

## Autoradiographic and Histological Studies of Postnatal Neurogenesis

### IV. CELL PROLIFERATION AND MIGRATION IN THE ANTERIOR FOREBRAIN, WITH SPECIAL REFER- ENCE TO PERSISTING NEUROGENESIS IN THE OLFACTORY BULB

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**ABSTRACT** The properties and fate of the cells of the subependymal layer of the anterior lateral ventricle and its rostral extension into the olfactory bulb were examined. In one experiment, histological analysis was made of this structure in a large group of rats, ranging in age from newborn to adults. It was established that the ventricular subependymal layer and its rostral extension are present as proliferative and migratory matrices throughout the period studied, with relatively little reduction in size from birth to adulthood. In another, autoradiographic study, the proliferation and migration of cells of this system, and their destination and mode of differentiation, were studied in rats that were injected with thymidine- $H^3$  at 30 days of age and killed at intervals ranging from 1 hour to 180 days. There was a declining gradient in cell proliferation in a caudorostral direction from a high level near the lateral ventricle to the absence of cell proliferation in the olfactory bulb. The labeled cells that were present in high proportion near the lateral ventricle in the rats killed 1–24 hours after injection had further multiplied and moved to the middle portion of the “rostral migratory stream” by the third day, and were located in the subependymal layer of the olfactory bulb by the sixth day after injection. By the twentieth day the labeled cells disappeared from the subependymal layer of the olfactory bulb and were distributed throughout the internal granular layer. The differentiated cells were tentatively identified as granular nerve cells and neuroglia cells. These results established that the major target structure of cell production in the subependymal layer of the lateral ventricle in young-adult rats is the olfactory bulb, with only moderate contribution made to the anterior neocortex and basal ganglia. It was postulated that the function of cell migration to the olfactory bulb is the renewal of its cell population.

Several investigators (Allen, '12; Bryans, '59; Globus and Kuhlbeck, '44; Opalski, '34; Rydberg, '32) reported the presence in adult animals and man of a mitotically active “subependymal layer” (Kershman, '38) around the ependymal wall of the anterior lateral ventricle. Because techniques were not available for tagging these cells, these earlier investigators could only speculate about their fate. The technique of thymidine- $H^3$  autoradiography, which can be used for tagging newly-forming cells in order to trace their destiny, was first addressed to this problem by Messier et al. ('58) and more particularly by Smart ('61). In a combined histological and autoradio-

graphic investigation, Smart examined the morphology of this subependymal layer in infant and adult mice. He established that the proliferative subependymal layer extended in adult mice from the anterior wall of the lateral ventricle rostrally into the olfactory bulb. Smart's results indicated that these cells give rise, in infant mice, to glia and neurons, but he failed to obtain evidence of migration in adult mice (excepting a few cells that seemed to “leak” into the corpus callosum). He postulated, in agreement with the hypothesis of previous investigators, that the mitotic activity of this layer in adults is an abortive phenomenon and the newly-forming cells

degenerate. A later study (Altman, '63) showed that in adult rats that were injected with thymidine- $H^3$  a large proportion of the cells were labeled in the subependymal layer of the lateral ventricle and in a subcallosal band issuing from it and situated over the caudate-putamen. The subsequent demonstration of cell migration in various brain regions in infant and adolescent rats, with evidence of their differentiation into neurons and glia (Altman, '66a, '66b, '67; Altman and Das, '65a, '65b, '66), raised the possibility that cell production in the subependymal layer of the anterior horn of the lateral ventricle is not an abortive phenomenon but that the cells that are produced there are incorporated into the architecture of the adolescent and adult brain. The observation that the forebrain continues to grow in the longitudinal direction in adult rats (Sugita, '17; Altman, Wallace, Anderson and Das, '68) raised the possibility that the cells proliferating at a high rate in the anterior forebrain are partly responsible for the continuing growth of the cerebral cortex.

#### MATERIALS AND METHODS

Brains from two groups of animals were used in this study. The first group consisted of 77 Long-Evans hooded rats ranging in age from birth to 90 days, as described in detail earlier (Altman, '69a). Some additional brains were included in this group, from rats ranging in age up to 150 days. All these brains were cut sagittally at 6 and 12  $\mu$  and were stained with cresyl violet or galloxyanin chromalum. The second group consisted of rats that were injected with thymidine- $H^3$  at 6 hours, 2, 6, 13 and 30 days of age and killed 1, 6 and 24 hours, and 3, 6, 20, 60, 120 and 180 days after injection. Details on the method of injection and on the autoradiographic procedure were presented in earlier papers of this series (Altman, '66a, '69a; Altman and Das, '66). In the present study we concentrated on an analysis of coronal autoradiograms from rats injected at 30 days of age. The qualitative and quantitative procedures used for the evaluation of this material are presented in the relevant sections below.

#### RESULTS

##### *Histological appearance of the subependymal layer of the anterior forebrain from birth to adulthood*

In sagittal sections of the forebrain of newborn rats the entire lateral ventricle is surrounded by a mitotically active ependymal layer. This layer is composed of rows of densely-packed, round, lightly-stained cells, with occasional mitotic cells seen near the lumen. The cell thickness of this layer and the location of mitotic cells near the lumen, reflecting interkinetic nuclear migration (Sauer, '35; Sidman et al., '59), indicate that at this stage of development the cells surrounding the lateral ventricle are not differentiated ependymal cells but undifferentiated cells of a germinal matrix, representing the *primary proliferative matrix*, or the primitive neuroepithelium (Altman, '69b).

Over the dorsal roof of the horizontal, or subcallosal, portion of the lateral ventricle (anterior and posterior horns) this neuroepithelium is about 6–8 cells thick in cross section. It is about 4–5 cells thick around the floor of the ventricle, over the dorsal border of the caudate-putamen and of variable thickness around the vertically oriented anterior portion of the lateral ventricle, the inferior horn.

This periventricular neuroepithelium is surrounded dorsally by a 14–18 cell thick layer, composed of less-densely packed, darkly staining, variably-shaped cells. This zone is the subependymal layer (Kershman, '38; Globus and Kuhlenbeck, '44); it represents a *secondary proliferative matrix* and is associated with certain cortical structures of the nervous system (Altman, '69b). Mitotic cells are seen scattered throughout the layer. From this dorsally situated subependymal layer radially-oriented streaks of cells were seen penetrating into the overlying corpus callosum and white matter, in apparent migration. This subependymal layer is not present around the neuroepithelium in the ventral floor of the horizontal component of the lateral ventricle, that is, over the basal ganglia. It is most prominent around the rostral wall of the anterior horn of the lateral ventricle. From the latter region issues a

rostrally oriented massive body of cells, 70–80 cells thick in cross section. It surrounds the narrow lumen that, at this age, connects the lateral and olfactory ventricles. This L-shaped structure, which first moves vertically and then horizontally, penetrates the laminated olfactory bulb (fig. 1). Mitotic cells are frequently seen in this rostral extension of the subependymal layer. In addition, two types of cells are most commonly seen: darkly-staining

spindle-shaped cells, which tend to be oriented parallel to the course of this band in sagittal section, as if streaming in it, and round, lightly-staining cells, among which mitotic ones are often seen.

The structure of the neuroepithelium and of the subependymal layer is similar in the 1–2 day old animals to that seen in newborns, with no indications of a reduction in their absolute size, although there is a relative reduction with respect

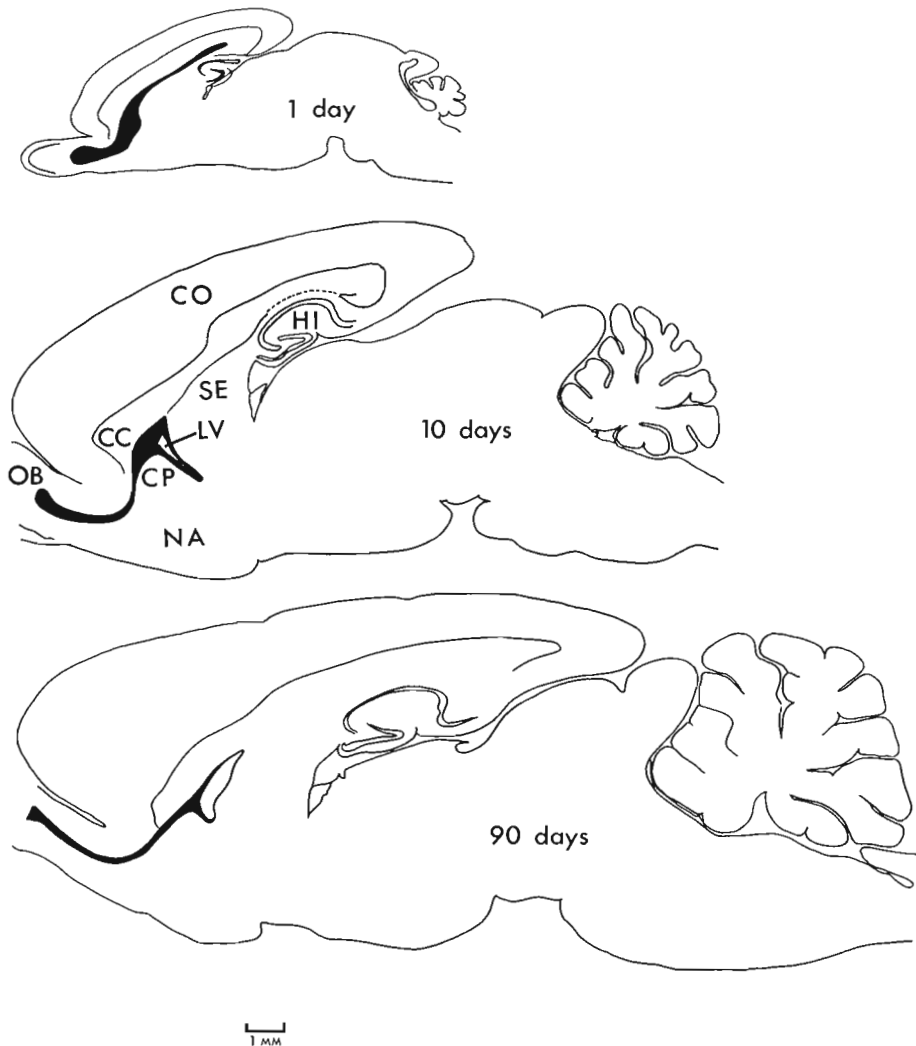


Fig. 1 Tracings of sagittal sections of the brain from rats of different ages to show the position of the subependymal layer and "rostral migratory stream" (black). CC, corpus callosum; CO, cerebral cortex; CP, caudate-putamen; HI, hippocampus; LV, inferior horn of lateral ventricle; NA, nucleus accumbens; OB, olfactory bulb; SE, septum.

to the rapidly growing forebrain. In the 3–4 day old animals there is a gradual decrease in the cell thickness of the neuroepithelium in the posterior horn of the lateral ventricle and this shrinkage begins caudally. At this age there is no decrease in the cell-thickness of the neuroepithelium and of the subependymal layer in the anterior and inferior horns of the lateral ventricle. In the 5–6 day old animals the neuroepithelium changes to a single-cell-thick ependyma in the caudal parts of the posterior horn, and the ventricular lumen gradually shrinks, with its former position being indicated by a band of cells that are similar in appearance to those seen in the subependymal layer. But there is still no sign of reduction in the thickness of the neuroepithelium around the inferior horn of the ventricle and the thickness of the subependymal layer is comparable to that seen in the newborn. Also around the floor of the anterior horn of the ventricle (over the basal ganglia where there is no subependymal layer) the neuroepithelium is still several cells in thickness.

In 1-week old rats the lateral ventricle is considerably withdrawn in a rostral direction and the posterior horn is surrounded by a single-celled wall of presumably differentiating ependymal cells. However, a prominent several-cells-thick neuroepithelium, with occasional mitotic cells near the lumen, is still present around the inferior horn of the ventricle. By this time the cell-thickness of the L-shaped rostral extension of the subependymal layer is reduced to about 40–50 cells, but because of its greatly extended length in the growing forebrain this may not represent a great reduction in the total cell population (fig. 2). In fortuitous sections, a narrow lumen, or cells resembling clusters of neuroepithelial cells without clear evidence of a lumen, is seen along the rostral extension of the subependymal layer, suggesting that the continuity between the lateral and olfactory ventricles is still maintained. In the dorsal aspect of this subependymal layer radially-oriented streaks of cells can be seen penetrating into the anterior portion, or genu, of the corpus callosum.

There is no evidence of reduction in the cell-thickness of the neuroepithelium

around the inferior horn of the lateral ventricle between 10–20 days and an occasional mitotic cell is still encountered near the lumen. Likewise, the rostral extension of the subependymal layer remains a massive band throughout this period (fig. 3a), 20–50 cells thick in cross section and composed predominantly of spindle-shaped cells (fig. 3b). In the rats aged 21–30 days the neuroepithelium is 3–8 cells thick around the inferior horn of the lateral ventricle, but mitotic cells are no longer seen near the lumen. Typically, mitotic cells are encountered at the interface of this layer and the adjacent subependymal layer, or occasionally in the outer border of the neuroepithelium itself, indicating a gradual transformation of this matrix. Interestingly, the neuroepithelium tends to be thicker in regions where the subependymal layer is vestigial or absent, as in the ventral part of the inferior horn, suggesting depletion of neuroepithelial cells at sites of intense production of subependymal cells. Gradually the surface area from which the subependymal cells arise becomes narrower and their main source becomes the tip of the anterior wall of the inferior horn, where the subependymal layer has a characteristic triangular shape (fig. 3c). At these ages the cell thickness of the rostral extension of the subependymal layer remains about 20–50 cells. In many animals a rich network of blood vessels and capillaries was observed around the rostral extension of the subependymal layer (fig. 3c).

In the 30–90 day old rats the cell-thickness of the neuroepithelium was reduced to about 3–4 cells, while the cell-thickness of the subependymal layer was about 20–35 cells. The observation made earlier, about the absence of mitotic cells in the “neuroepithelium” around the inferior horn, also held for these animals. In these animals, as in the previous group, the region of the “neuroepithelium” where the subependymal layer arises tended to be thinner at the site of origin of the rostral migratory stream than in its vicinity. In the latter structure spindle-shaped darkly-staining cells were interspersed with round, lightly-staining cells (fig. 3c), though in places they tended to form separate sheets with ill-defined boundaries. In some sec-

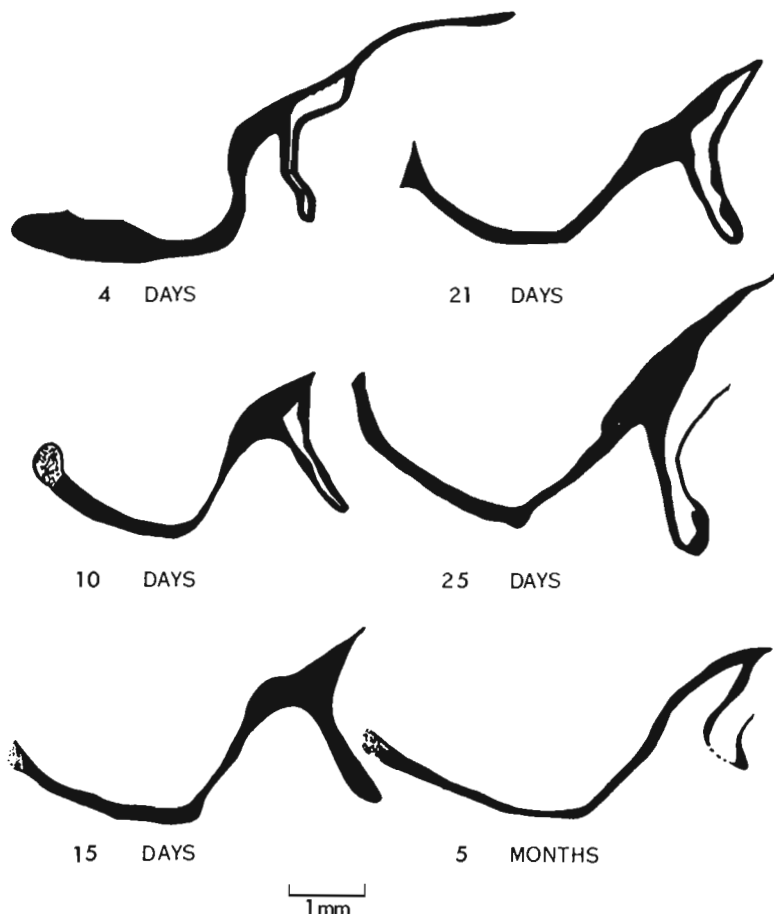


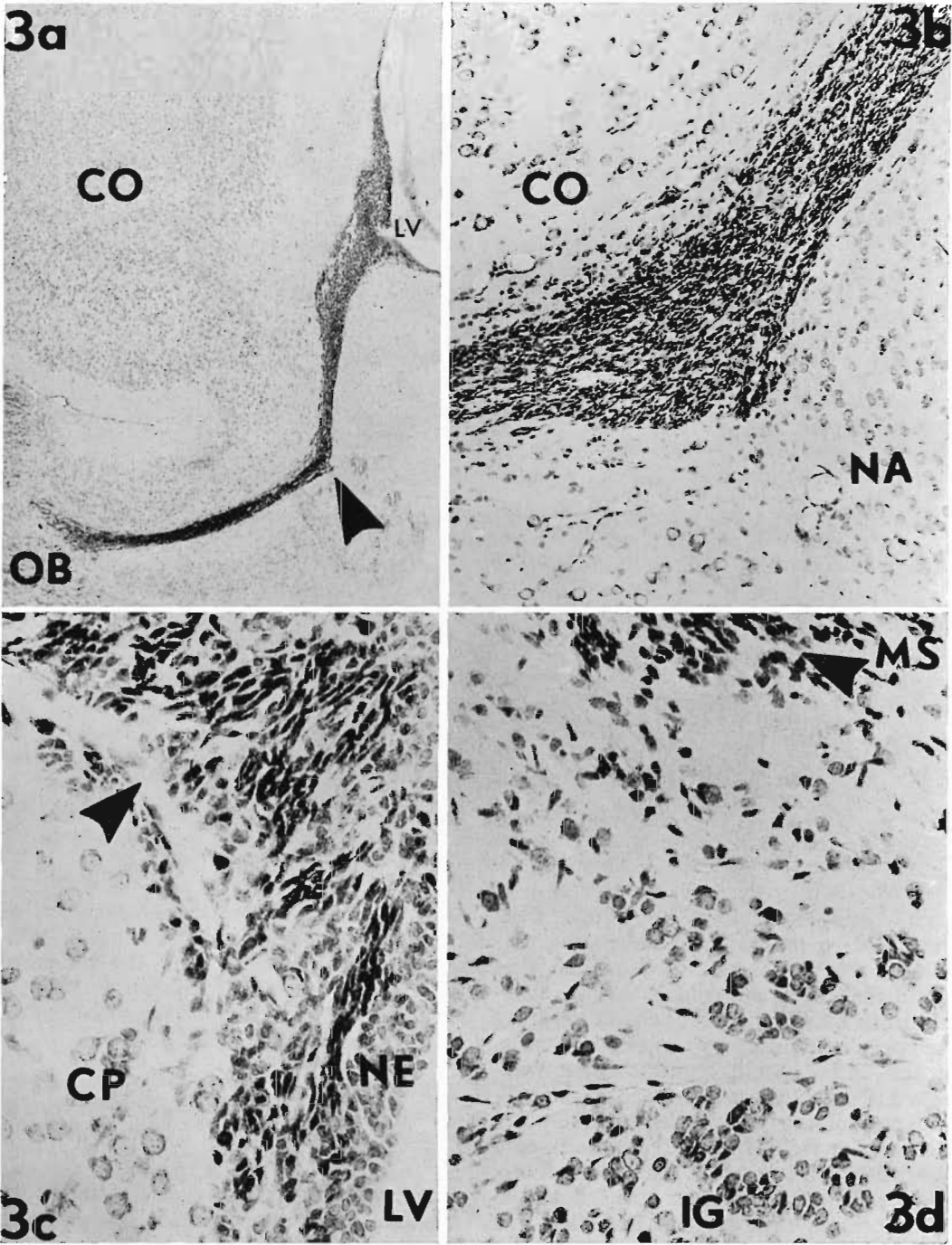
Fig. 2 Tracings of the subependymal layer of lateral ventricle and its rostral extension to show changes in its size as a function of age. In the older animals the terminal part of the subependymal layer in the olfactory bulb is outside the plane of sectioning.

tions, cells resembling ependymal cells were seen in the vertical, descending portion of the rostral extension of the subependymal layer, suggesting that a vestigial lumen may be present here, connecting the lateral and olfactory ventricles. Finally, in the 150 day old animals there were clear indications of a reduction in the cell-thickness of the "neuroepithelium" around the anterior wall of the inferior horn, to a two-cells thick layer, and the cell-thickness of the subependymal layer was reduced to 10–25 cells in cross section. The latter was still composed predominantly of spindle-shaped cells, with occasional mitotic cells seen among them.

In all animals this rostral extension of the subependymal layer of the lateral ventricle could be traced into the olfactory bulb. Here the spindle-shaped cells were seen penetrating into the laminated cortex of the olfactory bulb (fig. 3d).

*Autoradiographic analysis of cell movements in the anterior forebrain in rats injected at 30 days of age: migration to the olfactory bulb*

*Cell movements in the rostral migratory stream.* The subependymal layer of the anterior wall of the inferior horn of the lateral ventricle, and its rostral extension, the latter referred to intermittently as the



Figures 3

"rostral migratory stream," is shown in a sagittal section from a 21 day old rat in figures 3a,b. As described earlier, this massive band of cells is first oriented horizontally, in a subcallosal position, then dips downward and goes vertically for some distance over the rostral wall of the basal ganglia. In a ventral position it turns again and is oriented again horizontally, in a position directly above the intermediate olfactory tract; it is finally dispersed as it reaches the middle portion of the laminated cortex of the olfactory bulb. The majority of cells are spindle-shaped, oriented parallel to the direction of the band as a whole, suggesting that this extension of the subependymal layer is composed predominantly of migratory cells and that it represents a rostrally moving migratory stream of primitive cells. However, the presence of some mitotic cells, and a vestigial lumen with epithelial cells, which can be traced in fortuitous sections to the vestigial olfactory ventricle, indicate that this "stream" also contains proliferative elements.

We studied the dynamics of cell movements in the subependymal layer and its rostral extension in serially sectioned coronal autoradiograms. These autoradiograms were obtained from rats injected with thymidine- $H^3$  at 30 days of age and killed at intervals ranging from 1 hour after injection to 180 days of age. Whereas in animals killed 1, 6 and 24 hours after injection a large proportion of the cells were labeled in the subependymal layer in the anterior wall of the inferior horn of the lateral ventricle, labeled cells were not seen

in these animals more rostrally in the olfactory bulb (figs. 10, 14). Conversely, in animals that survived for three and six days after injection few labeled cells were seen in the former position, while a large proportion of the cells were labeled in the subependymal layer of the olfactory bulb (figs. 11, 12, 15). These qualitative observations seemed to confirm the impression that the rostral extension of the subependymal layer is indeed composed of a migratory stream of cells. Moreover, examination of the autoradiograms indicated that the labeled cells that reached the subependymal layer of the olfactory bulb disappeared from that position by the 20th day after injection (figs. 13, 16), at which time the labeled cells were concentrated in the internal granular layer of the olfactory bulb (fig. 16).

The following procedure was used for the quantitative determination of cell proliferation and migration in the subependymal layer of the lateral ventricle and in the rostral migratory stream issuing from it. Serially sectioned coronal autoradiograms, nominally separated by 180  $\mu$  from one another, were taken from animals that survived for 1 hour, 3, 6 and 20 days after injection. The proportion of labeled cells was established in all sections, in a region that extended from the anterior wall of the lateral ventricle to the tip of the olfactory bulb (fig. 5A), by counting and classifying as labeled or unlabeled all, or a representative sample, of the cells in the subependymal layer. To facilitate comparison with sagittally cut sections, the data were then transferred to a graph on the abscissa of which the interval represented the nominal distances between the coronal sections examined (fig. 5B). When plotting the data, the appearance of the subependymal layer and of the rostral migratory stream, which radically change their shape from one plane to the next and their position in the dorsoventral plane were checked to insure that corresponding points were plotted on the graphs. Where nominal distance and judged position did not quite match the latter was used for assigning the point along the abscissa.

The results are summarized in figure 5 for the animals that survived after injection.

Fig. 3 a. Low-power photomicrograph of the subependymal layer of the inferior horn of the lateral ventricle (LV) and the rostral migratory stream (arrow) in sagittal section in a 21 day old rat. Cresyl violet,  $\times 16$ .

b. The rostral migratory stream at higher magnification.  $\times 101$ .

c. "Neuroepithelium" (NE) and subependymal layer of the inferior horn of lateral ventricle (LV) in a coronal section near the caudate-putamen (CP).  $\times 256$ . Arrow points to blood vessel.

d. Terminal portion of the migratory stream (MS), or subependymal layer of olfactory ventricle, in the olfactory bulb. Note the many spindle-shaped cells above the internal granular layer (IG).  $\times 256$ . (CO, cerebral cortex; NA, nucleus accumbens; OB, olfactory bulb).

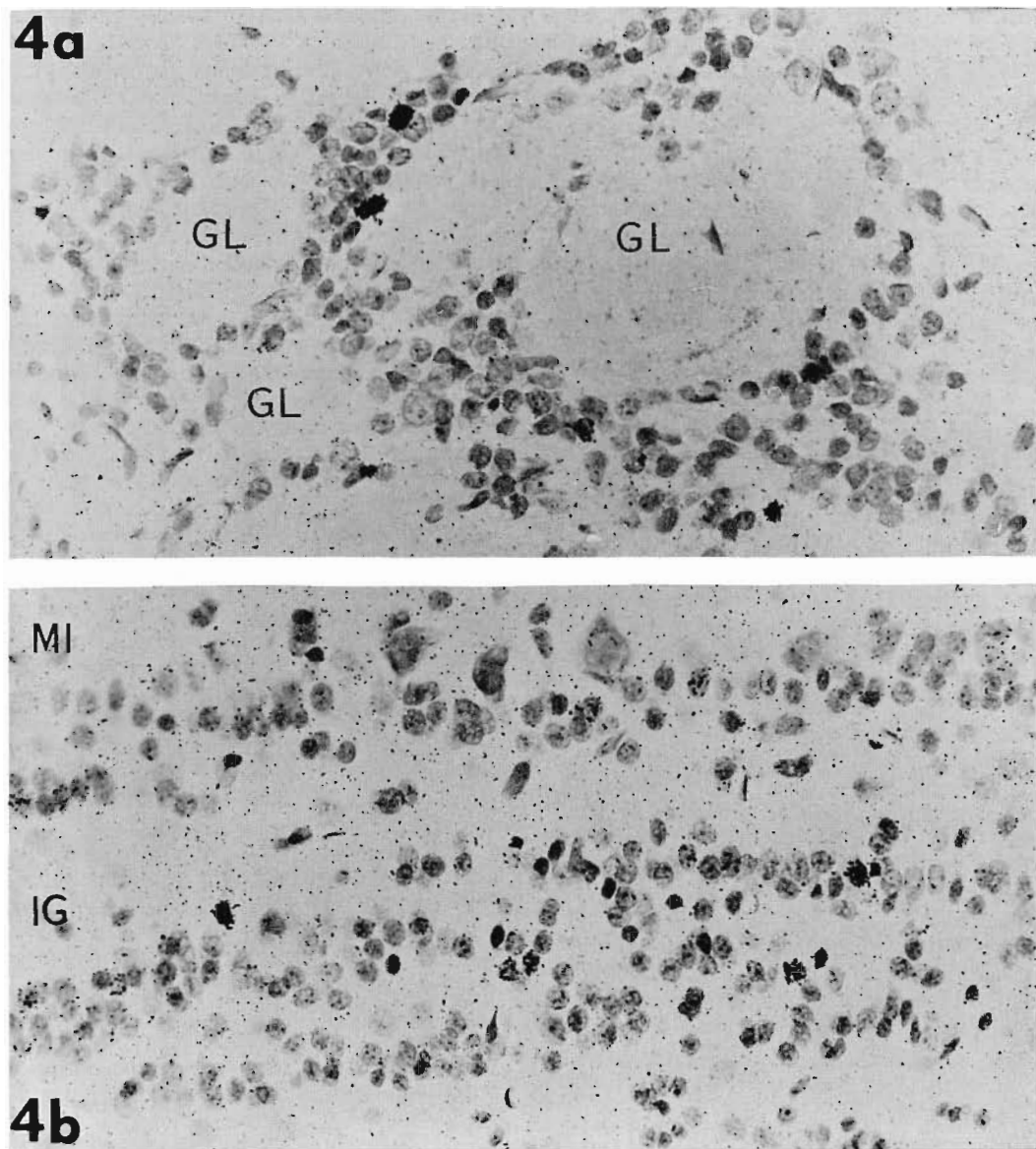


Fig. 4 High-power photomicrographs of autoradiograms of the olfactory bulb from a rat that was injected at 30 days and killed 120 days later. Note labeled cells around glomeruli (GL) in figure 4a, and labeled cells in the internal granular layer (IG) and an occasional one in the mitral cell layer (MI) in 4b. Gallocyanin chromalum,  $\times 400$ .

tion for one hour, three and six days. Because labeled cells were no longer seen in the subependymal layer of the lateral ventricle and its rostral extension in the animals that survived for 20 days (figs. 13, 16) these data were not plotted.

These quantitative results clearly show a caudo-rostral gradient in the proportion of labeled cells in the animal that was killed one hour after injection with thymidine- $H^3$ . The following observations may be added. While no labeled cells were seen



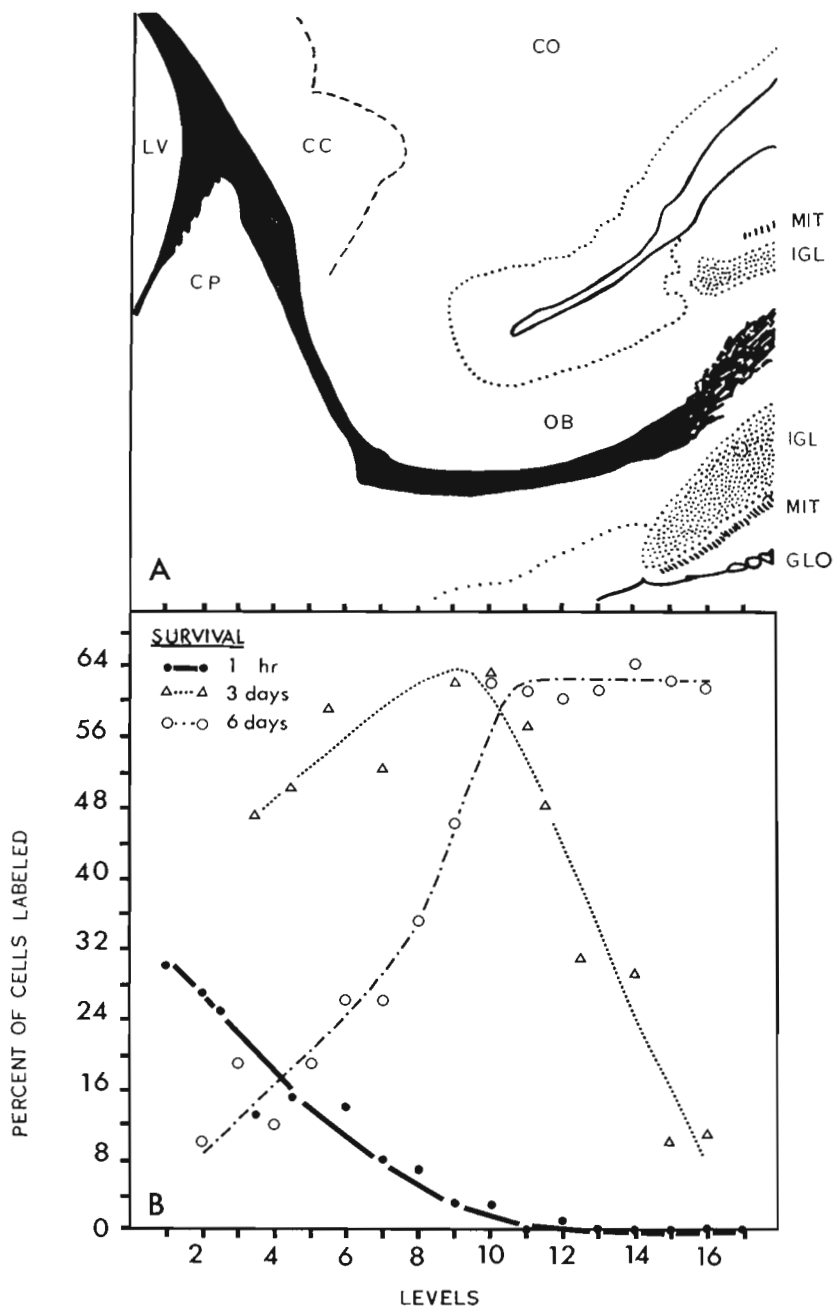


Fig. 5 A, tracing of the anterior forebrain from a young adult rat at a magnification designed to match the scale of the abscissa, with nominal distances of  $180 \mu$  between levels. CC, corpus callosum; CO, cerebral cortex; CP, caudate-putamen; GLO, glomerular layer; LV, inferior horn of lateral ventricle; MIT, mitral cell layer; OB, olfactory bulb. B, percentage of labeled cells obtained in coronal autoradiograms in the subependymal layer and rostral migratory stream, at the coronal levels indicated, in rats that survived for one hour, three and six days after injection. The continued multiplication of cells near the lateral ventricle and their subsequent migration into the olfactory bulb is indicated.

in the several cell thick neuroepithelium in the animals that survived for a short period after injection, many of the cells in the surrounding subependymal layer were intensely labeled along the anterior wall of the inferior horn of the lateral ventricle. The proportion of labeled cells fell sharply rostrally in the vertical limb of the migratory stream and virtually no labeled cells were encountered at the rostral tip of the stream in the subependymal layer of the olfactory bulb.

In the animals that survived for three days after injection a high proportion of intensely-, medium-, and lightly-labeled cells were seen in the subependymal layer of the rostral wall of the inferior horn of the lateral ventricle. This indicated continuing cell proliferation at this site, leading to an increased proportion of labeled cells (an increase from about 30 to 48%) combined with label dilution within the nuclei of many cells. However, the proportion of labeled cells was even higher rostrally in the middle portion of the migratory stream, indicating that many of the cells produced on the previous days have reached this region. Interestingly, the proportion of intensely-labeled cells was much higher at these rostral points, suggesting that these migratory cells were produced earlier (or have undergone fewer divisions) than the stationary ones still seen at this time near the lateral ventricle. Many intensely- and medium-labeled cells could also be seen by this time in the subependymal layer of the olfactory ventricle. Apparently, the first contingent of the cells multiplying around the lateral ventricle has reached the olfactory bulb. Whereas in the middle portion of the stream labeled cells were distributed throughout the cross section of the layer, in the olfactory bulb they were concentrated in the center with few labeled cells seen in the periphery (fig. 15). This suggested that migration is brisker in the center of the stream and that cells move first rostrally then radially.

In the animals that survived for six days after injection few labeled cells remained in the subependymal layer of the anterior wall of the inferior horn of the lateral ventricle. A "haze" of blackened silver grains, reflecting reduced label concentra-

tion just above background noise level, was seen in the subependymal layer of the lateral ventricle, suggesting that many of the cells were descendants of the originally-tagged cells but that the concentration of radioactive DNA was greatly reduced due to repeated divisions. This "haze" was less pronounced in the middle portion of the stream and there was an increase in the concentration of light- to medium-labeled cells in the rostral direction. In the terminal region of the migratory stream in the subependymal layer of the olfactory bulb, there was a high concentration of labeled cells, indicating that three to six days after their production the bulk of the cells have reached the olfactory bulb and replaced there the unlabeled cells that were produced prior to the injection.

*Cell movements and cell acquisition in the olfactory bulb.* As mentioned earlier, labeled cells were not seen (an occasional one excepted in some sections) in the subependymal layer of the olfactory bulb in the 30 day old animals killed 1, 6 and 24 hours after injection with thymidine- $H^3$ . The ependyma surrounding the narrow lumen of the olfactory ventricle, where visible, was also devoid of labeled cells. An occasional intensely-labeled cell was seen in the internal granular layer, and typically a few labeled cells were seen in the peripheral plexiform layer (situated above the mitral cell layer), and many more in the glomerular layer (fig. 14) and in the outer fibrillar layer. The latter observations suggested a low rate of local cell proliferation in the superficial layers of the bulb.

Labeled cells appeared in the subependymal layer of the olfactory bulb in the animals that survived for three days after injection (fig. 15) with the majority of the cells concentrated in the center part of the cross section of the subependymal layer. There was no appreciable increase at that time in the number of labeled cells in the internal granular layer. In the animals that survived for six days after injection there was considerable increase in the number of labeled cells in the subependymal layer with light-, medium- and intensely-labeled cells seen throughout the layer. A few labeled cells, generally in-

tensely-labeled ones, were also seen now at the base of the internal granular layer, as if early-arriving cells had begun their inward migration. The number of labeled cells in the superficial layers of the olfactory bulb was variable with little or no increase evident over animals that survived for shorter periods after injection.

A drastic change was observed in the animals that survived for 20 days after injection. No labeled cells were seen anymore in the subependymal layer of the olfactory bulb but there was an appreciable concentration of light-, medium- and intensely-labeled cells throughout the internal granular layer (fig. 16). Evidently, in the interval between 6–20 days after injection the labeled cells left the subependymal layer and migrated radially into the internal granular layer (fig. 6). A few labeled small cells were also seen in the mitral cell layer and there was an increase in the proportion of labeled cells in the superficial layers of the olfactory bulb, in particular the glomerular layer.

Apart from endothelial cells, three cell types could be distinguished in sections stained with gallocyanin chromalum or cresyl violet in the internal granular layer. (i) Darkly-staining spindle-shaped cells; these were not labeled 20 days after injection. (ii) Darkly-staining round cells, presumably neuroglia cells; these were often labeled. (iii) Somewhat larger, oval-shaped, lightly-staining cells, the long axis of the cells generally oriented at a right angle to the layers of the cortex of the olfactory bulb and often showing a nucleolus. These cells, which we tentatively identified as granule cells, were frequently labeled. In some instances the labeled cells formed groups, in others a single labeled cell was seen in the company of many unlabeled ones. No preferential position for the labeled cells could be seen within the internal granular layer or with respect to the olfactory bulb as a whole.

The proportion of labeled cells in the internal granular layer did not radically change in the animals that survived for 60 days after injection, but there was a definite decline in the animals that lived for 120 and 180 days after injection (fig. 6). The cells that were seen in the latter group

tended to be medium- to intensely-labeled. Some labeled cells also persisted in the glomerular layer but not in the outer fibrillar layer.

*Autoradiographic analysis of cell movements and cell acquisition in other forebrain structures*

The foregoing observations showed that the cells produced in prodigious numbers in the subependymal layer of the inferior horn of the lateral ventricle in 30-day old rats migrated along the rostral extension of the subependymal layer into the olfactory bulb and from there into the internal granular layer. Histological observations suggested that in the subcallosal portion of this matrix radially-oriented clusters of cells penetrated the corpus callosum. That the cerebral white matter might be another target structure or a migratory route of the cells produced in the subependymal layer was also indicated by the study of coronal sections which showed migratory cells fanning out laterally beneath the corpus callosum over the wall of the basal ganglia. Migration into the overlying white matter was also seen in the posterior cerebrum in surviving caudal fragments of the subependymal layer.

Therefore, it appeared worthwhile to examine whether or not the massive rostral migratory portion of the subependymal layer sends newly-formed cells in appreciable numbers to the corpus callosum and to the cortex and basal ganglia.

*Pattern of cell labeling in the corpus callosum.* The following procedure was used to determine whether or not cells migrate laterally in the corpus callosum from the paramedially placed subependymal layer. Matched coronal sections, de Groot ('59) coordinate A + 9.0, were selected from all the animals and the proportion of labeled cells were determined in four specified levels (medial, intermediate, dorsolateral, ventrolateral) of the corpus callosum (fig. 7). In all sampled regions at least 100 cells were counted on the right and left side. The data were pooled for the animals that lived for 1, 6 and 24 hours after injection, called "short survival" group; 3, 6 and 20 days, "in-

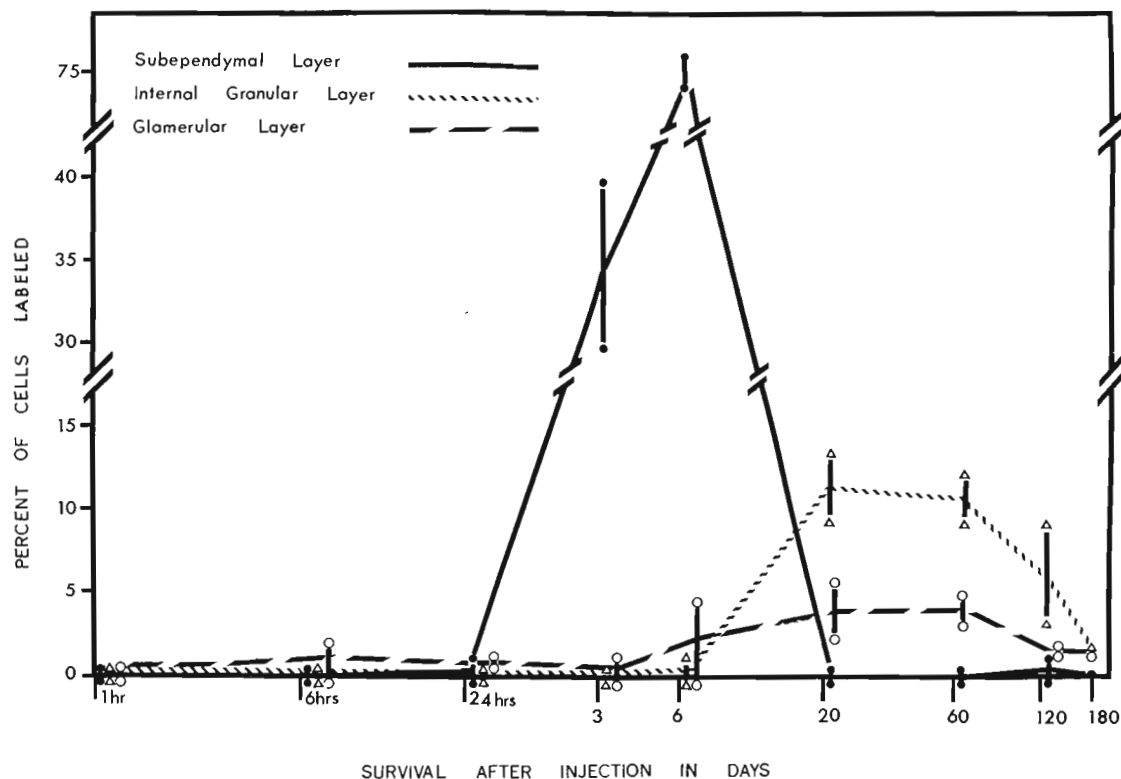


Fig. 6 Percentage of labeled cells in the olfactory bulb as a function of survival time after injection. Labeled cells appear first in the subependymal layer at three days and disappear by the twentieth day. In the internal granular layer they appear at the twentieth day after injection and decline gradually thereafter. The pattern is different and more variable in the glomerular layer.

intermediate survival"; and 60, 120 and 180 days, "long survival." The results indicate, first, a gradient, with higher concentration of labeled cells in the medial and intermediate portion of the corpus callosum than in its lateral parts. That is, the proportion of labeled cells was higher in the vicinity of the subependymal layer. However, because this gradient is also present in the short survival group, in which cell labeling reflects local proliferation, the contribution of the subependymal layer cannot be specified. Second, there was a rapid increase in labeled cells in the intermediate survival group, with a decline in the long survival group. This indicates a turnover of cells, due either to migration or cell death. The fact that there was never an increase in the proportion of labeled cells in the lateral regions with re-

spect to the medial segments of the corpus callosum suggests that the cells produced in the former position do not migrate laterally. Accordingly, we may conclude that if the subependymal layer contributes cells to the corpus callosum, this contribution cannot be a great one.

*Pattern of acquisition of labeled cells in the neocortex.* If the rostral migratory stream delivers cells in appreciable numbers to the cortex, it might be expected that the proportion of labeled cells will be much higher several days after injection in the anterior regions of the neocortex than in the posterior regions. Accordingly, labeled cells were counted in unit areas in the cortex in all the injected animals at six coronal levels, at approximate de Groot ('59) coordinates of A 10.6 (tip of anterior cortex), A 9.0 and A 7.4 (level of anterior

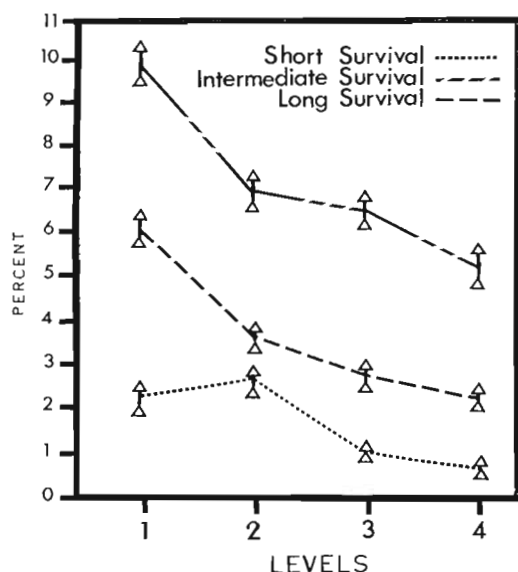


Fig. 7 Percentage of cells labeled in corpus callosum at four points sampled from the midline laterally.

commissure); referred to as anterior levels 1, 2 and 3. And also at A 5.8, A 4.2, and 2.2 (level of posterior commissure), referred to as posterior levels 4, 5 and 6. Moving 1500  $\mu$  from the midline, the number of labeled cells was determined in two adjacent, 500  $\mu$  wide vertical strips of the cortex on the right and the left side and the mean numbers of cells were plotted for square grids of 500<sup>2</sup>  $\mu$ . The results (fig. 8) indicated systematic differences in the number of labeled cells in terms of survival after injection: with intermediate concentration in animals surviving for a short period (1, 6 and 24 hours) after injection; high concentration in animals with intermediate survival (3, 6 and 20 days); and lowest concentration in animals with prolonged survival after injection (60, 120 and 180 days). This pattern indicates a gradual acquisition of labeled cells in the cortex and a slow turnover in the course of a period extending over several months. The data also indicate a somewhat higher concentration of labeled cells in the anterior parts of the neocortex. However, because the concentration of labeled cells is also higher during the first 24 hours after injection, the

higher number of labeled cells could be due to a higher rate of regional cell proliferation rather than to a higher rate of migration to the anterior neocortex.

*Pattern of acquisition of labeled cells in the caudate-putamen.* Because the rostral migratory stream issuing from the subependymal layer of the inferior horn of the lateral ventricle moves over the wall of the caudate-putamen we examined whether or not the latter complex is a major target structure of these newly-forming cells. Labeled cells were counted in two square areas of 500<sup>2</sup>  $\mu$  on the right and left side of the caudate-putamen at two anterior levels (A 8.6 and A 8.2), 1000 and 1500  $\mu$  from the midline, and approximately in the middle of the structure in the dorsoventral direction. The question we were raising was whether the caudate-putamen acquires new cells in higher numbers than does the neocortex. The results (fig. 9) show variable concentration of labeled cells in the animals that survived for brief periods after injection (1, 6 and 24 hours) and an appreciable increment by the sixth day. These cells, like those acquired by the neocortex, are slowly turning over, as indicated by the decline in the number of labeled cells in the animals that survived for longer periods after injection and also the considerable label dilution within cells. While the number of labeled cells is much higher after optimal survival period in the caudate-putamen than in the neocortex, it is by far below that seen (after 20 day survival) in the olfactory bulb. The cell contribution of the subependymal layer to the caudate-putamen is probably a moderate one.

#### DISCUSSION

Kershman ('38) and Globus and Kuhlenbeck ('44) were among the first investigators to draw attention to the existence of an extraependymal proliferative matrix in the cerebrum, what the former called the "subependymal zone," the latter the "subependymal cell plate." Kershman's view was that this proliferative matrix gave rise during embryonic development exclusively to neuroblasts (precursors of neurons). In this respect he considered them different from the cells of the external granular layer

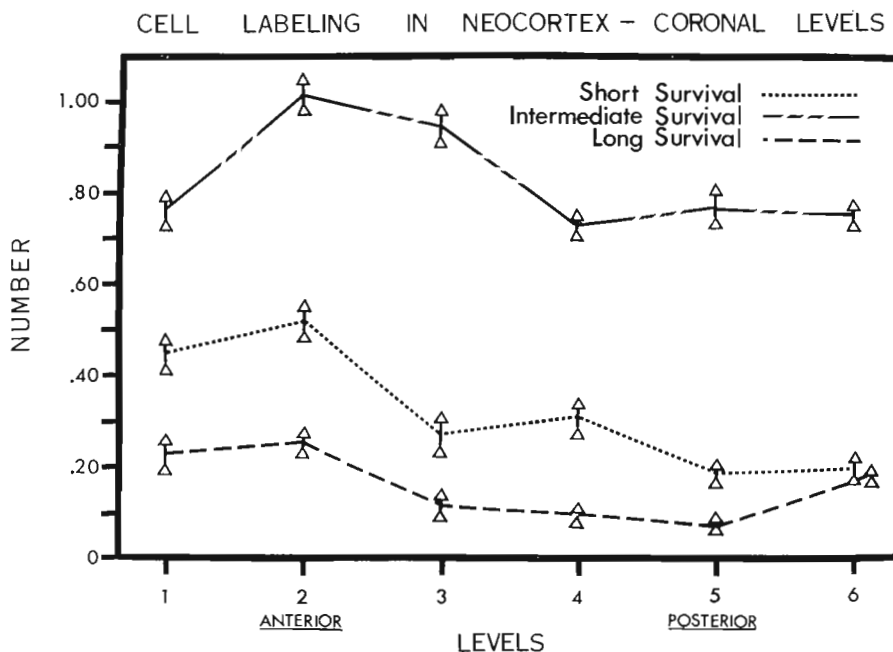


Fig. 8 Number of cells labeled in the neocortex at six points sampled in the anteroposterior direction.

of the cerebellar cortex which he thought to be bipotential "indifferent cells" (Schaper, 1894, 1897) or "medulloblasts" (Bailey and Cushing, '25) that could give rise to both neuroblasts and spongioblasts (precursors of neuroglia). In contrast, Globus and Kuhlenbeck, who pointed out the persistence of this layer in adults, postulated that the cells of the subependymal cell plate had the same properties as the neuroectodermal cells of the ependymal plate, with potency to produce both spongioblasts and neuroblasts. More recently, Smart ('61) investigated the properties of the "subependymal layer" of the anterior cerebrum in mice with thymidine- $H^3$  autoradiography. He postulated that in infant mice the "dark-nucleated cells" of this layer give rise to spongioblasts and the "light-nucleated cells" to neuroblasts. This is reminiscent of the theory of His (1889) with respect to the primitive ependyma according to which the larger, "germinal cells" lining the lumen of the neural tube are neuroblasts, whereas the other cells of the ependyma are "spongioblasts." It is

now established (Sauer, '35; Sidman et al., '59) that these two types of cells in the primitive ependyma represent phases in the mitotic cycle of undifferentiated neuroepithelial cells, and the location of "germinal cells" near the lumen is due to the migration of cell nuclei to the lumen during mitosis. The present study indicates (see further below) that in the subependymal layer the darkly-staining cells are the migratory elements and the lightly-staining cells are probably stationary pre- and postmitotic elements.

The subependymal layer of the anterior cerebrum is known to be present as a mitotic matrix in adult rats (Allen, '12; Bryans, '59), cats (Altman, '63), dogs (Fischer, '67), monkeys (Lewis, '68) and man (Opalski, '34; Globus and Kuhlenbeck, '44). In the study referred to, Smart traced this subependymal matrix to the olfactory bulb. However, on the basis of his autoradiographic studies, Smart ruled out the possibility of cell migration to the olfactory bulb and he concluded that, in adult mice, cell proliferation was an abor-

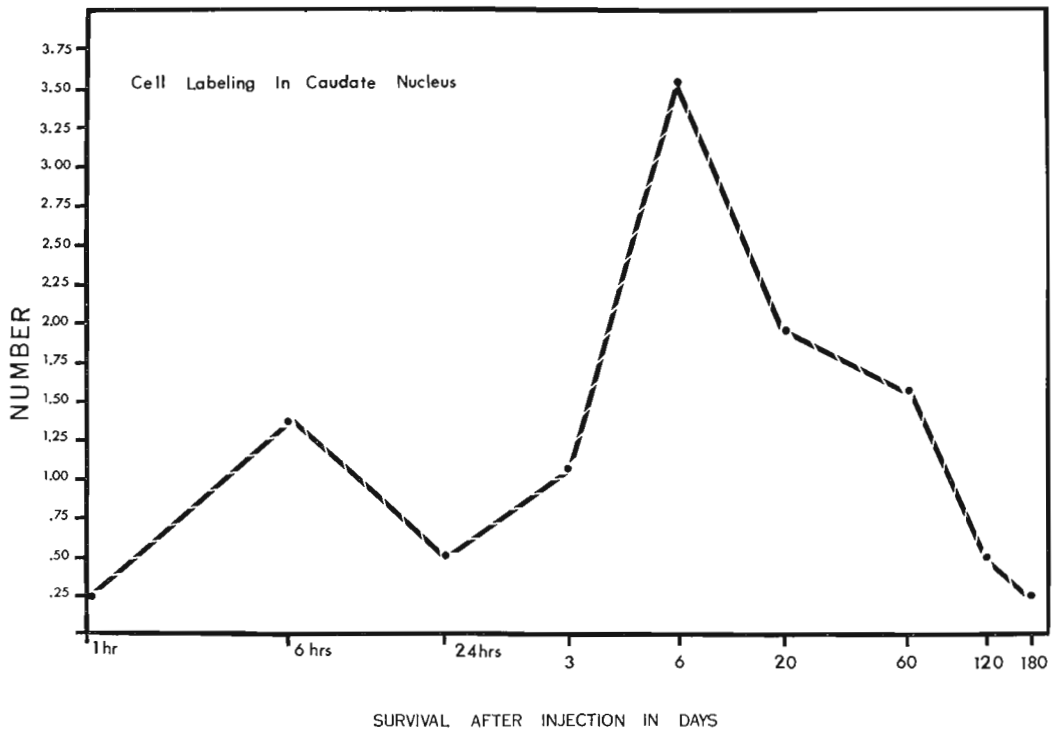


Fig. 9 Number of cells labeled in the caudate-putamen as a function of survival time after injection.

tive phenomenon. In previous papers of this series we have shown that during the first 2-3 weeks of life cell proliferation is quite brisk in rats along the entire subependymal layer of the anterior forebrain, extending from the rostral wall of inferior horn of the lateral ventricle to the olfactory bulb. Thus in the subependymal layer of the olfactory ventricle (Altman, '66a, see esp. fig. 3) about 24% of the cells were labeled in newborn rats killed six hours after injection; the proportion of labeled cells fell to about 12% in the two- and six-day old rats and to about 8% in the 13-day old rats. Because of the high rate of local proliferation in the olfactory bulb during these periods cell migration from more caudal parts of the forebrain could not be established as one cannot distinguish migratory cells from locally proliferating ones. Our observation that in 30-day old rats local cell proliferation in the subependymal layer of the vestigial olfactory ventricle came prac-

tically to a standstill made possible the study of cell migration in the anterior forebrain during this period.

Our observations in this study established that the cells produced in large numbers in the anterior wall of the inferior horn of the lateral ventricle migrated to the olfactory bulb. Regional cell proliferation in the subependymal layer showed a steep caudorostral gradient during this time, with the high rate of cell proliferation near the ependymal layer of the lateral ventricle (with about 30% of the cells labeled) falling to zero in the olfactory bulb. In the animals that survived for six days after injection the majority of labeled cells disappeared from the region of the lateral ventricle and about 65% of the cells located in subependymal layer of the olfactory ventricle were now labeled. Evidently cell proliferation was quite brisk at this age near the anterior wall of the lateral ventricles (shown by the increase in labeled cells and by the dilution of label

within cell nuclei) but a considerable proportion of the newly-formed cells left this site for the olfactory bulb.

Because regional cell proliferation at this age has essentially ceased in the subependymal layer of the vestigial olfactory ventricle, it is questionable whether the term "subependymal" should be retained for this region (as it implies a germinal matrix) or whether it should be considered a "stream" of migratory cells. Since the subependymal layer itself is composed of two types of cells, namely, proliferative and migratory cells, it may be argued that at different ages or different sites their proportion may be different, ranging from purely proliferative sites to purely migratory ones. The migratory stream that we have demonstrated in the anterior forebrain and the olfactory bulb would then represent a component of the subependymal layer composed at this age almost exclusively of migratory elements.

Proliferative and migratory elements of the subependymal layer are easily distinguished in autoradiograms as the former are labeled and the latter unlabeled in animals killed soon after injection with thymidine- $H^3$ . They are also easily distinguished in ordinary histological sections, provided that the migratory cells, which are elongated in the direction of migration, are cut parallel to their migratory route. For the subependymal layer of the anterior forebrain this requires sagittal sections; in coronal sections (which were used in Smart's study) these spindle-shaped elements appear as small darkly-staining, oval-shaped cells. (In the cerebellar cortex, where cell migration in the external granular layer is at right angles to the growing dendritic expanse of Purkinje cells, coronal sections are better suited for demonstrating spindle-shaped migratory cells; Altman, '69a).

In addition to demonstrating that cells in the rostral extension of the subependymal layer are migratory elements, we also showed that the newly-formed cells that reached the subependymal layer of the olfactory ventricle six days after injection have left this site by the twentieth day after injection and appeared then as cell constituents of the internal granular layer. About

12% of the cells of the internal granular layer were labeled at that time, which is comparable to that seen in animals injected at 13 days of age after 12 days survival, but considerably less than the percentage shown (over 30%) in the 13-day injection group after 20-day survival (Altman, '66a; see esp. fig. 2). Because in the present material there were no intermediate steps between 6 and 20 days survival after injection the exact date of their arrival and the speed of cell migration from the subependymal layer of the olfactory ventricle to the internal granular layer could not be determined. Conceivably, because the latter process involves neuronal differentiation the speed of migration could be lower here than in the migratory stream. Our previous study (Altman, '66a) indicated 12 days as the minimum transit time from the subependymal layer of the olfactory bulb to the internal granular layer in rats injected at 13 days of age.

With the staining technique that we used we could not distinguish with any certainty glia cells of the internal granular layer from granular nerve cells. Since both of the small cell types that we could distinguish were labeled, we assumed that the newly-formed cells could differentiate into both glia and neurons. Following a single injection of thymidine- $H^3$  about 12% of the cells were labeled 20 days later in the internal granular layer. If it were assumed that this high rate of cell gain is a purely acquisitive process the progressive growth of the olfactory bulb as a whole should be phenomenal. While we could not make an exact volumetric estimate of changes in the size of the olfactory bulb during this period, the available material indicated little or no change. Apparently, the persistent cell acquisition must serve the function of renewal of the cell population of the internal granular layer, an assumption that is supported by our data of a decline in the percentage of labeled cells in the internal granular layer in the animals that survived for a prolonged period after injection. If this interpretation is correct this would represent the first line of evidence of cell renewal in a neuronal population. Such renewal is not indicated for the other brain regions that we have stud-



ied so far. In the cerebellar cortex the proliferative matrix that gives rise to the basket, stellate and granule cells disappears after the end of three weeks (Altman, '69a) and our previous studies indicated that hippocampal neurogenesis is a cumulative process since we saw no signs of decline in the proportion of labeled cells as a function of survival after injection (Altman and Das, '65a, see esp. fig. 5).

Our auxillary studies regarding cell migration and acquisition in the corpus callosum, neocortex and caudate-putamen indicated that, at best, only a small contingent of cells that are produced in the subependymal layer of the inferior horn of the lateral ventricle are destined for these structures. It is interesting to note in this context that in these brain regions cell turnover appears faster than in the olfactory bulb with considerable label dilution within cells and earlier decline in the number of labeled cells after injection than that observed in the olfactory bulb. These two observations suggest that the cells migrating to these structures represent a rapidly turning over locally proliferating population, presumably precursors of glia cells (Altman, '66b).

We were particularly interested in the possible contribution of the rostral subependymal layer to the frontal neocortex and basal ganglia because data are available indicating a continuing growth of the forebrain in adult rats in the longitudinal direction (Sugita, '17; Altman, Anderson and Wright, '68). Indeed, we obtained some evidence that the growth of the forebrain along this dimension is influenced by various behavioral manipulations (Altman, Wallace, Anderson and Das, '68). Our present results, therefore, indicate that this late growth of the forebrain which is sensitive to behavioral influences is probably not due to cell acquisition since the rostral migratory stream makes relatively little contribution to extra-olfactory structures.

What is the role of the subependymal layer as a secondary proliferative matrix in mammalian neurogenesis? We have previously postulated (Altman, '69a,b) that also the subpial external granular layer of the cerebellum represents a secondary ger-

minal matrix. It is worth noting that both of these secondary matrices are a source of postnatally forming cells which differentiate into microneurons: the cells of the external granular layer into basket, stellate and granule cells in the cerebellum; the cells of the subependymal layer of the forebrain into granule cells in the hippocampal dentate gyrus and into granule cells of the olfactory bulb. While there are indications that the cells of these matrices are bipotential (giving rise also to neuroglia), a good proportion of them differentiate into short-axoned neurons. We may add to this that the cortices to which the secondary matrices contribute late-forming neuroblasts are in many respects different from the neocortex and allocortex, in which the recruitment of short-axoned neurons (possibly also from the subependymal layer) occurs earlier during ontogeny.

Inspection of a Nissl-stained sagittal section of the rat brain cut near the midline shows three cortical structures with extremely high cell density: the cerebellar cortex (due to high packing density of granule cells in the internal granular layer), the hippocampus (produced by the tight packing of granule cells in the dentate gyrus and of pyramidal cells in Ammons' horn) and the olfactory bulb (due to the presence of tight clusters or rows of granule cells in the internal granular layer). The *tight packing* of neurons in these *conspicuously laminated cortices* stands in sharp contrast to the *loose packing* of neurons in the neocortex and allocortex where there is an extensive network of neuropil (formed of neuronal and glial processes) among the perikarya of neurons. Indeed, electron microscopic studies showed that in these three regions the membranes of adjoining granule cells are in direct contact with each other over large surfaces (with the usual narrow extracellular cleft). This was observed in the cerebellar cortex (Gray, '61; Fox et al., '67), the hippocampal dentate gyrus (Blackstad and Dahl, '62; Blackstad, '63; Laatsch and Cowan, '62) and is also indicated for the granule cells of the olfactory bulb (see fig. 18 in Andres, '65). Glial processes, while present in some regions of

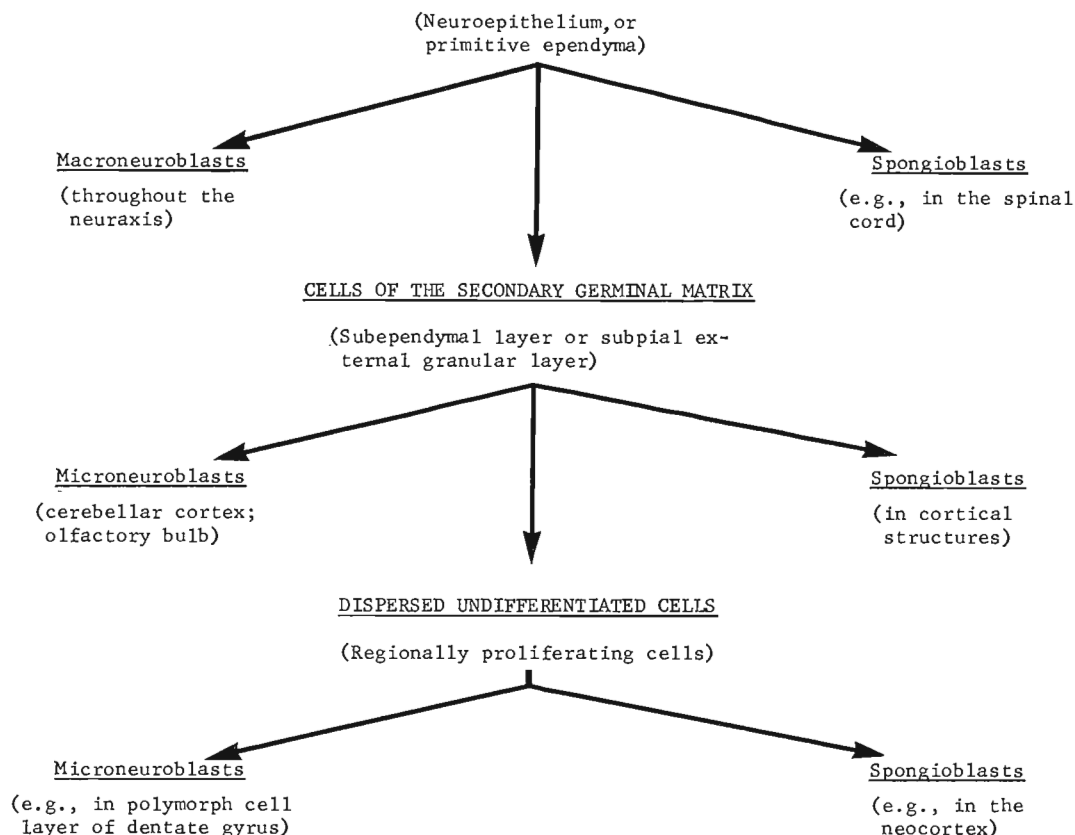
the perikarya of granule cells, are typically sparse. The functional significance of this arrangement is not known but these observations do point to structural peculiarities of these phylogenetically ancient cortices whose principal cell constituent, the vast population of granule cells, arise from secondary germinal matrices. Presumably the derivation of these granule cells from secondary germinal matrices is not a coincidence, nor is the survival of these matrices and the formation of granule cells for varying periods after birth.

We have previously postulated that the postnatal origin of granule cells in altricial mammals is related to a dependence of their differentiation (the connections they establish) on environment influences (Altman, '66a) and we raised the possibility that these microneurons may be responsible for neural "plasticity" or be the substrate of "memory" (Altman, '67). Since our present results indicate that the prolonged postnatal production of microneurons is characteristic only of the phylogenetically old, highly laminated and densely-packed cortices, the hypothesis can now be specified further to the effect that the "plasticity," or "memory," mediated by these postnatally-formed short-axoned neurons is associated with a circumscribed class of functions, namely, the acquisition of locomotor skills (cerebellum) and the fixation of behavior patterns relating to "affective, need-catering functions" (olfactory bulb, hippocampus) but not with memory processes related to "cognitive, instrumental functions" (Altman, '66c). Memory processes associated with these two classes of psychobiological functions are radically different from one another. The former, like imprinting, are dependent on developmental stages ("critical periods") and are highly resistant to extinction; that is, they become automatisms that are difficult to unlearn. In contrast, memory processes associated with cognitive functions (presumably mediated by phylogenetically newer brain structures) are less dependent on maturational stages and are easily altered by new experience. The neural mechanisms underlying these two types of memory processes may be different from one another and the postnatally-formed

microneurons would only be involved in the former.

In the histological analysis of our developmental material we observed that wherever there is a subependymal layer present in the forebrain there is a multiple-cell thick neuroepithelium (ependyma) around the ventricle. But we also noted that at some regions where a multiple-cell thick neuroepithelium is present in infant rats, as over the caudate-putamen, a subependymal layer is lacking. These two observations indicate, first, that the subependymal layer is a germinal matrix which is restricted to some brain regions, apparently those with a cortical organization (Smart, '61). Second, they suggest that where the subependymal layer is present, it is derived directly from the primitive neuroepithelium. These considerations, together with available knowledge about the embryonic development of the central nervous system, suggest the following sequence of events in mammalian neurogenesis (table 1). The primitive ependyma, or neuroepithelium, lining the lumen of the neural tube or brain ventricles is the ultimate source of all the neuroectodermal elements of the nervous system. The cells of the primitive neuroepithelium represent the primary germinal matrix which may give rise directly to neuroblasts (as, for instance, in the mantle layer of the spinal cord), to spongioblasts (as in the marginal layer of the spinal cord) and, when or where the primitive neuroepithelium loses its proliferative capacity and germinative potency, to differentiated epithelial cells that line the ventricles. In some brain regions, as in the cortices described, the primitive neuroepithelium gives rise to a secondary germinal matrix. Cells of this secondary matrix can differentiate into microneuroblasts (precursors of microneurons) or spongioblasts. The latter assumption is based on the observation (to be published) that practically all neuroglia cells of the cerebral cortex are formed after birth, and they apparently come from the massive subependymal layer present over the dorsal surface of the lateral ventricle (Altman, '66b). However, our studies also showed considerable local cell proliferation in such brain structures

TABLE 1  
CELLS OF THE PRIMARY GERMINAL MATRIX



as the white and gray matter of the cortex (Altman, '63, '66a, b). Obviously, cells with proliferative capacity are present at sites some distance removed from the germinal matrices, and glia cells could arise from this third source, the "dispersed undifferentiated cells," by local proliferation and differentiation. We may add to this that in at least one brain region, namely, the polymorph cell layer of the hippocampal dentate gyrus, locally multiplying cells (which ultimately derive from the subependymal layer) can give rise to micro-neurons (Angevine, '65; Altman and Das, '65a, b, '66). Recent electron microscopic studies appear to lend support to the assumption that undifferentiated cells are present in different regions of the brain some distance away from the germinal

matrices (Caley and Maxwell, '68a, b; Vaughn and Peters, '68).

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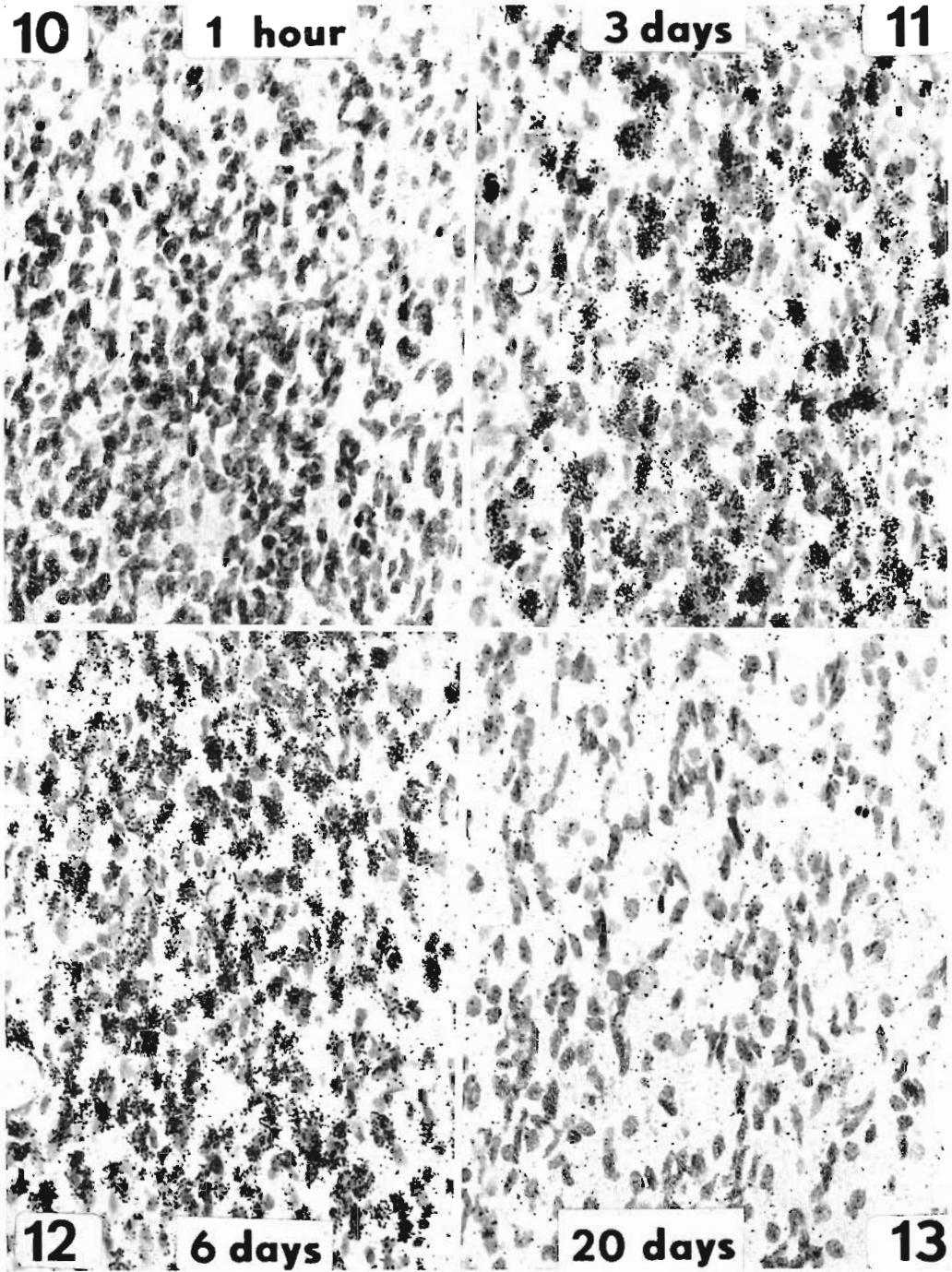
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PLATE 1

EXPLANATION OF FIGURES

- 10-13 High-power photomicrographs of autoradiograms of the subependymal layer in the olfactory bulb, from rats that were injected at 30 days of age and survived for different periods after injection. Galloxyanin chromalum,  $\times 400$ .



## PLATE 2

### EXPLANATION OF FIGURES

Photomicrographs of autoradiograms of the laminated cortex of the olfactory bulb in rats that were injected at 30 days and lived for different periods. GLO, glomerular layer; IG, internal granular layer; MI, mitral cell layer; SL, subependymal layer. Gallocyanin chromalum,  $\times 101$ .

- 14 From a rat that was killed one hour after injection. A few labeled cells are seen in the glomerular layer, but in general the bulb is free of labeled cells.
- 15 From a rat that was killed three days after injection. Note the high concentration of labeled cells in the subependymal layer. Labeled cells are not seen in the internal granular layer.
- 16 From a rat that was killed 20 days after injection. Labeled cells have disappeared from the subependymal layer and moved radially into the internal granular layer.



