Induction of deep layer cortical neurons in vitro

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SUMMARY

Transplantation studies suggest that the laminar fates of cerebral cortical neurons are determined by environmental signals encountered just before mitosis. In ferret, E29 progenitor cells normally produce neurons of layers 5 and 6. When transplanted during S-phase into an older ventricular zone, E29 progenitors produce neurons that change their fates and migrate to layer 2/3; however, cells transplanted later in the cell cycle migrate to their normal deep-layer positions even in an older environment (McConnell and Kaznowski, 1991). Here we utilize three culture systems to investigate the nature of the environmental signals involved in laminar specification. E29 cells were first cultured at low density to ascertain whether cell contact and/or short-range cues are required for deep layer specification. Neurons transplanted after a short time in low-density culture failed to adopt their normal fates and migrated instead to the upper layers. When crude cell contacts were restored by pelleting E29 cells together, most transplanted neurons cells became specified to their normal deep layer fates. Finally, E29 cells were transplanted after being cultured in explants that maintained the architecture of the cerebral wall. Explants allowed normal deep layer specification to occur, as transplanted cells migrated to layers 5 and 6. These results suggest that short-range cues induce multipotent progenitors to produce deep layer neurons.

Key words: induction, cerebral cortex, specification, commitment, pattern formation, neuron, cortical neuron

INTRODUCTION

As the structure responsible for the highest levels of cognitive function, the cerebral cortex has been an intensely scrutinized target of scientific investigation. The organization of the cortex into layers facilitates our understanding of its development and mature functions. Neurons in each layer tend to share several characteristics, including anatomy of projections, physiological properties and gene expression (reviewed in McConnell, 1995). The development of cortical layers proceeds in an organized manner (McConnell, 1988a). Neurons are generated in the ventricular zone (VZ), then migrate into the developing cortical plate. Layers are formed in an inside-out pattern, in which the deep layers are laid down first, followed by the addition of more superficial laminae. Thus, neurons within a given layer undergo their terminal mitotic divisions and migrate from the VZ to the cortical plate at similar times.

Transplantation experiments suggest that the cellular environment plays a critical role in layer formation. In ferret, embryonic day 29 (E29) progenitors normally produce neurons destined for the deep layers 6 and 5, while on postnatal day 1 (P1=E42), neurons of the upper layers 2 and 3 are being born (McConnell, 1988b; Jackson et al., 1989). Transplantation of E29 progenitors into P1 hosts has revealed that E29 cells are multipotent and can produce layer 2/3 neurons when transplanted during S-phase. However, when progenitors are transplanted later in the cell cycle, their progeny adopt the deep layer fates appropriate for their origin (McConnell and Kaznowski, 1991). The laminar fate of early progenitors can therefore be modified by environmental cues, but only during a sensitive period for specification that closes around the end of S-phase. By the time a young neuron is born, it has acquired a specific laminar fate that directs its migration to the appropriate layer.

The present study investigates the nature of the environmental cues that instruct early multipotent progenitors to produce deep or upper layer neurons. We used three cell culture systems to ask whether cell-cell interactions trigger the production of deep layer neurons. First, to investigate whether an active short-range cue is required for layer 6 specification, E29 cells were cultured at low density to deprive them of cell-cell contact and short-range diffusible cues. Second, to test whether specification can be reconstituted in culture, dissociated cells were reaggregated and cultured as pellets. Cell contact was preserved in pellets, but the organized neighbor-neighbor relations present in vivo were disrupted. Finally, to ensure that deep layer specification can occur in vitro, cells were cultured as intact explants maintaining the normal architecture of the VZ and cortical plate. After culturing progenitors using one of these three paradigms, transplantation into P1 ferrets was performed to assess the laminar fate choices made by postmitotic daughter neurons.

MATERIALS AND METHODS

Low-density cultures

The dorsal third of cerebral cortex from E29 ferrets (Marshall Farms, New York) was dissected into Hanks' Balanced Salt Solution (HBSS,

pH 7.4) and dissociated using procedures adapted from Huettner and Baughman (1986). Cells were incubated in papain (Worthington; 2 U/ml, 30 minutes, 32°C), washed with enzyme inhibitor solution [1 mg/ml bovine serum albumin (BSA); 1 mg/ml trypsin inhibitor (Boehringer Mannheim)], then centrifuged through 5 ml inhibitor solution (10 mg/ml BSA; 1 mg/ml trypsin inhibitor). Cells were diluted to ~1×10^5 cells/ml in Minimum Essential Medium (MEM; Gibco) without glutamine, then plated onto 150 mm Petri dishes at a density of ~110 cells/mm². Petri plastic was chosen as the minimally adhesive substrate that facilitated cell recovery after culture. After 4 or 6 hours of culture, cells were removed from dishes with a gentle stream of MEM followed by low Ca^{2+} salt solution. Cells were recovered by centrifugation and adjusted to the target concentration of 1.5-2.5×10^7 cells/ml for transplantation. Cell viability averaged 84%, as assessed by trypan blue exclusion.

[^{3}H]thymidine was administered at one of two times to cells cultured at low density. One set of cells was labeled with [^{3}H]thymidine (NEN; 1 μCi/ml enzyme solution) immediately after dissection during dissociation with papain, to label cells in S-phase when placed in culture. In separate experiments, embryos were labeled with [^{3}H]thymidine in utero (500 μCi in 0.1 ml saline/embryo) and left in place for 6 hours, to allow labeled progenitors to complete S-phase before dissociation (McConnell and Kaznowski, 1991). In some of the latter experiments, 5% fetal calf serum was added to the medium for the final 4 hours of culture.

Pellet cultures

E29 cortices were dissected, labeled with [^{3}H]thymidine during enzyme incubation and dissociated as above. To form pellets of ~3×10^6 cells each, 333 μl of cells (9×10^6 cells/ml) were centrifuged in a 1.5 ml microcentrifuge tube for 1 minute at 14,000 revs/minute in a tabletop microcentrifuge (Watanabe and Raff, 1992). Pellets were dislodged from tubes with a gentle stream of MEM, then cultured in 24-well plates (2 pellets/well in 600 μl MEM). After 6 hours, pellets were redissociated into single-cell suspensions for transplantation. Cell viability after the second dissociation averaged 97%. Alternatively, dissociated cells were reaggregated on a rotary shaker at 70 revs/minute for 18 hours (Garber and Moscona, 1972). Each 35 mm dish contained a total of 2×10^7 cells in 3 ml of MEM. After 18 hours, reaggregates were redissociated as above.

Explant cultures

E29 cortex was dissected into HBSS, then placed as explants into 24-well plates (2 explants/well in 600 μl MEM). [^{3}H]thymidine (1 μCi/ml MEM) was added during the first 30 minutes of culture, then was replaced with fresh MEM. After 6 hours, explants were incubated in 20 U papain for 15-20 minutes, dissociated and resuspended in 37°C HBSS to a target concentration of 1.5×10^7 to 2.5×10^7 cells/ml for transplantation. Cell survival in explant cultures averaged 75%.

Transplantation procedures

Transplantation procedures are described in McConnell (1988b). Briefly, P1-P2 host ferrets were anesthetized (5% halothane in O_2), and a small hole was made in the skull and dura overlying the occipital cortex of each hemisphere. Cells were loaded by pressure into a fine glass micropipette, which was lowered to a depth of ~1100 μm to target transplantations to the host VZ. In most cases, a single injection of 0.2-0.3 μl of cell suspension was made. In a few cases, 5 smaller injections (~0.075 μl each) were made between depths of 1060 and 1140 μm. Such 'rolling' injections yielded identical results to those of single, stationary injections.

Perfusions and histology

After survival times of 1 hour to 7 weeks, host ferrets were injected with an overdose of Nembutal, then perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde. Brains were sunk in 20% sucrose/4% paraformaldehyde, sectioned sagittally at 20 μm on a freezing microtome and mounted onto gelatin-subbed slides. Sections were processed for autoradiography as described previously (McConnell, 1988b) using Kodak NTB2 emulsion and a 6-week exposure, developed in Kodak D19, counterstained with cresyl violet and coverslipped with Permount. [^{3}H]thymidine-labeled cells that became terminally postmitotic after transplantation were heavily labeled ($\geq$20 silver grains/cell), whereas cells that divided again were lightly labeled (<20 grains/cell). The average number of silver grains per heavily labeled cell was similar (~40 grains/cell) in all four culture conditions, suggesting that cells in all conditions completed the cell cycle after transplantation.

Cell cycle kinetics

To compare the relative lengths of S-phase in culture, explants, pellets and low-density cultures were prepared simultaneously. Each received a 30 minute pulse of [^{3}H]thymidine (1 μCi/ml). Six hours later cultures were labeled with BrDU (6 μg/ml MEM, 30 minutes). To analyze cell cycle progression in vivo, E29 progenitor cells were labeled in utero with [^{3}H]thymidine (500 μCi/fetus). Fetuses were removed 6 hours later, and dorsal cortex was dissected and labeled with BrDU (6 μg/ml MEM, 30 minutes) in vitro. After BrDU labeling, cells in each condition were dissociated, plated onto slides coated with poly-D-lysine for 2 hours, then fixed in 70% ethanol. In addition, some intact explants and pellets were fixed in 70% EtOH or 4% paraformaldehyde to analyze the positions of labeled cells. Explants were embedded in paraffin and sectioned on a rotary microtome at 10 μm. Pellets were embedded in plastic and sectioned at 0.5-1.0 μm.

BrdU was visualized with mouse-anti-BrDU (Becton-Dickson; 1:100) and biotinylated anti-mouse secondary antibody (Vectastain) reacted with DAB. Slides were processed as above for autoradiography to visualize [^{3}H]thymidine-labeled cells. For each condition, numbers of cells labeled with [^{3}H]thymidine, BrDU, or both were calculated and compared to determine whether the kinetics of progression through S-phase were altered in culture.

RESULTS

Cells cultured at low density fail to adopt deep-layer fates

To ask whether cell-cell interactions and short-range cues are required for deep layer specification, progenitor cells were labeled with [^{3}H]thymidine immediately after the cortex was removed from E29 donors, just prior to low-density culture. These S-phase progenitors are multipotent: when transplanted directly into P1 hosts, ~85% of their daughters migrate into the upper layers 2 and 3 (McConnell and Kaznowski, 1991). In the present experiments, E29 progenitor cells were cultured for 4 or 6 hours at low density before transplantation into P1 host brains. This duration was chosen because previous experiments revealed that neurons become specified to a deep layer fate during the 4-6 hours after [^{3}H]thymidine labeling (McConnell and Kaznowski, 1991). Thus this 4-6 hour period of time in culture straddles an important cellular event that normally confers to E29 cells a deep layer identity, which persists even when cells are exposed to a novel environment. By removing cells from their normal environment, labeling them in vitro, then culturing them for 4-6 hours, we asked what phenotypic choices cells would make if deprived of normal environmental cues during the sensitive period for fate specification.

Cells were cultured at a target density of 110 cells/mm² (Fig. 1), which disrupts normal short-range interactions both among progenitors and with cells of the cortical plate. Not only are
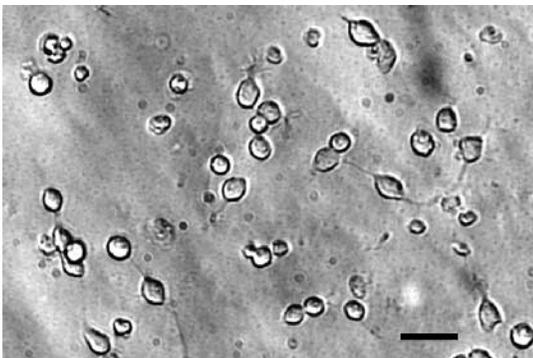
cell contacts minimal, but any diffusible factors produced are likely to be diluted in the relatively large volume of culture medium. Unless specifically noted, no growth factors, hormones or serum were added to the medium, in order to assess fate decisions made by cells in a neutral environment that supported cell survival for short periods in vitro.

After low-density culture, transplantation and a survival period of 4 to 6 weeks, host brains were processed and analyzed for the presence of cells that were heavily labeled with [3H]thymidine, which identified cells that became terminally postmitotic shortly after transplantation. A total of 123 cells that migrated away from injection sites and incorporated into the cortex were recovered from host brains. Cells labeled with [3H]thymidine had large pale nuclei and thus appeared to be differentiated neurons (Fig. 2A). Only one of these neurons established residence in the deep layers (Fig. 3A). Most transplanted cells were found in layer 2/3 (53%), where they were well-integrated into the surrounding cortex and were often found at the top of layer 2 (Fig. 2A). Transplanted cells in layer 1 (41%) sometimes displayed an unusual morphology for this layer, resembling large pyramidal neurons observed normally in deeper layers (Fig. 2B).
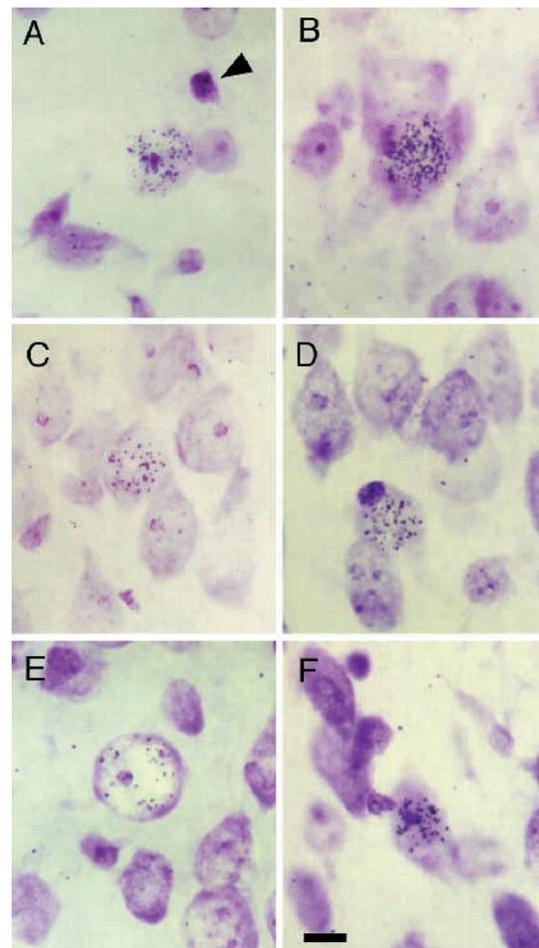
These results show that cells cultured at low density for 4-6 hours before transplantation into P1 hosts fail to adopt deep layer fates. We hypothesize that an active signal is required to induce cells to adopt their normal deep layer identities. While such cues are present in the normal E29 environment, they are disrupted by low-density culture, suggesting that signaling involves either cell contact or a short-range diffusible factor.

Low-density culture appears to 'erase' laminar specification

Because low-density culture disrupts interactions among cells undergoing the sensitive period for deep layer fate specification in vitro, we asked whether these culture conditions would have a similar effect on cells that have already received layer specifying signals in vivo. Ventricular cells were labeled with [3H]thymidine in utero at E29 and were left in place for 6 hours before being removed. Previous experiments have shown that when such cells are transplanted directly into P1 hosts, they behave as though they are committed to producing deep layer daughters (McConnell and Kaznowski, 1991). By culturing these cells at low density for 6 hours before transplantation, we


Fig. 2. Representative [3H]thymidine-labeled neurons recovered after transplantation into P1 hosts following 4-6 hours of culture. Cells labeled heavily with [3H]thymidine have large, pale nuclei characteristic of neurons, whereas glial cells (arrowhead in A) have small, dark cell bodies and were not labeled. (A,B) Cells cultured at low density migrated exclusively to the upper layers: (A) Layer 1 neuron. (B) Neuron in layer 2/3. (C,D) Cells cultured in pellets migrated to both deep and upper layers after transplantation: (C) pyramidal neuron in layer 2/3; (D) layer 6 neuron. (E,F) Cells cultured as explants migrated to deep layers following transplantation: (E) large neuron in layer 5; (F) layer 6 neuron. Scale bar, 10 μm.

Fig. 1. Cell-cell interactions are disrupted in low-density cultures (~113 cells/mm^2). Few cells make direct contact with neighbors. After several hours in culture, some cells extend neurites. Scale bar, 20 μm.

asked whether the acquisition of a deep layer fate represents a truly irreversible commitment, or whether the continued presence of environmental signals is needed to maintain a deep layer identity.

Surprisingly, low-density culture appeared to 'erase' what we had previously viewed as a commitment to a deep layer fate. Heavily labeled neurons (n=180) occupied every layer, with the majority residing in layers 1-4. Only 23% of transplanted neurons migrated to the deep layers (5, 6, and underlying white matter) after 6 hours of low-density culture, while almost half (47%) resided in layer 2/3 (Fig. 3B). These results contrast with those of McConnell and Kaznowski (1991), who found that about 90% of similarly labeled cells migrated to the deep layers when transplanted immediately into P1 hosts. These data demonstrate that progenitor cells, rather than being

Fig. 3. Laminar distributions of transplanted neurons. (A) Neurons were labeled with [³H]thymidine during dissociation, cultured at low density for 6 hours, then transplanted into P1 hosts (*n*=123 cells from 5 animals, 4 separate experiments). Heavily labeled neurons were found in the upper layers 1 and 2/3. (B) Neurons were labeled with [³H]thymidine in vivo and allowed to remain in situ for 6 hours. These specified cells were then removed, cultured at low density for 6 hours, then transplanted into P1 hosts (*n*=180 cells from 9 animals, 4 separate experiments). Heavily labeled neurons were found in all layers, especially the upper layers 1 and 2/3. (C) Neurons were labeled with [³H]thymidine during dissociation, cultured as pellets for 6 hours, then transplanted into P1 hosts (*n*=111 cells from 10 animals, 4 separate experiments). 67% of heavily labeled neurons migrated to layers 5, 6, or the white matter underlying layer 6. The remaining third migrated to layers 2/3 or 4, but never to layer 1. (D) Neurons were labeled with [³H]thymidine, cultured as explants for 6 hours, then transplanted into P1 hosts (*n*=99 cells from 5 animals, 3 separate experiments). Over 90% of heavily labeled neurons were found in layers 5, 6, and the underlying white matter. Abbreviations: uwm, white matter directly underlying layer 6.
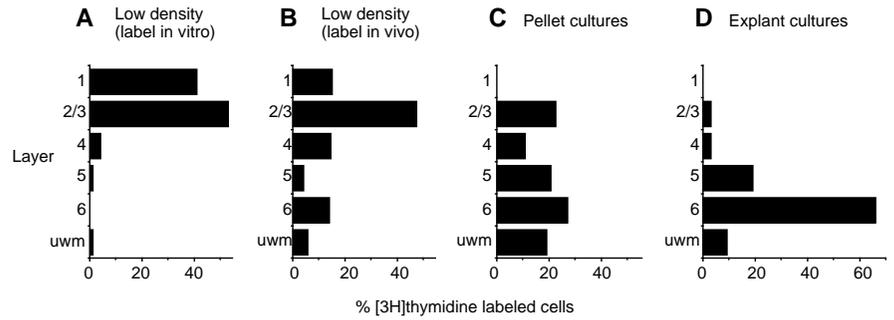
irreversibly committed to producing deep layer neurons, can under certain circumstances alter that fate. The disruption of deep layer specification suggests that a maintenance signal may be required for cells to remember or read out decisions made earlier in the cell cycle.

Serum contains factors that influence cell fate decisions in other culture systems (e.g., Raff et al., 1983); thus we asked whether serum could maintain laminar specification in vitro. E29 cells were labeled with [³H]thymidine, left in vivo for 6 hours, then placed in low-density culture; 5% fetal calf serum was added for the last 4 hours of the 6 hour culture period. (Addition of serum at the beginning of culture resulted in the formation of cell aggregates, restoring cell interactions.) Cells were then transplanted into P1 hosts. Of 30 heavily labeled neurons recovered from 3 hemispheres, 29 cells migrated to layers 1-4, and 23 of these were located in layer 2/3. No cells were found in layer 5, and only one was in layer 6. Although this is a small sample, these results suggest that, at the concentration and duration used here, fetal calf serum does not contain factors that maintain deep layer specification.

Pelleting cells restores the signals for deep layer specification

Because low-density culture disrupted deep layer specification, we asked whether restoring cell-cell interactions could enable cell specification to take place in vitro. We first investigated whether crude cell contacts and/or short-range signals are sufficient to instruct E29 cells to acquire and express deep layer fates. Pellets of cells were prepared by removing E29 cells, labeling them with [³H]thymidine during dissociation, then forming pellets either by brief centrifugation in a microcentrifuge (Watanabe and Raff, 1992) or by placing cell suspensions on a rotating shaker and allowing the cells to form reaggregates (Garber and Moscona, 1972). Cells were cultured for 6 hours (centrifuged pellets) or 18 hours (reaggregates), and then were redissociated into single-cell suspensions before transplantation into P1 hosts. Results obtained from the two methods for preparing cell pellets did not differ statistically (χ^2 test, *P*=0.56) and have been combined in the analysis below.

Cells in pellets appeared healthy and were closely apposed (Fig. 4). Progenitors that incorporated [³H]thymidine during

the initial dissociation were distributed randomly rather than in a discrete band, as are S-phase cells in control brains or cultured explants (Fig. 5A,B). Pellets thus lack the precise three-dimensional architecture of the normal cerebral wall. Although previous aggregation experiments using cortical plate cells suggested that specific cell types adhere preferentially to one another (Götz et al., 1996; Krushel and van der Kooy, 1993), we found no evidence in pellets for the selective sorting of cells according to their position in the cell cycle.

Although pelleted progenitors lack the normal neighbor relations present in vivo, restoration of cell contact or short-range signaling in pellets enabled the majority of labeled cells to adopt their normal deep layer fates after transplantation. The distribution of labeled neurons (*n*=111) is quantified in Fig. 3C. Two-thirds of the cells were found in the deep layers (5, 6, and underlying white matter; Fig. 2D); 11% migrated to layer 4, and 23% migrated to layer 2/3 (Fig. 2C). This bimodal distribution suggests that transplanted neurons actively choose
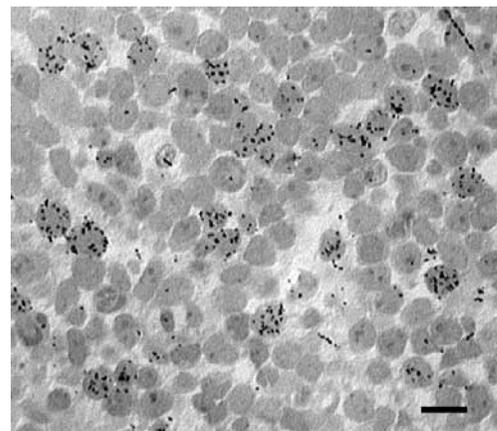
Fig. 4. A 1 µm section through a cultured pellet, revealing that pelleting cells together restores cell contact. S-phase cells that have incorporated [³H]thymidine (black grains) are mixed randomly with cells in other phases of the cell cycle. Pellets thus lack the architectural organization of the cerebral wall in vivo, where S-phase cells and mitotic cells occupy discrete regions of the VZ (Fig. 5). Scale bar, 10 µm.

a laminar position rather than migrate randomly to any layer. Random migration would be expected to deliver substantial numbers of cells to layer 4, which contains the greatest number of cells in any layer. In addition, cells cultured as pellets did not migrate to layer 1, a common fate after low-density culture.

Thus even a crude restoration of the normal environment can provide cues that specify the deep layer fates of most E29 progenitor cells. We hypothesize that signaling processes requiring either cell contact or short-range diffusible cues induce deep layer specification.

Deep layer specification is normal in explants

Most but not all labeled cells in pellets adopted deep layer fates after transplantation. Because pelleting cells disrupts their spatial organization, we wondered whether the normal, highly organized architecture of the VZ and cerebral wall are required for the normal specification of all progenitors. To explore this possibility, small pieces of the E29 telencephalon were cultured as explants that retain a relatively intact three-dimensional structure. The cellular constituents of these explants include VZ progenitors, newly postmitotic neurons of the preplate (subplate and marginal zone), and developing axons in the nascent intermediate zone. The cerebral wall is ~500 μm thick at this age, and both the ventricular and pial surfaces have direct access to culture medium.

The architecture of explants appeared normal after 6 hours in culture, as demonstrated by labeling both control brains and explants with [³H]thymidine, followed 6 hours later by a 30 minute pulse of BrDU. This double-labeling paradigm identified the positions of cells in S-phase at the beginning and end of the 6 hour period. Normal patterns of interkinetic nuclear migration were apparent in both in vivo controls and explants (Fig. 5): 6 hours after [³H]thymidine labeling, radioactively labeled cells had moved toward the lumenal surface in preparation for mitosis. BrDU-labeled cells, which were still in S-phase at the time of fixation, were found in the outer half of the VZ, the normal position of cells undergoing DNA synthesis (Sidman et al., 1959).

When E29 progenitors were labeled with [³H]thymidine at the beginning of the culture period, then transplanted into P1 brains after 6 hours in vitro, essentially all heavily labeled neurons adopted the deep layer fates expected for their origin (Fig. 3D): 92% (n=99) migrated to either layer 5 (Fig. 2E), layer 6 (Fig. 2F) or the underlying white matter. Lightly labeled neurons were also found in some brains. In accordance with previous findings (McConnell and Kaznowski, 1991), the granddaughters of E29 cells (which complete an entire cell cycle in the P1 host) adopted upper layer fates: 98% of lightly labeled neurons (n=154) acquired the fate typical of host neurons and migrated to layer 2/3. Thus when progenitors reenter the cell cycle they appear to experience a new sensitive period for laminar specification.

The behavior of E29 progenitor cells in explants is thus remarkably similar to that in vivo. Patterns of cell division and the nuclear movements that accompany cell cycle progression are maintained, and progenitors in explants produce deep layer neurons. Our findings suggest that the normal architecture of the cerebral wall in cultured explants allows the full preservation of signals for deep layer specification.

Progression through S-phase is slower in culture than in vivo

Because the sensitive period for fate specification is correlated with S-phase of the cell cycle (McConnell and Kaznowski, 1991), we investigated whether culturing cells at low density, as pellets, or as explants resulted in changes in the length of S-phase. Such changes might be correlated with the distinct behaviors of transplanted neurons following different culture conditions. For example, because neurons cultured at low density fail to adopt deep layer fates, one might hypothesize that S-phase lengthens in low-density cultures, thus lengthening the sensitive period for laminar induction and allowing cells to respond to cues in the host environment. Cell cycle kinetics were assessed by labeling cells in vivo or in each of the culture systems with a pulse of [³H]thymidine, followed 6 hours later by a pulse of BrDU. To determine whether cells progressed though S-phase at similar rates under different conditions, we calculated the percentages of [³H]thymidine-labeled cells that were also labeled with BrDU, a measure of the relative proportion of cells that were in S-phase at both the beginning and end of the culture period.

About 36% of cells labeled with [³H]thymidine in vivo also contained BrDU, suggesting that roughly a third of progenitors were still in S-phase 6 hours after [³H]thymidine was administered (Fig. 6). This rate of progression through S-phase is consistent with previous studies tracking DNA contents of BrdU-labeled cells over time (McConnell and Kaznowski, 1991). In contrast, nearly twice as many [³H]thymidine-labeled cells were also labeled with BrdU when cells were cultured as explants or pellets, or at low density (Fig. 6). These increases suggest that the rate at which cells progress through S-phase slows substantially in culture. S-phase is extended similarly in each of the three culture conditions, even though the fates of cells cultured as explants or pellets, or at low density are different. These experiments suggest that changes in the length of or rate of progression through S-phase cannot account for the distinct laminar phenotypes produced under different culture conditions. While we cannot rule out the possibility of subtle changes in cell cycle progression in cells cultured under different conditions, these findings suggest that the mechanisms that bring the sensitive period for laminar specification to a close are independent of DNA replication.

DISCUSSION

In this study, the specification of early cortical progenitor cells to a deep layer fate was examined in cells cultured at low density, as cell pellets and as intact explants. E29 ferret progenitors were labeled with [³H]thymidine in vitro to identify cells undergoing the sensitive period for laminar fate determination. Cells were cultured for 4-6 hours, during which time cells in vivo become specified to a deep layer fate (McConnell and Kaznowski, 1991), and were then transplanted into P1 hosts, in which upper layers are forming. The extent of deep layer specification obtained in vitro correlated with the intactness of the culture preparation. Progenitors cultured at low density showed no signs of deep layer specification and instead produced daughters that migrated to layers 1 and 2/3. When cell-cell interactions were restored by pelleting cells together

at the beginning of culture, two-thirds of labeled neurons adopted deep layer fates, and the rest migrated to the upper layers. Finally, when cells were cultured as explants that maintained the normal architecture of the cerebral wall, essentially all heavily labeled neurons adopted the deep layer fates typical of their origin. These results suggest that the acquisition of a deep layer fate is specified actively by an environmental signal. This signal, mediated by cell contact or short-range interactions, is present in explant preparations and to a lesser degree in pellets; signaling is disrupted completely by culturing cells at low density.

## Induction versus selective cell death

Transplantation experiments involving populations of cells invariably pose questions of interpretation. The fates exhibited by transplanted cells may result from the response of a uniform population to differing extrinsic cues, or from the preferential survival of previously specified subpopulations in different environments. A recent report suggests that large numbers of ventricular cells may undergo cell death in vivo (Blaschke et al., 1996). Nevertheless, we think it unlikely that our findings resulted from differential cell survival under different culture conditions. Such a possibility would require the existence of at least two specified subpopulations of cortical progenitors, one

committed to deep layer neurogenesis and the other to producing upper layer neurons. Upper layer precursors or their daughters would survive only when cultured at low density, whereas the deep layer precursors or their daughters would survive only in pellet or explant cultures. (The numbers of dead cells were comparable in all culture conditions.) In addition, to explain previous results (McConnell and Kaznowski, 1991), deep layer precursors would have to die selectively when cells are transplanted during S phase and upper layer precursors would fail to survive transplantation at any other stage of the cell cycle. However, in all these transplant experiments, lightly labeled cells that divided again after transplantation always migrated to the upper layers, revealing that progenitors competent to produce upper layer neurons have not been eliminated through cell death. Selective cell survival is thus unlikely to account for our results.

Molecular signals for deep layer induction

What is the nature of the signal(s) for deep layer induction? Both contact-mediated and diffusible cues play important roles in determining neuronal fates in other systems. These include the contact-dependent signaling processes mediated by sevenless and boss in the developing *Drosophila* eye (Hafen et al., 1987; Cagan et al., 1992), diffusible cues that promote the production of rod photoreceptors in rodent retina (Altshuler and Cepko, 1992; Watanabe and Raff, 1992; Altshuler et al., 1993; Kelley et al., 1995; Cepko et al., 1996) and a single protein, sonic hedgehog (Shh), that can act in either a contact-mediated or a diffusible fashion in the induction of ventral cells in spinal cord (Roelink et al., 1995; Tanabe et al., 1995). In the present experiments, cells cultured as pellets or explants clearly contact one another; we cannot, however, distinguish between a requirement for a contact-dependent signal or a locally acting diffusible cue in the induction of deep layer cortical neurons.

Several experiments have raised the possibility that cues for laminar induction may be generated through a series of interactions between progenitor cells and their progeny (Kim et al., 1991; Gillies and Price, 1993). Such a 'feedback' model
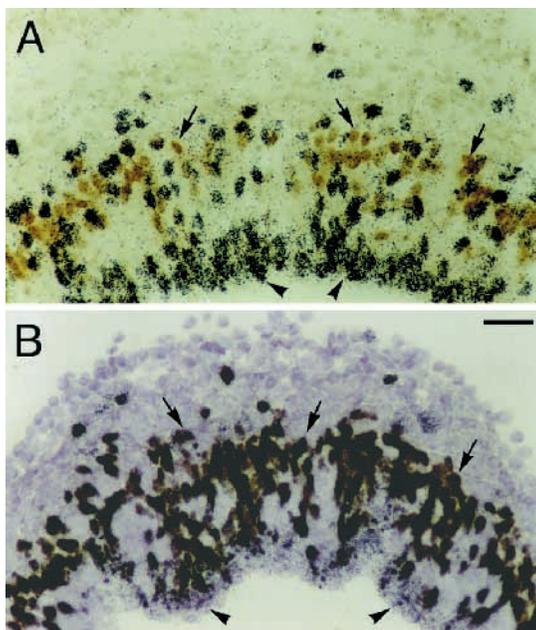
Fig. 5. Comparison of cell movements within the cerebral wall in vivo and in explants. (A) Section from an E29 ferret labeled with [3H]thymidine in utero, followed 6 hours later by a pulse of BrDU. During the 6 hours following [3H]thymidine incorporation in vivo, [3H]thymidine-labeled progenitors (black grains; arrowheads) move from the pial (top) surface of the VZ toward the lumen (bottom), where they undergo mitosis. BrDU-labeled cells (light brown cells; arrows), fixed immediately after labeling, are located in the pial third of the VZ, where cells undergo DNA replication. (B) Explants support interkinetic nuclear migration. BrDU-labeled cells (dark brown cells; arrows) are located in the outer third of the VZ, as in vivo, and [3H]thymidine-labeled cells (black grains; arrowheads) have moved toward the lumenal surface. The section in B was counterstained with cresyl violet. Scale bar, 30 μm.
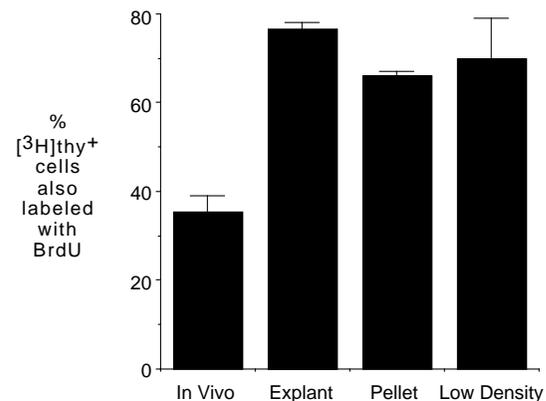
Fig. 6. Progression through S-phase slows in culture. Intact embryos or cultures (low density, pellet, and explant) were pulse-labeled with [3H]thymidine, followed 6 hours later with BrDU. This histogram shows the proportions of [3H]thymidine-labeled cells that were also labeled with BrDU. The fraction of double-labeled cells was much higher in all three culture systems than in vivo, indicating that progression through S-phase is slower in vitro than in vivo.

proposes that, as development progresses, differentiating neurons send signals back to the population of progenitors that produced them, triggering the production of the next layer (Reh and Tully, 1986). Differentiating neurons in the cortex may communicate directly with progenitors: axons and growth cones of cortical neurons travel along the upper (pial) surface of the VZ as they grow toward long-distance targets (Kim et al., 1991). Neurotransmitters released by growing axons can both depolarize progenitors and decrease DNA synthesis (LoTurco et al., 1995). The juxtaposition of growing axons and progenitors is likely maintained in explants, in which laminar specification occurs normally; however, feedback interactions may be disrupted when cells are dissociated and then reaggregated as pellets, resulting in imperfect specification. Such interactions could be mediated by either direct cell contact or by molecules, such as neurotransmitters, that are secreted but act over very short distances.

Are there default pathways for differentiation?

While results from transplanting cells cultured as pellets or explants support the conclusion that environmental signals actively induce neurons to adopt a deep layer fate, these experiments do not resolve the question of what mechanisms trigger the production of upper layer neurons. Does the generation of layer 2/3 neurons from early progenitors require an inductive signal, or is migration to layer 2/3 a default pathway followed in the absence of instructive environmental cues? We attempted to address these questions by culturing early progenitors at low density for 6 hours, then transplanting them into P1 hosts. The daughters of these progenitors migrated to layers 1 and 2/3.

One interpretation of these results is that migration to layer 2/3 represents a default fate chosen when cells fail to receive an environmental signal during the sensitive period for fate specification. Late progenitors in the P1 VZ are lineally committed to producing layer 2/3 neurons (Frantz and McConnell, 1996); it is possible that they achieve this commitment by losing their competence to respond to deep-layer inducing signals, thus reading out a preexisting default pathway in any environment. E29 cells, which have the potential to produce upper or deep layer neurons, may similarly respond to an environment devoid of layer specifying cues by producing layer 2/3 neurons. Alternatively, E29 cells may respond to inductive cues in P1 brains by changing their normal fates. Normal E29 progenitors adopt a deep layer identity in vivo by 4 hours after [3H]thymidine labeling (McConnell and Kaznowski, 1991). However, because this sensitive period for deep layer specification was defined in vivo, it is possible that depriving cells of environmental cues in low-density culture allows the sensitive period to be extended, so that progenitors retain their ability to respond to upper layer inducing cues in host brains. These possibilities remain to be teased apart experimentally.

The presence of transplanted neurons in layer 1 following low-density culture is puzzling. Layer 1 cells represent the exception to the 'inside-first, outside-last' pattern of layer formation; they are normally born at the beginning of neurogenesis, along with the subplate (Luskin and Shatz, 1985). Layer 1 thus constitutes an inappropriate laminar fate for either host or donor neurons. While it is possible that transplanted neurons actively choose to migrate into layer 1, their atypical pyramidal morphologies suggest an alternate interpretation. In the absence of inductive cues in low-density culture, it is plausible that cells become confused, fail to adopt any layer specific fate and accumulate at the outer surface of the cortex after migrating through all the layers without recognizing an appropriate laminar target. Migration to layer 1 was unique to cells cultured at low density; transplanted neurons were never found in layer 1 after explant or pellet culture, nor were they found in transplants performed previously without intervening culture periods (McConnell and Kaznowski, 1991).

The sensitive period for laminar specification

The sensitive period for deep layer specification is correlated normally with cell cycle progression. By the end of S-phase in vivo, E29 cells are specified to adopt deep layer fates (McConnell and Kaznowski, 1991), thus laminar determination may be coupled mechanistically to the process of DNA replication (McConnell, 1991). If S-phase were lengthened when cells were cultured at low density, but not as explants or pellets, it is conceivable that an extension of the sensitive period could explain the migration of cells to the upper layers after transplantation. Double-labeling experiments with [3H]thymidine and BrDU showed, however, that the rate of progression through S-phase is slowed to a similar degree in all three of culture paradigms used, yet cells adopted different laminar fates after transplantation. These experimental manipulations have thus decoupled S-phase, the sensitive period and laminar fate decisions, suggesting that DNA replication is not involved directly in laminar determination.

Laminar commitment re-examined

The notions of cell specification and commitment have proven useful in conceptualizing the processes by which cells develop their differentiated phenotypes. Specification is the acquisition by a cell of information that affects or instructs its future development, yet does not necessarily restrict the cell's potential to respond to inductive cues in its environment (Kimmel et al., 1991). Committed cells, in contrast, have lost the potential to differentiate along any but a single developmental pathway, even in the presence of cues that might have induced alternative fates at earlier times (Stent, 1985; Kimmel et al., 1991). In previous experiments, when E29 progenitors were transplanted late in the cell cycle into P1 hosts, they produced daughters that migrated specifically into the deep layers, as appropriate for their birthday. We concluded that these cells were committed to a deep layer fate (McConnell and Kaznowski, 1991). It was therefore surprising to find that the same population of cells, when subjected to 6 hours of low-density culture prior to transplantation, bypassed layer 6 and instead migrated predominantly to layer 2/3. For the majority of these cells, deprivation of cell contact and/or short-range cues seemed to erase their identity as deep layer neurons and to reveal a previously unseen potential to become upper layer neurons. This population of cells thus needs to be redefined as specified, but not irreversibly committed, to producing deep layer neurons.

We hypothesize the existence of additional signals that actively maintain specification and enable specified E29 cells to express a deep layer fate even when placed in an older environment. This signal must be present in both explant and pellet cultures, but is missing or disrupted when cells are cultured at low density. The nature of such a maintenance signal is unknown, nor do we know when or for what period of time such a signal must act. The signals that initially induce the pro-
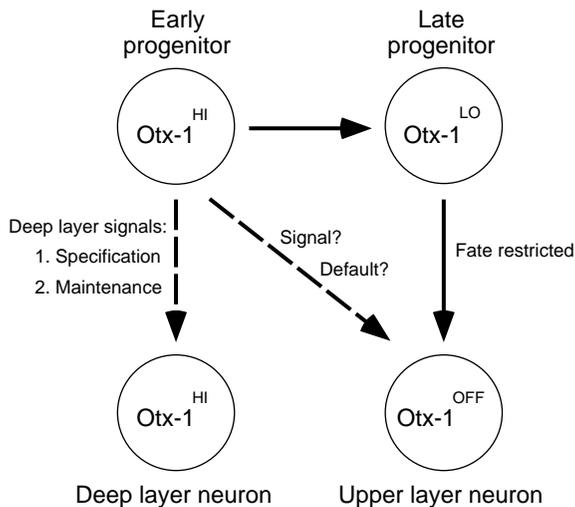
Fig. 7. A model for laminar specification, incorporating both the present and previous results. Early (E29) cortical progenitors express the homeodomain gene *Otx1* (Frantz et al., 1994) and are multipotent to produce either deep or upper layer neurons (McConnell and Kaznowski, 1991). Induction of deep layer neurons requires active signalling and additional signals are needed to maintain deep layer specification. The generation of upper layer neurons may either require inductive signals or may constitute a default fate. Late progenitors express *Otx1* weakly (Frantz et al., 1994) and are restricted to the production of layer 2/3 neurons (Frantz and McConnell, 1996). Thus, a combination of changing environmental factors and intrinsic changes in the competence of progenitors to respond to these signals results in the orderly production of the cortical layers during development.

duction of deep layer neurons are likely to be age-specific instructive cues; maintenance, however, is likely to be conferred by age-independent cell interactions, because E29 cells transplanted to P1 brains without an intervening period of culture become deep layer neurons. Thus maintenance signals are probably more generic or permissive cues that allow cells to carry out their specified programs for differentiation.

A model for laminar induction

Both this and previous studies reveal that E29 progenitors are multipotent and can produce either deep or upper layer neurons, depending on signals encountered during their final division. Transplants of late (P1) progenitors into a younger (E32) environment have revealed that the late progenitors, in contrast, are restricted to the production of upper layer neurons and are not competent to form the deep layers (Frantz and McConnell, 1996). The model illustrated in Fig. 7 proposes that a combination of environmental signals, which may change over time, and a progressive restriction of the developmental potential of older progenitors, work together to regulate the normal sequential production of the cortical layers. The present studies suggest that an active signal mediated by cell contact or short-range diffusible cues is required for the induction of deep layer neurons, although the molecular nature of such a signal is not known. Neither is it clear whether the generation of layer 2/3 neurons by early progenitors constitutes a default pathway for differentiation, or whether cues present in P1 brains induce cells to adopt this phenotype.

The restricted developmental potential of late cortical progenitors may result from cell-intrinsic changes that occur over time. One gene that may play a role in this process is the homeodomain gene *Otx1*, which is expressed by both early progenitor cells and their deep layer progeny in layers 5 and 6, but is not expressed at high levels by late progenitors or upper layer neurons (Frantz et al., 1994). It is plausible that these or similar changes in cell-intrinsic programs may be sufficient to restrict late progenitors to the production of upper layer neurons. However, the process by which the late progenitors lose their competence to produce deep layer neurons may not occur independently of environmental interactions; rather, one can envision a reciprocal interaction between environmental cues and gene expression. Environmental interactions during the course of development may cause changes in gene expression that, in turn, alter a cell's response to the environment. In their model of changing competencies during retinal development, Cepko et al. (1996) have underscored the significance of a cell's entire developmental history in determining its response to a signal. Changes in the response properties of a cell or lineage at different stages of development may help explain how a limited number of signaling cascades can participate in a wide variety of fate decisions.

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