Cerebral cortical specification by early potential restriction of progenitor cells and later phenotype control of postmitotic neurons

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INTRODUCTION

The mammalian cerebral cortex is subdivided into a variety of functional areas having characteristic patterns of neural connectivity and neurochemical organization. A great deal of attention has been directed toward understanding when and how characteristics of regional specificity are achieved during corticogenesis (Rakic, 1988; O'Leary, 1989; Kennedy and Dehay, 1993; Levitt et al., 1993, 1997; Arimatsu, 1994; O'Leary et al., 1994). It has been largely unknown, however, whether individual cortical progenitors proliferating in the ventricular zone are restricted to a local fate, and whether specification of progenitors and postmitotic young neurons is regulated by their regional environment.

Previously, we generated a monoclonal antibody, designated PC3.1, that recognizes a subset of neurons confined to the infragranular layers of lateral cortical areas in adult and early postnatal rats (Arimatsu et al., 1992). The PC3.1 antibody binds a 26 kDa protein, named latexin, which is encoded by the \( \text{lxn} \) gene that is transcribed in neurons of lateral cortex (Hatanaka et al., 1994; Jin et al., 1997). An independent biochemical study has recently revealed that latexin is able to inhibit the proteolytic activity of carboxypeptidase A in vitro (Normant et al., 1995). Many cortical latexin-immunopositive (+) neurons have the morphology of modified pyramidal cells and are glutamate+ (but not GABA+), suggesting that they are excitatory projection neurons (Arimatsu et al., 1999). The region- and lamina-specific distribution of the latexin-expressing subpopulation of glutamate+ neurons is a distinctive cellular feature that may contribute to the functional specialization of the lateral cortical areas.

We demonstrated that cortical specification occurs early by showing preferential generation of latexin+ neurons in cultures derived from a lateral, rather than a dorsal, portion of the developing cerebral wall (Arimatsu et al., 1992). Furthermore, the in vitro capacity to generate latexin+ neurons across the cerebral wall at embryonic day 13 (E13) and E16 matched well with the distribution profile of latexin+ neurons along both dorsal-ventral and rostral-caudal axes in the adult cortex (Arimatsu and Ishida, 1998). Since developing thalamic afferents grow into the cerebral cortex at E16 or later (Catalano et al., 1991) and the corticofugal axons do not exit the cerebral cortex before E13 (Blakemore and Molnar, 1990; De Carlos and O'Leary, 1992), we have concluded that the cerebral wall is prepatterned early by elements intrinsic to the cortex rather than by connectional interactions between cortical and extracortical structures. Consistent with these results, several studies have revealed characteristics of early cortical specification (Barbe and Levitt, 1991, 1992, 1995; Dehay et al., 1993; Ferri and Levitt, 1993; Cohen-Tannoudji et al., 1994; Ebrahim-Gaillard et al., 1994; Eagleson et al., 1997; Polleux et al., 1997; Tole et al., 1997). Thus, one of the most important questions remains: at what stage of corticogenesis are young neurons or their precursors determined to express a regional molecular phenotype? While it is possible that the fate of some
progenitors is determined early to generate neurons expressing region-specific traits, it is also possible that individual progenitors are multipotent and that certain early environmental cues subserve for later determination events. To address these points, we examined latexin expression in cells from different portions of the cerebral wall under controlled environmental conditions in vitro. Here we show evidence suggesting that both early restriction of developmental potential of progenitor cells and later phenotype control of competent postmitotic neurons contribute to the region-specific allocation of latexin-expressing neurons.

MATERIALS AND METHODS

Animals

Wistar rats were maintained under constant photoperiods (12L:12D; light on at 9:00 a.m.). Timed-pregnant rats were obtained by overnight mating. The day of identification of vaginal plug was designated E0. Birth usually occurred at E21 (=P0).

Monolayer culture

E13 fetuses were removed from dams anesthetized deeply with ether. The brains of the fetuses were taken out and placed in ice-cold L15 medium (GIBCO). Lateral and dorsal portions of the cerebral wall were dissected out at the rostral-to-middle level (Fig. 1A) and stored in cold L15 medium. It has previously been shown that, in slice cultures of E13 (and E16) cortices, latexin+ neurons appeared most prominently in those taken from rostral-to-middle portions of the lateral cerebral wall (Arimatsu and Ishida, 1998). The cortical sample was dissociated with papain (Pharmacia) and, after washing, the cell suspension was plated on an astroglial monolayer in wells of tissue culture chamber/slides (Lab-Tek, 79 mm²/well; 8x10⁴ cells/well). The cells were cultured at 37°C in 5% CO₂/95% air with a serum-containing medium consisting of 45% Dulbecco’s modified Eagle’s medium (GIBCO), 45% Ham’s F12 medium (GIBCO), 5% heat-inactivated horse serum (GIBCO) and 5% newborn calf serum (Mitsubishi Chemical). To eliminate dividing non-neuronal cells, 2.5 µM cytosine arabinoside (Sigma) was added to the culture medium for 24 hours at 5-6 days in vitro (DIV). The culture method was described previously (Arimatsu and Miyamoto, 1991). At appropriate DIV, the cells were fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.4 (5 minutes).

Reaggregated-cell culture

Dissociated cells from E13-P2 cerebral wall were prepared as described above. The cortical and other portions taken out for culture are shown in Fig. 1. For cells of E16-P2 animals, transverse slices of the lateral cerebral wall (A, E13; B, E16; C, E18-20; D, E21-P2). Filled and dotted regions represent the lateral (L) and dorsal (D) cerebral wall, respectively. Sep, septum. Str, Striatum.

Heterotopic (mixed-region) culture

To assess the regional difference in the developmental potential to generate latexin-expressing neurons, cells labeled with 5-bromo-2′-deoxyuridine (BrdU, Boehringer Mannheim) in vivo were mixed with 5-50× excess of unlabeled cells derived from either the same or different region (Fig. 2). The mixed cells were cultured in the monolayer or reaggregated-cell culture.

To prepare labeled E13 cells, a pregnant rat with embryos at E12 was injected subcutaneously with BrdU (50 mg/kg) at 8:00 p.m. At gestational day 13, the rat was injected twice with BrdU at the same dose (intraperitoneally; at 9:00 a.m. and 1:00 p.m.). Following 1 hour after the last injection, the rat was killed under deep anesthesia with ether and the fetuses were taken out. More than 80% of the dissociated cells from either lateral or dorsal cortex were BrdU labeled. To prepare BrdU-labeled cells from E16-P2 animals, pregnant rats at gestational day 15 were injected intraperitoneally with BrdU at the same dose at 2:00 p.m. and 8:00 p.m. Following an appropriate time period, the fetuses or neonates were killed as above. More than 60% of lateral cells and more than 80% of dorsal cells were labeled with BrdU when examined at E16.

Immunofluorescence staining

Cultured cells were fixed and stained for latexin-immunoreactivity with monoclonal antibody PC3.1 (mouse IgG1; Arimatsu et al., 1992). After incubation with PC3.1 antibody (15 µg/ml) in phosphate-buffered saline containing 5% normal goat serum and 0.1% Triton X-100, the cells were incubated with rhodamine- ( aw, in some case, fluorescein-) conjugated goat anti-mouse IgG (Chemicon, 1/50 dilution). For double labeling with anti-BrdU, cells stained with PC3.1 antibody were further treated with 4 N HCl for 20 minutes, neutralized with 0.5 M sodium borate (pH 8.6) and incubated sequentially with anti-BrdU monoclonal antibody (DAKO, 1/10 dilution) and fluorescein- (or rhodamine-) conjugated goat anti-mouse IgG. The number of latexin+ neurons out of 5x10^3 BrdU+ neurons was determined in each monolayer culture. For reaggregated-cell cultures, the number of latexin+ neurons out of 3x10^3 BrdU+ neurons was determined in every other section of each cell reaggregate.

Some cultures were stained using rabbit antiserum against neuron-specific class III β-tubulin isoform (1/500 dilution). The antiserum had been generated by immunizing synthetic 10-mer peptide corresponding to the carboxyl terminus of the protein (Burgoyne et al., 1988) with additional cysteine which was subsequently conjugated to keyhole limpet hemocyanin (Takiguchi-Hayashi et al., 1998). Cortical progenitor cells were stained with rabbit antiserum against the synthetic 10-mer peptide having the carboxyl-terminal sequences

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**Fig. 1.** Schematic illustration of the rat forebrain in the coronal plane, showing the locations from which the cerebral wall or other structures were dissected out for culture (A, E13; B, E16; C, E18-20; D, E21-P2). Filled and dotted regions represent the lateral (L) and dorsal (D) cerebral wall, respectively. Sep, septum. Str, Striatum.

Cells from the hippocampus and superior colliculus were also taken from E18 brains (not shown). Not proportional to the actual size.
of nestin (Lendahl et al., 1990). To identify a population of cortical pyramidal cells, cultures were immunostained with mouse monoclonal anti-calcium/calmodulin-dependent protein kinase II (CaMKII) antibody (Boehringer Mannheim, 10 μg/ml). Cortical nonpyramidal cells were labeled using rabbit anti-GABA polyclonal antibody (Sigma, 1/2000 dilution).

**Statistical analysis**
Significance of difference in the proportion of latexin+ and other neurons out of BrdU-labeled cells was assessed by t-test for independent samples.

**RESULTS**
Composition of E13 cortical progenitors is regionally distinct in developmental potential
It was previously shown that a fraction of cells derived from E13 lateral cerebral wall generated latexin+ neurons when cultured in a monolayer (Arimatsu et al., 1992). The initiation of latexin expression in vitro (at 10-14 DIV, equivalent to P2-6 in vivo) approximately corresponded to the timing at which latexin+ neurons appear in the cortex in vivo (Arimatsu et al., 1992). To clarify whether the developmental potential of cortical cells is regionally different already at E13, we here monitored latexin expression in cells in heterotopic monolayer cultures.

As reported previously (Ferri et al., 1996), cortical progenitor cells in the monolayer culture initially displayed a fairly prominent mitotic activity, but the activity declined rapidly: when cultured E13 cells (from either lateral or dorsal cortex) were incubated for 2 hours with 10 μM BrdU at 1, 2, 3 and 4 DIV, 11-14, 4-6, 1-2 and 0-1% of cells (progenitor cells plus young neurons, which were detected with a mixture of anti-nestin and anti-neuron specific β-tubulin antisera) were BrdU+, respectively.

The cells from either lateral or dorsal E13 cortex, which had been labeled with BrdU in vivo, were cultured with 10- or 50-fold excess of unlabeled L and D. (A-C) Photomicrographs showing a latexin-expressing neuron in the monolayer culture. An arrow points to a BrdU-labeled cell expressing latexin with the presence of 10x excess of unlabeled L. (A) Rhodamine-immunofluorescence for BrdU. (B) Fluorescein-immunofluorescence for latexin. (C) Phase contrast. Scale, 50 μm. (D) Proportion of BrdU-labeled neurons expressing latexin with 10x or 50x excess of unlabeled L and D. Filled and open columns represent BrdU-labeled L and D, respectively. Bars represent s.e.m. The number of cultures examined is shown near the bottom of each column. ***Significantly lower than BrdU-labeled L with the excess of unlabeled L (P<0.001).
C). At 17 DIV (equivalent to P9 in vivo), 2.57% ± 0.36 (s.e.m.) and 2.07% ± 0.41 of BrdU-labeled lateral cells were identified as latexin+ neurons in cultures containing excess of unlabeled lateral and dorsal cells, respectively (Fig. 3D). In contrast, far fewer BrdU-labeled dorsal cells expressed latexin when cultured with 10x excess of either lateral (0.30% ± 0.10) or dorsal (0.23% ± 0.10) cells. Essentially the same results were recorded with 50x excess of unlabeled lateral and dorsal cells; see, Fig. 3D). Since E13 rat cerebral wall comprises mostly proliferative progenitor cells, which will later differentiate to cortical plate neurons and glia (Berry and Rogers, 1965; Bayer and Altman, 1991), our findings suggest that the composition of cortical progenitor cells (at E13) is regionally distinct in its potential to generate latexin-expressing neurons.

Regional fate of cortical cells is influenced by environmental signals

While, in the monolayer culture, the proportion of lateral cortical cells expressing latexin was similar under different culture environments (i.e., with the excess of lateral and dorsal cells), it does not necessarily imply that the fate of individual progenitors is determined at E13, nor that cell-cell interactions are without effect on the fate of cortical cells. To address whether cell fate can be influenced by signals from extracellular sources, we performed heterotopic culture experiments using a reaggregated-cell culture system, in which a greater extent of cell-cell interactions would be expected than the monolayer culture. It was previously shown that certain developmental events in vivo, including cell proliferation and differentiation, are mimicked more appropriately in cell reaggregates or explants than in dispersed cells (e.g., Honneger, 1985; Temple and Davis, 1994; Ogawa et al., 1995). In our reaggregated-cell culture system, E13 cells retained high proliferation rates: at 1, 2, 3 and 4 DIV, about one third (35, 37, 35 and 29%, respectively) of lateral cortical cells took up immunohistochemically detectable BrdU following 2.5 hours incubation with 10 μM BrdU (more than 10^3 bisbenzimide-positive cells counted for each culture day). It was shown that cells that underwent cell division in the reaggregated-cell culture could generate latexin+ neurons. Thus, when cell aggregates of E13 lateral cortex were treated with 10 μM BrdU for 17 hours from the beginning of cultivation, 52% (59 out of 114) of latexin+ neurons were also BrdU+ at 22 DIV. The percentage was the same as neuron-specific class III β-tubulin+/BrdU+ cells (52%, 191 out of 367). Proliferation in vitro of prospective latexin+ neurons, however, was not extensive enough to dilute the BrdU-label to an immunohistochemically undetectable level. At 22 DIV, 90% of latexin+ neurons were also BrdU+ (820 out of 908) in reaggregates of E13 lateral cortical cells that had been labeled with BrdU in vivo at E12-13 (see Materials and methods). These results indicate that cell proliferation and differentiation of prospective latexin+ neurons occurs normally in this culture system, and that BrdU-labeled neurons are detectable after a long period in vitro.

A substantial fraction (2.66% ± 0.26, s.e.m.) of E13 lateral cortical cells became latexin+ neurons when cultured for 22 days in an aggregate consisting of only lateral cells (BrdU-labeled lateral cells plus 5x excess unlabeled lateral cells). However, in contrast to the monolayer culture, the proportion of latexin+ neurons was significantly reduced in the presence of 5x excess of dorsal cells to approximately one third (32.8%) of the value with purely lateral cells (Fig. 4A). A similar extent of reduction was observed in the presence of even fewer dorsal cells (only one sixth of total cells in culture). Since an additional experiment showed that the proportion of latexin+ neurons with 5x excess of dorsal cells remained at the same level (35.7%) even at 29 DIV (equivalent to P 21 in vivo) as that at 22 DIV, it seems unlikely that the reduction was due to a delay of general neuronal differentiation. These results suggest that environmental signals can exert a profound influence on the production of latexin-expressing neurons.

In contrast to lateral cortical cells, the number of dorsal cells becoming latexin+ neurons was, as in the monolayer culture, lowest even with the excess of lateral cells (Fig. 4A). The number of dorsal cells becoming latexin+ neurons was fewer than that of lateral cells under exactly the same environmental conditions (lateral:dorsal = 5:1; see, Fig. 4A). These results, together with those of the monolayer culture experiments, suggest that E13 dorsal progenitors do not have the capacity to become latexin+ neurons.
Environmental signals can influence fate of postmitotic neurons

To assess possible developmental changes in the responsiveness of lateral and dorsal cortical cells to their environment and those in the regional environmental signals affecting cell fate, we performed experiments of heterotopic reaggregate-cell cultures using cells at later developmental stages. BrdU-labeled cells from E16, E18, E19 and E20 cortices were cultured with 10× excess of unlabelled lateral or dorsal cells at the same age. As shown in Fig. 4B-D, the proportion of E16, E18 or E19 lateral cells becoming latexin+ neurons at the in vitro day equivalent to P14 in vivo was significantly lower with the excess of dorsal cells than with the excess of lateral cells, while the proportion of E20 lateral cells was at the same level with either dorsal or lateral cells (Fig. 4E). These results suggest that the fate of lateral cells has not been determined ultimately at E19 and that, at least until E19, there is a regional difference between dorsal and lateral cells in the ability to influence the production of latexin+ neurons.

In light of our previous results indicating that latexin+ cortical neurons become postmitotic around E15, irrespective of their tangential and laminar locations (Arimatsu et al., 1992, 1994), it is conceivable that the phenotype of competent precursors can be regulated even after their final mitosis. To examine whether the environmental influence detected in E18 cell-reaggregates targets postmitotic cells, the mitotic activity in reaggregates of unlabelled E18 lateral cells was determined during 0-1, 1-2 and 2-3 DIV by adding 10 μM BrdU to the culture medium for 20 hours (n=3 for each BrdU-treatment). Less than 1% of latexin+ neurons were BrdU+ in all these cultures when examined at 15 DIV (0.49%±0.08, 0.36%±0.25 and 0.07%±0.04, respectively; n>500 for each culture), indicating that most of the latexin+ neurons in cell-reaggregates did not undergo cell division during the culture period. This implies that the higher probability for E18 lateral cells to generate latexin+ neurons with lateral cells (Fig. 5B) was not due to an increased rate of proliferation of prospective latexin+ neurons, but rather due to their accelerated differentiation and/or survival of postmitotic neurons.

The effect of dorsal cells on the number of latexin+ cells was due probably not to delay of overt neuronal differentiation, since the effect on E18 cell aggregates was also evident at a later stage in vitro (22 DIV, equivalent to P19 in vivo; Fig. 5). The assumption was supported by the additional observation on the expression of CaMKII and GABA. CaMKII is strongly expressed in many cortical pyramidal cells with no marked regional heterogeneity (Terashima et al., 1995). GABA is expressed in nonpyramidal cells in the cortex. As shown in Fig. 5, the number of CaMKII+ and GABA+ neurons in the culture of E18 lateral cortical cell was not different with the excess of lateral and dorsal cells, indicating that the region-specific environmental influence is specific to latexin phenotype. Hence, we conclude that environmental signals are able to modulate specifically the generation of latexin+ neurons by influencing the fate of competent postmitotic cells.

In contrast to lateral cells, far less proportion of dorsal cells from E16-20 fetuses became latexin+ neurons even with the excess of lateral cells (Fig. 4B-E). This suggests persistent restriction up to E20 of the developmental potential in dorsal cortical cells.

Environmental signal that influence phenotype of postmitotic neurons is regionally and temporally regulated

The above isochronic culture experiments showed that the probability for E13-19 lateral cells to generate latexin+ neurons was lower with dorsal than lateral cells, and that the probability for E20 lateral cells was almost the same with dorsal and lateral cells (Fig. 4). We questioned whether the apparent discrepancy was due to the completion of fate determination of lateral cells around E20, or due to the loss of the regional environmental difference. To address this question, we performed heterochronic culture experiments using cells from E18-P2 animals. As shown in Fig. 6A, for both E18 and E20 (BrdU-labeled) lateral cells, the proportion of cells becoming latexin+ neurons with 10× excess of E18 dorsal cells was significantly lower than that with 10× excess of E18 lateral cells, whereas the proportion was almost the same with the excess of E20 dorsal and lateral cells. This suggests that the fate of lateral cells has not yet been determined ultimately at E20, and that the regional environmental difference is lost around E20. The terminal fate determination may occur during the early postnatal period, since the number of P2 (BrdU-labeled) lateral cells becoming latexin+ neurons was almost the same with both E18 dorsal and lateral cells (Fig. 6C). The proportion of E21 and P1 lateral cells becoming latexin+ neurons was different under different conditions (Fig. 6B,C).

Finally, as a step to characterize environmental signals modulating the generation of latexin+ neurons, we compared the effect of co-aggregation of cells from various sources outside the cortex on cultured cortical cells. As shown in Fig. 7, the generation of latexin+ neurons from E18 lateral cortical cells varied with the presence of excess cells from different extracortical sources. While, with 10× excess cells from the superior colliculus, lateral cortical cells generated latexin+ neurons at a level between those with lateral and dorsal cortical cells, they produced far less latexin+ neurons with 10× excess cells from the septum, striatum and hippocampus.

DISCUSSION

We have assessed regional differences within the cerebral wall in the context of (1) the developmental potential of progenitor cells, and (2) the environmental elements that can influence a neuronal phenotype. Individual cortical cells were labeled with BrdU in vivo, dissociated and exposed to a new environment in vitro and thus able to respond to very similar environmental signals as that labeled cells from different cortical region could respond. The experimental strategy allowed us to assess possible regional difference in the developmental potential of individual progenitors. On the contrary, when cells of the same potential (derived from the same region) were cultured in different regional environments, it was possible to identify the presence of regionally distinct environmental signals affecting a neuronal phenotype.

Based on the present data, we have drawn the following general conclusions. First, the region-specific distribution of latexin-expressing neurons in the mature cortex is substantiated during early corticogenesis by restriction of developmental potential in dorsal progenitor cells. Second, the phenotype of
competent lateral cortical cells can be regulated by regional environmental cues even after their final mitosis. These conclusions are consistent with the idea that cortical specification involves progressive cell fate determination toward a regional molecular identity.

**Early cortical specification by restriction of developmental potential in progenitor cells**

We have demonstrated that the cellular composition of E13 cerebral wall is regionally distinct by showing that the probability for E13 lateral cortical cells to generate latexin+ neurons was much greater than that for dorsal cortical cells under very similar or equivalent environmental conditions in vitro. Thus, (BrdU-labeled) lateral cells became latexin+ neurons 10-30\times greater in proportion than dorsal cells, when cultured with the excess of (unlabeled) lateral cells in monolayer and reaggregated-cell cultures. It is likely that, in most of progenitor cells in dorsal cortex, the developmental potential to generate latexin-expressing neurons has been restricted by E13, whereas at least a certain fraction of progenitors in lateral cortex is competent to choose such a differentiation pathway. Given that latexin-expressing neurons
become postmitotic around E15 (Arimatsu et al., 1992, 1994), the regional diversification of cortical progenitor cells should be independent of environmental elements during the final several rounds of the cell cycle. Accordingly, our findings not only contradict the view that the early cerebral wall is tabula rasa, but also our data are inconsistent with the assumption that early cortical progenitors share a broad spectrum of developmental potentials for any regional fates.

Based on the reports suggesting extensive mixing of cortical progenitors and of young neurons (e.g., Walsh and Cepko, 1992; O’Rourke et al., 1992; Fishell et al., 1993) and those showing early regional specification within the cerebral cortex (e.g., Barbe and Levitt, 1991; Arimatsu et al., 1992; Cohen-Tannoudji et al., 1994), one can hypothesize that “individual precursors would be multipotent” and that “the fate of a neuron is determined by” environmental cues acting either on the neuron itself or on its precursor” (Grove, 1992). It has been in fact shown in vitro that the combination of TGFβ/collagen type IV can regulate the differentiation of cortical progenitors to express the limbic system-associated membrane protein (LAMP), a region-specific neuronal phenotype (Ferri and Levitt, 1995; Ferri et al., 1996). Eagleson et al. (1997) have further shown that both limbic and nonlimbic cortical progenitors from E12-16 rats maintain competence to respond to the substratum-bound inductive signals, and that, once exposed to the signals during at least one complete cell cycle, they retain the memory for the molecular phenotype. Moreover, several recent fetal transplantation studies have shown that at least subpopulations of dorsal and ventral telencephalic progenitors have a certain degree of plasticity for their expression of regional traits (Fishell, 1995; Brüstle et al., 1995; Campbell et al., 1995; Olsson et al., 1997). In relation to the regional allocation of latexin-expressing neurons, however, the present study provides convincing evidence indicating that certain aspects of competence to express the region-specific phenotype have been set up within early cortical progenitors and maintained persistently throughout cortical development. This conclusion is consistent with the recent findings showing that major populations of cortical progenitors do not migrate extensively in the tangential plane (Tan and Breen, 1993; O’Rourke et al., 1997). Since many latexin+ neurons are glutamate+ and likely to correspond to a population of corticocortical pyramidal neurons (Arimatsu et al., 1999), the fate restriction in early progenitors shown here is particularly interesting in relation to the recent observations suggesting that precursors of cortical pyramidal neurons adopt

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**Fig. 7.** Effects of extracortical cells on generation of latexin-expressing neurons in E18 reaggregated-cell cultures. BrdU-labeled lateral cortical cells (L) from E18 fetuses were cultured in an aggregate containing 10x excess of unlabeled L, dorsal cortical cells (D), or cells from the septum (Sep), striatum (Str), hippocampus (Hip), or superior colliculus (SC) from E18 fetuses. The number of cultures is shown near the bottom of each column. ***Significantly lower than BrdU-labeled L with 10x excess of unlabeled E18 L (P<0.001). +++ Significantly lower than BrdU-labeled L with 10x excess of unlabeled E18 D (P<0.001). ### Significantly higher than BrdU-labeled L with 10x excess of unlabeled E18 D (P<0.001).

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**Fig. 8.** (A) Summary of experimental results in the present study. Relative probability for BrdU-labeled (*) lateral (L) and dorsal (D) cortical cells to generate latexin+ neurons with the excess of unlabeled L and D is shown. ND, not determined. (B) Proposed developmental events involved in the region-specific allocation of latexin-expressing neurons in the cerebral cortex. The developing cerebral wall has been regionally specified by E13, such that proliferative progenitor cells in the ventricular zone (VZ) of the dorsal sector have lost their potential to generate latexin-expressing neurons (open cells). At least certain progenitors in the lateral sector are competent to generate latexin+ neurons (hatched cells). They stop dividing around E15 and postmitotic young neurons migrate for a few days along the lateral cortical stream (LCS) for their final destination in the lateral cortical areas. Just corresponding to this time period (~E19), there exists regional environmental difference between dorsal and lateral cortices in the ability to influence the generation of latexin+ neurons. During the early postnatal period, the prospective latexin+ neurons lose their competence to respond to environmental signals and eventually differentiate to latexin-expressing neurons (filled cells).
The mechanisms producing the regional difference in early cortical progenitors are currently unknown. While this could be generated through a cell-autonomous process (Nakagawa et al., 1996), signals from intracortical or extracortical sources might provide cortical progenitors with inductive and/or repressive signals (Shimamura and Rubenstein, 1997). Previous studies have demonstrated that Emx2, a transcription factor with a homeodomain, is expressed in the developing cerebral wall with a dorsal-to-ventral gradient and that disruption of Emx2 gene in the mouse results in the loss of the hippocampal region (Simeone et al., 1992; Pellegrini et al., 1996; Gulisano et al., 1996; Yoshida et al., 1997). It is thus tempting to speculate that the regional expression of such developmental regulatory genes may be involved in the fate restriction of dorsal cortical progenitors.

**Control of fate of young neurons by regional environmental signals**

Another important finding in the present study is that regional environmental signals intrinsic to the cortex are able to modulate a neuronal fate even after their final mitosis. The probability that E13-19 lateral cortical cells would generate latexin+ neurons in vitro was consistently greater within isochronic cell aggregates containing excess lateral cells than those containing excess dorsal cells. The probability for even E20-P1 lateral cells was different under different environmental conditions (i.e., with E18 lateral versus dorsal cells), suggesting that the latexin phenotype of young cortical neurons is not terminally determined at P1, about 7 days following their final mitosis. This is in sharp contrast to the previous findings in developing cortex suggesting that certain neuronal phenotypes are determined around their final mitosis or before (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996; Eagleson et al., 1997). However, the number of cortical nonpyramidal cells expressing a particular neuropeptide changes during the late postnatal period (Parnavelas and Cavanagh, 1988; Obst and Wahle, 1995). It has been shown that afferent thalamocortical and corticocortical axonal innervation elicits a reduction in the percentage of neuropeptide Y-expressing neurons in organotypic slice cultures (Obst and Wahle, 1997). Moreover, in the retina, Ezzeddine et al. (1997) have demonstrated that, even during postmitotic period, prospective rod photoreceptors can change their fate to expressing phenotypes of bipolar cells in response to ciliary neurotrophic factor (CNTF). Our present findings may imply that the timing of the determination of certain traits is distinct for different phenotypes of cortical neurons. The developmental plasticity that extends into the postmitotic period is probably related to the relatively late onset of latexin expression in cortical neurons (Arimatsu et al., 1992).

The regional environmental difference for the production of latexin-expressing neurons was detected at E18-19, but not at E20. While E18-19 lateral cortex might contain specific signals that promote differentiation of latexin-expressing neurons than dorsal cortex, it is also possible that E18-19 dorsal cortex provides repressive signals that are not expressed by lateral cortex. On the contrary, although it seems highly unlikely that cortical cells are more susceptible to physiological cell death generally in dorsal cortex than lateral cortex (e.g., see Fig. 5), prospective latexin-expressing neurons may have been selectively eliminated (see, Miller, 1995; Blaschke et al., 1996; Thomaidou et al., 1997). It will be of special interest to perform co-aggregation experiments similar to the present study, using cells whose apoptosis is prevented in a general way (e.g., Kuida et al., 1996).

Interestingly, the time period when the regional environmental difference was evident closely overlaps that of migration of lateral cortical neurons. It has been proposed that young neurons born in the lateral sector of the cerebral wall migrate for 2-4 days through the lateral cortical stream (Bayer and Altman, 1991), a curving radial migratory pathway (Misson et al., 1991). As the birthdate of most latexin-expressing neurons in the cerebral cortex is E15 (Arimatsu et al., 1992, 1994), they would probably migrate from their birthplace to the final destination during E15-E19. Cortical cells during this time period were able to provide region-specific signals in vitro. Thus, it is possible that the temporally and regionally regulated environmental signals contribute to the region-specific allocation of latexin-expressing neurons in vivo. It is notable that even extracortical cells would not be neutral in affecting the phenotype of lateral cortical cells as the generation of latexin+ neurons varies with the presence of cells from different extracortical sources (Fig. 7). It is of particular interest that the probability of generating latexin+ neurons was greatly reduced in the co-aggregates with cells from telencephalic regions neighboring the neocortex, e.g., the hippocampus and striatum. These structures might also play some role in establishing the correct distribution of latexin+ neurons.

It is not known whether environmental cues can affect dividing progenitor cells in lateral cortex as well as postmitotic cells. Although the fate of E13 lateral progenitor cells was modulated in the heterotopic reaggregated-cell cultures, the effect could have derived from signals acting on postmitotic cells during later stages in vitro. Given that the progenitor cells are responsive to environmental signals, the phenotype-regulating activity might be mediated by cell-to-cell contact rather than by diffusible signals acting over a long distance, since the cell fate modulation occurred in the reaggregated-cell culture but not in the monolayer culture. It would be also possible that the differential environmental influence reflected different degrees of mitotic activity in these culture systems. The intimate relationship between the cell cycle and neuronal differentiation has been discussed elsewhere (Ross, 1996).

**Cerebral cortical specification by progressive acquisition of a regional neuronal phenotype**

It has been postulated that various neuronal traits are specified progressively during corticogenesis (Levitt et al., 1993; McConnell, 1995). The assumption is in accord with the apparently progressive acquisition of various phenotypic features in a single cortical neuron, such that certain molecular phenotypes (e.g., LAMP expression) appear relatively early, whereas others (e.g., certain cytoarchitectonic and connectional features, and neuropeptides expression) have a late onset. Furthermore, specificity for similar neuronal traits, such as laminar fate, occurs progressively among different cortical progenitors. Thus, while early progenitors are...
multipotent and committed to infragranular neurons during their final cell cycle (McConnell and Kaznowski, 1991), later progenitors are fated to generate only supragranular neurons (Frantz and McConnell, 1996). The present study further demonstrates that even a single molecular fate is not determined during a very limited time period, but established progressively during both proliferative and postmitotic phases of neuron production (Fig. 8). The progressive acquisition (restriction) of single molecular neuronal phenotypes in the developing retina has been demonstrated in a series of experiments (e.g., Watanabe and Raff, 1990; Alexiades and Cepko, 1997; Ezzeddine et al., 1997), in which it has been shown that subpopulations of progenitors display distinct biases towards the production of particular cell types, e.g., amacrine and horizontal cells, even though they are not committed but are able to produce other cell types, e.g., rod photoreceptors, under the influence of environmental signals. The progressive determination of neuronal fate is thus shared by developing cortical and retinal cells (Williams and Goldwitz, 1992; Ferri and Levitt, 1993; Frantz et al., 1994). The regulation of a regional neuronal fate during a prolonged time period of corticogenesis is consistent with a mechanism underlying the cooperative establishment of unique regional phenotypes during ontogeny and phylogeny.

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REFERENCES


