Prenatal Development of the Cerebellar System in the Rat

II. CYTOGENESIS AND HISTOGENESIS OF THE INFERIOR OLIVE, PONTINE GRAY, AND THE PRECEREBELLAR RETICULAR NUCLEI

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ABSTRACT Aspects of the prenatal development of the inferior olive, lateral reticular nucleus, nucleus reticularis tegmenti pontis, and of the pontine gray were investigated in the rat. The material used was the same as in the preceding study of cerebellar development, including histological preparations from normal and X-irradiated embryos at daily intervals (days E13 to E22), and autoradiograms from adult rats injected with two successive doses of ^{3}H-thymidine on overlapping days from days E13 + 14 on.

Neurons of the inferior olive form on days E13 and 14: those settling rostrally in the the principal nucleus somewhat ahead of those settling in the medial accessory nucleus caudally. The olivary neurons are generated in the neuroepithelium of the lateral recess of the fourth ventricle, migrate ventrally by way of the olivary migratory stream, split into two branches and provide cells to the olivary complex from its lateral and medial aspect. The migrating cells are differentiated, radioresistant elements. It was postulated that since the olivary migratory path and the course of olivary fibers (the inferior cerebellar peduncle) are on the same trajectory, climbing fibers could reach the surface of the cerebellum before the arrival of the radially migrating Purkinje cells.

Neurons of the lateral reticular nucleus form predominantly on days E13 and 14, of the nucleus reticularis tegmenti pontis on days E15-17, and of the pontine gray on days E17-19. The pontine neurons originate several days after but at the same site as the olivary neurons. The cells migrating in the pontine migratory stream are undifferentiated elements and follow a course different to that of their prospective axons in the middle cerebellar peduncle. The earliest forming pontine neurons settle near the pyramidal tract, the later arriving cells form cap-like shells around this core. Axonogenesis of pontine cells begins after the settling of the perikarya on day E19. The middle cerebellar peduncle appeared to approach the cerebellum on day E22, suggesting that pontine mossy fibers do not establish contact with cerebellar elements before the perinatal period, by which time the external germinal layer and the Purkinje cells have formed a cortical mantle over the entire cerebellum.

In an attempt to correlate the chronology of cytogenesis in some of the precerebellar nuclei with cell production and maturation in the cerebellum, it was hypothesized that mossy fibers of reticular origin exert influence on Purkinje cell dendrites in the lower parts of the molecular layer, whereas mossy fibers of pontine origin influence the upper parts of Purkinje cell dendrites. Reference is made to behavioral results indicating the mediation of dissociable functions by elements of the lower and upper molecular layer.

In the previous study of this series (Altman and Bayer, '78) we were concerned with the site and time of origin and the mode of distribution of the prenatally forming neurons of the rat cerebellum. It was determined that the neurons of the deep nuclei form between embryonic days 13 and 15 (E13-15) with a peak on day 14. The Purkinje cells arise in the same "collapsing" neuroepithelium of the cerebellar anlage between days E13-16, with a peak on day E15. Beginning on day E17 the external germinal layer forms posteriorly from one prong of the germinal trigone, and its cells gradually spread forward over the surface of the cerebellum. At the same time Purkinje cells begin their radial migration through the ranks of the differentiating deep neurons and aggregate superficially under the canopy formed by the external germinal layer (EGL) and an underlying, unidentified fibrous layer. The newly identified pale cells and the larger Golgi cells arise slowly from the "noncollapsing" cerebellar neuroepithelium beginning on day E19.

This second study of the series is concerned with cytogenesis and histogenesis of the major precerebellar nuclei of the hindbrain: the inferior olive, the lateral reticular nucleus, the nucleus reticularis tegmenti pontis, and the pontine gray. Among the early investigators of olivary and pontine neurogenesis we may refer to, and will later discuss, the contributions of His (1890), Essick ('07, '12) and Kooy ('17). Harkmark ('54) studied the site of origin and mode of migration of neurons of the olive and pons in chicks. The first detailed autoradiographic investigation of neurogenesis in the pons and nucleus reticularis tegmenti pontis was carried out in mice by Taber Pierce ('66), and a similar study of olivary neurogenesis was made by Ellenberger et al. ('69) in rats. In the present study we determined the chronology of the production, migration and settling of neurons in several precerebellar nuclei and attempted to relate them to similar events in the cerebellum. The time and site of origin of neurons of the inferior olive, the pons, the nucleus reticularis tegmenti pontis, and the lateral reticular nucleus were investigated in the same autoradiographic material that was used in the previous cerebellar study. Similarly, the route of migration and the time of settling of these cells, together with some indications of axonogenesis, were examined in the previously used normal and X-irradiated embryonic material.

MATERIALS AND METHODS

The previous paper (Altman and Bayer, '78) provides details about: (1) the normal and X-irradiated embryos used; (2) the schedules of embryonic injection (^{3}H-thymidine) of adults used for autoradiography; and (3) the procedure of "progressively delayed cumulative labelling" employed for the estimation of the proportion of cells differentiating (no longer labelled) on a given embryonic day. Procedural details relevant to specific brain regions are described in the appropriate RESULTS sections.

RESULTS

I. *The olivary nuclei*

Brief background. The olivary nuclear complex of the rat (Kooy, '17; Ellenberger et al., '69), like that of higher mammals, is divisible into three parts, the principal, the medial accessory, and the dorsal accessory nuclei (fig. 1). If the connections of the rat olive are similar to that of the cat and other mammals, it should receive afferents from the spinal cord (mostly lumbar regions; Brodal et al., '50), from the cerebral cortex (primarily from the motor area; Walberg, '56; Sousa-Pinto and Brodal, '69; Crill, '70), the caudate nucleus (Sedgwick and Williams, '67), and the cerebellar deep nuclei (Graybiel et al., '73; Martin et al., '76). The efferents of the olive are believed to be the major (if not exclusive) source of cerebellar climbing fibers (Szentágothai and Rajkovits, '59; Eccles et al., '67). Although this has been seriously questioned by several investigators, recent studies utilizing new techniques (for a review, see Desclin, '74) tend to corroborate this view. In contrast, the classical description of the topographic distribution of olivary fibers to the cerebellum, based on a retrograde degeneration study (Brodal, '40), was recently shown to be at least partly untenable (Armstrong et al., '74; Brodal et al., '76; Hoddevik et al., '76). Certainly the notion that those parts of the olive (rostral) that receive cerebral projections have selective connections with the cerebellar hemispheres, and those parts (caudal) that receive spinal afferents connect with the vermis (for a review, see Bloedel, '73) is not supported, and the entire matter requires re-examination.

Autoradiographic results. In matched sagittal sections neurons were classified as labelled or unlabelled (magnification, × 625; olivary subdivisions disregarded). The mean proportion of labelled olivary neurons was 99%

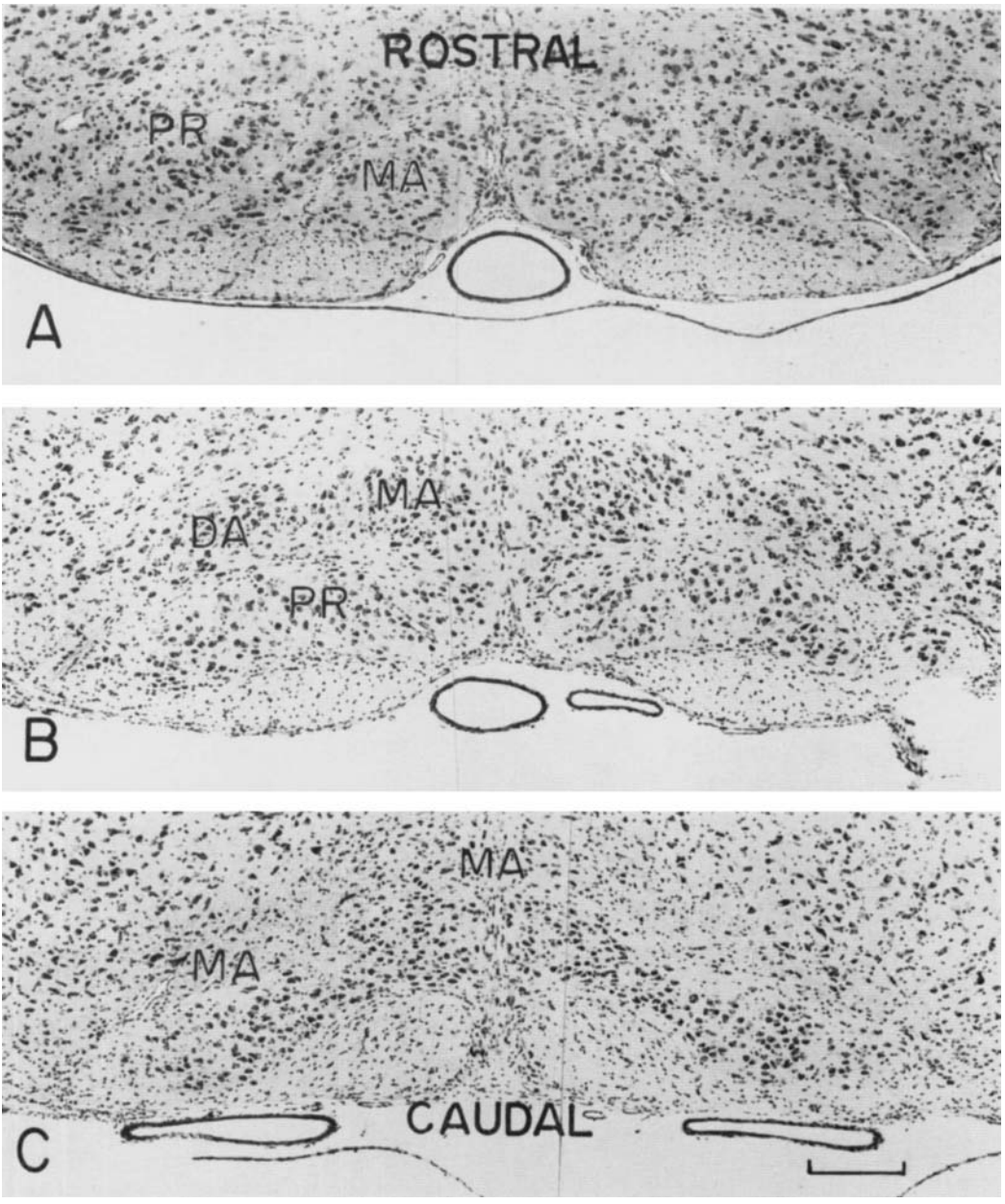


Fig. 1 Coronal section through the rostral (A), middle (B) and caudal region (C) of the inferior olivary nuclei. Nuclear designations after Kooy ('17). DA, dorsal accessory nucleus; MA, medial accessory nucleus; PR, principal nucleus. From a normal, 10-day-old (P10) rat. Hematoxylin-eosin. Scale: 200 μm.

in the E13+14 group (figs. 2A-B). In another group of E13+14 rats that received 4 injections at 12 hour intervals, all olivary neurons were labelled. Accordingly, it was concluded that in this material with two injections 99% was the maximal labelling efficiency and, accordingly, a correction factor of × 1.01 was applied to the data (fig. 3A). In the E14+15 group (fig. 2C) the proportion of labelled cells was 28%, but there were no labelled olivary

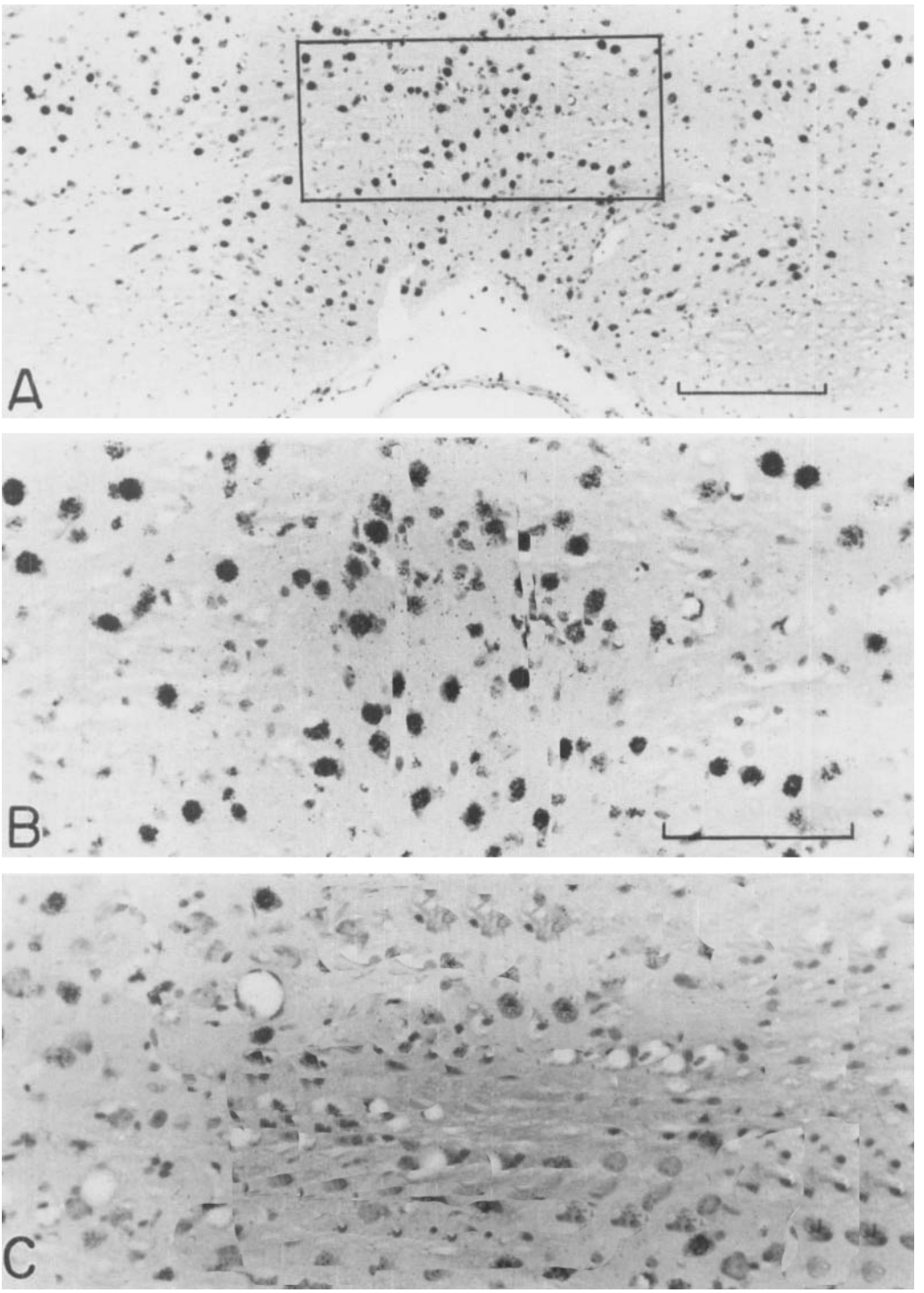


Fig. 2 Autoradiograms of the inferior olive. A, adult rat injected on days E13 + 14; B, higher magnification of the designated region in A; C, rat injected on days E14 + 15. Hematoxylin-eosin. Scales: A, 200 μm; B and C, 100 μm.

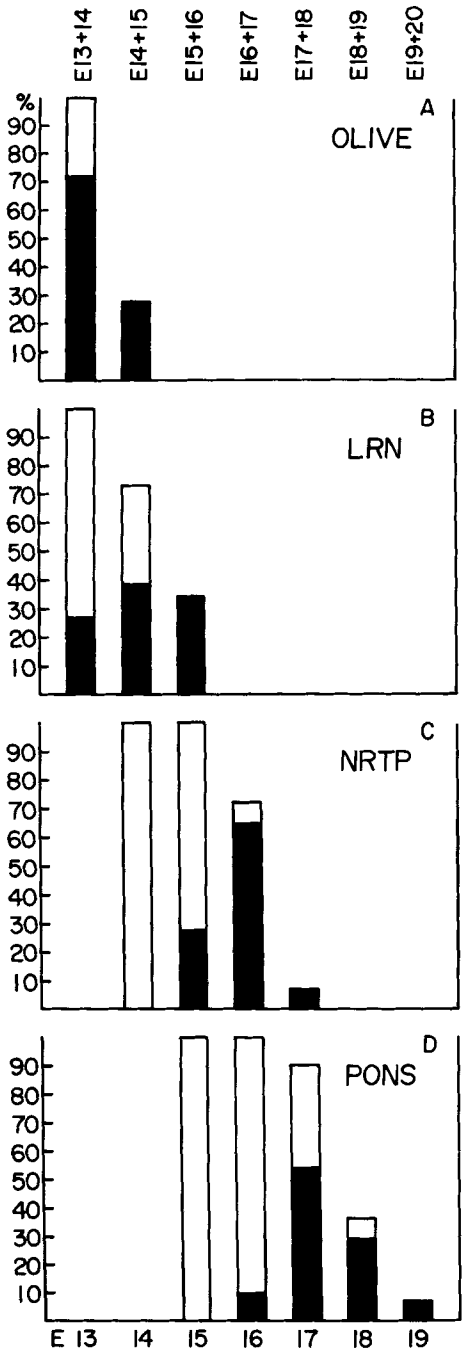


Fig. 3 Determination of the proportion of neurons formed on different days in the inferior olive (A), the lateral reticular nucleus (B), the nucleus reticularis tegmenti pontis (C), and the pontine gray (D). Empty bars indicate the proportion of cells (X correction factor; see text) in the injection groups indicated at the top; solid bars are the calculated percentages of cells formed on the day indicated at the bottom. For instance, olivary neurons formed on day E13 = (E13+14) − (E14+15).

cells in the E15+16 group (fig. 3A). It was concluded that in this material 72% of the olivary neurons formed on day E13, the rest on day E14.

Because the subdivisions of the olive are not easy to delineate in autoradiograms, the exact identification of the regions where many neurons were still labelled on day E14 proved to be difficult. In our judgment few neurons were labelled on day E14 in the principal nucleus, while those neurons labelled on day E14 in the medial accessory nucleus tended to concentrate caudally and ventromedially, and those in the dorsal accessory nucleus dorsolaterally. (This accords with the mode of migration of olivary cells and their pattern of settling, to be described below.)

Olivary neurons were not labelled in the E15+16 animals in which a certain proportion of deep cerebellar neurons, and many Purkinje cells, were labelled. The absence of labelled olivary neurons was noteworthy in one animal in which 44% of interpositus neurons and 47% of fastigial neurons were tagged, and Purkinje cell labelling at the same levels ranged between 78-79%. Apparently olivary cytogenesis comes to an end while the production of deep neurons and Purkinje cells is still in progress.

Embryological observations. In day E15 embryos the future site of the olive had a fibrous composition and was devoid of cell bodies. But in all day E16 embryos (fig. 4A) the olive was recognizable as a thin cigar-shaped structure, increasing in bulk by day E17 (figs. 4B-C). Near the pia and separated from the olive by a plexiform layer, was a compact layer of cells that parallelled in length the olive (fig. 4); this was identified in coronal sections as one of the two branches of the *olivary migratory stream.* The migratory stream of spindle-shaped cells was traced (fig. 5) from the lateral recess of the dorsal aspect of the fourth ventricle over the dorsal, then lateral aspect of the medulla, where it branched (fig. 5A). One branch, referred to by His (1890) as the *Olivstreife,* and by Ellenberger et al., ('69; fig. 11B) as the submarginal strand, penetrated the medulla and supplied cells to the olive throughout most of its length from a lateral position (fig. 6A). This branch remained recognizable in day E17 embryos (fig. 6B) but regressed considerably by day E18. The second branch (marginal strand) remained in an external position, curved around the base of the medulla across the midline (figs. 5, 6A-B). In day E16 embryos the marginal branch remained separated from the pons and was

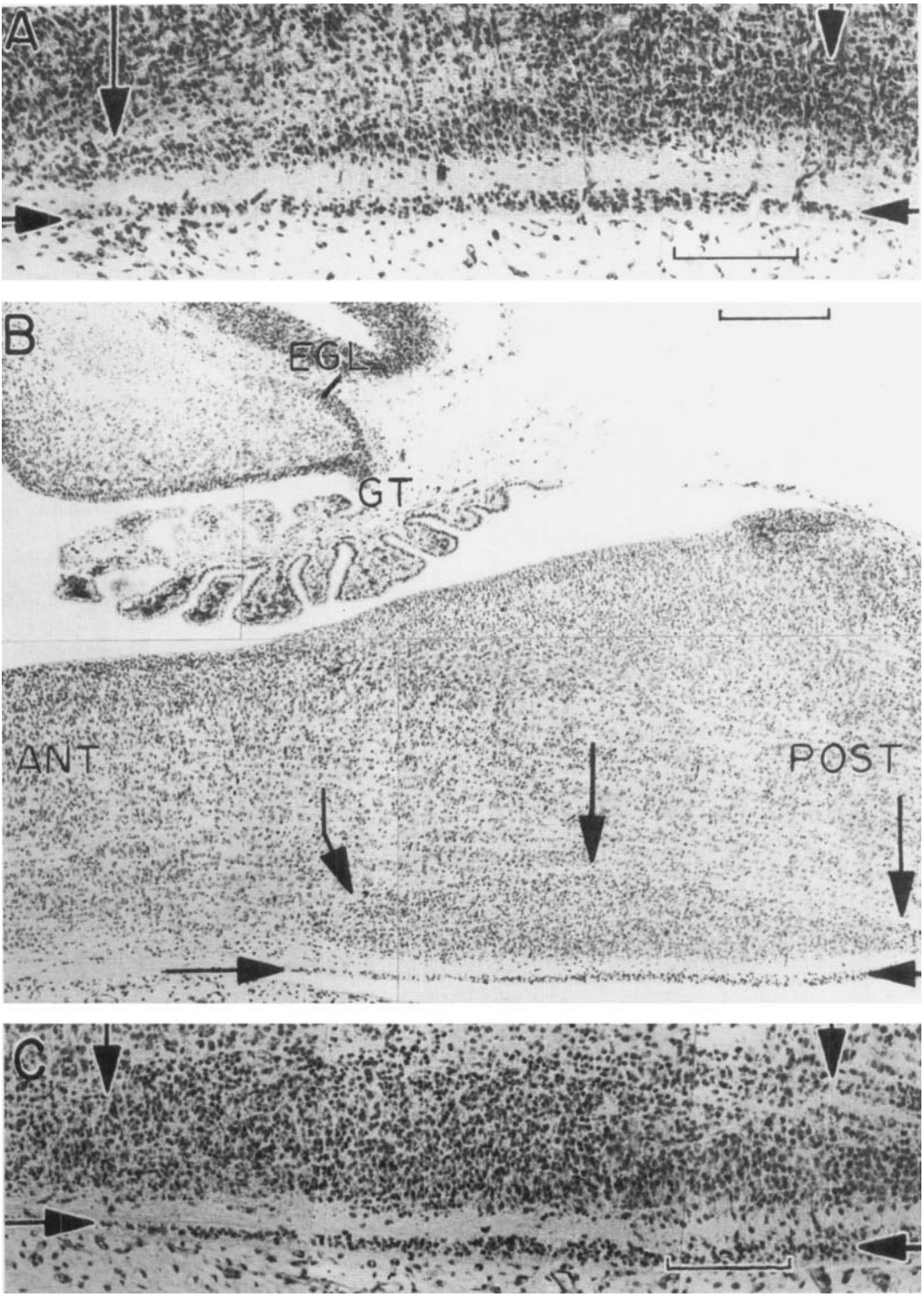


Fig. 4 Sagittal sections of the inferior olives: A, from a day E16 embryo; B and C from a day E17 embryo. Vertical arrows point to the olive, horizontal arrows to the marginal strand of the olivary migratory stream. Abbreviations: ANT, anterior; EGL, external germinal layer; GT, germinal trigone; POST, posterior. Scales: A and C, 100 μm; B, 200 μm.

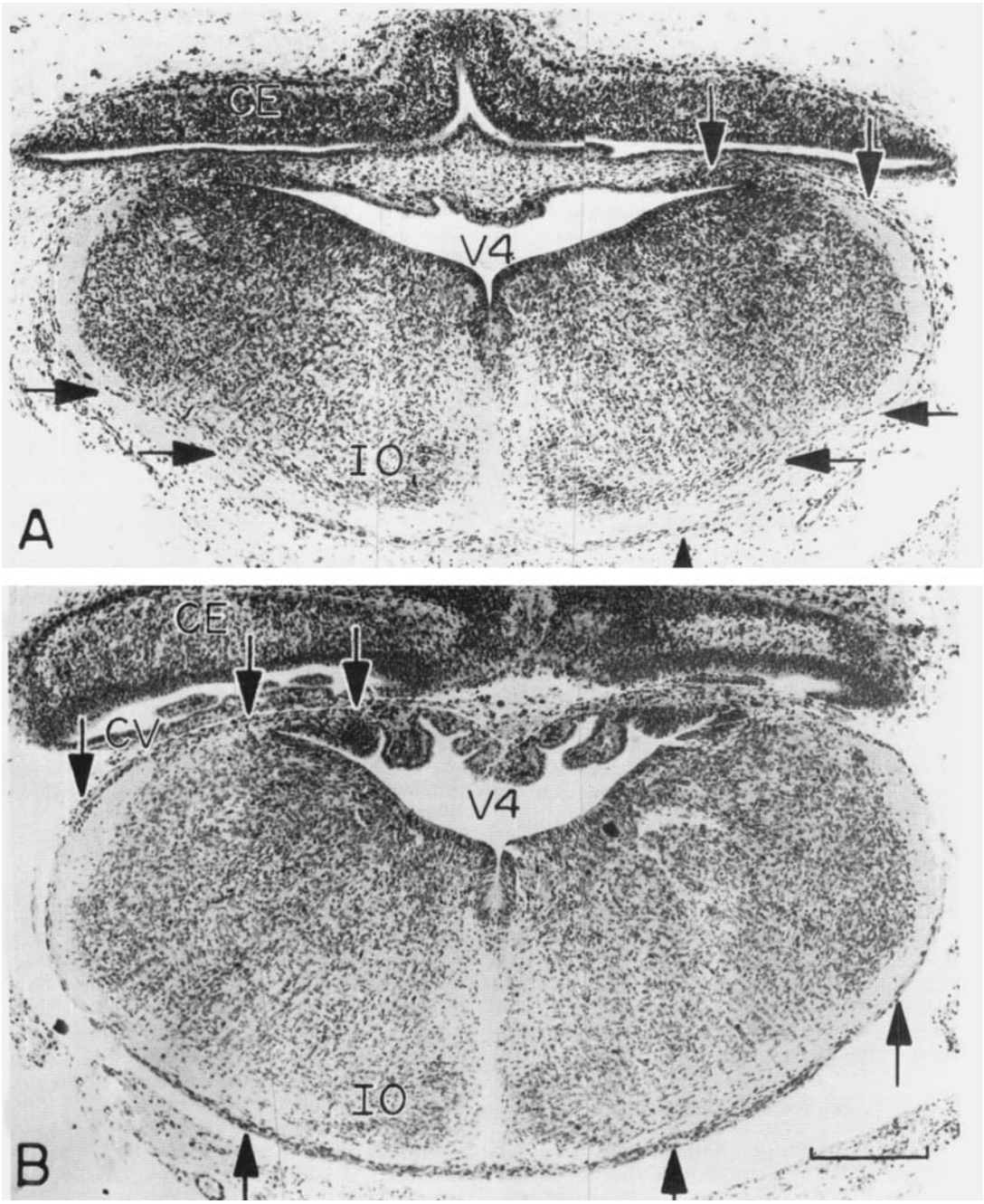


Fig. 5 Coronal sections through the primitive cerebellum (CE), cerebellar ventricle (CV), fourth ventricle (V4), medulla, and inferior olive (IO) of a day E16 (A) and day E17 (B) embryo. Downward directed vertical arrows point to the site of origin of the olivary migratory stream in the lateral recess of the fourth ventricle and the initial (dorsal) portion of the stream. Horizontal arrows in A delineate the submarginal migratory stream, from the point that it penetrates the medulla. Upward directed vertical arrows point to the marginal branch of the olivary migratory stream. Hematoxylin-eosin. Scale: 200 μm.

thickening caudally (fig. 5A); by day E17 cells began to stream upwards in large numbers near the midline to contribute cells to the caudal olive from its ventromedial and medial aspect (fig. 6B). The marginal band has regressed by day E18, though its remnants could still be seen in many E19 and E20 fetuses (figs. 6C-D).

This pattern of cell migration suggested the addition of cells to the more dorsally and rostrally situated parts of the olive from a lateral position somewhat earlier, and to the more caudally and medially situated parts (presumably to the medial accessory nucleus) from a ventromedial and medial position somewhat later. In line with this it was observed in E19 fetuses (fig. 6C) that olivary neurons were less mature (smaller and more tightly packed) in the medial accessory nucleus than in the principal nucleus; but by day E20 (fig. 6D) the difference was less evident. By the latter date the subdivisions of the olive resembled the adult pattern.

The spindle-shaped cells in the lateral and ventral aspects of the migratory stream appeared to be maturing elements in the sense that they tended to stain lightly and had recognizable nucleoli. In irradiated embryos these cells (unlike the primitive cells of the pontine migratory stream; see below) were radioresistant. This indicated that cells of the olivary stream are postmitotic elements, comparable to the radioresistant translocating granule cells in the cerebellar molecular layer (Altman and Nicholson, '71) which form their axons, the parallel fibers, before settling in the molecular layer. Another observation of importance (fig. 4B) was that the inferior olive had grown considerably in bulk by day E17, when the external germinal layer had just started to spread over the surface of the cerebellum and the radial migration of Purkinje cells had hardly begun (Altman and Bayer, '78).

II. *The lateral reticular nucleus*

Brief background. The lateral reticular nucleus (LRN) is situated in the rat in the ventral medulla, lateral to the olive and caudal to the facial nerve nucleus. It is composed of some small but mostly large reticular type of multipolar neurons that generally do not reach the size of the neurons of the adjacent, rostromedially situated nucleus gigantocellularis.

Brodal's ('43) retrograde degeneration study in the cat established that the projection of the LRN is to the cerebellum. Brodal proposed that the magnocellular portion projects to the hemisphere, the parvocellular portion to the vermis, and the subtrigeminal portion to the flocculonodular lobe. But recent anatomical and physiological studies have failed to confirm this pattern of projection. Matsushita and Ikeda ('75), using the Nauta and Fink-Heimer techniques, found that fibers from different parts of the LRN terminate chiefly (though not exclusively) in the ipsilateral anterior lobe, and in the lobus simplex and parts of the paramedian lobule. They also described a projection to the medial and intermediate deep cerebellar nuclei but not the lateral nucleus. Similar results were obtained by Künzle ('75) with the autoradiographic technique, except for the absence altogether of projections to the deep nuclei. Additional details were provided by P. Brodal ('75) who used the horseradish peroxidase technique. Physiological studies (Clendenin et al., '74) yielded similar results. The majority of LRN neurons are mono- and polysynaptically activated by secondary spinal afferents (Rosén and Scheid, '73; Kitai et al., '74) but there is also input from the deep cerebellar nuclei, the red nucleus, and the cerebral cortex (Zangger and Wiesendanger, '73; Clendenin et al., '74; Kitai et al., '74). The LRN axons terminate in the granular layer as mossy fibers (Sasaki and Strata, '67; Clendenin et al., '74). The conclusion drawn from these studies is that the projection of the LRN is to the classical spinal receiving areas of the cerebellum.

Autoradiographic results and embryological observations. In rats injected on days E13 + 14 over 90% of the LRN neurons were labelled (fig. 7). The unlabelled cells may have formed prior to day E13 but in accordance with the practice adopted in this study we assumed that this was our labelling efficiency in this region and, therefore, applied the correction factor of × 1.05 to the data (fig. 3B). The corrected labelling index in the E14 + 15 group was 73% and in the E15 + 16 group 34%. In most E16 + 17 rats LRN neurons were no longer labelled; in others only a rare labelled neuron was seen. It is concluded that LRN neurons form predominantly on days E13-15, with a slight peak indicated for day E14 (fig. 3B). The LRN, unlike the olive, was not identifiable as a discrete nucleus in the available embryonic material, suggesting perhaps

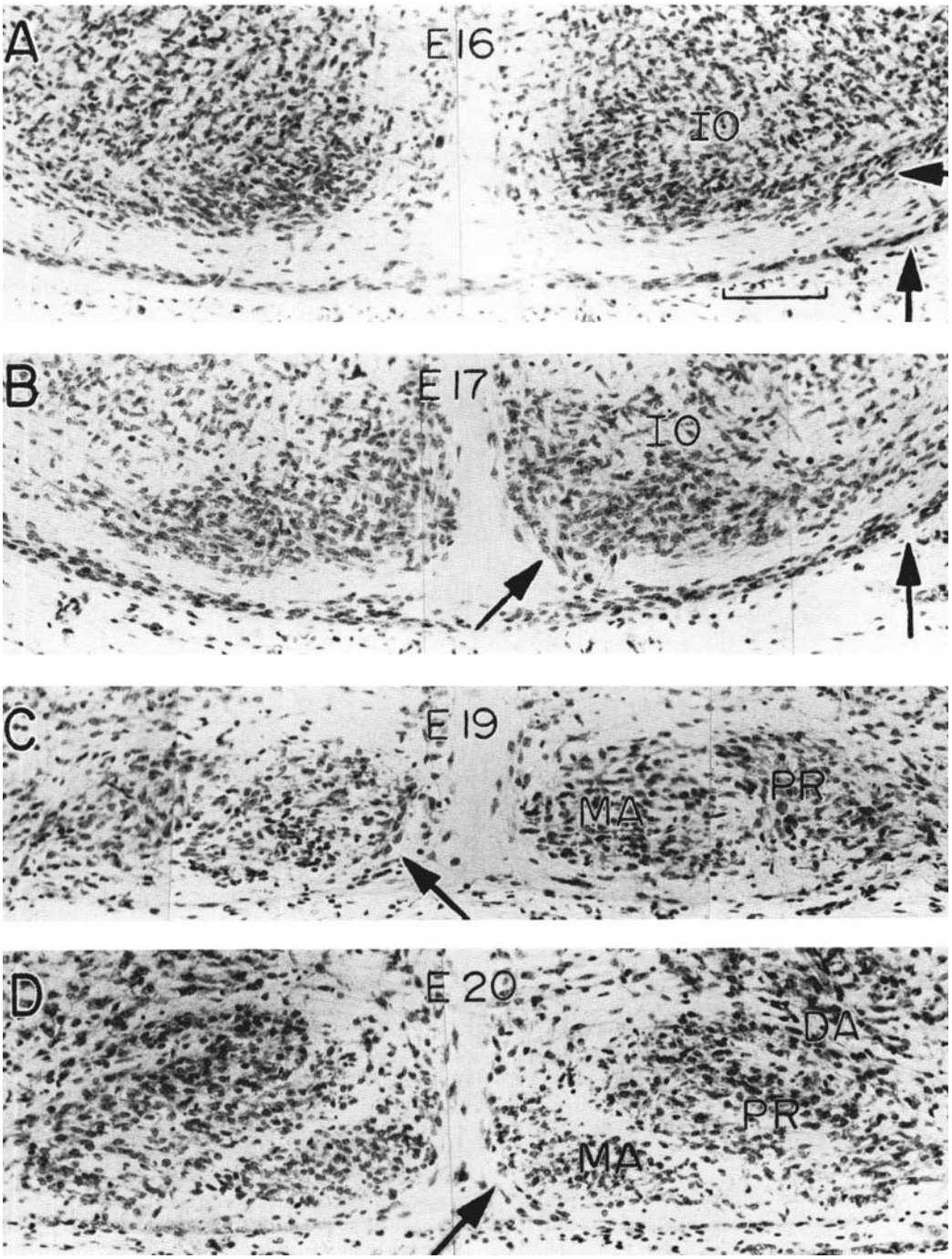


Fig. 6 Coronal section through the inferior olive (IO) at different ages (indicated at top center of each photograph). Horizontal and vertical arrows as in figure 5; oblique arrows point to the region where the cells of the marginal branch of the olivary migratory stream reach the medial accessory nucleus (MA). Note in C and D that cells of the latter are more primitive in appearance than cells of the principal nucleus (PR); DA, dorsal accessory nucleus. Hematoxylin-eosin. Scale: 100 μm.

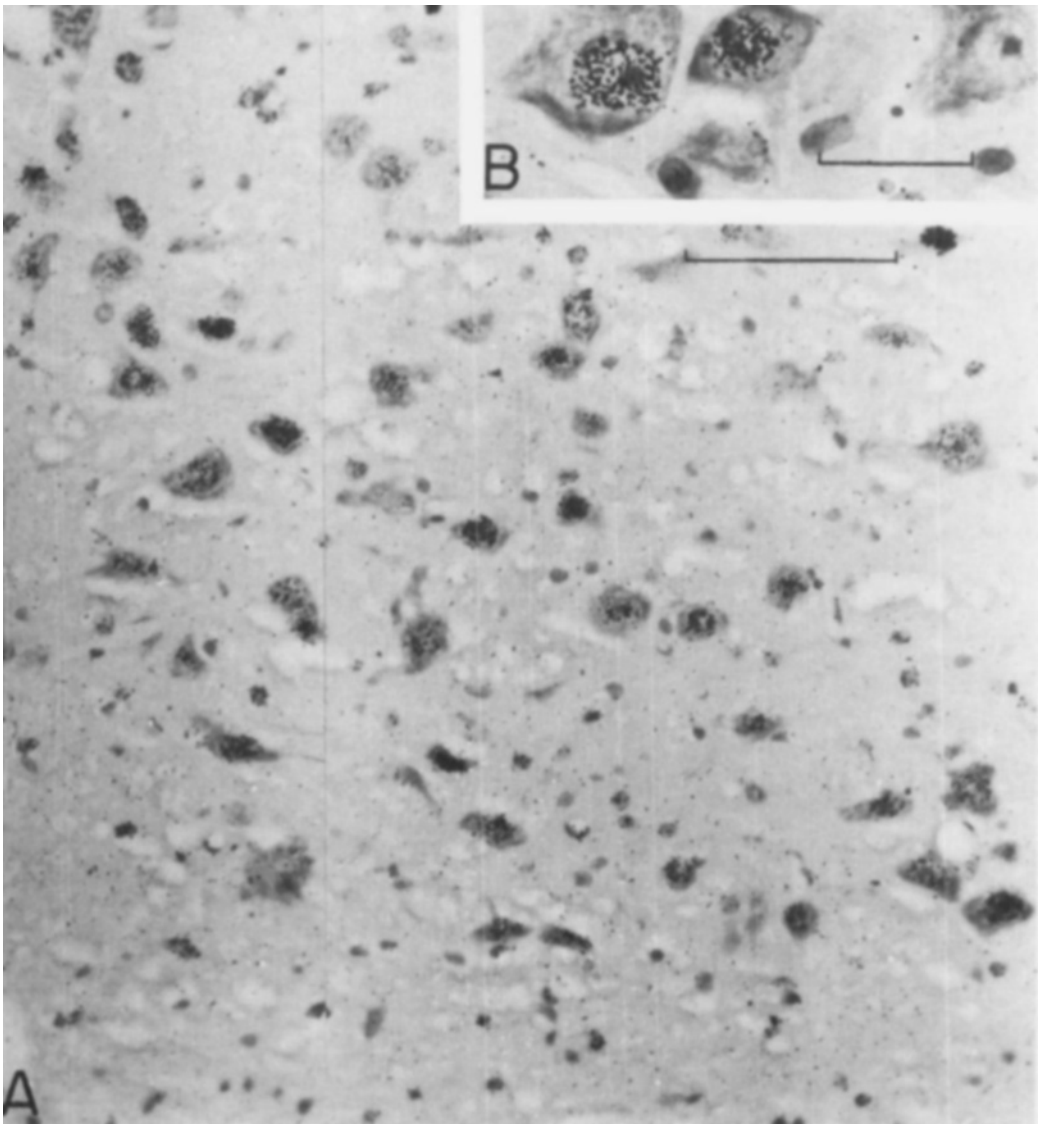


Fig. 7 Lateral reticular nucleus. A, autoradiogram from a rat injected on days E13+14. Most neurons are labelled. B, from a rat injected on days E15+16. Many neurons are still labelled. Scales: A, 25 μm; B, 100 μm.

a slow maturation of these early forming neurons.

III. *The nucleus reticularis tegmenti pontis*

Brief background. In the rat the nucleus reticularis tegmenti pontis (NRTP) is ovoid in shape. It is situated near the midline, dorsal to the pontine nuclei and separated from it by the pyramidal tract, but it does not extend laterally as far as the pons. Its typical cells are multipolar and larger than the pontine neurons; the cells in the dorsocaudal portion of the NRTP tend to be larger than those seen rostrally where the boundary between the pons and the NRTP becomes indistinct.

Studies in the cat by Brodal and his associates (Brodal and Brodal, '71; Brodal and Szikla, '72; Brodal et al., '72b) indicated that the NRTP, like the pons, receives afferents from various cortical regions, including the motor, premotor, and somatosensory areas. However the dominant source of its afferents is from

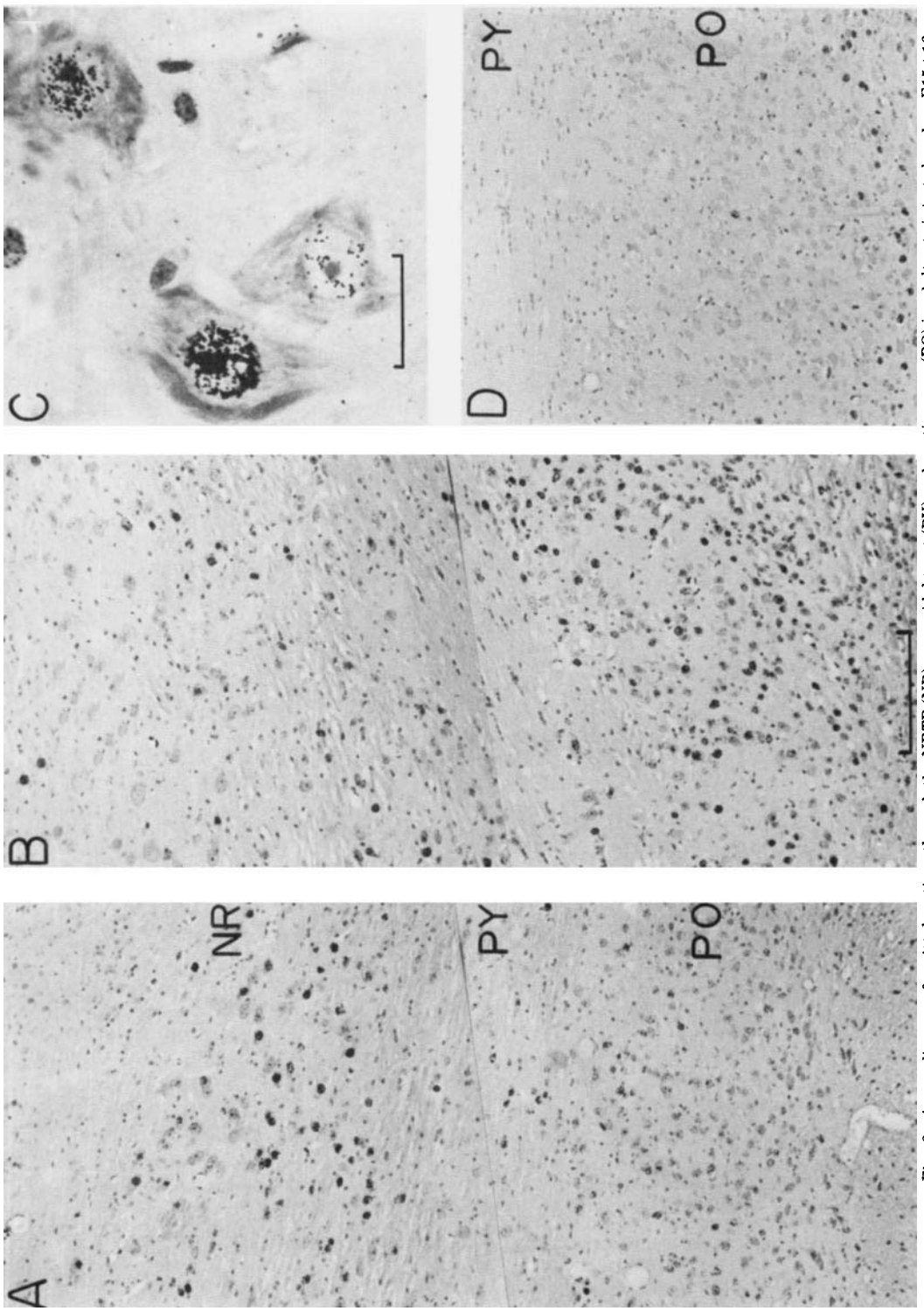


Fig. 8 Autoradiogram of sagittal sections through the NRTP (NR), pyramidal tract (PY) and pontine gray (PO) in adult rats injected on days E15 + 16 (A), E16 + 17 (B), and E18 + 19 (D; pons only). Note high proportion of well-labelled neurons in the NRTP in A, and their reduction in B. In A the pontine neurons are lightly labelled; in B many are intensely labelled; In D labelled cells are restricted to the external shell of the pons. C shows one lightly-labelled, and two well-labelled NRTP neurons in an adult rat injected on days E15 + 16. Scale in A, B and D, 100 μm; in C, 25 μm.

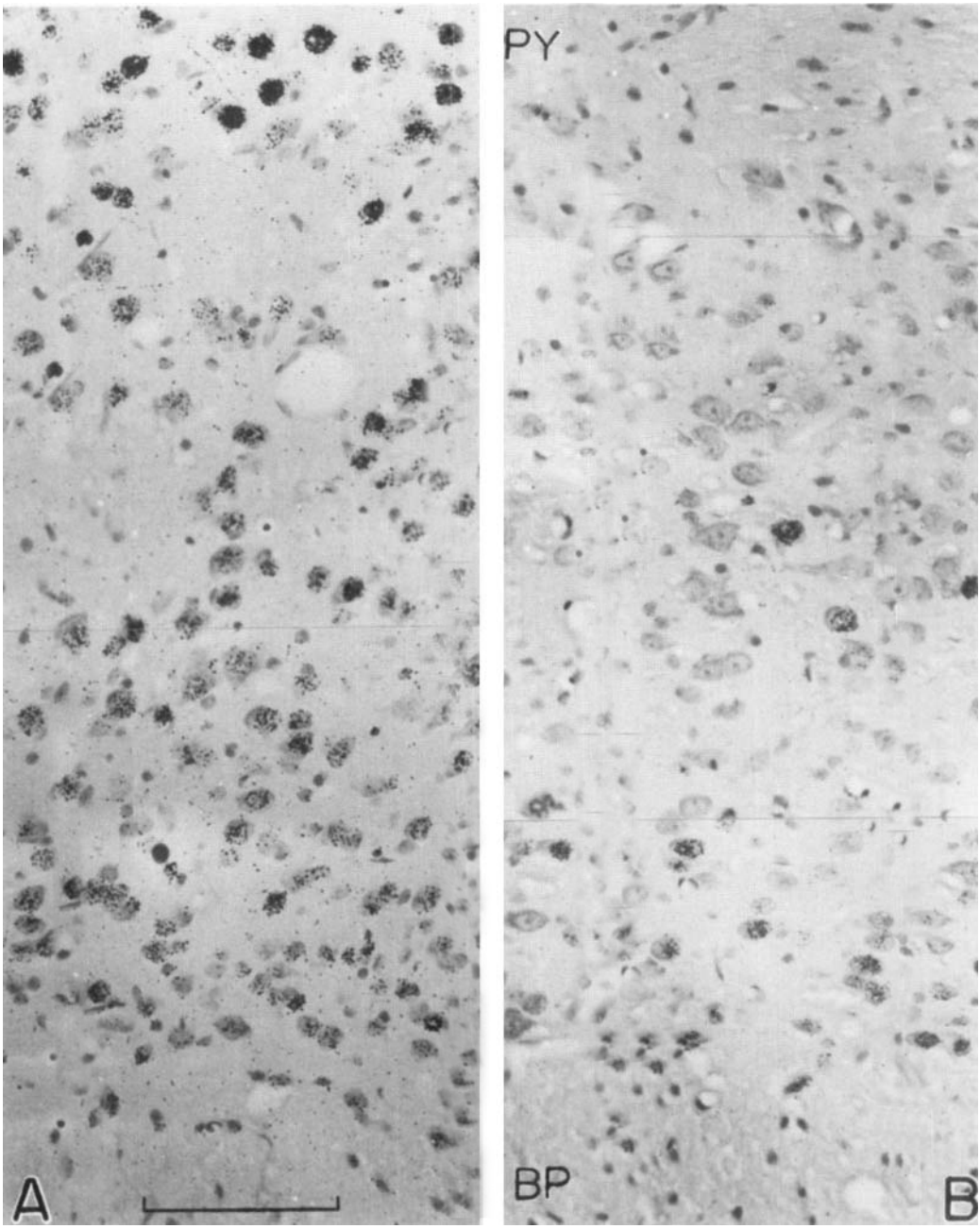


Fig. 9 Autoradiograms of the pontine gray in adult rats injected on days E16 + 17 (A) and E18 + 19 (B). In A all neurons are labelled: those in the depth of the pons (top) are well-labelled, the rest mostly lightly-labelled. In B few neurons are labelled in the depths of the pons adjacent to the pyramidal tract (PY) but many more superficially, adjacent to the brachium pontis (BP). Scale: 100 μm.

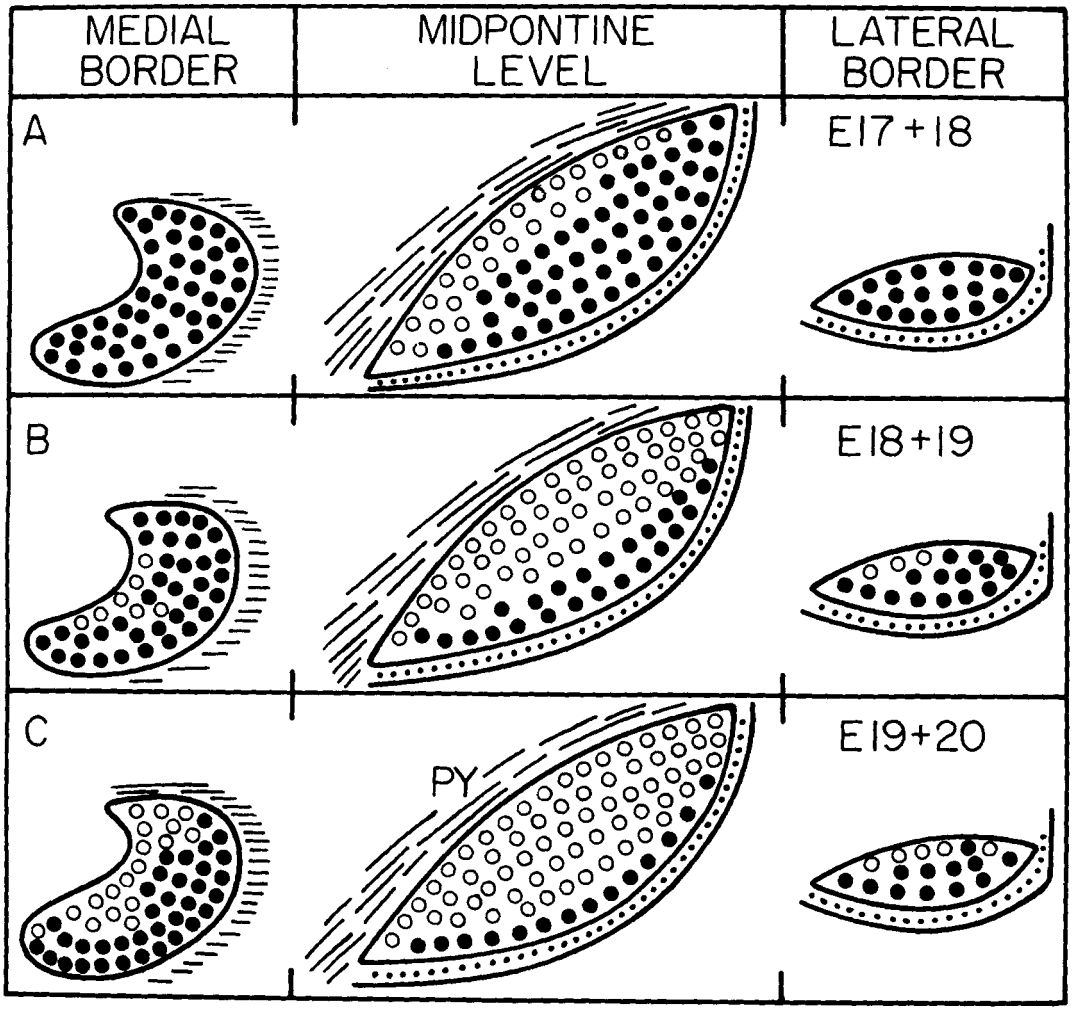


Fig. 10 Schematic illustration of the distribution of labelled (filled circles) and differentiated (empty circles) neurons at three sagittal levels of the pons (three columns) as a function of embryonic age at injection (rows A-C). The earlier differentiating neurons settle in the core of each pons adjacent to the pyramidal tract (PY), while the later forming neurons settle circumferentially.

the cerebellar deep nuclei, and from the vestibular nuclei, by way of the descending limb of the superior cerebellar peduncle. Most of its efferents, if not all, reach the cerebellum (Brodal and Jansen, '46) and terminate as mossy fibers (Bloedel, '73).

Autoradiographic results. In the E13+14 and E14+15 groups the majority of NRTP neurons were lightly labelled, and a few were intensely labelled. In the E15+16 group the proportion of intensely labelled cells increased (fig. 8A,C) and a rare large neuron was unlabelled. This indicated the incipient production of NRTP neurons.

In the E16+17 group about half of the NRTP neurons were no longer labelled dorsocaudally; elsewhere, particularly in the smaller-celled region anteroventrally the proportion of labelled cells was still high (fig. 8B). In the E17+18 rats most of the NRTP neurons were unlabelled. Most, though not all, of the labelled neurons were seen in the vicinity of the pyramidal tract and close to the midline where the boundary between the NRTP and

the pons is indistinct. No clear instances of NRTP neuron labelling were seen in the E18+19 group. For quantification matched sagittal sections were used at a level transecting the fastigial nucleus where the pyramidal tract clearly separated the NRTP from the pons. In a single section from each animal all neurons were classified at × 625. In the E15+16 group 96% of the cells were labelled and, therefore, a correction factor of × 1.04 was applied to the data. The results (fig. 3C) indicated that NRTP neurons are produced on days E15-17 with the peak proportion (65%) of cells produced on day E16.

Embryological observations. The site of origin of NRTP neurons could not be determined in the available material. The circumstance that these cells form predominantly on days E15-16, before the emergence of the pontine migratory stream (see below) renders this possible source unlikely. Possibly the NRTP cells form close to their settling region in the basal plate of the rhombic neuroepithelium. The aggregation of NRTP cells was first recognized on day E18, at a time when pontine cells were just beginning to settle. In newborn rats NRTP cells were dispersing and showing other signs of ongoing maturation.

IV. *The pontine gray*

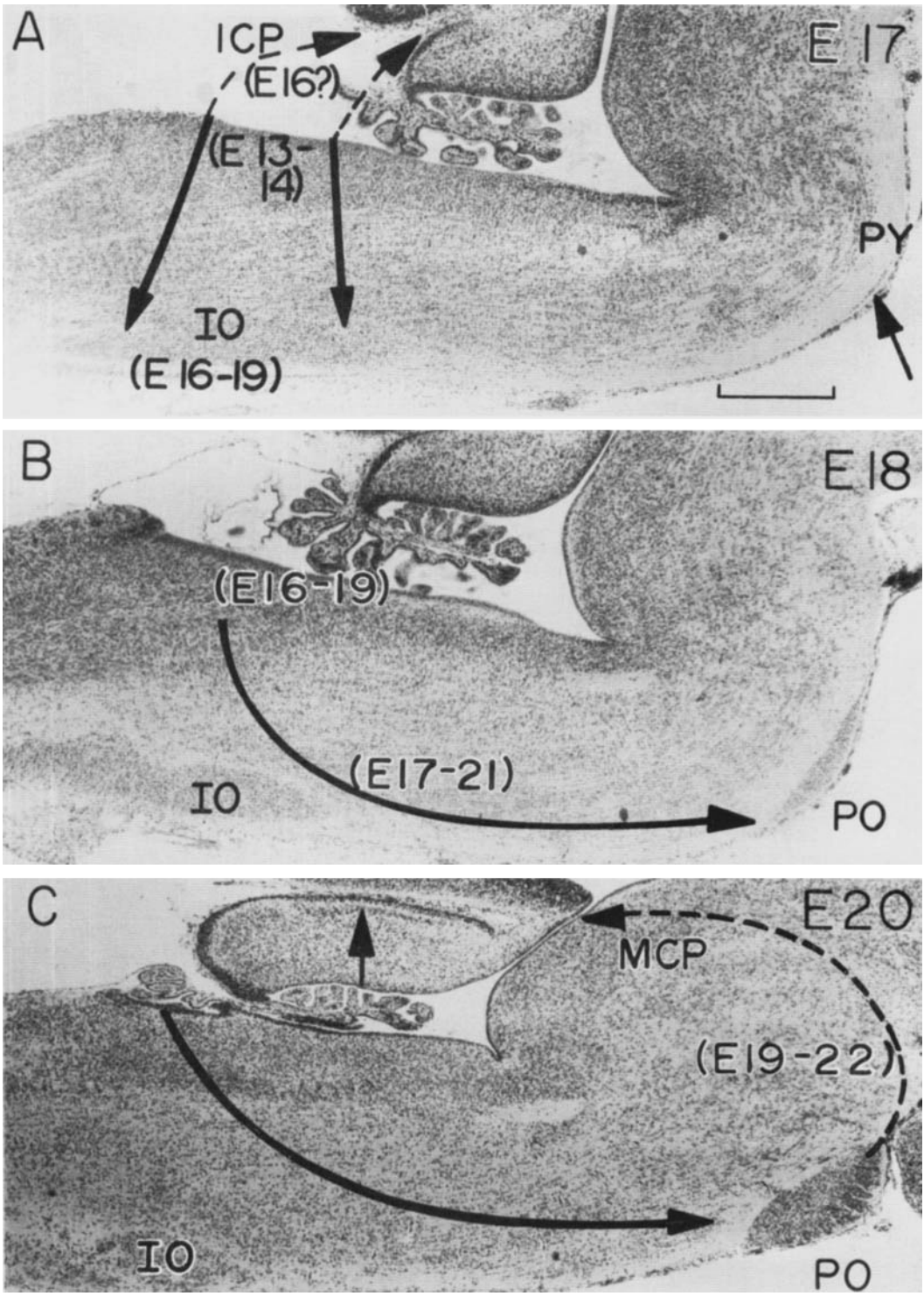
Brief background. The pontine gray of the rat is composed of fairly uniformly distributed neurons that form a pair of contiguous, concavo-convex structures around the descending fibers of the pyramidal tract. There is an irregular gradient in the size of the perikarya: the cells in the concavity (depth) of the pons tend to be larger than the cells that form a cap-like shell over its convexity (fig. 9).

The connections of the rat pons have not been described. According to anatomical and physiological studies made mostly in cats, the principal afferents of the pons are from the cerebral cortex, but there is a smaller projection from the deep cerebellar nuclei. The primary and secondary somatosensory areas, the motor and premotor areas, and the visual areas (P. Brodal, '72a,b) are among the major sources of cortical afferents and the projection pattern is a complex topographical one. Stimulation of the cerebral cortex (or of the medullary pyramids) activates pontine cells with a monosynaptic latency, and fast- and slow-conducting cortical fibers are selectively connected with fast- and slow-conducting pontine cells (Allen et al., '75). The cerebellar fibers of the pons are from the lateral and interpositus nuclei, and this projection, like the cortical projection, is topographically organized (Brodal et al., '72a). The pontine fibers terminate in the cerebellum as mossy fibers (Tsukahara et al., '68; Sasaki et al., '70).

Autoradiographic results. In the animals injected on days E14+15 all the neurons of the pons were lightly labelled. The pattern was similar in the E15+16 group, except that at midpontine sagittal levels (that is, at a level bisecting a single pons) a few neurons were intensely labelled adjacent to the pyramidal tract (fig. 8A). The concentration of intensely labelled cells in the latter position increased in the E16+17 group (fig. 9A), while in the E17+18 rats these cells were no longer labelled (fig. 10A). In contrast, in midline and lateral sections the majority of neurons were still labelled in the E18+19 group (fig. 10B); in autoradiograms bisecting the pons labelled cells were mostly restricted to the outer shell (fig. 9B). In the E19+20 rats labelled neurons became restricted at the lateral and medial boundaries of the pons to the shell of smaller cells over the exterior of the pontine gray (fig. 10C). In the E20+21 group only a rare neuron was labelled in some animals, indicating that pontine cytogenesis terminates essentially on day E19. From these observations we conclude that cells of the pontine gray form half-shells around the pyramidal tract from the inside outward as a function of their time of origin.

The proportion of labelled neurons was determined in matched sagittal sections through the approximate middle of the pons in a column at a right angle to the base of the

Fig. 11 Low-power photomicrographs of sagittal sections of the medullary and pontine region in rat embryos of different ages. A, day E17. Solid arrows show schematically the site of origin of inferior olivary neurons and the direction of the migratory stream to the olive (IO) over the *surface* of the medulla. Chronology indicated in brackets. Broken arrows indicate the direction of growth of olivary axons (climbing fibers) in the inferior cerebellar peduncle (ICP) *laterally*, with postulated time of growth. A few pontine cells beneath the pyramidal tract (PY) are shown by oblique arrow. See figure 12A for higher magnification. B, day E18. The site of origin of pontine neurons (same as that of the olivary neurons), their time of origin (later than that of olivary neurons), and gradual settling in the pontine gray (PO), C, solid line indicates the direction of the pontine migratory stream over the surface of the medulla; with estimated time of settling the pontine gray. Broken arrows outline the direction taken by pontine axons (mossy fibers) in the middle cerebellar peduncle (MCP). Note that Purkinje cells (small arrow in the cerebellum) are assembled beneath the EGL at the time pontine axonogenesis begins. Hematoxylin-eosin. Scale: 500 μm.


A
ICP
(E16?)
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PO

Figure 11

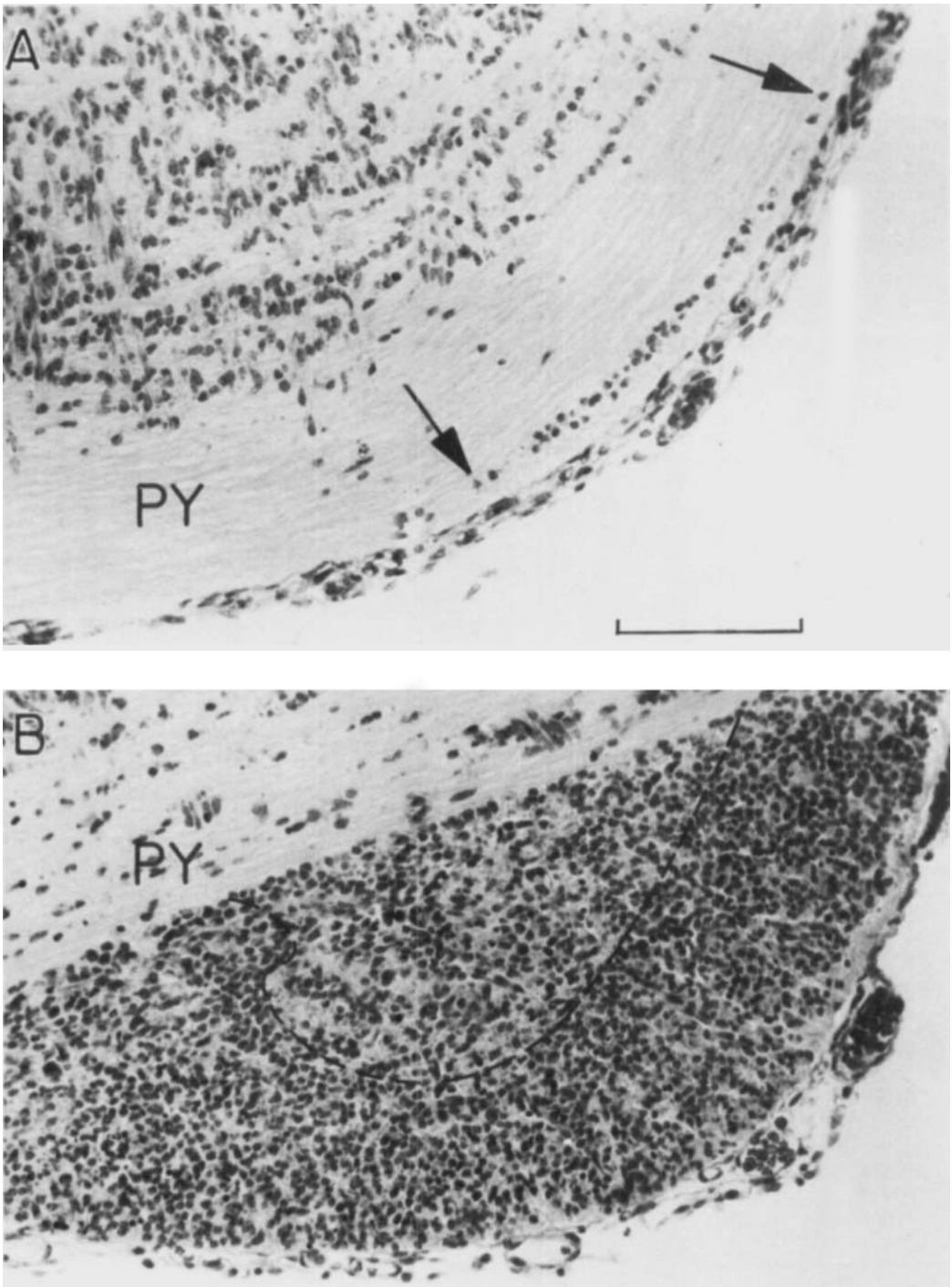

Fig. 12 Sagittal sections through the pontine gray. A, day E17. The pyramidal tract (PY) is contiguous with the pia but in some rats a few pontine cells (arrows) begin to settle. B, day E20. The pontine gray has grown considerably in size beneath the pyramidal tract. Note that the cells forming the outer band of the pons (arrows) are more densely packed (and are presumably less mature) than the cells in the core of the pons. Hematoxylin-eosin. Scale: 100 μm.

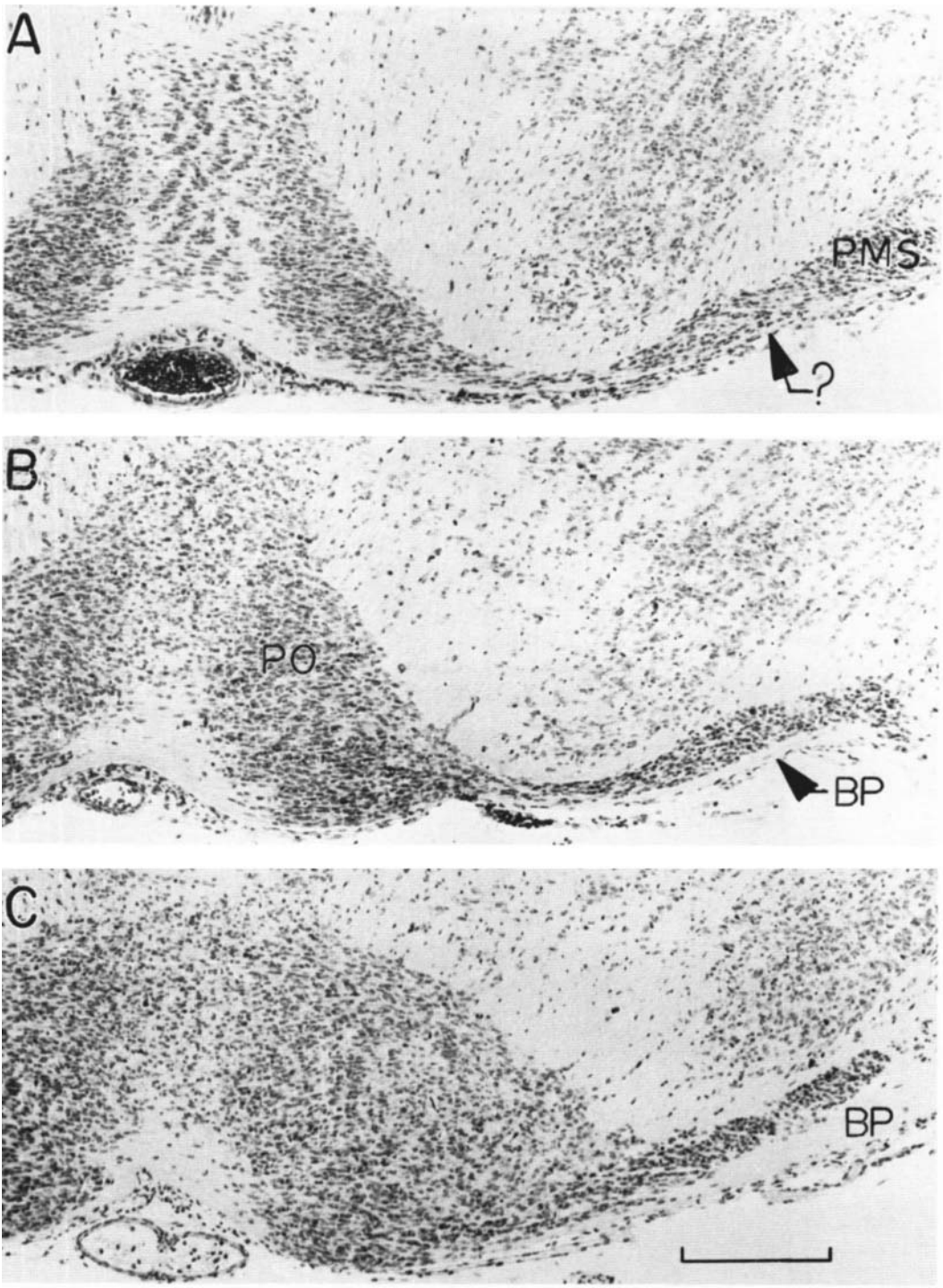

Fig. 13 The pontine gray (PO) and pontine migratory stream (PMS) in day E18 (A), E19 (B) and E20 (C) fetuses. The middle peduncle, or brachium pontis (BP) is not clearly discernible on day E18 (arrow with question mark in A) but grows in width from day E19 onward. Hematoxylin-eosin. Scale: 200 μm.

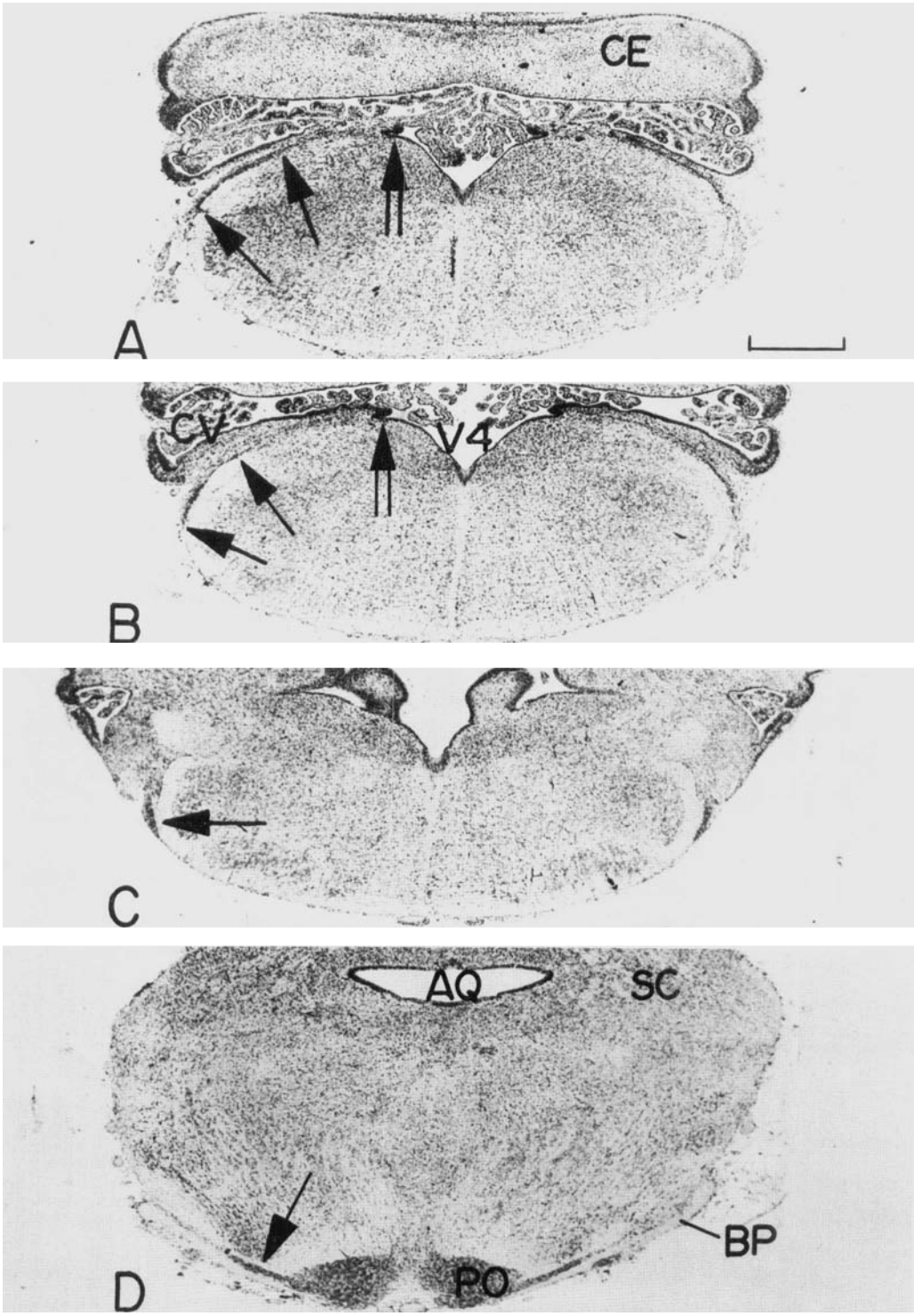

Fig. 14 Day E20 fetus. Coronal sections from caudal (A) to rostral (D) to show the site of origin (double arrow) of the pontine migratory stream (single arrow). The stream originates in the neuroepithelium of the lateral recess of the fourth ventricle (V4) not in the overlying transient cerebellar ventricle (CV). (See also figure 15). Abbreviations: AQ, aqueduct; BP, brachium pontis; CE, cerebellum; PO, pontine gray; SC, superior colliculus. Hematoxylin-eosin. Scale: 500 μm.

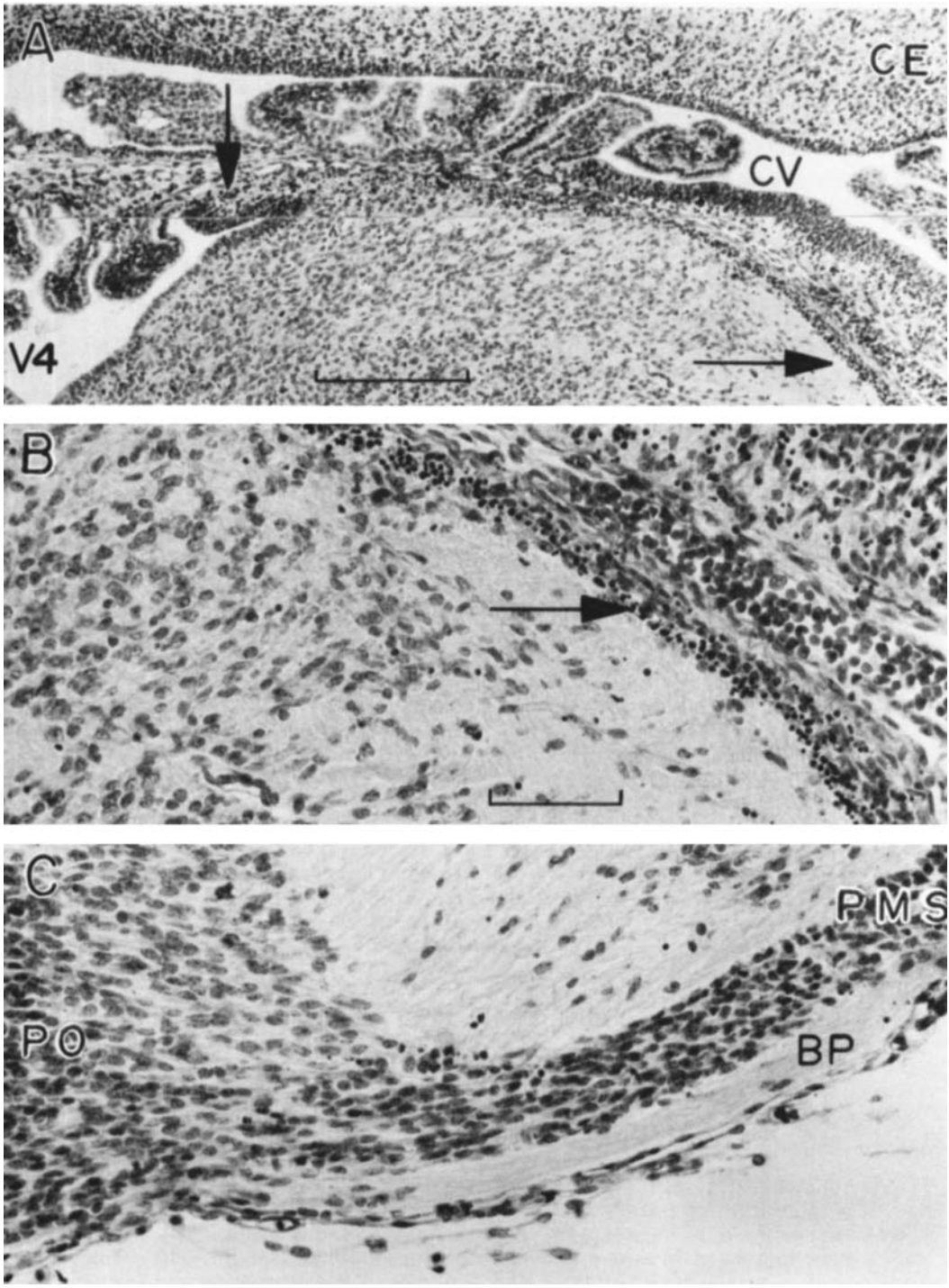

Fig. 15 Day E19, X-irradiated fetus. A, vertical arrow points to the source of the pontine migratory stream in the lateral recess of the fourth ventricle (V4). Cell pyknosis in the proximal portion of the stream (horizontal arrow) is more clearly discernible at higher magnification in B. Closer to the pons (PO), as shown in C, few cells of the pontine migratory stream (PMS) were killed by irradiation. Abbreviations: BP, brachium pontis; CE, cerebellum; CV, cerebellar ventricle. Hematoxylin-eosin. Scales: A, 200 μm; B and C, 100 μm.

pons. At least 100 cells were classified in each animal at × 625. In the E15 + 16 and E16 + 17 groups 97% of the cells were labelled and a correction factor of × 1.03 was applied to the data. The results (fig. 3D) indicated that peak formation time of pontine neurons (disregarding regional differences) was on days E17 and E18, with a small proportion of cells forming on days E16 and E19.

Embryological observations. In coronal sections the incipient pons was recognizable in some day E17 embryos (figs. 11A, 12A) as 1-2 row of cells beneath the bulky pyramidal tract. It was seen in all day E18 embryos as a semilunar structure (fig. 11B) that progressively grew in size on the succeeding days (figs. 11C, 12B). In coronal sections the developing pons (fig. 13) resembles a golf club with its handle pointing dorsolaterally. In coronal (fig. 14) and sagittal sections the "handle" was traced to be the terminal portion of a circumferential stream of spindle-shaped cells originating dorsocaudally in the neuroepithelium of the rhombic fourth ventricle (fig. 14). The site of origin of this *pontine migratory stream,* the pontobulbar body of Essick ('07, '12), is identical with that of the olivary migratory stream (figs. 5, 11).

Three differences were noted in the characteristics of these two migratory streams: (a) the olivary stream is prevalent on days E16-18, the pontine stream on days E17-20; (b) the olivary stream is directed ventrally and partly caudally (fig. 11A), the pontine stream ventrally and acutely rostrally (fig. 11B); (c) whereas the cells of the olivary stream are differentiating, radioresistant elements, cells of the pontine stream are primitive. Mitotic cells were seen in the pontine migratory stream near its ventricular source and over the dorsal and dorsolateral aspect of the medulla, though the frequency of mitotic cells was below that in the external germinal layer of the cerebellum. In fetuses aged days E18-20 most cells in the proximal portion of the pontine migratory stream were killed by X-irradiation (figs. 15A-B). The proportion of pyknotic cells declined along the path, and near the pons and the outer shell or base of the pons pyknotic cells were few in number (fig. 15C). This indicated that cells of the pontine migratory stream are differentiating *en route* to their target.

The pontine migratory stream was disappearing near its source in day E21 fetuses, and near the pons in day E22 fetuses; the stream was no longer recognizable in newborn rats. Between days E18-22 the pontine gray grew in bulk. In younger fetuses all pontine cells had a primitive appearance, although most of them were radioresistant. But by days E19-20 the cells situated in the interior of the pons tended to become somewhat paler, more dispersed and there was some neuropil discernible (figs. 12B, 13B-C, 15C). By the end of the embryonic period and in newborn rats even the cells in the outer shell of the pons lost their primitive appearance.

In day E19 fetuses the onset of pontine axonogenesis was indicated by the appearance of the brachium pontis (fig. 13B). It was composed of few fibers and could be recognized only near its origin underneath the pontine migratory stream (fig. 15C). On the following days the medial cerebellar peduncle grew in bulk (fig. 13C) and in day E21 fetuses the pontine fibers had pierced the migratory stream, apparently swinging dorsally. The earliest age that we could trace the brachium pontis to the cerebellum was on day E22 (fig. 16).

In summary, the following sequence of events was established. The pyramidal tract reaches the future site of the pons by day E17. Pontine cells begin to form in appreciable numbers on the same day in the dorsal medulla and in the proximal portion of the pontine migratory stream, and the cells that begin to arrive in large numbers on day E18 settle adjacent to the pyramidal tract. The later forming cells tend to form shells over the convexity of the pons. The brachium pontis emerges on day E19, presumably representing the axons of those neurons that settled on the previous day. Pontine fibers apparently do not reach the cerebellum until after the formation of the primitive cerebellar cortex (about days E20-21).

DISCUSSION

Previous studies have shown that there is a sequential order in the time or origin of the prenatally (Altman and Bayer, '78) and postnatally (Altman, '69a, '75) forming neurons of the rat cerebellum. The present study established that there is also a temporal order in the production and differentiation of neurons in four of the medullary precerebellar nuclei: the inferior olive, LRN, NRTP, and pons. Information was also gained about the migratory route and settling pattern of neurons in some of these structures and, in at least one instance, on the onset of axonogenesis. The attempt will be made here to correlate the time

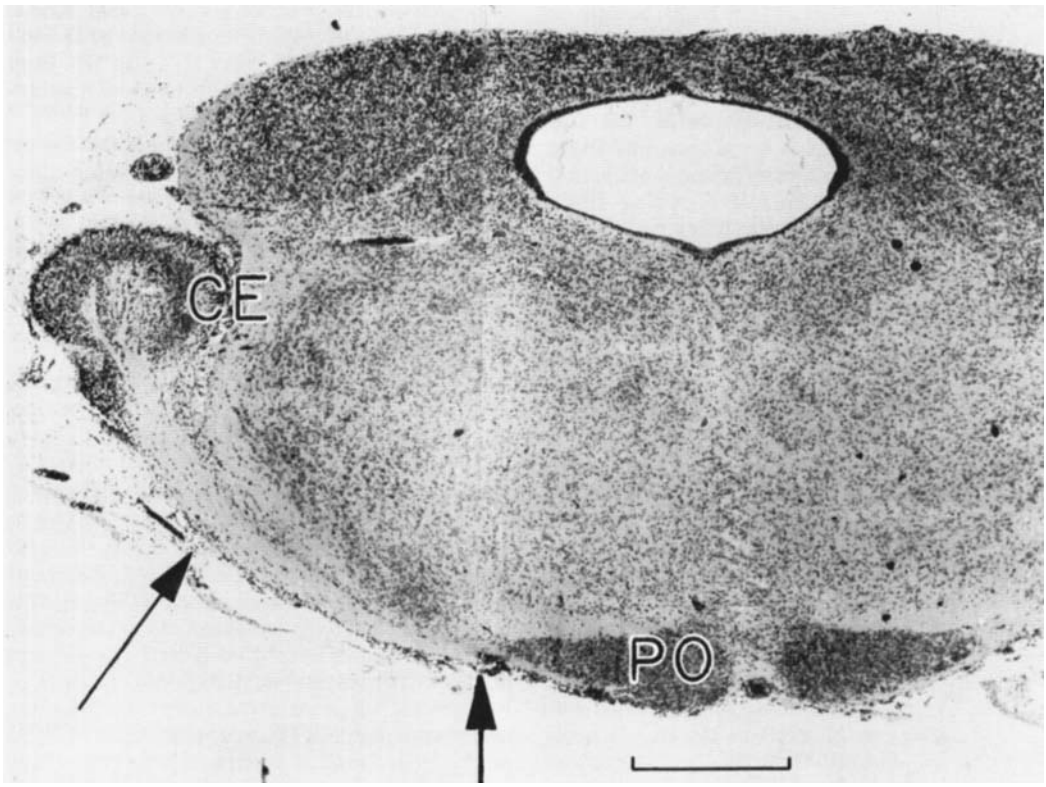

Fig. 16 Coronal section through the tectum of the mesencephalon and the pontine gray (PO) in a day E22 fetus. It was the earliest age that the brachium pontis (arrows) could be traced to the cerebellum (CE). Hematoxylin-eosin. Scale: 500 μm.

of origin of neurons in the cerebellum and the precerebellar nuclei, and examine the possible morphogenetic significance of this temporal orderliness in neuron acquisition.

The inferior olive

Neurons of the olive begin to differentiate at the same time (day E13) as do neurons of the deep cerebellar nuclei, but olivary cytogenesis is declining by the next day when the production of deep neurons peaks (Altman and Bayer, '78, fig. 14). Moreover, more than half of the Purkinje cells form on day E15, after the cessation of olivary neurogenesis. As olivary neurons may be the only source of cerebellar climbing fibers, this suggests the possibility of an early arrival of climbing fibers in the developing cerebellum. This study did not provide direct information about the onset of olivary axonogenesis, but the X-irradiation procedure suggested that the maturation of olivary neurons may already be in progress at the time they leave their site of origin in the medulla and before the onset of Purkinje cell migration (day E16). We observed that the spindle-shaped cells of the olivary migratory stream are radioresistant near their site of origin in the dorsal aspect of the medulla. In this they differed from the pyknotic (radiosensitive) cells of the forebrain rostral migratory stream (Altman et al., '68) the precursors of granule cells of the olfactory bulb (Altman, '69b). These are short-axoned elements that are not likely to produce axons until after their arrival in the olfactory bulb. The cells of the olivary migratory stream resembled, instead, the radioresistant young granule cells of the cerebellum (Altman and Nicholson, '71) that descend in the molecular layer after the production of the distal portion of their axons, the parallel fibers.

We noted in the previous study (Altman and

Bayer, '78) that when the EGL begins to spread over the surface of the cerebellum (day E17) a fibrous layer is present there and it subsequently separates the EGL from the radially migrating Purkinje cells. We suggested that this cannot be a true molecular layer with parallel fibers as granule cells have yet to differentiate. But if climbing fibers form at the medullary level on day E16, before and while the olivary cells are migrating ventrally, then they could reach the cerebellum by day E17. The early arriving climbing fibers could provide a guiding surface for the spreading EGL and arrest the migrating Purkinje cells. Ramón y Cajal ('60) found some evidence that climbing fibers establish early contacts with the transient perisomatic processes of Purkinje cells and, according to a recent study (West and del Cerro, '76), synapses are present in the molecular layer of the rat cerebellum as early as day E19.

The olivary migratory stream was originally recognized in man by His (1890) who distinguished a deep and superficial cell strand *(Grenzplatte* and *Olivstreife).* A similar distinction was made by Essick ('12) in several species, by Harkmark ('54) in the chick, and by Ellenberger et al. ('69) in the rat. Our observations indicated that the two migratory strands are branches of a single superficial stream that originates in the lateral recess of the dorsal medullary neuroepithelium. After separation laterally one branch penetrated and reached the olivary region from a lateral aspect (the deep or submarginal strand) while the other branch, continuing caudally and ventrally, circled around and reached the olivary region from a medial aspect (the superficial or marginal strand). The cells of the submarginal strand appeared to contribute cells to the principal and dorsal accessory nuclei, whereas the cells of the marginal branch to the medial accessory nucleus. Our embryological observations indicated that the submarginal branch supplied cells from a lateral aspect somewhat before the marginal stream supplied cells to the posteromedial region of the olive. In support of this, our autoradiographic data indicated that cytogenesis came to an end earlier in the principal nucleus (day E13) than in the medial accessory nucleus (day E14). This is in disagreement with Kooy's ('17) conclusion that in mammals the medial accessory nucleus develops first, the principal nucleus last, but agrees with the autoradiographic data of Ellenberger et al. ('69) that neurons of the principal olive form earlier (day E14 exclusively) than neurons of the medial accessory nucleus (day E14 and 15). However, there is a discrepancy here in absolute chronology as our data indicate days E13 and 14 for the same events. It may be pointed out that our dating was made in the same material in which the dating of the time of origin of Purkinje cells coincided with reports in the literature, with peak Purkinje cell formation on day E15 (Das and Nornes, '72; Schultze et al., '74) but no labelling of olivary neurons.

The precerebellar reticular nuclei

The time of origin of neurons of the LRN has not been reported before. Taber Pierce ('66) has studied the time of origin of neurons of the NRTP in mice. She suggested that NRTP neurons originate in the basal neuroepithelium of the pontine medulla and migrate ventrally but did not exclude the possibility that some of them originate in the rhombic lip and migrate by way of the pontobulbar body of Essick (the pontine migratory stream). Our autoradiographic studies established that the neurons of the LRN situated in the vicinity of the early-forming olive originate earlier than the neurons of the NRTP situated in the vicinity of the late-forming pontine gray. The earlier described differences in the connections of these two precerebellar reticular nuclei may be relevant in this context. The major input to the LRN is from the spinal cord and its major output is to the spinal receiving areas of the cerebellum; the LRN thus represents a spinal relay system (Clendenin et al., '74; Kitai et al., '74). In contrast, the afferents of the NRTP are partly from the cerebral cortex but more particularly from the cerebellum (by way of the descending limb of the superior cerebellar peduncle); representing a complex cerebellar-reticulo-cerebellar feedback system (Brodal et al., '72b; Brodal and Szikla, '72). It would be interesting to know whether or not the spinal afferents reaching the LRN arrive in the medulla earlier to establish synaptic contacts with their target cells than do the cerebellar fibers reaching the later forming neurons of the NRTP.

The pontine gray

The pontine neurons are the last-forming elements of the precerebellar nuclear system. This may be related to the relatively late arrival of corticofugal fibers. According to available autoradiographic evidence (Berry

and Rogers, '65; Hicks and D'Amato, '68; Bisconte and Marty, '75; Altman and Bayer, in preparation) the pyramidal cells of layer V of the cerebral cortex form about days E16-17. Since the cortical plate is just beginning to form on day E17 (presumably containing mostly layer VI cells) layer V pyramidal cells would be settling in the cortex on day E18 and thereafter (Altman and Bayer, in preparation). The fact that pyramidal tract fibers are present in appreciable concentration on day E17 (fig. 12A) suggests that the axons of layer V pyramidal cells have grown to a considerable length while their cell bodies are still migrating to the cortical plate.

Our results established that neurons of the pontine gray are produced at the same neuroepithelial site as the olivary neurons. But there is no overlap in their time of origin, since olivary cytogenesis ends on day E14 and pontine cytogenesis does not start until day E16. The precursors of pontine neurons migrated by way of the pontine migratory stream, the pontobulbar body of Essick ('07, '12) and Taber Pierce ('66). A very few cells reached the pontine level by day E17, within one day of the onset of their differentiation, and in larger numbers within two days (day E18). This accords with the 48 hour migration time deduced by Taber Pierce ('66) in the mouse. Our data indicated that pontine cytogenesis continued up to day E19 and the cells settled from the vicinity of the pyramidal tract outward. This pattern was the converse of the settling of NRTP neurons where it was the last forming neurons that settled near the pyramidal tract. But it is possible that the settling of NRTP neurons (most of which form by day E16) precedes the arrival of pyramidal tract fibers. Moreover, the NRTP neurons may be intimately related to afferent systems other than the pyramidal tract (see above).

Unlike the cells of the olivary migratory stream, which were radioresistant near their site of origin, the cells destined to reach the pons were mitotically active in the same position and were rendered pyknotic by X-irradiation in the proximal portion of their path. This indicated that the pontine migratory cells are undifferentiated. Indeed, the outgrowth of their axons, forming the middle cerebellar peduncle, did not start for 1-2 days after the settling of the first pontine neurons. An explanation of the difference in these two migratory systems may be the different courses followed to the cerebellum by olivary and pontine axons. The path of the olivary climbing fibers is on the trajectory of the olivary migratory stream (fig. 11A). Therefore, it would be economical if the outgrowth of olivary axons (whether decussating or not) started in the direction of the cerebellum before the cells migrated ventrally, spinning the proximal part of the axon *en route*. In contrast, it would represent a considerable caudal detour if the pontine fibers reached the cerebellum by way of the site of origin of pontine cells (fig. 11C). Instead, axonogenesis starts after the cells settle in the pons. They course for a short distance beneath the pontine migratory stream then swing dorsally, away from the migratory route which is directed more caudally (fig. 11C).

In contrast to the olivary afferents (climbing fibers) that we postulated to play a role in the embryonic morphogenesis of the cerebellum, the pontine afferents terminating as mossy fibers (Bloedel, '73) are not likely to be involved in the early phases of cerebellar development. Pontine neurons form essentially after the production of cerebellar deep neurons and Purkinje cells. In view of the additional delay in pontine axonogenesis, it is unlikely that morphogenetic influences are exerted by pontine mossy fibers before day E22 (fig. 16). It remains to be established when mossy fibers first contact deep neurons; evidently synapses will not be formed with granule cells until after their descent in the granular layer. Previous electron microscopic studies in the rat (Altman, '72c) indicated that the formation of rosettes and glomerular synapses starts on postnatal day 10-12.

Possible implications of the orderly generation of neurons of the cerebellum and of the precerebellar nuclei

Three ideas are prevalent about the morphogenetic mechanisms responsible for the specificity of axonal connections in the central nervous system: (1) chemical affinity between growing axons and their target structures; (2) the presence of guiding surfaces for the channelling of growth cones; (3) temporal order in the arrival of fibers such that the first arriving axons occupy the first available sites, later arriving fibers contact unoccupied or later forming sites. It is likely that all three of these mechanisms (and others as well) are involved in assuring the morphogenesis of selective connections, perhaps to different degrees at different sites and at different stages

of development. The data presented in this and the preceding paper are relevant to the second and third hypotheses. The fact that neurons of the cerebellum and of the precerebellar nuclei are produced sequentially in a precise temporal order allows for the possibility that their axons grow similarly in a precise order. Of course, the temporal relation between cytogenesis, on the one hand, and axonogenesis and dendrogenesis, on the other, need not be a linear or simple one, and definitive statements about the latter must be based on direct evidence gained with appropriate techniques.

Our earlier normative (Altman, '72a,b,c) and experimental (Altman, '76a,b,c) studies indicated that (a) the neurons of the molecular layer (basket and stellate cells) are generated sequentially from the bottom upward; (b) likewise, the parallel fibers of the descending granule cells are stacked from the bottom upward and form synapses in the same order; and (c) the upward expansion and normal branching of the Purkinje cell dendritic arbor is morphogenetically dependent on the orderly maturation of the stacked components of the molecular layer. Is the order of maturation of the cerebellocortical interneuronal system morphogenetically coupled with the orderly maturation of the precerebellar nuclei? If this is so, and there is a stratification in the mossy fiber-parallel fiber relay to the dendritic arbor of the Purkinje cell, what may be the functional significance of such a stratification?

Figure 17 summarizes some known and some postulated chronological relations in precerebellar and cerebellocortical neurogenesis. (Since the growth of climbing fibers follows the expanding Purkinje cell dendrite, it will be assumed that these afferents from a single source, the inferior olive, are not part of the stratified input system.) We mentioned earlier that mossy fiber synapses with granule cells first appear in the rat about postnatal day 10-12 (Altman, '72c). There is experimental evidence (Altman, '73) that the growth of mossy fibers in the cerebellar cortex is arrested upon contact with granule cells; when granule cell production and descent is delayed by transient decimation of the EGL by X-irradiation, the mossy fibers continue their growth into the molecular layer and form synapses there with reciprocally arrested granule cells in ectopic zones. Therefore, if there is a chronological relationship between cytogenesis and axonogenesis, the first complement of descending granule cells, those with parallel fibers in the lower molecular layer, should contact mossy fibers from early differentiating precerebellar nuclei, whereas later descending granule cells, with parallel fibers in the middle or upper molecular layer, should contact mossy fibers from late-forming precerebellar nuclei. Since we have little information at present about the time of origin of neurons in the spinal precerebellar nuclei, this hypothetical relationship of the chronologically stratified parallel fiber system of the molecular layer will be restricted to three sequentially produced precerebellar medullary nuclei: the LRN, the NRTP and the pontine gray (fig. 17).

The hypothesis implies that mossy fiber input from the LRN should have preferential influence on the lower branchlets of Purkinje cells and on basket cells, whereas mossy fiber input from the pons should influence the upper dendritic field of Purkinje cells. The hypothesis is testable as it predicts, for instance, that current density profiles in the molecular layer will differ as a consequence of stimulation of the LRN versus the pontine gray. Relevant in this context are behavioral studies from our laboratory which indicate that interference with the development of the entire parallel fiber system of the molecular layer (schedules of repeated focal X-irradiation of the cerebellum started within a few days after birth) produces profound motor deficits (Anderson and Altman, '72) and deficits in spatial discrimination learning (Bulut and Altman, in preparation). In contrast, interference with the development of the upper molecular layer (X-irradiation started on postnatal day 12) produces no gross motor deficits but profound alterations in activity level (i.e., hyperactivity, as measured in an open field or activity wheels) and spatial learning deficits in either a T maze or a multiple water maze (Pellegrino and Altman, submitted for publication; Browne and Altman, work in progress). Our anatomical and behavioral data led to the tentative hypothesis that the input to the lower molecular layer (with its postulated predominant spinal connections) is concerned with the executive task of *motor coordination,* whereas the upper domain of the Purkinje cell dendrite (with its possible selective cerebral connections) is concerned with the legislative task of *action coordination.*

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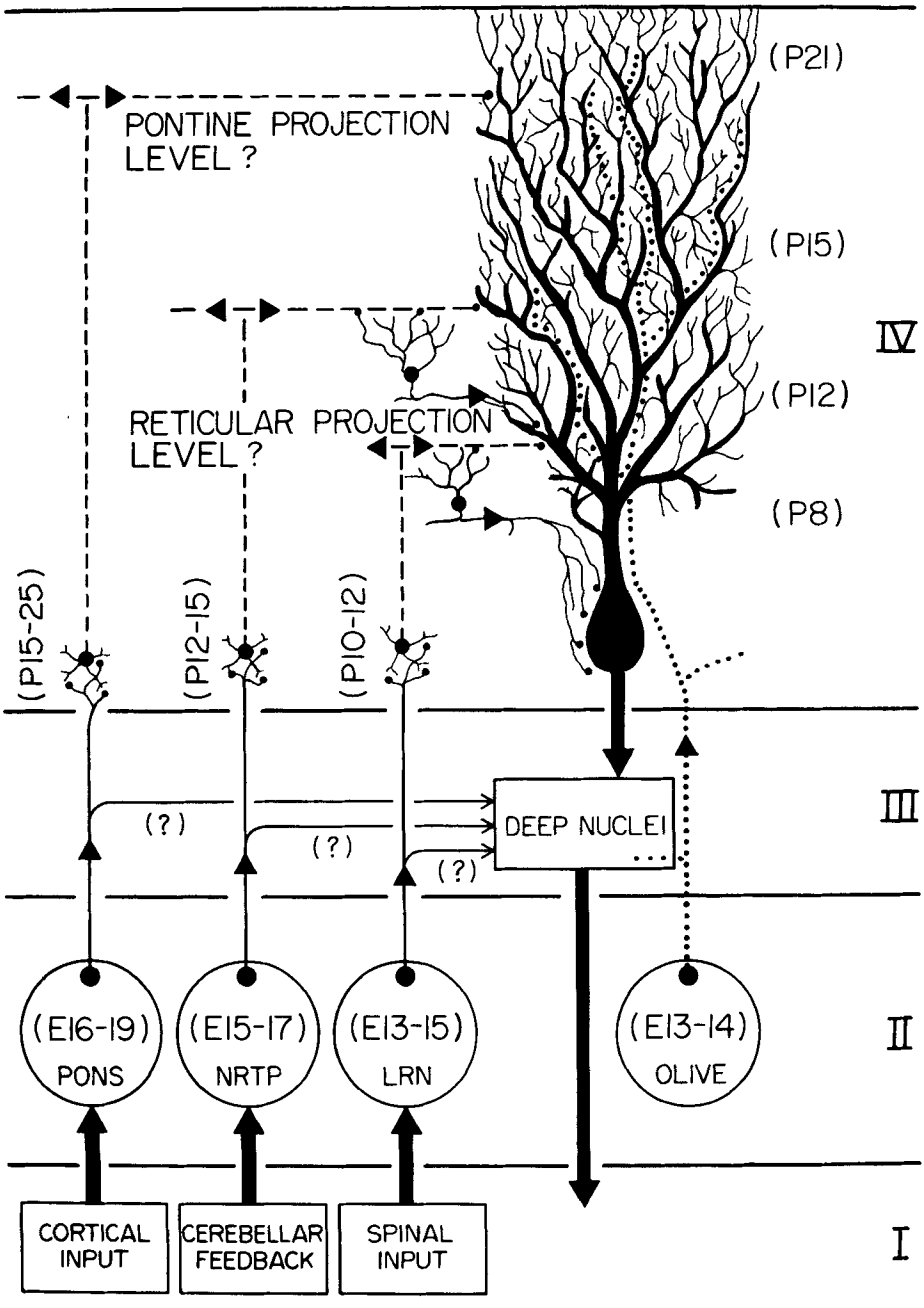

Fig. 17 Summary diagram of some temporal relationships between cerebellar and precerebellar morphogenesis, and of some correlations in the development of this system. Roman numerals on the right panel indicate the four levels of this system: I, extracerebellar input lines or feedback loops: II, some of the medullary precerebellar nuclei; III, the deep cerebellar nuclei; and IV, the cerebellar cortex. Bracketed numbers at level II refer to the embryonic days of the time of origin of neurons (based on the present study). Bracketed numbers on the right at level IV refer to the estimated postnatal days of maturation of the Purkinje cell dendritic arbor (based on Altman, '72b); on the left they refer to the estimated dates of granule cell synapse formation with mossy fibers (based on Altman, '72c). We have no data regarding the establishment of connections with the deep cerebellar nuclei (level III). The indicated selective connection of the reticular nuclei and the pontine gray with the lower and upper domain of Purkinje cell dendrites is hypothetical.

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