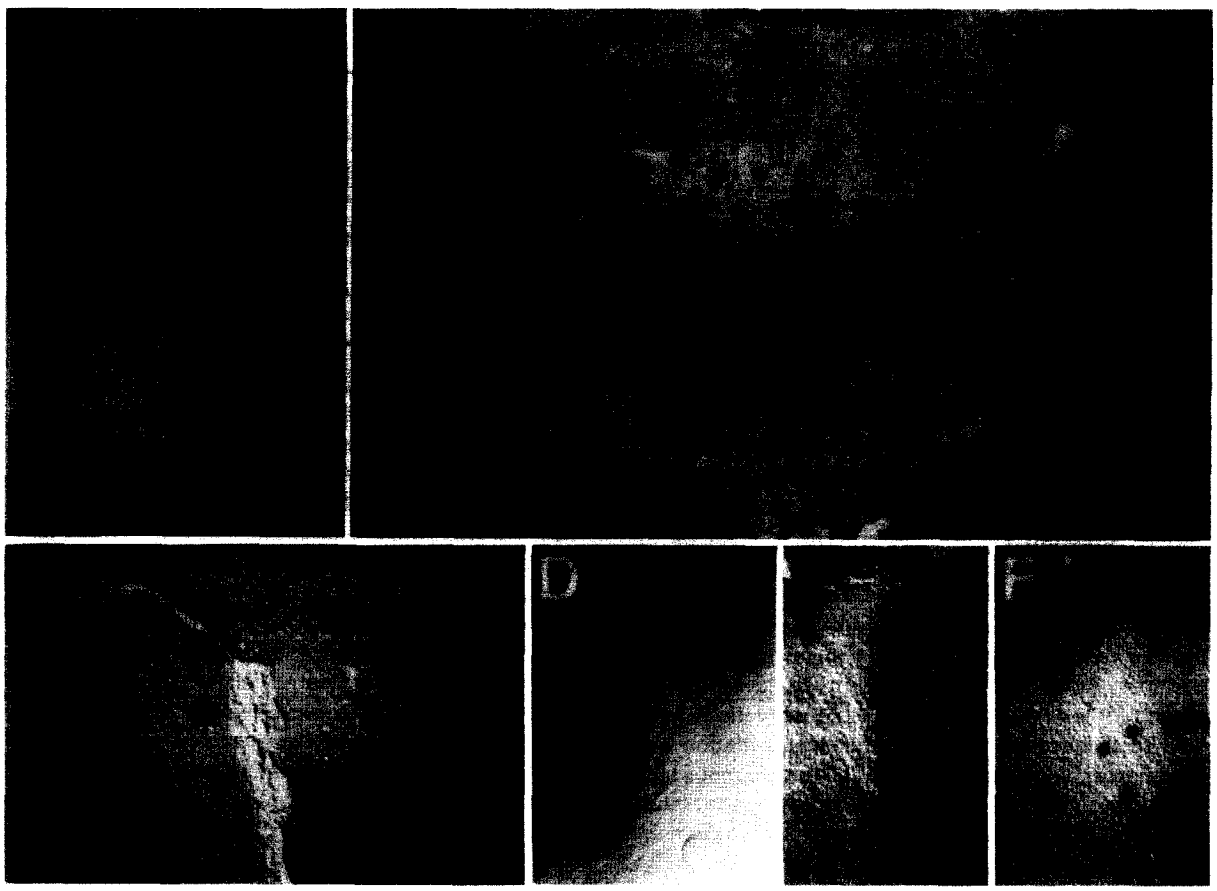

Figure 1. Incorporation of Mouse Neural Precursors into the Developing Rat Brain

In situ hybridization with a digoxigenin-labeled probe to mouse satellite DNA, performed 2 weeks after implantation of precursor cells derived from mouse E14 medial ganglionic eminence into the telencephalic vesicle of an E18 rat. Within an individual animal, incorporated cells were found in many brain regions. Shown here are parietal cortex (A), thalamus (B and E), septum (C), striatum (D), and periaqueductal gray (F). (B) and (C) display the symmetrical distribution of the incorporated cells across both hemispheres. In thalamus, the transplanted cells showed a strong accumulation in the late-generated midline structures such as the rhomboid and reuniens nuclei (B, arrows) and the paraventricular nucleus (E). Note the selective incorporation of the transplanted cells into the paraventricular thalamic nucleus and the absence of hybridization signal in the adjacent areas (E). Asterisks mark the ventricle.

ferring from their distribution, cells moving into cortex followed the radial trajectory characteristic for cortical neuroblasts (Rakic, 1972). In several stillborn animals, perpendicular streams of donor cells were detected between the ventricular wall dorsal to the presumptive stratum oriens and the CA1 sector, the genuine pathway of developing hippocampal pyramidal neurons (Altman and Bayer, 1990) (data not shown). Other regions frequently populated by donor-derived neurons leaving the lateral ventricles included the septum (see Figures 1C and 3G), striatum (see Figures 1D and 3H), thalamus, and hypothalamus. When compared to data obtained by in vivo birthdating with tritiated thymidine, the integration sites showed a good correlation with the endogenous neurogenetic gradients. In both thalamus and hypothalamus, neurogenesis is known to proceed in an outside-in pattern (Altman and Bayer, 1979, 1986). Accordingly, donor-derived neurons in thalamus accumulated in the late-developing, medially situated paraventricular, rhomboid, and reuniens nuclei (see Figures 1B, 1E, and 3I). In hypothalamus, they were preferentially located in the periventricular area and the mammillary body (see Figures 3K and 5). A similar correlation with host neurogenesis was noted in the tectum, where cells originating from the tectal neuroepithelium migrated in large numbers into the inferior colliculus (see Figures 3L and 3M; cessation of neurogenesis: E21), with few cells settling in the earlier-developing superior colliculus (Altman and Bayer, 1981a, 1981b). Cells entering the brain from the aqueduct and the fourth ventricle were also found to migrate into the midbrain central gray (see Figure 1F) and the dorsal pontine area.

Surprisingly, neurons derived from medial ganglionic eminence, lateral ganglionic eminence, and cortex incorporated across the entire spectrum of the target areas. Taking into account the considerable interindividual differences in the numbers of incorporated neurons (Table 1), we found no preferential integration pattern for either of the three donor populations. Animals implanted at E16 exhibited donor-derived neurons in the same brain regions as those transplanted at E18 (Table 1). We noticed, however, subtle differences in cell distribution within the respective areas. Following the lateral to medial neurogene-

Table 1. Integration of Mouse Neurons and Astrocytes into Embryonic Rat Brain

	E14 Cortex[a]					E14 MGE				E14 LGE					
Integration Site	E16/ P0[b]	E16/ P12	E18/ P6	E18/ P13	E18/ P35	E16/ P7	E16/ P14	E18/ P13	E18/ P13	E16/ P7	E16/ P14	E16/ P14	E16/ P14	E18/ P6	E18/ P13
Neurons															
Cortex	68	246	>500[c]	23	5	3	70	323	369	12	7	7	431[c]	7	29
Hippocampus	20	53	25	48	240	10	6	64	24	3	1	1	15	8	11
Striatum	1	3	70	3	7	2	11	47	20	9	4	7	34[c]	9	11
Septum	4	9	2	3	10	2	21	43	22	45	1	9	21	2	12
Thalamus	17	20	10	6	107	2	2	94	20	22	0	24	4	3	14
Hypothalamus	4	26	0	0	7	5	6	25	19	20	31	23	2	13	18
Central gray	7	31	27	25	101	38	36	83	113	35	39	13	1	15	23
Inferior colliculus	4	8	14	1	32	18	14	53	31	198	4	2[d]	3	6	15
Dorsal Pons	4	25	7	18	25	8	1	17	69	11	4	171	1	13	39
Glia															
Cortex	0	63	96[c]	17	6	3	5	6	2	30	22	5	214[c]	7	8
Hippocampus	0	43	0	26	52	41	0	14	28	0	0	9	3	4	19
Striatum	0	8	0	5	14	0	0	8	2	0	13	0	>500[c]	0	0
Septum	25	155	0	116	58	30	3	34	4	44	38	35	63	7	32
Thalamus	0	63	24	124	>500	0	0	268	69	109	41	>500	57	20	73
Hypothalamus	28	>500	22	101	87	46	58	87	37	86	81	212	120	42	45
Tectum	5	>500	101	>500	>500	>500	>500	>500	>500	>500	>500	>500[d]	4	120	77
Tegmentum	0	37	33	>500	>500	6	4	52	137	45	20	39	0	130	11
Pons and brain stem	0	>500	9	105	173	5	0	23	37	0	20	0	0	8	41

Average number of cells per 50 μm section, based on five sections per region. Each column represents one transplanted animal. Recipient rats had received an intraventricular injection of 600,000 cells on E16 or 1,000,000 cells on E18. Only areas exhibiting consistent donor cell integration are listed. Neurons were identified by X-Gal histochemistry. Astrocytes were detected using an antibody to the mouse-specific glial antigen M2 (Lagenaur and Schachner, 1981; Lund et al., 1993). Neurons derived from embryonic cortex, medial ganglionic eminence (MGE), and lateral ganglionic eminence (LGE) incorporated into various regions along the anteroposterior and ventrodorsal axes. The cell distributions showed considerable intra- and interindividual variations, and no preferential integration pattern was discernible for either of the three donor populations. P, postnatal day.

[a] Donor tissue.

[b] Time of implantation/time of analysis.

[c] Unilateral distribution, possibly injection-related.

[d] Area exhibiting structural damage.

tic gradients in diencephalon and inferior colliculus, neurons originating from E18 injections generally assumed more medial positions than those implanted at E16. Such a correlation between regional neurogenetic gradients and neuronal integration patterns was also visible in cortex and hippocampus. In both areas, neurogenesis is known to follow an inside-out gradient, with neurons generated late in development occupying positions most remote from the germinative zone (Angevine and Sidman, 1961; Bayer and Altman, 1991). Although donor-derived neurons were generally scattered across several cortical laminae, an accumulation in upper layer positions was noted in animals implanted at E18 (see Figure 2A). Similarly, cells transplanted into E18 recipients integrated preferentially into the concave side of the hippocampal CA1 sector, a location typically occupied by neurons generated between E18 and the end of gestation (Altman and Bayer, 1990) (see Figure 2C). These observations suggest that, independently of their origin, heterotopically placed neuronal precursors respond to environmental cues regulating cell migration and are even able to reproduce the subtleties of neuronal fine positioning exhibited by their endogenous neighbors.

Differentiation of Transplanted Neuroblasts

Within their target areas, donor-derived neurons acquired phenotypes indistinguishable from the surrounding host cells. Neurons settled in cortex exhibited both pyramidal (Figures 2A, 3A, and 3B) and nonpyramidal (Figure 3D) morphologies. Figures 3A and 3B show integrated neurons derived from cortical and ganglionic precursors, respectively. Both of these cells, identified by virtue of their lacZ expression, exhibit morphological features indistinguishable from those of their neighbors, i.e., a large, radially oriented cell body with an apical dendrite and a large nucleus. Figure 3D demonstrates a donor-derived cell with the morphology of a cortical interneuron. The proximal dendrites, visualized immunohistochemically with an antibody to calbindin (see below), show the multipolar orientation frequently encountered in intrinsic neurons (Feldman and Peters, 1978). Acquisition of local phenotypes was obvious, too, in hippocampus. Here, donor-derived neurons within the CA1–CA3 fields had large pyramidal morphologies (see Figure 2C), whereas those in the dentate granular layer displayed the small ovoid appearance typical for dentate granule neurons (see Figure 2B). Orientation of the dendritic shaft was in both cases identical to that of surrounding host neurons (see Figures 2B and 2C) In the hippocampus, as in cortex, donor-derived cells were also found to acquire interneuron morphologies (data not shown). Neurons indistinguishable in shape and size from the surrounding host cells were detected as well in septum, striatum, diencephalon, midbrain, and the dorsal pons (Figures 3G–3M).

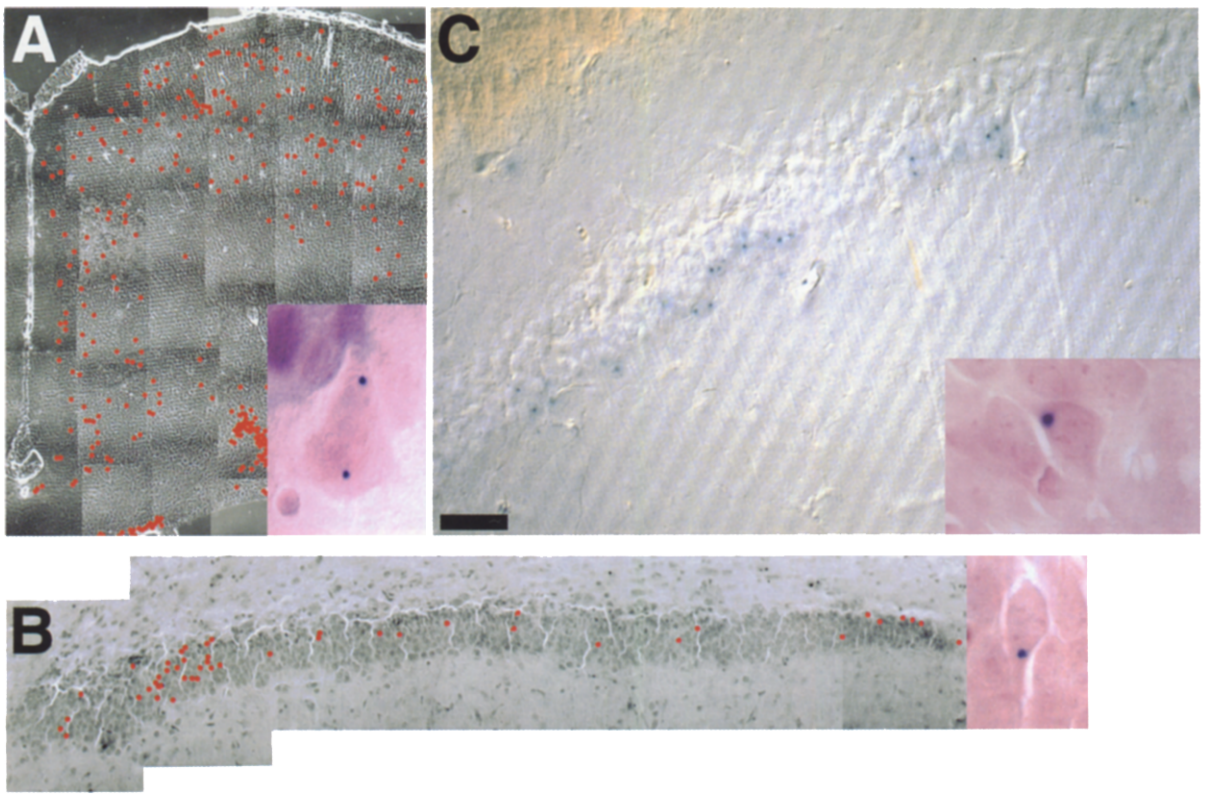

Figure 2. Detection of LacZ-Positive Donor Cells in Cortex and Hippocampus

Neuroepithelial precursor cells derived from TgR(ROSA26) mice (Friedrich and Soriano, 1991) were injected into the telencephalic vesicles of embryonic rats (50 μm vibratome sections, X-Gal histochemistry; insets were counterstained with hematoxylin and eosin).

(A) Cingulate and adjacent motor cortex of a 2-week-old rat that had received an intraventricular injection of precursors derived from E14 medial ganglionic eminence at E18 (montage; lacZ-positive cells with neuronal morphology are marked with red dots). The inset shows a donor-derived neuron with a pyramidal morphology. The granular X-Gal signal is typical for neurons derived from TgR(ROSA26) transgenic mice (Friedrich and Soriano, 1991). In this animal, 88% of donor-derived cortical neurons were found in the upper two-thirds of the cortical mantle, reaching a local density of 824 cells/mm^3. This distribution is typical for neurons generated late in cortical development (Bayer and Altman, 1991), suggesting that the transplanted neurons adopt the migration behavior of their endogenous counterparts.

(B) Integration of E14 mouse cortical precursors into the rat dentate granular layer 19 days after intrauterine transplantation on E16 (montage of the infrapyramidal leaf of the granular layer; lacZ-positive cells with neuronal morphology are marked with red dots). The integrated neurons display the typical morphology and orientation of granule cells (inset).

(C) Cells derived from the E14 cortical neuroepithelium of a TgR mouse and transplanted into the telencephalic vesicle of an E18 rat have integrated into the CA1 field of the hippocampal pyramidal cell layer. As in neocortex, neurogenesis in the hippocampal pyramidal cell layer follows an inside-out gradient, and positions at the concave side are typically occupied by neurons generated between E18 and the end of gestation (Altman and Bayer, 1990) (5-week-old animal). The inset shows that the integrated cells acquire a phenotype appropriate for hippocampal pyramidal cells. Bar, 50 μm.

The large number of differentiated neurons produced a prominent axonal network within the host brains (Figure 3C). Using an antibody to the mouse-specific antigen M6 (Lagenaur et al., 1990), axons were detected in all areas harboring donor-derived neurons and along their trajectories. In cortex, the majority of the visualized axons followed a perpendicular path. However, axonal processes could also frequently be traced running parallel to the pial surface for several hundred micrometers. Being detectable at all levels of the host brain including the brain stem, donor-derived axons reached their highest densities in cortex, corpus callosum, striatum, thalamus, and inferior colliculus. While M6 was clearly localized to the axons of mouse-derived neurons, some neurons also showed membranous dendritic labeling. In these cases, confocal laser microscopy allowed complete reconstruction of the entire cell. The example in Figure 3M shows a computer reconstruction of a neuron in the inferior colliculus, derived from the medial ganglionic eminence. When compared to data obtained by Golgi impregnation of the inferior colliculus, cell size, orientation, and dendritic organization were compatible with the LF ("less flat") type of neuron described by Malmierca et al. (1993).

The limbic system–associated membrane protein (LAMP) was one of the markers used to determine whether the implanted cells, in addition to the morphology, also exhibit expression patterns characteristic of their target region. LAMP is expressed on limbic system neurons but not on neurons in nonlimbic areas such as the sensorimotor cortex (Levitt, 1984). We found strong LAMP expression on donor-derived neurons situated in hippocampus and cingulate cortex (Figure 3F). In contrast, neurons in the sensorimotor cortex of the same recipients were consistently LAMP negative (Figure 3F, inset). The calcium-binding

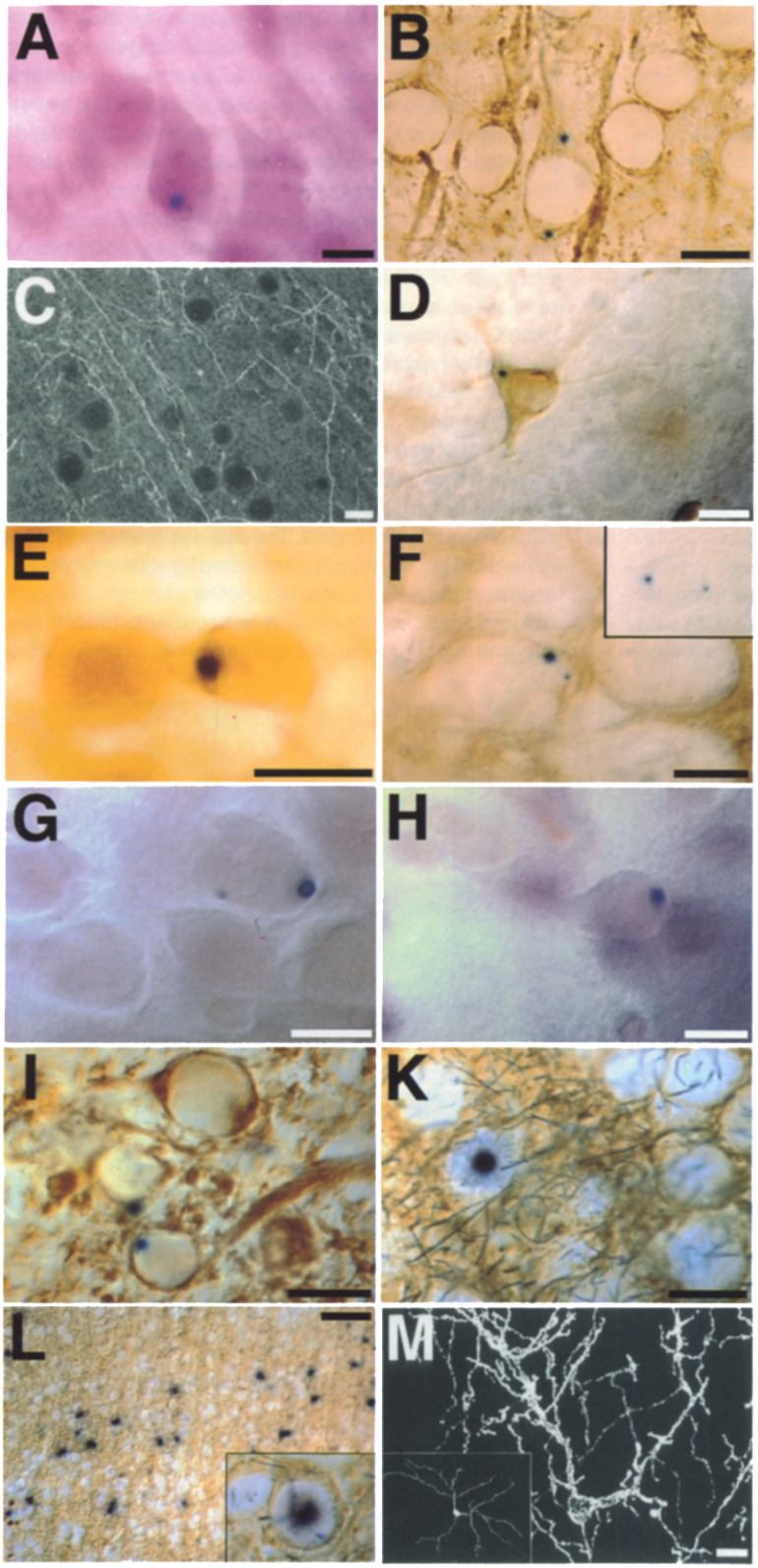

Figure 3. Phenotypic Features of Neurons Derived from E14 Mouse Neuroepithelial Precursors Implanted into the Ventricular System of the Developing Rat Brain

(A and B) Pyramidal-shaped neurons in the frontal cortex of newborn rats that had received an intraventricular injection at E18 of lacZ-positive precursor cells derived from E14 cortex (A) or medial ganglionic eminence (B). Sections were processed for X-Gal histochemistry and counterstained with hematoxylin and eosin (A) or double labeled with an antibody to MAP2 (B). Both neurons show the localized granular X-Gal signal characteristic for cells derived from TgR(ROSA26) transgenic mice (Friedrich and Soriano, 1991).

(C) Visualization of donor-derived axons in layer VI of the parietal cortex, 15 days after intraventricular injection of mouse E14 cortical precursors. Immunofluorescence using an antibody to the mouse-specific antigen M6 (Lagenaur et al., 1990).

(D) Cortical interneuron in a 13-day-old rat, 18 days after intrauterine implantation of cells derived from E14 TgR(ROSA26) medial ganglionic eminence. X-Gal histochemistry followed by immunohistochemical detection of calbindin-D28k, a calcium-binding protein frequently expressed by cortical interneurons (Baimbridge et al., 1992).

(E) Calbindin-positive dentate granule neuron in the dentate granule cell layer of a 12-day-old rat transplanted with TgR(ROSA26) cortical precursors on E16.

(F) Neurons derived from E14 TgR(ROSA26) medial ganglionic eminence integrate into the CA1 field of the recipient hippocampus and show strong expression of the limbic system–associated membrane protein (LAMP). The inset in the upper right corner of the picture shows a donor-derived neuron in the motor cortex, taken from the same section. Note the absence of LAMP expression.

(G) LacZ-positive neurons in the dorsolateral aspect of the septum (same animal as in [F]) counterstained with hematoxylin and eosin.

(H) Striatal neuron, derived from E14 cortical precursors, 16 days after intrauterine transplantation (X-gal histochemistry, counterstained with hematoxylin and eosin).

(I) Donor-derived neurons in the host thalamus (nucleus reuniens), 16 days after implantation of progenitors from the medial ganglionic eminence. Cells are identified by X-Gal histochemistry and immunohistochemistry with an antibody to MAP2.

(K and L) DNA in situ hybridization with an oligonucleotide probe to mouse satellite DNA strongly labels the central nucleolus of MAP2-positive donor-derived neurons in the medial preoptic area (K) and inferior colliculus (L). This animal was transplanted at E16 with cells derived from the lateral ganglionic eminence. A detail of (L) is shown in the inset.

(M) Computer reconstruction of an M6-positive donor-derived neuron in the inferior colliculus, 2 weeks after transplantation of precursors from the medial ganglionic eminence. The inset shows the outline of a Golgi-impregnated neuron in the inferior colliculus exhibiting a very similar morpholgy (adapted from Malmierca et al., 1993).

Bars, 10 μm (A–K and M), 50 μm (L).

protein calbindin, too, is found in only a subset of CNS neurons. This protein is typically present in hippocampal granule neurons and cortical interneurons (Sloviter, 1989; Baimbridge et al., 1992). Indeed, we detected strong calbindin expression in donor-derived neurons that had integrated into the hippocampal granular layer and acquired the typical orientation and morphology of dentate granule neurons (Figure 3E). Donor cells with morphologies of cortical interneurons exhibited calbindin-positive dendritic arbors indistinguishable from their endogenous neighbors (Figure 3D).

Distribution of Nonneuronal Cells

In addition to neurons, large numbers of astrocytes were found in the host brains (see Table 1). Since glial cells derived from TgR(ROSA26) mice expressed only low levels of histochemically detectable lacZ, the presence of donor-derived astrocytes was confirmed immunohistochemically with an antibody to glial fibrillary acidic protein (GFAP) and subsequent DNA in situ hybridization with a probe to mouse satellite DNA (data not shown). For detection of mouse astrocytes in the large numbers of serial sections, an antibody to the mouse-specific glial antigen M2 (Lagenaur and Schachner, 1981) proved more practical. The species specificity of the M2 antigen has been exploited in many xenograft paradigms for the identification of mouse astrocytes in a rat background (Zhou et al., 1990; Zhou and Lund, 1992, 1993; Lund et al., 1993). In accordance with these previous studies, we detected M2 only on the surface of cells with characteristic astroglial morphologies (Figures 4A and 4B). When used in conjunction with cell type–specific markers, M2 colocalized exclusively with GFAP but not with neuronal antigens such as microtubule-associated protein 2 (MAP2; data not shown). The M6 antigen, primarily used for the detection of donor-derived axonal profiles (see Figure 3C), was also consistently present on donor-derived GFAP-positive cells with astrocytic morphology (Figures 4C and 4D). In addition, M6 was expressed on the processes of few galactocerebroside-positive cells with oligodendroglial phenotype and on GFAP- and galactocerebroside-negative cells with immature glial morphologies. The species specificity of the M2 and M6 antibodies was confirmed by subsequent DNA in situ hybridization (data not shown).

Surprisingly, donor-derived astrocytes exhibited specific and highly reproducible integration patterns within

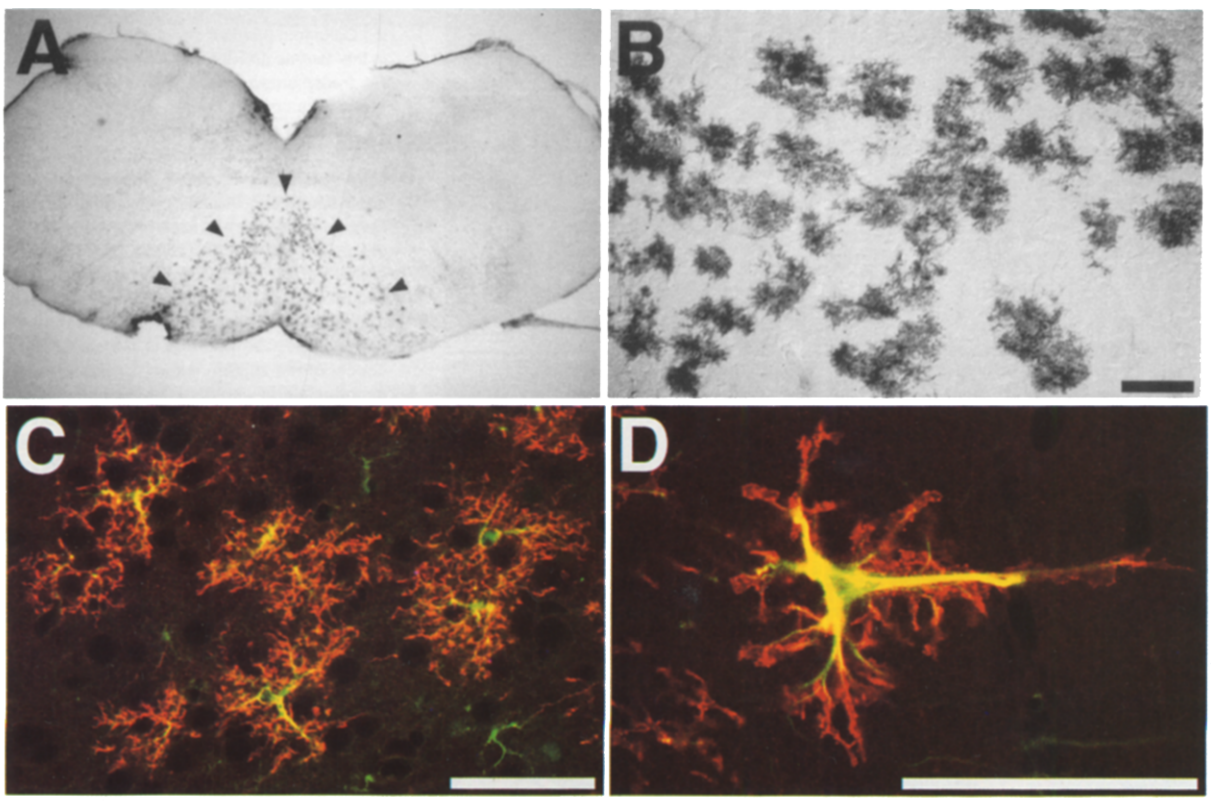

Figure 4. Detection of Donor-Derived Astrocytes Using Antibodies to Mouse-Specific Antigens

(A and B) Eighteen days after intrauterine transplantation of E14 cortical precursors, donor-derived astrocytes expressing the M2 antigen have integrated into various host brain regions, including the brain stem (A) and the septum (B). Arrows in (A) delineate the pyramidal decussation.

(C) Mouse astrocytes in the horizontal limb of the diagonal band of Broca, an area frequently populated by donor-derived glia. This 1-week-old animal was transplanted with cortical precursors at E16.

(D) High power image of a donor-derived astrocyte in the corpus callosum, 2 weeks after intraventricular injection of neural precursors from the lateral ganglionic eminence. Cells in (C) and (D) are double labeled with antibodies to M6 (red) and GFAP (green) and visualized by confocal laser microscopy.

Bars, 50 μm.

the host brains. Generally, they were found to incorporate into the same brain regions as neurons but showed a more widespread distribution. For example, large numbers of astrocytes were found in the lateral thalamic and hypothalamic areas, whereas integrated neurons were restricted to the medially situated diencephalic nuclei. Similarly, astrocytes in the tectum populated both the inferior and superior colliculi, whereas neurons incorporated preferentially into the inferior colliculus. An exception was the cerebral cortex, where astrocytic cells were largely confined to the medial cingulate area. However, since gliogenesis in the neocortex continues postnatally (Levison et al., 1993), this distribution might be merely due to the early time point of analysis. Prominent accumulations of astrocytes were consistently present in the septal area (Figure 4B), often continuing into the adjacent nucleus of the horizontal limb of the diagonal band (Figure 4C). In, addition, donor-derived astrocytes invaded the gray and white matter of tegmentum, ventral pons, and brain stem, i.e., areas not typically populated by neurons (Figure 4A). In most regions, astrocytes far outnumbered the integrated neurons (see Table 1). A synopsis of the glial and neuronal distribution patterns is shown in Figure 5.

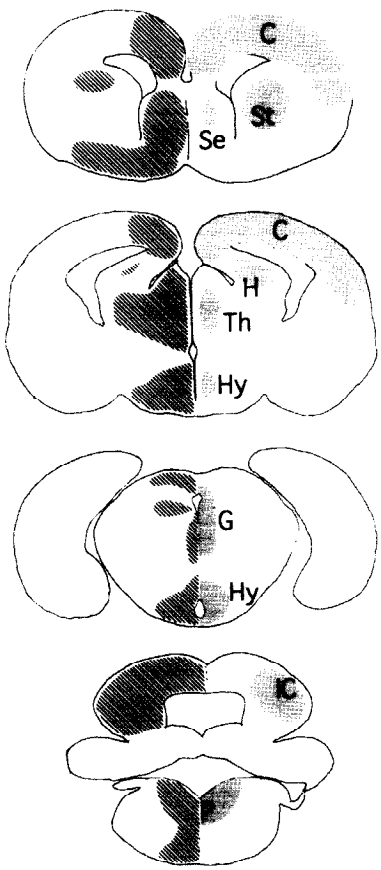

Figure 5. Schematic Representation of the Main Integration Sites of E14 Mouse Neural Precursors Implanted into the Telencephalic Vesicles of E16–E18 Rat Brains

The distribution of donor-derived neurons and astrocytes is shown on the right and left, respectively. Independently of their origin, neurons derived from embryonic cortex or ganglionic eminences incorporated into various regions across the anteroposterior and ventrodorsal axes. Integration was largely restricted to areas undergoing neurogenesis at and after the time of implantation and occurred most efficiently in brain regions with large neurogenetic matrices and protracted neurogenesis until late gestation (i.e., the cerebral cortex [C] and the hippocampal formation [H]; Bayer, 1980; Bayer and Altman, 1991). Neurons originating from the diencephalic neuroepithelium incorporated preferentially into the late-generated midline structures of thalamus (Th) and hypothalamus (Hy) (Altman and Bayer, 1979, 1986). Neuronal precursors originating from the neuroepithelium around the aqueduct and the fourth ventricle settled in the periaqueductal gray (G), dorsal pons (P), and tectum. In tectum, neurons incorporated consistently into the inferior colliculus (IC; cessation of neurogenesis: E21), with very few cells settling in the earlier-developing superior colliculus (Altman and Bayer, 1981a, 1981b). Astrocytes appeared to populate the host brain along the same entry sites as neurons but in general showed a more widespread distribution. An exception was the cortex, where astrocytes were largely confined to the medial cingulate area. Prominent accumulations of astrocytes were found in septum (Se) and the adjacent nucleus of the horizontal limb of the diagonal band, in the medial and lateral thalamus and hypothalamus, and across the entire tectum. The illustration shows four coronal sections through an E20 rat brain (modified from Altman and Bayer, 1995). St, striatum.

Discussion

Implications for Developmental Neurobiology

Our data demonstrate that neural precursor cells transplanted into the ventricular system of the embryonic CNS enter the brain parenchyma and incorporate into various telencephalic, diencephalic, and mesencephalic target sites. Neurons derived from the transplanted cells distribute along endogenous migratory pathways and integrate preferentially into areas undergoing neurogenesis at or after the time of implantation. Although the precursor cell suspensions were prepared from distinct regions of the dorsal and ventral telencephalic neuroepithelium, the cells were found to populate regions at all levels of the host brain. Cells derived from the ganglionic eminences incorporated into cortex and hippocampus. Conversely, cortical precursors settled in ventral areas such as the striatum and septum. Migration into heterotopic areas was not restricted to the ventrodorsal axis. Cells from cortex and ganglionic eminences efficiently populated various sites along the anteroposterior axis, including such remote areas as the caudal tectum. These observations indicate that heterotopically placed neuronal precursors can respond to environmental cues regulating cell migration and adopt the migration behavior of their new neighbors. The fact that precursors from the ventral and dorsal telencephalon were found to incorporate similarly into the various host brain regions suggests that this ability is not limited to a locally restricted precursor cell population. It is, at present, unclear whether this phenomenon results from a selective or an instructive mechanism and whether the cells capable of heterotopic integration represent the majority or only a subset of the donor cell population. The interindividual differences in the number of integrated neurons did not allow us to resolve these issues conclusively. It is likely that the developing neuroepithelium contains a mixture of precursors at different developmental stages. Cells able to integrate heterotopically might represent only a small fraction of the donor cell population. Comparing the num-

ber of integrated neurons with those found in the periventricular reaggregates, we estimate that, depending on the individual animal, only 1%–20% of the surviving neurons incorporate into the brain parenchyma. The varying numbers of incorporated neurons did not permit us to discern possible subtle differences between the three donor sources (i.e., medial and lateral ganglionic eminences and cortex). However, our data unequivocally demonstrate that precursors from all three areas incorporate consistently across the entire spectrum of the target regions (see Table 1). If environmental factors guide the migration of transplanted neurons, one might expect different integration patterns at different developmental time points. As a first step in this direction, we compared animals transplanted at E16 with those injected at E18. In both series, neurons integrated into the same brain areas but showed subtle differences in local cell distribution paralleling the regional neurogenetic gradients. A more extensive series of transplantions involving various recipient ages will delineate age-dependent migration patterns.

Following migration, donor-derived neuronal progenitors differentiated into mature phenotypes. They expressed neuronal antigens and extended intricate dendritic and axonal processes, resulting in a dense innervation of the host brain. Within the various telencephalic, diencephalic, and mesencephalic integration sites, the donor-derived neurons exhibited the size, orientation, and morphology of their endogenous neighbor cells and were only detectable by virtue of their genetic label. The acquisition of characteristic morphologies such as those of hippocampal pyramidal or granule neurons suggests that the transplanted cells undergo region-specific differentiation. This interpretation is supported by immunohistochemical findings showing appropriate expression of antigens restricted to certain neuronal subpopulations, e.g., LAMP and calbindin. An extensive series of neuroanatomical and neurochemical studies will be necessary to cover the aspect of region-specific differentiation in a comprehensive fashion. Fine structural features, expression of homeotic genes and neurotransmitters, synaptic connectivity, and electrophysiological properties will have to be examined to delineate the extent of this adaptation process. This is especially true for regions that, using conventional histological techniques, do not exhibit unequivocal and characteristic neuronal morphologies and contain a multitude of neuronal subpopulations expressing distinct but not region-specific antigens.

Integration of donor cells was not restricted to the neuronal lineage. Abundant astrocytes were consistently detected in the host brains in a very stereotyped distribution (see Figure 5). Unlike in the adult brain, where astrocytes migrate predominantly along fiber tracts (Zhou and Lund, 1993), they efficiently populated both white and gray matter in highly reproducible patterns (see Table 1). Similar observations have been made following transplantation of astrocytes into the neonatal brain (Zhou et al., 1990; Zhou and Lund, 1992, 1993). Together, these findings indicate that astrocyte migration, like neuronal migration, is regulated predominantly by local cues. Since little is known about the migration of glial precursors in the embryonic brain, this transplantion paradigm, with its potential to trace small subsets of progenitors reliably, might yield useful information on the genesis and distribution of astrocytes in the developing CNS. Analysis of recipient animals at later time points should allow similar observations on donor-derived oligodendroglial cells, which were encountered only infrequently in this study. The grossly overlapping distributions of donor-derived neurons and astrocytes could indicate that many of the cells entering the host brain represent multipotential precursors that then give rise to neurons and glia. Alternatively, precursors already committed to either of the lineages might populate the host brain via the same entry sites. Combination of the in utero transplantation strategy with retroviral labeling of the donor cells could delineate to what extend the integrating cells are committed with respect to their lineage. Donor-derived astrocytes generally outnumbered the neurons. However, since glial cells retain their proliferative potential, these numerical relations are difficult to interpret. In vivo birthdating experiments using tritiated thymidine or bromodeoxyuridine will assess cell division following transplantation and show whether cells need to divide and thus be "born" in the new environment to be able to respond appropriately to local signals (O. B. et al., unpublished data).

The results of this study show that regional and cellular diversity of neuronal cytoarchitecture can arise when precursors derived from defined aspects of the embryonic neuroepithelium (i.e., the dorsal or ventral telencephalon) are exposed to different environmental cues. This indicates that non-cell-autonomous signals play a major role in neuronal migration and differentiation. While our data do not exclude the possibility that precursor cells in distinct regions of the neuroepithelium exhibit preferential traits, they clearly demonstrate that these regions share a population of neuronal progenitors that is not committed to locally restricted migration and differentiation pathways. This view accommodates and extends the interpretation of several heterotopic transplantation experiments in the developing rodent cortex that show acquisition of region-specific axonal connections, cytoarchitectonic features, and protein expression (Stanfield and O'Leary, 1985; McConnell, 1988; O'Leary and Stanfield, 1989; Barbe and Levitt, 1991; Schlaggar and O'Leary, 1991; Fishell, 1995). Clonal analysis of neuronal precursors in vivo suggests that recruitment to a local phenotype is, in fact, an integral part of cortical development. The widespread dispersion of clonally related neuronal precursors across large areas of the developing cortex indicates that these cells are not specified regarding their ultimate areal fate by a lineage-based mechanism (Walsh and Cepko, 1992). Our results suggest that neuronal recruitment is not restricted to the cortex but can occur in various locations along the ventrodorsal and anteroposterior axes. In fact, the specificity of this recruitment phenomenon is most apparent in areas such as the thalamic midline nuclei or the inferior colliculus. Whereas these regions typically contained large numbers of incorporated donor cells in a small volume, neighboring areas generated before the time of implantation were devoid of donor-derived neurons. The observation

that cortical and ganglionic precursors incorporate into the same developing brain regions supports the idea that neuronal precursors in different parts of the neuroepithelium may share equivalent developmental potentials.

The aquisition of a target site–specific migration behavior and the generation of local phenotypes following transplantation of cortical and ganglionic cell suspensions, even at late developmental stages (e.g., E18), differ from results obtained by heterotopic transplantation of tissue fragments in the embryonic avian and neonatal rodent brain. Although these studies show that some tissue fragments acquire a local phenotype when transplanted early in development (Alvarado-Mallart et al., 1990), many regions were found to maintain their phenotype in heterotopic positions (Martinez et al., 1991; Cohen-Tannoudji et al., 1994). However, in both cases the fate of a single cell could be determined by its microenvironment. Cells within tissue fragments are transplanted along with their microenvironment and therefore fail to "adapt" to their new location. In contrast, grafted dissociated cells are individually exposed to a new environment and are thus able to respond to host signals.

A Novel In Vivo Tool

Generation of brain chimeras by intrauterine transplantation allows the design of brains composed of neurons with different genetic backgrounds and developmental histories. Such heterochronic and heterotopic combinations of genetically distinct cell populations within a functional brain are not possible using common embryonic stem cell–based chimeras. This new approach will define cell-autonomous programs and environmental factors in CNS development by combining the classic embryological strategies of heterotopic and heterochronic cell displacement with modern mouse genetics. The efficient and highly localized cell transfer allows the targeted introduction of genetically distinct neurons into defined brain regions. Although we have described only results obtained by transplantation of E14 donor tissue into E16–E18 recipients, this paradigm already suffices to target brain regions such as the neocortex, the hippocampus, the midline thalamus and hypothalamus, and the tectum. Selection of younger or older recipient animals may increase or restrict the number of amenable regions. Such a chimera strategy could be useful to study the molecular mechanisms underlying cell migration and differentiation and to refine the analysis of a growing repertoire of genetically modified animals. Transgenic mice and mice generated by homologous recombination (knockout mice) have become indispensable tools in the study of brain development and function (Zimmer, 1992; Soriano, 1995). There are, however, significant obstacles limiting full exploitation of these powerful technologies. First, the embryonic or perinatal lethality of a large number of homozygous knockout mice precludes an analysis of the CNS phenotype at later developmental stages (Forrest et al., 1994; Hynes, 1994; Li et al., 1994). Second, the restriction of transgene expression or gene targeting to defined brain regions and developmental stages remains problematic. Third, targeted deletions of genes considered essential for cell viability and function frequently yield only inconspicuous phenotypes, an observation that has been attributed to early compensatory events (Weintraub, 1993; Malenka, 1994; Routtenberg, 1995). Intrauterine transplantation of neural precursors derived from transgenic or knockout donors (both exemplified in this study by TgR(ROSA26) mice, i.e., a promoter trap strain carrying the *lacZ* gene; Friedrich and Soriano, 1991) might help to overcome these obstacles. Since the chimeric animals were found to mature normally, and no signs of rejection were detected up until the age of 4 weeks, this approach allows the long-term study of neural cells derived from knockout mice with embryonic or early postnatal lethality far beyond the natural lifespan of these animals. Introduction of genetically modified precursor cells into defined areas of the developing brain permits localized gene targeting or transgene expression with comparatively little effort, and the competition of knockout and wild-type cells in embryonic neural chimeras may unravel deleterious effects possibly compensated in an organism harboring a germline deletion.

Experimental Procedures

Donor Animals

Wild-type C57BL/6NCR embryos (DCRT, Frederick, MD) and embryos generated by mating wild-type C57BL/6NCR mice with homozygous TgR(ROSA26) transgenic mice (Jackson Laboratory, Bar Harbor, ME) were used as donor animals. The TgR(ROSA26) strain was obtained after insertion of a promoter trap construct carrying the E. coli *lacZ* gene into an unknown locus and exhibits strong lacZ expression in all developing mouse tissues (Friedrich and Soriano, 1991). When processed for X-Gal histochemistry, a characteristic granular staining is detected in all CNS cell populations up until at least the age of 1 year (data not shown).

Intrauterine Transplantation

Pregnant mice were sacrificed at day 14 of gestation by cervical dislocation, the embryos were removed, and the fetal brains were dissected using a stereomicroscope. Medial and lateral ganglionic eminences and the cortical neuroepithelium (including cingulate cortex and the presumptive hippocampus) were prepared separately and transferred into ice-cold Hanks' balanced salt solution (HBSS) containing 1 g/l glucose. Tissue fragments were washed 3 times in HBSS, enzymatically dissociated (0.125% trypsin, 0.1% DNase in HBSS, 10 min at room temperature), and gently triturated to obtain a single-cell suspension. Cell viability routinely ranged between 80% and 95%, as determined by the trypan blue exclusion method. Timed pregnant Sprague–Dawley rats were anesthetized (80 mg/kg ketamine–HCl, 10 mg/kg xylazine) and placed on a 37°C plate. The uterine horns were exposed, the telencephalic vesicles of the embryos were identified under transillumination, and 0.1×10^6 to 1×10^6 cells (0.1×10^6 cells/μl HBSS) were injected into the telencephalic vesicle using a glass capillary. Injected embryos were placed back into the abdominal cavity for spontaneous delivery. The percentages of live-born transplanted animals were 60% and 80% for E16 and E18 recipients, respectively. Donor-derived neurons were found in 56 out of 64 analyzed recipients.

X-Gal Histochemistry

Live-born recipient animals (age 1–36 days) were deeply anesthetized and perfused with 2% paraformaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA in 0.1 M PIPES buffer. Brains of stillborn recipients were fixed by immersion for 12 hr at 4°C. Vibratome sections (50 μm) were permeabilized in phosphate-buffered saline containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.01% Nonidet P-40, followed by incubation in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 1 mg/ml X-Gal (Sigma) at 37°C for 6–10 hr. X-Gal histochemistry typically produced one or two strongly labeled granula within the perikaryon of donor-derived neurons.

Immunohistochemistry

Transplanted brains were characterized using monoclonal antibodies to MAP2 (1:200 dilution; Sigma), GFAP (1:200 dilution; ICN), calbindin-D28k (1:200 dilution; Sigma), M2 and M6 (1:10 dilution; Lagenaur and Schachner, 1981; Lund et al., 1985), and LAMP (1:100 dilution; Levitt, 1984). For double labeling, immunohistochemistry was performed either before DNA in situ hybridization or after X-Gal histochemistry. If followed by immunohistochemical detection of membranous antigens, no Nonidet P-40 was used during X-Gal histochemistry. For characterization of donor-derived astrocytes, selected sections were subjected to double-immunofluorescence analysis using antibodies to M2, M6, and GFAP. Antigens were visualized by appropriate fluorophore- or peroxidase-conjugated secondary antibodies. Sections were analyzed on Zeiss Axioplan and Axiovert microscopes featuring confocal laser attachments.

In Situ Hybridization

Sections were digested with pronase (Sigma; 25 μg/ml in 2× SSC, 5 mM EDTA [pH 8.0]) for 15 min at 37°C, dehydrated, and subsequently denatured in 70% formamide, 2× SSC for 12 min at 85°C. Following additional dehydration in ice-cold ethanols, the sections were hybridized overnight at 37°C with a digoxigenin end-labeled oligonucleotide probe to the mouse major satellite (Hörz and Altenburger, 1981; 30–60 ng/section in 65% formamide, 2× SSC, 250 μg/ml salmon sperm DNA [pH 7.0]). After washing in 2× SSC and 0.1× SSC (37°C, 30 min each), hybridized oligonucleotides were visualized using an alkaline phosphatase–linked antibody to digoxigenin (Genius system, Boehringer).

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