Prenatal Development of the Cerebellar System in the Rat

I. CYTOGENESIS AND HISTOGENESIS OF THE DEEP NUCLEI AND THE CORTEX OF THE CEREBELLUM

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ABSTRACT Prenatal cerebellar development was investigated with three approaches. In normal embryos sectioned in three planes morphological and cytological changes were determined at daily intervals beginning on embryonic day 13 (E13). A similar series of X-irradiated embryos was used to study changes in neuroepithelial organization and in the location of primitive (radiosensitive) or differentiated cells. Finally, to quantify the time of origin of different classes of cerebellar neurons with the progressively delayed labelling procedure, we used autoradiograms from adult rats whose mothers were injected with two successive daily doses of ^{3}H-thymidine on overlapping days from day E13 on.

The cerebellar anlage was delineated in the dorsal metencephalon by the collapse of its ventricular lining after X-irradiation. This "collapsing neuroepithelium" was located laterally on day E13, then it spread medially and reached the midline on day E16. Deep nuclear neurons began to differentiate on day E13, with two-thirds forming on day E14; Purkinje cell formation peaked on day E15, with a few cells still forming on day E16. It was postulated that the deep nuclear neurons settled first in the superficial "nuclear zone," and that the Purkinje cells gathered temporarily in the underlying "transitory zone," adjacent to the collapsing neuroepithelium.

In the next period of cerebellar development four major events were recognized. (1) Beginning on day E17 the cells of the nuclear and transitory zones became intermingled. It was postulated that the Purkinje cells were migrating radially through the ranks of the stationary deep nuclear neurons and assembled under the spreading canopy of a fibrous plexus and the external germinal layer. (2) It was also on day E17 that the external germinal layer began to form as one of the prongs of the "germinal trigone" in the posteroventral aspect of the cerebellum. On the succeeding days the external germinal layer spread over the surface of the cerebellum; in the vermis in a rostral direction. (3) Two cell types destined to settle in the future granular layer, the pale cells and the Golgi cells, began to form at a relatively slow rate on day E19. Chronological considerations suggested that they were generated in the regressing, noncollapsing neuroepithelium of the cerebellar ventricle. (4) From the beginning (day E17) of its genesis posteroventrally, the primitive cerebellar cortex bridged the midline. As the fused cortex spread rostrally, the vertical ventricular cleft separating the underlying portions of the cerebellum became shallower and then disappeared; the process was completed in the anterior cerebellum by day E22. By the time of birth the maturation of the neurons of the deep nuclei appeared advanced but the maturation of the prenatally produced neurons of the cortex does not start until after birth when a new class of neurons is generated in the external germinal layer.

The postnatal development of the cerebellum in rodents and other animals became a favorite topic of morphological investigations nearly a century ago (Ramón y Cajal, '60) and has remained the subject of many descriptive and experimental studies to this day (for a review of recent literature in mice, see Rakic, '74; in rats, Altman, '75, '76). However, surprisingly few studies are available regarding the prenatal development of the rodent cerebellum (Miale and Sidman, '61; Korneliussen, '68; Das and Nornes, '72; Pierce, '75) and, to our knowledge, there is no comprehensive study available, in a single species and from one laboratory, of the ontogeny of the entire cerebellar system. The term "cerebellar system" refers to the cerebellum together with those associated structures, or "precerebellar nuclei," that project exclusively or predominantly to the cerebellum.

The first paper of this series deals with the early histogenesis and cytogenesis of the deep nuclei and cortex of the rat cerebellum. The subject of the subsequent paper is the histogenesis and cytogenesis of the precerebellar nuclei of the brain stem: the inferior olive, the lateral reticular nucleus, the nucleus reticularis tegmenti pontis, and the pontine nuclei. In these two studies we utilized an extensive collection of material prepared with three techniques. Developmental changes in the appearance and size of the structures referred to were determined in normal embryos, sectioned in different planes, at daily intervals from embryonic day 13 (E13; when the cerebellar anlage first becomes recognizable) until day E22 (the day before birth). A parallel collection was available from embryos that were irradiated with 200 R X-ray shortly before removal from the uterus. Because this radiation dose selectively kills proliferating, migrating and undifferentiated cells (Hicks, '58; Hicks and D'Amato, '66) we were able to identify sites of cell proliferation and the location of differentiating cells that were spared by the irradiation. In addition, this technique aided us in a better delineation of the anlage of the cerebellum, and the growth pattern of its neuroepithelium, as a result of its distinguishable radiosensitivity. Finally, we examined autoradiograms of adult rats that were injected with multiple doses of ^{3}H-thymidine on overlapping successive days beginning on day E13. Using the procedure of "progressively delayed cumulative labelling" (Bayer and Altman, '74) this material allowed us to estimate with accuracy the time of origin of neurons in the structures examined.

MATERIALS AND METHODS

Normal and experimental embryonic material

Dated, sperm-positive Purdue-Wistar female rats were used. The day of sperm-positivity was counted as day one of gestation. Fetuses from control females were undisturbed prior to their removal while those from the experimental females were exposed to a single dose of 200 R from a Maxitron 300 kV unit 6 hours earlier. Fetuses from one or more control and experimental females were removed on gestation days 13, 14, 15, 16, 17, 18, 19, 20, 21 and 22 and immersed in Bouin's fluid for 24 hours. The brains of fetuses aged 17-22 embryonic days (E17-E22) were dissected before embedding. A total of 261 fetuses was prepared and examined (table 1).

TABLE 1

Number of embryonic cerebella examined

Age	Normal	X-irradiated
E13	8	21
E14	7	20
E15	11	20
E16	10	14
E17	14	16
E18	6	11
E19	10	12
E20	11	16
E21	10	17
E22	16	11
Subtotal	103	158
TOTAL	261	

The majority of these fetuses or brains were embedded in paraffin and sectioned in the sagittal, coronal and horizontal planes at 6 μm. All sections were saved in fetuses aged E13-E14; every fifth section in fetuses aged E15-E16; every 10th section in fetuses aged E17-E22. Alternate sections were stained with cresyl violet and hematoxylin-eosin. A few fetuses were embedded in methacrylate and cut on a Sorvall Porter-Blum JB-4 microtome at 3 μm; these were stained similarly.

The most symmetrically cut and best preserved sagittal, coronal and horizontal sections of the cerebellum were examined microscopically and photographed at selected intervals. The prints were aligned and attached to each other to form long folding strips. This arrangement allowed us to compare the structure of the cerebellum in different planes of

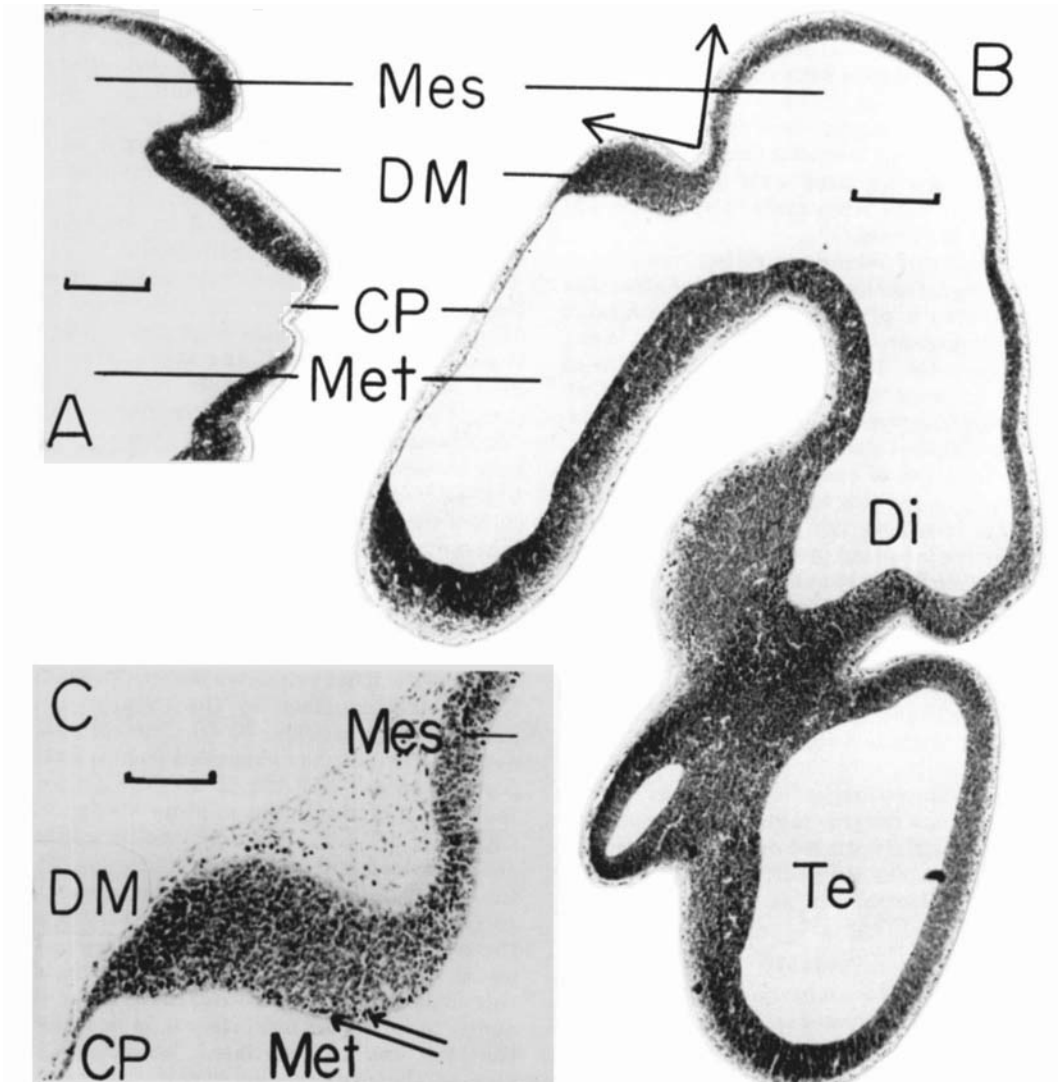


Fig. 1 Normal, 13-day old embryos. A. Horizontal section through the dorsal metencephalic plate (DM). B. Sagittal Section through the brain. C. Higher magnification of the dorsal metencephalic plate in sagittal section; arrows point to mitotic cells near the lumen. Abbreviations: CP, primordium of choroid plexus; Di, diencoele; Mes, mesencoele; Met, metencoele; Te, telencoele. Relationship of the posterior wall of the mesencephalon to the cerebellar plate indicated by angle in B. Scale in A and B, 250 μm; in C, 100 μm.

sectioning within the same ages and across different ages in both normal and experimental embryos.

Embryonically tagged adult material. Purdue-Wistar pregnant females were injected subcutaneously with two successive daily doses of ^{3}H-thymidine (specific activity, 6.0 C/mM; dose, 5 μc/g body weight) on the following gestational ages: E13+14, E14+15 . . . E21+22. Their progeny were killed at the constant postnatal age of 60 days by cardiac perfusion with 10% neutral formalin. The brains were embedded in paraffin, sections were cut at 6 μm serially in the 3 planes and every 15th section was saved. Successive sections were stained with cresyl violet and hematoxylin-eosin for examination without nuclear emulsion or were prepared for autora-

diography. The latter procedure has been described elsewhere (Altman, '69). Briefly, deparaffinized sections were coated with Kodak NTB-3 emulsion in the dark, exposed for 90 days with a dessicant, developed with D-19, and stained with hematoxylin-eosin. In the present study we used only sagittally cut matched sections from male rats, 6 cerebella per injection group.

Cell counting was done either visually at 625 × magnification with the aid of an ocular grid or with a projection system integrated with a Summagraph X-Y digitizer and a Wang 2200 computer. The latter procedure allowed us to determine areal changes in selected regions as a function of age and correlate cell labelling with cell size (see RESULTS for details). The estimation of the proportion of cells differentiating (ceasing to divide) on a particular day was based on the progressively delayed cumulative labelling procedure. The rationale of this procedure is that as long as virtually all the cells of a selected brain region can be labelled (in the populations studied here this can be accomplished with two successive daily injections) all the cells are considered to be precursors that have not started to differentiate. When with delayed onset of injections all cells can no longer be tagged, the proportion of cells that can no longer be labelled as a result of a single day delay is taken to be the complement that differentiated on the previous day. For instance, the cells differentiating on day E17 are determined as follows: E17 = (E17+18) − (E18+19).

RESULTS

I. *Histological examination of normal and irradiated embryos*

(a) *Early events in the cerebellar anlage*

Day E13. The traditionally designated anlage of the cerebellum is represented by paired plates of neuroepithelial cells over the dorsolateral aspect of the metencoele (fig. 1A). These dorsal metencephalic plates are caudally continuous with the primordium of the choroid plexus which, at this stage of development, is a monocellular sheet stretched across the roof of the metencoele (fig. 1B); they are rostrally linked by way of an isthmus with the primordium of the tectum. In normal embryos the cells are primitive in appearance, and many of those situated near the lumen are mitotically active (fig. 1C). However, in irradiated embryos (fig. 2) three zones may be distinguished in the dorsal metencephalic plates: (i) a zone of radioresistant primitive cells that include cells near the lumen that are undergoing mitosis; (ii) a substantial intermediate zone of pyknotic cells; and (iii) a thin superficial zone of radioresistant, apparently differentiating cells. There is an exception to this pattern in the lateral aspect of the plates (fig. 2) where also the cells situated near the ventricle are killed by irradiation and the ventricular wall collapses.

Day E14. The primordium of the choroid plexus invaginates into the metencoele (fig. 3B). In the paired plates of normal embryos two changes are noted: the appearance of a zone of differentiating cells (the classical mantle layer) and of a *superficial fibrous layer* (the classical marginal layer). There is also a hint of a second, or *intermediate fibrous layer* (fig. 3A). In irradiated embryos most of the cells of the differentiating zone are radioresistant, and the collapsing component of the neuroepithelium expands.

Day E15. In the dorsal telencephalic plate cell differentiation is far more advanced anteriorly than posteriorly (fig. 4). In irradiated embryos the more primitive posterior region is further distinguished by the collapse of its neuroepithelium (figs. 5C-D). The collapsing neuroepithelium has expanded from a lateral position on day E13 (fig. 2) to the vicinity of (but not including) the midline by day E15 (figs. 5A, 5C, 5/II). Neuroepithelial collapse characterized not only the posterior aspect of the dorsal telencephalic plates, but also other cortical structures, such as the tectum and the cerebral cortex, and a few other regions to be discussed later (figs. 5A-B). On the basis of this consideration and autoradiographic evidence to be presented later, it is postulated that the less differentiated "collapsing" region of the metencephalic plates represents the cerebellar anlage proper. If this is correct, then the cerebellar anlage would begin to form on day E13 laterally and expand from there gradually towards the midline.

The differentiating component of the cerebellar plate is divided by the intermediate fibrous layer into two zones (fig. 4). We shall call the differentiating region adjacent to the superficial fibrous layer the *nuclear zone,* and the gradually emerging and more slowly differentiating region below the intermediate fibrous layer, the *transitory zone.* Autoradiographic and other lines of evidence will be offered below that the cells aggregated in the former region may be neurons of the deep nu-

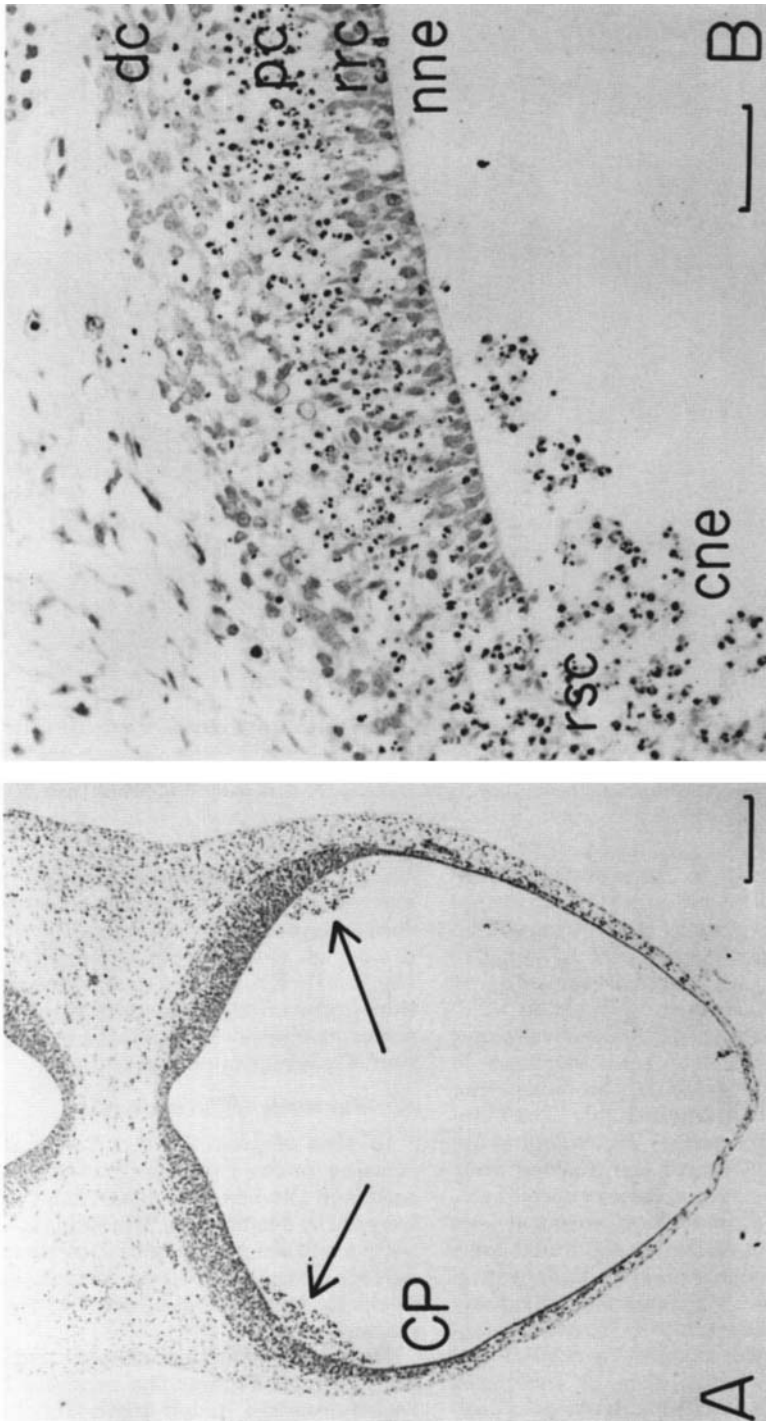


Fig. 2 Horizontal section through the dorsal metencephalic plates in a 13 day-old X-irradiated embryo. A. Collapsing neuroepithelium (arrows) is restricted to the lateral aspect of the plates. B. Part of the collapsing (cne) and noncollapsing (nne) neuroepithelium at higher magnification. Abbreviations: CP, primordium of choroid plexus; dc, zone of differentiating cells; pc, zone of pyknotic cells; rrc, radioresistant cells of neuroepithelium; rsc, radiosensitive cells of neuroepithelium. Methacrylate-embedded material. Scale in A, 250 μm; in B, 50 μm.

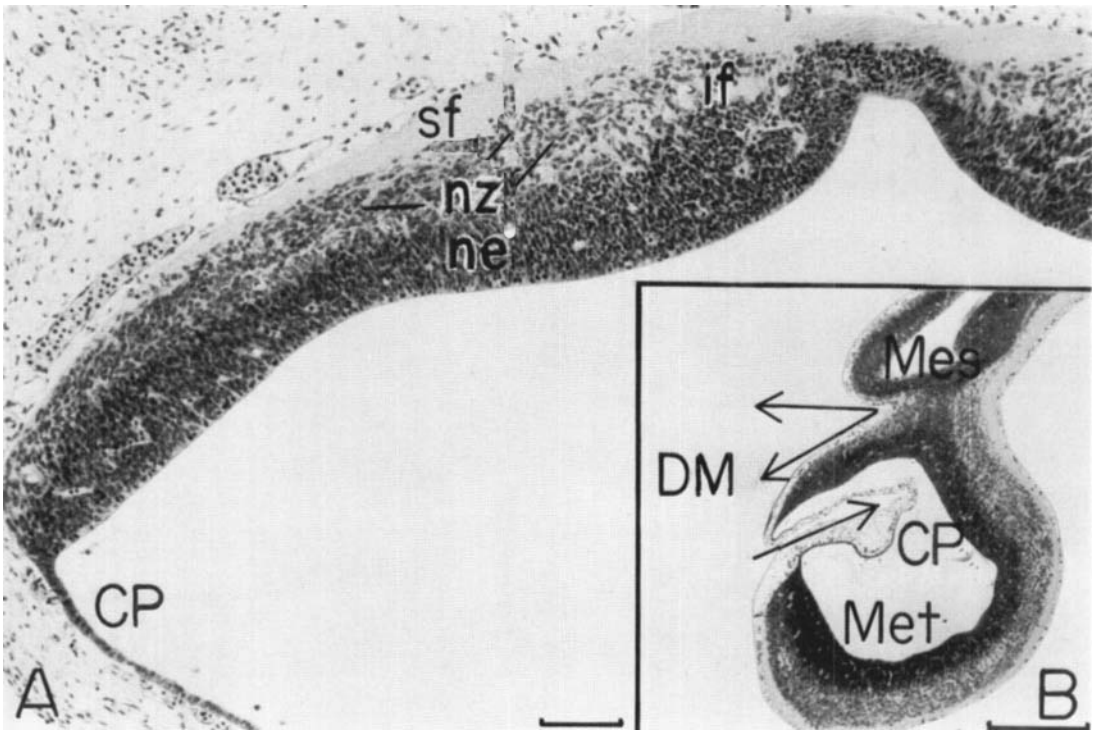


Fig. 3 Dorsal metencephalic plates in normal 14 day-old embryos. A. Horizontal section; B, sagittal section, Compare angle (arrows) of the metencephalic plate to the mesencephalon in this figure with that in figure 1B. Abbreviations: CP, primordium of choroid plexus; DM, dorsal metencephalic plate; if, traces of intermediate fibrous layer; Mes, mesencoele; Met, metencoele; ne, neuroepithelium; nz, differentiating cells of nuclear zone; sf, superficial fibrous layer. Scale in A, 100 μm; in B, 500 μm.

clei, whereas the cells in the underlying transitory zone would be premigratory Purkinje cells. Examination of serial sections suggested that the nuclear zone is divisible by day E15 into a lateral crescent-shaped portion and into a less clearly defined medial cell group.

Day E16. In normal embryos most changes are gradual. The nuclear zone increases in size, and its two components, the lateral and medial, are clearly distinguishable. The transitory zone has particularly grown and is apparently pushing outward the nuclear zone. The only new phenomenon seen in normal embryos is the appearance of an inconspicuous neuroepithelial cap in the ventrocaudal margin of the metencephalic plates. (Beginning on day E17 this region will generate the rapidly expanding external germinal layer.) In contrast to the gradual changes in normal embryos, a sudden change seen in irradiated embryos is the cessation of neuroepithelial collapse (fig. 6). This we refer to as *juxtaventricular transformation.* However, as an important and consistent exception, the neuroepithelium does collapse posteromedially (fig. 6B); this is the region that did not collapse on day E15 (fig. 5/II). Evidence will be presented later that juxtaventricular transformation (except posteromedially) is correlated with the cessation of production of Purkinje cells.

(b) *Migration of Purkinje cells*

In this section we describe observations relating to the radial migration of Purkinje cells and the onset of differentiation of neurons of the deep nuclei. The formation and dispersion of the external germinal layer over the surface of the cerebellum, which takes place at the same time, will be described in the subsequent section.

Major morphological changes begin on day E17. In most regions the neuroepithelium is rapidly reduced in cell depth (fig. 7A) and at the same time the segregation of the nuclear

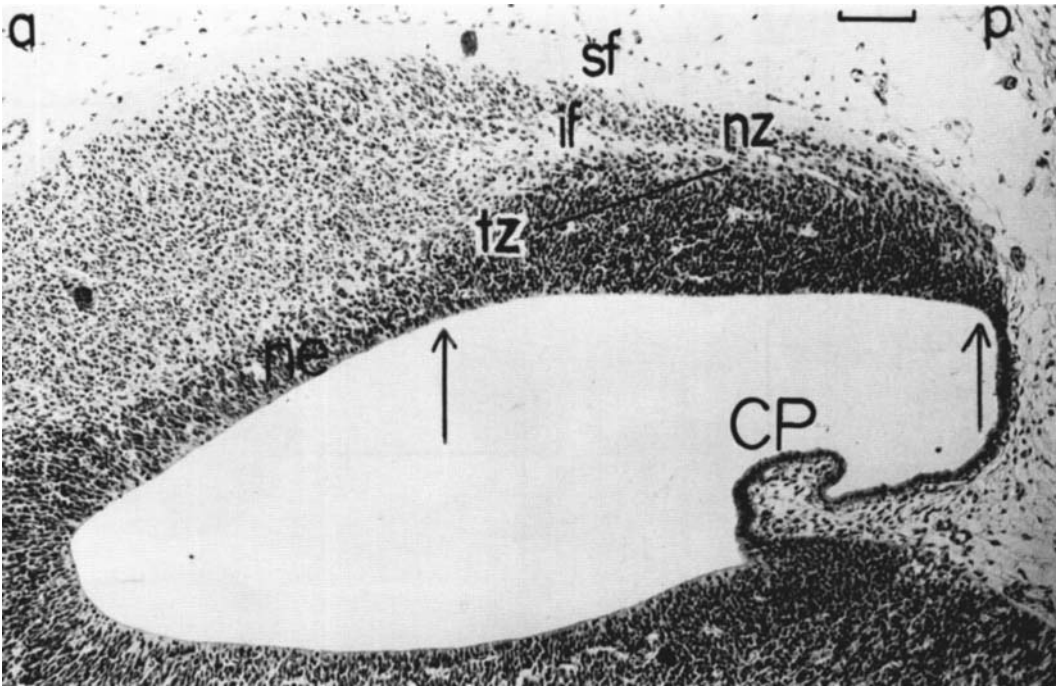


Fig. 4 Normal, 15 day-old embryo. Dorsal metencephalic plate in sagittal section; lateral aspect. Abbreviations: a, anterior; CP, primordium of choroid plexus; if, intermediate fibrous layer; ne, neuroepithelium; nz, nuclear zone; p, posterior; sf, superficial fibrous layer; tz, transitory zone. Arrows outline the boundaries of the presumed cerebellar anlage, the region where the neuroepithelium collapses after X-irradiation (see fig. 5D). Scale, 100 μm.

zone and transitory zone becomes posteriorly indistinct. For several days the corpus of the cerebellum appears as an amorphous mass of cells. We suggest that the Purkinje cells have begun to migrate *en masse* from the transitory zone, and are temporarily intermingled with the stationary cells of the nuclear zone. This process takes several days (fig. 10) and by day E20 (fig. 7D) the spatial relation between deep nuclear neurons and Purkinje cells heralds the adult pattern insofar as the latter are not distributed superficially.

During the same period the intermediate fibrous layer of the cerebellar anlage becomes unrecognizable. What actually happens to the disposition of fiber tracts could not be determined in the available material. Relationships are complicated by the appearance of two large, somewhat intermingled tracts over the dorsolateral and anterolateral aspects of the cerebellum, and by the fiber bundles that distribute themselves in the sagittal and transverse planes. In irradiated embryos there are few pyknotic cells in the core of the cerebellum (the stationary deep nuclear neurons and migrating Purkinje cells are apparently radioresistant) but they are numerous in the external germinal layer (fig. 8).

(c) *Formation of the external germinal layer*

While in most regions the cerebellar neuroepithelium is regressing by day E17, it is actually expanding in the wedge-shaped posteroventral margin and the lateral protuberance of the cerebellum. In this continuous region the neuroepithelium has three prongs: an inferior, a horizontal and a superior (fig. 7C). We shall call this region the *germinal trigone* (fig. 7C). The inferior prong produces the cells of the choroid plexus. At this period the choroid plexus grows considerably and virtually separates the metencoele into two communicating compartments, the fourth ventricle and the transient cerebellar ventricle (fig. 7). The horizontal prong is the regressive ventral portion of the cerebellar neuroepithelium. The superior prong gives rise to the external germinal layer that is beginning to emerge on day E17. Thereafter, the external germinal layer rapidly expands over the surface of the

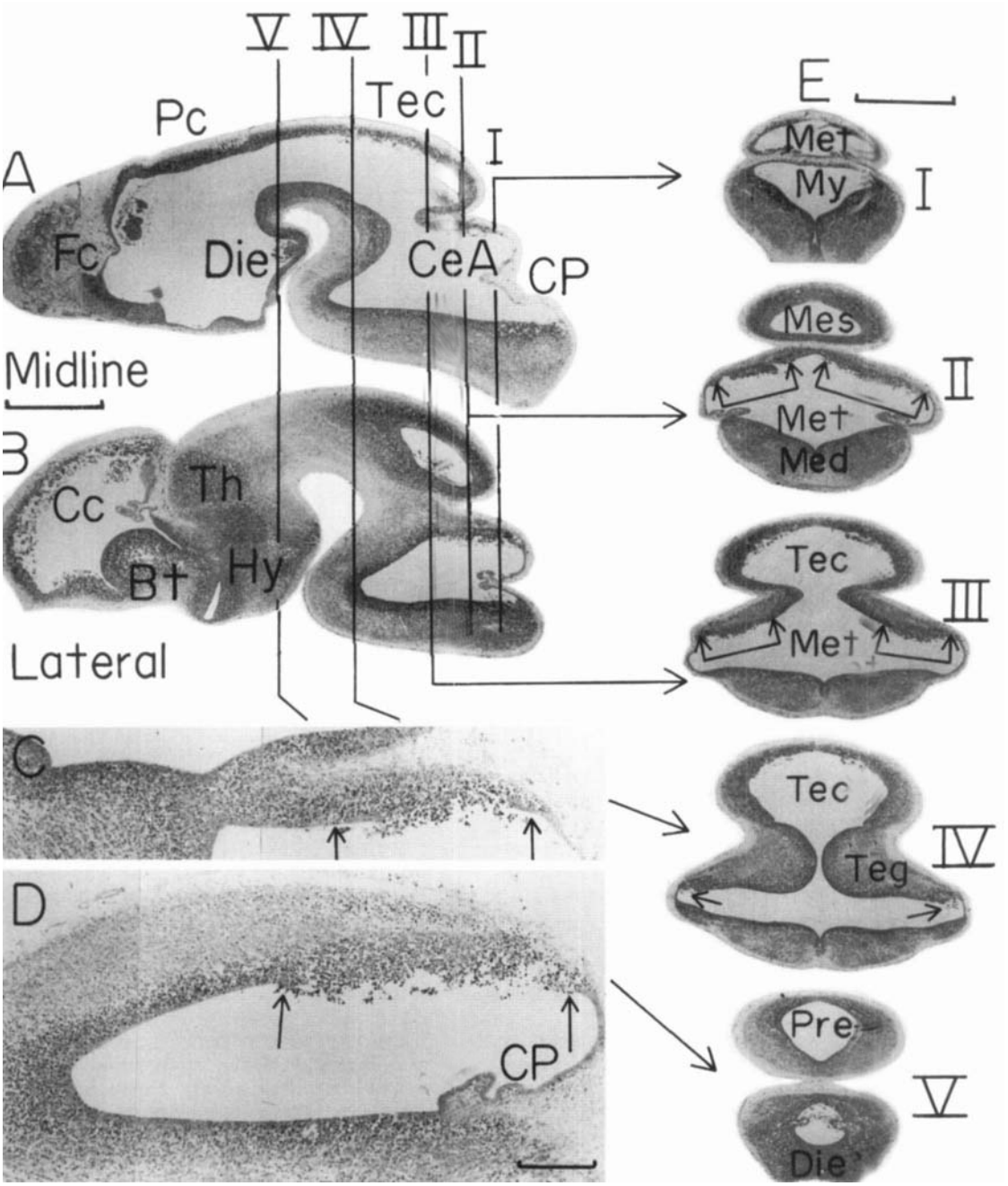


Fig. 5 X-irradiated, 15 day-old embryos. A, midsagittal section through the brain; B, parasagittal section from the same animal. C and D, midsagittal and parasagittal sections, respectively, through the cerebellar anlage to show expansion of the collapsing neuroepithelium (arrows) to the vicinity of the midline. EI-V, coronal sections from another X-irradiated animal corresponding to the levels indicated by I-V in A and B. In addition to the cerebellar anlage (CeA), collapsing neuroepithelia are seen in the cerebral cortex (Cc), the anterior aspect of basal telencephalon (Bt), a restricted region of the diencephalon (Die), and in the tectum (Tec). Abbreviations: CP, primordium of choroid plexus; Fc, falx cerebri; Hy, hypothalamus; Med, medulla; Mes, mesencoele; Met, metencoele; My, meyelencoele; Pc, posterior commissure; Pre, pretectum; Teg, tegmentum; Th, thalamus. Scales in A, B, and E, 1 mm; in C and D, 200 μm.

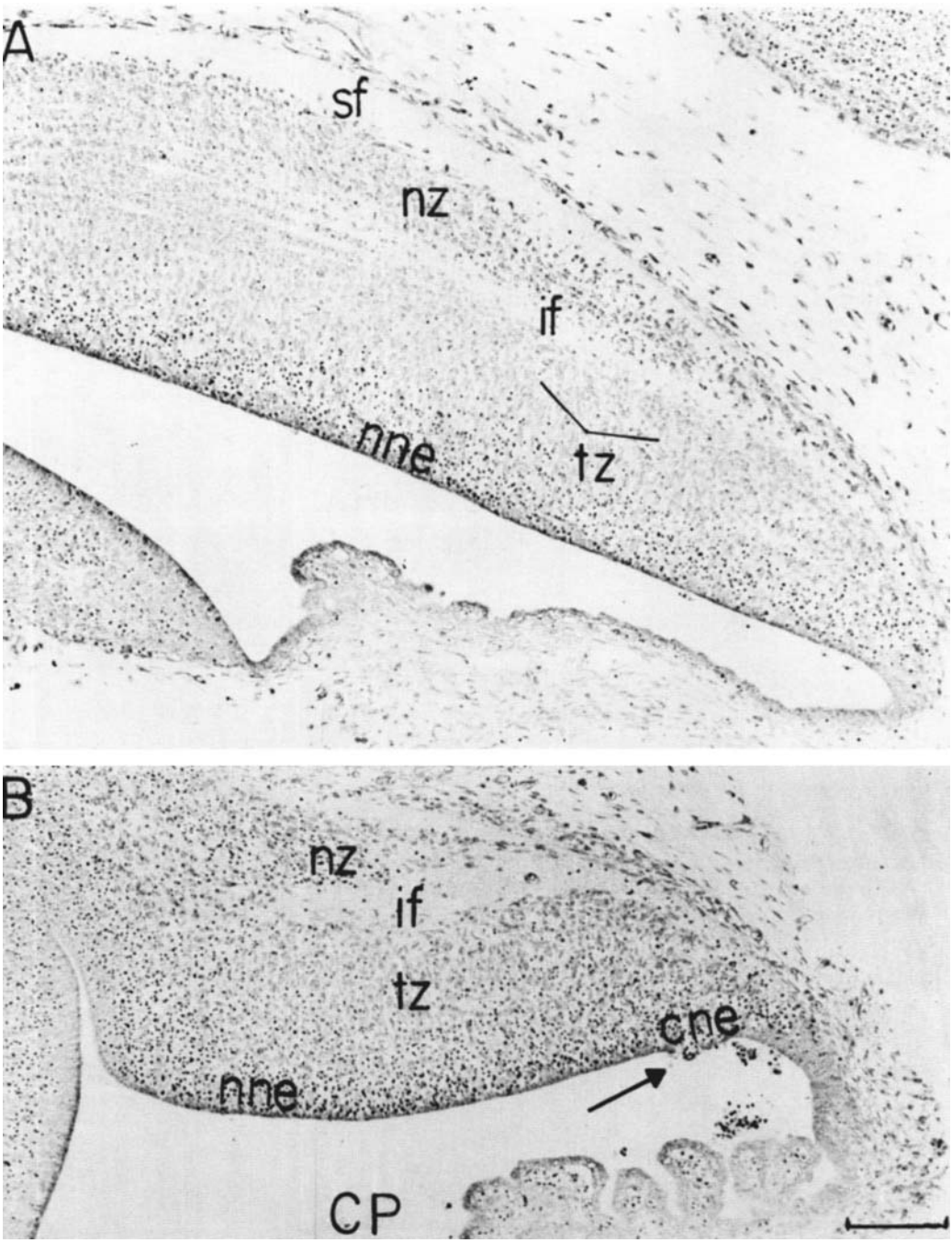


Fig. 6 X-irradiated, 16 day-old embryo. A, cerebellar anlage in parasagittal section; B, in midsagittal section. The transitory zone (tz) has increased considerably in bulk (compare with fig. 4). Pyknotic cells are abundant in the noncollapsing neuroepithelium (nne), scarcer in the transitory zone and the nuclear zone (nz). The collapsing neuroepithelium (cne) is restricted at this age to the posteroventral aspect of the cerebellar anlage near the midline (B). Abbreviations: CP, choroid plexus; if, intermediate fibrous layer; sf, superficial fibrous layer. Scale, 100 μm.

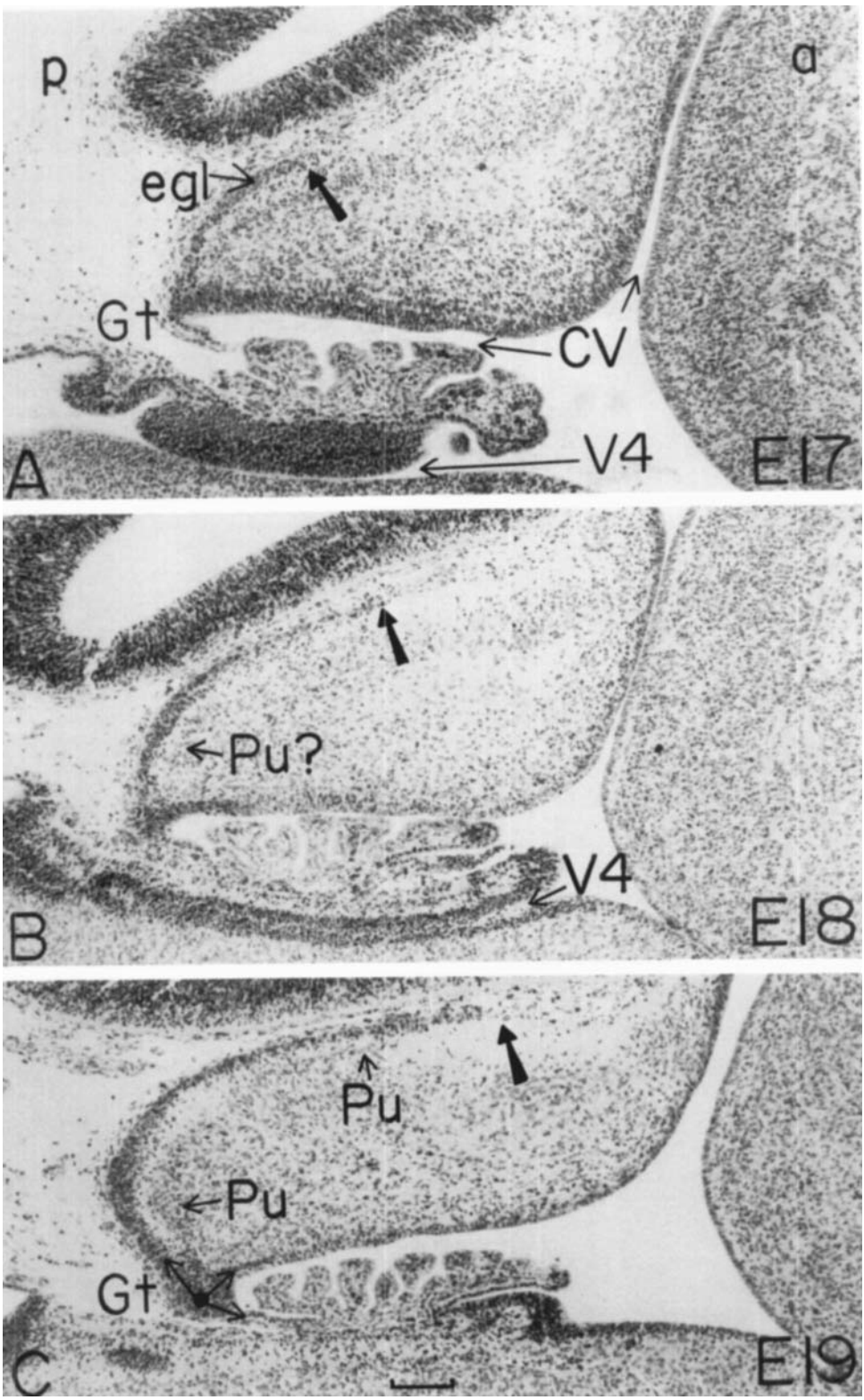
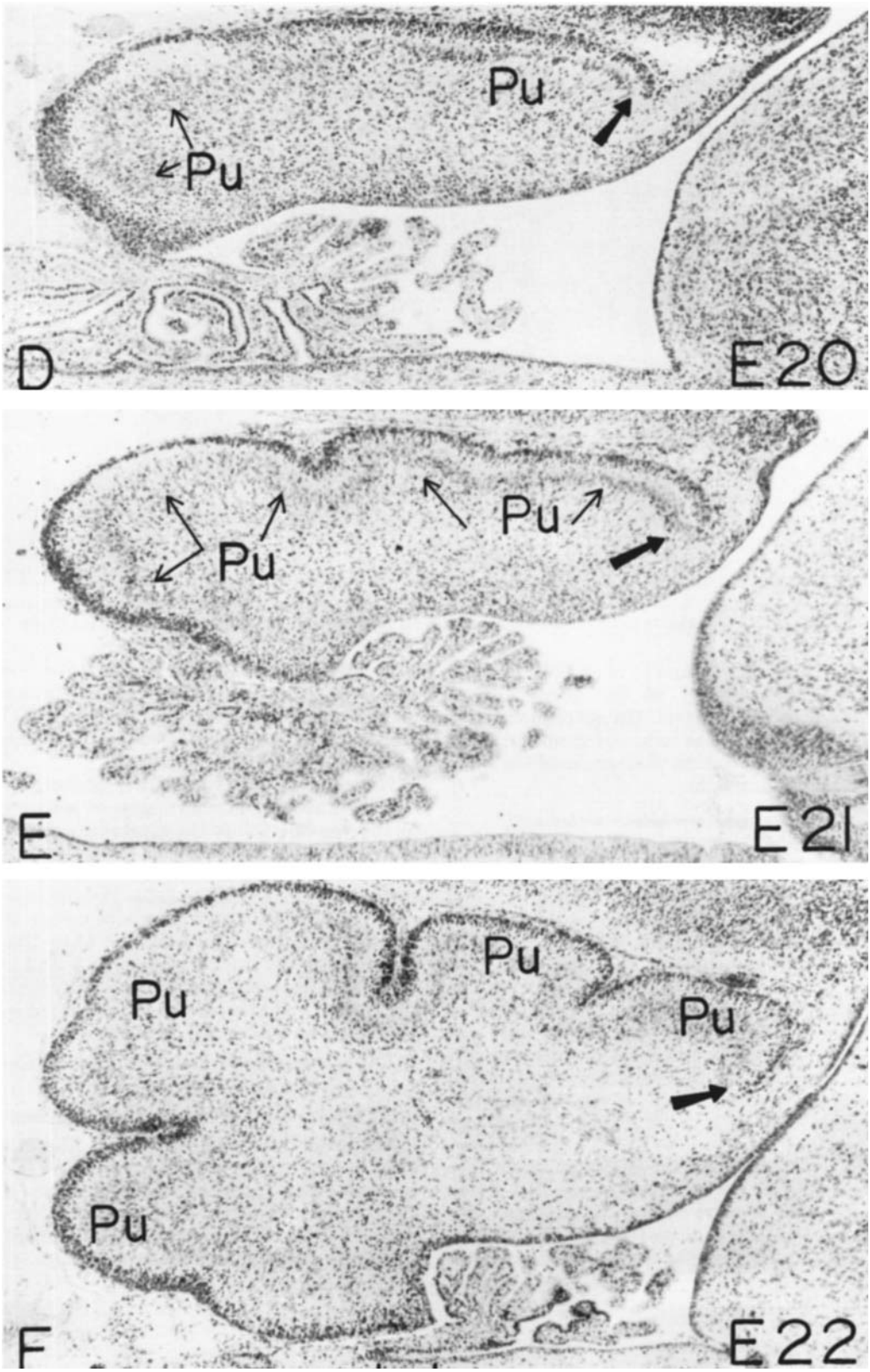


Fig. 7 Low-power photomicrographs of midsagittal sections of the developing cerebellum of normal rat fetuses aged 17, 18, 19, 20, 21 and 22 days. Note progressive daily expansion of external germinal layer (egl) from the posterior germinal trigone (Gt) in the anterior direction (large arrows). A gradual accumulation of Purkinje cells (Pu) beneath the expanding



external germinal layer may also be seen. Abbreviations: a, anterior; CV, cerebellar ventricle; p, posterior; V4, fourth ventricle. Scale, 100 μm.

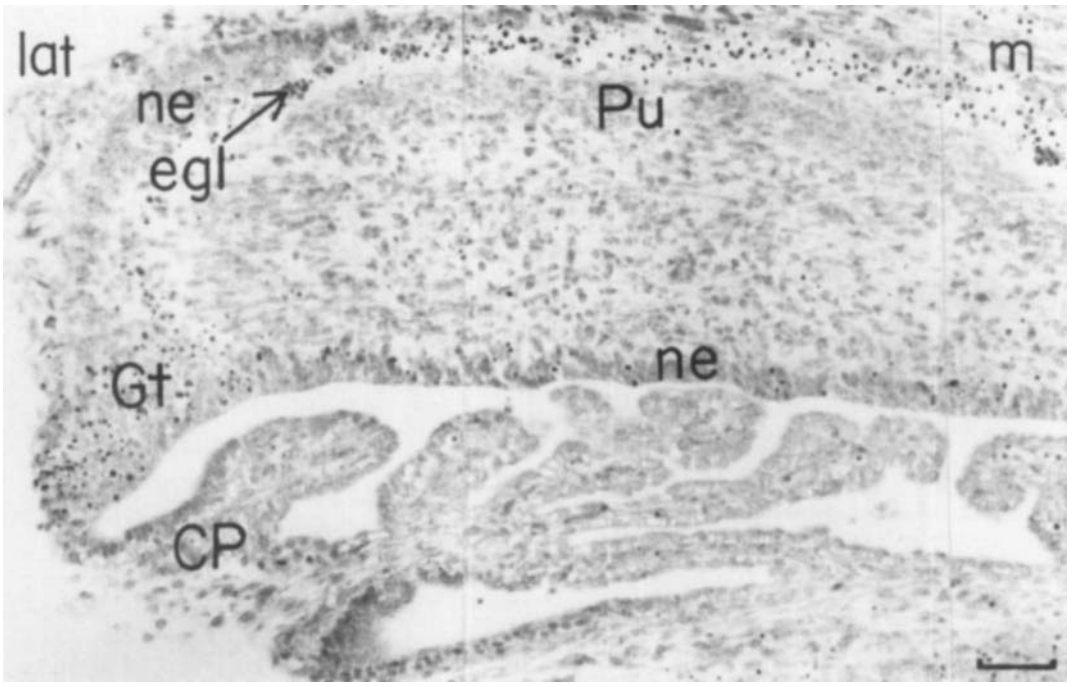


Fig. 8 X-irradiated, 18 day-old cerebellum; posterior coronal section. Abbreviations: CP, choroid plexus; egl, external germinal layer; Gt, germinal trigone; lat, lateral; m, medial; ne, neuroepithelium; Pu, Purkinje cells. Scale, 50 μm.

cerebellum (fig. 7) and by day E20 (fig. 7D) the anterior aspect of the cerebellum is covered by this matrix. On subsequent days the expansion of the external germinal layer continues penetrating the fissures of the gradually emerging folia.

(d) *The primitive cerebellar cortex and cerebellar fusion*

Reference was made earlier to the spreading of the "collapsing neuroepithelium" on day E16 to the midline in the posteroventral margin of the cerebellum. When the external germinal layer arises in the same region on day E17 it stretches continuously across the midline (fig. 11A). Anterior to this germinal region a fibrous band likewise bridges the midline (fig. 11B); this is the primitive molecular layer. On the following days the external germinal layer spreads over the primitive molecular layer and the Purkinje cells gather beneath it (fig. 11C). We do not know the composition of the primitive molecular layer. It is not likely to be composed of parallel fibers since it is present before the external germinal layer and because the cells of the latter remain undifferentiated, or radiosensitive (fig. 10B), for several days. Similarly, the fiber composition of the white matter, which separates the primitive cerebellar cortex from the maturing deep nuclei (fig. 9B, 10C), remains to be determined.

The bulk of the cerebellum beneath the fused primitive molecular layer is separated by the vertical cleft of the cerebellar ventricle (fig. 11B). As the external germinal layer spreads over the surface of the cerebellum rostrally and the Purkinje cells gather underneath to form the fused primitive cortex, the vertical cleft becomes shallower, then disappears (fig. 11C). In this manner fusion of the corpus of the cerebellum follows the fusion of the cortex, and the process is completed rostrally by day E22 or the day of birth.

II. *Autoradiographic examination of the time of origin of cerebellar neurons*

(a) *Time of origin of deep nuclear neurons*

The autoradiograms were from the cerebella of adult male rats whose mothers were injected with two successive daily doses of ^{3}H-thymidine on days E13+14, E14+15, E15+16, E16+17 and E17+18. Six sagittally sectioned cerebella were quantified in each

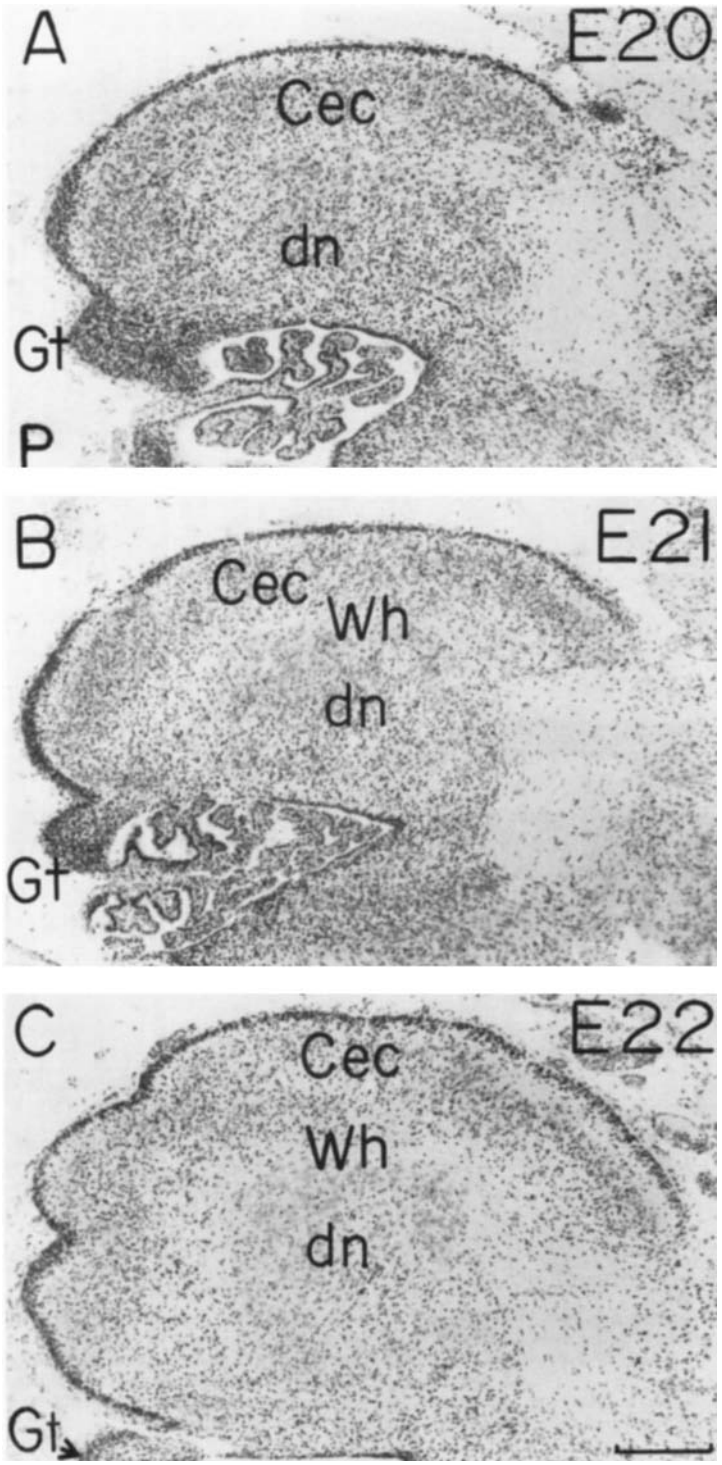


Fig. 9 Low-power photomicrographs of parasagittal sections of the developing cerebellum in normal rat fetuses aged 20 (A), 21 (B) and 22 (C) days (compare with figs. 7D-F). By day E21 the white matter (Wh) clearly separates the primitive cerebellar cortex (CeC) from the underlying deep nucleus (dn). Abbreviations: Gt, germinal trigone; p, posterior. Scale, 200 μm.

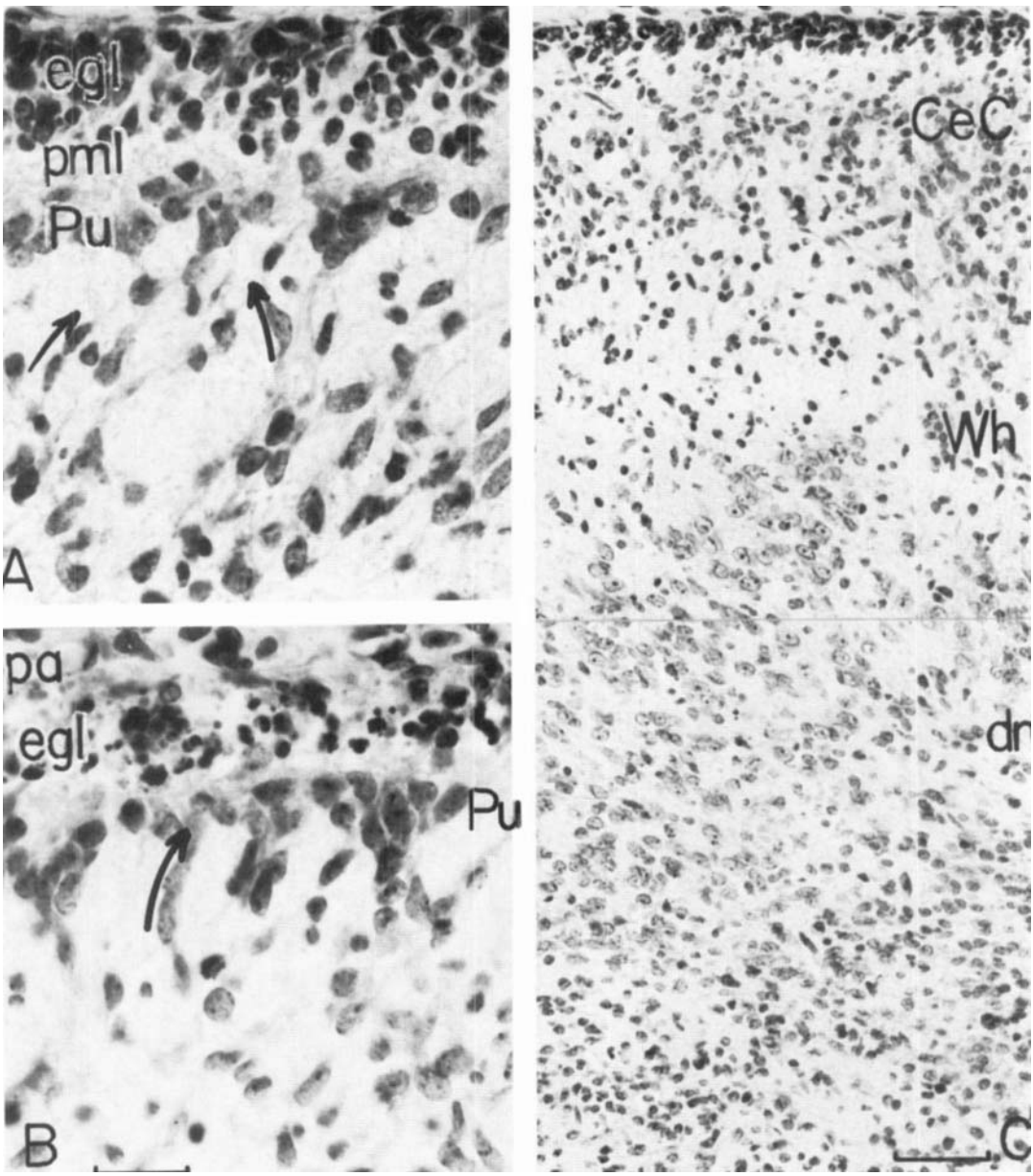


Fig. 10 The cerebellar cortex in 20 day old normal (A) and X-irradiated (B) fetuses. Cells throughout the external germinal layer (egl) are pyknotic in the irradiated fetus. This suggests that differentiating bipolar cells, and therefore parallel fibers, should not be present in the primitive molecular layer (pml). Arrows point to some of the presumed migrating Purkinje cells. C. The cerebellar cortex (CeC) in a normal 21 day old fetus with white matter (Wh) and deep nucleus (dn). While the Purkinje cells are primitive in appearance, the deep nuclear neurons are maturing. Abbreviations: pa, pia-arachnoid membrane; Pu, Purkinje cells. Scales, in A and B, 20 μm; in C, 50 μm.

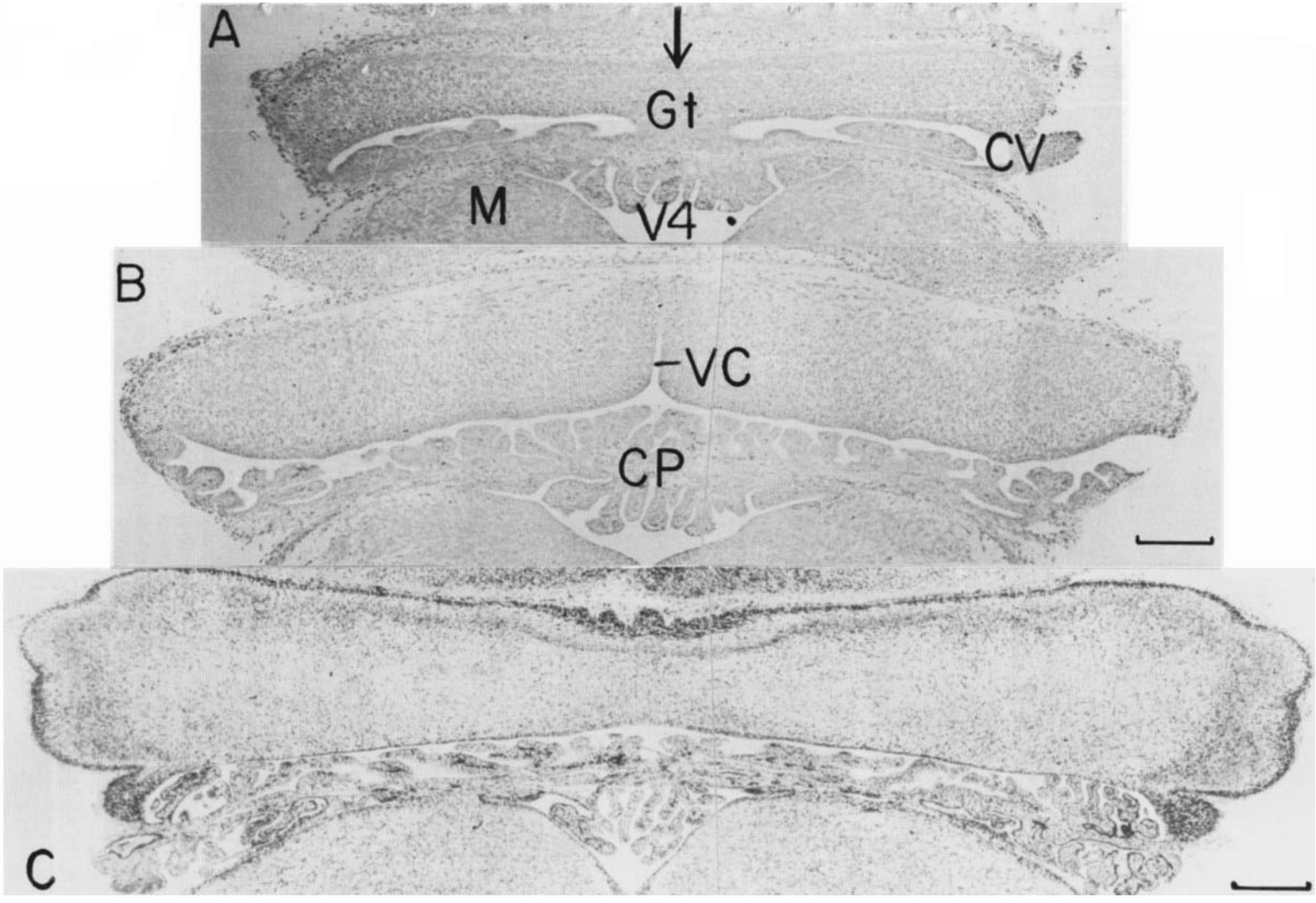


Fig. 11 A. Coronal section through the fused posteroventral margin of the cerebellum (arrow) and the germinal trigone (Gt) in a normal 17 day old embryo. B. A more anterior section in the same animal as A, showing the fused superficial fibrous layer, and the vertical ventricular cleft (VC) that separates the two halves of the underlying cerebellum. C. The same coronal level as in B in a 21 day old embryo. Note that the ventricular cleft is no longer visible beneath the primitive cerebellar cortex. Abbreviations: CP, choroid plexus; CV, cerebellar ventricle; M, medulla; V4, fourth ventricle. Scales, in A and B, 200 μm; in C, 300 μm.

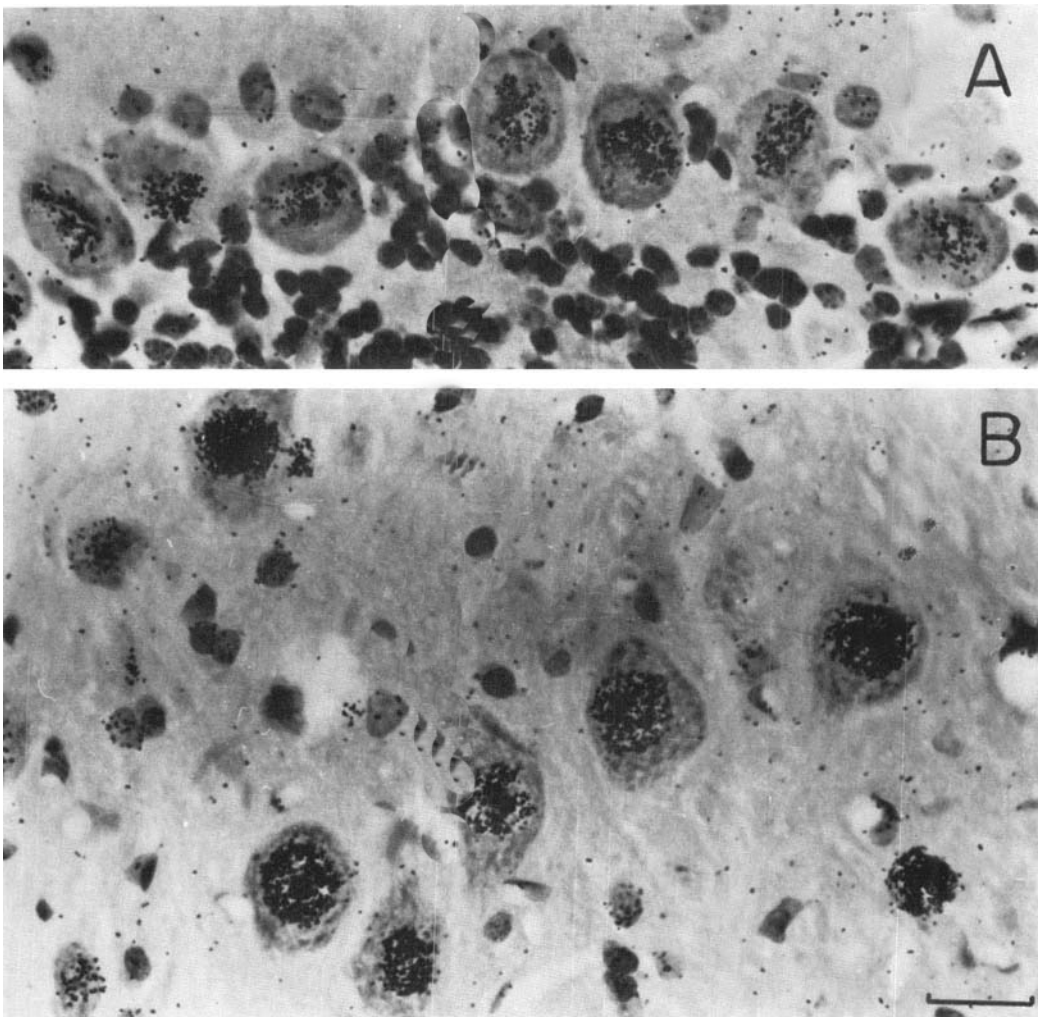


Fig. 12 From a single autoradiogram of a 60 day-old rat injected on days E13+14. In this animal virtually all Purkinje cells (A) and deep neurons (B) were labelled. Hematoxylin-eosin. Scale, 20 μm.

injection group. In all animals 100 neurons (over 15 μm) with nucleoli were classified as labelled or unlabelled in each of the three deep cerebellar nuclei. The medial (fastigial) nucleus was sampled in serial sections in which it became first recognizable passing from the midline laterally; the intermediate (interpositus) nucleus at a level where the facial and trigeminal motor nuclei were transected; and the lateral (dentate) nucleus in sections where it became identifiable, moving from the lateral aspect of the cerebellum medially. Usually 2-3 sections were scanned to obtain 100 neurons in each deep nucleus.

Maximal proportion of labelled deep nuclear neurons were obtained in the E13+14 group (fig. 12), about 95% (range 93-96) at each level. (Since all deep neurons were labelled in rats whose mothers received four injections on days E13+14, it was concluded that with two injections 95% was our optimal labelling efficiency in this region. Accordingly, we applied a correction factor of × 1.05 to all values.) The proportion of tagged deep nuclear neurons fell moderately in the E14+15 group, precipitously in the E15+16 group (fig. 13), and none could be labelled in the E16+17 or E17+18 groups. The proportion of deep neurons formed

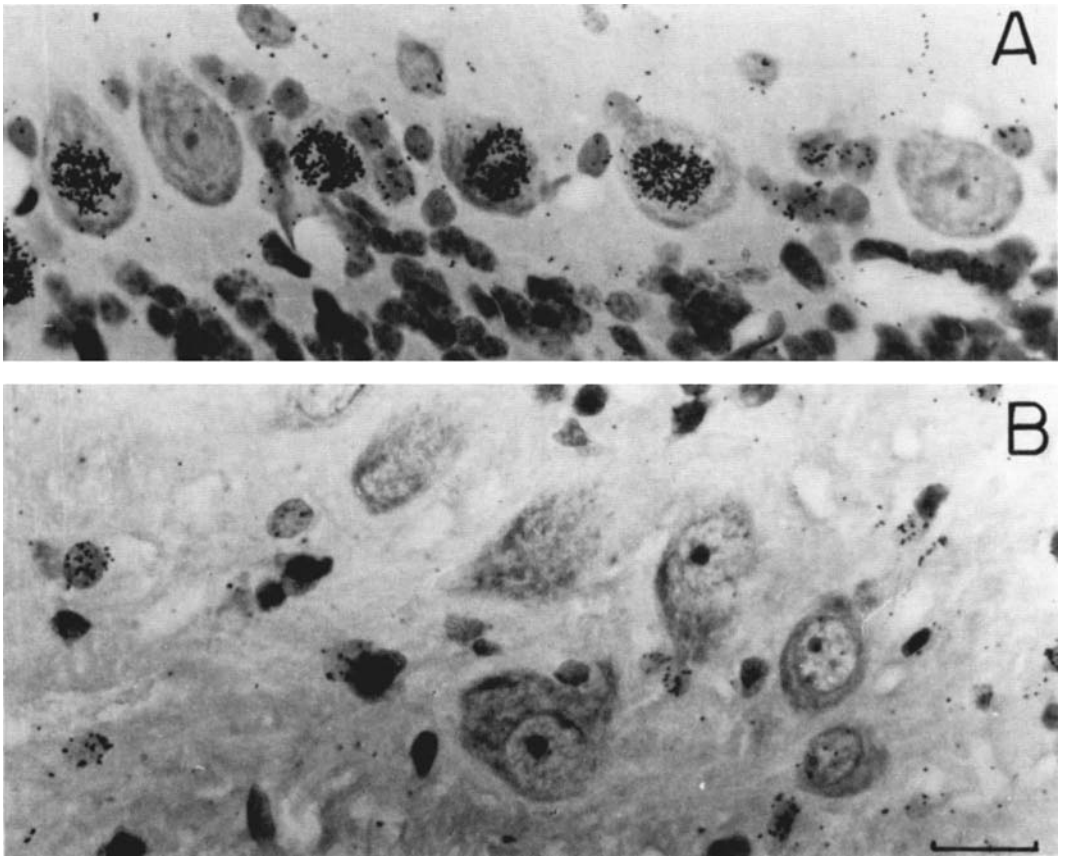


Fig. 13 From a single autoradiogram of a 60 day-old rat injected on days E15+16. In this animal a high proportion of Purkinje cells (A) but few deep neurons (in the illustrated region, B, none) were labelled. Hematoxylin-eosin. Scale, 20 μm.

on days 13, 14 and 15 were determined according to the formula given in the METHODS section. The results (fig. 14) indicate a moderate lateromedial gradient: on terminal day E15 only 6% of deep neurons were labelled in the lateral nucleus, while 26% of them were still labelled in the medial nucleus.

The following procedure was used to investigate possible differences in the time of origin of deep nuclear neurons of different sizes. In two animals from each of the relevant injection groups (E13+14, E14+15, E15+16) cerebellar autoradiograms were selected at approximately 360 μm intervals from the midline laterally (providing about 7-8 sagittal levels through the deep nuclei). The deep nuclei were photographed at × 476 magnification and montages were prepared. The boundaries of nucleated perikarya were traced with the cursor of a Summagraph digitizer, each cell was classified as labelled or unlabelled, and the information was fed into a Wang 2200 computer. To exclude glia cells, those cells with an area smaller than 200 sq. μm were rejected (which must have included a class of the smallest neurons). The results of over 250 cells so classified indicated considerable differences in the proportion of labelled deep neurons as a function of age at injection but no differences were detected in any of the groups at any level with respect to cell size. Figure 15 summarizes the results after the classified cells were dichotomized as "small" or "large" (smaller or larger than 650 sq. μm).

(b) *Time of origin of Purkinje cells*

Virtually all Purkinje cells, like deep neurons, were labelled in the E13+14 group (fig. 12). But the calculations indicated (fig. 14) that, except laterally, few Purkinje cells were

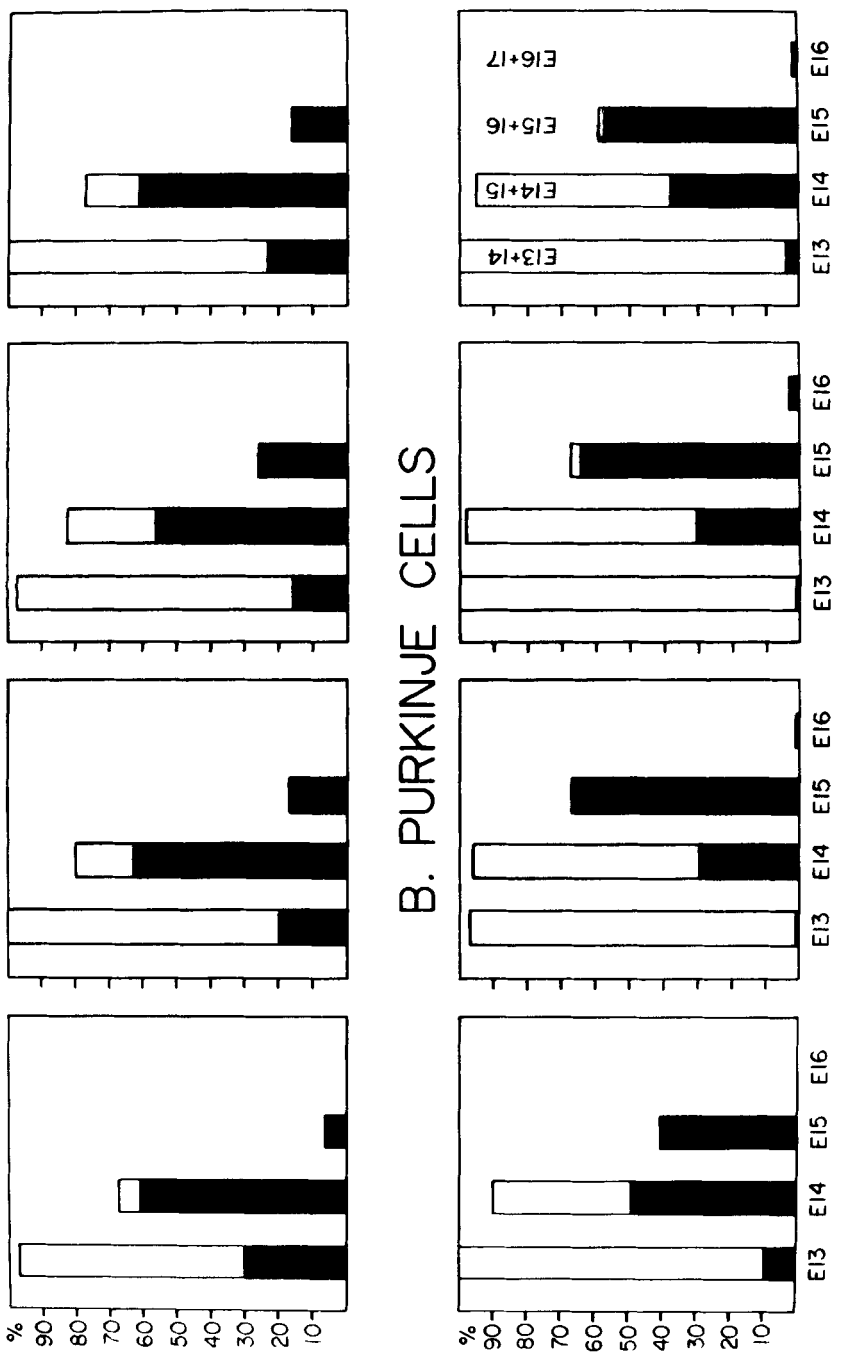


Fig. 14 Proportion of labelled deep neurons (A) in the dentate (I), interpositus (II) and fastigial (III) nuclei, and their summed average (IV). B. Proportion of labelled Purkinje cells at the same sagittal levels and the summed average. Empty bars show the actual proportion of labelled cells in the E13+14, E14+15, and E15+16 groups (6 cerebella per group), respectively. Solid bars indicate the estimated proportion of cells differentiating on the day specified on the abcissa.

differentiating on day E13 as virtually all of them were labelled in the E14+15 group. In contrast to the peak formation of deep neurons on day E14, Purkinje cells formed in the highest numbers on day E15 (the difference is illustrated in figs. 12-13). The Purkinje cells that formed on day E16 were preferentially localized in the nodulus and ventral uvula. However, beyond the lateral level indicated in figure 14, labelled Purkinje cells were also seen in the E16+17 (but not E17+18) group in the flocculus. This suggests that the flocculonodular lobe may be receiving some of the latest forming Purkinje cells. The lateromedial gradient was slight (fig. 14) and no other systematic lobular differences could be recognized.

(c) *Time of origin of pale cells*

Elsewhere (Altman and Bayer, '77) we described a new cell type, called pale cells, with preferential distribution in the granular layer of the nodulus, ventral uvula, lingula, flocculus, and paraflocculus. These cells, which are larger than granule cells and smaller than Golgi cells, start to form on day E19 and are produced through the perinatal period. Because over 60% of the pale cells arise on days E19 and 20, before the external germinal layer is completely dispersed over the cerebellar surface, it was suggested that they originate in the noncollapsing neuroepithelium of the cerebellum.

(d) *Time of origin of Golgi cells*

To guard against the inclusion of pale cells, Golgi cells were scanned in matched sections of the cerebellar hemispheres (where pale cells are rare). Only cells embedded in the granular layer, larger than 10 μm, and having a recognizable nucleus were classified as labelled or unlabelled (fig. 16). In each cerebellum 100 such cells were classified. A maximal labelling efficiency of 88-89% was obtained in the E18+19 and E19+20 groups, respectively. Accordingly, a correction factor of × 1.12 was applied to the data. The results indicated (fig. 17) that Golgi cell differentiation begins on day E19 and continues at a slow pace until after birth. The Golgi cells that formed as late as P2 were often located superficially near the layer of Purkinje cells.

DISCUSSION

Delineation of the cerebellar anlage

Following irradiation of 13-day-old embryos

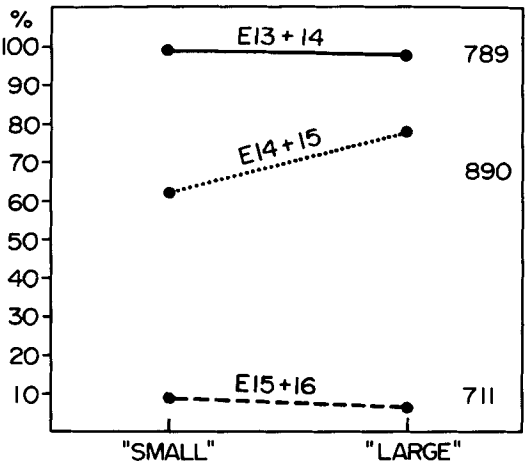


Fig. 15 The proportion of labelled "small" (200-649 sq. μm) and "large" (650-1100 sq. μm) deep neurons in three injection groups. The total number of cells so classified in each group is indicated on the right.

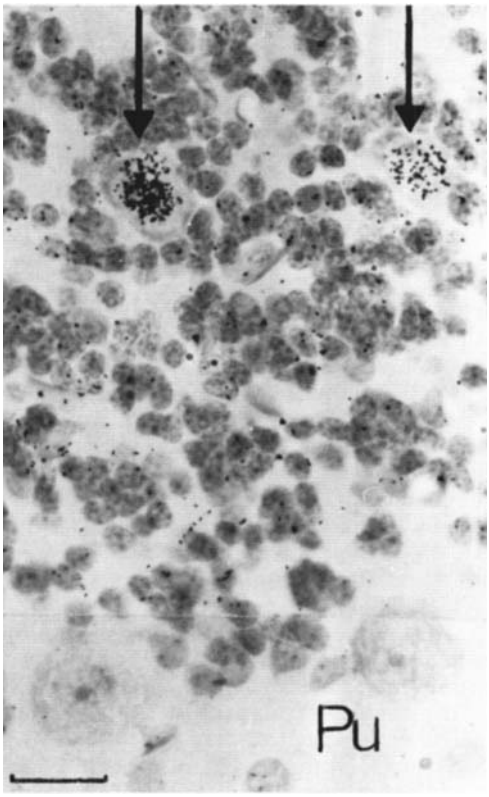


Fig. 16 A lightly and intensely labelled Golgi cell (arrows) in an animal injected on days E18+19. The earlier-forming Purkinje cells (Pu) and the post-natally forming granule cells are not labelled. Hematoxylin-eosin. Scale, 20 μm.

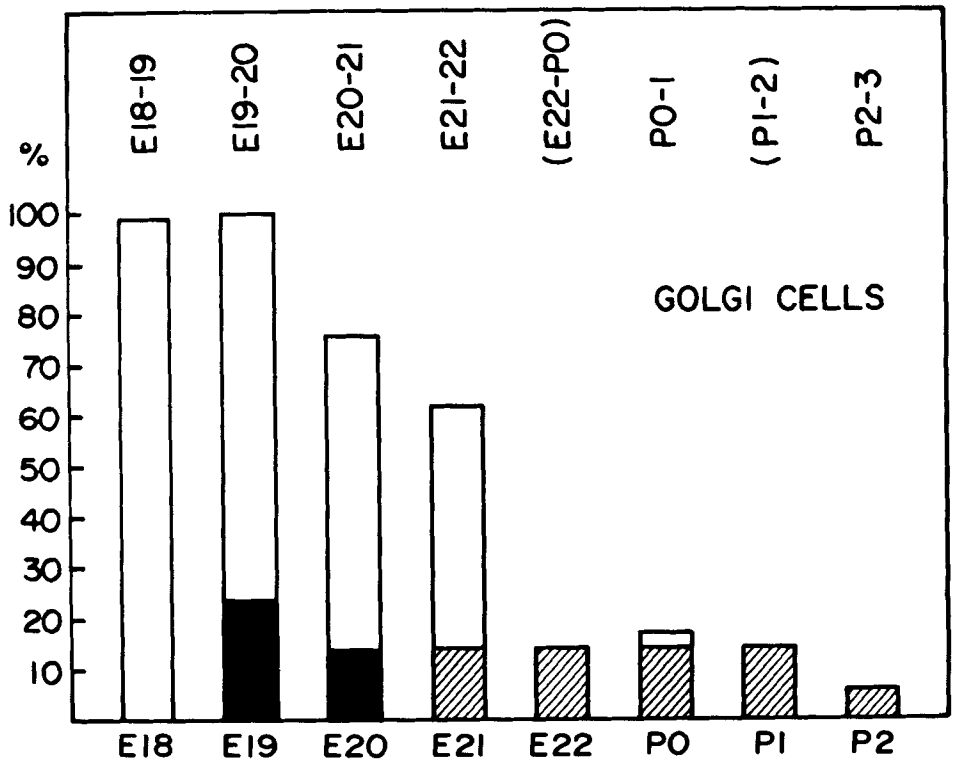


Fig. 17 Proportion of labelled Golgi cells in successive injection groups (designated at the top). Empty bars show the actual proportion of labelled cells; solid bars indicate the estimated proportion of cells differentiating on days E19 and E20. Because certain injection groups were missing (E22+P0 and P1+2) the values beyond day E21 (hatched) are extrapolated.

with a single dose of 200 R X-ray many of the neuroepithelial cells in the intermediate zone of the dorsal metencephalic plates were killed while cells (many of them mitotic) in the zone bordering the lumen were spared. An exception to this was a small lateral region, adjacent to the attachment of the primordium of the tela choroidea. In this circumscribed area the cells lining the ventricle were also killed, with the result that the ventricular wall collapsed and the pyknotic cells were shed into the ventricle. This area of collapse became somewhat extended medially by embryonic day 14, and by day 15 it reached the vicinity of the midline, such that virtually the entire neuroepithelium collapsed after X-irradiation. By day 16 the thinning neuroepithelium no longer collapsed except in a narrow zone across the midline, that is, at the opposite end where this phenomenon started on embryonic day 13. By day 17 the cerebellar neuroepithelium was regressing in all regions except the ventral and lateral aspect of the caudal margin of the cerebellum where the so called germinal trigone gave rise to the subpial external germinal layer.

The phenomenon of ventricular collapse after X-irradiation was first described by Hicks and his collaborators (Hicks et al., '59; Hicks and D'Amato, '66). We saw in X-irradiated, day E15 embryos (fig. 5A-B) that in some regions of the neuraxis the neuroepithelium collapsed, while in others, in spite of an abundance of pyknotic cells, the neuroepithelial wall remained intact. The phenomenon is a consistent one, and is both site and age dependent; for instance, neuroepithelial collapse in the cerebral cortex prevails until day E18 (Altman and Bayer, in preparation). Figure 5 shows that in addition to the cerebral cortex, collapse characterizes a limited region of the basal telencephalon, the anlage of the superior colliculus, and a small, circumscribed region of the diencephalon. There is no ventricular collapse in day E15 embryos in most regions of the basal telencephalon, dien-

cephalon, mesencephalon and metencephalon. The first hypothesis that we entertained, and it requires certain qualifications (Altman, Bayer and Peters, in preparation), has been that neuroepithelial collapse is associated with regions that generate structures with a laminated or cortical organization (Altman and Bayer, in preparation). The corollary of this general hypothesis is that the collapsing region in the dorsal metencephalic plate outlines the anlage of the cerebellum.

This hypothesis is reconcilable with the autoradiographic results. The presence on day E13 of a small, laterally situated collapsing neuroepithelium coincides with the onset of production of deep nuclear neurons. The bulk of these neurons are formed by the end of day E14 (about 85% of the total; fig. 14A) before the collapsing neuroepithelium has spread far medially. Since there is only a slight hint of a lateromedial gradient in the production of deep nuclear neurons, it is assumed that most of them originate in the lateral aspect of the cerebellar anlage. The larger population of Purkinje cells begins to differentiate in appreciable numbers on day E14 but many more are formed on day E15 (fig. 14B), when the collapsing neuroepithelium has become greatly enlarged and approximates the midline. Finally, the persistence of the collapsing neuroepithelium in the posteroventral aspect of the cerebellar anlage on day E16 was correlated with the production of a small complement of Purkinje cells in the lobules derived from this region, i.e., the nodulus and flocculus. Accordingly, we may tentatively conclude that the collapsing neuroepithelium of the dorsal metencephalic plates outlines the cerebellar anlage, and that the development of juxtaventricular radioresistance in this region ("juxtaventricular transformation") signals the end of production of deep nuclear neurons and Purkinje cells. The postulated neuroepithelial transformations are summarized in figure 18.

Time of origin and mode of dispersion of deep nuclear neurons and Purkinje cells

In their pioneering study in mice, Miale and Sidman ('61) concluded that "Purkinje cells and neurons of roof nuclei form simultaneously in the primitive ependyma of the young embryo . . ." (p. 277) on days E11-13. The chronology of cerebellar development is different in the mouse than the rat and there may also be a species difference in the time of origin of these two cell types. But since we ourselves have found considerable overlap on day E14, the demonstration of a temporal separation may require quantification. As stated, our results show that while the bulk of deep neurons are formed on day E14, more than half of the Purkinje cells form on day E15. To our knowledge there is no published data on the time of origin of deep nuclear neurons in the rat but our dating of the time of origin of Purkinje cells is in agreement with other reports (Das and Nornes, '72; Schultze et al., '74). In a recent study, Pierce ('75) reported that in the mouse deep nuclear neurons are formed predominantly on day E11, with some small neurons arising thereafter. In our quantitative study in the rat we could not demonstrate a difference in the time of origin of large versus small deep nuclear neurons. However, our survey excluded the smallest of deep neurons.

Our demonstration of a temporal difference in the production of deep nuclear neurons and Purkinje cells was the basis for distinguishing the two differentiating fields of the cerebellar anlage as the "nuclear zone" and the "transitory zone." The superficial nuclear zone could be identified in irradiated embryos as early as day E13, and it was prominent by day E14. This zone could be composed of deep nuclear neurons, most of which differentiate by this day. We suggested that the next wave of differentiating cells leaving the neuroepithelium formed the transitory zone beneath the intermediate fibrous layer; since this zone appeared on day E15 it was presumed to be composed of Purkinje cells. The existence of at least two differentiating fields was described by previous investigators. Schaper (1894; quoted by Hannaway, '67) distinguished between a superficial and deep mantle zone, and Korneliussen ('68) described two major fields, "Migr. A" and "Migr. B", with several subdivisions. According to Korneliussen, Migr. B produces the deep nuclear neurons (p. 391). No explicit reference was made to the site of original Purkinje cells, but the implication was that they arise from Migr. A ". . . caudally and laterally at the attachment of the tela, its cells migrating superficially rostralwards . . ." (p. 386). This fits our description of the germinal trigone, which does not appear in most embryos until day E17 (compare figs. 6B and 7A), that is, until *after* the production of Purkinje cells.

The idea that Purkinje cells migrate super-

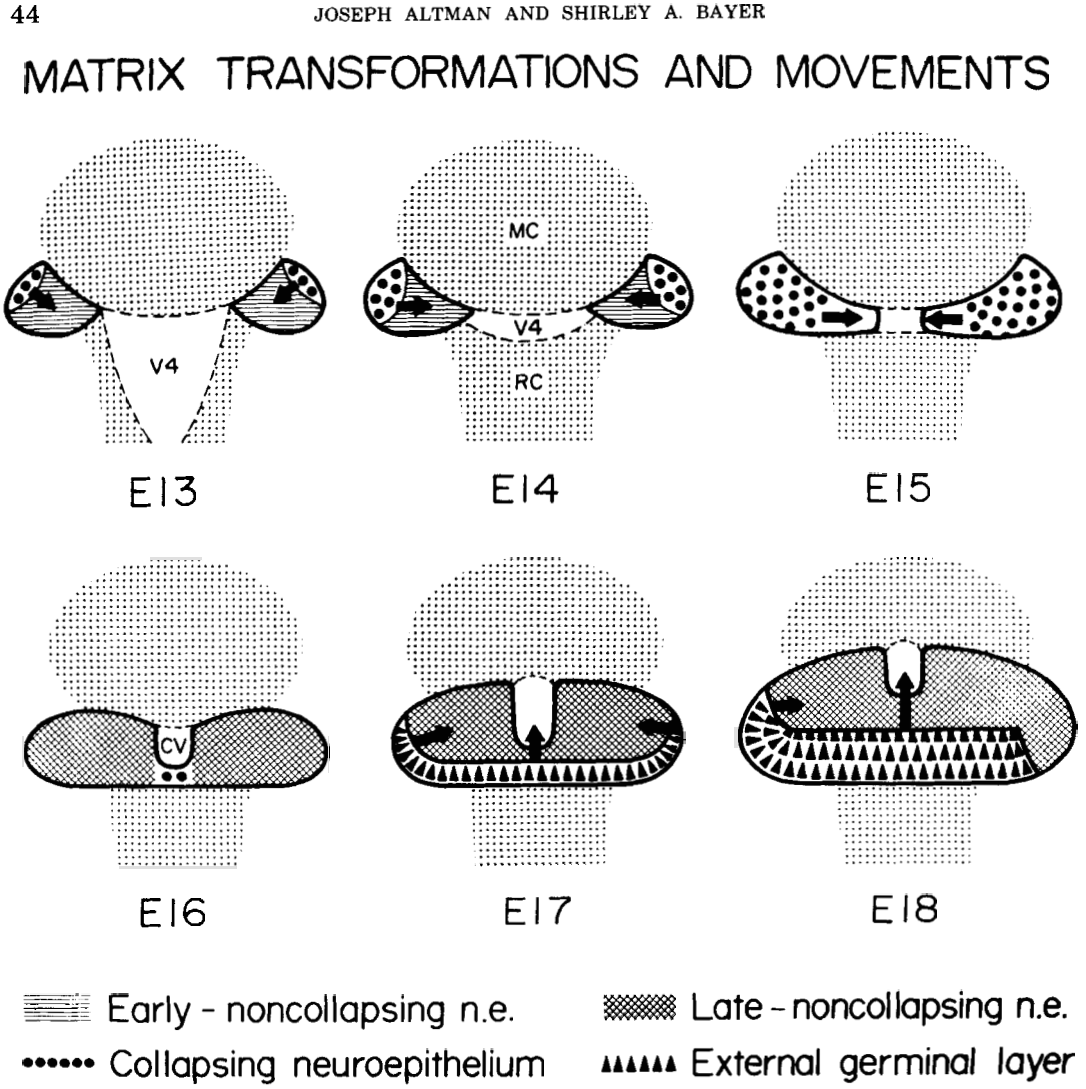


Fig. 18 Schematic illustration of the major transformations of the metencephalic germinal matrices between embryonic days 13 and 18. Arrows in upper row show the spread of the collapsing (presumably cerebellar) neuroepithelium from a lateral position medially over the early, noncollapsing (presumably noncerebellar) metencephalic plates. Arrows in bottom row indicate the movement of the external germinal layer from a posteroventral and ventrolateral position over the surface of the cerebellum. The transformed (or late, noncollapsing) neuroepithelium persists until after birth. The two halves of the cerebellum are initially separated by the fourth ventricle (V4), later by its subdivision, the cerebellar ventricle (CV). As the external germinal layer, and with it the primitive cerebellar cortex, spreads rostrally as a single sheet across the midline, the cerebellar ventricle recedes and gradually the cerebellum as a whole is transformed into a fused body. Abbreviations: MC, mesencephalon; RC, rhombencephalon.

ficially has never been seriously considered. Therefore, the Purkinje cells must move radially through the ranks of the somewhat earlier forming deep nuclear neurons. We have presented cytological evidence of the abundance of radially oriented cells in the body of the cerebellum during this period (fig. 10A-B). Radial migration of Purkinje cells would also explain the intermingling of elements of the nuclear and transitory zones beginning on day E17. These hypothetical events, together with the accumulation of Purkinje cells near the surface of the cerebellar cortex beginning on day E18 are summarized in figure 19.

Derivatives of the germinal trigone and of the regressing neuroepithelium

Hanaway ('67) proposed that the cells of the external germinal layer, like the Purkinje

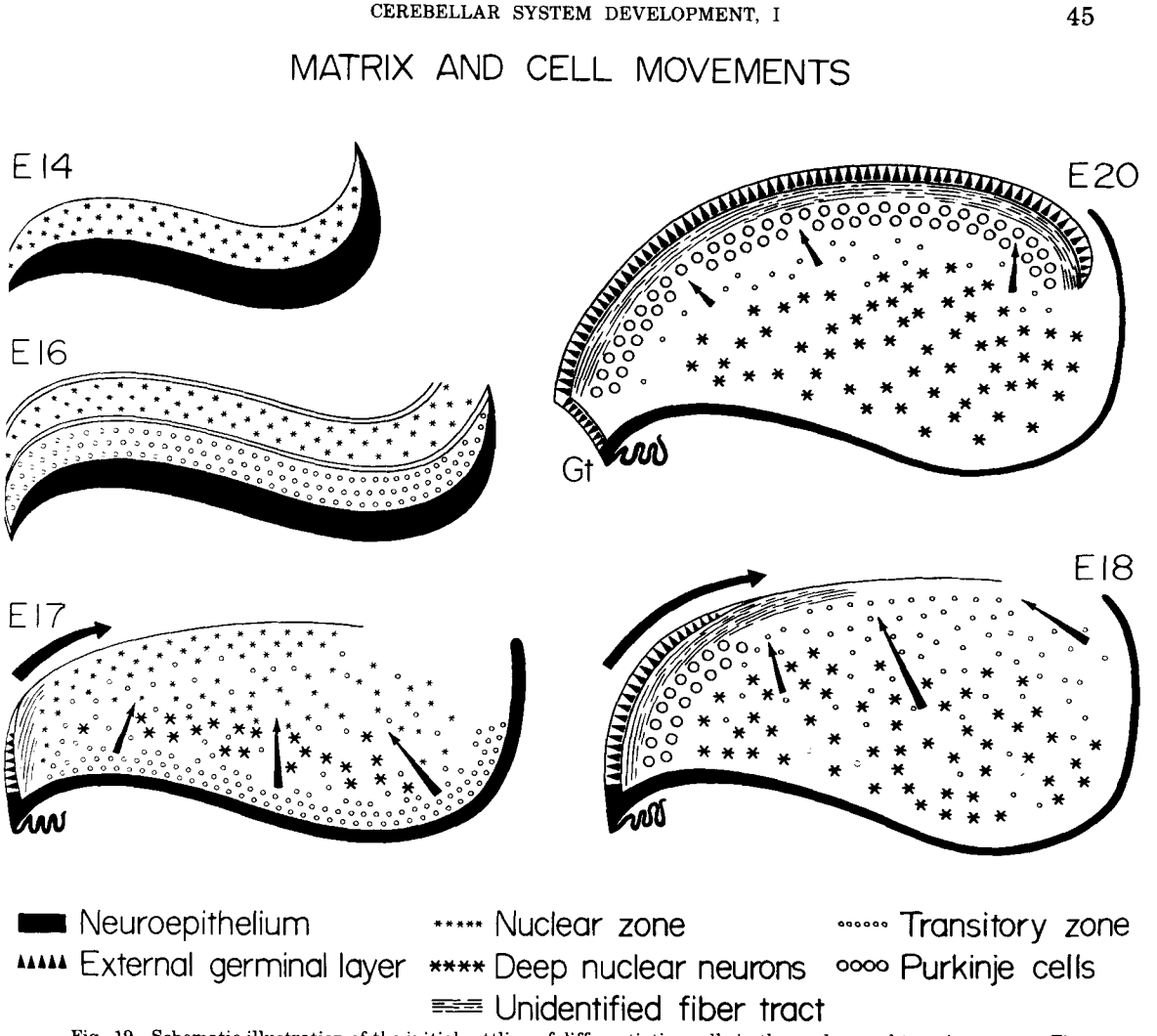


Fig. 19 Schematic illustration of the initial settling of differentiating cells in the nuclear and transitory zones. The somewhat earlier forming and faster differentiating cells of the nuclear zone are postulated to be the stationary deep nuclear neurons, the later maturing, larger population of the transitory zone are postulated to be the Purkinje cells. The Purkinje cells migrate radially toward the surface and gather underneath a canopy, formed of an unidentified fibrous layer and cells of the external germinal layer, that spread superficially in a rostral direction. The origin of the external germinal layer posteroventrally in the germinal trigone (Gt) is also illustrated.

cells (Ramón y Cajal, '60) migrate radially. Hanaway argued that if migration were superficial "a center of proliferation should be found in the ventrolateral region" (p. 5) but "at no time during development can a proliferation center be found in the ventrolateral angle of the fourth ventricle" (p. 1). Our demonstration of the active germinal trigone counterindicates Hanaway's argument. Our results agree with the earlier conclusion of Miale and Sidman ('61) that the "transient external granular layer arises by proliferation of cells of the lateral caudal cerebellar surface lining the fourth ventricle. These cells migrate over the surface and continue to proliferate abundantly . . ." (p. 227), with the qualification that the laterally situated cells are deployed medially over the hemispheres while the caudally situated cells spread rostrally over the surface of the future vermis. Incidentally, the spreading of the external germinal layer over the surface of the cerebellum from its posterior pole anteriorly may explain the paradoxical fact that the posteroventral nodulus, which is among the last lobules to acquire its full complement of Purkinje cells, is among the earliest to mature postnatally both in terms of granule cell

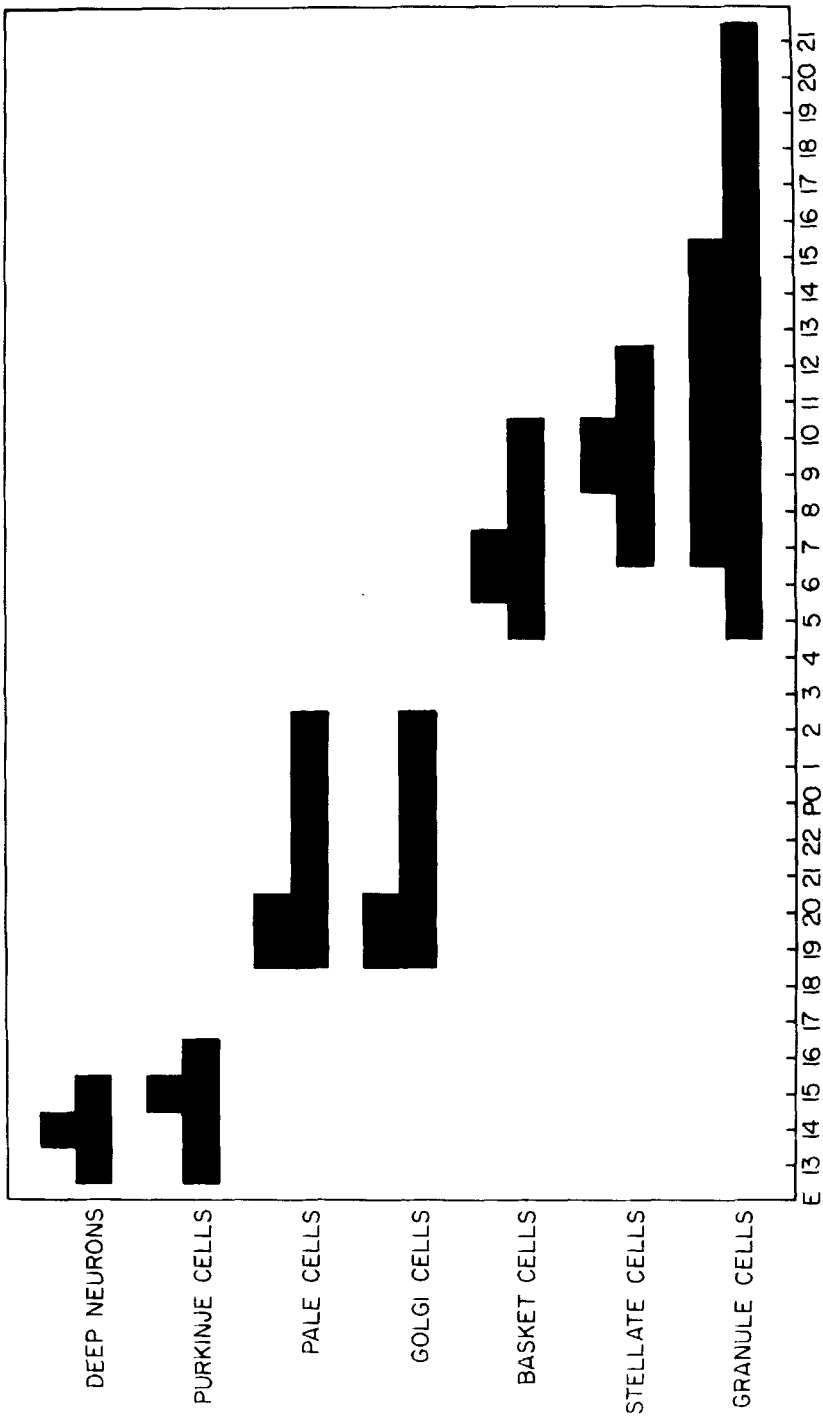


Fig. 20 Summary diagram of autoradiographic results of the sequential, nonoverlapping origin of three classes of cerebellar neurons: (1) the deep neurons and Purkinje cells; (2) the pale cells and Golgi cells, and (3) the basket, stellate and granule cells. An overlapping sequential origin is also indicated for some cell types within the groups. The graph accurately reflects the time span of cell origin; however, the proportion of cells formed on a given day is schematic (highlighting the peak formation time of different neurons).

acquisition and Purkinje cell maturation (Altman, '69).

The germinal trigone may be identical with the "rhombic lip" of His (1891). Ellenberger et al., ('69) have reviewed the prevailing confusion in the literature about the identity of the rhombic lip. They concluded that the "rhombic lip of the type seen by His in human material was not found in the lateral aspect of the medullary neuroepithelium of the rat" (p. 79). In a recent review, the germinal trigone, as here defined, was designated by Sidman and Rakic ('73, fig. 10B) as the rhombic lip. If this identification is correct then the classical hypothesis that all cells of the cerebellum are derived from the rhombic lip is not supported by our studies in the rat. The "rhombic lip" forms on embryonic day 17 *after* the production of both the deep nuclear neurons and Purkinje cells. If the term "rhombic lip" is preferable to "germinal trigone" it should be described as the source of the cell population of the external germinal layer.

Our autoradiographic data indicate that two classes of cells, the recently identified pale cells (Altman and Bayer, '77) and the larger Golgi cells of the granular layer, start to form on day E19. It is likely that both of them are derived from the regressing neuroepithelium rather than the external germinal layer. The latter does not reach the anteroventral aspect of the cerebellum, such as the lingula, until about day E22 (fig. 7F) where both pale cells and Golgi cells have started to differentiate several days earlier. It is probable that the glia of the cerebellum are likewise derived from the radioresistant regressing neuroepithelium. But because of their continuing multiplication, very few glia cells can be labelled even with multiple prenatal injections.

Investigators have for long puzzled over the nature of the midline fusion of the cerebellar plates (Ingvar, '18; Hochstetter, '29). Our observations showed that the process begins on day E16 in the rat, when in the caudal portion of the cerebellum and adjacent to the attachment of the tela choroidea, the "collapsing" neuroepithelium reaches and bridges the midline. It is from this fused neuroepithelium that the germinal trigone is derived on day E17 and which in turn produces the external germinal layer. The fused external germinal layer spreads over a similarly situated fibrous layer of unknown origin. (The possibility that they are climbing fibers will be discussed in the succeeding paper; Altman and Bayer, '78). The Purkinje cells piling up underneath the primitive molecular layer add to this fused canopy. As this cortical sheet spreads forward, the vertical ventricular cleft that separates the two halves of the cerebellum progressively retreats in the rostral direction and virtually disappears by day E22. The fusion of the cerebellar plates is thus attributable to the growth of a single cortical sheet over the lateralized deep nuclei.

Our earlier autoradiographic (Altman, '69) and radiation (Altman, '75) studies indicated that the microneurons of the cerebellar cortex are formed after birth in a precise temporal order. Combining these results with our present demonstrations, it appears that the neurons of the cerebellum as a whole are formed in a chronological sequence over three nonoverlapping periods (fig. 20): the neurons of the deep nuclei and the Purkinje cells between days E13-16; the pale cells and the Golgi cells between day E19 and the perinatal period; the basket and stellate cells in succession between the second half of the first and the end of the second postnatal week (Altman, '72), and the granule cells throughout this period but more than half of them during the third week (Altman, '69; and unpublished observations). The cells derived directly from the ventricular neuroepithelium evidently form mostly prenatally while the cells derived from the external germinal layer form postnatally. The largest cells (deep nuclear neurons, Purkinje cells) are formed before the smallest cells (stellate and granule cells). But there are exceptions. In the deep nuclei, larger cells and smaller cells differentiate at the same time. In the cortex, many of the small pale cells differentiate before the much larger Golgi cells, and before the similarly-sized or larger basket cells. It is probably not size per se but other factors that are indirectly associated with perikaryal size that govern the precise temporal order of neuronal cytogenesis. We shall return to this question in the second paper of this series (Altman and Bayer, '78) where we shall attempt to relate the sequential origin of neurons of several precerebellar nuclei to that of the cerebellum.

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