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DEVELOPMENT OF THE ENDOPIRIFORM NUCLEUS AND THE CLAUSTRUM IN THE RAT BRAIN

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Abstract—Long-survival [^{3}H]thymidine autoradiography was used to quantitatively determine the time of origin of neurons in the endopiriform nucleus and the claustrum in rats killed on postnatal day 60 after their dams received two consecutive daily injections of [^{3}H]thymidine on embryonic day E13 and E14, E14 and E15, ... E21 and E22. The claustrum originates late, on E15 and E16, and has a strong gradient in the longitudinal direction, posterior (older) to anterior (younger). In contrast, the endopiriform nucleus originates early, on E14 and E15, and lacks a longitudinal gradient but has a strong one in the vertical direction, ventral (older) to dorsal (younger). Sequential-survival [^{3}H]thymidine autoradiography was used to qualitatively determine the germinal sources and settling sites of endopiriform and claustral neurons in embryonic rats. The dams received a single injection of [^{3}H]thymidine on either E14 (to heavily label older endopiriform neurons) or E16 (to heavily label younger claustral neurons) and were killed in sequential 24-h intervals. Neurons in the presumptive endopiriform nucleus settle within two to three days after their peak time of neurogenesis while those in the presumptive claustrum take approximately five days to settle after their peak.

It is postulated that endopiriform neurons are generated in the palliostriatal ventricular angle, the neuroepithelium that forms a wedge between the primordia of the neocortex and the basal ganglia, and that claustral neurons are generated in the neocortical neuroepithelium. Divergent developmental patterns between the endopiriform nucleus and the claustrum support the anatomical evidence that these nuclei have different connections. Furthermore, neurogenetic gradients in the claustrum correlate with the pattern of anatomical connections between the claustrum and the neocortex.

Two prominent structures lie outside the external capsule beneath the insular cortex and the piriform cortex (PC). They were considered a single entity in the earliest drawings by Willis in 1672, in the first written description by Vicq d'Azyr in 1786, and Burdach named both of them the claustrum (CL) in the early 19th century.[41] Following that tradition, Gurdjian[21] referred to both structures as the CL in his descriptive study of the rat striatum. However, Loo[35] carefully examined the opossum brain and was the first to distinguish the CL proper, a dorsal compact group of large cells beneath the extreme capsule in the insular cortex, from the endopiriform nucleus (EP), a ventral accumulation of large cells scattered among fibers in the core of the piriform lobe. But for several years after Loo's work, many descriptive anatomical studies continued to include the EP as part of the CL.[23,25,26,34,57,59] The term EP again reappeared in Krettek and Price's[29–31] work on the anatomical connections of the basal forebrain in rats and cats. Since the late '70s several anterograde and retrograde tract tracing anatomical studies have documented the differential connectivities of the EP[17,20,22,24,36,49,54,58] and the CL.[10,11,16,28,32,33,38,42,43,45,47,50,52,53,55]

All the embryonic studies done thus far have lumped the EP and the CL together as the CL. The early literature concerning the embryonic origin of the CL has been reviewed by Filiminoff.[18] Meynert, Brodmann and DeVries proposed that the CL was a derivative of the cortex, while Landau and Spiegel linked its origin to the basal ganglia. Cajal thought that the CL was too isolated from the cortex to be considered completely cortical in origin, and Rose thought that both cortical and basal cells intermingled in the CL. Both Macchi[37] and Filiminoff[18] postulated that the CL has a cortical origin.

In this paper, [^{3}H]thymidine autoradiography is used for the first time to study development of the EP and the CL. Two basic questions are addressed. Firstly, do the EP and the CL have different neurogenetic patterns? The data based on long-survival [^{3}H]thymidine autoradiography in adult brains were used to indicate the times of origin of the two structures. Secondly, do the EP and the CL have different embryonic developmental patterns? Observations in the embryonic brain with sequential-survival [^{3}H]thymidine autoradiography were used to trace the migratory paths of neurons from their putative germinal sources to their settling sites in the EP and the CL.

EXPERIMENTAL PROCEDURES

Long survival [^{3}H]thymidine autoradiography

The experimental animals were the offspring of Purdue–Wistar timed-pregnant rats given two subcutaneous injections of [^{3}H]thymidine (Schwarz–Mann; sp. act. 6.0 Ci/mM; 5 μCi/g body wt) to insure that cells originating after the onset of the injections will be detected as labeled (compre-

Abbreviations: BG, basal ganglia; CL, claustrum; CP, cortical plate; DEP, dorsal endopiriform nucleus; E, embryonic day; EP, endopiriform nucleus; LCS, lateral cortical stream; NC, neocortex; PC, piriform cortex; VEP, ventral endopiriform nucleus.

hensive labeling). The injections (given between 08:30 and 9:00) to an individual animal were separated by 24 h. Two or more pregnant females made up each injection group. The onset of the [^{3}H]thymidine injections was progressively delayed by one day between groups (E13 and E14, E14 and E15, . . . E21 and E22) so that the proportion of neurons generated at daily intervals could be determined. The day the females were sperm positive was designated E1. Normally, births occur on E23, which is also designated as postnatal day (P)0. All animals were perfused through the heart with 10% neutral formalin (pH 7.4) on P60. The brains were kept for 24 h in Bouin's fixative, then were transferred to 10% neutral formalin until they were embedded in paraffin. The brains of at least six animals from each injection group were blocked coronally at the stereotaxic angle of the atlas of Pellegrino *et al.*[48]. Every 15th section (6 μm) through the entire brain was saved. Slides were coated with Kodak NTB-3 emulsion, exposed for 12 weeks, developed in Kodak D-19, and post-stained with hematoxylin and eosin.

Coronal sections were selected for quantitative analysis at five anteroposterior levels of the EP[48] (A9.4–A5.4; drawings, Fig. 2) and at four anteroposterior levels of the claustrum (A9.4–A6.4; drawings, Fig. 9). Cells were counted microscopically at $\times$ 312.5 in unit areas of a 10 $\times$ 10 ocular grid (0.085 mm^2/square). For quantification, all neurons within a designated area were divided into two groups, labeled or unlabeled. Cells with silver grains overlying the nucleus in densities above background levels were considered labeled (Fig. 1). In our material, background noise is very low (approximately two to four grains per grid square), and a cluster of six to 12 silver grains over a nucleus (approximately 3 $\times$ background) were enough to identify a labeled cell. Because of the comprehensive labeling and the long exposure period, only a slight proportion of the neurons were lightly labeled. Obvious endothelial cells (crescent-shaped cells with pale nuclei surrounding capillaries) and glial cells (small cells with densely staining nuclei and indefinite nucleoli) were excluded from the counts. The proportion of labeled cells (percentage labeled cells/total cells) was then calculated from the data.

The determination of the proportion of cells arising (ceasing to divide) on a particular day used a modification of the progressively delayed comprehensive labeling procedure[4] and is described in detail elsewhere.[5] Briefly, a progressive reduction in the proportion of labeled neurons from a maximal level ($>95\%$) in a specific population indicates that the precursor cells are producing postmitotic neurons. By analysing the rate of decline in labeled neurons, one can determine the proportion of neurons originating over blocks of days (or single days) during development. Data and calculations for the ventromedial EP (bottom graph, Fig. 2) are shown in Table 1.

Throughout the quantitative analysis, it was noted that even slight regional differences in cell labeling were consistent within animals. For example, the CL tended to have a lower percentage of labeled neurons posteriorly than anteriorly. That indicates that a higher proportion of posterior cells has earlier birthdays than anterior cells. Parametric statistical tests, such as a non-nested analysis of variance, look only at the degree of divergence between groups, and slight consistent differences within animals are disregarded. Consequently, the non-parametric sign test[12] was used to analyse cell labeling patterns. The sign test determines the consistency of sequential neuron production between paired locations within individual animals. The comparisons are grouped into three categories:
(i) $X > Y$, " $-$ " comparison; (ii) $X < Y$, " $+$ " comparison; (iii) $X = Y$, "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). The graphs throughout this report show the more variable group data rather than consistent trends in data from individual animals. Consequently, some of the statistically significant neurogenetic gradients are not conspicuous in the group data.

Sequential survival [^{3}H]thymidine autodiography

The experimental animals were embryos from Purdue-Wistar timed-pregnant rats given a single subcutaneous injection of [^{3}H]thymidine (Schwarz–Mann; sp. act. 6.0 Ci/mM; 5 μCi/g body wt) between 09:00 and 11:00. The day of sperm positivity is designated as E1. Several dams were injected for each day between E13 and E21. Survival times in each injection group varied from one day to several days. In the dams injected with [^{3}H]thymidine on, for example, E15 one dam was killed one day later on E16, another was killed two days later on E17, and so on, until the last dam was killed on E22. All injection groups were treated as the E15 group. All dams were anesthetized with pentobarbital (35 mg/kg) before their embryos were removed and were killed by immersion in Bouin's fixative. After 24 h, the embryos were transferred to 10% neutral formalin until the time of embedding in either paraffin or methacrylate. The blocks were serially sectioned (every 10th saved) at 6 μm (paraffin) or at 3μm (methacrylate) in the coronal, sagittal and horizontal planes. For [^{3}H]thymidine autoradiography, the slides were coated with Kodak NTB-3 emulsion, exposed for six weeks (paraffin sections) or 12–18 weeks (methacrylate sections), developed in Kodak D-19 and post-stained with hematoxylin.

RESULTS

The endopiriform nucleus

Neurogenesis. The EP at a midcoronal level in a rat exposed to [^{3}H]thymidine on E15 and E16 and killed on P60 is shown in Fig. 1. Nearly all neurons are unlabeled ventromedially indicating that these cells originated before E15. In contrast, there are many heavily labeled cells dorsolaterally indicating that these cells originated on or after the day when the injections began (E15). This labeling pattern provides qualitative evidence for a ventromedial (older) to dorsolateral (younger) neurogenetic gradient within the EP.

To quantify this neurogenetic gradient, the proportion of labeled neurons was determined in ventromedial and dorsolateral parts of the EP at five levels, from A9.4 anteriorly to A5.4 posteriorly (drawings, Fig. 2). Because the sign test indicated that neurons are generated simultaneously at all levels in the anteroposterior plane, Fig. 2 shows combined data. E14 is the peak day of neuron origin ventromedially, while E15 is the peak dorsolaterally ($P < 0.0001$).

Neurogenesis in the EP as a whole (data for ventromedial and dorsolateral parts were combined) was compared with neurogenesis of the deep neurons (layer III) in the PC at level A6.4 (Fig. 3). On average, neurons in the EP are older (more are generated on or before E14) than the deep neurons in the PC (more are generated on or after E15; $P < 0.002$).

Embryonic development. Because the neurogenetic data from the long-survival [^{3}H]thymidine autoradiography (Figs 2, 3) indicate that most neurons in the EP (especially those in the ventromedial part)

Abbreviations used in the figures

AC	anterior commissure
BG	basal ganglia
bg	neuroepithelium of the basal ganglia
BT	basal telencephalon
DL	dorsolateral
IM	intercalated mass of the amygdala
im	presumptive intercalated mass of the amygdala
iz	intermediate zone
LOT	lateral olfactory tract
LV	lateral ventricle
nc	neuroepithelium of the neocortex
ne	neuroepithelium
psv	palliostriatal ventricular angle
r	reservoir of the lateral cortical stream
ST	striatum
VM	ventromedial

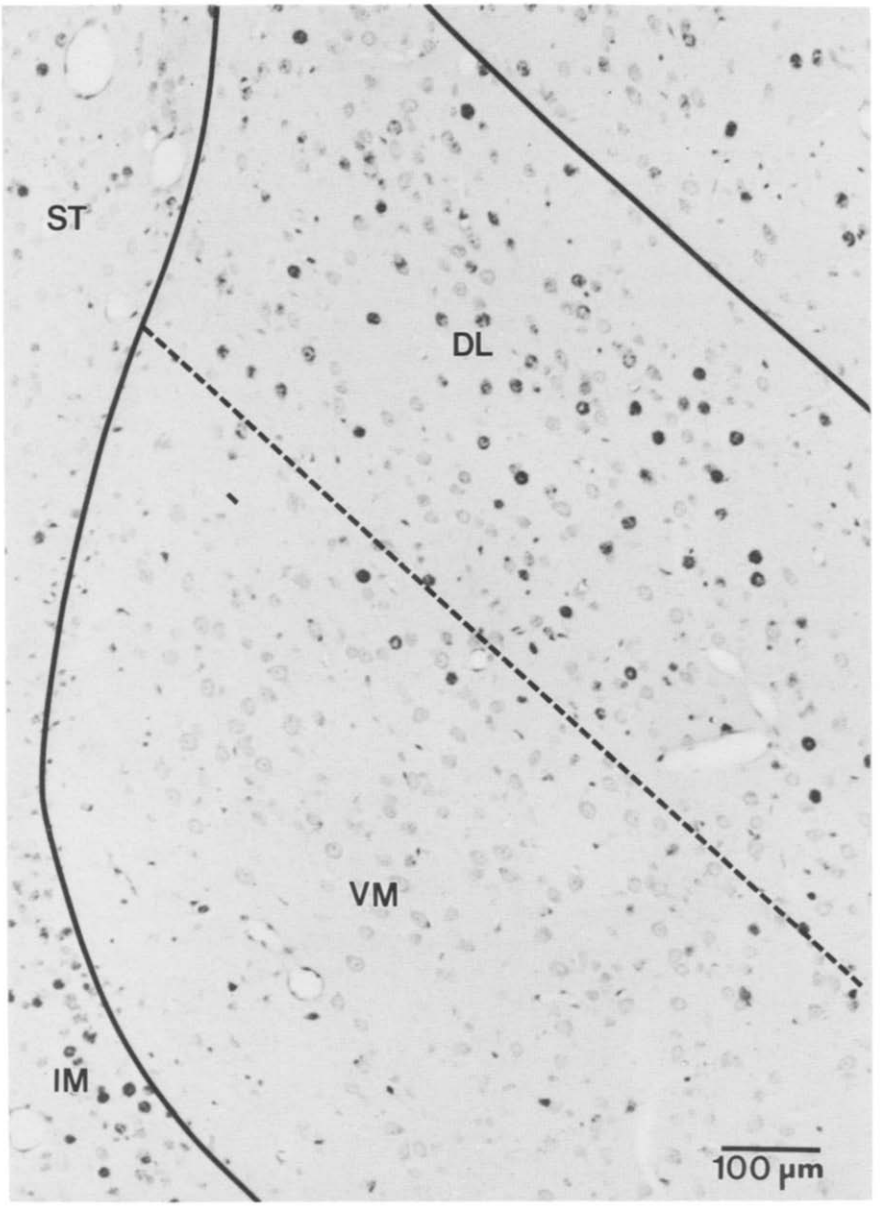


Fig. 1. [^{3}H]Thymidine autoradiogram of the EP in an adult rat that was exposed to the radiochemical on E15 and E16 (dorsal is at the top and medial is to left; 6-μm paraffin section, hematoxylin and eosin stain). Cells with intense black dots contain radioactive label. Most labeled neurons are in the dorsolateral rather than in the ventromedial part.

originate on E14, the embryonic development of the EP was studied in sequential-survival [^{3}H]thymidine autoradiograms of embryonic rat brains after a single exposure to [^{3}H]thymidine on E14. Since there were no differences in time of neuron origin in the antero-posterior plane, one midcoronal level was analysed in detail on E15 (Fig. 4), E17 (Fig. 5), E19 (Fig. 6) and E21 (Fig. 7).

Table 1. Neurogenesis of the ventromedial endopiriform nucleus

Injection group	*N*	Percentage labeled cells (mean ± S.D.)	Day of origin	Percentage cells originating*
E12–E13	6 (A)	100	E12	2.69 (A, B)
E13–E14	8 (B)	97.31 ± 0.75	E13	14.14 (B, C)
E14–E15	7 (C)	83.17 ± 6.36	E14	54.32 (C, D)
E15–E16	6 (D)	28.85 ± 15.67	E15	25.11 (D, E)
E16–E17	12 (E)	3.74 ± 0.65	E16	1.56 (E, F)
E17–E18	9 (F)	2.18 + 0.61	E17	2.18 (F, G)
E18–E19	6 (G)	0 ± 0	E18	—

The data for the ventromedial neurons in the EP are given as an example of how they are derived for presentation in the bar graphs used throughout the paper. *n*, Number of animals analysed in each injection group. The percentage labeled cells for each injection group gives the group means ± S.D. for the raw data counts percentage of labeled cells to total cells in individual animals). The standard deviations are typical of the variability seen throughout data collection. The percentage cells originating column lists the data that are presented in the bar graph (Fig. 2, bottom). To get the height of the bar on E14, for example, the proportion of labeled cells in injection group E15–E16 (entry D, column 3) is substracted from the percentage labeled cells in injection group E14–E15 (entry C, column 3) to get the proportion of cells originating during the day on E14 (54.32%).

*Bottom graph, Fig. 2.

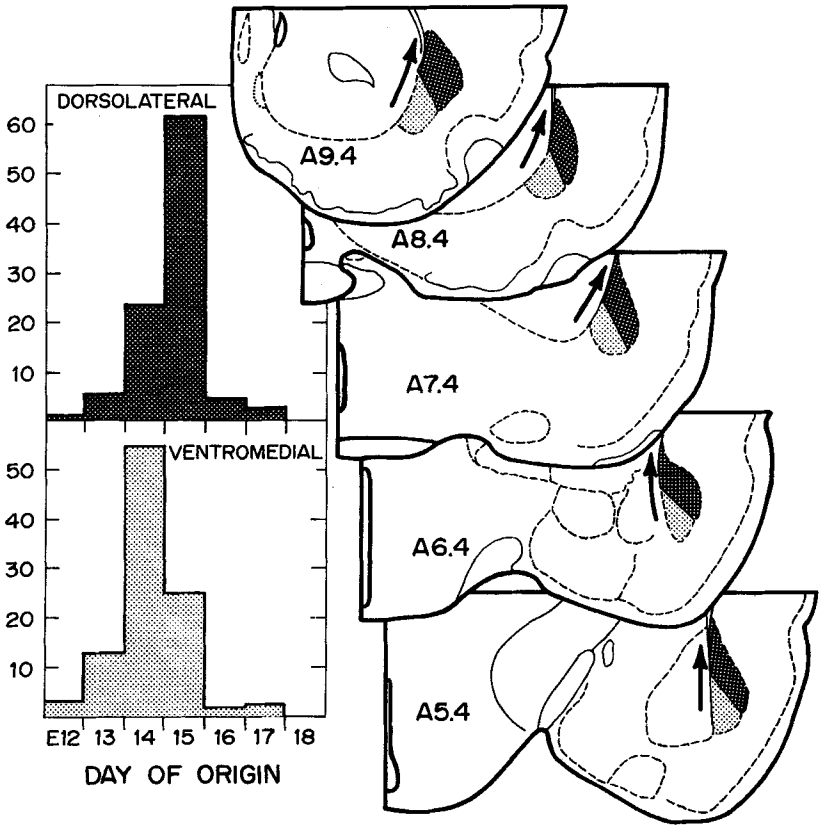


Fig. 2. Time of origin of neurons in the EP as determined in long-survival [^{3}H]thymidine autoradiograms similar to the one shown in Fig. 1. Drawings indicate the sections analysed (levels A9.4–A5.4[48]). Shaded areas indicate the regions where cells were counted. Bar graphs are the proportion of neurons that originate during single days of embryonic life. The calculations for the bottom graph are shown in Table 1. On average, ventromedial neurons are generated earlier than dorsolateral neurons (arrows in drawings).

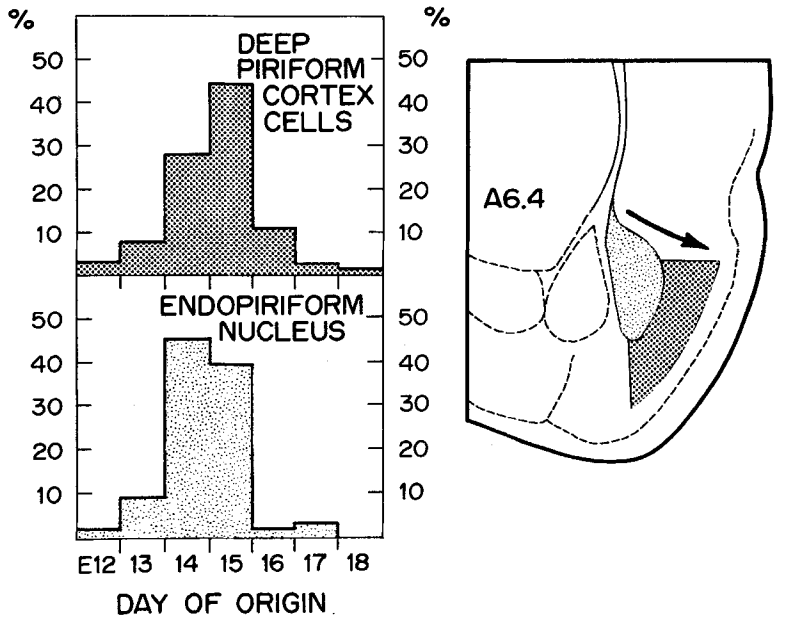


Fig. 3. Timetables of neurogenesis in the EP and the deep cells of the PC at level A6.4. Shaded areas in the drawing indicate where the neurons were counted to compile the quantitative data. Bar graphs are the proportion of neurons generated during single days of embryonic life. Neurons in the EP tend to be slightly older than neurons in the deep PC (arrow in drawing).

On E15 (Fig. 4), one day after the [^{3}H]thymidine injection, many heavily labeled cells are seen outside of the neuroepithelium (dashed outline, Fig. 4A) in the neocortical intermediate zone, within the primordium of the basal ganglia (BG), and deep to the PC. The presumed settling site of neurons in the EP lies just outside of the neuroepithelium between the neocortex (NC) and the basal BG (where the two arrows are converging in Fig. 4B). The heavily labeled cells here are presumed to be a mixture of young postmitotic neurons that have moved out of the adjacent neuroepithelium (straight arrow, Fig. 4B) and of young neurons that have migrated ventrally (curved arrow, Fig. 4B) from the intermediate zone of the neocortex.[7,8] However, at this early stage none of the heavily labeled cells can be identified as future endopiriform neurons. Young neurons (unlabeled) that were generated before E14 accumulate in a thick band beneath the pial surface in the basal telencephalon (BT). These neurons may ultimately settle in the magnocellular basal telencephalic nuclei and anterior amygdaloid area, many of which originate on E12 and E13.[1,2]

On E15, there are some labeling patterns in the neuroepithelium that deserve mention. In the neocortical neuroepithelium, there are scattered heavily labeled superficial cells, but most cells are lightly labeled, indicating a high rate of cell proliferation. In contrast, the BG neuroepithelium contains many heavily labeled cells, indicating a low rate of cell proliferation. An unusually dense cluster of heavily labeled superficial cells (dashed line, Fig. 4B) is seen in the neuroepithelium between the neocortex and the lateral part of the BG. These are either slowly cycling proliferating cells (leading to less label dilution) or postmitotic neurons sequestered in the neuroepithelium.[7] We emphasize this cluster because it is seen bilaterally in every coronally sectioned E15 embryo one day after an E14 [^{3}H]thymidine injection.

On E16 (not shown), two days after the [^{3}H]thymidine injection, many of the heavily labeled cells have migrated farther outward and some have reached the superficial parts of the basal telencephalon and the PC. But there is still no evidence of a cluster of heavily labeled cells that could be identified as the EP.

By E17 (Fig. 5), three days after the [^{3}H]thymidine injection, there is a hint of the accumulation of a few rounded heavily labeled cells (circled outline, Fig. 5A; short arrows, Fig. 5B) in the depths of the developing PC. These cells are surrounded by smaller, spindle-shaped young neurons that appear to be migrating (long curved arrows, Fig. 5) downward and out to the PC. Heavily labeled cells are now accumulating in the PC. On E18 (not shown), the stream of cells migrating from dorsal to ventral has increased, but the accumulation of heavily labeled cells in the presumptive EP remains indistinct.

It is not until E19 (Fig. 6), five days after the E14 [^{3}H]thymidine injection, that the EP (outlined in Fig. 6B) becomes obvious as a cluster of heavily labeled neurons (short arrows, Fig 6B). But there are still many lightly labeled cells that appear to be migrating through the medial part of the nucleus. The large numbers of cells migrating ventrally from the NC are now concentrated in a narrower path, the

lateral cortical stream[7,8] (LCS; long curved arrows). Many of the migrating cells are accumulating in a reservoir just medial to the formative EP, while others are migrating outward toward the PC. On E20 (not shown), the EP becomes more distinct as the cells migrating downward from the cortex begin to dwindle.

On E21 (Fig. 7) seven days after the E14 [^{3}H]-thymidine injection, the EP becomes still more distinct with its large accumulation of heavily and lightly labeled neurons (outlined in Fig. 7B). Now there are many fewer cells migrating (arrows) in the LCS. However, many labeled cells still remain in the reservoir. By this time, a cluster of lightly labeled claustral neurons (CL) can be seen just dorsal to the EP.

The claustrum

Neurogenesis. The CL at anterior and posterior levels in a rat exposed to [^{3}H]thymidine on E16 and E17 and killed on P60 is shown in Fig. 8 . There are more labeled cells posteriorly than anteriorly. That indicates that most posterior neurons originated before E16, while many anterior neurons originated on or after E16. In contrast to the ventral to dorsal neurogenetic gradient in the EP, the CL has a distinct posterior (older) to anterior (younger) neurogenetic gradient.

The birthdates of claustral neurons were determined at four levels, from A9.4 anteriorly to A6.4 posteriorly[48] (drawings, Fig. 9). There is a sharp peak in neurogenesis on E15 at level A6.4 (bottom graph). An increasing proportion of neurons is generated on E16 at levels A7.4 and A8.4 (two middle graphs), and at level A9.4 (top graph); more neurons originate on E16 than on E15. The sign test indicated that this trend is significant between levels A6.4 and A7.4 ($P < 0.003$), between levels A7.4 and A8.4 ($P < 0.042$) and between levels A8.4 and A9.4 ($P < 0.027$).

The CL and the entire EP is shown at level A8.4 in a rat that was exposed to [^{3}H]thymidine on E16 and E17 and was killed on P60 (Fig. 10). There are no labeled neurons in the ventral EP (VEP), only a few in the dorsal EP (DEP), but many in the CL. This indicates a ventral (older) to dorsal (younger) neurogenetic gradient within the EP and between the EP and CL. These qualitative observations are quantitatively documented in Fig. 11. Neurons in the VEP originate mainly on E14, those in the DEP originate mainly on E14 and E15, while those in the CL originate mainly on E15 and E16 ($P < 0.0001$ to $P < 0.004$, sign test).

Embryonic development. To follow the embryonic development of the CL with sequential-survival [^{3}H]thymidine autoradiography, it was important to time the [^{3}H]thymidine injections so that the early-generated endopiriform neurons and late-generated claustral neurons could be distinguished from each other by their labeling patterns. Because the neurogenetic timetables indicated that E16 is a time of

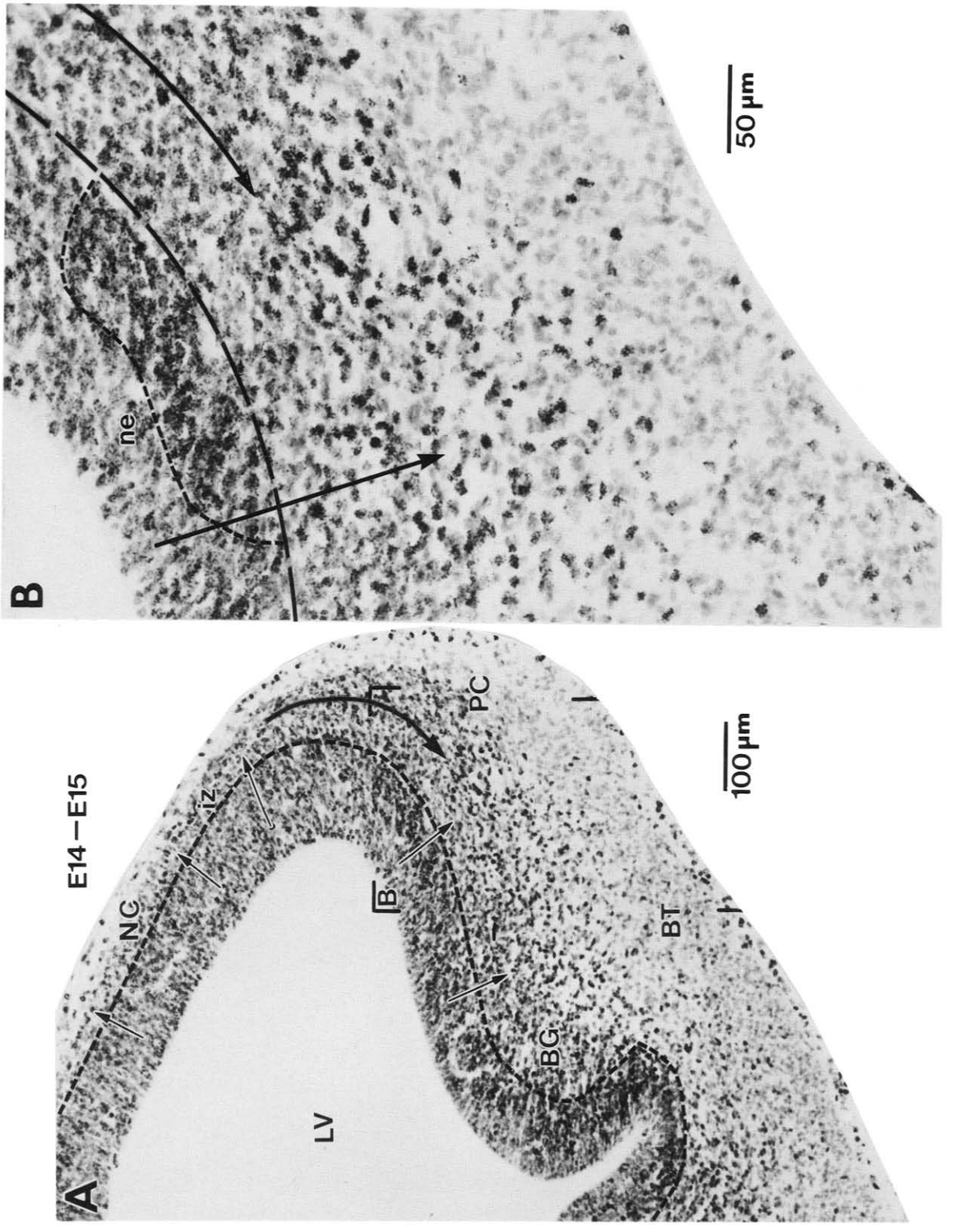
Fig. 4. Autodiagram of the coronally sectioned ventrolateral telencephalon on a rat embryo that was exposed to [^{3}H]thymidine on E14 and survived to E15 (6-μm paraffin section, hematoxylin stain). (A) Low magnification view. Many heavily labeled cells have migrated outward (straight arrows) from the neuroepithelium, and some are migrating downward (curved arrow) from the neocortical primordium toward the primordia of the PC and the basal telencephalon. (B) High magnification view of the area marked with brackets in A. There is a large cluster of heavily labeled cells in the superficial part of the neuroepithelium (surrounded by a dashed line) in the angle between the BG and the NC. The settling site of EP neurons (many of which have already originated by E15) is presumed to be the area between the converging arrows.

Fig. 5. The same as in Fig. 4 in a rat embryo that was exposed to [^{3}H]thymidine on E14 and survived to E17 (6-μm paraffin section, hematoxylin stain). (A) Low magnification view. Labeled cells are still migrating downward (arrows) from the NC. Dashed line indicates the lateral border of the striatum; circled outline is the presumed settling site of EP neurons. (B) High magnification view of the area marked with brackets in A. Long curved arrows represent the trajectories of migrating young neurons. Short arrows indicate the rounded heavily labeled cells that may be settled EP neurons.

Fig. 6. The same as Fig. 4 in a rat embryo to [^{3}H]thymidine on E14 and survived to E19 (6-μm paraffin section, hematoxylin stain). (A) Low magnification view; (B) high magnification view of the area marked with brackets in A. A more concentrated stream of migrating cells (curved arrows), the LCS is moving downward from the neocortex along the lateral border of the striatum (dashed outline). Many lightly labeled cells are accumulating in a pod-shaped structure called the reservoir at the base of the LCS while others are migrating outward to the PL. The large heavily labeled cells (short arrows) just ventrolateral to the reservoir can now be more clearly identified as settled EP neurons (solid outline).

Fig. 7. The same as Fig. 4 in a rat embryo exposed to [^{3}H]thymidine on E14 and survived to E21 (6-μm paraffin section, hematoxylin stain). (A) Low magnification view; (B) high magnification view of the area marked with brackets in A. Now fewer cells are migrating downward in the LCS but many are still in the reservoir. The EP (solid outline) is clearly seen ventral to the cluster of claustral neurons.

Fig. 8. [^{3}H]Thymidine autoradiograms of the CL in an adult rat that was exposed to the radiochemical on E16 and E17 (dorsal is at the top and medial is to the left; 6-μm paraffin sections, hematoxylin and eosin stain). Many neurons at level A9.4 (A) are labeled, while most neurons are unlabeled at level A6.4 (B).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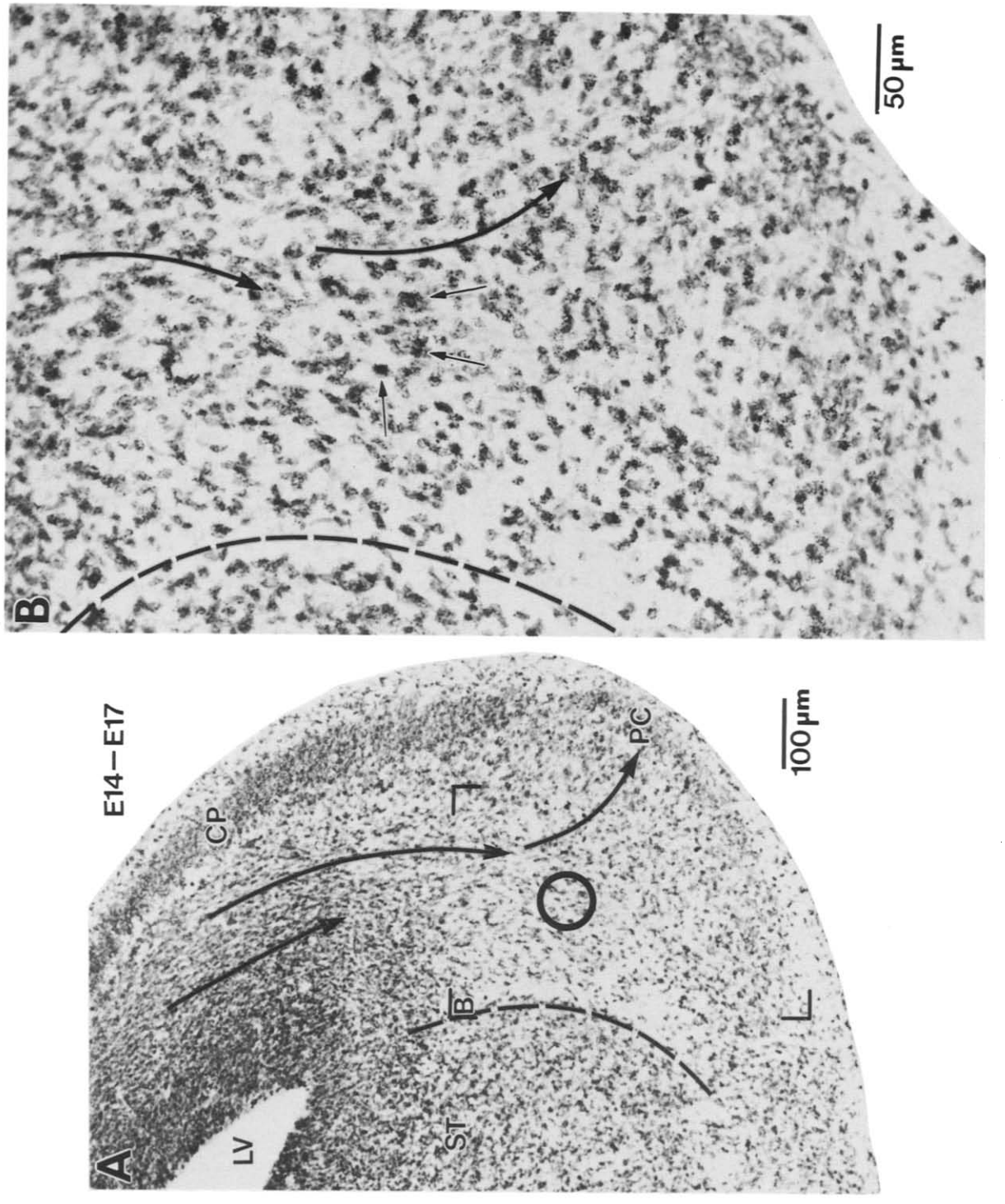
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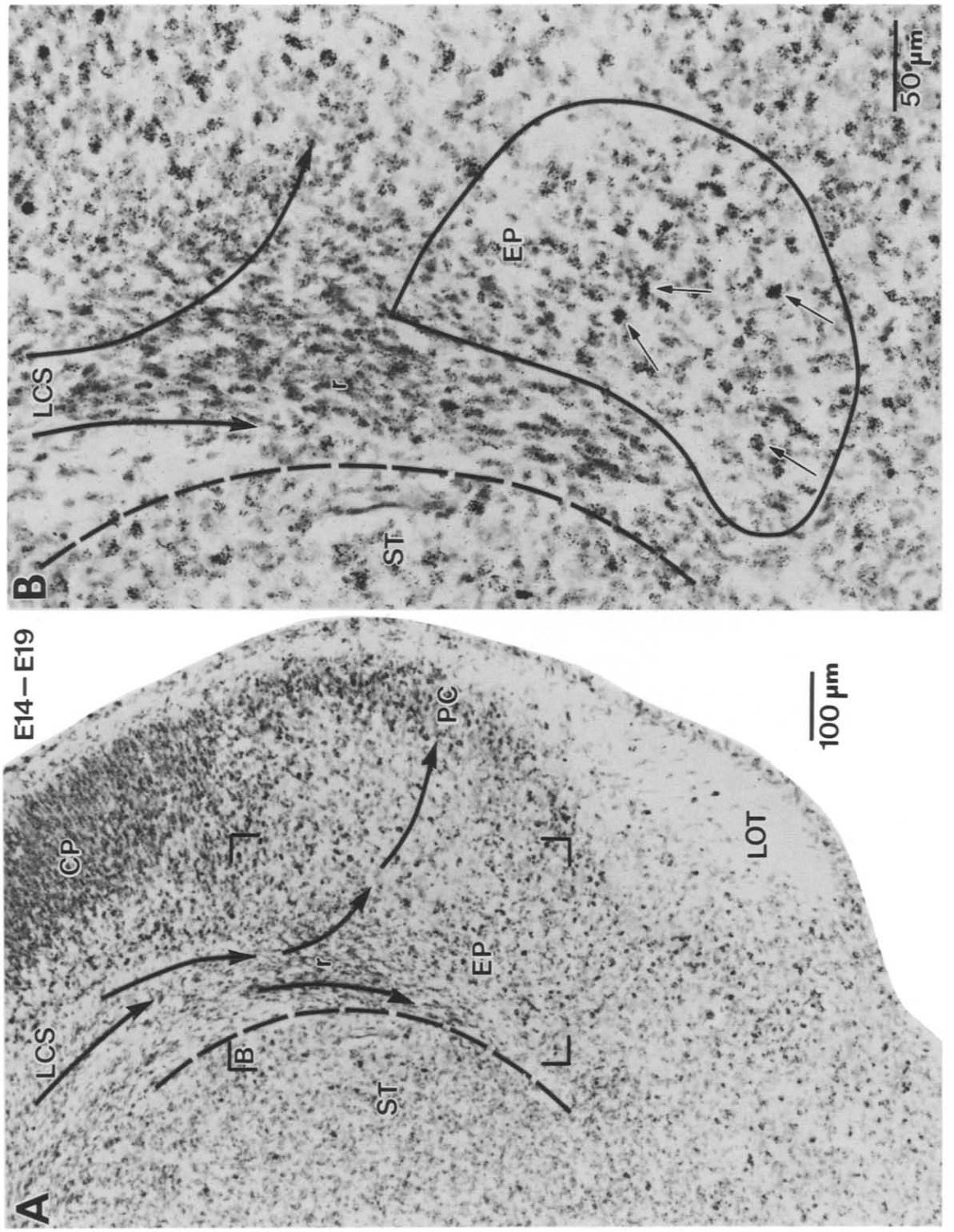

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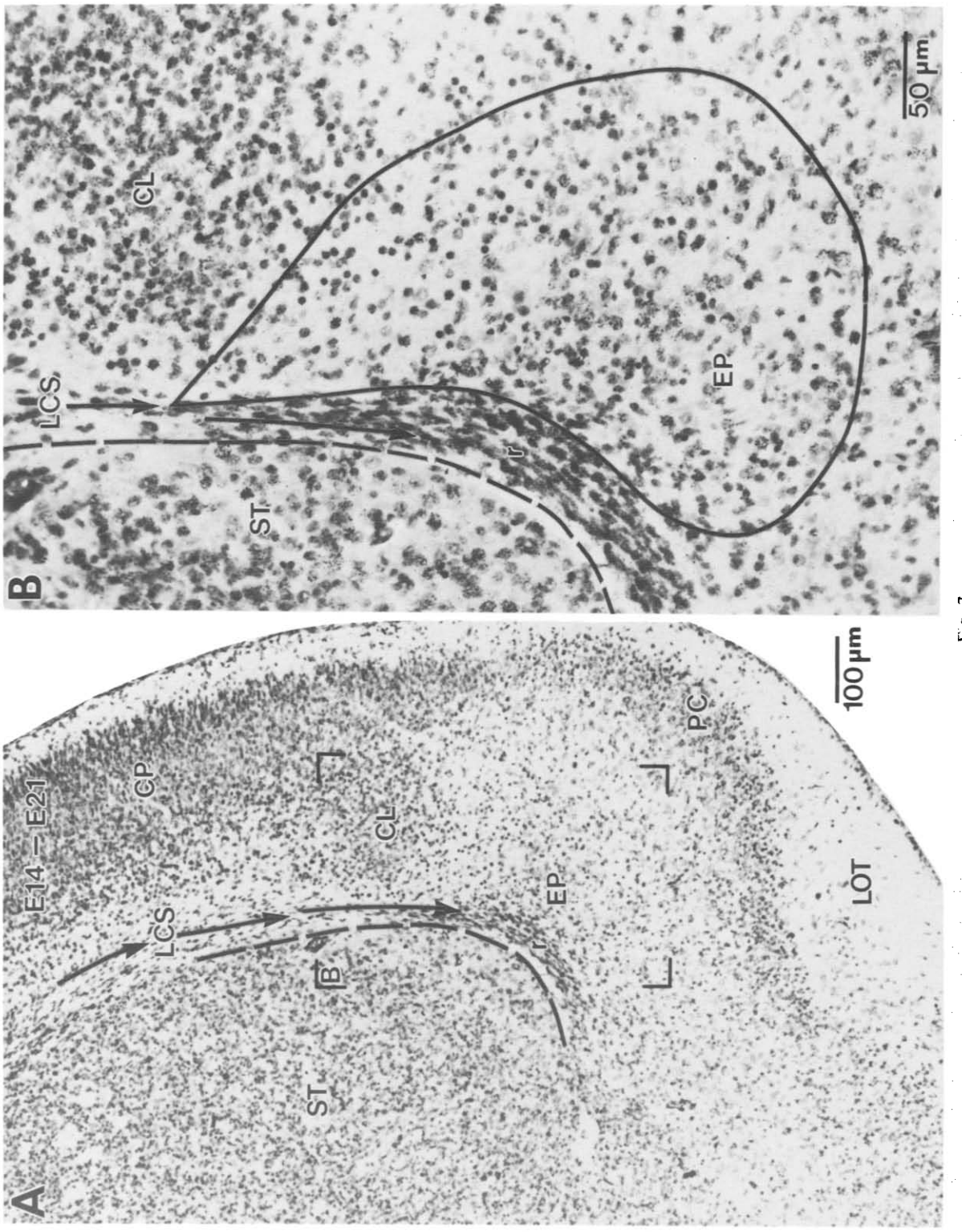

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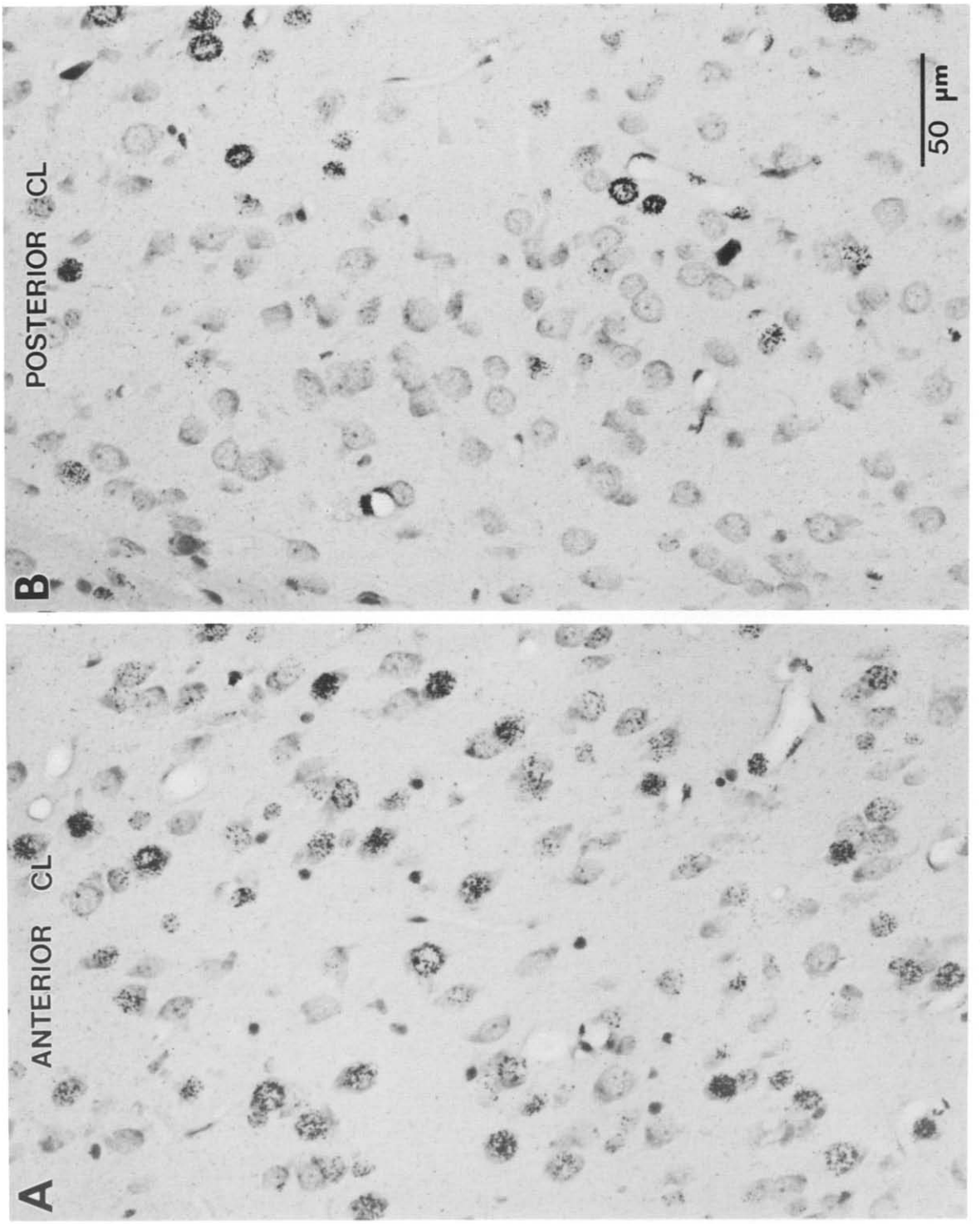

Fig. 8.

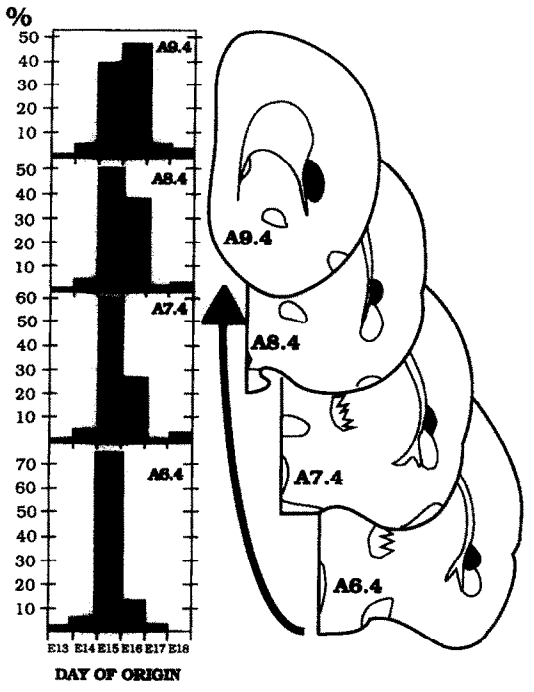

Fig. 9. Time of origin of neurons in the CL as determined in long-survival [^{3}H]thymidine autoradiograms similar to the ones shown in Fig. 8. Drawings indicate the sections analysed (levels A9.4–A6.4). Shaded areas indicate the regions where cells were counted. Bar graphs are the proportion of neurons that originate during single days of embryonic life. On the average, posterior neurons are generated earlier than anterior neurons (large arrow outside drawings).

considerable claustral neurogenesis (Figs 9, 11) and negligible endopiriform neurogenesis (Figs 2, 3, 11), the embryonic development of the CL was studied in sequential-survival [^{3}H]thymidine autoradiograms after a single exposure to [^{3}H]thymidine on E16. Both anterior and posterior levels were studied because of the posterior (older) to anterior (younger) neurogenetic gradient. Detailed observations were made on E18 (Fig. 12), E20 (Fig. 13) and E22 (Fig. 15).

By E18 (Fig. 12), two days after the E16 [^{3}H]thymidine injection, many labeled young neurons are migrating ventrally (curved arrows) out of the NC in the LCS at both anterior (Fig. 12A) and posterior (Fig. 12B) levels. Some of the migrating young neurons are leaving the LCS (outward pointing curved arrows) and are penetrating the ventrolateral cortical plate (CP) and the deep layers of the PC. No clusters of cells can be recognized as the CL at either level. On E19 (not shown), migrating young neurons are still prominent in the LCS, and the CL is still not recognizable at anterior and posterior levels.

By E20 (Fig. 13), four days after the [^{3}H]thymidine injection, the LCS has become more compact, and many cells are accumulating in its reservoir both anteriorly and posteriorly. At the posterior level, unlabeled and labeled cells (circled outline, Fig. 13B) begin to cluster beneath the CP, just in the location where claustral neurons will settle. That cluster of cells is shown at higher magnification in Fig. 14, where some heavily labeled cells appear to be migrating into it (curved arrow) from the LCS. These may be migrating and settling claustral neurons. A large group of mostly unlabeled cells lie ventral to the cluster (solid outline, Figs 13B, 14); these must be the endopiriform neurons. At the anterior level (Fig. 13A), no such cluster can yet be identified. On E21, five days after the [^{3}H]thymidine injection, the cluster of presumptive claustral neurons becomes more distinct at the posterior level, is becoming recognizable at the middle level (Fig. 7), but cannot yet be seen at the anterior level in most animals and is only an indistinct cluster in a few more advanced animals.

By E22 (Fig. 15), six days after the [^{3}H]thymidine injection, the CL is easily identified in low magnification sections both anteriorly (Fig. 15A) and posteriorly (Fig. 5B). High magnification views of the clusters show that the posterior one is smaller (dashed outline, Fig. 15D), and contains fewer labeled neurons than the anterior one (dashed outline, Fig. 15C), conforming to the neurogenetic gradient seen in adults (Fig. 9).

DISCUSSION

The neurogenetic timetables presented in Figs 3 and 9 show that the EP and the CL have different times of origin and divergent neurogenetic gradients. The CL originates late, on E15 and E16, and has a strong gradient in the longitudinal direction, from posterior (older) to anterior (younger). In contrast, the EP originates early, on E14 and E15, and lacks a longitudinal gradient but has a strong one in the vertical direction, from ventral (older) to dorsal (younger). The embryonic observations indicate that the neurons in the EP settle within two to three days after the peak time of neurogenesis (Fig. 5) whereas neurons in the CL settle between four to six days after the peak (Figs 13–15). The postulated events that take place during embryonic development are shown in Fig. 16. In the following discussion, we summarize the evidence supporting the hypothesis that the EP and the CL originate from different neuroepithelial sources, and have different settling patterns.

Neuroepithelial sources and migratory patterns of endopiriform neurons and claustral neurons

Endopiriform nucleus. Endopiriform neurons have unique features that provide clues to their embryonic development. They are the earliest generated neurons in the piriform lobe, with a peak time of production on E14 (Figs 2–3), and they settle deep within the piriform lobe, just outside of the external capsule that demarcates the striatal border (Fig. 1). The ventrolateral telencephalon on E14 (Fig. 16) consists mainly of a thick neuroepithelium (stippled layer) surrounded by a thin layer of differentiating cells (clear layer). There is only a slight depression in the neuroepithe-

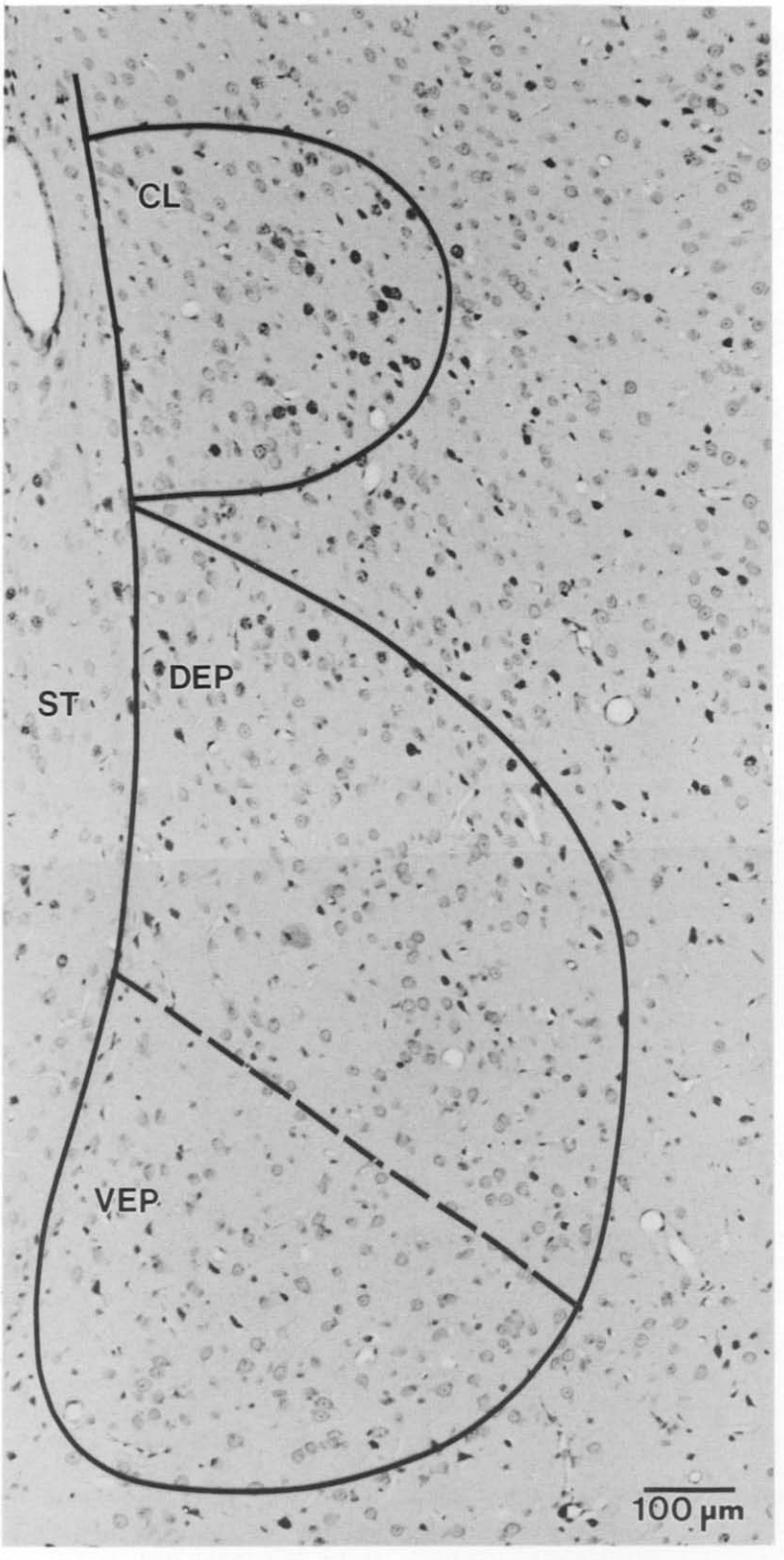

Fig. 10. A low magnification [^{3}H]thymidine autoradiogram that includes the VEP and DEP beneath the CL at level A8.4 in an adult rat that was exposed to the radiochemical on E16 and E17. There are no labeled neurons in the VEP, only a few in the DEP, and many in the CL. Note that the few darkly staining shrunken cells scattered in VEP and DEP are fixation artifacts and are not labeled neurons.

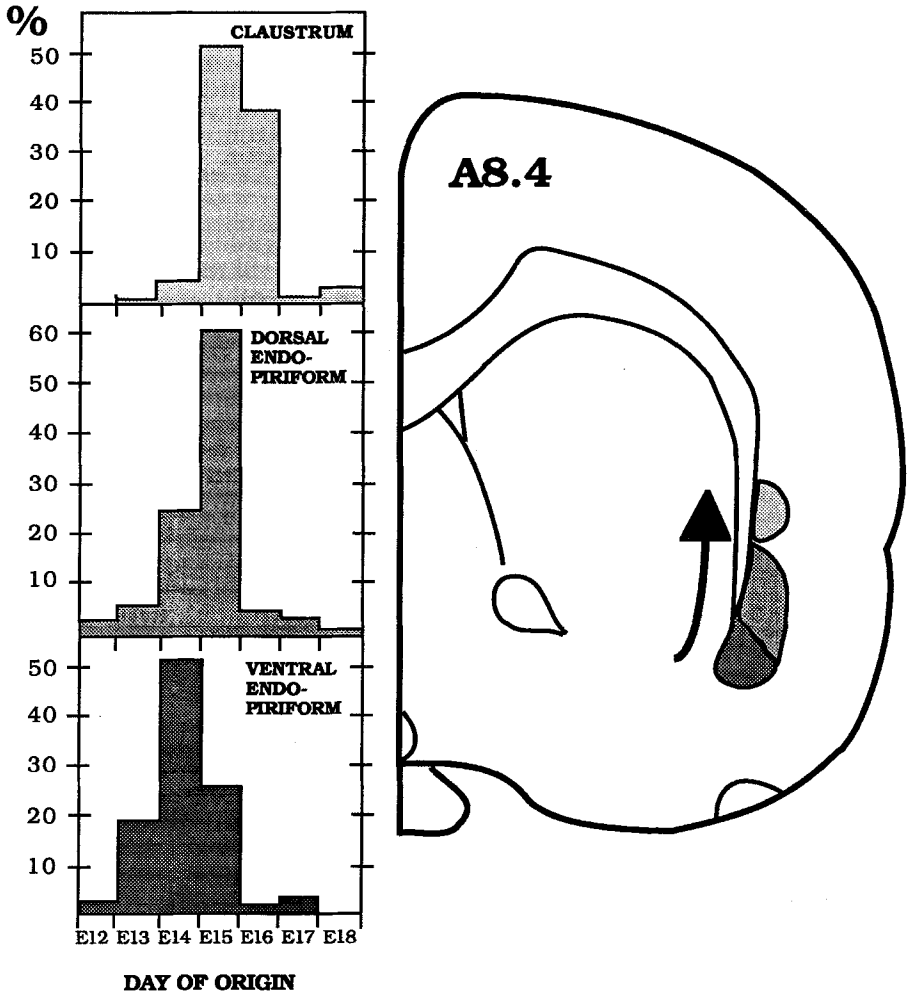

Fig. 11. Timetables of neurogenesis in the VEP and DEP and the CL at level A6.4. Shaded areas in the drawing indicate where the neurons were counted to compile the quantitative data. Bar graphs are the proportion of neurons generated during single days of embryonic life. There is a ventral (older) to dorsal (younger) neurogenetic gradient in the locations measured (arrow in drawing).

lium marking the border between the lateral ganglionic eminence, which will give rise to the striatum (BG), and the pallium, which will give rise to the NC. Källén[27] called this area dv (the ventral enlargement of the roof plate), and Filiminoff[18] called it the palliostriatal ventricular angle (psv on E14, Fig. 16). Both of these investigators surmised that the CL (including the EP) arises in that germinal source. The observations in this study support the hypothesis that the EP is generated in the palliostriatal ventricular angle, but we postulate that another neuroepithelial site is the source of the CL.

With regard to the neuroepithelium in the palliostriatal ventricular angle, two different sequences of events are possible. Firstly, young endopiriform neurons may simply migrate out radially shortly after their generation on E14 and settle immediately on E15. The many heavily labeled cells (birthdays on E14) already outside of that neuroepithelial site on E15 (Fig. 4B) support this hypothesis. However, the neuroepithelium in the palliostriatal ventricular angle has an unusually dense cluster of heavily labeled superficial cells (dotted outline, Fig. 4B; closed circles in psv on E15, Fig. 16) that may indicate another sequence of events. These heavily labeled cells may be postmitotic young endopiriform neurons that are sequestered in the neuroepithelium for approximately one day before migrating outward on the next day. Such sequestering of postmitotic neurons has been postulated to occur in the developing neocortex.[7] One day after a [^{3}H]thymidine injection during early cortical development (say on E14 and E15) heavily labeled cells are more commonly seen in the superficial part of the neocortical neuroepithelium, and lightly labeled cells predominate near the ventricular lumen. We postulated that the early generated subplate neurons are sequestered in the neuroepithelium for approximately one day before migrating outward.[7] It is interesting that endopiriform neurogenesis (Fig. 2) and subplate neurogenesis[6] have similar timetables,

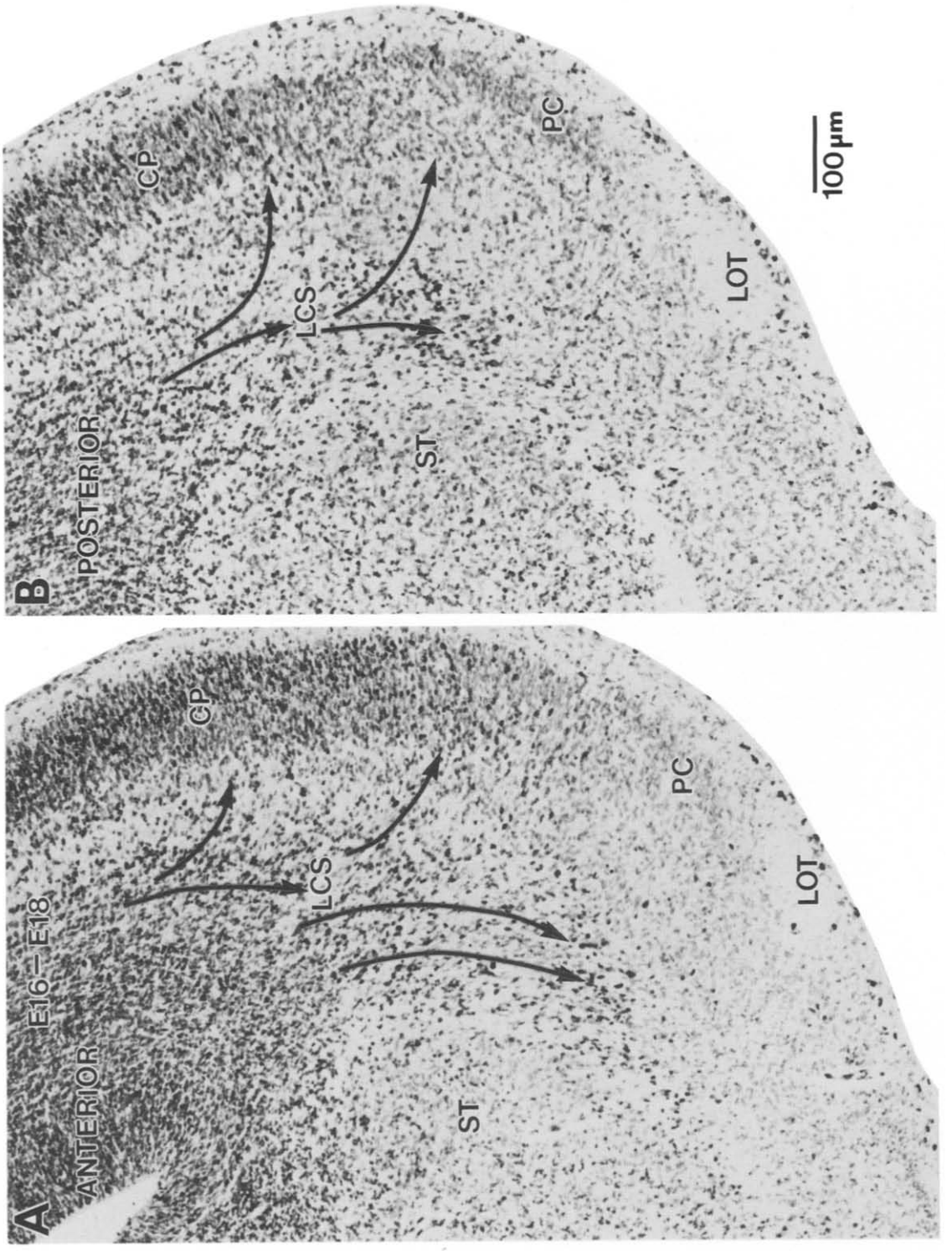

Fig. 12. Autoradiogram of the coronally sectioned ventrolateral telencephalon at anterior (A) and posterior (B) levels of the future site of the CL in a rat embryo that was exposed to [^{3}H]thymidine on E16 and survived to E18 (6-µm paraffin sections, hematoxylin stain). Arrows represent the trajectories of migrating young neurons in and away from the LCS. No settled claustral neurons can be discerned at either level.

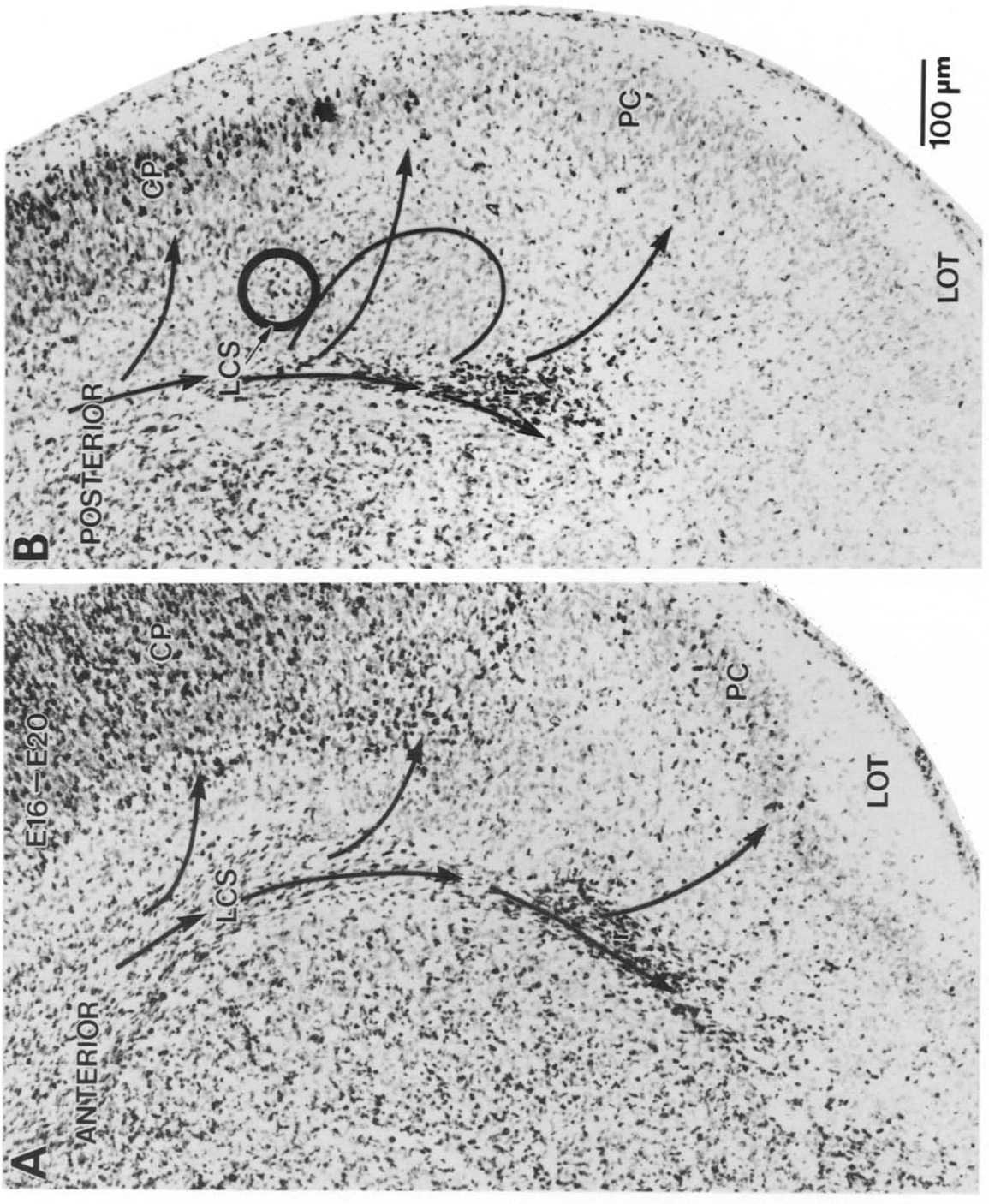

Fig. 13. The same as in Fig. 12 in a rat embryo that was exposed to [^{3}H]thymidine on E16 and survived to E20 (6-μm paraffin sections, hematoxylin stain). At the posterior level (B), a small cluster of presumptive claustral neurons (circled outline) is located dorsal to the EP (solid outline).

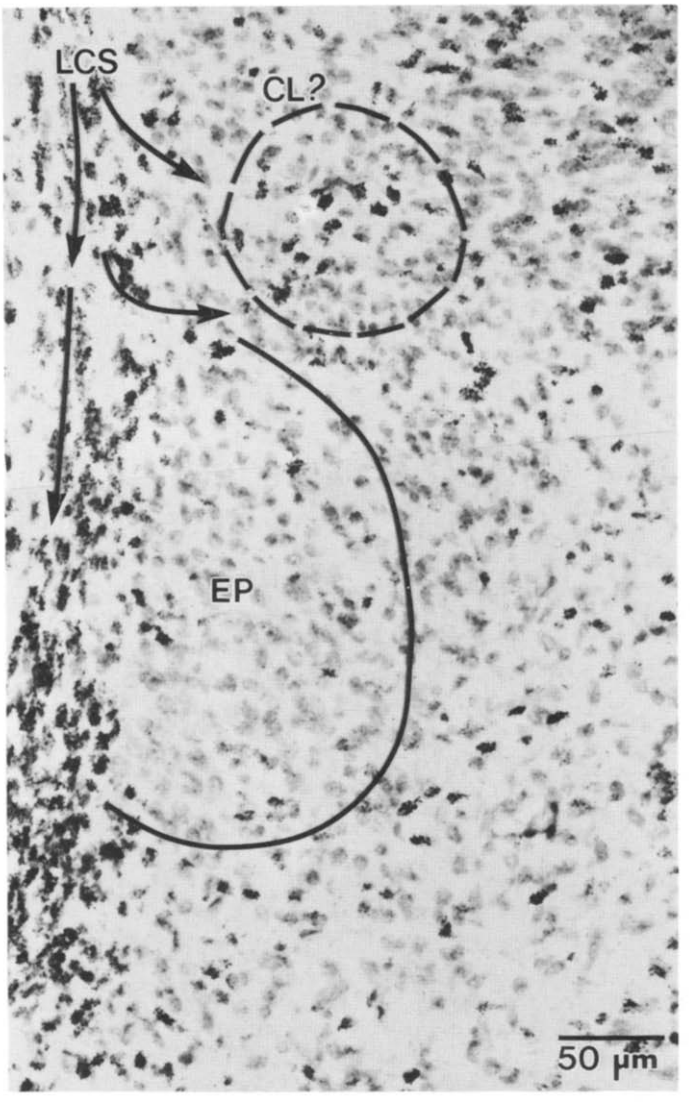

Fig. 14. High magnification view of the cluster of presumptive claustral neurons (dashed outline) in Fig. 13B. A few claustral neurons are heavily labeled, while most endopiriform neurons are unlabeled. Arrows represent the trajectories of migrating cells in and leaving the LCS.

both occurring mainly on E14 and E15. The unusually dense cluster of heavily labeled cells in the neuroepithelium of the palliostriatal ventricular angle on E15 (Fig. 4B) may reflect the higher number of endopiriform neurons that are sequestered there in contrast to the more sparse distribution of subplate neurons in the neocortical neuroepithelium.

If we accept that endopiriform neurons are sequestered in the neuroepithelium, then what are the heavily labeled young neurons already outside of the palliostriatal ventricular angle on E15? Again, turning to our developmental study of the neocortex,[7] we found neurons in layers VI–II to immediately move outside of the neuroepithelium and accumulate (sojourn) in layer-specific bands in the neocortical intermediate zone the first day after their generation. According to the data presented in Fig. 3, many layer III PC neurons are also generated on E14. We propose that the layer III PC neurons are already outside of the neuroepithelium on E15 and subsequently migrate radially to settle (closed squares, Fig. 16).

On the following days, growth of the BG displaces the palliostriatal ventricular angle upward.[7] That would cause the neurons generated on E14 to settle ventrally, possibly as early as E16 (large closed circles, Fig. 16) and the neurons generated on E15 to settle dorsally by E17 (large open circles, Fig. 16). That sequence of events could account for the ventral (older) to dorsal (younger) neurogenetic gradient in the EP.

Because the neuroepithelium in the palliostriatal ventricular angle shares many labeling patterns with the neuroepithelium in the NC and is a likely source of deep neurons in the PC, it may be classified as a cortical primordium rather than a ganglionic one.

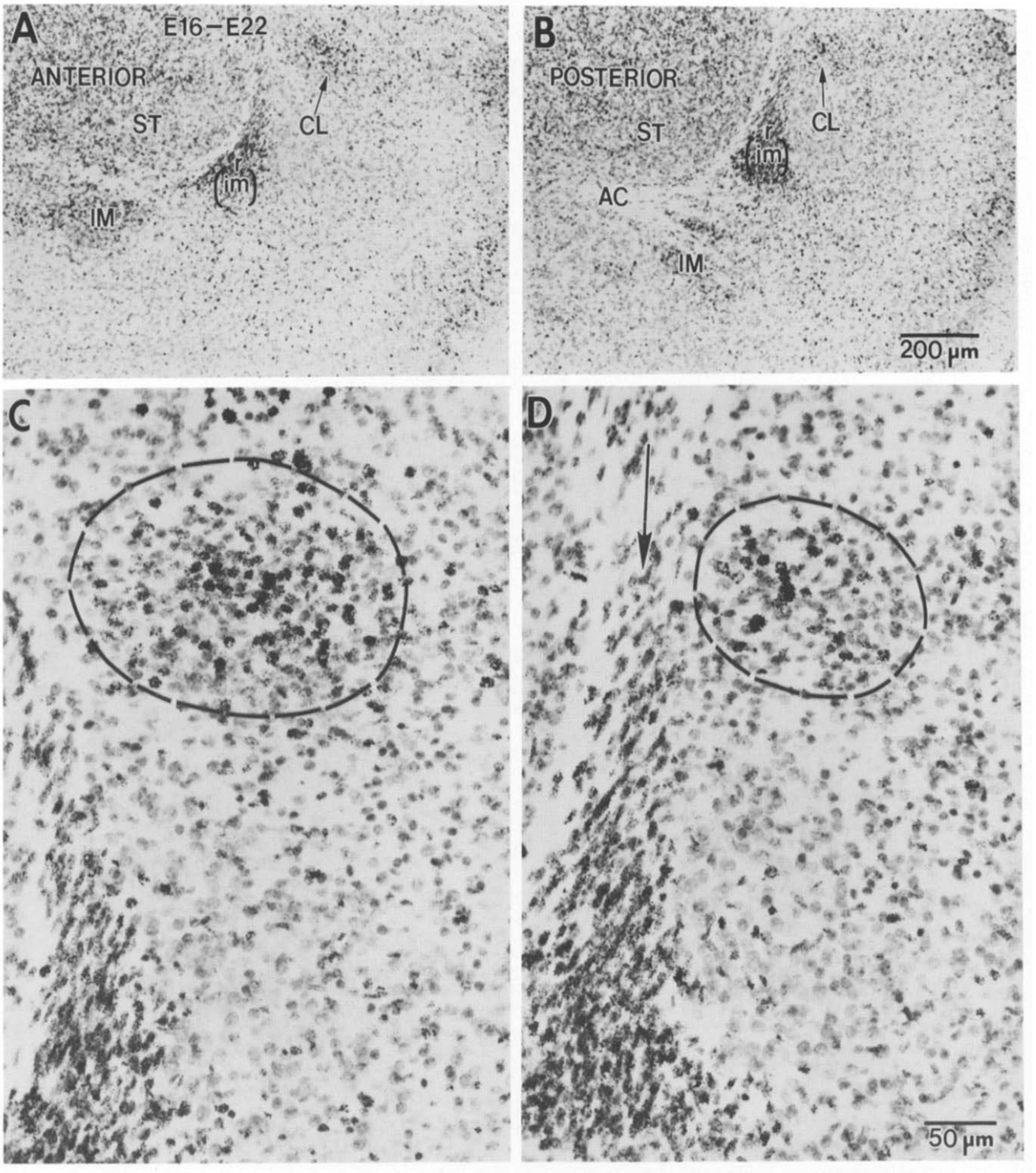

Fig. 15. Low and high magnification views of the CL in the ventrolateral telencephalon at anterior (A, C) and posterior (B, D) levels in a rat embryo exposed to [^{3}H]thymidine on E16 and survived to E22, the day before birth (6-μm paraffin sections, hematoxylin stain). The CL is larger anteriorly (dashed outline, C) than it is posteriorly (D) and contains a higher proportion of labeled cells, in accordance with the posterior (older) to anterior (younger) neurogenetic gradient (Fig. 9).

Even though the EP has anatomical connections with the BG (see below), it is most unlikely that any of its neurons originate in the BG neuroepithelium and migrate laterally to settle there. The external capsule, which demarcates the boundary of the striatum, is already definite on E17 (dashed line, Fig. 5), and there are very few cells migrating across it.

The claustrum. The fact that claustral neurons are recognizable much later after their generation (four to five days, Figs 13–14) than endopiriform neurons (three days, Fig. 5) strongly suggests that claustral neurons migrate over a longer distance before settling. As shown in Fig. 16, we postulate that claustral neurons are generated in the neocortical neuroepithelium, posterior neurons mainly on E15 (closed rectangles) and anterior neurons mainly on

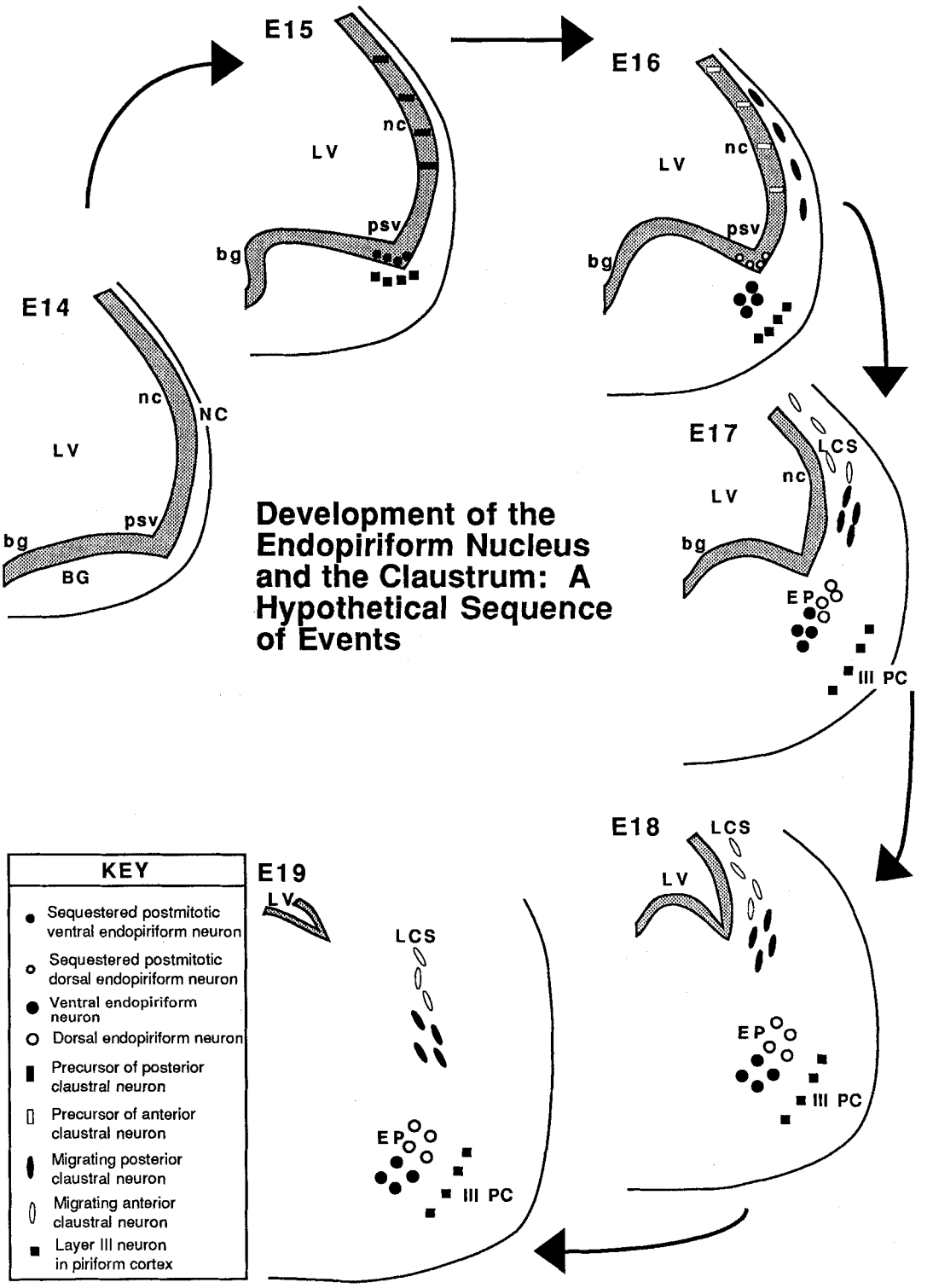

Fig. 16. A diagrammatic summary of the postulated events that occur in the ventrolateral telencephalon during development of the EP and the CL. The neuroepithelium is the stippled layer; young postmitotic neurons migrate and settle in the clear layer outside the neuroepithelium. The endopiriform neurons are generated in the palliostriatal ventricular angle on E14 and E15, are sequestered there for one day (closed circles, E15; empty circles, E16), and settle in a ventral (first) dorsal (last) gradient (closed circles, E16; closed and open circles in EP, E17). Claustral neurons are generated in the neocortical neuroepithelium on E15 (closed rectangles) and E16 (open rectangles), move out of the neuroepithelium one day later (closed ellipses, E16; open ellipses, E17) and migrate in the LCS for several days before settling between E20 and E22 (not shown). Closed squares outside of palliostriatal ventricular angle on E15 are postulated to be young neurons that will settle in layer III of the PC.

E16 (open rectangles). After following cell movements with cholinergic histochemistry[51] and with sequential-survival [^{3}H]thymidine autoradiography,[8] it has been found that many neurons generated in the neocortical neuroepithelium settle outside of the NC, in the PC and in basal telencephalic sites (as yet undetermined, unpublished observations). It is certainly possible that some of these migrating cells settle in the CL; ventrally migrating claustral neurons in the LCS from E16 to E19 are shown in Fig. 16. The posterior CL does not appear until E20 (Fig. 13B), and that delay would correlate with the extra time needed for claustral neurons to migrate in from a more distant germinal source. In fact, migrating cells can be seen leaving the LCS and penetrating the cluster of presumptive claustral neurons (Fig. 14). Additional support for a cortical origin comes from Golgi studies. The majority of claustral neurons resemble cortical pyramidal cells[9,39,57] with one main spiny dendrite coming from the apex of the cell body.

The embryonic observations show that the posterior CL appears later than the anterior CL (Figs 13–15), correlating with the claustral neurogenetic gradient (Fig. 9). Claustral neurons settle deep in the lateral limbic cortex (insular and gustatory areas). A peculiarity of the lateral limbic cortex is that neurons in layers V and VI have a posterior (older) to anterior (younger) neurogenetic gradient,[3,7] matching the neurogenetic gradient in the CL. Claustral neurons and deep limbic neurons may be generated in similar locations in the neocortical neuroepithelium, and migrate in the anteroposterior plane as well as in the dorsoventral plane before settling.

Differential connectivities of the endopiriform nucleus and the claustrum. Loo's[35] parcellation of the EP and the CL as separate brain areas is supported not only by the divergent developmental patterns described here but also by the accumulating evidence that they have different anatomical connections. The EP gets input from structures that are monosynaptically linked to the main olfactory bulb, such as the anterior olfactory nucleus,[36] the olfactory tubercle,[17] the posterior PC,[22] the lateral entorhinal cortex,[30,58] and the ventral agranular insular cortex.[49] Additional input to the EP comes from the lateral amygdaloid nucleus[31] and from the intralaminar thalamic nuclei.[46] The efferent projections of the EP are also strongly olfactory, including a direct projection to the main olfactory bulb.[54] Several efferents reciprocate afferents, such as the ones to the anterior olfactory nucleus,[24] the olfactory tubercle,[17] the PC,[22,31] and the lateral entorhinal cortex.[29] Some efferents go to the BG, including the ventral putamen,[30] the nucleus accumbens,[20] and there are widespread projections to several amygdaloid nuclei.[31] In contrast, the CL has connections with all parts of the NC and limbic cortex. Most of its input comes from cortical layer VIa,[16] especially from the visual areas[11,28,32,43,52] and from cingulate areas.[32] In turn, the CL has extensive projections to sensory and motor areas of the NC.[10,11,32,33,38,42,44,45,47,50,53,55] There are also widespread projections from the CL to the limbic cortex, including the cingulate cortex,[14,15,19,33,40] the medial prefrontal cortex[15,56] and the perirhinal/insular cortex.[13,40]

The well-documented finding that the claustral projection to the NC is topographic can be related to the posterior (older) to anterior (younger) neurogenetic gradient in the CL (Fig. 9). Topographic projections were first reported by Narkiewicz[44] in the cat and have been confirmed not only in the cat,[32,38,45] but also in the monkey,[47] the tree shrew[10] and the rat.[33,42,53,55] The studies in rats indicate that posterior claustral neurons project to posterior targets in the visual cortex, while progressively more anterior claustral neurons project to parietal and frontal cortical targets.[33,42,53,55] This relationship suggests the possibility that the earlier generated posterior claustral neurons are programmed to send axons to different targets than the later generated anterior claustral neurons.

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