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Autoradiographic and Histological Studies of Postnatal Neurogenesis

III. DATING THE TIME OF PRODUCTION AND ONSET OF DIFFERENTIATION OF CEREBELLAR MICRONEURONS IN RATS

JOSEPH ALTMAN
Laboratory of Developmental Neurobiology, Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

ABSTRACT In one experiment rats were injected with single does of thymidine-H^3 at 6 hours, 2, 6 and 13 days of age, or with multiple doses during two periods of early life, and were killed when 2, 4 and 6 months old. In the autoradiographic analysis attention was focused on the time of origin and differentiation of basket, stellate and granule cells in different regions of the cerebellar cortex. In another, non-radioactive study, in rats ranging in age from newborn to adult, the development of the cerebellum was studied with quantitative and qualitative histological techniques.

In the sagittal plane the area of the cerebellum increases over 20-fold from birth to 21 days. This increase is primarily due to the growth of the cerebellar cortex, much of the increment in the area of the subcortical regions occurring after 21 days. During the first week the growth of the different layers, excepting the proliferative external granular layer, is sluggish. During this period, the cells of the external granular layer do not differentiate but provide stem cells to this growing proliferative matrix. These proliferating and migrating cells of the external granular layer are the precursors of the basket, stellate and granule cells of the cortex; glia cells probably arise from cells multiplying locally. The first cells to differentiate are situated in the lower half of the molecular layer and include basket cells. Stellate cells differentiate later, with a peak at the end of the second week. The bulk of granule cells differentiate during the second and third weeks, with 25–80% of them, depending on the region, being formed between 11–21 days. These differences, together with several histological criteria (thickness of external granular and molecular layers, appearance of Purkinje cells) were used for constructing regional developmental maps of the cerebellar cortex. Granule cells differentiate in the depth of vermian fissures before they do over the exposed surfaces of the lobes; the ventral lobes (lingula and nodulus) mature before the anterior lobes; and the last maturing vermian lobes are the tuber, declive and culmen. The hemispheres, with some exceptions, mature later than the vermis, with the paraflocculus being among the last maturing structures.

In two previous papers (Altman and Das, '66; Altman, '66) we traced the fate of labeled cells in the brains of rats that were injected with thymidine-H^3 at 6 hours, 2, 6 and 13 days of age and were killed after injection at intervals ranging from 6 hours to 20 days (limited attention was given in the second paper to material from animals that survived for 60 days). This paper deals with cell labeling in the cerebellar cortex in animals with the same injection history but with prolonged survival periods of 60, 120 and 180 days after injection. Also included is an analysis of autoradiographic material from a few animals that were given multiple injections during the first or second week of life that, likewise, were allowed to survive for several months after injection. Finally, use is made of a large collection of histological material from rats ranging in age from birth to maturity.

We shall be concerned, first, with establishing the schedule of production of different cell types (in particular basket, stellate and granule cells) in the cerebellar cortex by estimating what proportion of these cells are formed during different periods after birth. Second, we shall try to reconstruct the order of their recruitment, or interdigitation, into the complex cytoarchitectonics of the cerebellar cortex

by dating the onset of their differentiation. Third, consideration will be given to differences in the date of maturation of the different subdivisions of the cerebellum.

Autoradiograms from animals with long survival time after injection are particularly suitable for determining the type of cells formed during different periods because ample time is available for the labeled cells to differentiate and settle in their ultimate stations. In such material, degree of labeling (from intense, indicating high concentration of labeled DNA, to light) can be used to infer the time of differentiation of different cell types if the assumption is made that the onset of specialization is associated with the cessation of cell proliferation (that is, that differentiated neurons do not multiply). In animals injected at particular ages, those cells that are intensely labeled may be assumed to have begun to differentiate soon after injection, whereas cells that are lightly labeled apparently were not ready to differentiate at that time but continued to multiply, as indicated by the label dilution.

Autoradiograms from animals that were given a single injection at a specified age are not suitable for determining the proportion of cells formed during that period unless the date of injection coincides with the gradual cessation of cell proliferation. If cell proliferation was still brisk in that population at the time of injection, an unknown proportion of descendants of originally tagged cells will appear unlabeled due to label dilution produced by repeated divisions. To be able to estimate in such populations the proportion of cells produced in a given period, it is necessary that the animal is injected several times during that period with thymidine-H^3 in order to re-label the cells that continued to multiply. Owing to these considerations, we also made use in this study of autoradiograms from animals that were given multiple doses of injections.

In addition, we evaluated a large collection of histological sections of the cerebellum from uninjected rats, ranging in age from birth to adulthood, in order to obtain collateral information about cerebellar development. In this material, attention was paid to the differential rate of development of different lobes of the vermis, and to the different maturation history of different cortical layers and cell types within lobes.

MATERIALS AND METHODS

In all essential details the standardized autoradiographic procedures used in previous studies of this series were also employed in this study. Laboratory-bred, Long-Evans hooded rats were injected at 6 hours, 2, 6 and 13 days of age intraperitoneally with 10 μc/gm body weight of thymidine-H^3 (specific activity, 6.7 C/mM; radiochemical dissolved in isotonic saline, 1 mC/ml). Pairs of animals were allowed to survive for 60, 120 and 180 days after injection, at which time they were killed by cardiac perfusion with 10% neutral formalin solution (table 1). The removed brains were further fixed in formalin, then dehydrated in a graded series of alcohols and embedded in Paraplast. Serial coronal sections were cut at 6 μ, and 3 consecutive sections out of 30 were pre-

TABLE 1

Age and number of animals

Age in days	No. of animals
0	3
1	4
2	2
3	2
4	2
5	2
6	0
7	3
8	2
9	1
10	7
11	4
12	3
13	5
14	4
15	4
16	4
17	1
18	2
19	3
20	4
21	4
22	1
23	1
24	1
25	1
30	3
90	4
Total	77

served. Of the preserved sections, two sets were stained with gallocyanin chromalum, and one of these was coated in the dark with melted Kodak NTB-3 nuclear emulsion, dried, and exposed with a dessicant at 5°C for 91 days.

Use was also made of autoradiograms from pairs of rats that were injected repeatedly on alternate days with thymidine-H^3 from birth to the eighth day, or at daily intervals from 11 to 16 days, and killed at three and one-half to four months of age. Because of the very high concentration of labeled cells, in this material we prepared autoradiograms with short exposure period (30 days) as well as with standard exposure (91 days).

In the supplementary study we evaluated qualitatively and quantitatively the cerebella of 77 uninjected rats, ranging in age from newborn to adult, as summarized in table 2. These brains were embedded in Paraplast and sections chosen for gross morphological evaluation were cut at 12 μ and stained with cresyl violet; those for histological and cytological evaluation were cut at 6 μ and stained in the same manner. Because of drastic developmental changes in the structural appearance of the cerebellum itself, landmarks in the thalamus were used for matching the sections. The plane chosen was one situated laterally from the medial habenular nucleus, in which the stria terminalis is conspicuous over the dorsal and rostral border of the thalamus and the fornix forms a continuous ventral band. This plane is about 1000 μ from the midline in the adult rat and corresponds to the plane illustrated in figure 62 in the atlas of König and Kippel ('63). Tracings were made of the cerebellar vermis and the different layers at this level with the aid of a modified Leitz projection apparatus at 65 × magnification (fig. 1). Measurements were made on these outlines with an Ott compensating planimeter of the total area of the sagittal section of the cerebellar vermis and of the areas occupied by the external granular layer, the molecular layer, the internal granular layer (including the layer of Purkinje cells), and of the remaining subcortical regions, which included the medullary layer and the deep cerebellar nuclei. In all instances the areas were scanned twice and the means were plotted.

TABLE 2

Survival after injection and number of animals

Age at injection	Age when killed in days		
	60	120	180
6 hours	2	2	0
2 days	2	2	2
6 days	2	2	2
13 days	2	2	2
30 days	2	2	2

RESULTS

1. *Gross-morphological quantitative data*

The postnatal growth of the cerebellum in the sagittal plane is considerable (fig. 1).

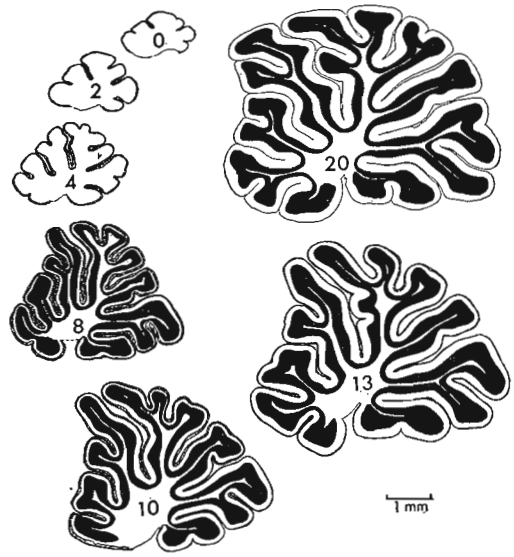

Fig. 1 Tracings of sagittal sections of the cerebellum (in a plane specified in the Materials and Methods section) from rats of different ages; constant magnification. *Outer band,* external granular layer; *black,* internal granular layer; *white above black,* molecular layer; *white below black,* subcortical regions. For designations of lobes and major fissures see figure 11a. Numbers refer to age of animals in days.

Figure 2 summarizes the planimetric results regarding the growth of the cross section of the cerebellar vermis in the plane specified earlier, together with an analysis of the variable growth of the different layers or components of the vermian cortex

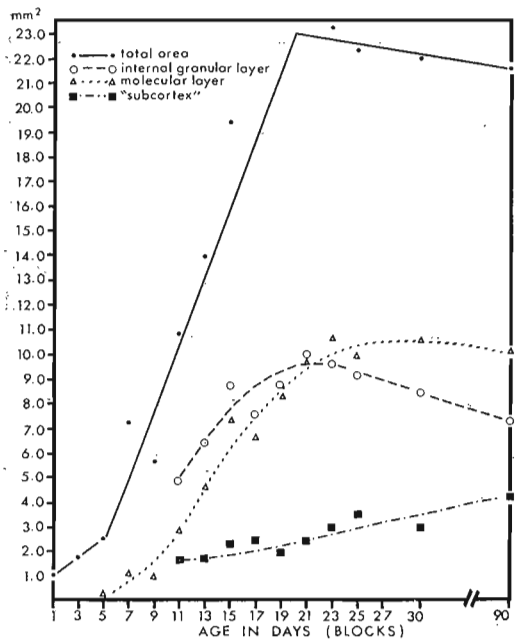

Fig. 2 Planimetric measurements of the areal growth of the cerebellum (in matched sagittal sections) together with a laminar analysis. Area occupied by external granular layer is plotted separately in figure 3. Each point represents means from several animals.

and subcortical regions. The growth pattern of the transient external granular layer is plotted in figure 3. Because of an over 12-hour uncertainty in dating the age of the animals (those born after working hours in the evening were considered "newborn" next morning), and also because the gestation periods of the animals were not taken into consideration, the data are presented in blocks of two days.

The cross section of the cerebellum increases over 20-fold from days 0–1 to 20–21 days of age. There is apparently a moderately rapid growth period between 0–5 days, and an extremely rapid one between 5–21 days. There is a slight decline between 23–90 days. We could not determine the growth pattern of the internal granular layer before ten days of age because of the uncertainty of its boundaries with the medullary layer and, because early in life it is predominantly composed of Purkinje cells and Bergmann glia cells, and thus would not provide a meaningful picture of the developmental pattern of granule cells. The internal granular layer, which in all measurements included the Purkinje cells, increases rapidly between 10–21 days, then declines appreciably to the ninetieth day. The area occupied by the molecular layer is minimal between 7–9 days. But beginning about the tenth day there is an extremely rapid rise, almost matching that of the cerebellum as a whole. The growth of the molecular layer continues until about 23 days of age and it remains essentially unchanged thereafter. There was no apparent growth in the subcortical regions between 11–18 days which is the period of fast growth of the cerebellar cortex, but a sluggish increase was noted thereafter, continuing up to 90 days. (The "subcortical regions" refers to the medullary layer, and the area occupied by the deep cerebellar nuclei and their surrounds. Its value was obtained by deducting the area occupied by the other layers, including the external granular layer, from the total area of the cerebellum in cross section.)

The growth pattern of the transient external granular layer, which is the proliferative matrix of the cerebellar cortex, is summarized in figure 3. Because of the difficulty in making accurate planimetric measurements at the magnification used (× 65) of the external granular layer in the older animals (in which it becomes very thin and fragmented) the bulk of the data presented is based on material gained from animals aged 0–1 to 14–15 days. The results indicate a rapid growth during the first week in the area occupied by (or in the cell population of) the external granular layer. During the second week an asymptotic level is apparently maintained (the variability observed makes this uncertain), and there is a rapid decline during the third week.

2. *Chronological, histological and cytological observations*

In the qualitative and semi-quantitative histological evaluation of the non-autoradiographic, developmental material described in the previous section, attention was paid: (a) to the differential growth of the different layers of the cortex in sagittal sections of the vermis; (b) to regional differences in this respect in different lobes

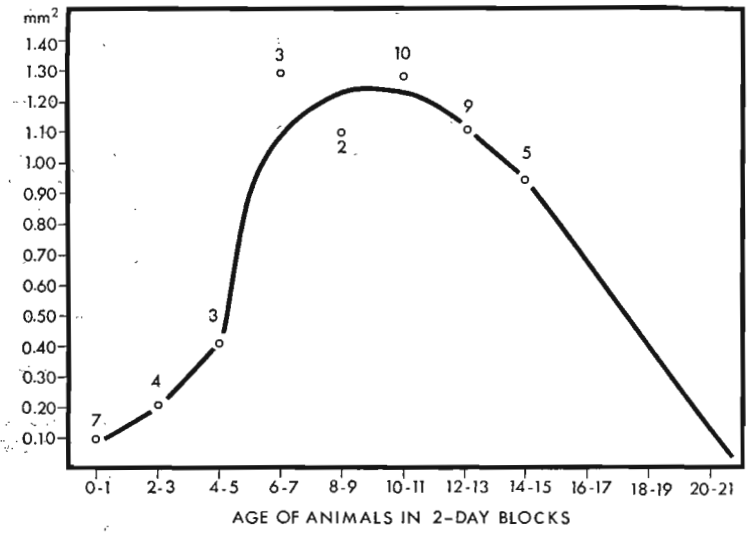

Fig. 3 Area occupied by the external granular layer in sagittal section. Numerals refer to the number of animals used in each block.

of the vermis (for a classification of lobes, see fig. 11a); (c) to changing cell composition of these layers during development; and (d) to changes in the morphological appearance of the cells themselves.

The cerebellum of the newborn and 1-day old rat is extremely small and immature morphologically. Though the major fissures of the vermis are recognizable, most of them are quite shallow. Over the surface of the cerebellum the external granular layer forms a sheet 3–5 cells in thickness, and at its base the roof of the fourth ventricle is covered by a mitotically active several-cell thick neuroepithelium. The molecular layer is either absent (its position is recognizable as the interface between the external granular layer and Purkinje cell layer) or it is a thin white band which in places contains some scattered cells. Below this zone is a 3–5 cell-thick layer composed primarily of small, round, pale nuclei, devoid of visible cytoplasm; these cells are the undifferentiated Purkinje cells. Scattered among these cells, and forming a separate zone, are small dark cells, presumed to be the precursors of cerebellar glia cells, particularly Bergmann cells (see below). Even at this early age some local differences were discernible in the vermis. For instance, Purkinje cells are larger and lightly stained (and an occasional one has some cytoplasm) around fissura prima; they are smaller, darker and more densely packed in the posterior vermis.

On the following days (2–5 days) there is a considerable growth in the cross section of the cerebellum, with an increase in the thickness of the external granular layer and a considerable deepening of the major fissures of the vermis. In the 4–5 day old animals the external granular layer is 8–12 cells thick in many regions (fig. 12), with mitotic cells quite frequently seen in the upper three-fourths of the layer. The neuroepithelium over the dorsal wall of the fourth ventricle is still about three cells thick and appears mitotically active. In sagittal sections a gap is seen, formed by invagination of the choroid plexus, between the neuroepithelium and the external granular layer covering the nodulus. In many regions the Purkinje cells have become larger and display what appears to be the first cytological sign of their differentiation, the formation of an apical cytoplasmic mitre or cone over the nucleus, pointing upward towards the surface of the cerebellar cortex (fig. 12). This is best seen at this age in the anterior vermis, particularly lingula and centralis, around the depth of fissura prima and in the nodulus in the posterior vermis. This

change is interpreted to herald the development of the apical dendrite system of the Purkinje cells. Associated with this change, a slight increase is observable in the width of the molecular layer in the early-maturing parts of the vermis and the internal granular layer as a separate entity is beginning to be recognizable. This is most conspicuous in the nodulus (where, however, the molecular layer is still quite undeveloped).

The thickness of the external granular layer is maintained over the rapidly increasing surface of the cerebellar cortex in the 7–8 day-old rats (figs. 13, 14, 19, 20) with suggestive signs of reduction in the 8-day old in some regions, such as fissura prima. A conspicuous change in the faster developing portions of the vermis (lingula and centralis anteriorly, nodulus and uvula posteriorly, and around the fissura prima) is the appearance of many vertically oriented, slender, spindle-shaped cells in the molecular layer (fig. 14), apparently descending through the layer and moving into the internal granular layer. These cells often have thin visible processes (fig. 14), some in contact with one another, others in apparent contiguity with the thick shaft of Purkinje cells dendrites. At these early maturing regions, the Purkinje cells form a closely-packed single row of cells, and are characterized by extreme increase in the size of their apical cytoplasm (figs. 14, 19, 20). Some lightly-stained cells are also seen in the lower parts of the developing molecular layer, presumably representing (see below) differentiating basket cells. By this time a distinct internal granular layer is also present and there is considerable increase in the width of the molecular layer. These changes are not seen at this age in the more slowly developing parts of the vermis, the declive and culmen anteriorly, and the pyramis and tuber posteriorly.

In the 9–11 day-old rats there is a considerable reduction in the width of the external granular layer (to 3–6 cells) in some regions, particularly the nodulus, while in other regions (in general, over the dorsal surface of the vermis) the thickness of the layer is unchanged (fig. 16). In most regions there is a change in the zonal composition of the layer, with a decrease in the width of the proliferative zone and an increase in the migratory zone. In the regions where there was a decrease in the thickness of the external granular layer there was a concomitant increase in the width of the molecular layer. In the early-maturing regions, as in the nodulus or the depth of fissura prima (fig. 16) two types of cells are abundant in the molecular layer: vertically-oriented spindle-shaped cells throughout the layer, and horizontally-oriented cells, some showing signs of differentiation, in the bottom third of the layer. The former are presumably migrating precursors of granule cells and other cell types, the latter, differentiating basket cells. In these regions the Purkinje cells have begun to assume a new appearance, the apical cones have disappeared but there is some increase in basal cytoplasmic stainability. There is an evident increase in the width of the internal granular layer in all parts of the vermis, with signs of increases in the concentration of cells in the medullary layer. In contrast, in the late-developing parts of the vermis, as in the dorsal portions of the tuber, declive and culmen, the molecular layer is still thin, with only a few migratory cells; the Purkinje cells are closely aggregated and are either undifferentiated or showing the early signs of differentiation, that is, the formation of apical cones.

In 12–13 day-old rats the thickness of the external granular layer is greatly reduced over the nodulus (1–2 cells thick), it is intermediate in width in the depth of fissura prima (fig. 17) and several other regions (3–6 cells thick), but is still of maximal thickness over the declive, tuber and pyramis. In early-maturing regions, there is great increase in the number of differentiating basket cells in the lower half of the molecular layer, while in the upper half horizontally-oriented, spindle-shaped, undifferentiated cells are seen, presumably representing precursors of stellate cells (see below). The number of vertically-oriented, spindle-shaped cells remains high throughout the molecular layer, and Purkinje cells either show an increase in basal cytoplasmic stainability or have begun to show adult appearance. In late-maturing regions the differentiation of the molecular layer has not begun, and Purkinje cells are still densely packed, many of them

showing exaggerated apical cytoplasmic growth.

In 14–15 day-old rats the thickness of the external granular layer is reduced to a single row of cells over the nodulus; it is about 2 cells thick over the lingula; 3–4 cells thick over centralis; about 5 cells thick over many parts of uvula; 5–7 cells thick over pyramis; and still about 8–10 cells thick over tuber. In the earliest-maturing regions the differentiation of stellate cells in the upper half of the molecular layer has begun, and the proportion of dark, horizontally-oriented, spindle-shaped cells is reduced at the expense of pale, round or polymorphic cells. Many Purkinje cells show increased basal cytoplasmic stainability. In other regions many differentiated basket cells are seen, but the upper part of the molecular layer is occupied by undifferentiated cells. In all regions, vertically-oriented, spindle-shaped cells, the presumed precursors of granule cells, are present in large number.

Vertically-oriented, spindle-shaped cells were seen in large numbers also in the 16-day old rats (fig. 18), although there is a general decrease in the thickness of the external granular, including over tuber, where it is reduced to about four cells. In the early-maturing regions the differentiation of basket cells appears completed, that of stellate cells is in progress, and the soma of Purkinje cells shows adult appearance. However, in the dorsal vermis the molecular layer is still undeveloped and Purkinje cells appear immature.

In 18–19 day old rats the external granular layer is reduced to a row of single cells over most regions of the vermis. It is fragmented over the nodulus and lingula, in an apparent process of dissolution, though it is still about four cells thick over some parts of the dorsal vermis. In all except the latest-maturing regions, stellate cells are well-differentiated; but vertically-oriented, spindle-shaped cells are still present in appreciable numbers.

In 20–21 day-old rats, some scattered cells resembling those of the external granular layer are seen near the surface of nodulus, part of the uvula and over centralis and lingula. A continuous row composed of single cells is seen over parts of uvula and pyramis, while a 2-cell-thick continuous matrix remains over tuber and the posterior part of declive. The molecular layer appears mature in the entire vermis, though vertically-oriented, spindle-shaped cells are seen throughout the cortex. These migratory elements are fewer in number in early-maturing regions, where the external granular layer is in process of dissolution, and are very common in late-maturing regions. In the latter regions, as in tuber, also some horizontally-oriented, spindle-shaped cells are seen in the upper parts of the molecular layer, suggesting that the differentiation of stellate cells may still be in progress.

In the 22–23 day-old rats, few isolated small, dark cells, representing the dissolving external granular layer, are seen over most regions of the vermis, and in some no vestiges of it remain, with the exception of the tuber, declive and the depth of some fissures, where a discontinuous row of single cells still remains. Vertically-oriented, spindle-shaped cells are seen in the molecular layer in variable numbers over the entire vermian cortex.

In 24–25 day-old rats vestiges of the external granular layer, in the form of widely-scattered cells remain over tuber and declive; elsewhere cells belonging to this germinal matrix are no longer discernible. Vertically-oriented, spindle-shaped cells are still seen in the early-maturing regions in small number, and they are quite numerous in the late-maturing regions. Indeed, even in the 30-day old rats, in which all vestiges of the external granular layer are gone throughout the vermis, occasional vertically-oriented, spindle-shaped cells are still seen. This suggests that the migration of cells formed in the external granular layer through the molecular layer goes on for several days, possibly up to one week after its dissolution.

3. *Qualitative and quantitative autoradiographic results*

A summary of the injection and sacrificing schedule of the adult rats utilized in this part of the study was presented in table 2. In addition, autoradiograms from a group of adult rats injected repeatedly with thymidine-H^3 at two distinct developmental periods, as described earlier, were also evaluated. Where not otherwise speci-

fied, the analyses were carried out in the pyramis or lobe VIII of Larsell ('52) in the posterior cerebellar vermis (fig. 4). All the autoradiograms were obtained from serial sections cut in the coronal plane. To facilitate comparison with the data described above, the results were in some instances transferred onto sagittal tracings of a normal adult cerebellum, as shown in figures 11a,b.

Cerebellar neurogenesis during the first eight days of life

Cell labeling in rats injected at six hours of age. In animals injected soon after birth and killed 60, 120 and 180 days later, a very high proportion of lightly-labeled cells were seen in the internal granular layer and in the molecular layer. Only an occasional intensely- or medium-labeled cell was seen in the internal granular layer, with some variations in the same section in different lobules (e.g., more intensely-labeled granule cells were present in the nodulus than in the pyramis). Medium labeling characterized many of the cells situated around the soma of Purkinje cells, presumably Bergmann glia cells, and also cells situated in the lower half of the molecular layer, some of which were tentatively identified as basket cells. Intensely-labeled Bergmann glia cells and basket cells were seen in higher numbers in some regions, such as the nodulus, than in others, as the pyramis. Cells in the upper half of the molecular layer, which included stellate cells, tended to be very lightly-labeled or unlabeled. Purkinje cells were never labeled. Likewise, the great majority of Golgi cells were unlabeled, though occasionally a well-labeled Golgi cell was encountered in some regions, such as the tuber.

These observations suggested that very few granule cells and stellate cells have begun to differentiate on the first day of life or soon thereafter, but somewhat more Bergmann glia cells and basket cells. Also, differentiation in some regions of the vermis began before it started in other regions.

Injection at the age of two days. The pattern of cell labeling in the animals injected at two days was essentially similar to that seen in animals injected immediately after birth. Very few intensely-labeled cells were seen in the internal granular layer (though slightly more than in the newborn group) and there was a gradient, with declining label concentration over cell nuclei, from the interface of the molecular layer (the position of Bergmann glia cells) toward its surface (the position of stellate cells) (see fig. 30, in Altman, '66).

Injection at the age of six days. The proportion of labeled granule cells greatly increased in this group, with many medium-labeled cells and a low but increasing number of intensely-labeled cells. Many cells around Purkinje cells, presumably Bergmann glia cells, were intensely-labeled, others were unlabeled. Cells in the lower half of the molecular layer, including recognizable basket cells, showed medium or intense labeling; cells in the upper half of the molecular layer were generally lightly-labeled (fig. 7a). Labeled Golgi cells were not encountered.

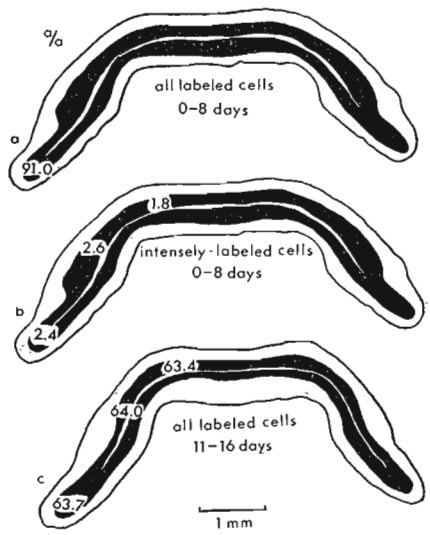

Fig. 4 Tracings of the pyramis in matched coronal sections from adult rats. Numbers refer to the percentage of cells found labeled in the internal granular layer in the indicated regions of the pyramis. Upper two tracings from an animal injected with repeated doses from birth to eight days; lower tracing from an animal injected with repeated doses from 11–16 days. *Black*, internal granular layer. See also figure 10c for percentage of cells found labeled in the latter animal in the pyramis in a different section.

These results indicated that many of the Bergmann glia cells have either differentiated before the sixth day or were differentiating toward the end of the first week of life. A considerable proportion of the basket cells were also preparing to differentiate at this period. A few granule cells were also differentiating but not yet the stellate cells.

Multiple injection from birth to eight days. Intensive study was made of autoradiograms from the cerebellum of a rat injected with standard doses of thymidine-H^3 at 0, 2, 4, 6 and 8 days and killed at 120 days. The pattern of cell labeling in this animal was expected to reflect the cumulative pattern for animals injected over the first week of life (and shortly thereafter).

Practically all the granule cells, in all regions of the cerebellum (91% in the pyramis), were labeled (fig. 4a). Most of these cells were lightly labeled; a small proportion, 2–3% in the pyramis (fig. 4b), were intensely-labeled. An occasional intensely-labeled Golgi cell was seen, but the majority was unlabeled. Virtually all the cells in the molecular layer were labeled,

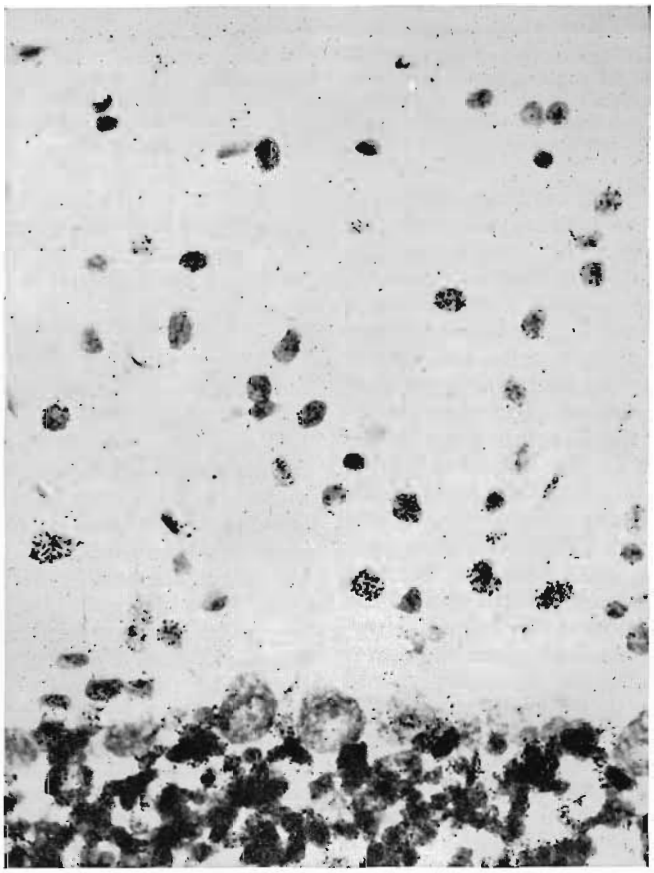

Fig. 5 Photomicrograph of an autoradiogram (short exposure of 30 days) showing a portion of the pyramis from an adult animal injected repeatedly between birth and eight days. Note light labeling of cells in the internal granular layer (lower portion of picture); intermediate labeling of several cells in the molecular layer in the vicinity of Purkinje cells (these cells would be intensely-labeled with standard, 91 day exposure of audoradiograms); and lightly- or unlabeled cells in the upper part of the molecular layer. (In this and subsequent black-and-white photomicrographs labeling in the internal granular layer is difficult to identify due to the intense staining of granule cells.) Gallocyanin chromalum, × 400.

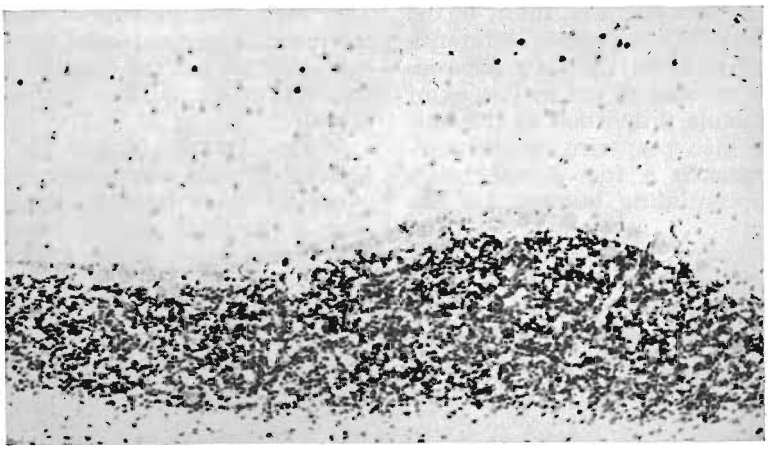

Fig. 6 Autoradiogram (standard exposure) from the pyramis of a rat injected at 13 days of age and killed when four months old. Note that in the molecular layer intensely-labeled cells are concentrated in the upper region, few cells are labeled below that zone. These labeled cells are tentatively identified as stellate cells. Many intensely-labeled cells are present in the internal granular layer. Gallocyanin chromalum, × 101.

with a gradient of label concentration over cell nuclei from the base of the layer toward its surface (fig. 5). Bergmann glia cells were intensely- or medium-labeled; basket cells tended to show medium labeling, stellate cells were lightly labeled. More intensely-labeled granule cells and basket cells were encountered in some regions of the vermis than in others; and more in the vermis, including the pyramis than in the hemispheres, such as the flocculus.

These observations indicate that virtually all the cells of the granular layer and molecular layer, the Golgi cells excepted, are derived from cells that are formed after birth (only a small proportion of the Golgi cells are of postnatal origin). However, few of the cells formed in the external granular layer cease to multiply, and presumably begin to differentiate, in the first eight days of life. The Bergmann glia cells, which are derived from locally proliferating cells around Purkinje cells, rather than the external granular layer (Altman, Anderson and Wright, '68), do differentiate during this period and so do many of the basket cells. But very few of the granule cells and possibly none of the stellate cells have commenced to differentiate. Since there is a very high rate of cell proliferation in the external granular layer during this period (fig. 3), it must be assumed that the major outcome of this activity is the enlargement of the proliferative compartment of the external granular layer with an increasing number of stem cells produced for later differentiation.

Cerebellar neurogenesis from 11 to 21 days

Injection at the age of 13 days. In animals of this group killed at 60, 120 and 180 days of age, cells in the lower two-thirds–three-fourths of the molecular layer, including basket cells, were typically unlabeled, whereas many cells in the upper one-third–one-fourth of the layer, including some tentatively identified as stellate cells, were intensely labeled (figs. 6, 7a,b). Near the layer of Purkinje cells many intensely-labeled Bergmann glia cells were seen, but the majority of these was unlabeled. In the internal granular layer, lightly-, medium- and intensely-labeled cells were seen in high concentration.

Fig. 7 Autoradiograms (standard exposure) of the cerebellar cortex in uvula of six month old rats. A, from a rat injected at six days of age. Note accumulation of intensely-labeled cells in the lower one-third of molecular layer. In autoradiograms with short exposure some of these cells were tentatively identified as basket cells. B, from a rat injected at 13 days. Note accumulation of intensely-labeled cells in upper one-third of molecular layer. Some of these are presumed to be stellate cells. Gallocyanin chromalum, × 256.

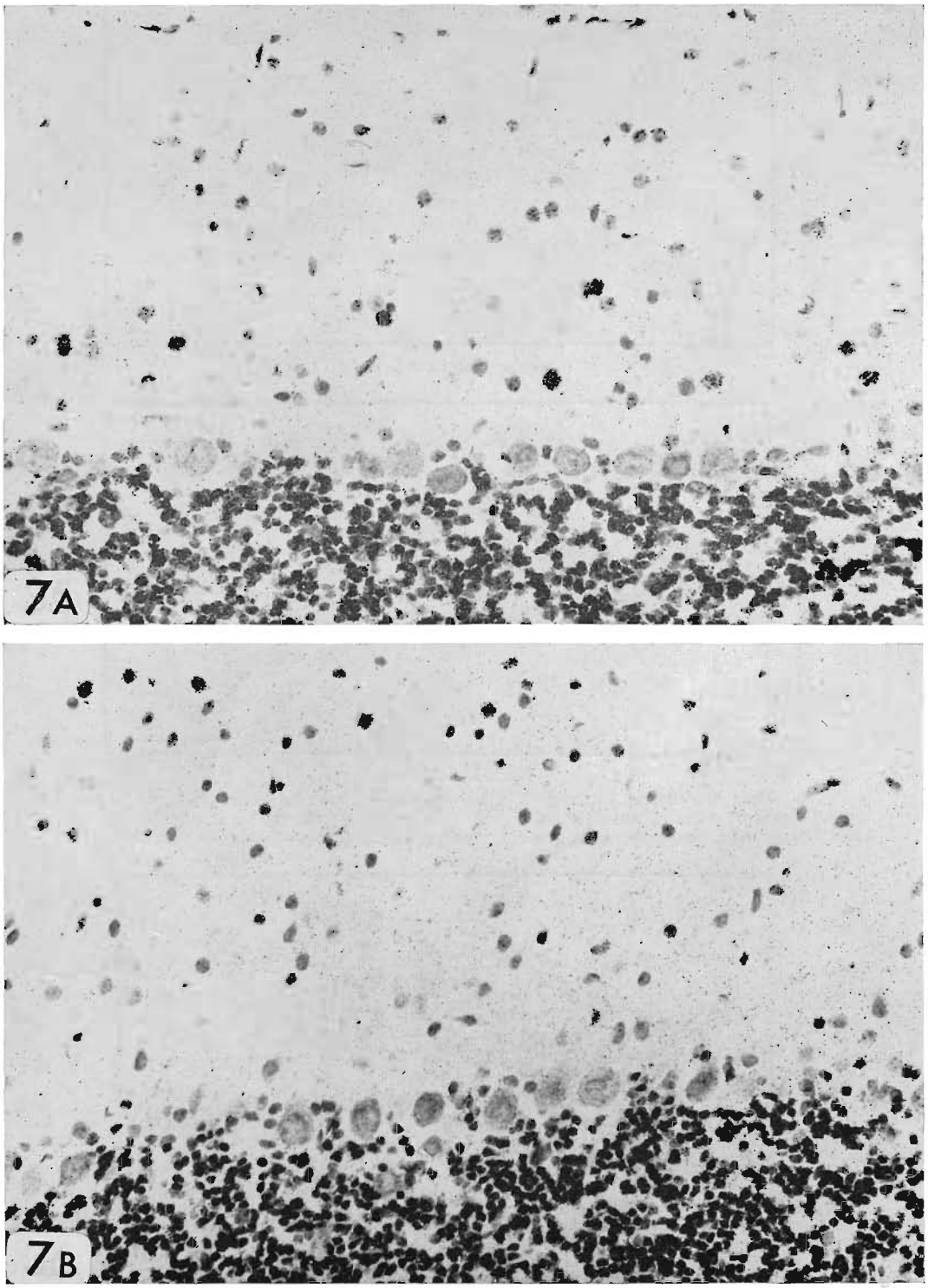

Figure 7

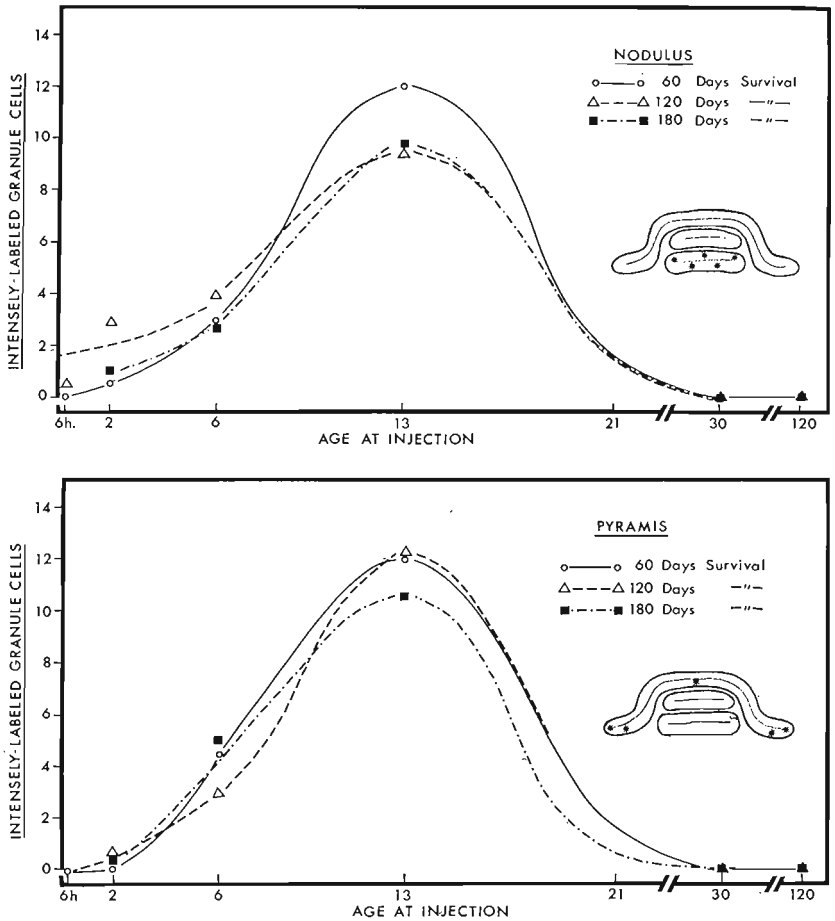

Fig. 8 Mean number of intensely-labeled cells in the internal granular layer in nodulus and pyramis in pairs of animals, aged two, four and six months, as a function of age at time of injection. Sampling sites (stars) schematically indicated; sample size 130^2 μ.

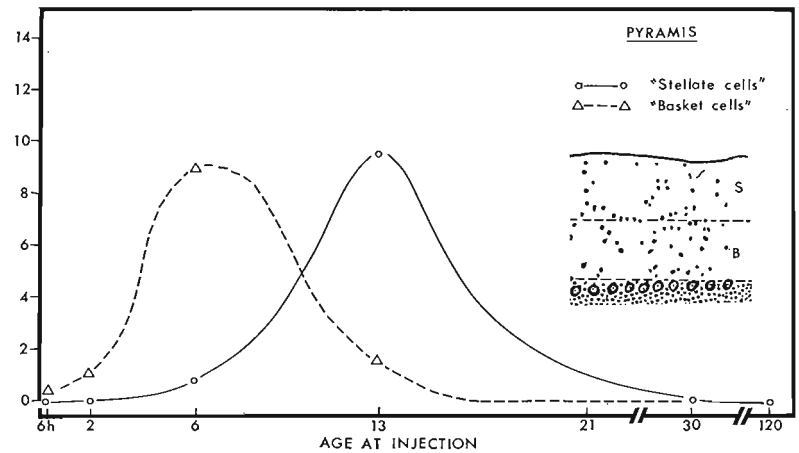

Fig. 9 Mean number of intensely-labeled cells in the upper ("stellate cells") and lower ("basket cells") half of the molecular layer in the pyramis in pairs of rats injected at different ages and killed when four-months old. Size of sampled area specified in text.

These results suggested that the formation of "basket cells" ceased before the thirteenth day of life; that many of the "stellate cells" were still being formed; that while many of the granule cells were differentiating, the precursors of many others were still actively proliferating.

Two quantitative procedures were utilized to estimate the proportion of cells of different types formed before, during or after the thirteenth day. In one procedure the numbers of intensely-labeled cells were counted in animals injected at 6 hours, 2, 6 and 13 days of age on the assumption that these numbers provide indices of the proportion of cells that were beginning to differentiate on these days. The mean number of intensely-labeled cells in the internal granular layer in the nodulus and pyramis, based on five samples of square areas 130 μ × 130 μ in each animal, is summarized in figure 8. It may be seen that few or no intensely-labeled cells were present in either area in animals injected at six hours of age; that a few were seen in the nodulus in animals injected at two days of age; that there was an increase in the number of intensely-labeled cells in both areas in the group injected at six days of age and a peak was reached in the animals injected at 13 days of age. Autoradiographic material was not available at 21 days but because at this age the external granular layer has essentially disappeared it is assumed that the zero level seen in animals injected at 30 and 120 days is actually reached at about 21 days.

This procedure was also employed to obtain an estimate of the concentration of intensely-labeled cells in the molecular layer. In the pyramis, in animals that survived until 120 days of age, the molecular layer was bisected with the aid of an ocular grid parallel to the surface of this structure (fig. 9). The intensely-labeled cells encountered in the upper half of the layer (which included many stellate cells) and those encountered in the lower half (which included many basket cells) were counted separately. In all animals 20 130 μ-long strips were sampled. Because the sampled area in the granular layer (fig. 8) was larger, and also the packing density of cells is higher, we plotted for the two zones of the molecular layer the mean numbers of intensely-labeled cells calculated for strips 650 μ-long. The results indicate, in agreement with our previous qualitative description, that the differentiation of cells in the lower half of the molecular layer, including that of basket cells, with a peak at six days, antedates the differentiation of cells in the upper half, including stellate cells, with a peak at 13 days (fig. 9). The differentiation of granule cells apparently coincides with that of stellate cells.

In another procedure the total number of labeled cells, including lightly-labeled ones, was counted. Because cell proliferation is so brisk during the first two weeks

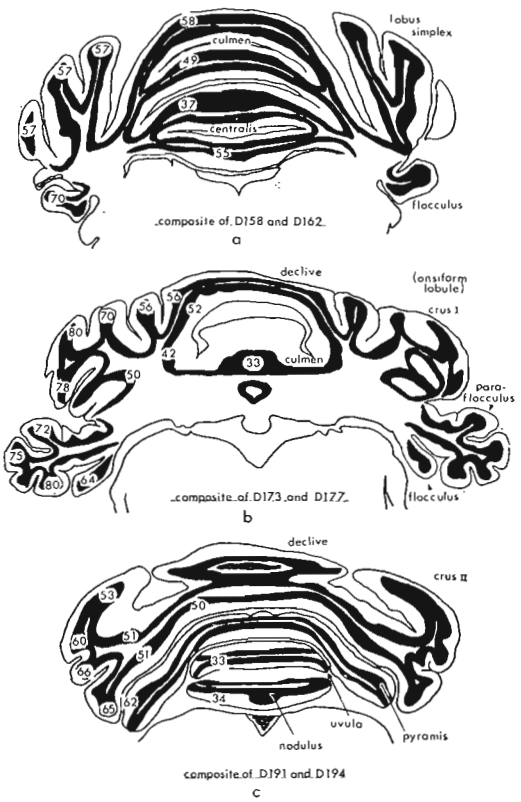

Fig. 10 Tracings of three coronal sections of the cerebellar cortex from a rat injected with repeated doses from 11–16 days and killed as an adult. For the planes of sectioning compare with figure 11a; a, tracing of section D162; b, D177; c, D194. The numbers indicate the mean percentage of labeled cells in the internal granular layer (black) at the sites indicated. The means represent pooled data from the right and left side from two adjacent sections, as indicated.

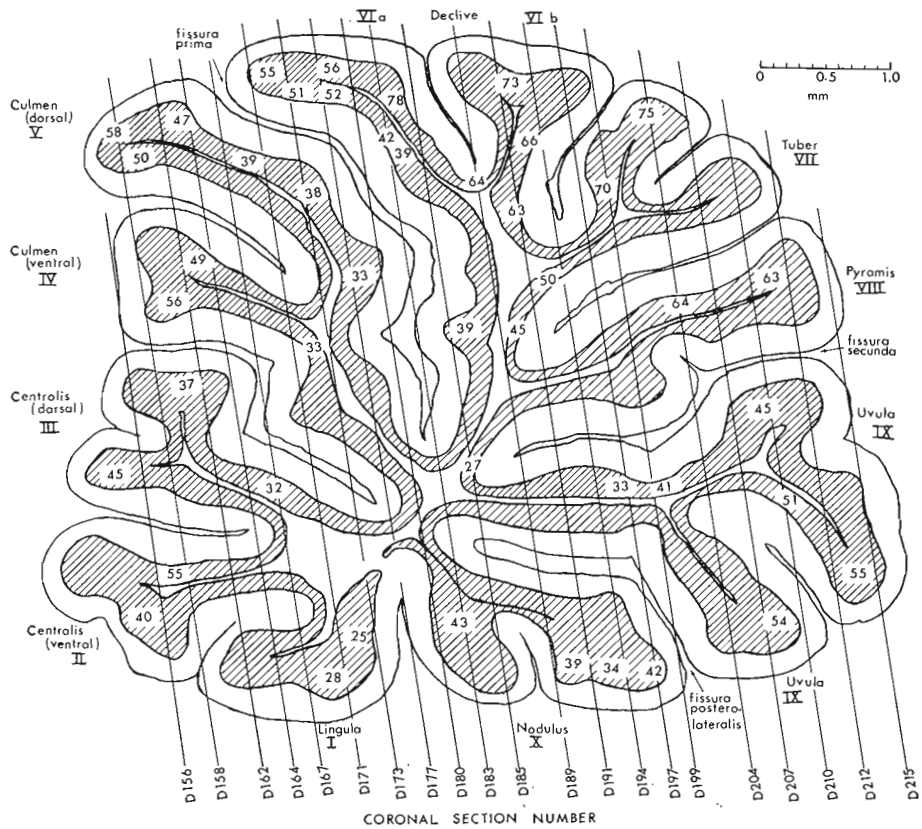

Fig. 11a Summary of percentage of cells found labeled in the internal granular layer (diagonal lines) in the animal injected from 11–16 days in samples taken near the midline in 21 coronal sections. The data from coronal sections were transferred to a sagittal tracing of an adult rat cerebellum to indicate differential rate of acquisition of granule cells after 11 days in different parts of the vermis. 11b (next page) schematic presentation of the results shown in a, with an arbitrary tripartite classification of the vermis on basis of percentage of labeled granule cells, into early-maturing (light diagonal lines), intermediate (heavy lines) and the late-maturing regions (black).

of life many cells that are tagged at 6 hours, 2 or 6 days of age are bound to lose their labeling. Therefore, this procedure cannot be used with these groups as the number of labeled cells provides too low an estimate of the number of cells formed during these periods. Usable data, however, were obtained with this procedure in the 13-day group. In both animals that were injected at 13 days and lived to the age of 60 days, 45% of the 2,000 cells counted in the pyramis were labeled. The fact that many of these cells were lightly-labeled, indicated that cell proliferation continued for some time after the injection in a portion of the population. Accordingly, the percentage obtained (nearly one-half) may be considered a low estimate of the proportion of granule cells formed after the thirteenth day of life, or the third week of cerebellar neurogenesis. (Compare this result with data summarized in fig. 4.)

The late differentiation of granule cells was also indicated by a detailed quantitative analysis of autoradiograms from an animal injected at daily intervals with thymidine-H^3 between 11–16 days of age.

Multiple injection from 11 days to 16 days. Serial autoradiograms were available from two rats that were repeatedly injected with standard doses of thymidine-

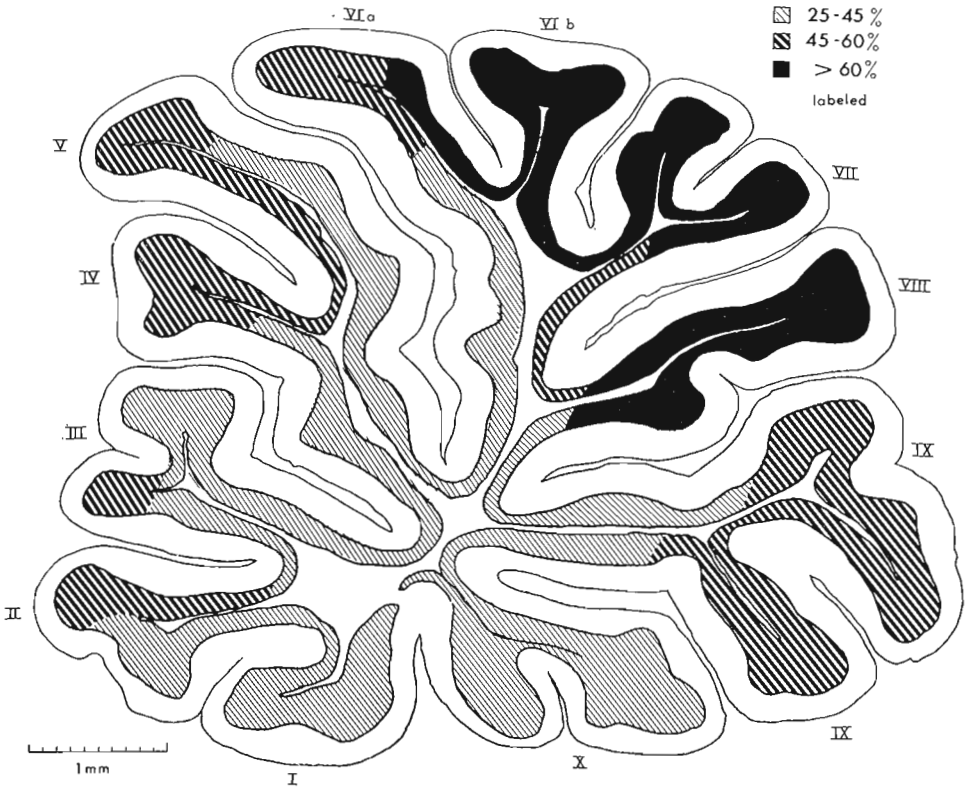

Figure 11b

H^3 on days 11, 12, 13, 14, 15 and 16 and were killed at 100 days of age. The entire cerebellum of one animal was evaluated quantitatively in an attempt to establish possible regional differences in the rate of differentiation of granule cells. In the planes indicated in figure 11a, a total of 21 coronal sections were studied in the following way. Tracings were made of these sections at 50 × magnification with a modified Leitz (Aristophot) projector. In each section several samples were taken bilaterally at 640 × magnification in square areas 130 μ × 130 μ, and the proportion of labeled and unlabeled cells in the internal granular layer was determined. A total of 230 such square areas were sampled, with several samples for each major subdivision of the cerebellar cortex, and their sites were marked on the tracings (fig. 10).

Because these animals were injected daily from the eleventh day until the sixteenth day, when proliferative activity begins to decline considerably, the proportion of labeled cells encountered at a given site can be taken to represent a fair (or only slightly deflated) estimate of the number of cells produced and differentiating from the eleventh day until the cessation of cerebellar neurogenesis about the twenty-first day. As illustrated in figure 10, considerable regional differences exist in the proportion of cells formed in the cerebellum from the eleventh day onward. In some regions, as in the nodulus and uvula (fig. 10c), and a portion of culmen (fig. 10b), less than 40% of the cells were formed during this period, indicating that over 60% were formed before the end of the tenth day of life. In other regions, such as the paraflocculus (fig. 10b), between 70–80% of the cells of the granular layer were formed during the second half of the period available for postnatal cerebellar neurogenesis. In general, the examples shown in figure 12 indicate that a larger

proportion of cells are formed from the eleventh day on in the cerebellar hemispheres than in the vermis, suggesting that the vermis begins to mature earlier, or is ontogenetically "older," than the hemispheres. But there are many exceptions to this generalization. Thus, some regions of the vermis (e.g., dorsal culmen; fig. 10a) are comparable to some regions of the hemispheres (e.g., lobus simplex). In the vermis, the declive, tuber and pyramis were found to be late-forming; and among the exceptionally late-forming regions in the hemispheres were crus I (ansiform lobule) and the paraflocculus.

In order to obtain a better understanding of the pattern of regional cell production and differentiation in the vermis of the cerebellum, all the data obtained from coronal sections in the medial portion of the vermis were transcribed onto a tracing of a typical sagittal section of the cerebellum of an adult rat, as shown in figure 11a. To further facilitate identification of a possible developmental pattern in the ontogenesis of the granular layer of the cerebellar cortex, we have arbitrarily grouped areas in terms of percentages obtained ranging from very early-forming regions (25–45% labeled between 11–16 day), intermediate regions (45–60% labeled), to late-forming regions (more than 60% labeled) (fig. 14). The results permit the following generalizations. In most regions the sulci of the lobes are formed before the gyri. Exceptions are the lingula (I) and nodulus (X) (or the ventral vermis) which are early-forming in their entirety, and the tuber (VII) and part of the declive (VIb) (or the dorsocaudal vermis) which are late-forming.

DISCUSSION

Three major experimental approaches were undertaken in this study to obtain information about the postnatal development of the cerebellar cortex: (a) a gross morphological investigation of growth in sagittal plane of the cerebellum as a whole, and of the various layers of the cerebellar cortex, from birth to maturity; (b) histological and cytological analyses of developmental changes in the cell composition of the cortical layers, and in the structure of the cells themselves; and (c) autoradiographic analyses of the location and identity of labeled cells in the cerebellar cortex of adult rats which were injected with single or multiple doses of thymidine-H^3 early in life. In many respects the results were complementary and the attempt will be made here to formulate a tentative picture of some aspects of postnatal cerebellar neurogenesis.

The phenomenal growth of the cerebellum as a whole in sagittal plane, which is over 20-fold between 1–21 days of age, is primarily due to the growth of the cerebellar cortex. The growth of the cerebellum was less rapid from birth to five days, than between 5–23 days, thereafter there were indications of some reduction. Little growth was indicated for the subcortical regions of the cerebellum (which in this plane is made up largely of the medullary layer) between 10–21 days, the time when it could be reliably measured. There was some increase in the area of the subcortical regions between 21–90 days, presumably reflecting a growth due to myelination.

Analysis of differential laminar contribution to the growth of the cerebellar cortex gave the following results. The molecular layer could not be measured, due to its thinness, before five days. There was minimal increase in its area between 5–9 days, but there occurred thereafter a rapid growth phase, which lasted until 23 days, leading to a nearly 10-fold increase. The growth of the molecular layer lagged behind and outlasted that of the internal granular layer. The growth of the latter could be determined with accuracy only after 10 days of age and it showed an appreciable decline between 21–90 days.

These results are in good agreement with the quantitative data of Haddara and Nooreddin ('66) in mice. They found that the cerebellar cortex increases 20-fold between birth and 15 days, the time when the external granular layer disappears in the mouse. (The increase was slow in the first 5 days, rapid thereafter.) They found only a 4-fold increase in the white matter, indicating that the major contribution to cerebellar growth was made by the development of the cerebellar cortex. They did not observe any decline in the area of the cerebellum as a whole in adults, though

they do report (see their fig. 5) a decrease in the thickness of the "cerebellar cortex" (without laminar analysis) after 15 days. Like in this study, they also observed an increase in the white matter after maturation of the cerebellar cortex.

The growth of the cerebellar cortex is directly or indirectly related to the migration of cells produced at a high rate in the subpial, external granular layer. In the newborn rat there are virtually no cells in the thin slit that represents the molecular layer, and the layer below it consists mostly of densely packed, undifferentiated Purkinje cells (formed before birth) and locally proliferating cells that are presumed to be precursors of glia cells. It may be safely concluded that virtually all the stellate, basket and granule cells are formed after birth. The areal growth of the external granular layer is brisk between birth and five days and it increases rapidly between 5–7 days. This initial exponential growth suggests that the cells produced remain in the proliferative compartment (the upper proliferative zone of the external granular layer) as reproductive stem cells during the first week. This ascending phase presumably reflects a developmental period during which the stock of proliferating cells is enlarged, and is manifested both as the expansion of the area of the external granular layer over the growing surface of the cerebellum and in the increase of the thickness of the layer, as seen histologically. During this period (or, at least, up to 5 days) the increase in the area of the cerebellum as a whole is modest, suggesting that its rapid growth has to await or is associated with the commencement of migration and differentiation of cells produced in the external granular layer. This interpretation is supported by our autoradiographic results which indicated that few of the cells that were produced in the external granular layer differentiated during the first week, with the exception of cells located in the lower half of the molecular layer.

The quasi-equilibrium indicated for the area of the external granular layer during the second week of life corresponds to the period when there is a rapid growth in the surface area of the cerebellar cortex but when, at least in the early maturing regions, there is a decline in the thickness of the external granular layer. Our earlier studies with rats injected at birth, 2, 6, and 13 days of age, which survived for 6 hours only after injection, indicated (see especially fig. 12 in Altman, '66) that the proportion of labeled cells in the external granular layer is only slightly lower at 13 days than it is at 2 or 6 days and is nearly as high as at birth. This indicates that the rate of cell production is not appreciably reduced during the second week of life. From a kinetic point of view the lack of growth in the population of this compartment can be accounted for by assuming that the rate of cell production is matched by the rate at which cells disappear from this compartment. Histological evidence indicated that at the beginning of the second week, vertically-oriented, spindle-shaped cells begin their migration through the molecular layer in large numbers and we also observed the slow accumulation of differentiating cells in the lower half of the molecular layer and in the internal granular layer. The gross morphological data showed that during the second week there was rapid increase in the area occupied by both the molecular and internal granular layers. Finally, the autoradiographic evidence indicated that during this period stellate cells and granule cells differentiated in large numbers, with a peak reached at the end of the second week (13 days). Accordingly, the first week is primarily taken up with the accumulation of undifferentiated cells (during which there is relatively little growth in the area occupied by the cerebellar cortex, notwithstanding the considerable growth of the external granular layer itself). Then, during the second week, both cell production and cell differentiation are very brisk. Finally, during the third week, which is characterized by the rapid decline in the area occupied by the external granular layer over the vermis, cell differentiation predominates over cell production, with more cells leaving the proliferative compartment than are reproduced, leading to the eventual dissolution of the proliferative layer. The histological evidence indicated that during this period the upper half of the molecular layer, with its stellate cells, are differentiating, and

the autoradiographic evidence showed that, (varying in different regions) 25–80% of the granule cells differentiated after the eleventh day. Fujita, Shimada and Nakamura ('66) reported that in the mouse more than 50% of the granule cells migrate from the external granular layer after ten days of life, and 81–92% of them are produced later than seven days of age. In this context we may mention that these workers' estimate of less than one day for the transit of granule cell precursors through the molecular layer (in the mouse) is not in agreement with our previous autoradiographic findings (in the rat) indicating a minimum of three days and a maximum of six days (see especially fig. 11 in Altman, '66), and with our present histological observation of the presence of spindle-shaped, migratory cells in the molecular layer up to one week after dissolution of the external granular layer.

Cytological observations indicated several phases in the differentiation of Purkinje cells. At birth, when they are densely aggregated between the external granular layer and the medullary layer, they appear undifferentiated, with little or no cytoplasm surrounding their round, pale nuclei. After a few days, a unipolar cytoplasmic growth is seen in the form of a mitre, or cone, which is typically oriented toward the surface, or the cells of the external granular layer. It is presumed that this growth signals the first step in the differentiation of the Purkinje cell, the development of its apical dendrite system. It is tempting to assume an interaction between the Purkinje cells and cells in the migratory zone of the external granular layer, a hypothesis which is supported by our observation that if the external granular layer is subtotally eradicated at three days of age by low-dose x-irradiation (Altman, Anderson and Wright, '69) the unipolar "apical cones" become randomly oriented with respect to the surface of the cortex at 4–5 days of age. These findings, furthermore, suggest that the first step in the differentiation of Purkinje cells (the outgrowth of apical cone) does not require the presence of the external granular layer but that its *directed* growth perpendicular to the surface does. This is supported by our Golgi studies of the development of Purkinje cells after x-irradiation (to be published).

The second step in the differentiation of Purkinje cells, as revealed in Nissl-stained material, is dissolution of the apical cone and the development of increased stainability of the basal cytoplasm, in the lower portion of the cell. It is conceivable that this heralds the development of basal processes, perhaps the rich recurrent collateral system of Purkinje cell axons. Either this second stage lasts for a short period, because only a moderate proportion of the Purkinje cells show it in a given region, or it may not be a universal stage in the development of Purkinje cells. Finally, as the last stage, the Purkinje cell assumes adult appearance. In some cerebellar regions this occurs as early as the end of the second week, in others not until the end of the third week. These changes in the development of Purkinje cells are presumably associated with changes in their relation with the differentiating basket, stellate and granule cells. The rapid growth of the molecular layer must be due to the increasing arborization of Purkinje cell dendrites, together with the formation of more and more parallel fibers, and to the increase in the number and in the processes of basket and stellate cells. Our quantitative analyses indicated that the areal growth of the molecular layer initially lags behind that of the internal granular layer, but then overtakes and surpasses it, and continues for several days after the external granular layer has essentially disappeared. These data suggest that the growth of the processes of the nerve cells located in the molecular layer continues after the acquisition of new cells has come to an end. The significance of the sequential recruitment of basket and stellate cells, indicated by this study, remains to be established.

Another result that deserves consideration is the difference in the maturation of different regions of the cerebellar cortex. The histological and autoradiographic results appeared essentially to provide converging results. Lobes identified as early-forming structures, such as the nodulus, were found to be distinguished by the following characteristics: (a) early decline in the width of the external granular layer; (b) early maturation of Purkinje cells;

(c) early increase in the width of the molecular layer; and (d) relatively low proportion of labeled granule cells in animals injected with repeated doses of thymidine-H^3 after 11 days of age. The opposite was observed in late-forming structures, such as the tuber in the vermis, and in many parts of the cerebellar hemispheres. Conceivably, these differences in regional maturation of the cerebellar cortex are related to differences in the functional maturation of the body structures that are represented in the different lobes of the cerebellum, but the exact nature of this relationship remains to be worked out. In at least one earlier study (Raaf and Kernohan, '44) regional differences in the maturation of the cerebellum in man could not be demonstrated.

Our earlier studies (Altman, '66; Altman, Anderson and Wright, '68) showed that in addition to the external granular layer, two other proliferative sites exist in the postnatally developing cerebellum, one in the medullary layer and the other around the Purkinje cells. It is difficult to determine the fate of the cells produced in these two latter regions, but it appears reasonable to assume that the cells multiplying at a low rate in the medullary layer give rise to locally differentiating glia cells, and there is suggestive evidence that the cells multiplying around the Purkinje cells differentiate as Bergmann glia cells. If these assumptions are correct, then it would follow that the cells of the external granular layer are precursors primarily of microneurons, namely, basket, stellate and granule cells but they could conceivably also give rise to the glia cells of the internal granular and molecular layers. These considerations have bearing on the old controversy whether the cells of the external granular layer are pluripotential "indifferent cells" (Schaper, 1897) and can give rise to both glia and neurons, or whether, as is more commonly assumed, neurons and glia arise from different precursors. Evidence is available that the large neurons of the deep cerebellar nuclei and Purkinje cells arise sequentially during embryonic development, from cells of the primitive neuroepithelium (Miale and Sidman, '61). The Golgi cells themselves, the last of which are formed about the time of birth, may arise directly from the primitive neuroepithelium. It is conceivable, as I have argued elsewhere (Altman, in press), that this primary proliferative matrix gives rise in some brain regions to secondary germinal matrices (as the external granular layer in the cerebellum and the subependymal layer in the forebrain) and also to dispersed stem cells which can proliferate locally (as the cells that multiply in the medullary layer of the cerebellum). In each of these proliferative systems, developmental potency (or "indifference") is gradually reduced. Cells of the primitive neuroepithelium may give rise directly to macroneurons and indirectly to microneurons and neuroglia; cells of the secondary germinal matrix directly to microneurons and indirectly to dispersed stem cells; dispersed stem cells may give rise in some regions to neuroglia and in others (as in the polymorph layer of the hippocampal dentate gyrus; Angevine, '65; Altman and Das, '65, '66) to microneurons.

ACKNOWLEDGMENTS

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PLATE 1

EXPLANATION OF FIGURES

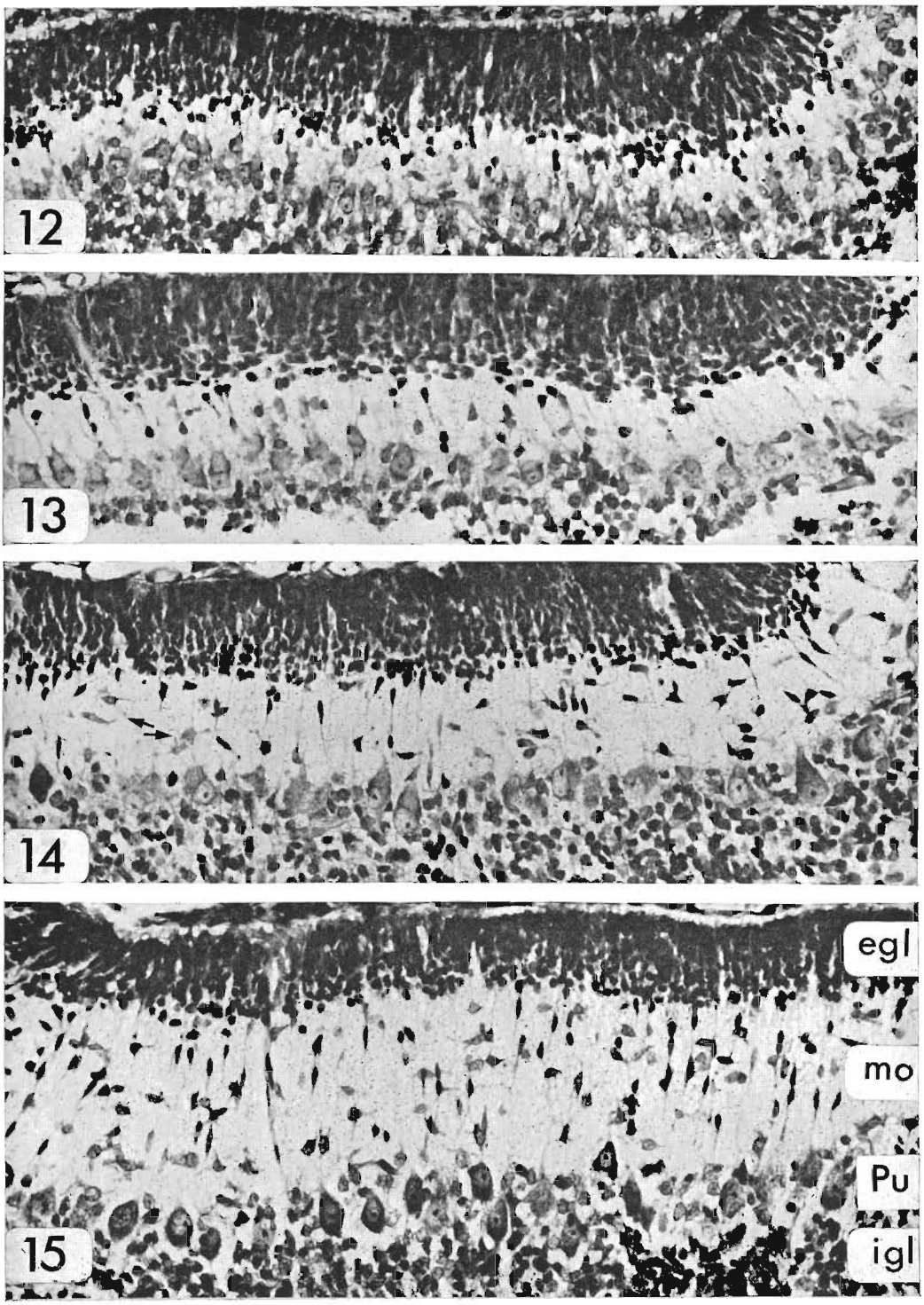
Photomicrographs of a portion of the cerebellar cortex at the depth of fissura prima in rats of different ages. Abbreviations: e.g.l., external granular layer; i.g.l., internal granular layer; mo, molecular layer; Pu, Purkinje cells. Cresyl violet, × 256.

12 From a five-day old rat. Note that maximal width of external granular layer is attained. Molecular layer is thin with a few scattered, mostly roundish cells. Some Purkinje cells have apical cytoplasmic cones.

13 From a seven-day old rat. There is some increase in width of molecular layer and in size of Purkinje cells.

14 From an eight-day old rat. Vertically-oriented, spindle-shaped cells with processes in apparent migration through molecular layer. Note also some light-staining cells with processes on the left side (arrows).

15 Ten-day old rat. Note decrease in width of external granular layer and great increase in width of molecular layer with many vertically-oriented spindle-shaped cells. Some lightly-staining, differentiating cells (basket cells?) are seen throughout the layer. Apical "mitre" of Purkinje cells no longer visible in this region. A few Purkinje cells show moderate increase in the stainability of the basal cytoplasm.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egl
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PLATE 2

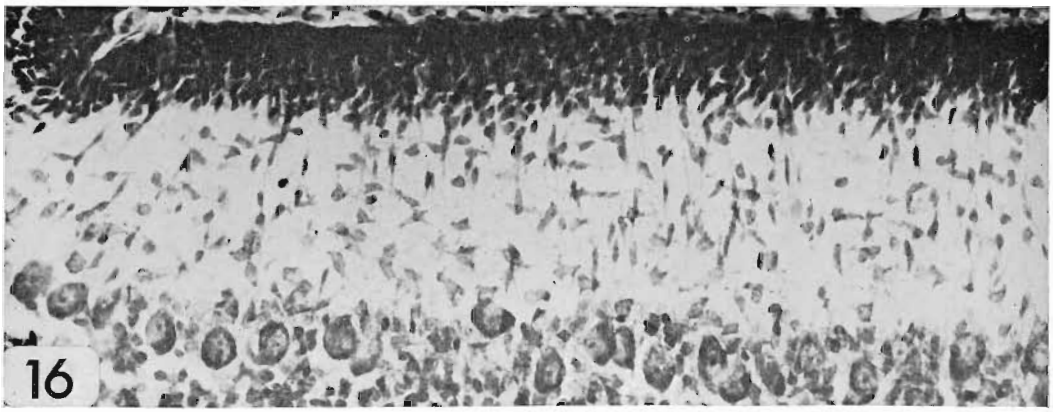
EXPLANATION OF FIGURES

Photomicrographs of a portion of the cerebellar cortex at the depth of fissura prima. Continuation of plate 1; for details see plate 1.

16 From an 11-day old rat. Note the appearance of some horizontally-oriented cells in the upper half of the molecular layer and the many lightly-stained, presumably differentiated cells in the lower half.

17 From a 13-day old rat. Further decrease in the width of external granular layer and increase in the width of molecular layer. Horizontally-oriented spindle-shaped cells pronounced in upper half of molecular layer; numerous differentiated basket cells seen in lower half of molecular layer.

18 From a 16-day old rat. Note reduction of external granular layer to a two-cell thick sheet and the presence of differentiated cells throughout the molecular layer, the uppermost zone excepted. Vertically-oriented, spindle-shaped cells persist throughout the molecular layer for many days.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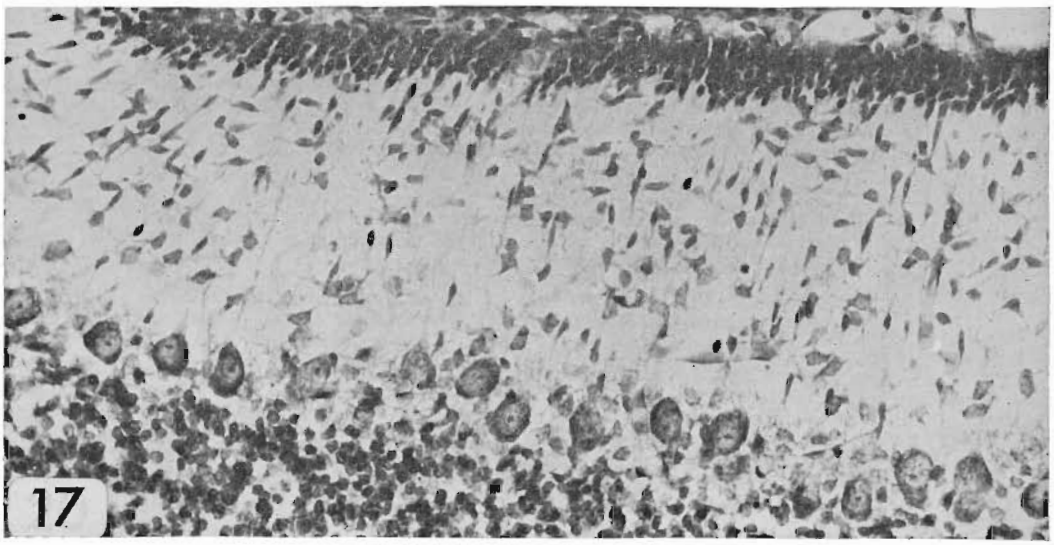
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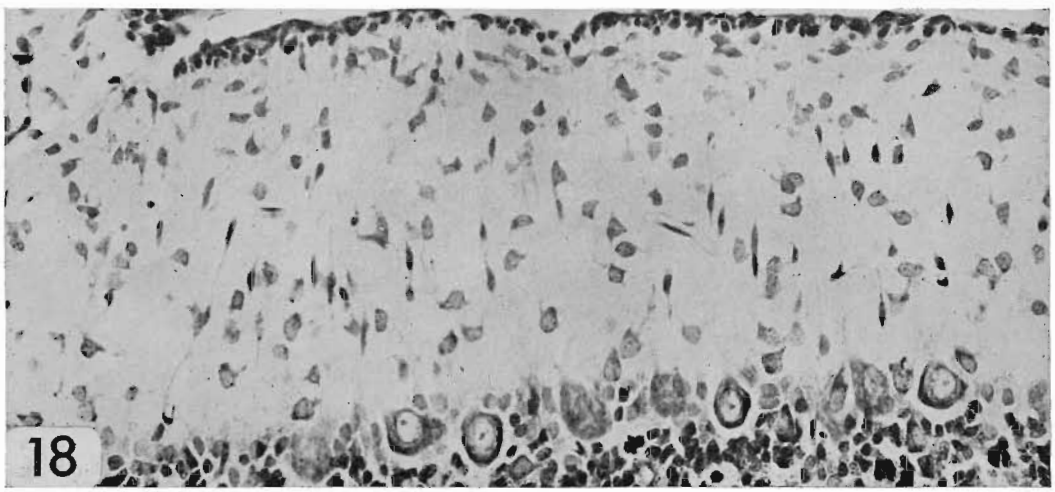
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PLATE 3

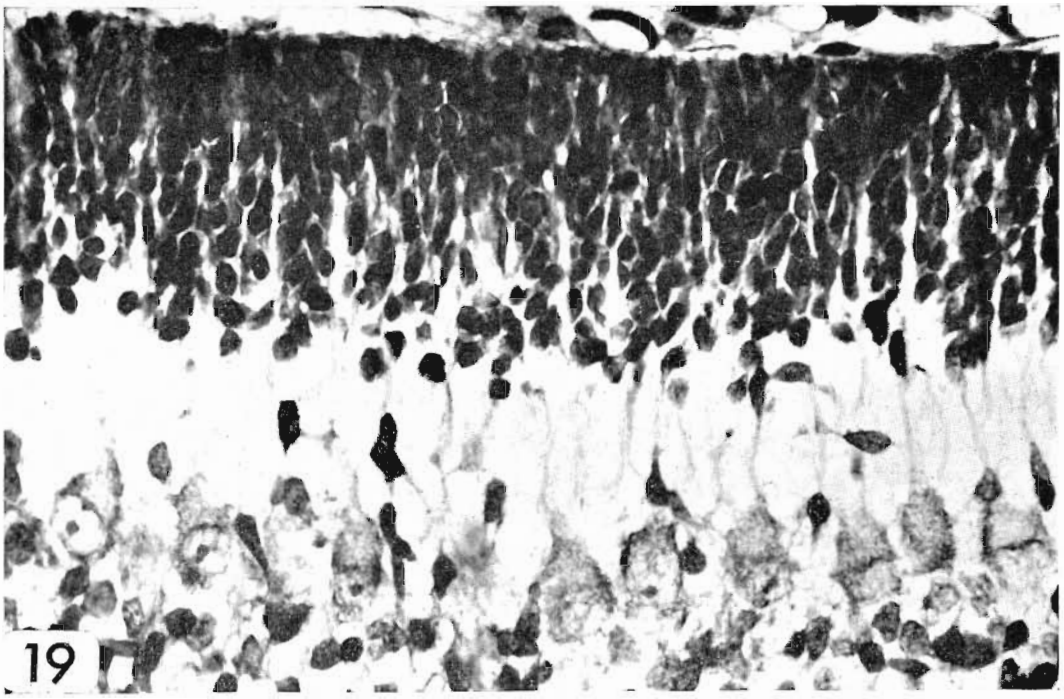
EXPLANATION OF FIGURES

Comparison of the appearance of the apical cones of the developing Purkinje cells in seven-day old rats in sections cut at right angles to one another with respect to the orientation of cells in the migratory zone (lower half) of the external granular layer. mz, migratory zone, pz, proliferative zone of external granular layer. Cresyl violet; × 640.

19 Note that cells in the migratory zone of the external granular layer are oval-shaped. This region is sectioned perpendicular to the direction of cell migration within the layer.

20 Note that in this region the cells of the migratory zone are spindle-shaped; sectioning here is parallel to the direction of cell migration.

The latter plane, which is parallel to the long axis of the folium, is presumed to be the direction in which the parallel fibers (the bifurcating axon terminals of mature granule cells) are laid down, at a right angle to the planar surface of Purkinje cell dendrites. Accordingly, these observations indicate that these "apical" cones are truly conical in shape and not flattened at this early stage of development as will be later the dendrites that they will give rise to.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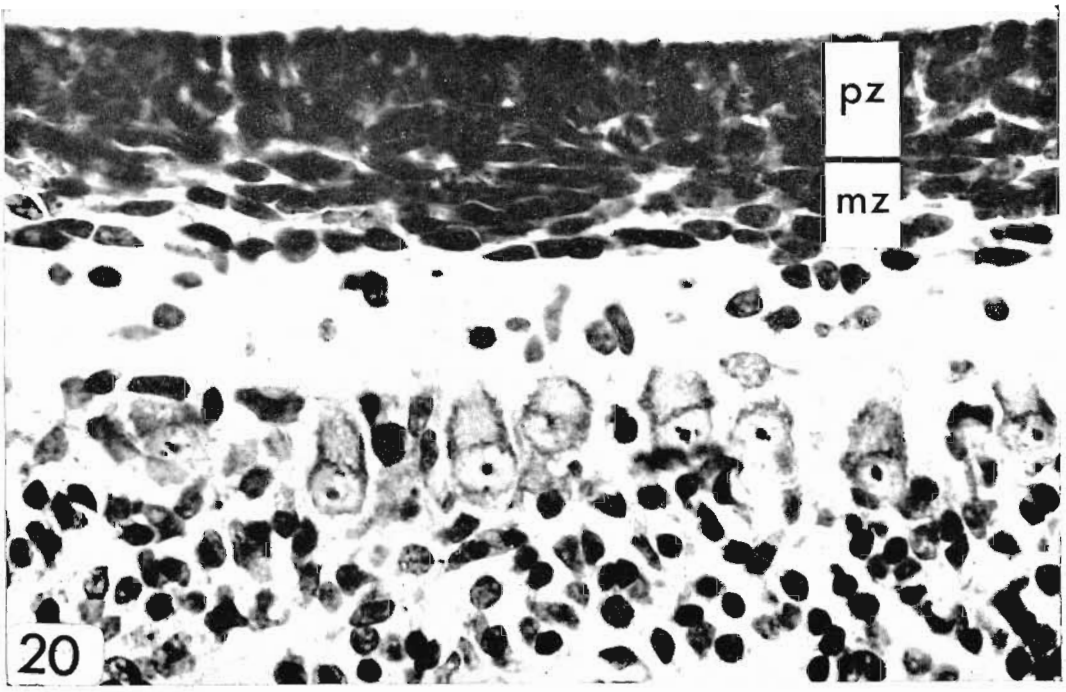
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20