Vertical Compartmentation and Cellular Transformations in the Germinal Matrices of the Embryonic Rat Cerebral Cortex

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Cellular compartmentation was studied in the germinal matrices (the primary neuroepithelium and the subventricular zone) of the rat cerebral cortex at successive stages of embryonic development. Three sets of materials were used: methacrylate-embedded brain sections from normal embryos, autoradiograms from embryos labeled with [3H]thymidine, and methacrylate-embedded sections from embryos exposed to 200 R X-ray. Examination of normal specimens showed that between Embryonic Day 12 (E12) and E15 the cortical germinal matrix consists only of a primary neuroepithelium. By Day E16, a subventricular zone has formed in the early developing ventrolateral aspect of the cortex. The subventricular zone grows in depth for several days, while at the same time the depth of the neuroepithelium decreases. Examination of short-survival thymidine radiograms revealed that the labeled cells do not form a continuous band in the neuroepithelium but aggregate in patches reminiscent of bunches of grapes strung one a line. It is postulated that this vertical periodicity is due to the alternation of cell aggregates with short and long cell cycle times. Finally, examination of the cortical neuroepithelium in rats exposed to 200 R X-ray showed that there is an alternation of radiosensitive (collapsing) and radioresistant (intact) patches that roughly correspond in size to the labeled and unlabeled patches seen in autoradiograms. Additional observations concern the onset of local cell proliferation in the white matter at late stages of fetal development and the transformation of the neuroepithelium into a matrix producing ependymal cells.

MATERIALS AND METHODS

Animals. Purdue-Wistar rats bred in our laboratory were used. Adult females were paired with males in the evening and vaginal smears were taken the next morning. Females found sperm-positive were isolated in maternity cages and were considered to have reached Gestational Day 1. At daily intervals, dams ranging in gestational ages between Days 12 and 21 were either injected with [3H]thymidine or were irradiated with X-ray to study normal and abnormal developmental phenomena in their offspring. An untreated group of pregnant females was the source of control embryos.
Injection of $[^3H]$thymidine. The dams were injected subcutaneously with a single dose (5 μCi/g body weight) of $[^3H]$thymidine (Schwarz-Mann, spec act, 6.0 Ci/mmol) between 9:00 and 11:00 AM and killed 2 h after injection.

Radiation procedures. The radiation source was a 300-kV Maxitron unit with a 1.5-mm copper filter. Individual dams were placed in a glass container (20 × 10 × 6.5 cm) covered with a perforated clear vinyl lid and exposed to 200 R at approximately 50 R/min (measured with a Victoreen 250-roentgen probe).

Histological and autoradiographic procedures. The dams were anesthetized at the designated times, and the embryos or fetuses were removed and immediately killed by immersion in Bouin’s fluid. After 24 h, the specimens were transferred for storage into 10% neutral formalin. The specimens were blocked coronally, sagittally, or horizontally and embedded in either paraffin or methacrylate. The paraffin blocks were serially sectioned at 6 μm and the methacrylate blocks at 3 μm, and every 10th section was saved. Successive sections were either stained with cresyl violet and hematoxylin-eosin or first immersed in Bouin’s fluid. After 24 h, the specimens were transferred for storage into 10% neutral formalin, developed in Kodak D-19, and exposed to 200 R at approximately 50 R/min (measured with a Victoreen 250-roentgen probe).

Materials available for analysis. The collection of short-survival thymidine radiograms used in this study consists of 94 paraffin- or methacrylate-embedded specimens. The X-irradiated specimens were drawn from a collection consisting of 115 methacrylate-embedded embryos and fetal brains (E12 Xr–E22 Xr). Finally, use was made of relevant materials in our large histological collection of normal developing brains.

RESULTS

The Segregation of the Ventricular and the Subventricular Zones: The Primary Neuroepithelium and the Secondary Germinal Matrix

Examination of methacrylate embedded specimens of normal rat embryos indicates that on Embryonic Day 13 (E13)¹ (Fig. 1A) the germinal matrix of the cerebral cortex is composed of a single germinal zone and the great majority of mitotic cells are situated in a periventricular position (arrows in Fig. 1A). This is the primary neuroepithelium. The only change on Day E14 is the formation of the primordial plexiform layer of the cortex (designated as layer I), consisting of an extracellular gridwork (channel 1; ch1 in Fig. 1B) with a few scattered horizontally oriented cells (the presumed Cajal-Retzius cells; arrows in Fig. 1B). By Day E15, when the neuroepithelium reaches its maximum width, mitotic cells begin to appear within the neuroepithelium (not shown) and, in particular, at its upper margin (arrows in Fig. 1C).

Unlike the periventricular mitotic cells, which tend to have vertically oriented cleavage planes, the intraventricular mitotic cells tend to have horizontally oriented cleavage planes. At this stage of development, a subventricular zone is still not evident in the formative cerebral cortex even in its most advanced ventrolateral aspect. By Day E16 two new cellular strata appear in the early developing regions of the cerebral cortex: (a) the partially formed cortical plate (CP in brackets in Fig. 2A) underneath layer I; and (b) the subventricular zone (sv), a secondary germinal matrix with relatively high numbers of mitotic cells (double arrows in Fig. 2A). There are also some intraepithelial mitotic cells present at this stage. The cleavage plane of the subventricular mitotic cells, like those of intraepithelial mitotic cells, is usually horizontally oriented. Conceivably, the subventricular mitotic cells are progeny of the intraepithelial mitotic cells, i.e., cells of the primary neuroepithelium that do not undergo interkinetic nuclear migration. By Day E17 the cortical plate is more clearly defined (CP in Fig. 2B) and separated from it by a new gridwork (channel 2; ch2 in Fig. 2B) the intermediate zone (iz) begins to form. The boundary between the intermediate zone, which is devoid of mitotic cells, and the subventricular zone, which contains many mitotic cells, is not well defined. For several days there is an increase in the thickness of the subventricular zone (sv in Figs. 3A–3C) but there is a steady reduction in the thickness of the primary neuroepithelium (ne). By Day E21 the subventricular zone (sv in Fig. 3D) also begins to diminish in size and there is an apparent reduction in its mitotic activity.

Cellular Heterogeneity in the Cortical Neuroepithelium

Columnar heterogeneity seen 2 h after labeling. In short-survival radiograms of rats labeled with $[^3H]$thymidine from Day E12 onward and killed 2 h after injection (Figs. 4A–4D), heavily labeled cells are concentrated in a band away from the lumen, traditionally identified as the neuroepithelial synthetic zone (sz). The synthetic zone has a ragged internal border with heavily labeled cells aggregated in sawtooth-shaped patches, reminiscent of bunches of grapes strung on a line. One possible explanation for this periodicity is that the neuroepithelium is made up of alternating cell populations (microzones) with short or long cell cycle times (21). The microzone containing the labeled cells extending toward the lumen may be composed of more fast cycling cells, while the adjacent unlabeled microzone may be com-

¹ Abbreviations used: an, anterior; CF, callosal fibers; ch1, channel 1; ch2, channel 2; chp, choroid plexus; CP, cortical plate; E, embryonic; ez, ependymal zone; I, layer I (marginal layer); iz, intermediate zone; lv, lateral ventricle; mz, mitotic zone; ne, neuroepithelium; pc, pyknotic cells; SP, subplate; sv, subventricular zone; sz, synthetic zone; TH, thalamus; WH, white matter.
FIG. 1. Cellular organization of the developing rat cerebral cortex on Days E13 (A), E14 (B), and E15 (C). Arrows in A point to periventricular mitotic cells; arrows in B point to horizontal cells (presumed Cajal-Retzius cells) in layer I; arrows in C point to superficially located intraepithelial mitotic cells. Methacrylate; hematoxylin–eosin.
posed of more slow cycling cells. Whether or not this interpretation is correct, our thymidine radiographic observations suggest that there is columnar cellular heterogeneity within the cortical neuroepithelium.

Columnar heterogeneity following X-irradiation. Periodicity within the neuroepithelium is seen more clearly following X-irradiation. In rat embryos exposed to a single dose of 150–200 R X-ray and killed 6 h after irradiation a high proportion of the multiplying and migrating cells of the nervous system is killed, while the mature cells are spared (2, 10, 12). The pyknotic debris of dead cells abound in the neuroepithelium throughout the central nervous system, in particular in the band corresponding to the synthetic zone. In most brain regions the cells adjacent to the lumen are spared. But in circumscribed areas, cells near the lumen are also killed. As a
FIG. 3. Cellular organization of the germinal matrices of the developing rat cerebral cortex on Days E18 (A), E19 (B), E20 (C), and E21 (D). Note the progressively diminishing depth of the neuroepithelium (ne) and the initial increase and subsequent decrease in the depth of the subventricular zone (sv). Arrows point to subventricular mitotic cells. Methacrylate; hematoxylin-eosin.
FIG. 4. Autoradiograms of the developing cortex from rats that received [³H]thymidine on Days E12 (A), E13 (B), E15 (C), and E15 (D) and were killed 2 h after injection. The patchy distribution of labeled cells is evident throughout this period. (A similar pattern on Day E16 is shown in Fig. 7A.) (A–C) methacrylate; (D) paraffin.
result, the ventricular wall collapses and the dead cells of the neuroepithelium, as well as those in the adjacent premigratory and migratory areas, spill into the ventricle.

The cerebral cortex (together with a few other cortical structures) shows a unique feature in that, during a specific period of development (especially between Days E15 and E18), the collapse of the neuroepithelium is limited to alternating patches (Fig. 5B). With progressive cortical development, the total area of collapse shrinks from ventral (Fig. 6B) to dorsal (Fig. 6A). This parallels the same gradient in cortical development, as judged by the width of the cortical plate and intermediate zone (CP and iz in Fig. 6B). The alternating collapsing and surviving neuroepithelial patches appear to be similar in size (Figs. 5B, 7B), irrespective of the plane of sectioning; to
FIG. 6. Horizontal sections through the cerebral cortex of an X-irradiated Day E17 rat from dorsal (A) to ventral (B). Note that the entire cortex shows periodic collapse dorsally but the collapse is absent in much of the cortex ventrally. Methacrylate; hematoxylin-eosin.

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the alternating microzones of labeled and unlabeled cells seen in short-survival radiograms (Figs. 5A and 7A). This evidence supports our hypothesis that the cortical neuroepithelium is composed of at least two cell populations aggregating in alternating columns. Our data do not directly reveal which of the two patches is the more radiosensitive one, whether it is the patch containing a higher proportion or a lower proportion of labeled cells.

Transformation of the Neuroepithelium

The high concentration of labeled cells in the synthetic zone of the neuroepithelium, as seen in short-survival radiograms of Day E12-E18 rats (sz in Figs. 4A-4D, 7A, and 9A), is progressively reduced on Days E19 (sz in Fig. 9B), E20 (sz in Fig. 8), and E21 (sz in Fig. 9C). There is also an apparent reduction in the width of the synthetic zone during this period. This event may be correlated with the progressive decline in cortical neuron production, which comes to an end on Day E21 (5).

Concurrent with the reduction in the proportion of labeled cells in the synthetic zone there is an increase in the proportion of labeled cells in the mitotic zone. Whereas in the short-survival radiograms of rats labeled with [3H]thymidine between days E12 and E18 few labeled cells are seen in the mitotic zone (mz in Figs. 4, 7A, and 9A), there is a progressive increase in such cells in E19 (Fig. 9B), E20 (Fig. 8), and E21 rats (Fig. 9C). We postulate that these cells, which apparently do not undergo interkinetic nuclear migration, are locally multiplying ependymal cells (ez in Figs. 8 and 9C).

Local Glial Cell Proliferation

Another change taking place during this period is the increase in cell proliferation outside the germinal matrix. While an occasional labeled cell is not difficult to
find outside the germinal matrix in short-survival radiograms of younger embryos, there is a substantial increase in "locally" multiplying cells within the intermediate zone and the cortical plate from Day E20 onward (arrows in Figs. 8 and 9C). The gradient in the concentration of these cells from the base of the formative cortex to its surface suggests that these locally multiplying cells leave the germinal matrix, and diffuse outward, at about this time.

**DISCUSSION**

*The Cellular Heterogeneity of the Primary Neuroepithelium*

The patchy organization of the cortical neuroepithelium is not evident in conventional Nissl-stained sections. Indeed, the alternation of patches with high and low concentrations of heavily labeled cells that we describe in this paper has not been previously reported by others who have used thymidine autoradiography to study cortical neurogenesis. Possibly this is due to the circumstance that even though these patchies are visible in many sections, irrespective of the plane of sectioning, the periodicity is not discernible in all sections. Our tentative interpretation is that the cortical neuroepithelium is composed of columns of differentially labeled cells arranged in an irregular checkerboard pattern but that this pattern is apparent only in those sections that are cut parallel to the alignment of the columns. Diagonally cut sections will show a more uniform (all high or all low) labeling pattern. To test this hypothesis it will be necessary to three-dimensionally reconstruct the patches from serial thymidine radiograms.
In contrast to this new thymidine radiographic observation, the alternation of collapsing (more radiosensitive) and noncollapsing (less radiosensitive) neuroepithelial patches following X-irradiation was previously noted by Hicks (10), see his Fig. 3) and by Berry and Eayrs (6), see their Fig. 2). Hicks related this selective neuroepithelial effect to the formation of cortical "rosettes" in animals that survived for 24 h or longer after irradiation. The roughly equal size of the differentially labeled radiographic microzones and of the alternating radiosensitive and radioresistant patches suggests that the two techniques reveal the same phenomenon.

Waechter and Jaensch (21) described two proliferative cell types in the rat cortical neuroepithelium, one with fast and the other with slow turnover rates. Accordingly, the patchy labeling pattern seen in short-survival radiograms could indicate that fast cycling cells and slow cycling cells form separate aggregates in the cortical neuroepithelium. If so, which of the two aggregates is more radioresistant? The following considerations suggest that it is the slow cycling cells which remain intact after irradiation. (a) There is evidence that the lengthening of the cell cycle is due to the prolongation of the G1—postmitotic, presynthetic—phase (13, 18). (b) As a consequence of interkinetic nuclear migration, cells in the G1 phase should be preferentially concentrated beneath the synthetic zone of the neuroepithelium. Finally, (c) it is beneath the synthetic zone where radioresistant and radiosensitive cells alternate following X-irradiation; in the synthetic zone itself, the pyknotic cells tend to form a continuous band (Figs. 6 and 7B). The confirmation of this hypothesis will require an experimental investigation using a combination of [3H]-thymidine labeling and X-irradiation.

The two types of proliferative cells segregated in the cortical neuroepithelium could represent either the stem cells of two distinct classes of neurons or two distinct classes of stem cells, one for neurons and the other for glia. It is possible, for instance, that early during development the stem cells of the early generated cortical neurons (those that will form the lower cortical layers) multiply at a fast rate, while the stem cells of later differentiating cortical neurons (those that will settle in the upper layers) are quiescent. This would explain why neuroepithelial periodicity is evident in the cortical neuroepithelium up to Day El8 but not thereafter even though cortical neurogenesis (the generation of neurons for the superficial layers) continues in the rat up to Day E21 (5, 7, 8, 11). An alternative possibility is that neuroepithelial mosaicism is the basis of the well-known columnar organization of the neocortex. But the evidence currently available for chronoarchitectonic differences among the cortical columns (16, 20) is weak. The possibility that the aggregates of fast and slow cycling cells and of radiosensitive and radioreistant cells represent stem cells of neurons and glia deserves serious consideration. The alternation of neuronal and glial stem cells in
FIG. 9. Autoradiograms of the developing cortex from rats that received \[^{3}H\]thymidine on Days E18 (A), E19 (B), and E21 (C) and were killed 2 h after injection. Note the progressive reduction in labeled cells in the synthetic zone (sz) of the neuroepithelium and the increase in periventricular labeled cells, presumably ependymal cells (ez in C). Arrows in C point to locally multiplying (presumed glial) cells. Methacrylate; hematoxylin-eosin.
the neuroepithelium is suggested by immunocytochemical evidence in the developing monkey cortex (14). As immunocytochemical markers are now becoming available that can distinguish the precursors of neurons and glia in the rodent brain, this issue ought to be clarified soon. The question of whether it is the precursors of neurons or of glia that are cycling faster and are more radio-sensitive could also be addressed.

The Subventricular Zone

The cortical subventricular zone represents a secondary germinal matrix in which a population of stem cells proliferates outside the neuroepithelium proper. Smart (19) proposed that the displacement of some proliferative cells from the ventricular lumen to the interior of the neuroepithelium and to the subventricular zone represents little more than a mechanism to accommodate the growing number of mitotic cells without congestion near the lumen. But we favor the interpretation that the cells proliferating in the subventricular zone represent a discrete subpopulation of cells. Several observations support this view. First, subventricular zones have a limited distribution in the developing brain and do not seem to be preferentially associated with singularly thick neuroepithelial regions. In the thalamic neuroepithelium, which was the focus of Smart's investigation, the subventricular zone is limited to a circumscribed region which, according to our investigations (3), is the source of neurons of the ventral nuclear complex. Most active thalamic neuroepithelial regions are devoid of a subventricular zone. In many developing brain regions (the thalamus is an example) the temporary increase in cell proliferation apparently leads to the lobulation of its ventricular surface (the formation of neuroepithelial eversions and inversions), presumably as a mechanism to accommodate the mitotic cells near the lumen. This requirement may be absent in the developing cerebral cortex because the expansion of its neuroepithelial surface is relatively unimpeded by other structures around it. The second consideration favoring the idea that the subventricular zone is composed of a distinct population of cells is our observation that the growth of the cortical subventricular zone is preceded by the appearance, in fairly large numbers, of mitotic cells within the corpus of the neuroepithelium. These cells are apparently not undergoing interkinetic nuclear migration and may be destined to form the subventricular zone. Finally, our observation that a high level of proliferative activity continues in the subventricular zone after the primary neuroepithelium has begun to shrink (Fig. 3) suggests that it is not a high level of neuroepithelial cell proliferation per se that causes mitotic activity to take place away from the lumen.

The problem of the morphogenetic significance of the cortical subventricular zone is complicated by the observation, described in the next paper (4), that it contains a large population of putative young neurons that pause here for a while prior to their migration to the cortical plate. Are the proliferative cells of the subventricular zone part of this population of young neurons, with the implication that some of them are not fully differentiated and retain their proliferative capacity? Relevant in this context is the evidence for compartmentation in another type of secondary germinal matrix, the superficial external germinal layer of the cerebellar cortex. The latter, which is well known as the source of the cerebellar microneurons, is composed of two compartments, a subpial proliferative zone and a deeper premigratory zone (1). The premigratory zone is composed of horizontally oriented bipolar cells, interpreted as the future granule cells in the process of extruding the terminal portion of their axons, the parallel fibers. Importantly, Zagon and McLaughlin (22) showed recently that the premigratory zone of the external germinal layer contains some mitotic cells, suggesting that the segregation of premigratory and postmitotic cells in the proliferative and premigratory zones is not as sharp as previously thought. If this observation in the developing cerebellar cortex is applicable to the developing cerebral cortex, this would mean that the "premigratory" compartment of the subventricular zone is composed not only of young neurons but also of some neuronal precursor cells with proliferative potentials.

The Progressive Transformation of the Neuroepithelium

The cortical neuroepithelium is the ultimate source of neurons and glia as well as of the specialized ependymal cells that line the lateral ventricle of the maturing cortex. Past studies concerned with the development of other brain regions have concurred that neurons differentiate first and ependymal cells differentiate last (they can be labeled in most brain regions several days after neurogenesis has ceased). Gial cells appear to occupy an intermediate position insofar as the production of a contingent of "early glial cells" (like the radial glial cells) overlaps with the generation of neurons, whereas the production of a contingent of "late glial cells" (in particular oligodendrocytes and a class of astrocytes) continues beyond the termination of neurogenesis. This sequence suggests a progressive change in the stem cell composition of the neuroepithelium. The present study indicates that at least five stages may be distinguished in the transformation of the germinal matrix of the cerebral cortex. During the first stage there is only a neuroepithelium present but no subventricular zone. During the second stage mitotic cells appear within the corpus of the neuroepithelium and soon thereafter the subventricular zone forms. During the third stage the neuroepithelium begins to shrink and the expanding subventricular zone becomes the dominant germinal matrix. These three stages are chronologically related to the sequential
production of different classes of cortical neurons. But neither the exact nature of this relationship nor the role of the germinative matrix in the production of satellite cells during this period is currently understood. During the fourth stage a population of locally multiplying, presumed glial cells becomes dispersed throughout the intermediate zone and the cortical plate. Finally, during the fifth stage the receding neuroepithelium is transformed into a germinative source that does not display interkinetic nuclear migration; this presumably represents the stage when it begins to produce ependymal cells.

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