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Progressive restriction in fate potential by neural progenitors during cerebral cortical development

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SUMMARY

During early stages of cerebral cortical development, progenitor cells in the ventricular zone are multipotent, producing neurons of many layers over successive cell divisions. The laminar fate of their progeny depends on environmental cues to which the cells respond prior to mitosis. By the end of neurogenesis, however, progenitors are lineally committed to producing upper-layer neurons. Here we assess the laminar fate potential of progenitors at a middle stage of cortical development. The progenitors of layer 4 neurons were first transplanted into older brains in which layer 2/3 was being generated. The transplanted neurons adopted a laminar fate appropriate for the new environment (layer 2/3), revealing that layer 4 progenitors are multipotent. Mid-stage progenitors were then transplanted into a younger environment, in which layer 6 neurons were being generated. The transplanted neurons bypassed layer 6, revealing that layer 4 progenitors have a restricted fate potential and are incompetent to respond to environmental cues that trigger layer 6 production. Instead, the transplanted cells migrated to layer 4, the position typical of their origin, and also to layer 5, a position appropriate for neither the host nor the donor environment. Because layer 5 neurogenesis is complete by the stage that progenitors were removed for transplantation, restrictions in laminar fate potential must lag behind the final production of a cortical layer. These results suggest that a combination of intrinsic and environmental cues controls the competence of cortical progenitor cells to produce neurons of different layers.

Key words: Fate determination, Neurogenesis, Lineage, Cerebral cortex, Migration, Ferret

INTRODUCTION

The mammalian cerebral cortex is a multilayered structure derived from cells of the neural tube (reviewed in McConnell, 1995). The cortex is organized into six layers, each of which contains neurons with similar morphologies and projection patterns (Gilbert and Kelly, 1975; Gilbert and Wiesel, 1985). Cortical neurons are generated from proliferating progenitor cells located in the ventricular zone, which lines the lateral ventricle. Postmitotic neurons migrate away from the ventricular zone and coalesce into distinct layers in the developing cortical plate. The cortical layers are generated in an inside-first, outside-last pattern (see McConnell, 1988a, for review): the neurons of the deep layers 6 and 5 arrive first in the cortical plate, and are followed by cells of layers 4, 3 and 2, which take on successively more superficial positions. Because the "birthday" of the neurons in each layer can be marked with [^{3}H]thymidine (Angevine and Sidman, 1961), one can predict accurately the laminar fate of neurons born on a certain day. This close correlation between cell birthday and laminar fate has enabled an analysis of the timing of commitment to specific laminar phenotypes during development.

Transplantation experiments have examined the laminar fate potential of both early and late cortical progenitor cells (McConnell, 1988b; McConnell and Kaznowski, 1991; Frantz and McConnell, 1996). Transplantation of early progenitors, normally destined to generate layer 6 neurons, into the brains of older hosts has revealed that the laminar fate of early progenitors is determined just prior to a neuron's final mitotic division. Cells transplanted late in the cell cycle, shortly before mitosis, are specified to generate neurons that migrate to layer 6 even in older host brains (McConnell and Kaznowski, 1991). In contrast, progenitors transplanted early in the cell cycle, during S-phase, are multipotent, generating neurons that migrate to the upper layers 2 and 3. Consistent with these findings, retroviral lineage studies have demonstrated that early progenitors are normally multifated, giving rise to clones that span several layers of the cortex following multiple cell divisions (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Reid et al., 1995, 1997). The transplantation experiments provide direct evidence that these early progenitor cells are multipotent.

The developmental potential of cortical progenitors is altered, however, by the end of neurogenesis. Retroviral lineage studies have shown that progenitors infected during late neurogenesis normally produce neurons that populate only the upper layers (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Reid et al., 1995, 1997). Transplantation experiments examined directly the competence

of upper-layer progenitor cells to produce neurons of other layers (Frantz and McConnell, 1996). In these experiments, late progenitors were transplanted during S-phase into younger hosts, in which deep-layer neurons were being generated. The vast majority of the transplanted neurons migrated to layer 2/3, appropriately for their origin. Even more astonishing, transplanted cells migrated to their cell-autonomous position even after one or more rounds of cell division in the younger host environment. Late progenitors are thus no longer competent to respond to environmental cues that specify deep-layer formation, and are instead lineally committed to the production of upper-layer neurons.

These results indicate that the competence of cortical progenitors to produce neurons of different phenotypes is altered over time during development: early progenitors are multipotent, but late progenitors are restricted in their fate potential. The process of development is ripe with examples of progenitors that change their competencies over time. A classic example of progressive restriction lies in the hematopoeitic system, in which stem cells generate a multitude of cell types but accomplish this through the generation of distinct subsets of lineage-restricted cells. These lineage-restricted cells respond to different growth factors that modulate self-renewal (or lineage expansion) and the generation of various differentiated progeny (Cross and Enver, 1997; Metcalf, 1998). Within the nervous system, neural crest cells also appear to exhibit progressively restricted competencies in a process termed "neuropoeisis" (Anderson, 1989). Transplantation experiments show that, while neural crest cells are multipotent early in development, late-emigrating crest cells have a restricted developmental potential (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991; Artinger and Bronner-Fraser, 1992). In vitro studies have identified neural crest stem cells capable of both self-renewal and the generation of neurons and glia (Morrison et al., 1999). Sublineages committed to the production of distinct subsets of crest derivatives have also been identified in vitro, including progenitors for the sensory (Greenwood et al., 1999), autonomic (Sommer et al., 1995) and glial (Shah et al., 1994) sublineages (reviewed by Anderson et al., 1997). In contrast to the production of committed sublineages by hematopoeitic and neural crest cells, a common progenitor in the retina appears to generate different cell types at different times during development (Turner and Cepko, 1987; Turner et al., 1990), similarly to cortical neurons. Cepko et al., (1996) have proposed that retinal progenitors progress through a series of state changes: at each stage, a progenitor can produce only one or a few phenotypes and movement from one state to the next occurs over time within a lineage.

Little is understood about the cellular nature of changes within cortical progenitors over time, how these changes impact fate potential, or what mechanisms drive the changing competence of cells to respond to fate-inducing cues. Here we ask whether the restriction in laminar fate potential by late progenitors is acquired suddenly or progressively, by challenging cortical progenitor cells at middle stages of neurogenesis to adopt either earlier- or later-generated fates. Such transplanted cells lie temporally between early, multipotent deep-layer progenitors and the late, committed precursors to upper-layer neurons. We find that laminar fate potential becomes restricted in a progressive, stepwise manner during cortical development: mid-stage progenitors can produce phenotypes generated normally at later times during development, but are incompetent to produce earlier-generated phenotypes. Cortical progenitors thus appear to start off with a broad potential to produce all of the layers and gradually lose the competence to produce earlier-generated phenotypes. Finally, we show that the loss of potential to produce a particular layer lags behind the final production of that layer, suggesting that fate restriction does not occur through a purely cell-autonomous mechanism such as counting mitotic divisions.

MATERIALS AND METHODS

Animals

Transplants were performed on timed-pregnant ferrets (*Mustela furo*, Marshall Farms, NY), in which cortical neurogenesis extends into postnatal life (Jackson et al., 1989). Gestation lasts 41 days; the day of birth is postnatal day (P) 0. Heterochronic transplants were performed by injecting cells from embryonic day (E) 36 fetuses into host brains at two ages: 50 ferrets from 6 litters received transplants on E30, and 19 ferrets from 3 litters received transplants on P2. Isochronic transplants of E30 cells into E30 hosts were performed on 145 ferrets from 16 litters.

Birthdating

To study the normal development of neurons generated on E30 and E36, fetuses were injected with [^{3}H]thymidine (500 μCi /0.1 ml) in utero (McConnell, 1988b). Kits were born naturally and survived until maturity (6 weeks postnatal). To study the positions of layer 6 and layer 5 neurons on E36, embryos were birthdated as above on E30 or E33 and killed on E36.

Transplantation

To isolate E36 progenitor cells, 400 μm coronal slices were made from E36 donor brains (Roberts et al., 1993). The ventricular zone was dissected from the cortical plate using fine knives and dissociated enzymatically with papain (6 U/ml, 20-45 minutes). 1 μCi [^{3}H]thymidine/μl was added during enzyme incubation to label progenitors in S-phase, a stage at which early (E29) progenitors are multipotent (McConnell and Kaznowski, 1991). Dissociated cells were concentrated in Hank's Buffered Salt Solution at a density of approx. 5×10^6 cells/ml.

Transplantation into younger hosts was performed as in Frantz and McConnell (1996). Briefly, pregnant ferrets were anesthetized on E30 with halothane (1-4% in O_2). The uterine horns were exposed and fetal heads were extruded through incisions in the uterine musculature. Cell injections (1-2 μl/injection, approx. 15,000 cells) were made into the lateral ventricle of each hemisphere, enabling incorporation of injected cells into the host ventricular zone (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995). Host animals were delivered by Caesarian section on E41 and cross-fostered to lactating jills. For transplantation into older hosts, P2 ferrets were anesthetized with halothane, their heads were stabilized with molding clay, and cells were injected into the ventricular zone through a glass micropipette using a hydraulic microdrive (McConnell, 1988b). All hosts were raised to maturity (6 weeks postnatal).

Control transplants of E36 cells into E36 recipients were not attempted. Because the ability of cells to incorporate into the ventricular zone from the lateral ventricle is lost over time (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995), performing these experiments would require that cells be targeted directly into the ventricular zone, an extremely difficult feat when both the fetal head and injection pipette must be stabilized by hand.

Perfusions and histology

Ferrets were killed with an overdose of Nembutal and perfused through the heart with 4% paraformaldehyde. Brains were removed, sunk in 20% sucrose, and sectioned sagittally at 20 μm on a freezing microtome. Sections were mounted and dried on gelatin-subbed slides, defatted in xylenes, dipped in 2% gelatin, coated with Kodak NTB2 nuclear track emulsion and incubated in the dark at 4°C for 6 weeks. Slides were then developed with Kodak D-19 developer, fixed with Kodak Rapid Fix and counterstained with cresyl violet. Transplanted cells labeled with [^{3}H]thymidine were identified by aggregations of silver grains over cell nuclei. Heavily labeled cells contained at least half the maximal number of silver grains observed (typically ≥20 silver grains). Lightly labeled cells, which presumably have undergone cell division in the host environment after transplantation, showed less than half the maximal number of silver grains (<20 silver grains) (McConnell, 1988b). Neurons were readily identifiable by their large nuclei, pale cytoplasm and proximal dendrites (McConnell, 1988b). One brain from an isochronic transplant (E30 into E30) contained [^{3}H]thymidine-labeled cells with abnormal morphologies (small, round, lacking processes) concentrated in layers 1 and upper 2/3. This brain was excluded from further analysis.

Microscopy and camera lucida reconstructions

Sections were visualized with a Nikon Optiphot microscope and viewed in dark field using Fiber-Lite illumination (Micro Video Instruments, Inc.). Camera lucida reconstructions were made using a Nikon drawing tube. Reconstructions were scanned into the computer and re-traced using Canvas 5.0. For quantification of the fraction of transplanted cells in each layer, only those cells that migrated into the cortex were included (McConnell and Kaznowski, 1991).

RESULTS

To ascertain whether cortical progenitors become progressively restricted in their fate potential during the course of development, we transplanted progenitor cells from the ferret at mid-stages of cortical neurogenesis, during the time of layer 4 formation, into host brains of different ages.

Identification of the progenitors of layer 4 neurons

We first identified a stage at which progenitors might display a state of competence intermediate between that of early (multipotent) and late (committed) precursors. Previous studies of ferret neurogenesis suggested that cells of the middle layer (layer 4) of the cortex are generated on and around E36 (Jackson et al., 1989). To confirm this, and to provide a baseline for comparison with transplant results, we injected [^{3}H]thymidine into E36 fetuses and examined the final positions of cells generated on that day by performing autoradiography on the mature (6 weeks postnatal) cortex. The laminar positions of both heavily and lightly labeled are tabulated in Fig. 1. Most heavily labeled cells (82.3%) born on E36 were found in layer 4, with a small percentage (16.1%) in layer 2/3. Lightly labeled cells, which undertook one or more additional rounds of cell division after labeling, were shifted toward more superficial positions, with 55.4% in layer 4 and 41.4% in layer 2/3. These data are consistent with the later birthdates of neurons in progressively more superficial positions (Angevine and Sidman, 1961; Jackson et al., 1989), and confirm that E36 progenitors normally generate neurons destined for layer 4.

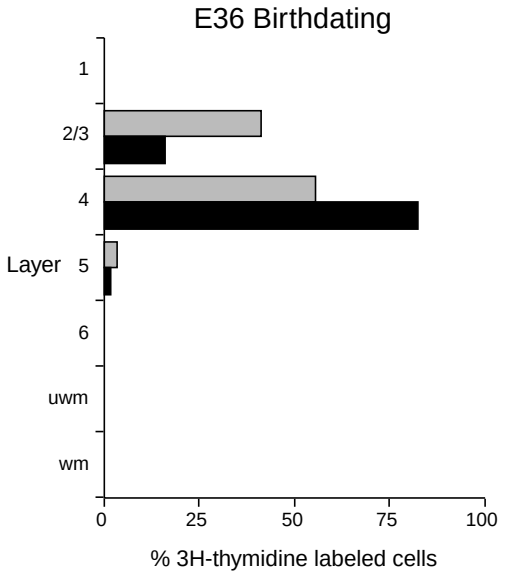

Fig. 1. Laminar positions of neurons born on E36 in the ferret. Black bars show the final laminar positions of cells heavily labeled with [^{3}H]thymidine (*n*=373), and gray bars show the positions of lightly labeled cells (*n*=249). Most heavily and lightly labeled cells were found in layer 4, with smaller fractions migrating to layer 2/3. Abbreviations: wm, white matter; uwm, white matter directly underlying the cortex (remnant of embryonic subplate zone).

Layer 4 progenitors are multipotent when transplanted into older host brains

To assess the developmental potential of presumptive layer 4 cells, E36 progenitors were first transplanted forward in developmental time into brains in which layer 2/3 neurons were being born. Previous isochronic control transplants of P2 progenitors into P2 hosts showed that the transplanted cells migrated exclusively to layer 2/3, the position appropriate for both their origin and their new environment (McConnell, 1988b). For the current experiments, cerebral ventricular cells were dissected on E36 and labeled with [^{3}H]thymidine during dissociation, immediately before transplantation. This procedure radioactively tags cells in S-phase, a time when early progenitors are multipotent (McConnell and Kaznowski, 1991). Labeled cells were injected into the ventricular zone of P2 hosts, which were killed 6 weeks later. Their brains were sectioned and [^{3}H]thymidine-labeled neurons were visualized using autoradiography. As in prior experiments (McConnell, 1988b; McConnell and Kaznowski, 1991; Frantz and McConnell, 1996; Bohner et al., 1997), the transplanted cells migrated away from the ventricular zone, integrated into the cortex and assumed morphologies typical of normal cortical neurons (not shown).

If the progenitors of layer 4 neurons were restricted in their laminar fate potential, they should migrate to layer 4, the fate characteristic of their birthday. If the cells were multipotent, they should alter their normal fates and migrate to layer 2/3 along with newly generated host neurons. As shown in a representative camera lucida reconstruction, nearly all heavily labeled transplanted neurons were located in layer 2/3 (Fig. 2A). Most transplanted cells were found in the visual and splenial areas of neocortex, although small numbers were

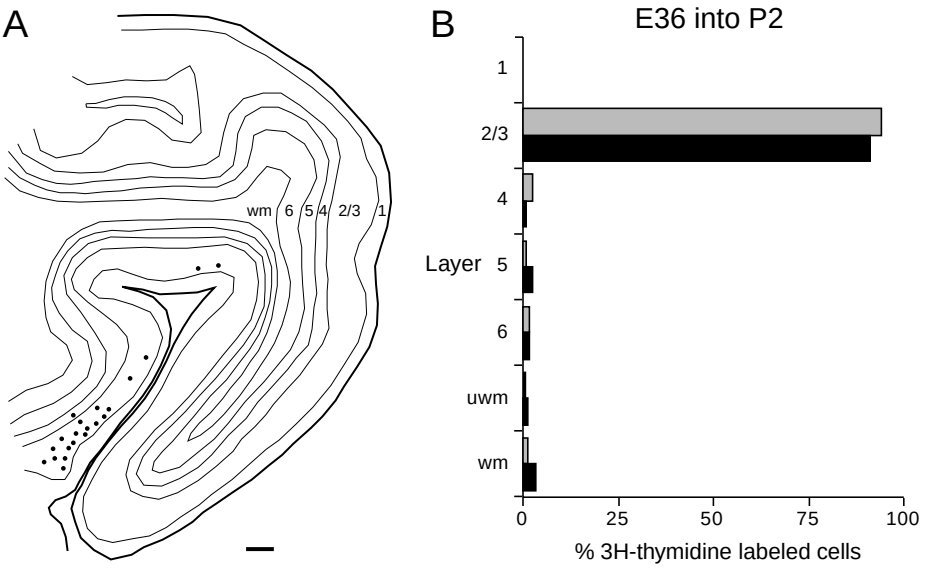

Fig. 2. Results of heterochronic transplantation of E36 progenitors into P2 hosts. (A) Camera lucida reconstruction of the posterior (visual) cortex of an adult host brain. Neurons heavily labeled with [^{3}H]thymidine (black dots) were found almost exclusively in layer 2/3. Sagittal section; dorsal is up, anterior to the right. Scale bar, 500 μm. (B) Final laminar positions of [^{3}H]thymidine-labeled, transplanted neurons. Black bars, heavily labeled neurons (*n*=260); gray bars, lightly labeled neurons (*n*=320). The vast majority of both heavily and lightly labeled cells occupied layer 2/3, the normal position of neurons generated on P2. Abbreviations as in Fig. 1.

recovered in more anterior neocortical areas and in entorhinal cortex. Cells from all neocortical areas were included in our analysis, but those from entorhinal cortex were excluded.

The laminar positions of both heavily and lightly labeled neurons were quantitated from 6 animals in 2 independent experiments (Fig. 2B). The large majority (91.2%) of heavily labeled neurons abandoned their normal fates and migrated to the laminar destination typical of the host environment, layer 2/3. Lightly labeled cells, which went through an additional round of cell division in the host, also changed fates and migrated to layer 2/3. These results demonstrate that E36 progenitors can alter their normal laminar phenotype and adopt an upper layer position, suggesting that E36 progenitors are capable of generating laminar fates normally generated on or after E36. This finding is in accordance with lineage studies suggesting that E36 progenitors will normally produce not only layer 4 neurons but also the progenitor cells that populate layer 2/3 (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Reid et al., 1995, 1997).

Layer 6 neurons are generated on and around E30

Although E36 cells are competent to produce layer 2/3 neurons, which are normally generated later during development, we also wanted to transplant these cells backward in developmental time and assess their ability to produce earlier-generated layers. If E36 cells were fully multipotent, they should be competent to change fates and migrate to the deepest cortical layer. But if E36 cells were partially restricted in fate potential, they might bypass the deepest layers and instead home to the middle-layer positions typical of their origin. Previous birthdating studies of ferret neurogenesis suggested that neurons at the base of the cortical plate, in layer 6, are born on and around E30 (Jackson et al., 1989). To confirm this, we injected [^{3}H]thymidine into E30 fetuses and assessed the positions of labeled cells at maturity (Fig. 3). The majority of heavily labeled cells (89.6%) born on E30 were in layer 6, with a small percentage (2.5%) in layer 5. Lightly labeled cells were also found predominantly in layer 6, as expected (Jackson et al., 1989). No layer 4 neurons were generated at this age, suggesting that E30 hosts would provide an ideal environment into which to transplant E36 progenitor cells.

Control isochronic transplantation of E30 progenitors into E30 hosts

To ensure that the transplantation of cells into E30 brains does not on its own initiate a laminar fate change, control experiments were performed in which E30 ventricular cells were transplanted isochronically into E30 hosts. Most transplanted cells were recovered in visual neocortex, although some cells were found in more anterior regions (Fig. 4A). As expected, the majority of heavily labeled, transplanted neurons migrated to layer 6 (Fig. 4A,B). In addition, many labeled cells

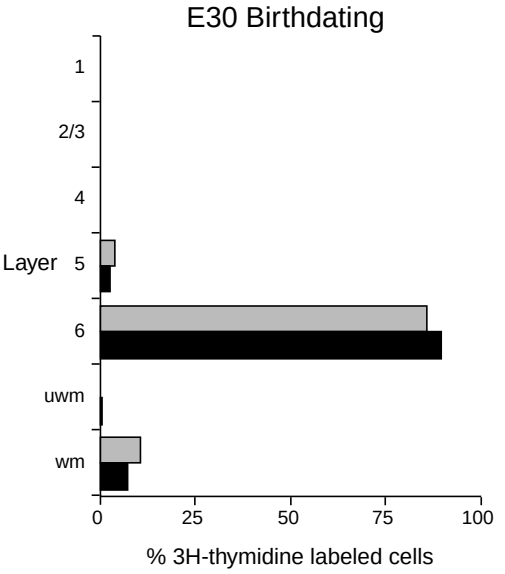

Fig. 3. Laminar positions of neurons born on E30 in the ferret. Black bars, heavily labeled neurons (*n*=163); gray bars, lightly labeled neurons (*n*=154). Both heavily and lightly labeled cells shared similar distributions, with nearly all cells occupying layer 6. Abbreviations as in Fig. 1.

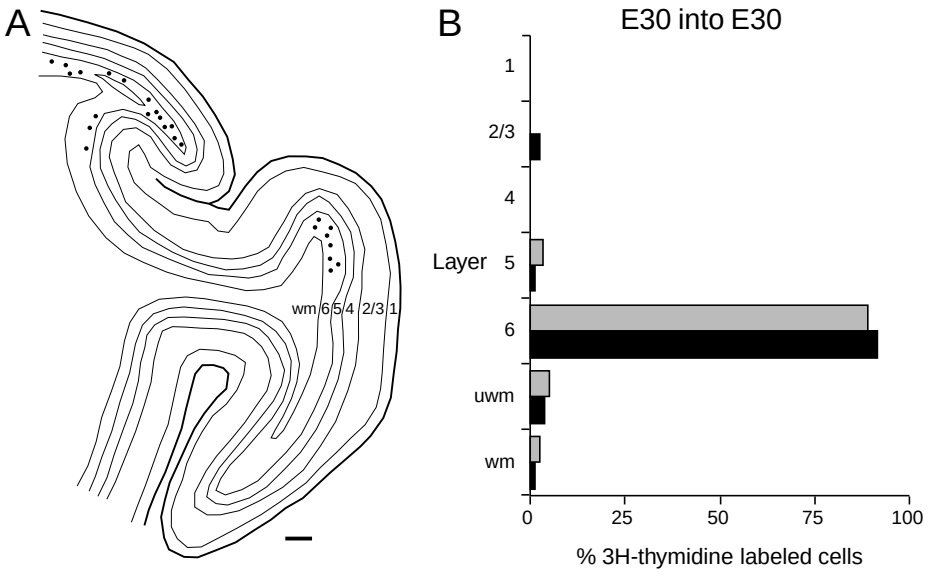

Fig. 4. Results of isochronic transplantation of E30 progenitors into E30 hosts. (A) Camera lucida reconstruction of the posterior cortex of an adult host brain. Heavily labeled neurons (black dots) were found almost exclusively in layer 6. Sagittal section; dorsal is up, anterior to the right. Scale bar, 500 μm. (B) Final laminar positions of [^{3}H]thymidine-labeled neurons. Black bars, heavily labeled neurons (*n*=81); gray bars, lightly labeled neurons (*n*=119). Nearly all labeled transplanted neurons migrated to layer 6, the layer generated normally on E30. Abbreviations as in Fig. 1.

were found in or near the ventricular zone in sites characterized by a mildly disrupted cytoarchitecture (not shown). These incorporation sites are found commonly when injections of cells are made into the lateral ventricle (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995; Frantz and McConnell, 1996).

The laminar distribution of [^{3}H]thymidine-labeled cells that migrated into cortex was tabulated from 8 animals in 3 independent experiments (Fig. 4B). The large majority of heavily labeled neurons (91.4%) migrated to layer 6, the layer generated on E30 and the layer to which the neurons would migrate normally. These results demonstrate that transplantation per se does not initiate a fate change in cortical neurons.

Layer 4 progenitors are unable to generate layer 6 neurons

To ascertain whether presumptive layer 4 cells are restricted from adopting a layer 6 fate, E36 progenitors were transplanted into E30 host brains. Ventricular cells were dissected from E36 donors, labeled with [^{3}H]thymidine in vitro and transplanted into the lateral ventricles of E30 hosts. We predicted that, if E36 progenitors were restricted in their fate potential to layers produced on or after E36, their daughters should bypass layer 6 and instead migrate to layer 4. If multipotent, the transplanted cells should populate layer 6. Our results suggest that fate potential is restricted, but in a manner more complex than hypothesized. As in the other transplants, labeled neurons were found throughout neocortex, but occupied primarily the visual and splenial areas (Fig. 5A). Most heavily labeled cells were found in layers 2/3, 4 and 5; however, almost no transplanted neurons were present in layer 6.

Quantification of the laminar distribution of transplanted neurons in 10 animals from 3 experiments (Fig. 5B) revealed that only 2% of cells migrated to layer 6, the layer generated in the host environment. The near-absence of labeled cells in layer 6 indicates that E36 progenitors are no longer competent to respond to cues present at E30 that specify the formation of layer 6 neurons (Bohner et al., 1997) and thus have become restricted in their laminar fate potential. However, we hypothesized that a restricted E36 progenitor should produce daughters that migrate exclusively to layer 4, the layer generated on E36. Instead, many transplanted neurons migrated to layer 5 (40.8%; Fig. 5B), a position appropriate for neither the donor nor the host environment. A third of heavily labeled neurons migrated to layer 4 (33.3%), and 23.8% migrated to layer 2/3, a somewhat higher number than produced normally on E36 (16.1%; Fig. 1). Lightly labeled cells had a similar distribution, though slightly more cells migrated to layer 2/3. These laminar distributions indicate that E36 progenitors are restricted from generating layer 6 neurons, but are still competent to make all other layers of the cortical plate, including layer 5, the genesis of which is normally complete by E36.

Positions of deep-layer neurons during layer 4 neurogenesis

Either intrinsic or extrinsic mechanisms could alter the developmental potential of cortical progenitors over time. For example, a cell-intrinsic mechanism such as counting cell divisions might progressively alter the capacity of E36 progenitors to generate deep-layer neurons. However, our data contrast with the simplest cell-intrinsic models; [^{3}H]thymidine birthdating reveals that neither layer 6 nor layer 5 neurons are generated normally on E36 (Fig. 1), yet only the potential to make layer 6 neurons has been lost. If progenitors were simply counting cell divisions, they should no longer be competent to generate layer 5 cells. An alternative model is that extrinsic signals, possibly arising from maturing neurons in the cortical plate, could affect progressive changes in progenitors via signals that result in a loss of competence (McConnell, 1995). Such a “feedback” model might predict that on E36, the migration of layer 6 neurons into their final positions is complete, enabling layer 6 neurons to signal back to the ventricular zone and restrict further production of layer 6. This model might also account for the maintenance of the potential to produce layer 5 neurons: if layer 5 was not formed fully at E36, no signal to halt layer 5 production would have been sent; thus E36 progenitors would remain competent to generate layer 5.

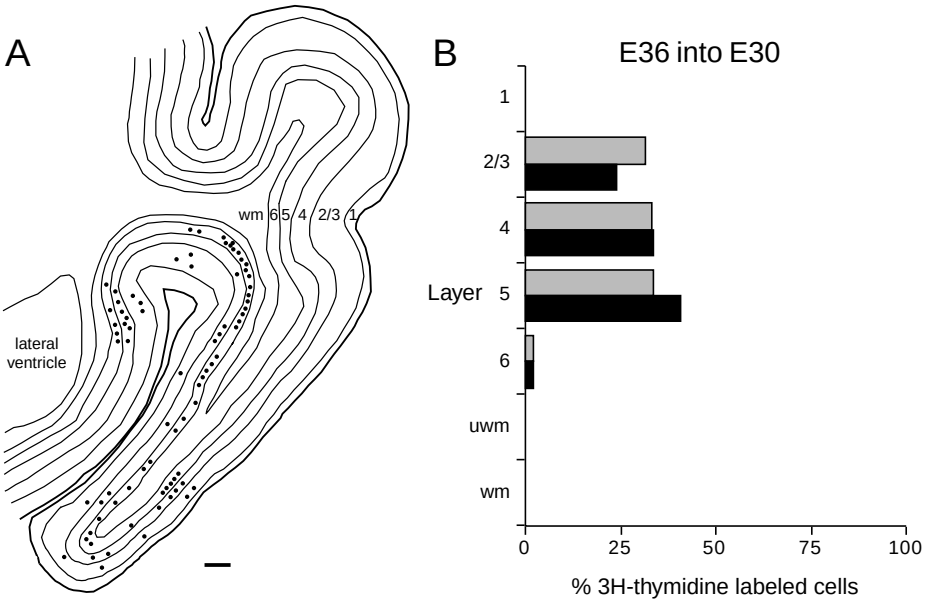

Fig. 5. Results of heterochronic transplantation of E36 progenitors into E30 hosts. (A) Camera lucida reconstruction of the posterior cortex of an adult host brain. Heavily labeled neurons (black dots) were found predominantly in layers 4 and 5, with a small fraction in layer 2/3. No labeled cells were visible in layer 6. Saggital section; dorsal is up, anterior to the right. Scale bar, 500 μm. (B) Final laminar positions of [^{3}H]thymidine-labeled neurons. Black bars, heavily labeled neurons (n=201); gray bars, lightly labeled neurons (n=339). Both heavily and lightly labeled cells were found predominantly in layers 5, 4 and 2/3, but not in layer 6. Abbreviations as in Fig. 1.

A simple prediction of this feedback hypothesis is that, on E36, layer 6 is fully formed and in place, whereas the neurons of layer 5 are still coalescing into a layer. To test this, we injected [^{3}H]thymidine on either E30, when layer 6 neurons are born, or E33, to label layer 5 cells (Jackson et al., 1989), and assessed on E36 the positions of [^{3}H]thymidine-labeled cells. On E36, cells born on E30 had completed their migration into layer 6 and were located in the cortical plate (Fig. 6A). In contrast, cells born on E33 had not coalesced into a layer on E36, and were still migrating through the intermediate zone en route to the cortical plate (Fig. 6B). These results are consistent with the hypothesis that on E36, layer 6 neurons have signaled progenitors to lose the potential for further production of layer 6, but that migrating layer 5 neurons have yet to provide a feedback signal. A direct test of this hypothesis might involve the targeted ablation of layer 6 neurons prior to layer formation, followed by an examination of the developmental potential of progenitor cells in the ventricular zone.

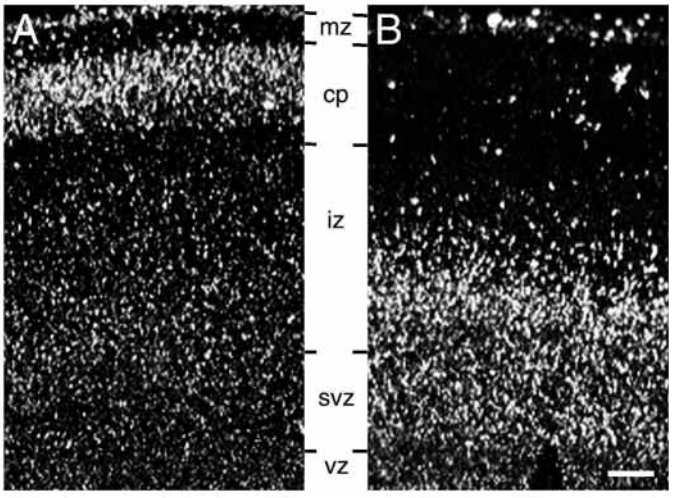

Fig. 6. Laminar positions of cortical cells labeled with [^{3}H]thymidine on E30 or E33 and examined on E36. Sagittal sections viewed in dark field. (A) Cells labeled with [^{3}H]thymidine on E30 have taken up residence in layer 6 of the cortical plate by E36. (B) On E36, cells labeled with [^{3}H]thymidine on E33 are still migrating through the intermediate zone, en route to their final positions within layer 5. Abbreviations: vz, ventricular zone; svz, subventricular zone; iz, intermediate zone; cp, cortical plate; mz, marginal zone. Scale bar, 100 μm.

DISCUSSION

Although progenitor cells early in cortical development are multipotent with respect to the laminar fates they can generate (McConnell and Kaznowski, 1991; Bohner et al., 1997), progenitors at late stages are committed to the production of neurons destined for the most superficial layers (Frantz and McConnell, 1996). In this study, we demonstrate that progenitors during a middle stage of neurogenesis are partially restricted in their fate potential, suggesting that a restriction in competence is acquired progressively during development. In the ferret, E36 progenitors normally produce neurons destined for cortical layer 4. When transplanted heterochronically into older hosts, neurons derived from the transplanted progenitors could change their laminar fates and migrate to layer 2/3. E36 progenitors are therefore fully competent to acquire a later-generated fate. In contrast, E36 cells transplanted into E30 hosts, during layer 6 neurogenesis, did not adopt the laminar fate typical of the younger environment: barely any labeled cells migrated to layer 6. E36 progenitors are thus partially restricted in the laminar fates that they can produce. Interestingly, while these progenitors could not adopt a layer 6 fate, they did retain the potential to produce another earlier-generated phenotype, that of layer 5. The migration of transplanted neurons to both layer 4 and layer 5 was a surprising outcome, because layer 5 represents a fate typical of neither the host (E30) nor the donor (E36), but is rather a phenotype generated between these two times in development.

Cellular mechanisms underlying changes in developmental competence

The loss by E36 cells of the potential to produce layer 6 neurons could result from systematic changes over time in a uniform population of progenitors. Alternatively, the ventricular zone might contain distinct subpopulations of progenitors, each capable of producing a small subset of laminar phenotypes, that get depleted at different times during development. If there existed a separate pool of progenitors whose sole purpose was to generate layer 6, and this pool was depleted by E36, we might witness an apparent restriction in fate potential over time that actually resulted from the loss of progenitors predestined for layer 6. Although it is difficult to rule out this possibility definitively, we do not favor this interpretation for several reasons. First, retroviral lineage-tracing experiments in both ferret and rodent have shown that early progenitor cells give rise to clones spanning many cortical layers (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Reid et al., 1995, 1997), whereas progenitors restricted to layer 6 production would generate clones occupying only layer 6. In ferret, retroviral infection of early progenitors results in clones containing single neurons or large, multicell clones containing both glia and neurons, with the latter spanning several layers (Reid et al., 1997). These data suggest that cortical progenitors divide asymmetrically during neurogenesis, each division typically producing a postmitotic neuron and another progenitor cell. Asymmetric divisions in the ferret ventricular zone have been visualized directly by time-lapse confocal microscopy of living brain slices: horizontally oriented cleavages generated a pially oriented (basal) daughter that migrated away, presumably to become a neuron, and an apically located sister that remained in the ventricular zone (Chenn and McConnell, 1995). Collectively these data suggest that early progenitors are multifated and progressively generate many laminar phenotypes through asymmetric cell divisions. This notion is strengthened further by results of transplanting early (E29) progenitors into older host brains. Whereas heavily labeled cells migrate to layer 6 if transplanted around or after the time of mitosis, these cells can change fates if transplanted earlier in the cell cycle (McConnell and Kaznowski, 1991). These results show that E29 progenitors are directly competent to form upper-layer neurons.

Intrinsic changes in ventricular cells may trigger changes in the cells' responsiveness to inductive cues that create distinct laminar phenotypes. Indeed, we have observed molecular changes in progenitors over time. For example, early progenitors express high levels of the homeodomain transcription factor *Otx1*,which is maintained by the deep-layer progeny of these cells. At later times, when progenitors have lost the competence to produce deep-layer neurons, *Otx1* expression in the ventricular zone is markedly diminished (Frantz et al., 1994). This and other examples of changes in gene expression by progenitors (Burrows et al., 1997; A. Okada and S. K. McC., unpublished) provide independent evidence of the changing and dynamic nature of the ventricular zone.

Progressive restrictions in cell fate potential in the developing nervous system

The nervous system provides several examples of uncommitted progenitor cells that restrict their fate potential over time. Neural crest cells are multipotent early in development and give rise to diverse progeny (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991). Early premigratory crest cells can differentiate into a wide range of derivatives in culture or upon transplantation. A small number of cells appear to retain this multipotency, serving as self-renewing neural crest stem cells (Stemple and Anderson, 1992). However, many crest cells at later stages show partial restrictions in fate potential: for example, late-emigrating crest cells can differentiate into melanocytes but not adrenergic neurons (Artinger and Bronner-Fraser, 1992), and postmigratory crest progenitors expressing the receptor tyrosine kinase c-RET are committed to neurogenesis even when challenged with glial growth factor, which promotes glial differentiation in early crest cells (Shah et al., 1994; Lo and Anderson, 1995). These and other examples suggest that initially multipotent neural crest cells progressively restrict their developmental potential through the production of lineage-restricted precursor cells (Anderson, 1993; Anderson et al., 1997).

In the CNS, it is not clear whether the changing competence of progenitors to respond to extrinsic signals is a unique property of particular neuronal sublineages, or whether neurogenesis is relatively independent of lineage diversification for specifying neural fates. Although self-renewing neural stem cells have been identified in the CNS, they are relatively rare (reviewed by Barres, 1999). Progenitors in regions such as cortex and retina may represent restricted sublineages derived from stem cells, but lineage-tracing studies indicate that both cortical and retinal progenitors can produce glia and many different types of neurons in vivo (Turner and Cepko, 1987; Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Cepko et al., 1993; Reid et al., 1995, 1997). In vitro studies suggest that retinal progenitors derived from animals at different ages are biased intrinsically to generate cell types appropriate for that developmental stage: early progenitors produce ganglion cells and later progenitors make rod photoreceptors (Reh and Kljavin, 1989). Retinal progenitors are not, however, committed to the production of a single cell type at each age, because the cells can respond to extrinsic growth factors and alter their fate (e.g., Levine et al., 1997). Despite this plasticity, the range of fates open to a retinal cell at any particular time is apparently quite narrow; at each stage, the cell is competent to produce only a few cell types (Cepko et al., 1996). Once a cell has passed through one stage of competence, it appears unable to go back and produce cell types generated at earlier stages.

Cortical progenitors alter their competencies in a manner distinct from that seen in the retina. Our studies suggest that cortical progenitors start off with a broad potential to produce neurons of any layer, then gradually lose their competence to produce earlier-generated phenotypes. Thus, instead of moving through discrete state changes, as proposed for the retina (Cepko et al., 1996), cortical progenitors seem to progress through an ever-narrowing funnel of possible fates. It is not clear why two layered regions of the CNS should differ in this regard, although one might argue that the retina must produce a greater diversity of functional phenotypes (e.g., light-transducing photoreceptors, non-spike-generating interneurons, and spike-generating ganglion cells) compared to cortex, and thus requires a distinct strategy for the generation of such diverse cell types.

Cell-autonomous and extrinsically driven changes in competence

A number of mechanisms could drive changes in fate potential over time, but these generally fall into two classes: cell-autonomous and induced. Cell-autonomous changes could be accomplished if progenitors counted divisions internally, with the number of divisions determining their laminar competence. Similarly, progenitors might progressively dilute or accumulate an intrinsic factor required in threshold amounts for the production of particular fates. An intensively studied case of a cell-intrinsic clock or timer is the oligodendrocyte-type 2 astrocyte (O2-A) progenitor cell, which differentiates synchronously into oligodendrocytes after several symmetric divisions in culture, even in the continued presence of mitogen (Temple and Raff, 1986; Raff et al., 1988; Raff, 1989; Lillien and Raff, 1990). The cell-intrinsic timer seems to measure time and not cell divisions per se (Gao et al., 1997), and depends on the accumulation of both thyroid hormone receptor, which enables cells to respond to the differentiation-inducing effects of thyroid hormone (Gao et al., 1998), and the cyclin-dependent kinase inhibitor p27 (kip1), which promotes cell cycle exit (Durand et al., 1997). An alternative mechanism by which progenitor cells might acquire a restricted fate potential involves exposure to extrinsic cues (Anderson, 1993). Inductive signals influence the commitment of neural crest stem cells to various sublineages. For example, brief exposure to glial growth factor suppresses neuronal differentiation by multipotent rat neural crest cells, but longer exposures commit cells to a glial fate (Shah et al., 1994; Shah and Anderson, 1997). In contrast, bone morphogenetic protein 2 promotes neurogenesis by neural crest stem cells (Shah et al., 1996; Shah and Anderson, 1997; Lo et al., 1998). These studies provide compelling parallels with the hematopoeitic system, in which peptide factors also trigger the production of committed sublineages of progenitors (Metcalf, 1998).

Our data suggest that it is unlikely that a solely cell-intrinsic mechanism, such as counting divisions, specifies fate decisions in the developing cerebral cortex. If cortical cells were simply counting mitotic divisions, E36 cells (which normally produce only neurons destined for layer 4 and above) would have already counted past the divisions that enable the production of layers 5 and 6. While our finding that E36 progenitors are restricted from making layer 6 cells is congruent with such a possibility, the observation that E36 cells can still produce layer 5 neurons is inconsistent with a strict counting mechanism.

We think it more likely that extrinsic signals effect cell-intrinsic changes in progenitor cells, which alter the progenitors' ability to respond to inductive cues encountered subsequently. In both cortex and retina, progenitor cells show altered patterns of gene expression over time, indicative of intrinsic changes that correlate with alterations in fate potential

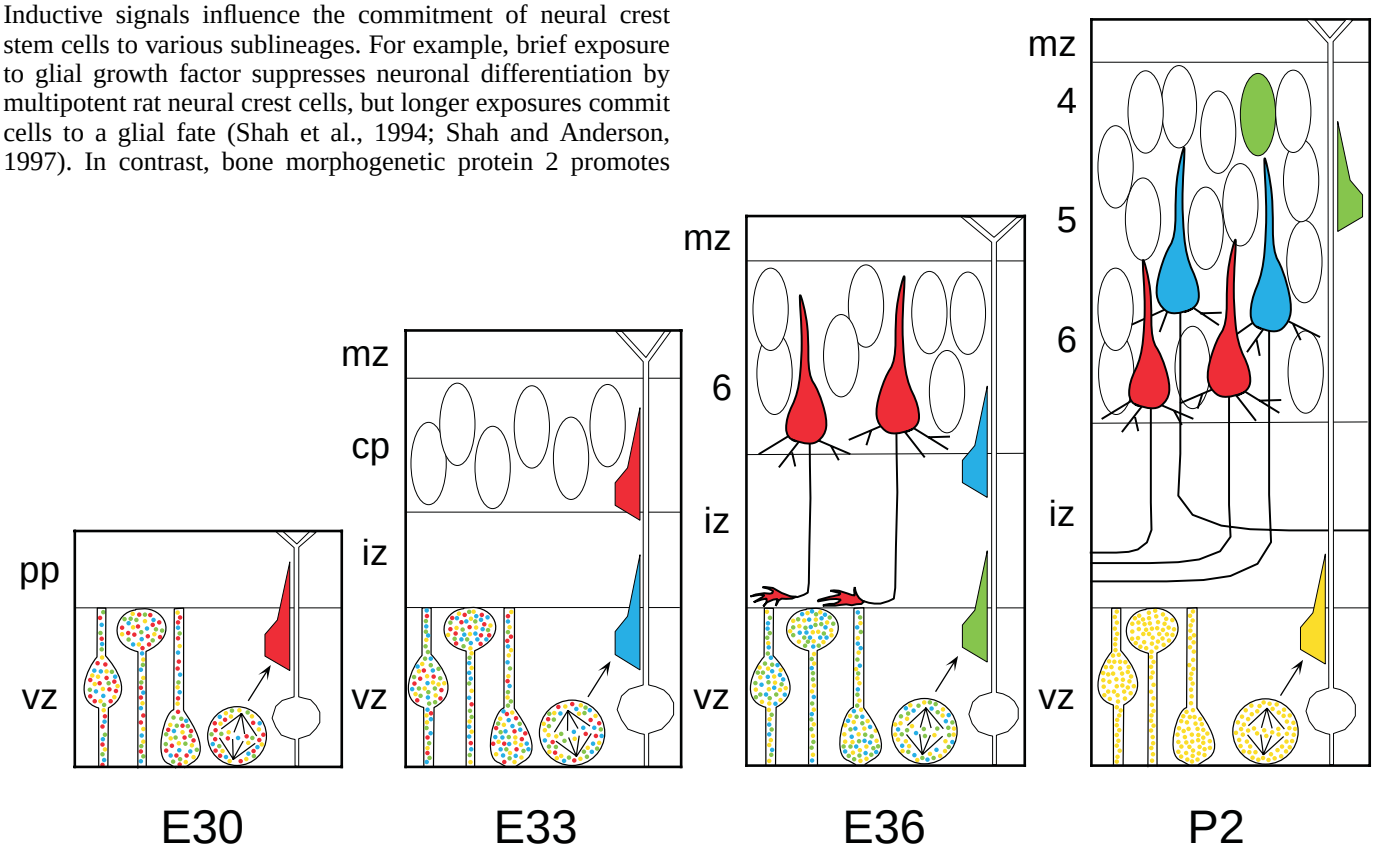

Fig. 7. Model of how a cortical layer, once fully formed, might provide a feedback signal to progenitor cells in the ventricular zone, resulting in a loss of potential by the progenitors from further generation of that layer. The dotted patterns represent each cell's potential to produce neurons of different layers (layer 6, red; layer 5, blue; layer 4, green; layer 2/3, yellow). On E30, ventricular cells are generating layer 6 neurons (red), but have the potential to produce all the layers. On E33, progenitors are signaled to begin generating layer 5. By E36, progenitors are signaled to generate layer 4 neurons. The progenitors have lost the potential to make layer 6 neurons but retain the ability to generate layers 5, 4 and 2/3 (blue, green, yellow dots). At this same age, layer 6 neurons have reached the cortical plate (Fig. 6) and extend axons along the ventricular surface (Kim et al., 1991), whereas layer 5 neurons (blue) are still migrating to the cortical plate. On P2, progenitors are committed to the production of upper layer neurons (yellow) (Frantz and McConnell, 1996). Abbreviations: pp, preplate; remainder as in Fig. 6.

(Lillien and Cepko, 1992; Frantz et al., 1994; Alexiades and Cepko, 1997; A. Okada and S. K. McC., unpublished). Feedback signals from postmitotic neurons may play a role in generating these changes and in inhibiting further production of a particular cell type. Coculture experiments have demonstrated that the presence of amacrine cells specifically inhibits embryonic retinal progenitors from producing amacrine cells in vitro (Belliveau and Cepko, 1999). Similarly, ganglion cells secrete a factor that actively inhibits embryonic progenitors from choosing the ganglion cell fate (Waid and McLoon, 1998). Collectively these studies suggest that the number of amacrine and ganglion cells are regulated by the feedback inhibition of progenitor cells by postmitotic neurons (Reh and Tully, 1986).

We propose that similar mechanisms not only regulate the production of certain classes of neurons in developing cortex, but in a second step also impinge directly on progenitors to alter their developmental potential (Fig. 7). In the first step, inhibitory feedback from the production of layer 6 neurons rapidly triggers the cessation of layer 6 production and/or the promotion of layer 5 neurogenesis (Fig. 7: E33). This signal would be relayed to progenitors before layer 6 neurons have migrated into place. However, the permanent loss by E36 ventricular cells of the potential to produce layer 6 neurons correlates temporally with the completed migration of postmitotic neurons into layer 6. At this time, layer 5 neurons are still migrating into place and E36 progenitors retain the capacity to generate layer 5 cells. We thus hypothesize a second step in which cortical neurons provide inhibitory feedback to the ventricular zone, but only after completing their migration into the cortical plate (Fig. 7: E36). Feedback signals could be sent to the ventricular zone via the axons of differentiating neurons, which extend radially to the top of the ventricular zone, make a sharp turn and travel along the ventricular surface for a short distance (McConnell et al., 1989; Kim et al., 1991). A permanent loss of competence could be triggered by novel feedback signals, or the axons might express the same signals presented earlier, which thus exert a cumulative effect. Although these hypotheses have yet to be tested experimentally, feedback models for the regulation of neurogenesis are particularly plausible for laminated structures like retina and cortex: each generates cells that migrate over relatively short distances, permitting short-range influences between differentiating neurons and progenitors. In contrast, cells of the neural crest migrate over large distances and through varied terrain, enabling them to utilize local environmental signals to induce specific fates (Anderson, 1993).

A remaining mystery is why E36 progenitors produce neurons that migrate to layer 5 when transplanted into E30 hosts, an environment in which layer 5 has yet to be formed. One possibility is that E36 cells have undergone cell-intrinsic changes that preclude the accurate interpretation of signals which normally induce layer 6 (Bohner et al., 1997). Perhaps these inductive cues are not ignored, but are interpreted instead in a novel manner, resulting in the production of layer 5 neurons. However, only about a third of the transplanted cells migrated into layer 5; the remainder were distributed in layers 4 and 2/3, fates appropriate for cells generated at later times in development. It is possible that the E36 cells transplanted into younger hosts fail to receive an interpretable signal that specifies a fate, and thus occupy at random any layer except layer 6. A final possibility is that the E36 ventricular zone is heterogeneous with respect to the developmental potential of each progenitor cell: perhaps some cells have lost the potential to produce layer 6 neurons and therefore migrate to layer 5, while others have lost the potential for making both layers 5 and 6 and thus end up in layer 4. Regardless of which hypothesis is correct, it is important to emphasize that E36 progenitors as a population are perfectly capable of responding to cues that induce a later-generated fate (layer 2/3) when transplanted into an older host brain during S-phase. Collectively our data suggest that the cues that induce different laminar fates change over time and that the ability of progenitors to respond to these cues is altered progressively as well. The phenotype that results will thus be the product of the signals present in the environment and the ability of the progenitor to interpret these cues accurately.

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