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CHAPTER 2

Early development of the cerebral cortex

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Introduction

As a biological system, the mammalian cerebral cortex represents unrivaled functional and architectural diversity. The organization of the cerebral cortex is particularly striking in its extensive cellular variety, precise laminar stratification, and a regionalization that belies functional specialization. While anatomical studies have uncovered the gross morphological changes associated with forebrain development (see Bayer and Altman, 1991 for a review), they have left many unanswered questions with regard to the cellular and molecular processes that generate the structure most responsible for complex cognitive and perceptual behavior. As a result, there has been little consensus about the most basic aspects of cortical progenitor cell behavior such as the patterns of cell division by which cortical precursors produce various cellular phenotypes in the appropriate numbers and ratios to populate the regionally heterogeneous cerebral cortex.

In the developing embryo, daughter cell fates are frequently influenced by the immediate environment of the dividing progenitor cell (including cell-cell contact, cell-matrix contact and the binding of diffusible factors to cellular receptors). On the other hand, other progenitors appear to be indifferent to such environmental influences and demonstrate a commitment to a particular pattern of differentiation. A cell is said to be committed when it has acquired the information that ultimately dictates the phenotypes or fates of its daughter cells (McConnell, 1991). While the molecular basis of cellular commitment is poorly understood, investigators have sought to determine its role in the generation of cellular and regional diversity. Might some or all mitotic progenitors, for instance, demonstrate a commitment to producing certain cell types? Are dividing cells allocated and committed to the production of particular cortical layers or cortical regions? While these simplest of developmental questions were first considered over 100 years ago by cortical histologists, they continue to elicit opposing points of view from developmental biologists and remain the subject of debate.

Overview of cortical anatomy and development

In recent years, innovative approaches to the study of cortical development have dramatically increased our understanding of the manner in which germinal zone progenitors generate the tremendous cellular and structural diversity of the mature cerebral cortex. The cortex is organized horizontally into functional areas and vertically into cortical layers or laminae. Each functional area within the cortex represents a variation on the laminar theme. Each of the cortical layers is recognizable across cortical regions and displays similar connectivity to its counterparts in other cortical areas, but its size (absolutely and relative to the other cortical layers) and cellular composition vary according to the functional domain in which it is observed. Neurons within a regional domain make connections and receive projections that reflect their functional specialization, as well as cortical layer. Visual cortex, for example, makes reciprocal connections with the lateral geniculate nucleus, the

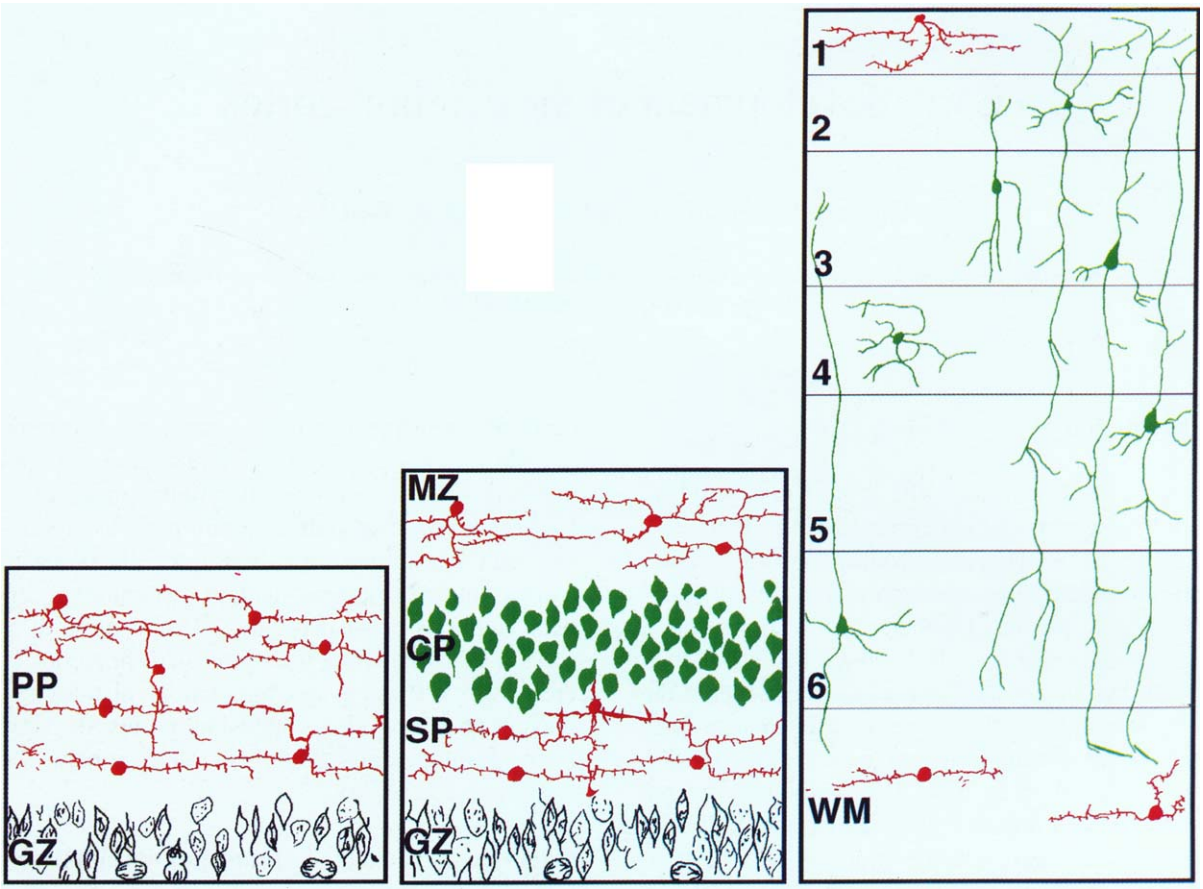

Fig. 1. A summary of cerebral cortical development represented schematically by drawings of cortical cross-sections at very early (left), intermediate (center) and late stages of development (right). These drawings correspond roughly to the E12 (left), E16 (center), and adult rat (right) sections. The cerebral cortex arises from progenitor cells within the germinal zone (GZ) lining the lateral ventricles. Early in neurogenesis these cells divide to form the preplate (PP) cells. These early formed neurons eventually differentiate to become subplate neurons and the Cajal-Retzius neurons of layer I, also known as the marginal zone (MZ). Over the course of development, other neurons arising from the germinal layer migrate to a position within the preplate, eventually splitting the preplate into layer I (I) superficially and the subplate (SP) subjacently. The intervening neurons arrive to form the cortical plate (CP) in an inside-out fashion. Layer six (6) neurons arrive first, then layer five (5) and so on.

major visual nucleus of the thalamus. In addition to being functionally specific, these connections are also layer-specific. As in other cortical regions, the visual projections to subcortical targets are made by deep layer (V and VI) neurons and thalamic afferents are received primarily by neurons in layer IV.

Birthdating experiments have demonstrated that the layers of the cerebral cortex are formed in an orderly sequence. The deepest (subplate) and most superficial (I), layers are formed first as the earliest-born neurons migrate a relatively short distance to form the "preplate," or primordial plexiform layer (Marin-Padilla, 1971), a structure subsequently invaded and divided by later-formed cortical plate neurons and glia (Fig. 1). These newer cells add to the cortical plate in an "inside-out" sequence. Cogenerated neurons migrate past previously generated neurons to form layers VI through II. Layer II represents the home of the most recently generated neurons and derives from the most superficial layer of the cortical plate (Angevine and Sidman, 1961). The superficial cells of the divided preplate lie just beneath the

pial covering of the brain, above layer II, and in the mature brain are referred to as layer I or the marginal zone. The deep preplate cells reside in the eventual white matter as the subplate or layer VIb/VII cells (Allendoerfer and Shatz, 1994).

All cortical neurons are the progeny of cells that line the lateral ventricle as a pseudostratified neuroepithelium or ventricular zone (Boulder Committee, 1970). The cells of the neuroepithelium are known as precursor cells or progenitor cells. These cells attach apically via cell processes that extend to the pial surface and attach basally in the ventricular zone. At the earliest stages of cortical development most progenitor cells appear to undergo cell division leading to extensive cellular proliferation (Takahashi et al., 1993). The position of progenitor cell nuclei within the neuroepithelium corresponds to their stage in the cell cycle. The nuclei of mitotic progenitors occupy a relatively superficial (basal) position within the neuroepithelium during DNA replication (S phase), but descend through G2 phase and divide apically, near the ventricular lumen. This coupling of cell cycle stage and nuclear position is known as interkinetic nuclear migration (Angevine and Sidman, 1961).

Transplantation studies

One approach to examining progenitor cell commitment to producing progeny with specific cortical phenotypes involves transplanting ventricular zone cells to novel environments and assaying the fates of their progeny. Laminar position, like cell morphology and neurotransmitter expression, represents one aspect of cell phenotype within the differentiated cerebral cortex. The simple correlation between birthdate and neuronal laminar identity has proven advantageous to studies designed to assess whether or not progenitor cells demonstrate commitment to producing progeny destined for a particular cortical layer (McConnell, 1985, 1988, 1990; McConnell and Kaznowski, 1991). Donor progenitor cells, labeled by injecting pregnant ferrets with tritiated thymidine, were harvested and dissociated before transplantation to the germinal zones of developing host animals. Weeks later, the labeled progeny were visualized by autoradiography to determine their laminar position (McConnell, 1988). When cortical progenitors, originally isolated from an immature brain at the time when progenitors would normally produce layer VI cells, were transplanted into an older brain that is producing layer II cells, progenitors were expected to either demonstrate an intrinsic commitment to produce neurons destined for layer VI, or demonstrate the capacity to respond to the novel host environment by instead producing layer II neurons. About half of all tritiated thymidine-labeled progenitors produced progeny which migrated preferentially to layers V and VI, layers appropriate to age of the donor cortex, illustrating an early commitment of some progenitor cells to populate specific layers in the developing cortex. That commitment appeared to be maintained even after the donated progenitor cells were exposed to a novel host environment (McConnell, 1988).

Subsequent experiments demonstrated that the commitment of mitotic progenitors to produce neurons populating specific laminae is regulated by the environment in a cell cycle-dependent manner. Progenitors that undergo S phase in the new host brain (as measured by new DNA synthesis) adopt the same fate as endogenous precursors, while progenitor cells that progress to G2/M phase before transplantation produce cells which occupy layers reflective of the donor brain's developmental stage (McConnell and Kaznowski, 1991). Laminar fate, therefore, seems to be specified by temporally-regulated environmental cues between the S and M phases of the cell cycle. When the experiment is performed in the reverse with postnatal day 1 (P1) progenitors transplanted to E29 brains, neurons failed to appear in the deep layers of the cortex (Frantz and McConnell, 1996). Therefore, progenitors from older brains may differ from early progenitors in that they lack the ability to respond to cues promoting deep layer differentiation. These results imply that during the course of neurogenesis, progenitor cells respond to changing environmental signals to produce neurons which come to reside in progressively more superficial laminae over the course of neurogenesis and which differentiate accordingly.

Transplant experiments involving entire slabs of developing cortex provide evidence for spatial cues within the ventricular zone that influence the commitment of progenitors to produce neurons with area-specific phenotypes. Limbic associated membrane glycoprotein (LAMP) is a recently cloned member of the immunoglobulin superfamily and is expressed specifically in developing limbic cortices such as prefrontal, perirhinal, and entorhinal cortex (Levitt, 1984; Barbe and Levitt, 1991, 1992; Pimenta et al., 1995). While progenitors within the ventricular zone have not been shown to express LAMP, migrating postmitotic neurons destined for limbic regions do express LAMP. Reciprocal transplants of limbic (perirhinal) and LAMP-negative nonlimbic (somatosensory) cortex were made at E12, E14, and E17. E12 transplants would have contained most actively dividing progenitor cells while E14 and E17 transplants would have contained progressively fewer dividing cells. Following grafting, the remaining progenitor cells, which normally would have produced exclusively LAMP-positive neurons (limbic progenitors) or exclusively LAMP-negative neurons (neocortical progenitors), were able to respond to changing spatially-regulated cues and produce neurons with the molecular phenotype appropriate to the new environment (Levitt, 1995). Thus it appears that there is some malleability of progenitor commitment to producing neurons with regional or domain-specific phenotypes as well as laminar fate at earlier stages, but not at later stages. Similar transplantation experiments which studied a transgenic insertion marker specific for somatosensory cortex also showed relative resistance to re-specification of this regional marker at later stages of neurogenesis, but did not analyze effects at very early stages (Cohen-Tannoudji et al., 1994).

Cell lineage studies with retroviral vectors

Tracing cell lineages represents a conceptually simple approach to understanding the manner in which cellular diversity is achieved in the mature cortex. The potential usefulness of lineage tracing has been best illustrated in the unpretentious nematode, *Caenorhabditis elegans*, where the organism's entire stereotyped lineage, from the first egg cleavage through final differentiation, has been carefully recorded. As a result, studies of *C. elegans* development have greatly increased our understanding of cellular diversification, the nature of progenitor cell commitment, and the identification of specific gene defects which alter cell fate (Desai et al., 1988). Elucidating the patterns of cell division that characterize cortical development and defining the regulatory mechanisms which control cell proliferation and differentiation have the potential to greatly increase our understanding of the generation of cellular and regional diversity in the cerebral cortex.

One approach to tracing cell lineage involves infecting ventricular progenitors with modified retroviruses (Sanes et al., 1986; Turner and Cepko, 1987). Retroviruses used in studies of cell lineage have been rendered replication-incompetent, and modified by the insertion of a reporter gene that can be visualized histochemically. Within an infected progenitor cell, viral RNA is reverse transcribed to DNA and is incorporated into one of the host chromosomes. The viral sequence is thereafter replicated along with host DNA, and transmitted during cell division to progeny cells. These progeny express the reporter gene as well and are identifiable in histological preparations. When a single retrovirus is used to infect dividing progenitors, the progeny of multiple infections will carry the same histochemical marker gene. It is therefore impossible to definitively distinguish cells that arise from different progenitors and those that arise from a common progenitor. For this reason investigators have focused their efforts on clusters of labeled cells and, based on the reasonable assumption that sibling cells would occupy nearby positions in the adult cortex, have used proximity as the basis for assigning clonal relationships among labeled cells.

The earliest studies employing retroviral markers in the cerebral cortex (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988) emphasized clusters, sometimes involving cells in multiple cortical laminae or sometimes composed of homogenous cell morphologies. One study (Luskin et al., 1988) also suggested that sibling

neurons migrated along similar radial paths to nearby positions within the cortex. Subsequent retroviral studies emphasized the fact that clusters tended to be composed of similar cell types. Initially, neurons and glia were shown to occur in distinct clusters in the adult mouse brain (Luskin et al., 1988). Retroviral infection of E15–E16 rat progenitors likewise revealed distinct astrocyte, neuronal clusters, or mixed neuron-oligodendrocyte clusters (Williams et al., 1991; Grove et al., 1993). Spatially distinct localization of neurons and glia was later confirmed by studies in which cells expressing the retrovirally encoded β-galactosidase reporter gene were injected with Lucifer yellow to facilitate morphological identification, and were stained for MAP2 or GFAP reactivity (Grove et al., 1992, 1993). These results were interpreted as evidence for early glia-neuronal divergence during corticogenesis as well as the existence of distinct neuronal and glial progenitors.

Further restrictions within the neuronal and glial (Parnavelas et al., 1991; Luskin et al., 1993) cell lineages were suggested by subsequent retroviral studies. Retroviral infection of E16 rat progenitors resulted in cell clusters that were overwhelmingly composed of exclusively oligodendrocytes, astrocytes, pyramidal neurons, or nonpyramidal neurons (Parnavelas et al., 1991; Luskin et al., 1993). The occurrence of pyramidal neuron and nonpyramidal neuron morphologies in separate homogenous cell clusters (Parnavelas et al., 1991) was confirmed immunohistochemically when antibodies raised against glutamate and GABA, respectively the major pyramidal and nonpyramidal neurotransmitters, recognized non-overlapping retrovirally-labeled clusters. This result appeared to imply subclass-specific progenitors each giving rise to multiple morphologically and neurochemically similar neurons (Mione et al., 1994). The pyramidal-nonpyramidal neuron dichotomy bolstered the view that committed ventricular zone progenitors give rise to a limited range of cell types and that cell lineage plays a pivotal role in determining functionally important phenotypic attributes of mature cortical cells.

Recently, in retroviral studies of primate neurogenesis, labeled cells were found to occur in horizontally or vertically oriented cell clusters (Kornack and Rakic, 1995). Horizontally oriented clusters were twice as common as vertically oriented clones and were postulated to be the progeny of infected progenitors which divided symmetrically to form multiple progenitors within the proliferative zone. Subsequent symmetric division of these secondary progenitors would form "cousin" postmitotic progeny thought to migrate along nearby radial glia to the same cortical laminae (Kornack and Rakic, 1995). These horizontally oriented clusters appear similar to the majority of retrovirally labeled clusters observed in the rat. Vertically oriented sibling clusters were interpreted as resulting from progenitors dividing in a stem cell fashion to produce a series of postmitotic neurons within a confined region that each migrated radially to the layer appropriate to its birthdate. Vertically, or radially oriented clones were first reported in the mouse (Luskin et al., 1988), but have also been observed occasionally in the rat (Price and Thurlow, 1988; Walsh and Cepko, 1988).

All of the retroviral studies discussed above relied heavily on the assumption that clonally related cells come to occupy geographically similar positions in the adult cortex. The distribution of clones within the cortex, at least in the rat (Walsh and Cepko, 1992), is in fact unpredictable. This unpredictability poses a serious limitation to conclusions made on the basis of single retrovirus experiments, since widespread sibling cells cannot be recognized as sibling cells. Thus accurate definition of clonal relationships requires a method that distinguishes intermingled and dispersed cortical clones.

Cell lineage and the retroviral library innovation

Using a large number of retroviruses whose proviral sequences can be distinguished by polymerase chain reaction represents a conceptually simple solution to the problem of differentiating cells of multiple clones. Retroviruses can be engineered to contain non-coding DNA inserts of variable size and sequence. These inserts can be amplified from

individual retrovirally-labeled cells dissected out of histochemically stained tissue sections. If the number of retroviruses overwhelmingly exceeds the number of infective events, it is unlikely that the same DNA insert will be present in more than one progenitor cell, much less multiple progenitors. Therefore the problem of differentiating related and unrelated cells has been largely eliminated, since each clone is uniquely identified by the specific DNA insert contained in each retrovirally labeled cell of that clone.

The retroviral library is introduced into developing brains by injection into the lateral ventricles of fetal rats, just like the simple retroviral supernatant. After the animal has been allowed to develop normally, labeled cells are studied by microscopic examination of tissue sections, and cell morphologies and positioning within the cortex are recorded by photography and camera lucida drawings. Finally, clonal analysis is performed by preparing DNA samples from labeled cells for amplification by PCR. The coverslips are removed from histological slides, and small fragments of tissue containing the nucleus of each labeled cell are dissected-out in small chunks of tissue. The tissue samples are then digested, and a nested PCR protocol is employed to amplify the DNA inserts that identify distinct clones. The resultant PCR products are separated by size on agarose gels. Each PCR product is digested with a standard battery of five restriction enzymes to generate a fingerprint (Walsh and Cepko, 1992).

Experiments employing retroviral libraries have shown that progeny of a single progenitor may reside, not only in different cortical layers, but also in distinct functional domains (Walsh and Cepko, 1992; Reid et al., 1995). The wide dispersion of clonally related progeny seems at odds with evidence that the vast majority of postmitotic neurons migrate radially from the ventricular zone (Rakic, 1972; O'Rourke et al., 1992). On the other hand, the migration of ventricular progenitor cells (Fishell et al., 1993), or postmitotic cells within the VZ and SVZ, coupled with the curved geometry of the rapidly expanding cerebral cortex, suggests that progeny generated at different points along the ventricle might be expected to occupy multiple cortical regions. Likewise, spatially-regulated cues within the ventricle might influence the phenotypes of progeny cells produced by a single progenitor as it travels to different locations in the ventricular zone.

A disadvantage of clonal analysis with PCR is that PCR is successful only for 40–65% of histochemically labeled cells. Consequently, many cells cannot be assigned to a clone and are essentially ignored. For this reason, the number of sibling cells per clone, and the distribution of sibling cells, is systematically underestimated. The success of PCR in any given cell appears to be random, however, and analysis of a large number of PCR-defined clones in multiple experiments tends to reveal common patterns of clonal dispersion and clonal composition. Retroviral clonal analysis represents a snapshot of development at a certain time point, and retroviral studies alone cannot definitively demonstrate the cellular processes that produce that picture. The direct imaging of dividing and nondividing cells in living tissue, on the other hand, has offered surprising insights into migratory and mitotic behavior of germinal zone cells. Initial application of retroviral libraries (Walsh and Cepko, 1992) showed that cortical sibling cells could be highly separated from one anther, but it has taken further experimentation to determine how that wide dispersion occurs.

In vitro imaging of cortical progenitors

The innovative use of modern time lapse video microscopy has allowed investigators to directly visualize progenitor cell behavior within the ventricular zone (Fishell et al., 1993; Chenn and McConnell, 1995) and migrating postmitotic neurons (O'Rourke et al., 1992). Video microscopic studies have demonstrated that ventricular zone cells display extensive lateral movement within the ventricular zone between mitoses (Fishell et al., 1993). In explants prepared from the forebrains of E15 mice, Di-I labeled cells in the ventricular zone of the cerebral cortex were seen migrating within the cortical neuroepithelium in short bursts of 5–20 μm for an average rate of 10–100 μm per hour. Some of these labeled cells were observed to di-

vide suggesting that they were cortical progenitor cells; the resulting daughter cells then migrated with non-correlated trajectories within the ventricular zone. Often one or more cells exited the ventricular zone, though it could not be determined if they were undergoing interkinetic migration or becoming postmitotic and leaving via radial glia. In general, cortical ventricular zone cells migrated in a random manner, but, tended not to cross over the cortico-striatal border and instead migrated along this longitudinal border in a rostral or caudal fashion (Fishell et al., 1993). A similar phenomenon of ventricular zone dispersion was indicated by retroviral library analysis following fetal injection and short survival times, since sibling cells were sometimes widely dispersed but still confined to the proliferative layers (Walsh and Cepko, 1993). The movement of cells in the proliferative regions appears to be an adequate explanation for the widespread clonal dispersion seen with retroviral libraries. However, only more recently has the widespread clonal dispersion in the cortex been reconciled with description of retrovirally labeled cell clusters of uniform phenotype.

Improved staining and clonal assignment with the AP retroviral library

Retrovirally-encoded beta-galactosidase often failed to produce cellular labeling adequate for cell type identification (Walsh and Cepko, 1992), making it very difficult to relate patterns of clonal dispersion to the formation of cell phenotypes. Therefore a second retroviral library coding for alkaline phosphatase (AP) was designed for enhanced cell type identification. Unlike retrovirally-encoded β-galactosidase, retrovirally-encoded AP frequently produced a "Golgi-like" filling of neuronal processes. Retrovirally-encoded AP was seen to produce intense labeling of cellular processes, allowing morphological identification of >90% of labeled cells as neuronal or glial by standard morphological criteria. The morphologies of retrovirally labeled neurons were similar to those seen in postmortem staining of fixed tissue (e.g. Werner et al., 1985). While an unambiguous classification of all cortical neuronal types cannot be made on the basis of morphological criteria alone (Peters and Jones, 1988), we found that AP staining allowed identification of morphological subtype in ≈80% of labeled neurons. Clonal analysis was performed by PCR amplification of retrovirally-encoded DNA tags (Walsh, 1995).

Clonal structure revealed by the AP retroviral library

Cortical clones labeled by injection of the AP-encoding retroviral library at E15 or E17 shared two strikingly different clonal patterns: clustered and widespread clones. Fifty-two percent of clones labeled by E15 injections were clustered. Clones were considered to be clustered if they consisted of single cells, or single clusters of cells grouped within 1 mm (Fig. 2). Clones that contained sibling cells scattered over >1.5 mm were defined as widespread, and constituted 48% of the clones, a proportion comparable to that seen in studies using a β-galactosidase-encoding library (Walsh and Cepko, 1993) and included the majority of retrovirally labeled neurons (73%).

Clustered clones, like the cell clusters analyzed in earlier retroviral experiments, contained from 3 to >20 glia, or 2–4 morphologically similar neurons within one, and occasionally two, cortical laminae (Fig. 2). Many single-neuron clones were likely part of clusters of morphologically similar cells for which PCR was only successful in one cell. The finding of clustered neuronal clones confirmed earlier descriptions of retrovirally labeled clusters (Parnavelas et al., 1991; Grove et al., 1993; Luskin et al., 1993) which have suggested considerable morphological and biochemical homogeneity among closely clustered retrovirally labeled cells. Our analysis of AP-labeled cells confirmed earlier suggestions that clustered clones contain mainly cells of similar morphology (Parnavelas et al., 1991; Grove et al., 1993; Luskin et al., 1993).

Although widespread clones contained sibling cells over great distances, the relative location of widespread sibling cells was not completely random. When we analyzed the intercell distances among sibling cells, we found that neuronal sib-

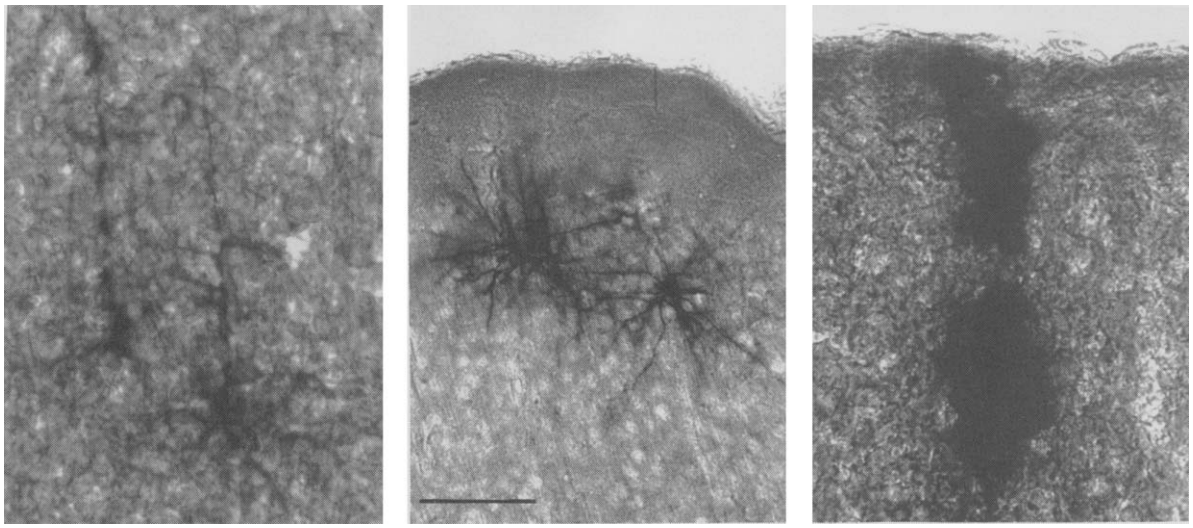

Fig. 2. Three AP-retrovirally labeled cell clusters, typical of those found either in single clusters or as subunits of widespread clones, are illustrated. Two deep layer pyramidal neurons (A), two layer II multipolar neurons (B) and a cluster of several astrocytes spanning cortical laminae (C) demonstrate the relationship between cell morphology and cell clustering within clones. Closely spaced sibling cells tended to occupy similar conical laminae and adopted similar morphologies even though those cell clusters were often part of larger, morphologically diverse clones. The scale for all three panels is the same and is indicated by a scale bar in (B) which indicates 100 μm.

lings in widespread clones were spaced preferentially at certain intervals along the rostrocaudal axis. Sibling cells were located most commonly between 0 and 0.5 mm from each other. On the other hand it was very rare for sibling cells to be spaced 1.0–1.5 mm apart. Siblings were also commonly spaced 2–3 mm or even 4–6 mm apart, suggesting a periodic spacing of sibling cells corresponding to 2–3 mm in the adult brain. Since unrelated retrovirally-labeled cells did not display a similar periodic spacing in the cortex, a mechanism intrinsic to cortical progenitor cells seems to determine the periodic spacing of their progeny. Nearby sibling cells, whether they were members of a single cluster clone or widespread clone, showed similar laminar location and morphology (Fig. 2).

While clustered sibling cells showed similar location and morphology, widely dispersed siblings often showed different phenotypes. Within a single widespread clone, sibling cells localizing to distinct forebrain structures, such as the hippocampus or neocortex, took on locally appropriate, though widely divergent, morphologies. In addition, some widespread clones contained cells in widely different cortical laminae. Furthermore, 2/18 widespread clones labeled at E15 included neurons in one region and glia in another region confirming an observation of occasional neuron-glia clones observed in vivo with the beta galactosidase-encoding retroviral library (Walsh and Cepko, 1992; Walsh and Cepko, 1993) and in vitro (Williams et al., 1991; Davis and Temple, 1994). Finally, 8/18 widespread clones labeled at E15 contained neurons with nonpyramidal morphology in one neocortical location, and pyramidal morphology in another. Of 18 widespread clones (13 from E15, 5 from E17), 17 (94%) contained cells with multiple phenotypes defined either as (i) being located in neocortex plus non-neocortical areas, (ii) being pyramidal plus nonpyramidal neurons, or (iii) being neurons plus glial cells.

When retroviral labeling was performed at E17 rather than E15, the structure of cortical clones was systematically different. Widespread clones were almost four-fold less common as a percent-

age of the total number of clones (12.5 versus 48%), a difference significant at $P < 0.002$ (Chi-squared test). Widespread clones were not only unusual after E17 injection, but they also contained few neurons. All 5 of the widespread clones labeled with the AP-encoding library contained only two cells each. All widespread clones labeled at E17 with a previous β-gal-encoding library also contained two cells each (Walsh and Cepko, 1992). Widespread clones labeled at E17 also accounted for a much lower percentage of the total number of retrovirally labeled neurons (29 versus 73%), suggesting that retroviral injections later in development label progenitors at later stages of neurogenesis.

A hierarchical model of cortical cell lineage

Clones labeled at E15 or E17 distributed in the cortex as clustered and widespread clones, definable both by location and by cell morphology. Clustered clones imply a progenitor that does not migrate widely as it divides to produce a cluster of a relatively uniform cell type (Grove et al., 1993) suggesting that some progenitors undergo multiple rounds of cell division, producing the same or similar cell types each time. In contrast, widespread clones consisted of nonrandomly distributed, clonal subunits, each subunit being otherwise indistinguishable from a clustered clone. A subunit is defined as one or more sibling cells located within 1 mm of one another and separated from other sibling cells by >1.5 mm.

The widespread dispersion of subunits is most easily explained by the migration of progenitor cells. This migration has been suggested by prior retroviral studies as well as direct in vitro observation (Fishell et al., 1993; Walsh and Cepko, 1993). The periodic spacing of subunits would then be determined by the rate and trajectory of progenitor cell migration and the length of the progenitor's cell cycle. The distance through which a progenitor moves need not be large in order to generate the 2–3 mm periodicity seen in the adult brain. The 2–3 mm periodicity at P15 corresponds to ~0.5 mm in the E18 cortex, or perhaps 100–250 μm per cell cycle (20–24 h) in the E18 ventricular zone (Waechter and Jaensch, 1972). This rate of movement is well within the range observed by Fishell et al. (1993).

A reasonable model of cell lineage in the cortex postulates that migratory, multipotential progenitors located in the cortical ventricular zone divide asymmetrically in a stem cell fashion to produce a nonmigratory cell and generate a multipotential cell (Fig. 3). The nonmigratory cells produced by divisions of the multipotential cell can themselves differentiate, or divide 1–4 times to generate multiple cells. Some nonmigratory cortical progenitors appear to generate single neuronal or glial types over multiple cell divisions and therefore may represent sublineages, secondary progenitors restricted to producing a single cell type. Since retroviruses appear to integrate into only one of the two original daughter cells, infecting a multipotential progenitor at E15 would result, with equal probability, in retroviral integration into the nonmigratory or the migratory daughter cell. Integration into the nonmigratory daughter would produce labeling in a single cluster or single cell (see Fig. 3), whereas integration into the migratory daughter would label a widespread clone. This might account for the consistent labeling of ~50% clustered and 50% widespread clones in this report and a previous one (Walsh and Cepko, 1993). In accordance with this model, each clonal subunit would correspond to a cell cycle of the migratory progenitor, with the observed maximum number of subunits (4) close to the estimated number of remaining neurogenetic cell cycles between E16–E20 (Waechter and Jaensch, 1972). Retroviral injections at E17 would infect a more mature progenitor with fewer remaining cell divisions. Regardless of which of the two daughter cells were labeled by the retrovirus, widespread clones would be labeled less commonly, and the widespread clones would contain fewer subunits, as was observed.

Clusters of retrovirally labeled cells with uniform phenotypes can now be recognized as terminal branches of widely dispersed lineages. A transition from a multipotential to a potentially more restricted progenitor fits well with recent in vitro studies and previous transplantation studies

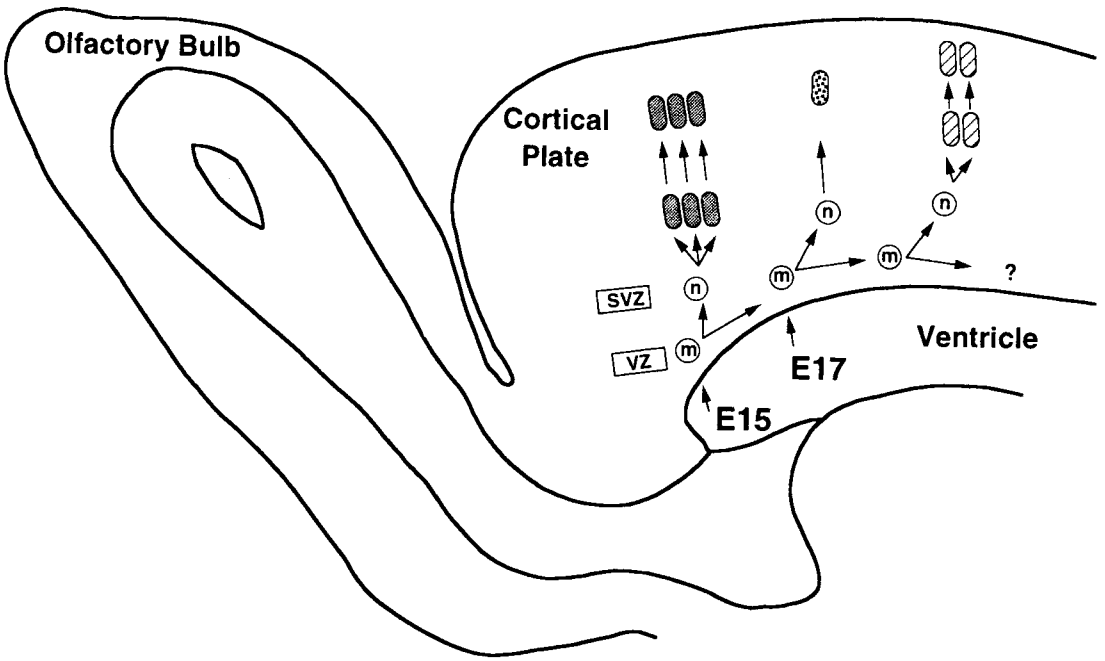

Fig. 3. A hypothetical model of cell lineage in the mammalian cerebral cortex is depicted along a schematically rendered saggital section through the E22 rat forebrain. Cerebral cortical cells are derived from the two proliferative tones (VZ, SVZ) and most neocortical neurons migrate radially from the proliferative zones to the cortex proper. The behavior of one multipotential cell is illustrated. The multipotential cell (m) migrates as it divides, sequentially producing three non-migratory progenitors at spatio-temporal intervals and regenerating a multipotential cell in "stem cell" fashion. Each non-migratory progenitor then behaves essentially independently, dividing multiple times (shading), directly differentiating (stippling) or dividing once (cross-hatching), to form three distinct cell clusters. Since cells in the rodent cortex are added in a roughly inside-out sequence, the oldest non-migratory progenitors would tend to form deeper neurons, and the newer non-migratory progenitors would tend to form more superficial neurons. Infection of the multipotential precursor at E15 would result, with equal probability, in integration of the viral DNA into the migratory or the non-migratory daughter integration into the non-migratory daughter would label a single clustered clone (or single cell), whereas integration into the migratory daughter would label a widespread clone consisting of several subunits. In contrast, infection at E17 would label a progenitor with fewer remaining cell divisions. Therefore widespread clones would be rare and small following E17 injections.

suggesting early cortical progenitors are multipotential, but are iteratively committed to produce progeny with specific phenotypes.

In vitro evidence for multipotential progenitors

Parallel evidence for the existence of a transition from multipotential to more restricted progenitor comes from in vitro analysis. Consistent with early retroviral data, most progenitors in vitro give rise to single cells or small cell clusters. A significant minority of progenitors, however, display a very different behavior in vitro (Davis and Temple, 1994; Williams and Price, 1995). When single ventricular zone progenitors were dissociated from E12 and E14 cortices and then followed in culture over several days (Davis and Temple, 1994), roughly 50% of E14 progenitors and 56% of E12 progenitors soon differentiated into a single neuron, while 26% (E14) and 21% (E12) of ventricular progenitors differentiated into pairs of neurons. Notably, the small size of neuronal clones in single-cell culture corresponded closely to the results obtained from retroviral labeling experiments in vivo (Reid et al., 1995). Purely astrocytic or oligodendroglial clones were, however, much more rare in culture than in some in vivo experiments suggesting that the in vitro conditions lacked certain glial differentiation factors present in vivo.

In vitro experiments also demonstrated that a significant minority of progenitors (approximately 9% at both E12 and E14) give rise to large mixed

neuronal and glial clones (average size of these mixed clones was 25 progeny for E14 progenitors and 29 progeny for E12 progenitors) (Davis and Temple, 1994). More surprisingly, the remaining 7% of ventricular zone cells continued to divide in culture for several weeks and generated greater than 100 progeny of mixed cell types. Some of these clones included all three of the major cortical cell types. Based on their multipotentiality and ability to divide several times, these cells were termed stem cells. When these cultures were subcloned many of the progeny of the original VZ cell also divided several times and gave rise to large clones. After 6 days in culture the average size of E12 stem cell clones was significantly greater than E14 stem cell clones (65.2 versus 33.3) This result suggests that these progenitors possess an internal clock and that E12 derived stem cells may undergo the cell cycle more rapidly, differentiate later, undergo less cell death, or divide with more symmetric divisions (Davis and Temple, 1994).

A separate study that examined the progeny of E12 and E14 progenitors in culture also suggested the existence of a population of asymmetrically dividing, multipotential progenitors, and a second small population (<5%) of progenitors that gave rise to small clones containing both neurons and glia (Williams and Price, 1995). Together these studies suggest that multipotential cells may be the ancestors of secondary cortical progenitors cells that exhibit more limited proliferation and more limited progeny cell fates. Secondary progenitors, in contrast to multipotential progenitors, isolated from E14 cortices appeared to be indistinguishable from E12 secondary progenitors with regards to the number and range of cell types that they generated (Davis and Temple, 1994). The age-independent behavior of the presumptive secondary progenitors in vitro correlates well with data obtained with the AP retroviral library discussed previously.

Molecular signals governing proliferation and differentiation

The emerging patterns of cell lineage in the mammalian cerebral cortex, in which multipotential stem cells produce more committed progenitors, has formal similarities to cell lineage patterns in the CNS of *Drosophila*, and there is parallel evidence that many molecular signaling mechanisms are conserved as well. *Drosophila* CNS neurons are derived from multipotential neural stem cells called neuroblasts (NB's). Each NB can form from any one of 4–6 neuroectodermal cells at a given position (Goodman and Doe, 1993). These cells are essentially equivalent, and are themselves defined as a group by the expression of proneural genes such as those of the *achaete-scute* complex (AS-C) (Jan and Jan, 1995). Once a given cell differentiates into a NB, lateral inhibition (or lateral specification (Artavanis-Tsakonas et al., 1995)), mediated by neurogenic genes including *Delta* and *Notch*, prevents adjacent cells within the group from also becoming NBs. Activation of the Notch receptor in cells adjacent to the incipient NB (by binding of Delta) activates expression of genes such as *Enhancer of split*, whose protein products act in the nucleus to down-regulate expression of AS-C genes. Once formed, the NB undergoes a series of defined, asymmetric, stem cell mitoses to generate ganglion mother cells (GMCs). The GMCs differ morphologically from the NBs, and have a much more limited developmental potential. The GMCs generally divide once to form two daughter cells, typically two identifiable neurons that are distinct from each other morphologically. The asymmetry of the NB/GMC appears to be determined largely by the asymmetrical inheritance of the protein products of the *numb* and *prospero* genes (Rhyu et al., 1994; Jan and Jan, 1995; Spana and Doe, 1995), and perhaps additional genes not yet identified.

Recently, vertebrate homologues of many key *Drosophila* neurogenic genes have been identified and studied. A vertebrate homologue of *Delta* suppresses neurogenesis by binding to Notch1, a vertebrate homologue of Notch (Chitnis et al., 1995; Henrique et al., 1995). Activated Notch1, or increased levels of Notch1 expression, suppresses neurogenesis in a cell-autonomous fashion (Austin et al., 1995; Dorsky et al., 1995). In myogenic precursors, activated Notch1 activates the expression of *HES-1*, a vertebrate homologue of *Enhancer of*

split (Jarriault et al., 1995). *HES-1*, in turn, directly inhibits neurogenesis in the cerebral cortex when overexpressed with retroviral vectors (Ishibashi et al., 1994). Finally, targeted disruption of vertebrate *HES-1* produces mice with severe defects in CNS development, coupled with precocious expression of *MASH-1*, also suggesting that *HES-1* normally inhibits expression of mammalian *AS-C* homologues, and hence neuronal fate (Ishibashi et al., 1995). Therefore, there appears to be amazing conservation of the *Notch* signaling pathway in vertebrates, although there are many uncertainties that still need to be resolved.

In addition to vertebrate homologues of neurogenic genes, there have also been a number of secreted proteins that appear likely to play a role in regulation of cortical neurogenesis in vertebrates. EGF stimulates the proliferation of forebrain precursor cells (Reynolds and Weiss, 1992), and bFGF stimulates the proliferation of nestin-positive neuroepithelial cells isolated from the hippocampal germinal zone and acts as a survival factor for GFAP-positive astrocyte precursors (Vicario-Abejon et al., 1995). bFGF also appears to acts as a differentiation factor for calbindin-positive hippocampal neurons. Rapid induction of calbindin expression independently by bFGF and/or NT-3 in vitro suggests that they act at early stages of hippocampal neuron differentiation by inducing signal pathways which specify a particular neuronal subtype. Likewise, NT-3 and BDNF stimulated the differentiation of hippocampal neuroepithelial cells into MAP-2 positive neurons (Vicario-Abejon et al., 1995).

Basic FGF has also been shown to have stimulatory effects on stem cells derived from rat cerebral cortex (Ghosh and Greenberg, 1995). In vitro studies of cortical progenitors isolated from whole embryonic cortex showed that these cells exhibit tyrosine phosphorylation of MAP kinase and cAMP response element binding protein (CREB) in response to stimulation with bFGF, BDNF, NT-3 or NT-4; however differentiated neurons appear to lose responsiveness to bFGF (Ghosh and Greenberg, 1995). These studies also demonstrated the potential of bFGF-stimulated neuroepithelial cells to differentiate into neurons and oligodendrocytes in the absence of serum, or neurons and astrocytes in the presence of serum. The ability of an unidentified serum factor to promote astrocyte over oligodendrocyte differentiation has been observed in similar cortical cell culture studies (Williams and Price, 1995). NT-3 appeared to antagonize the proliferative effects of bFGF on the progenitor cells but promoted neuronal differentiation while BDNF increased cortical neuron survival.

Whether or not bFGF and the neurotrophins and other growth factors play a similar role in vivo is difficult to assess since mice with engineered mutations in many of these genes (NT-3, BDNF, TrkC, and TrkB) fail to show obvious cerebral cortical phenotypes (for review see Klein, 1994) perhaps as a result of receptor promiscuity demonstrated by many growth factor families. Mice that carry mutations in multiple neurotrophin genes or multiple receptors may however show cortical phenotypes.

Finally, neurotransmitters themselves have recently been implicated in controlling and perhaps directing neurogenesis of cortical precursors. Both GABA and NMDA agonists depolarize cortical precursor cells in vitro and inhibit DNA synthesis (LoTurco et al., 1995). Furthermore, in vitro evidence suggests that GABA exposure may alter the progeny of dividing cortical progenitor cells (Price, 1995). These observations are particularly interesting in light of the finding that GABA-ergic neurons are located in the VZ and SVZ, and that processes of early-differentiating neurons from other cortical layers project through the VZ and SVZ (Kim et al., 1991). Therefore, the anatomy of growing cortical axons would allow for the regulation of cortical development by released neurotransmitters in the proliferative regions (McConnell, 1991).

Conclusion

Lineage analysis and transplantation experiments in the cerebral cortex have produced some surprising findings, but ones that correlate in interesting ways with developing knowledge of neural progenitor behavior in vitro. The lineage patterns hold

promise to form a framework for interpreting the actions of a burgeoning variety of molecules isolated in the developing cortex.

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