Development of the Diencephalon in the Rat

I. AUTORADIOGRAPHIC STUDY OF THE TIME OF ORIGIN AND SETTLING PATTERNS OF NEURONS OF THE HYPOTHALAMUS

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ABSTRACT Groups of pregnant rats were injected with two successive daily doses of ^{3}H-thymidine from gestational day 13 (E13+14) until the day before birth (E21+22). This double labelling procedure was combined with an injection schedule of a single day delay between groups (E13+14; E14+15; E15+16..). The two injections assured the comprehensive labelling of practically all neurons of a given structure prior to the onset of their differentiation (comprehensive labelling), whereas the progressive daily delay in injections made it possible to estimate the proportion of neurons formed in various regions of the hypothalamus on a single day. Hypothalamic areas or nuclei were assigned into four classes on the basis of their cytogenetic isochronicity. Structures composed of the earliest arising (class 1) neurons constitute a lateral tier that includes the lateral preoptic and lateral hypothalamic areas, and the lateral mammillary nucleus. Structures composed of early arising (class 2) neurons form a heterogeneous collection of nuclear systems, including the paraventricular, internuclear and supraoptic magnocellular neurons, and several intermediate tier nuclei of the anterior and posterior hypothalamus. The late arising (class 3) and latest arising (class 4) nuclei constitute a periventricular system anteriorly and a more extensive region posteriorly. The latter two nuclear systems may constitute the hypophysiotropic area of the hypothalamus. The nuclei of the mammillary system, which are produced sequentially, are distinguished from other hypothalamic structures by their more rapid generation time. Internuclear labelling gradients were used to infer the neuroepithelial site of origin and settling pattern of neurons. Common sites of origin were indicated for the following structures: the magnocellular neurohypophysial neurons; the neurons of the dorsomedial and ventromedial nuclei; and the neurons of the tuberomammillary and arcuate nuclei. The sites of origin of these groups of nuclei were related to specialized ventricular linings in the mature hypothalamus.

This paper is the first of a series dealing with the development of the rat diencephalon. The objective is to provide a comprehensive description of the ontogeny of the major diencephalic nuclei and systems by gathering information about the time of origin of cells, their site of origin, route of dispersion, pattern of settling, and mode of differentiation. The time of origin of identified neurons is determined quantitatively by using autoradiograms of adult rats in which proliferating cells were tagged with ^{3}H-thymidine during embryonic or perinatal development. Inferences about the site of origin and mode of settling of identified classes of neurons is made in selected cases by correlating embryonic and autoradiographic data. There are considerable age-dependent differences along the third ventricle in the size and shape of germinal neuroepithelia, with apparent peak levels of activity at one site when there is a decline at another. The resultant neuroepithelial mosaic provides the first clue in relating autoradiographically dated, specific classes of neurons

to their possible sites of production. The presence of a zone of primitive cells or a stream of migratory cells, which can be rendered pyknotic by low-level X-irradiation, often link the presumed production sites and settling regions. Finally, gradients in labelling patterns within a nucleus or nuclear system (lateromedial, mediolateral, dorsoventral, ventrodorsal, etc.) are interpreted as directional "arrows" of settling patterns. The onset of neuronal differentiation is deduced from the cessation of radiosensitivity at a particular locus following X-irradiation, and the mode of cell differentiation is studied in a developmental series of embryonic and postnatal brains that were prepared with the Golgi technique. The initial three papers of this series deal with the development of the neurons of the hypothalamus and the cells of the specialized ventricular linings of the "endocrine hypothalamus." Subsequent papers will deal with the ontogeny of the circumventricular organs of the diencephalon, and the development of the thalamus and subthalamus.

The autoradiographic investigation of the time of origin of neurons of the hypothalamus has been a neglected subject. The two reports presently available are the pioneering work by Ifft ('72) in the rat, and a study by Shimada and Nakamira ('73) in the mouse. There is a considerable literature on the embryonic development of the hypothalamus and it will be referred to in the second paper of this series (Altman and Bayer, '78b).

MATERIALS AND METHODS

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. The progeny of at least two dams/injection group 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 three planes and every fifteenth section was saved. Successive sections were stained with cresyl violet and hematoxylin-eosin for examination without nuclear emulsion or were prepared for autoradiography. The latter procedure has been described elsewhere (Altman, '64). Briefly, deparaffinized sections were coated with Kodak NTB-3 emulsion in the dark, exposed for 90 days with a desiccant, developed with D-19, and stained with hematoxylin-eosin.

Coronal sections from six male rats per injection group were used for quantitative purposes. The proportion of labelled to unlabelled neurons was determined in identified areas or nuclei of the hypothalamus at approximately five levels (A7.4, 6.6, 5.8, 5.6, and 3.8) according to the stereotaxic atlas of de Groot ('59). We consulted also the atlas of the hypothalamus of the rat by Christ ('69) and of the mouse by Broadwell and Bleier ('76) and made some modifications (figs. 5-9). Each area or nucleus was scanned at 625 × magnification with the aid of an ocular grid oriented to traverse strips through a given structure, at a right angle to gradients of cell labelling where such was indicated. In all instances a minimum of 100 (up to several 100) cells were classified in each structure at a given level per animal. The estimation of the proportion of cells differentiating (ceasing to divide) on a particular day was based on the progressively delayed comprehensive 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 2 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 E15 are determined as follows: E15 = (E15+16) − (E16+17).

The quantitative data obtained about the time of origin of different nuclear groups allowed us to identify *internuclear* gradients through the hypothalamus. As a graphic aid to visualize these gradients we attempted to construct "isochronic maps," taking into consideration not only the time of onset or cessation of cytogenesis but also their time span (rapid or prolonged). The categories of this classification, which were arrived at post hoc, are described in the relevant sections of the RESULTS. Some quantitative information was also obtained about *intranuclear* gradients in the longitudinal direction in those structures that spanned through two or more of the coronal levels scanned. But, in general, the presence or absence of gradients in any direction

Abbreviations

a, anterior
AC, anterior commissure
AM, amygdala
ANB, anterobasal nucleus
ca, caudal
CP, cerebral peduncle
DMH, dorsomedial nucleus (hypothal.)
do, dorsal
DPA, dorsal preoptic area
F, fornix
IMN, intermediate mammillary nucleus
LAE, laminated epithelium
LHA, lateral hypothalamic area
LMN, lateral mammillary nucleus
LPA, lateral preoptic area
ME, median eminence
MFB, medial forebrain bundle
MHA, medial hypothalamic area
MMn, medial mammillary nucleus
MPA, medial preoptic area
mp, mammillary peduncle
MPN, median preoptic nucleus
OC, optic chiasma
OT, optic tract
PAR, paraventricular nucleus
  mc, pars magnocellularis
  pc, pars parvicellularis
PER, periventricular area
PMD, premammillary nucleus, pars dorsalis
PMN, principal mammillary nucleus
  c, pars centralis
  v, pars ventralis
pmt, principal mammillary tract
PMV, premammillary nucleus, pars ventralis
POH, posterior hypothalamic nucleus
PPA, preoptic periventricular area
ro, rostral
SCN, suprachiasmatic nucleus
SMN, supramammillary nucleus
SON, supraoptic nucleus
STN, subthalamic nucleus
TAN, tanycyte-lined region, third ventricle
TUM, tuberomammillary nucleus
ve, ventral
VMD, ventromedial nucleus, pars dorsalis
VMV, ventromedial nucleus, pars ventralis
ZI, zona incerta

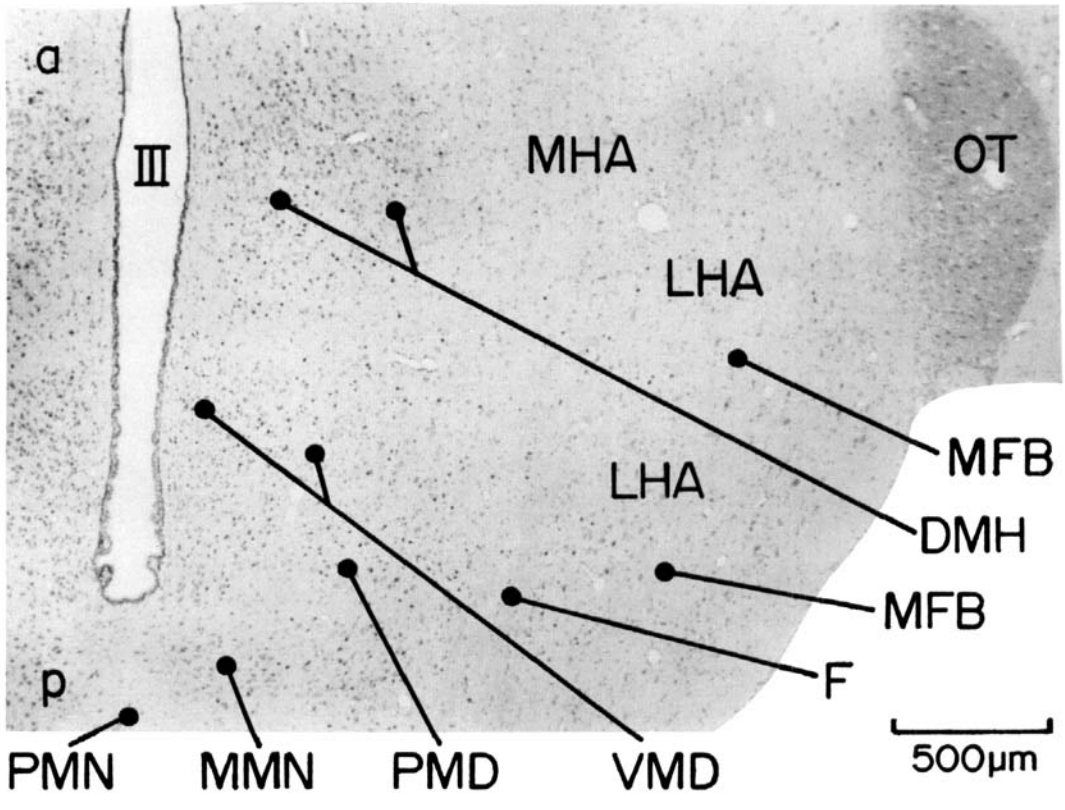

Fig. 1 Low power autoradiogram of a dorsal horizontal section through the hypothalamus of a 60-day-old rat injected on days E15+16. A portion is shown at higher magnification in figure 2.

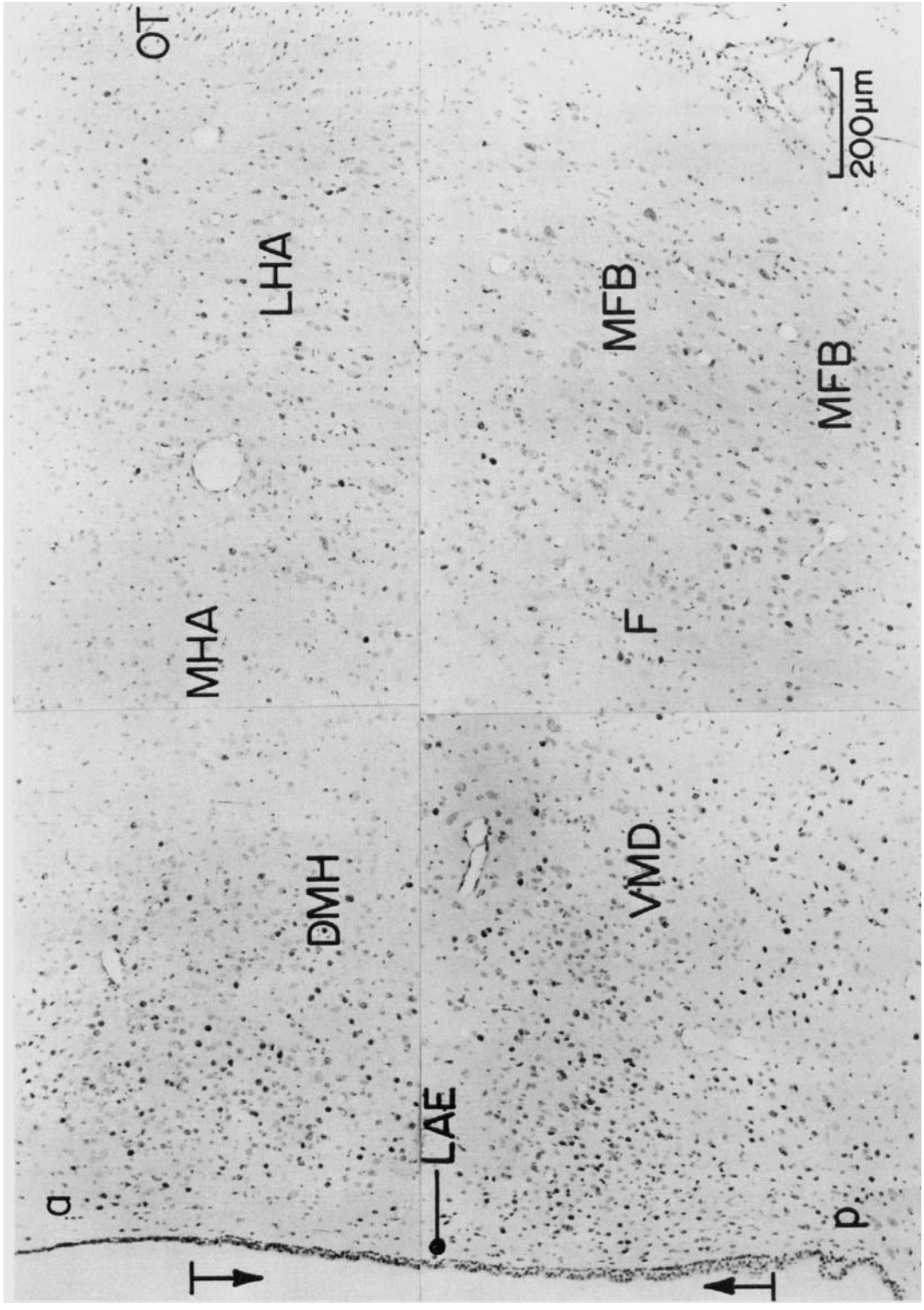

Fig. 2 The general lateral-to-medial gradient of labelling is shown in this horizontal section of a E15+16 rat. Arrows outline the extent of the laminated epithelium of the third ventricle.

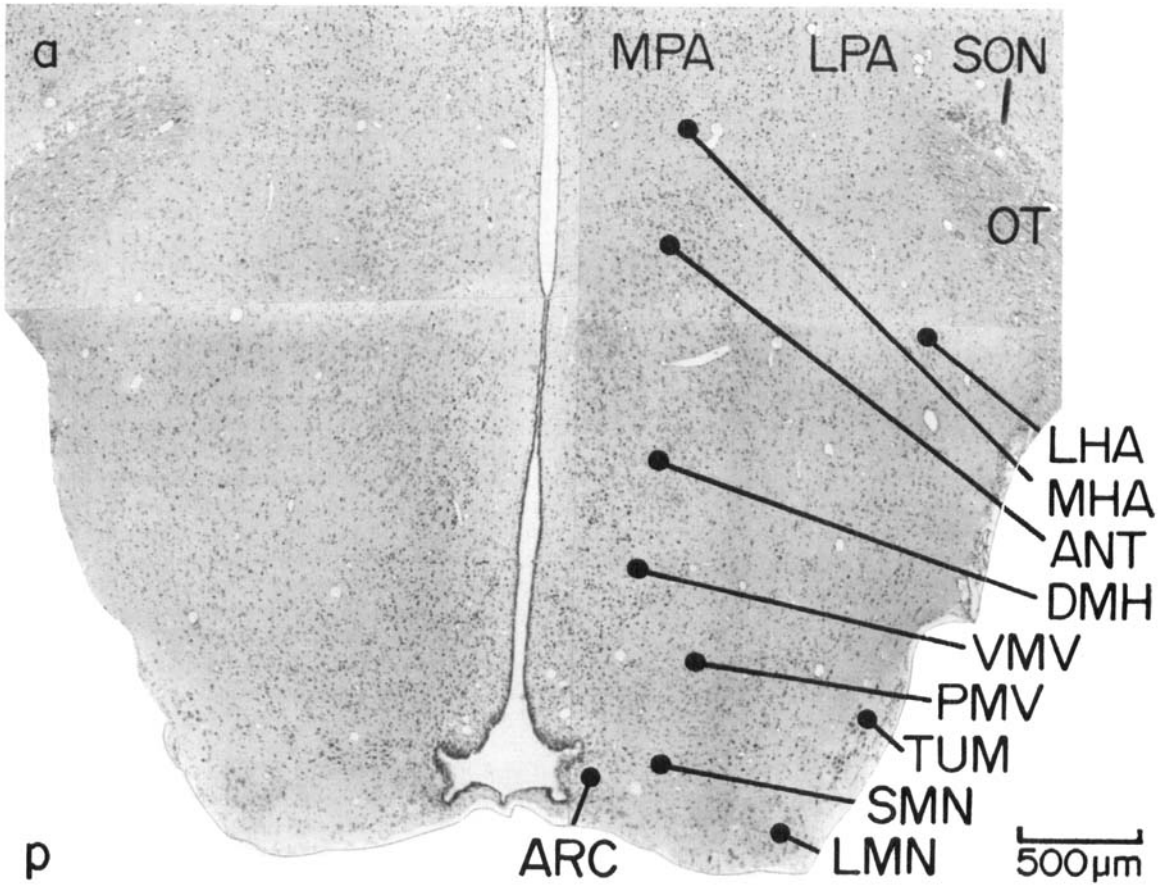

Fig. 3 Low power photomicrograph of a ventral horizontal section from a rat injected on days E16+17. A portion is shown at higher magnification in figure 4.

was studied qualitatively and documented photographically in sections cut in the coronal, sagittal and horizontal planes.

RESULTS

1. *General autoradiographic observations*

In practically all regions of the hypothalamus most neurons were intensely or lightly labelled in animals injected on days E13+14. But regional differences began to appear in labelling patterns in rats injected on days E14+15 and thereafter. As an illustration, figures 1-2 show the labelling patterns in a horizontal section through the mid-dorsal hypothalamus in a day E15+16 animal. Few neurons are labelled in the lateral and medial hypothalamic areas but many in several more medially situated nuclei: the dorsomedial, ventromedial (pars dorsalis), premammillary (pars dorsalis), and medial mammillary nuclei. At this level in this injection group the observations suggest a lateral-to-medial generation gradient. However, in a more ventrally sectioned horizontal level in a day E16+17 rat the gradient appears to be a more complex one (figs. 3,4). For instance, in two adjacent laterally situated, large-celled nuclei of the caudal hypothalamus, the neurons of the lateral mammillary nuclei are unlabelled (its neurons form on days E13-15; fig. 9) whereas most neurons of the tuberomammillary nucleus are labelled (its neurons form on days E15-18; fig. 8). In general, the labelling patterns of all nuclei showed low variability between animals of the same injection history.

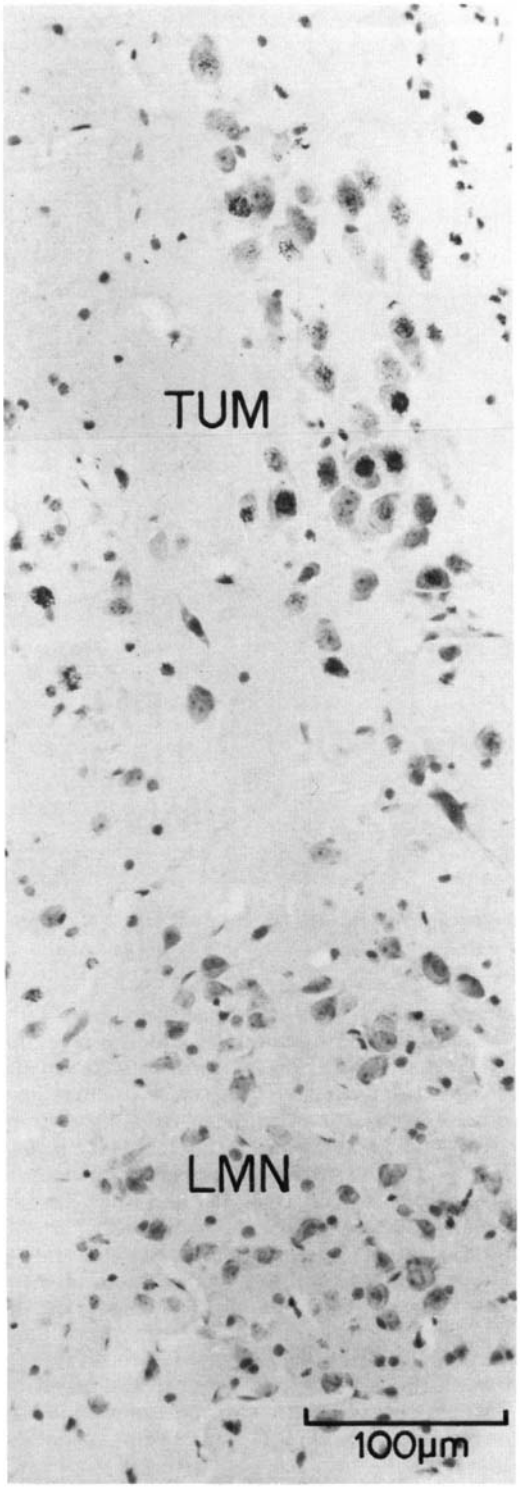

Figure 4

2. *Quantitative autoradiographic data*

Level A7.4. This was the most rostral level examined (fig. 5A). Clearly delineated hypothalamic or preoptic nuclei at this level are the supraoptic, suprachiasmatic and median preoptic nuclei. Less clearly delineated are the lateral, medial and dorsal preoptic areas. The results (fig. 5B) indicated that neuron formation began *before* day E13 ("E12") and terminated on day E15 in the lateral and dorsal preoptic areas; these were classified as the earliest-forming, or class 1, hypothalamic regions. In the supraoptic and median preoptic nuclei, and the medial preoptic area neuron formation began on day E13 and was completed either on day E15 or E16; these were designated as early-forming, or class 2 regions. Finally, in the suprachiasmatic and preoptic periventricular nuclei neurons were still forming in appreciable numbers on day E17; these structures were designated as late-forming, or class 3 regions. Outlines encircling the contemporaneously forming, or isochronic, structures suggest a topologically distorted lateral-to-medial internuclear gradient at this level of the hypothalamus.

Level A6.6. At this level (fig. 6A) four regions were evaluated: the magnocellular part of the paraventricular nucleus, the anterior hypothalamic nucleus, the lateral hypothalamic area, and the arcuate nucleus (rostral portion). Labelling pattern in the periventricular nucleus was judged to be similar to that more rostrally. Neuron formation (fig. 6B) in the lateral hypothalamic area was similar to that of the dorsal preoptic area rostrally (class 1). The magnocellular paraventricular nucleus resembled the magnocellular supraoptic nucleus (class 2). The anterior nucleus neurons were also classified as class 2, although the proportion of neurons formed on day E16 was much higher than in the previous class 2 nuclei considered. The arcuate nucleus neurons were classified as class 3. The results suggest again a lateral-to-medial internuclear gradient.

Level A5.8. Neurons at this level (fig. 7) were classified as follows: lateral hypothalamic area, class 1; ventral portion of the ventromedial nucleus, class 2; dorsal portion of the ventromedial nucleus, the dorsomedial nucleus, and the arcuate nucleus, class 3. The in-

Fig. 4 At this level the gradient is caudal-to-rostral as the lateral mammillary nucleus neurons are unlabelled, the tuberomammillary neurons are labelled.

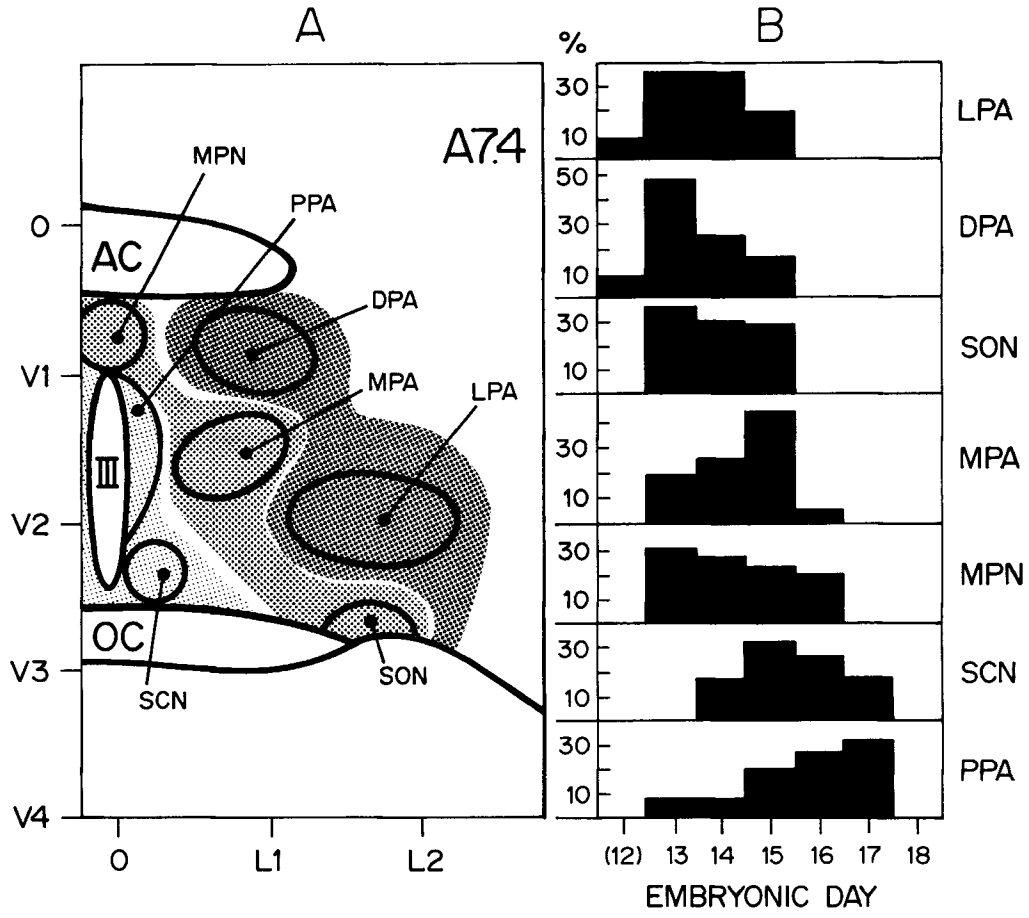

Fig. 5 Percentage of cells formed on days indicated (B) and internuclear gradient (A) at anterior level A7.4 (de Groot, '59).

ternuclear lateral-to-medial gradient was partly attributable to the intranuclear gradient in the ventromedial nucleus.

Level A4.6. Neuron origin at this level (fig. 8) differed from that more rostrally. There were no class 1 structures (although the nonhypothalamic zona incerta and subthalamic nucleus were so classified laterally; work in progress). The following nuclei were categorized as class 2: posterior hypothalamic, premammillary dorsalis, and premammillary ventralis. These structures had the same pattern of cell acquisition as the ventral part of the ventromedial nucleus more rostrally, forming an isochronic vertical plate (fig. 7A). There were no class 3 structures at this level. The neurons of the large-celled tuberomammillary nucleus, and of the arcuate nucleus were classified as very-late forming, or class 4 neurons (onset of neurogenesis day E15 or 16, cessation on day E18 or thereafter). We can conceptualize the internuclear gradient at this level as lateral-to-medial, if we include the zona incerta and substantia nigra, or as dorsal-to-ventral, if hypothalamic structures alone are considered.

Level A3.8. This was the most caudal hypothalamic level examined (fig. 9). The large-celled lateral mammillary nucleus is composed of class 1 neurons. The intermediate-sized neurons of the medial mammillary nucleus form much later, but before the small-sized neurons of the supramammillary and principal mammillary nuclei. Because of the uniquely rapid pattern of cell formation in all but the lateral mammillary nucleus (with 60-

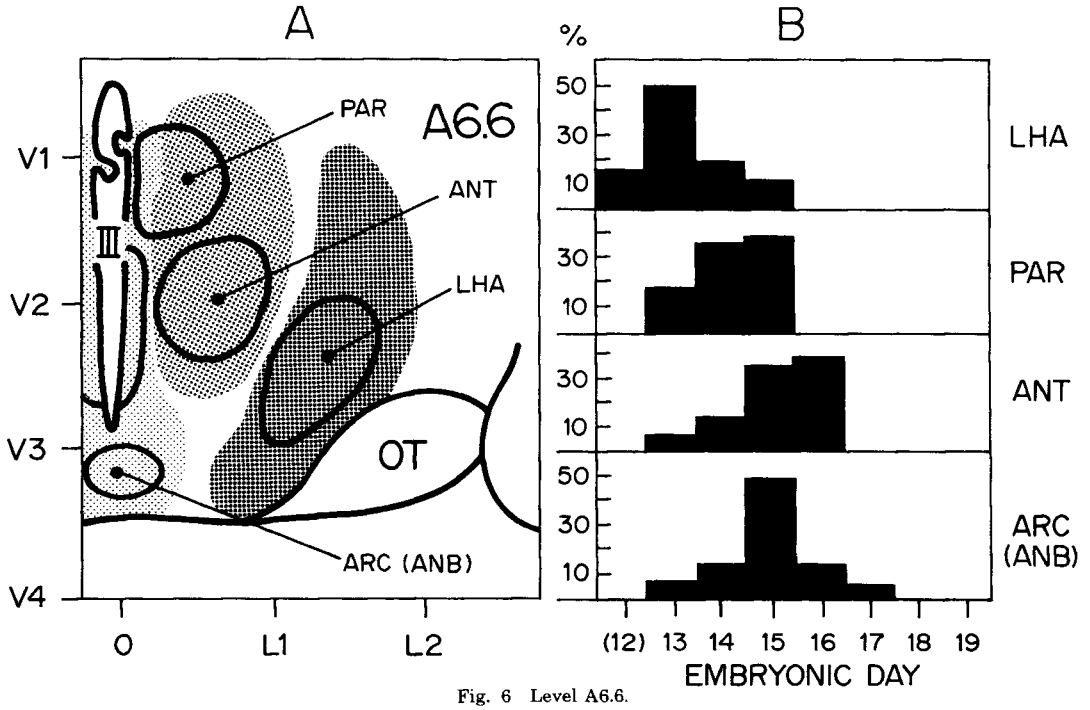

Fig. 6 Level A6.6.

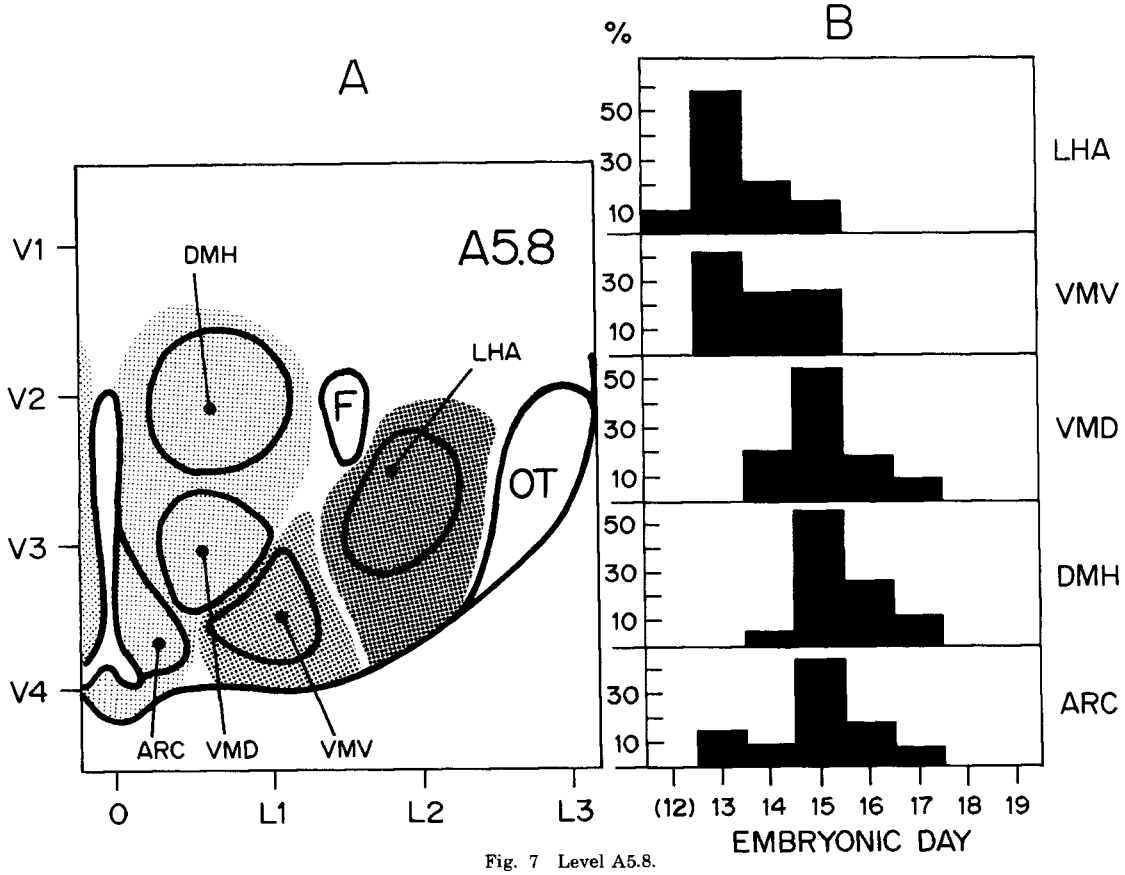

Fig. 7 Level A5.8.

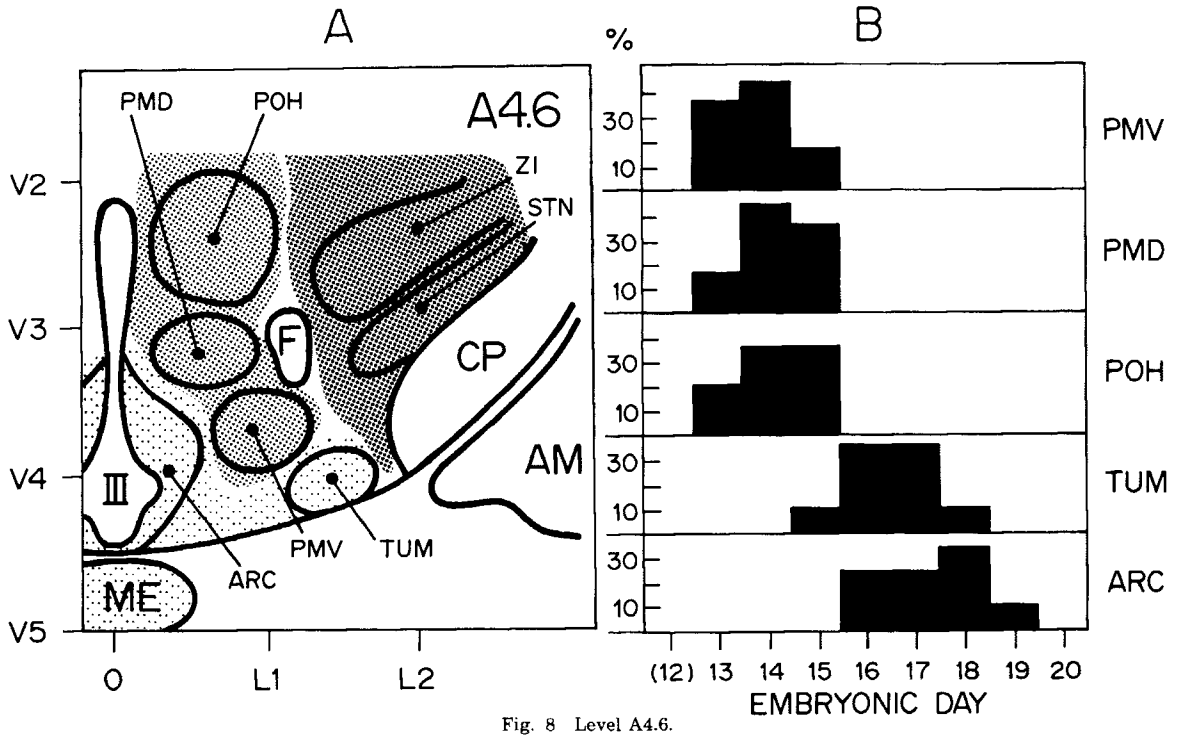

Fig. 8 Level A4.6.

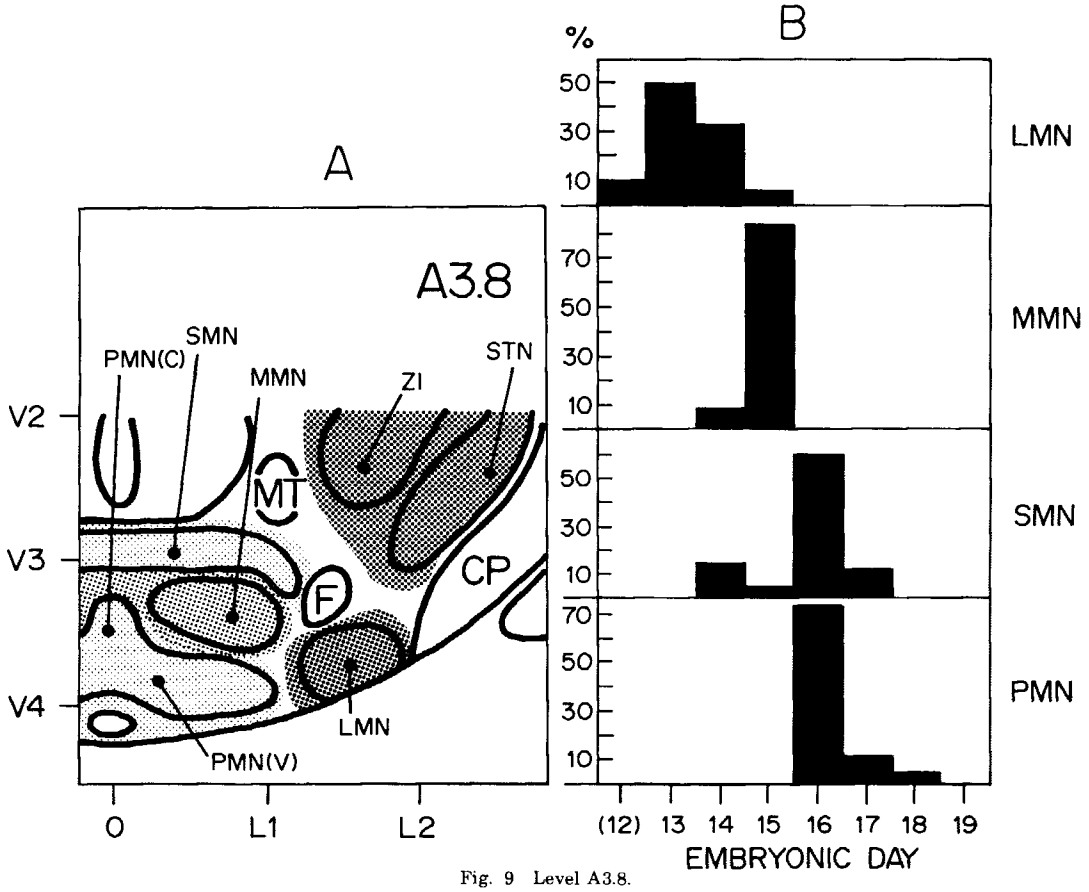

Fig. 9 Level A3.8.

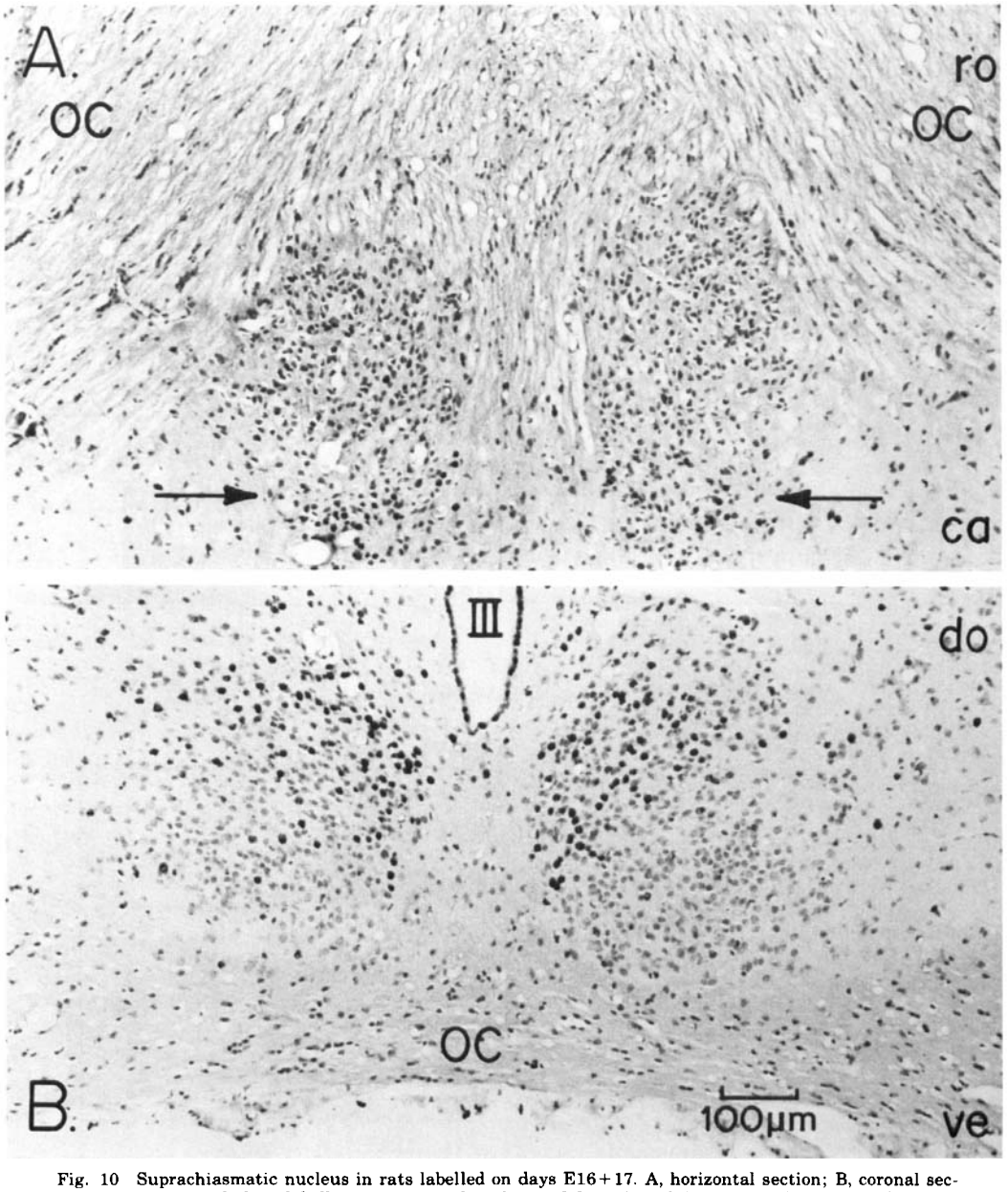

Fig. 10 Suprachiasmatic nucleus in rats labelled on days E16+17. A, horizontal section; B, coronal section. In the horizontal plane labelling is restricted to the caudal portion of the nucleus (arrows in A); in the horizontal section to its dorsomedial aspect.

80% of the cells forming on a single day), the cells of the medial mammillary nucleus are designated as class 2(M) neurons, the cells of the supramammillary and principal mammillary nuclei as class 3(M) neurons. The lateral mammillary nucleus fits the common lateral-to-medial internuclear gradient, but the other mammillary nuclei appear to form a dorsally and ventrally oriented sandwiched pattern.

3. *Intranuclear gradients*

Suprachiasmatic nucleus. In this nucleus

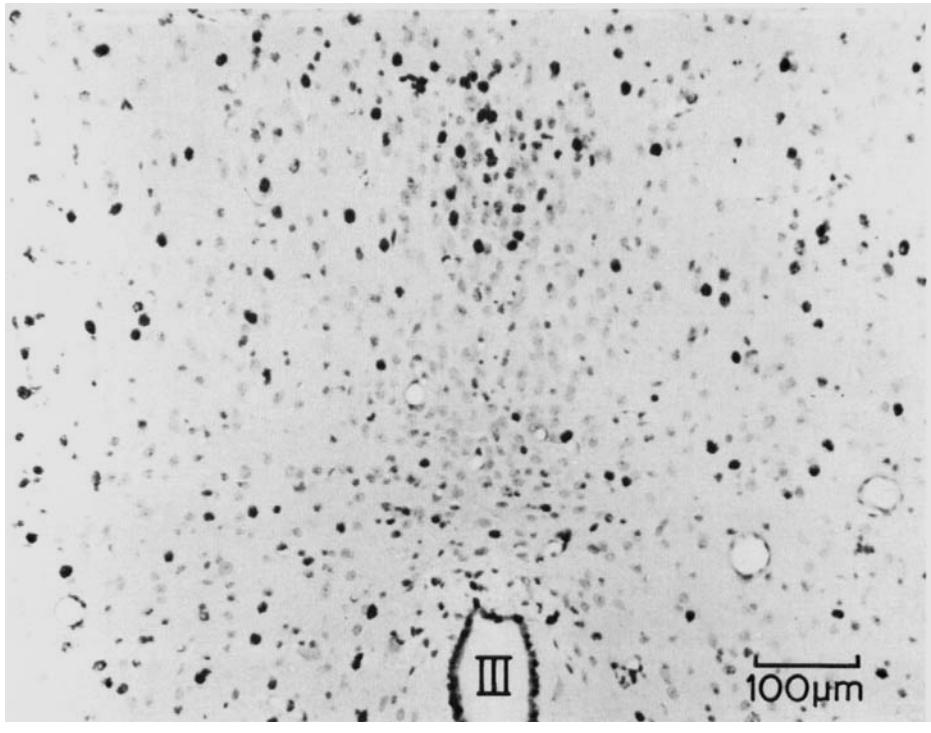

Fig. 11 Median preoptic nucleus in rat labelled on days E15+16.

a consistent gradient was observed. In horizontal sections the pattern is rostral-to-caudal, and in E16+17 rats labelled cells are mostly restricted to the caudal tip of the nucleus (fig. 10A); in coronal sections the gradient is oblique lateroventral-to-mediodorsal (fig. 10B). It appears that the earliest forming cells settle anteriorly and ventrally in the wedge-shaped region of the optic chiasma, and the last forming cells remain farthest from this region near the floor of the third ventricle.

Median preoptic nucleus. The consistent, though not pronounced, gradient in this small dorsal nucleus is ventral-to-dorsal (fig. 11). This is the opposite of what would be expected if its cells originated in the underlying roof of the anterior third ventricle neuroepithelium. Suggestive embryonic evidence will be presented in the next paper (Altman and Bayer, '78b) that the nucleus is not diencephalic but originates from the overlying, anteriorly situated neuroepithelium of the foramen of Monro.

Supraoptic nucleus. Cell formation comes to an end in this nucleus on day E15 (fig. 5). In E14+15 rats almost all the neurons are labelled in the ventromedial aspect of the nucleus close to the optic chiasma whereas many of the cells farther dorsolaterally are unlabelled. In E15+16 rats most of the neurons are unlabelled in the latter region (figs. 12B,D) but there is a fair concentration of them in the vicinity of the optic tract fibers ventromedially (figs. 12A,C). This labelling pattern suggests that the earliest forming supraoptic neurons settle farthest from the optic tract fibers and the chiasma, and the latest arriving elements end up closer to the optic tract. This is the converse of the pattern noted in the suprachiasmatic nucleus.

Internuclear magnocellular neurons. Cells similar in appearance to the neurosecretory neurons of the supraoptic and paraventricular nuclei, referred to as internuclear cells (Defendini and Zimmerman, '78), were regularly seen in small scattered groups around blood vessels in an imaginary trajectory between the supraoptic nucleus and the dorsal aspect of the hypothalamic third ventricle (figs. 13A,B). Many of these cells were labelled in the E15+16 animals (figs. 14A,B) and a

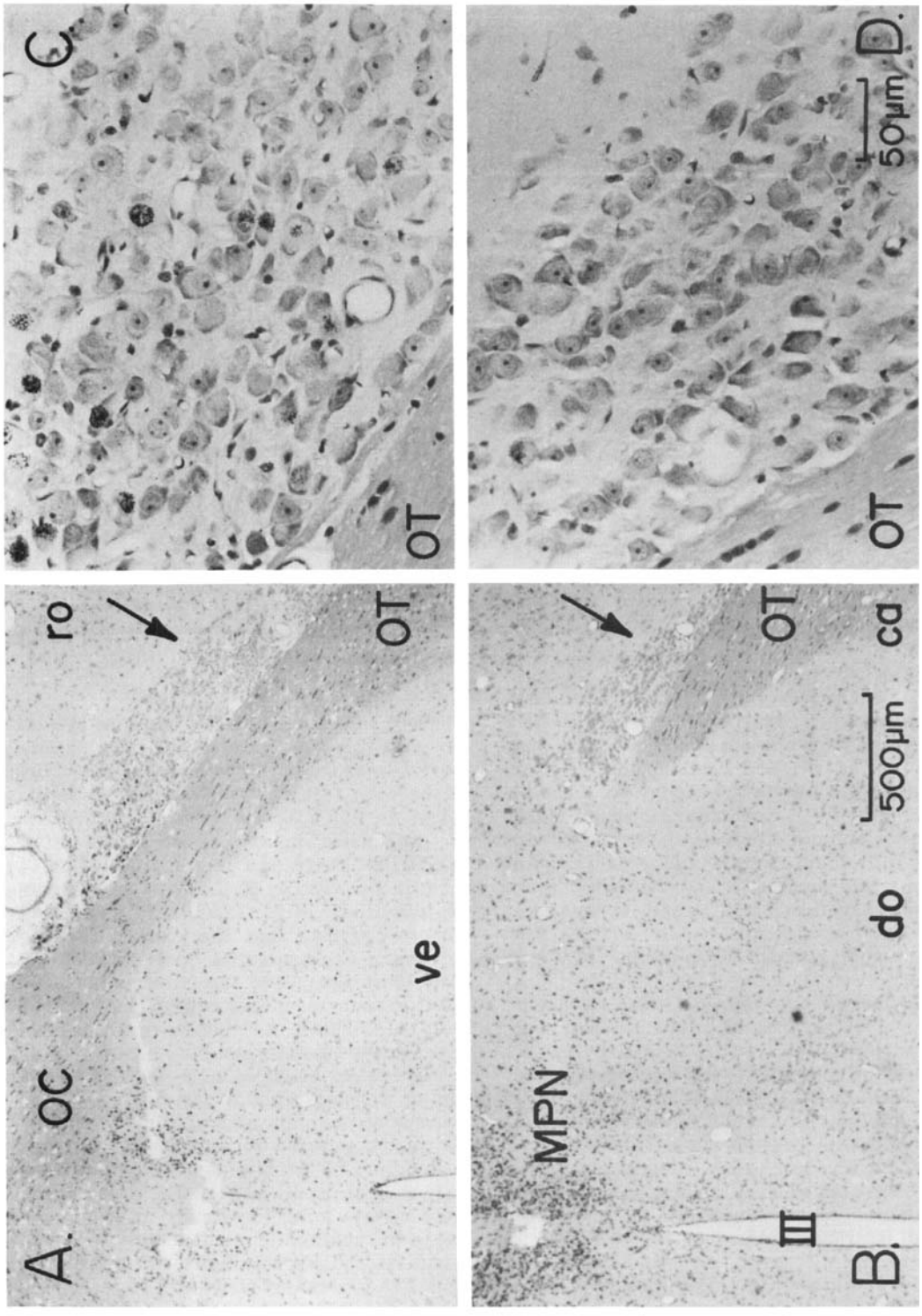

Fig. 12 Labelling pattern in the supraoptic nucleus (arrows in A and B) in a rat injected on days E15+16. Labelled cells still present in ventromedial aspect of the optic tract (A and C) but not dorsolaterally (B and D).

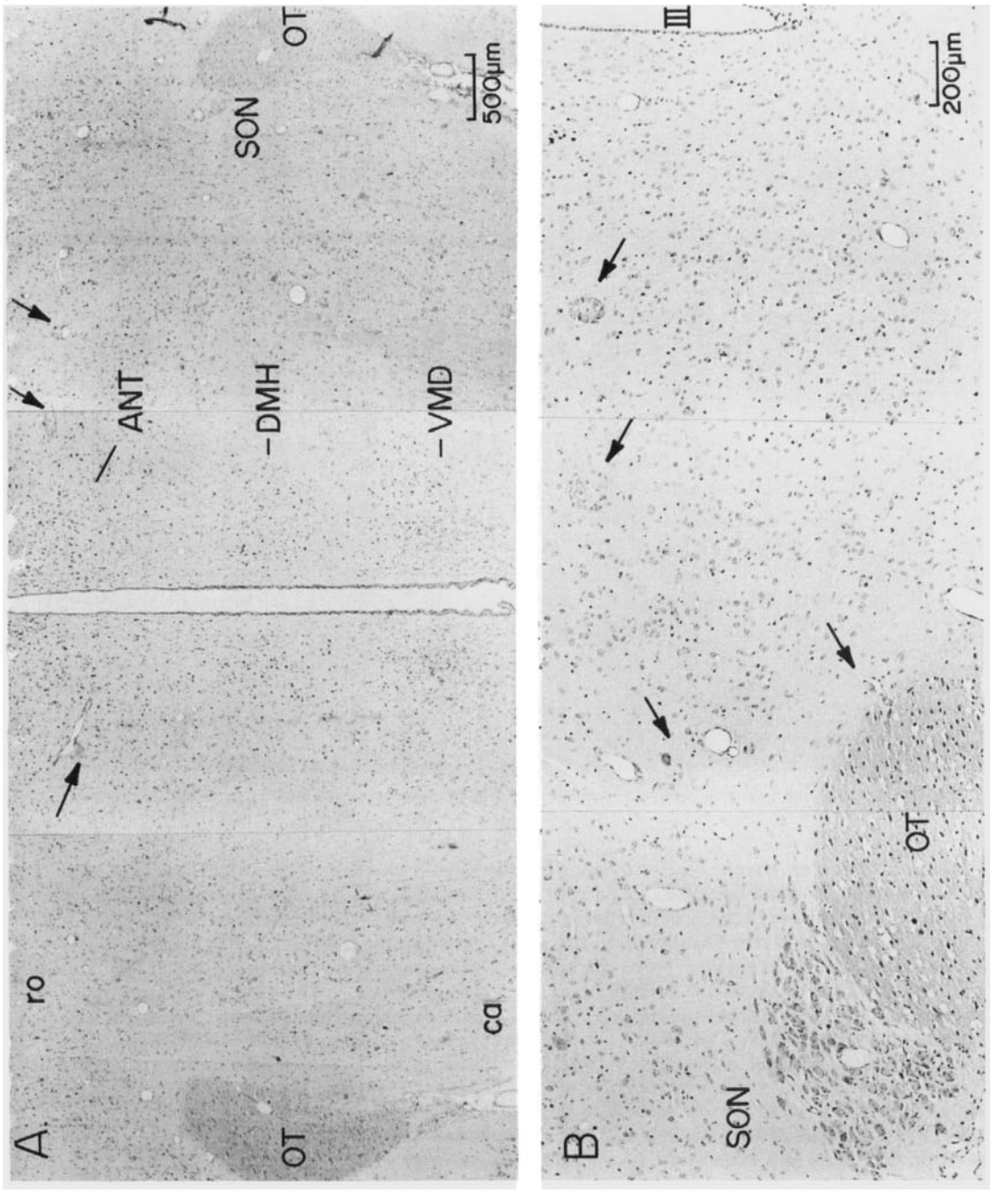

Fig. 13 A. Horizontal section from a rat injected on days E15 + 16 showing clusters of internuclear neurons (arrows). The cells are on a trajectory between the dorsally situated paraventricular nucleus (not shown) and the supraoptic nucleus. B. The internuclear neurons (arrows) at a higher magnification in a nonautordiographic section.

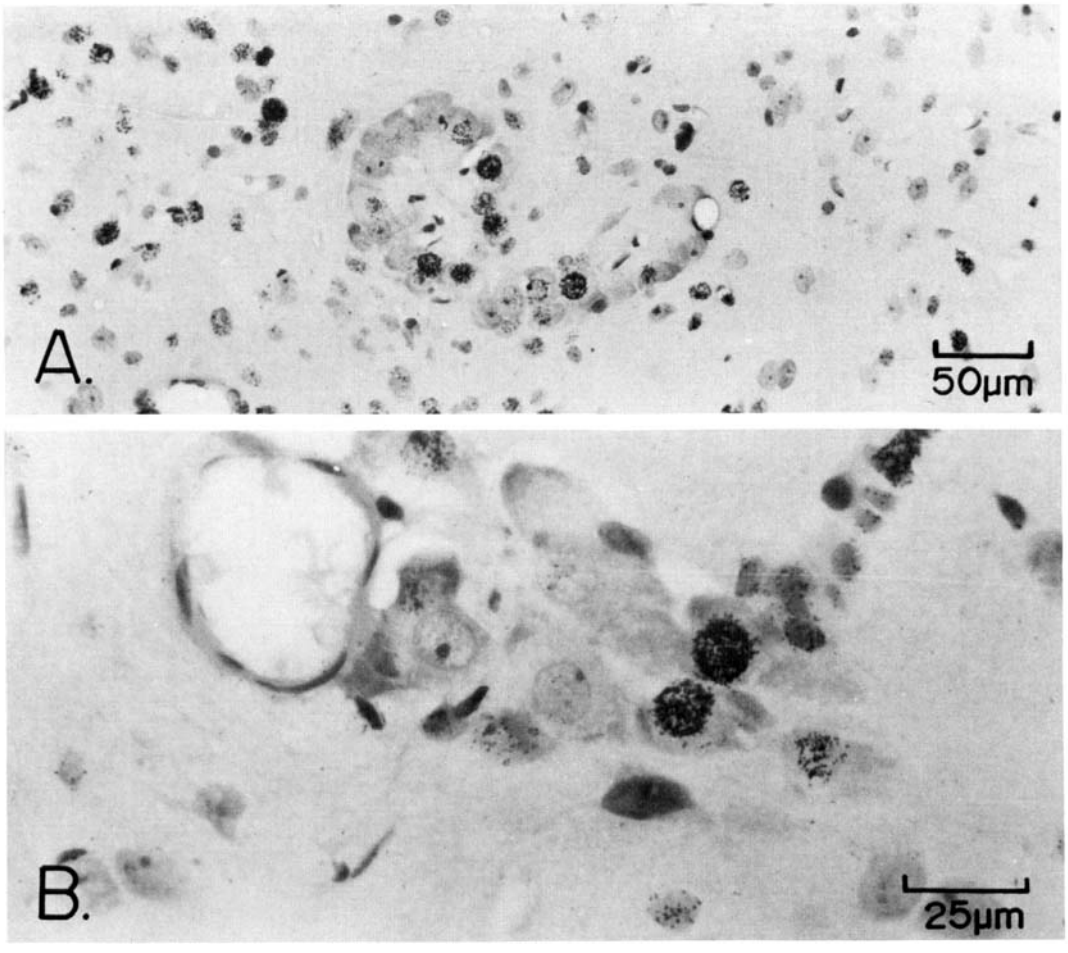

Fig. 14 Labelled and unlabelled internuclear magnocellular neurons from a rat injected on days E15+16; at two magnifications.

rare labelled cell was encountered in some E16+17 animals. Related to these were the cells seen in the medial aspect of the optic tract (fig. 13) or apposed to a blood vessel that traversed the body of the optic tract (figs. 15A,B). No gradient could be established between mediodorsally and lateroventrally situated clumps of internuclear neurons.

Paraventricular nucleus. The shape and exact position of the magnocellular portion of the paraventricular nucleus vary in different animals, which makes it difficult to establish whether or not there is a consistent gradient present. In many instances a dorsolateral-to-ventromedial gradient seemed to be indicated. This is illustrated in figure 16 from an E15+16 rat, which shows the sparsity of labelled cells in a dorsal horizontal section, and their relative abundance in a more ventrally cut section. But there is a clear lateral-to-medial gradient in this region with respect to the late forming parvicellular component of the nucleus adjacent to the ventricle (fig. 16).

The neurons of the supraoptic, internuclear and paraventricular nuclei, constituting the magnocellular hypothalamo-neurohypophysial secretory system, arise largely concurrently. Suggestive embryonic evidence will be presented in the next paper (Altman and Bayer, '78b) that they arise from the same neuroepithelial matrix (as outlined in adults by the specialized convoluted ependymal lining, fig. 16) and that the neurons of the supraoptic nucleus migrate, along the trajec-

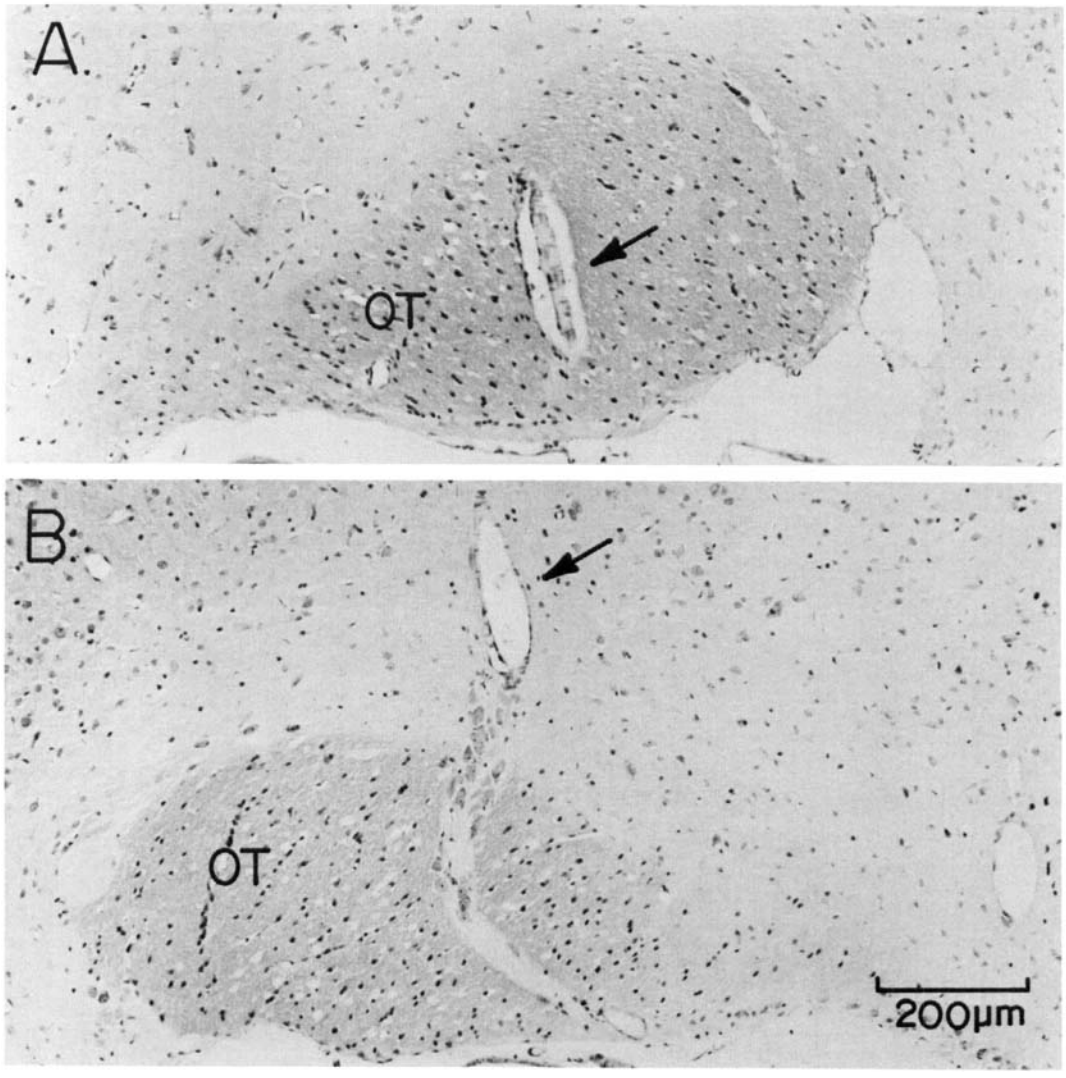

Fig. 15 The relationship between magnocellular neurons of the supraoptic nucleus and blood vessels is illustrated in these two neighboring sections in which the two jointly penetrate the optic tract (arrows).

tory outlined by the internuclear neurons, at the time the fibers of the optic tract grow towards the dorsal thalamus.

Ventromedial nucleus. In coronal sections two parts of the ventromedial nucleus could be distinguished, the ventrolaterally situated pars ventralis and the dorsomedially situated pars dorsalis. As indicated by the quantitative data (fig. 7) and illustrated in figure 17, the cells of pars ventralis form largely before the cells of pars dorsalis. Therefore, the nucleus as a whole displays a ventrolateral-to-dorsomedial gradient of formation. There was also an indication of an outside-in gradient along the oblique column formed by the two parts of the nucleus (fig. 17A). In E17+18 rats (fig. 17B) mostly cells adjacent to the "laminated epithelial" wall were labelled.

Dorsomedial nucleus. In coronal sections the dorsomedial nucleus forms an oblique column roughly at a right angle to the ventromedial nucleus. The gradient of cell labelling (fig. 18) is dorsolateral-to-ventromedial; the mirror image of that of the ventromedial nu-

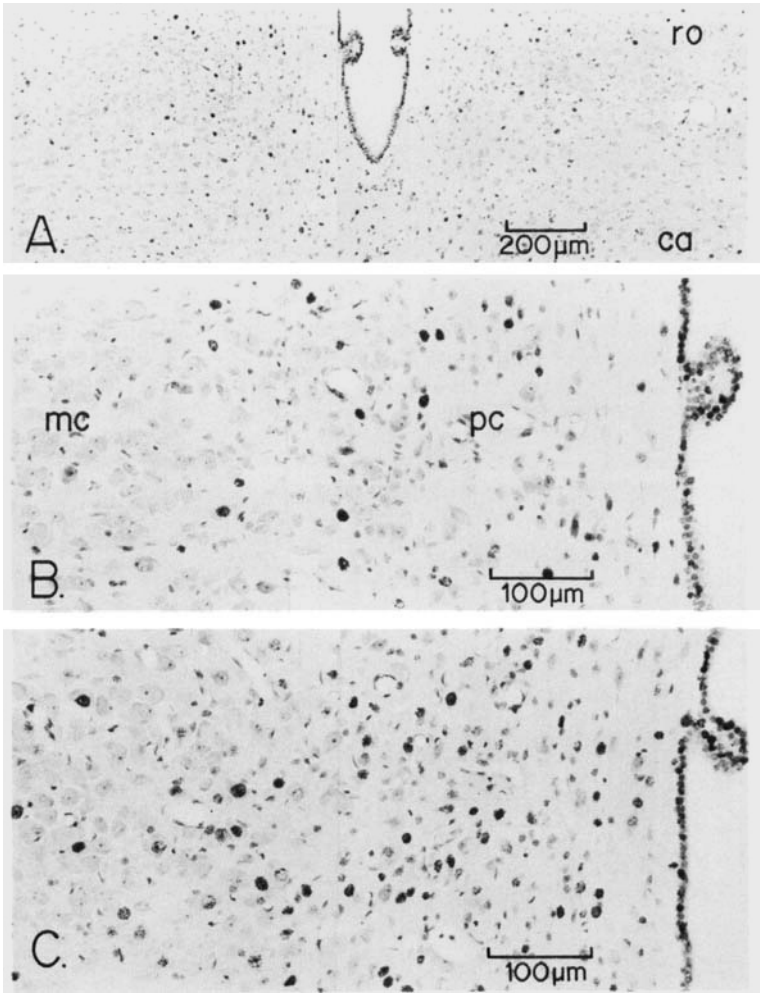

Fig. 16 A. Horizontal dorsal section through the paraventricular nucleus adjacent to the "convoluted ependyma" from a rat injected on days E15+16. B shows a portion of A at higher magnification. C is from a ventral section, showing a higher proportion of labelled magnocellular neurons.

cleus. These observations suggest that the neurons of these two nuclei may arise in the same neuroepithelial region, characterized in the adult by the "laminated epithelium," by moving outward ventrolaterally and dorsolaterally.

Arcuate nucleus. The quantitative data (figs. 6-8) suggested that the arcuate nucleus, as usually identified (Christ, '69), has at least two developmentally distinct components, a relatively early forming rostral portion (peak on day E15) and a late forming caudal part (peak on day E18). The rostral region may be distinguished by two additional criteria: it is composed of larger cells than the caudal arcuate nucleus and, unlike the latter, it is not surrounding a tanycyte-lined but an ependyma-lined ventricular region. On the basis of these considerations, and additional embryonic evidence to be described in the succeeding paper Altman and Bayer, '78b), we suggest that the anterior arcuate nucleus is a separate region and will tentatively refer to it as the *anterobasal nucleus.*

Posterior nucleus and premammillary nuclei. These early forming nuclei contribute to the lateral-to-medial gradient present in the upper three-fourths of the hypothalamus at

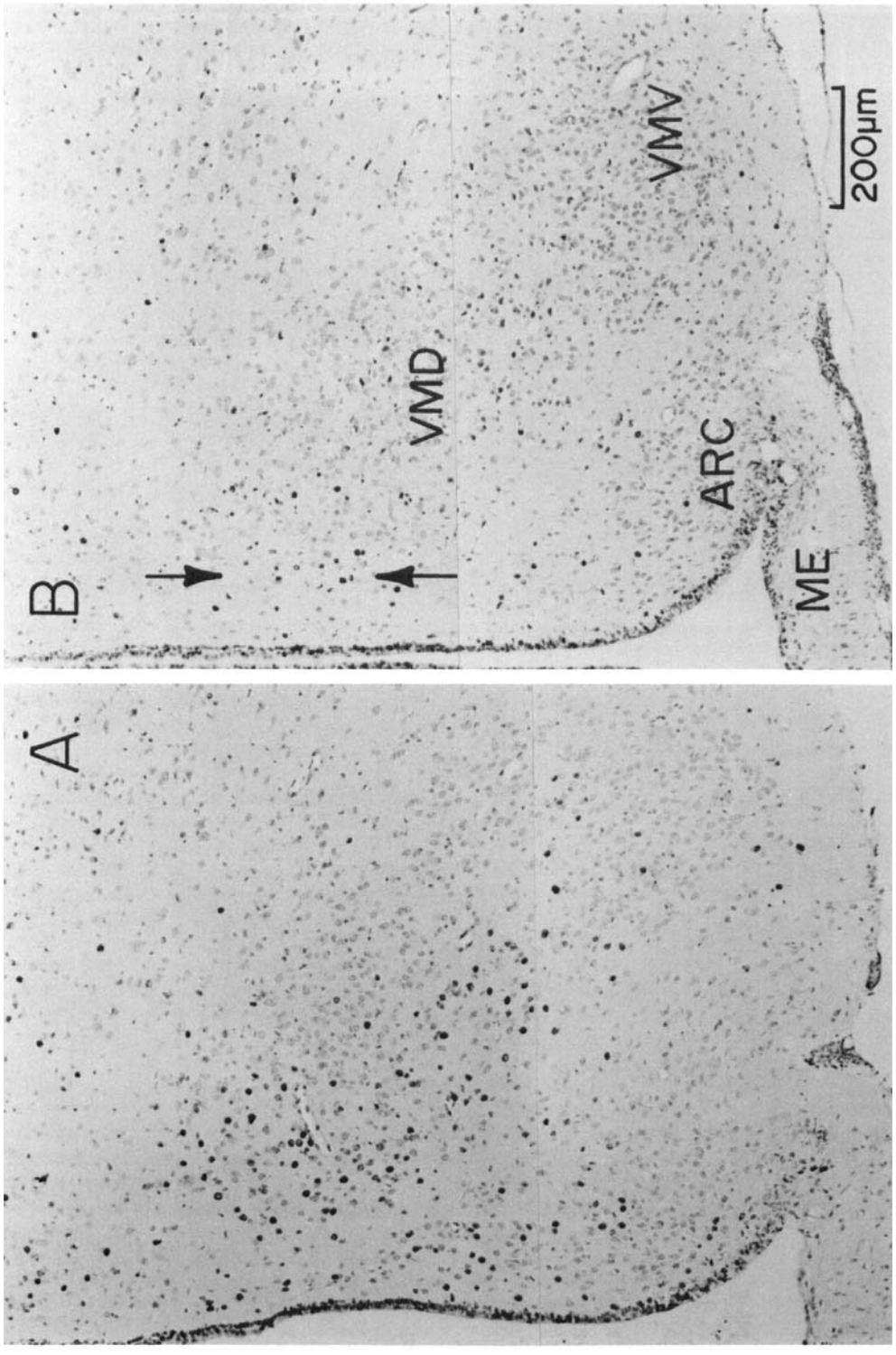

Fig. 17 Coronal sections through the ventromedial nuclei (pars dorsalis and pars ventralis) from a rat injected on days E16+17 (A) and days E17+18 (B). In the latter, cells tended to be labelled near the midline (arrows) adjacent to the laminated epithelium.

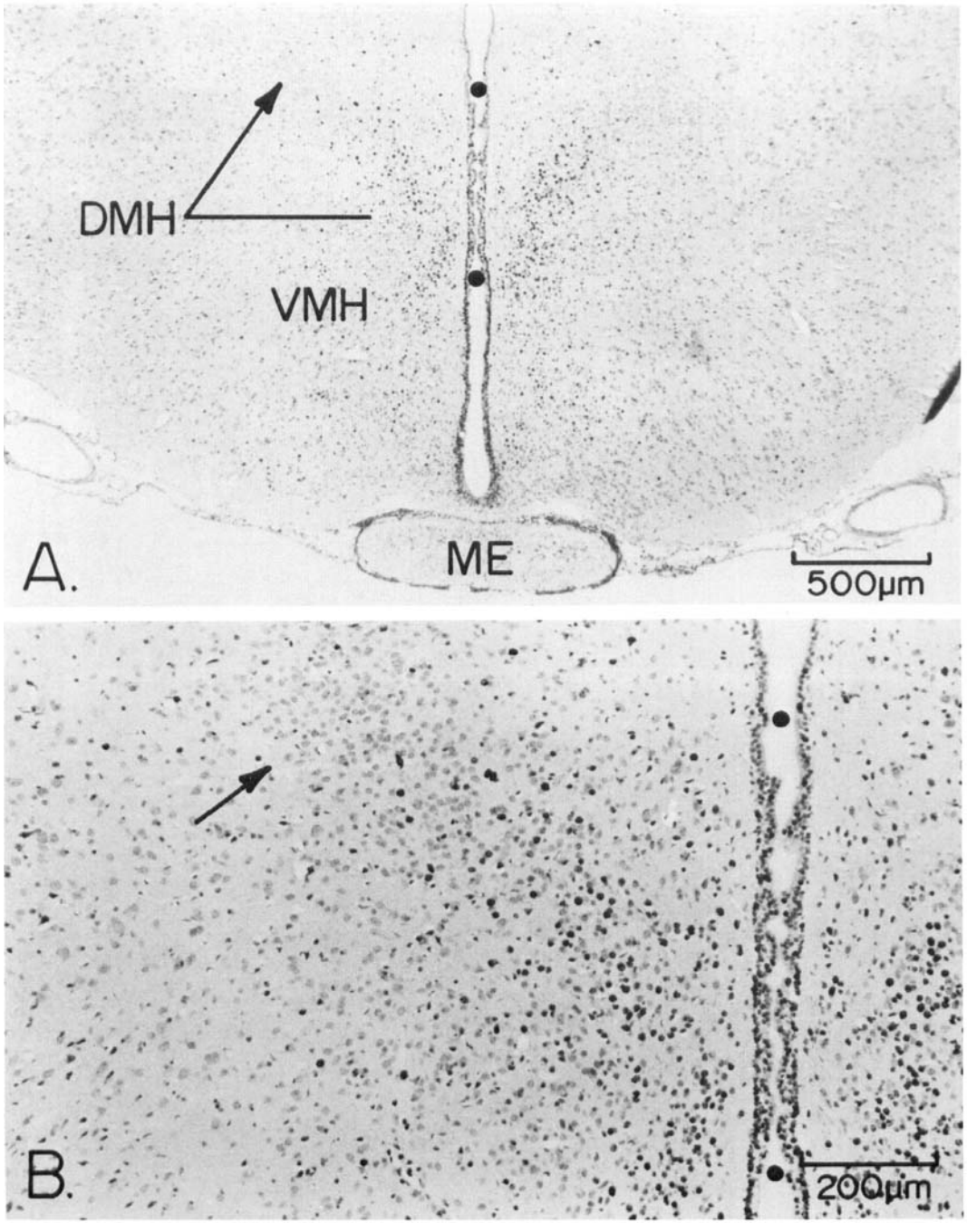

Fig. 18 Coronal section through the dorsomedial and ventromedial hypothalamic nuclei of a rat injected on days E17+18. The two dots in A and B outline the extent of the laminated epithelium of the third ventricle, representing the medial meeting point of the two nuclei. Note the scarceness of labelled cells (arrows) in the dorsolateral aspect of the dorsomedial nucleus.

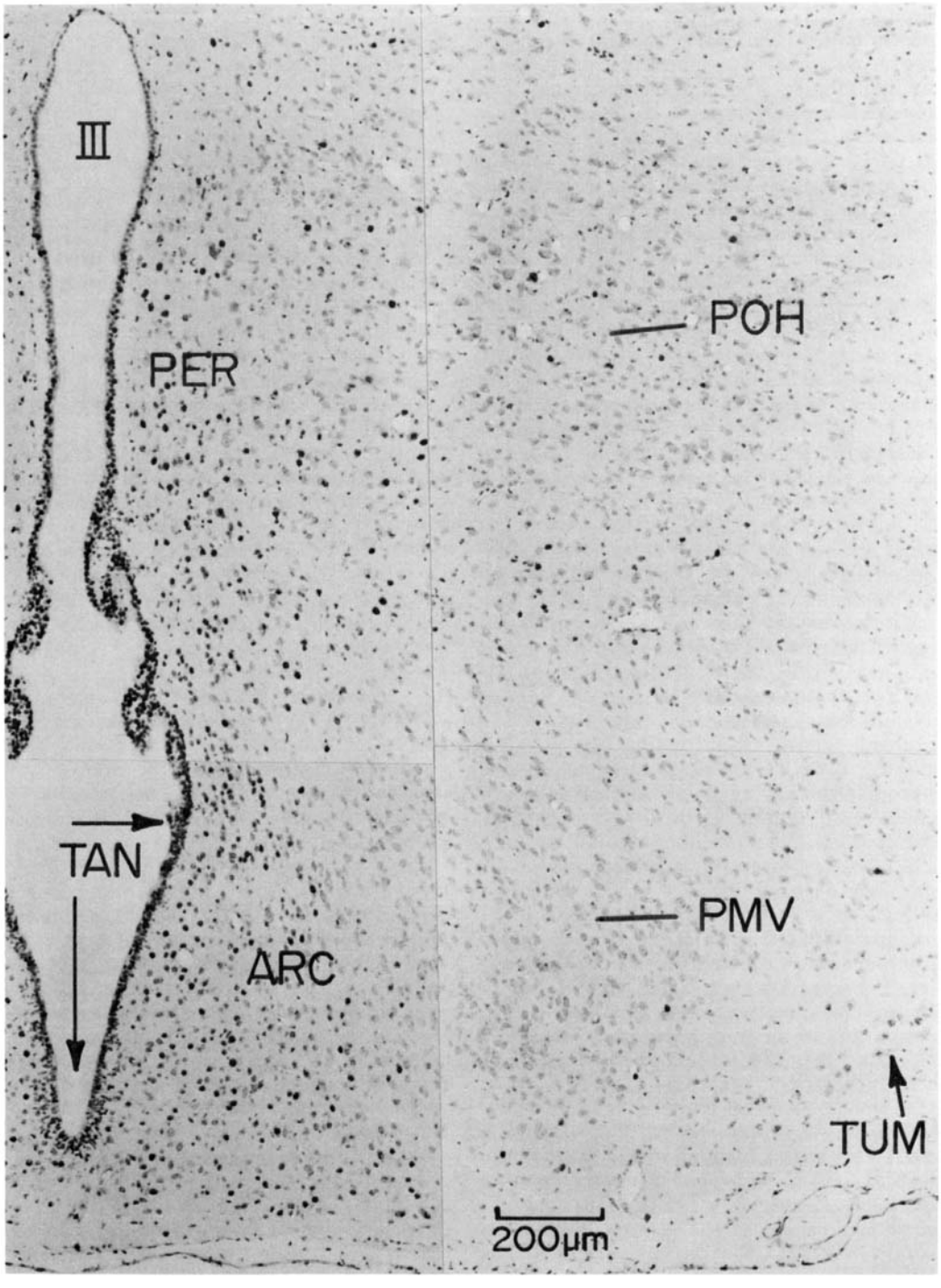

Fig. 19 Coronal section through the posterior hypothalamic nucleus of a rat injected on days E16 + 17. Arrows point to the tanycyte-lined portion of the third ventricle related to the arcuate nucleus.

coronal level A4.6 (fig. 8). Since the neuroepithelium of the floor of the third ventricle (see below) appears to generate the late forming neurons of the posterior arcuate and tuberomammillary nuclei, it is more likely that the posterior and premammillary nuclei arise from the more dorsally situated parts of the third ventricle neuroepithelium. However, an intranuclear gradient could not be discerned in these nuclei (fig. 19) to point to their site of origin.

Tuberomammillary nucleus. The neurons of the tuberomammillary nucleus (fig. 4) which resemble the neurosecretory cells of the magnocellular hypothalamo-neurohypophysial system, were classified, together with the arcuate nucleus, as very-late forming, or class 4 cells (fig. 8). The temporal and topographic relationship between these two posterior nuclei, and the contiguity of both with the tanycyte-lined portion of the third ventricle (fig. 20) suggests the possibility that this large-celled nucleus may be part of the "parvicellular" (Szentágothai, '64; Halász, '72) or hypothalamo-adenohypophysial endocrine system.

Mammillary nuclei. On the basis of cytological differences we subdivided the mammillary nuclear complex of the rat into the lateral nucleus (large cells; figs. 21A,D); the medial nucleus (median cells; figs. 21A,C); the principal nucleus (small cells; figs. 21A,B); and the dorsally situated, crescent shaped supramammillary nucleus (horizontally oriented, small, spindle shaped cells; fig. 21A). Our quantitative autoradiographic data indicated that the lateral mammillary nucleus is composed of class 1 (M) cells; the medial mammillary nucleus of class 2 (M) cells; and the principal mammillary and the supramammillary nuclei of class 3 (M) cells (fig. 9). Additional observations suggested that the principal nucleus has two parts: a paired ventral portion and an unpaired central portion (fig. 22). Anteriorly the central and ventral parts form an inverted T; more posteriorly only the ovoid ventral portion is present. In addition, our autoradiographic observations suggest a fourth component in the posterior part of the complex which is formed of early forming small cells (fig. 22C); we shall tentatively refer to this region as the *intermediate mammillary nucleus.* No intranuclear gradient could be discerned in the early forming lateral, medial and intermediate nuclei, but a lateral-to-medial gradient is suggested for the crescent-shaped supramammillary nucleus and a ventral-to-dorsal gradient for the posterior part of the principal mammillary nucleus. These observations suggest two separate neuroepithelial foci, a dorsal site of origin for the supramammillary nucleus and a ventral one for the principal nucleus. Suggestive support for this hypothesis will be presented in the succeeding paper (Altman and Bayer, '78b).

4. *Some statistical data*

An examination of our quantitative results indicated that the sequence of neuron generation in a group of hypothalamic nuclei was often more clearly indicated in the data from individual animals than in the pooled data. This apparent variability between animals might be due to differences in the exact age of the pooled individuals or their exact developmental stage (differences were often noted within littermates). Accordingly, we employed a statistical procedure, Conover's ('71) sign test, to determine the consistency of sequential neuron production in pairs of hypothalamic nuclei regardless of the chronological grouping of the individual animals. This test is based on paired comparisons (X, Y) within individual animals. The comparisons are grouped into three categories: (1) $X > Y$, "$-$" comparison; (2) $X < Y$, "$+$" comparison; (3) $X = Y$, "0" comparison. The zero comparisons are discarded and, depending on the total number of remaining "$-$" and "$+$" comparisons, either a binomial distribution or a normal approximation is used to calculate probabilities (p's).

Since various hypothalamic nuclear groups arise from different regions of the diencephalic neuroepithelium, their simultaneous or sequential generation was not a major concern. What we deemed interesting was to determine whether or not structures that appear to arise from the same neuroepithelial matrix are generated simultaneously or sequentially. Similarly, we inquired about the temporal pattern of cell production in nuclei which have been classified in the past as components of one system.

The results indicated that within some of the nuclear groups that we classified as constituting a single class there were components that had significantly different generation times. Thus, while there were no differences between the class 1 neurons of the lateral hypothalamic area at level A6.6 (fig. 6) and A5.8 (fig. 7), the neurons of the dorsal and lateral preoptic areas (fig. 5; also classified as class 1)

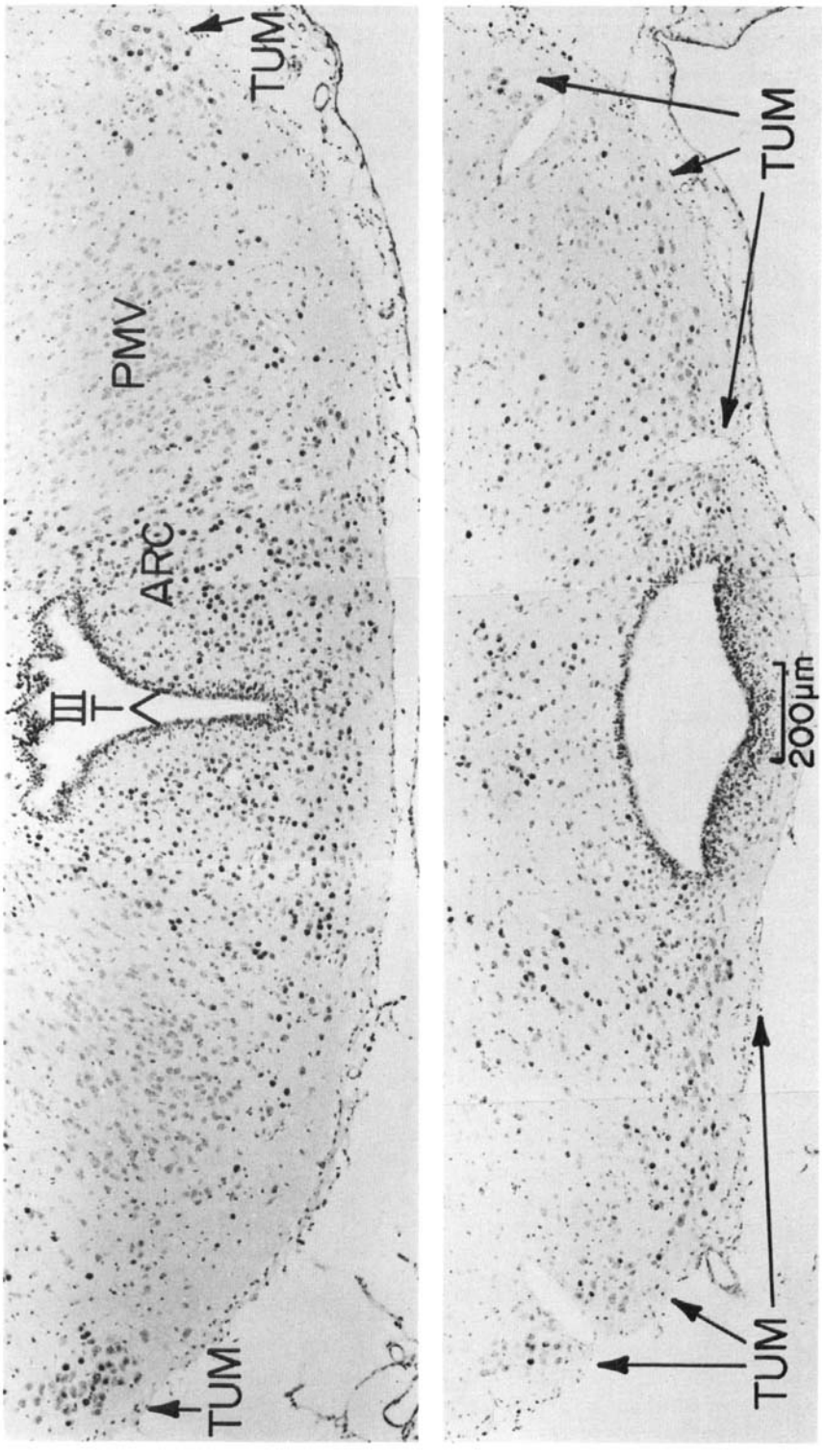

Fig. 20 Coronal sections through the tuberomammillary nucleus (arrows) at a rostral (A) and caudal (B) level from a rat injected on days E16+17. Caudally the tuberomammillary nucleus neurons approximate the tanycyte-lined (T) portion of the third ventricle.

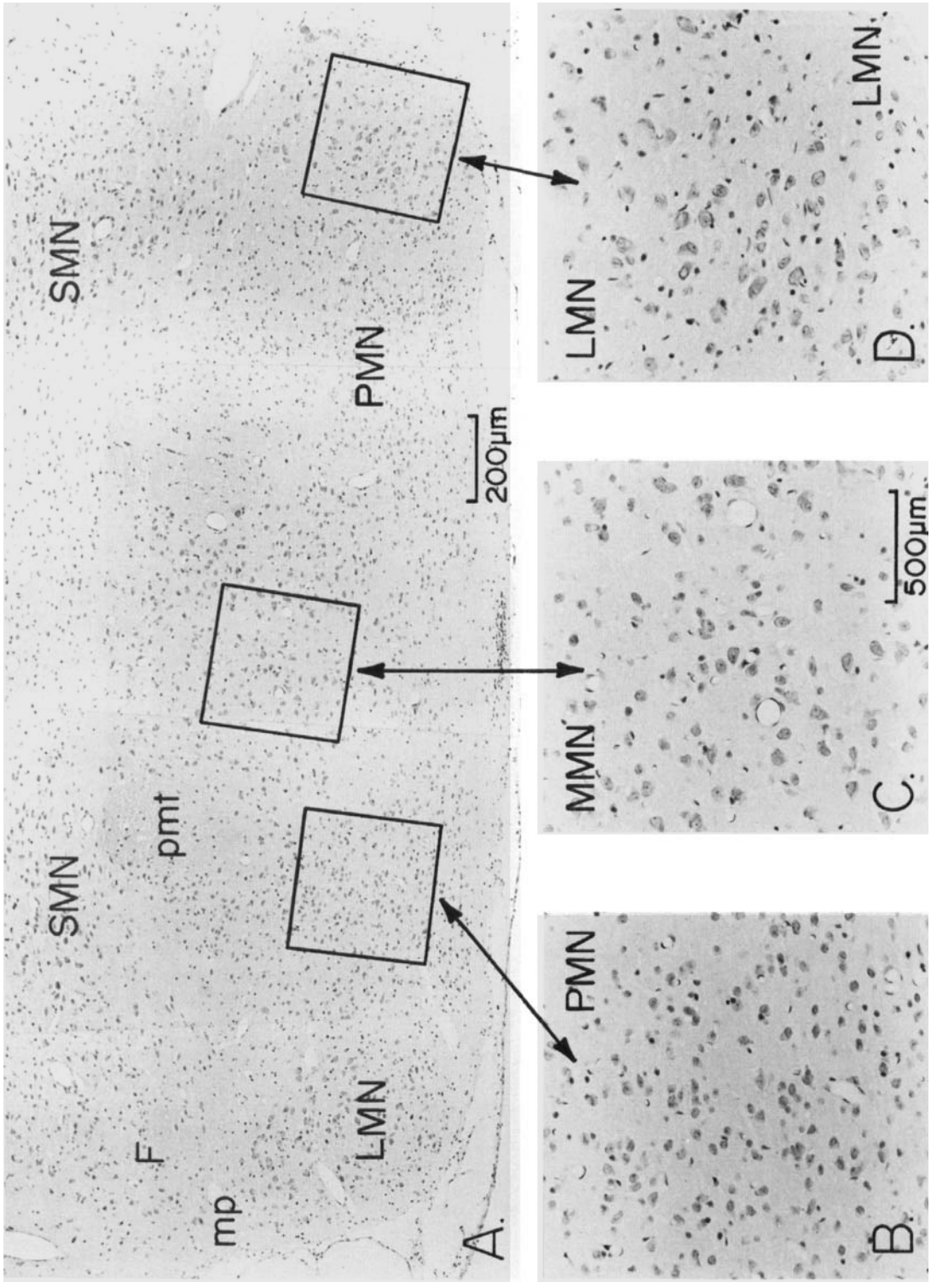

Fig. 21 Coronal section (nonautoradiographic) through the mammillary complex (A) with enlarged views of three of its nuclear components (B-D).

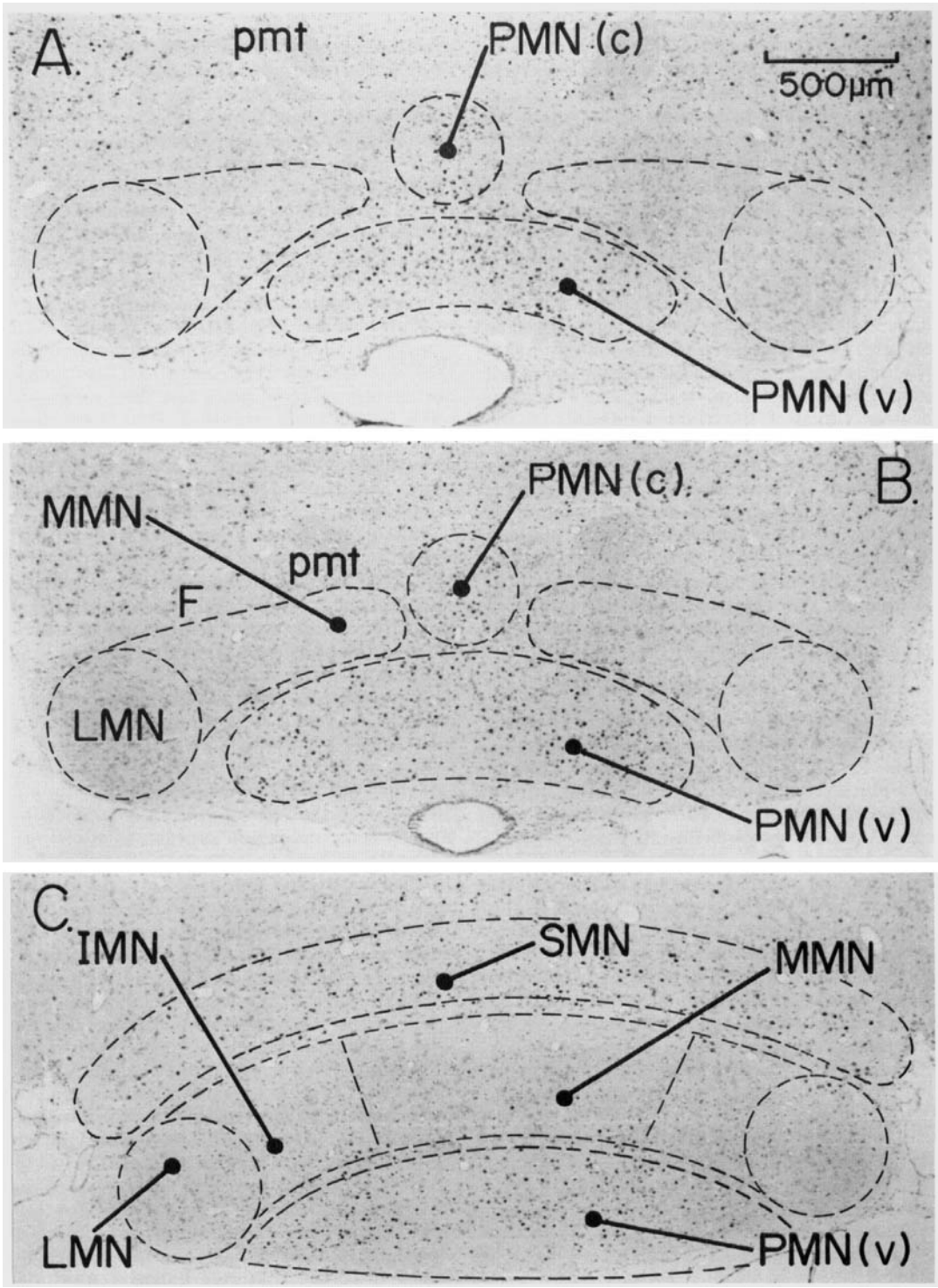

Fig. 22 Coronal sections (A-C, rostral to caudal) through the mammillary complex of a rat injected on days E16+17. The sandwiched pattern of gradient is pronounced caudally (C).

formed significantly later ($p < 0.05$) than the lateral hypothalamic area. These results indicate a rostral-to-caudal gradient in the lateral tier region. The neurons of the supraoptic and paraventricular nuclei (figs. 5, 6) which we classified as class 2 neurons and postulated to arise from the same neuroepithelial site, were also found to be sequentially generated. The distal neurons of the supraoptic nucleus are generated significantly earlier ($p < 0.01$) than the neurons of the paraventricular nucleus which apparently remain near their generation site. This conforms to a modified lateral-to-medial gradient that is often observed as an internuclear pattern within a single hypothalamic nucleus. A similar lateral-to-medial gradient was indicated by comparing the generation times of the class 4 neurons of the tuberomammillary nucleus and the caudal (A4.6) arcuate nucleus; two nuclei that we postulated to originate at the same neuroepithelial sites. The laterally situated large neurons of the tuberomammillary nucleus arise significantly earlier ($p < 0.01$) than the smaller neurons of the arcuate nucleus. But, in general, the statistical results confirmed the classification of hypothalamic neurons into four broad cytogenetic classes.

DISCUSSION

In his pioneering study of the time of origin of neurons of different nuclei of the rat hypothalamus, Ifft ('72: p. 193) concluded: "Laterally placed nuclei were found to arise on the fourteenth day of gestation while medial nuclei, in general, arose on day 16. Final cell division occurred over a period of time but the majority took place on the days indicated. Apparently a lateromedial gradient exists in the hypothalamus, and possibly a dorsoventral gradient." Ifft used a procedure in which estimates of the time of origin of neurons were based on the proportion of lightly and intensely labelled cells as a result of a single injection of ^{3}H-thymidine in different animals on successive days. We have obtained a more complex labelling gradient and, in some cases, fundamentally different datings. We attribute this to our use of the technique of progressively delayed, comprehensive labelling, which makes possible to account for the generation of practically all neurons in a given brain region and the precise estimation of the percentage formed on a given day.

Time of origin of hypothalamic nuclei

Our data indicated that neurons of the various hypothalamic nuclei form over a protracted period; some in three days, many more in four. An exception to this was the relatively fast generation time of some of the mammillary nuclei which, like many nuclei of the thalamus (work in progress) are generated more rapidly, with a peak on a single day. In figure 23 we have arranged the hypothalamic nuclei examined in terms of the time of origin of their neurons, as class 1, 2, 3 and 4 structures. We have excluded the rapidly forming mammillary nuclei (fig. 9) as a functionally relatable single system; the median preoptic nucleus (which is probably a telencephalic structure); and the ill-defined anterior hypothalamic nucleus. This classification of 20 hypothalamic regions into a few isochronic systems has brought together structures that have been associated before on the basis of structural or functional considerations and others that may have little else in common except their similar time of origin.

Among the class 1 (or earliest forming) structures, the lateral and dorsal preoptic areas, and the lateral hypothalamic area could constitute a single lateral system that is intimately related to the medial forebrain bundle (Nauta and Haymaker, '69). However, it is unlikely that the isochronic lateral mammillary nucleus is part of the same system. Among the class 2 structures it is reasonable to distinguish between a rostral and a caudal system as arising from different portions of the third ventricle neuroepithelium. Among the rostral class 2 structures the magnocellular paraventricular and supraoptic nuclei are evidently related as secretory neurons that produce oxytocin, vasopression and neurophysins that are transported by axoplasmic flow to the neurohypophysis (for recent reviews, see Clementi and Ceccarelli, '70; Stutinsky, '74; Defendini and Zimmerman, '78). We shall provide suggestive embryonic evidence in the next paper (Altman and Bayer, '78b) that these two nuclei, together with the scattered internuclear magnocellular neurons, arise from a common neuroepithelial locus, and thus constitute a single developmental unit. However, we have no evidence that the isochronic medial preoptic area is in any way related to the secretory magnocellular system. As regards the caudally situated class 2 structures, we can say little as there is a paucity of data about the connections or functions of the posterior hypothalamic nucleus and the premammillary nuclei. It is conceivable that some or all of these class 2 struc-

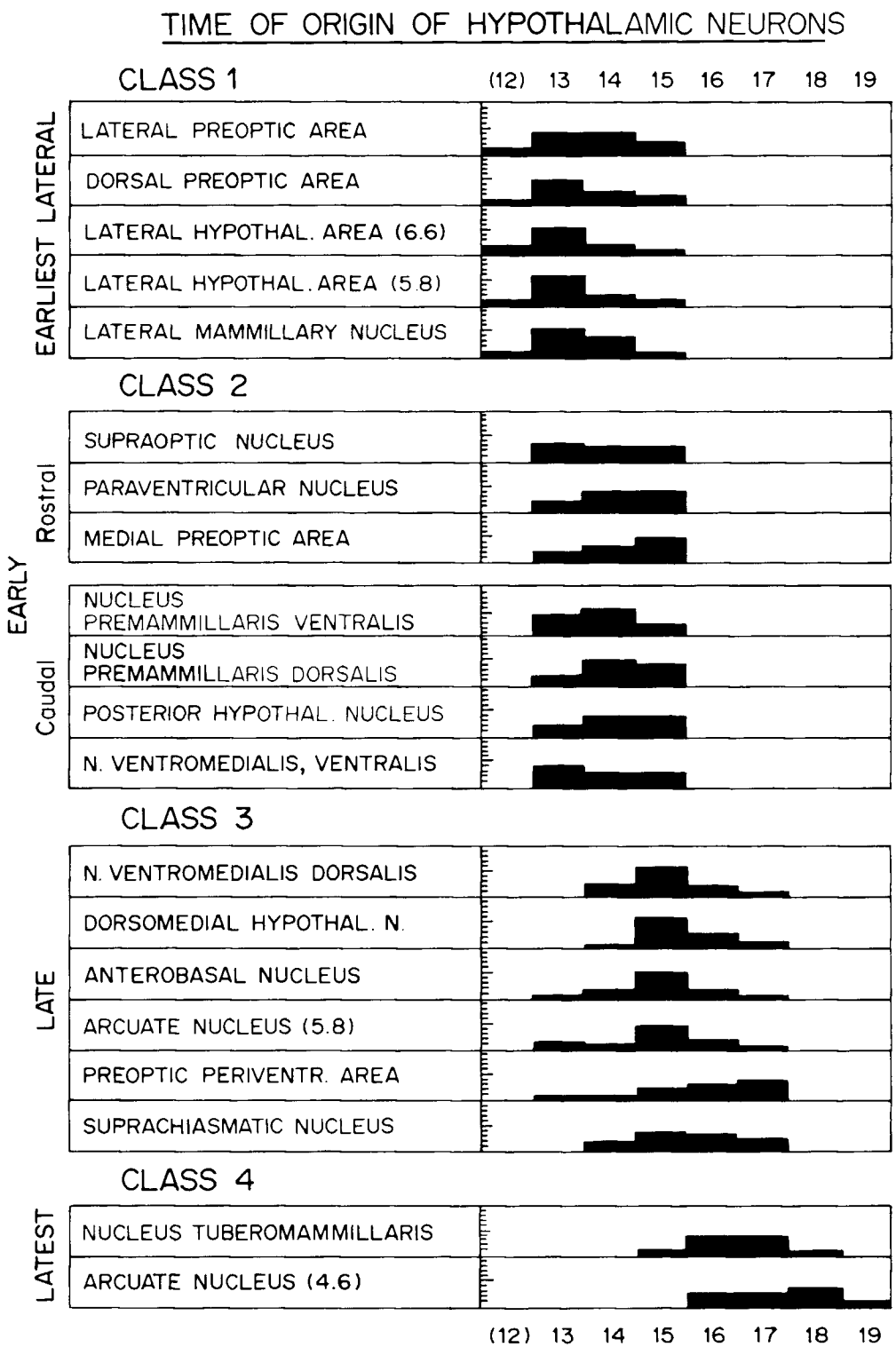

Fig. 23 Summary diagram of isochronic and heterochronic hypothalamic structures.

tures form an interposed tier between the earliest forming lateral system and the late forming, class 3 "parvicellular" nuclei.

It has been suggested by several investigators (Szentágothai, '64; Flament-Durand, '65; Mess et al., '70; Halász, '72; Martini, '74; Renaud, '78) that some or all of the nuclei listed as class 3 structures in figure 23 constitute a single functional system, variously named as the parvicellular endocrine system, the hypophysiotrophic area, or the source nuclei of the tuberoinfundibular pathway. These are the structures that control the activity of the anterior pituitary by means of releasing and inhibitory factors secreted by them. Our autoradiographic data suggest that, from a developmental point of view, this "parvicellular" system may have two separate, or heterochronic, components and that one member of the class 4 structures, the tuberomammillary nucleus, is made up of large neurons. Apparently parts of these two systems arise from and are related to different ventricular regions: the dorsomedial and ventromedial nuclei to the "laminated epithelium," the arcuate nucleus to the tanycyte-lined ventricle (Altman and Bayer, '78c).

Intra- and internuclear gradients

Gradients may provide clues about the settling patterns of neurons and other aspects of the developmental process. Where a lateral-to-medial gradient is present in the diencephalon the interpretation is that the earliest forming neurons of the midline third ventricle were displaced outward by later generated neurons, forming an "outside-in" pattern (Angevine, '70). Where the gradient is different as in the case of the dorsomedial and ventromedial hypothalamic nuclei (being dorsolateral-to-ventromedial and ventrolateral-to-dorsomedial, respectively) the developmental "arrows" of the two nuclei suggest a common site of origin and their fanning out for some reason to form a mirror-image pattern. Where the gradients were more complex (as in the case of the median preoptic nucleus) we deduced an origin from different ventricular sites. Of course, such deductions have to be supported by embryonic observations and this will be attempted in the next paper (Altman and Bayer, '78b).

In addition to passive displacement, gradients may be generated by active cell migration. The settling of the early forming neurons of the supraoptic nucleus and of the later forming neurons of the tuberomammillary nucleus in the ventrolateral wall of the diencephalon was attributed to such migration, and embryonic evidence will be presented in the next paper (Altman and Bayer, '78b) that supports the migration of the cells of the supraoptic nucleus. In this context it is of interest that settling patterns of neurons of the supraoptic nucleus differ from that of the suprachiasmatic nucleus: the earliest generated neurons of the former settle away from the fibers of the optic tract, those of the latter settle near them. We have previously noted a similar relationship in the medulla in that the earliest forming neurons of the nucleus reticularis tegmenti pontis settled distal to the pyramidal tract whereas those of the pontine gray proximal to it (Altman and Bayer, '78a). In that context we suggested that the settling of the first arriving neurons of the pons near the pyramidal tract reflects their intimate relation to the fibers of this system. Conceivably the same applies to the neurons of the suprachiasmatic nucleus, which receive retinal input (Moore, '73; Nishino et al., '76) and have been implicated in the regulation of circadian rhythms (Moore and Eichler, '72; Ibuka and Kawamura, '75; Zucker et al., '76; Saleh and Winget, '77). By implication the neurons of the supraoptic nucleus are not functionally related to the fibers of the optic tract.

Whereas gradients within nuclei may reveal the site of origin and settling patterns of their neurons, and possibly their relationship to certain fiber tracts, gradients between nuclei could reflect the hierarchic ordering of constituent structures within larger systems. But such interpretations are risky because in many instances heterochronicity might reflect, instead, the lack of structural relationship between contiguous nuclei. This dilemma may be illustrated in the case of the mammillary complex. It is composed of class 1 (lateral mammillary), perhaps class 2 (intermediate mammillary), class 3 (medial mammillary) and class 4 (supramammillary and principal mammillary) nuclei. Do all these nuclei, not all of which seem to be derived from the neuroepithelium of the mammillary recess, constitute a single structural or functional system in some kind of hierarchic relation to one another? It is possible that these different nuclei are differentially related to the afferents of the fornix and to the efferents of the mammillothalamic and mammillotegmental tract and their subdivisions. We shall return to this

question in the next paper (Altman and Bayer, '78b). It is interesting to note that the rapid generation of neurons of several of the mammillary nuclei resembles that of many thalamic nuclei (work in progress) and differs from the protracted generation of most hypothalamic nuclei.

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