

Hippocampal Development in the Rat: Cytogenesis and Morphogenesis Examined with Autoradiography and Low-level X-irradiation

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ABSTRACT The cytogenesis and morphogenesis of the rat hippocampus was examined with the techniques of ^3H -thymidine autoradiography, cell pyknosis produced by low-level X-irradiation, and quantitative histology.

The procedure of progressively delayed cumulative labelling was used for autoradiography. Groups of rats were injected with four successive daily doses of ^3H -thymidine during non-overlapping periods ranging from birth to day 19. They were killed at 60 days of age, and the percentage of labelled cells was determined. The pyramidal cells of Ammon's horn and the polymorph cells of the dentate gyrus are not labelled postnatally, confirming earlier conclusions of their prenatal origin. In the dentate gyrus, 85% of the granule cells are formed postnatally, with 45% forming during the first week. The majority of the small cells of the dentate molecular layer and of the Ammonic strata oriens, radiatum, and lacunosum-moleculare are formed during the second week.

As an aid to locating the proliferative compartments of the hippocampus during development and to characterize the time course of both cell differentiation and morphological development, rats ranging in age from gestation day 17 to postnatal day 103 were killed six hours after one exposure to 200r X-rays, and the pyknotic cells were counted. Cell pyknosis in Ammon's horn reaches a maximal level prenatally and declines rapidly during the early postnatal period. Cell pyknosis in the dentate gyrus reaches its highest level during the second postnatal week and declines gradually with some radio-sensitive cells still present in the adult. Immature granule cells are also at their highest level during the second postnatal week, while mature granule cells gradually accumulate to attain asymptotic levels at around two months of age. The alignment of the pyramidal cells to form the characteristic curvature of Ammon's horn occurs shortly after pyramidal cell cytogenesis is completed. Conversely, the sharp fold in the dentate gyrus is apparent from the day of birth onward, before the completion of granule cell cytogenesis. Possible mechanisms for the morphological development of the dentate gyrus along with a consideration of the possible migratory route of granule cell precursors is also discussed.

Although the anatomy of the adult mammalian hippocampus has been studied extensively, few reports have dealt with hippocampal cytogenesis and morphogenesis. Developmental analyses are not only useful for their own sake but can also be used to increase our understanding of the role individual components play in producing adult morphology and physiology. Fortunately, the rodent hippocampus exhibits radical changes in both cell

acquisition and gross morphology during a time (late prenatal and early postnatal periods) when two powerful techniques, autoradiography with ^3H -thymidine and low-level X-irradiation, are easily applied. One of the concerns of this study is the specification of the time of origin and maturation of different hippocampal cells. The majority of neurons of the mammalian brain are formed before birth. However, the formative period of some neu-

ronal populations extends to postnatal ages — especially in altricial species such as the rat. The biological significance of postnatal neurogenesis is not known and has been questioned. But, in the case of the hippocampus, some evidence is available that its late morphological maturation is associated with delayed "behavioral maturation" as an adaptive phenomenon (Altman et al., '73). It has also been shown that, if the acquisition of the postnatally-formed neurons of the dentate gyrus is prevented by exposure to low-level X-irradiation, the animals display behavioral symptoms comparable to surgical removal of the entire hippocampus (Bayer et al., '73). In light of these, the accurate dating of the time of origin of various neuronal populations in the hippocampus is potentially useful information.

³H-thymidine autoradiography has been used for the past 10–12 years in attempting to document the time of origin of various neurons in the mammalian brain. In the hippocampus, precursors of the entire Ammon's horn pyramidal cell population can be labelled during prenatal stages in both the mouse (Angevine, '65) and rat (Hine and Das, '74). Since these cells cannot be labelled when ³H-thymidine is injected postnatally (Angevine, '65; Altman and Das, '65a,b), it can be safely concluded that they are of prenatal origin. In contrast to this unequivocal finding, a large proportion of the granule cells of the dentate gyrus become labelled (many of them quite heavily) when injections are made either prenatally (Angevine, '65; Hine and Das, '74) or postnatally (Altman and Das, '65a,b, '66; Altman, '66). Because most of these cells can be labelled with postnatal injections, it must be concluded that they are of postnatal origin. However, in the absence of the latter findings, the conclusion would have been drawn that the majority of granule cells are of prenatal origin. For example, Angevine ('65) found intensely labelled granule cells in mice scattered throughout the midsection of the granular layer when injections were given on embryonic day 18, postnatal day 0, or postnatal day 5. This points to the pitfall of dating neuron origin on the basis of single flash labelling alone, even if the criterion used is intense or heavy label-

ling. The latter is taken as evidence of absence of label dilution and is interpreted as a sign that the cells so labelled have commenced to differentiate soon after the injection. But in the light of the observations referred to and the evidence to be presented, this conclusion is not warranted, presumably because cells may enter a dormant phase for several days or longer and resume their limited divisions after such a delay.

The experiments reported here utilize multiple injections of ³H-thymidine in a new procedure which we call "progressively delayed comprehensive labelling." Since repeated divisions of a group of labelled cells results in loss of detectable labelling, it is necessary to inject ³H-thymidine several times if all the cells formed after a certain date (or the entire population) are to be labelled. This is the cumulative labelling procedure. If the cumulative labelling in different groups of animals is progressively delayed, one can determine the proportion of cells that were formed before that date (those that can no longer be labelled) and the proportion that was formed thereafter. This procedure has allowed us to specify the proportion of granule cells formed over blocks of days (in theory over any specified period) within the period of granule cell acquisition.

The second procedure used in this study was irradiation of the developing hippocampus with low-level X-ray. Dividing and migrating cells of the fetal (Hicks and D'Amato, '66) and infant (Altman et al., '68; Altman and Nicholson, '71) rat nervous system die within a few hours after exposure to low-level (100–200r) X-irradiation. Cells that are either differentiating or are already mature are killed only by much higher doses (Hicks, '58). Given these differences, it follows that by killing young animals shortly after low doses of X-irradiation, one can "tag" various proliferating and migratory regions by subsequent histological preparation and quantification of the dead (pyknotic) cells. In this study, systematic irradiation of the hippocampus during various stages of its development (pre- and postnatal) were used to quantitatively monitor changes in the germinal compartment of the hippocampus. Finally, the autoradiographic and X-irradiation data were correlated with

qualitative observations and quantitative determinations obtained from conventionally prepared histological material.

MATERIALS AND METHODS

Purdue-Wistar male rats were cross fostered and raised six to a litter. For the long-survival autoradiographic studies, two pups each received four successive injections of ^3H -thymidine (New England Nuclear, NET-027; 5 $\mu\text{Ci/gbw}$) on days 0–3 (day 0 = day of birth), 4–7, 8–11, 12–15 and 16–19; they were killed at 60 days of age. For the short-survival autoradiographic studies, two pups each were killed 6 hours after a single injection of ^3H -thymidine (10 $\mu\text{Ci/gbw}$) on days 0, 2, 6, 13 and 30. All X-irradiated animals were killed six hours after a single exposure to 200r from a Maxitron 300kV unit. Two pregnant females each were irradiated on gestation days 17, 19 and 21 (day 1 = day of sperm positivity). Eighteen pups each were irradiated on days 0, 1, 3, 5 and 7. Six pups each were irradiated on days 9, 12, 15, 18, 21, 30 and 70. Finally, two animals were irradiated at 103 days of age. All neonate, juvenile and adult animals were killed by transcardiac perfusion with 10% neutral formalin. The brains were carefully removed, placed in Bouin's fixative for 24 hours and were embedded in paraffin after further fixation in 10% neutral formalin. Fetal brains were preserved in Bouin's fixative and 10% neutral formalin before embedding. Serial sections (6 μ) of the hippocampal region were cut in either the sagittal, coronal or horizontal planes. Those slides prepared for autoradiography were dipped in Kodak NTB-3 emulsion; exposure time was ten weeks.

For the long survival autoradiographic analysis, the percentage of labelled cells in both the dentate gyrus and Ammon's horn were based on counts in anatomically matched sagittal sections (L 1490 μ ; König and Klippel, '63). All cells with reduced silver grains over the nucleus in densities obviously above noise level were considered labelled.

Using matched coronal sections (approximately A 3.0 mm; De Groot, '59) from the brains of a series of irradiated animals aged from one to 103 days, pyknotic, immature granule cells and mature

granule cells were counted in the dorsal hippocampus. The presence of small, spherical, and densely stained bodies were taken to indicate recent cell death. In this study, one spherical body was counted as one pyknotic cell. Figure 11 shows this cell type. Labelled cells were also counted in matched coronal sections from the brains of the animals in the short survival autoradiographic series — part of the collection used by Altman and Das ('65b) in their study of hippocampal neurogenesis.

Homologous sagittal sections from brains of a second series of irradiated animals ranging in age from gestation day 17 to postnatal day 5 were used to count pyknotic cells in three zones of the hippocampus. The L 1490 μ level (König and Klippel, '63) best corresponds to the homologous sections used in younger animals. Zone I (fig. 14) included the stratum oriens and alveus extending from the subiculum to the midpoint of the curvature of the stratum pyramidale. Zone II was bounded mainly by the dentate stratum granulosum and contained the hilus; pyknosis in the CA4 region of Lorente de Nó ('34) was excluded from the sample area. Zone III was the fimbria. The sagittal length of the stratum pyramidale was also measured in this series. Sagittal length is defined as the extension of the stratum pyramidale from a point directly above the center of the fimbria to the termination of CA4 in the dentate hilus (fig. 15).

RESULTS

Time of origin of cells: Autoradiographic assessment in adults

In an attempt to label all cells formed after specified ages (figs. 1–4), groups of animals were given successive daily injections of ^3H -thymidine over blocks of four-day periods (0–3, 4–7, 8–11, 12–15, 16–19 days). It is assumed that all the cells labelled by this cumulative procedure were formed after the onset of the injections, while most of the nonlabelled cells were formed before. As the onset of injections was progressively delayed, the number of labelled cells decreased. The difference between the numbers of cells formed during any two injection periods was taken to reflect the quantity of cells formed during the interval.

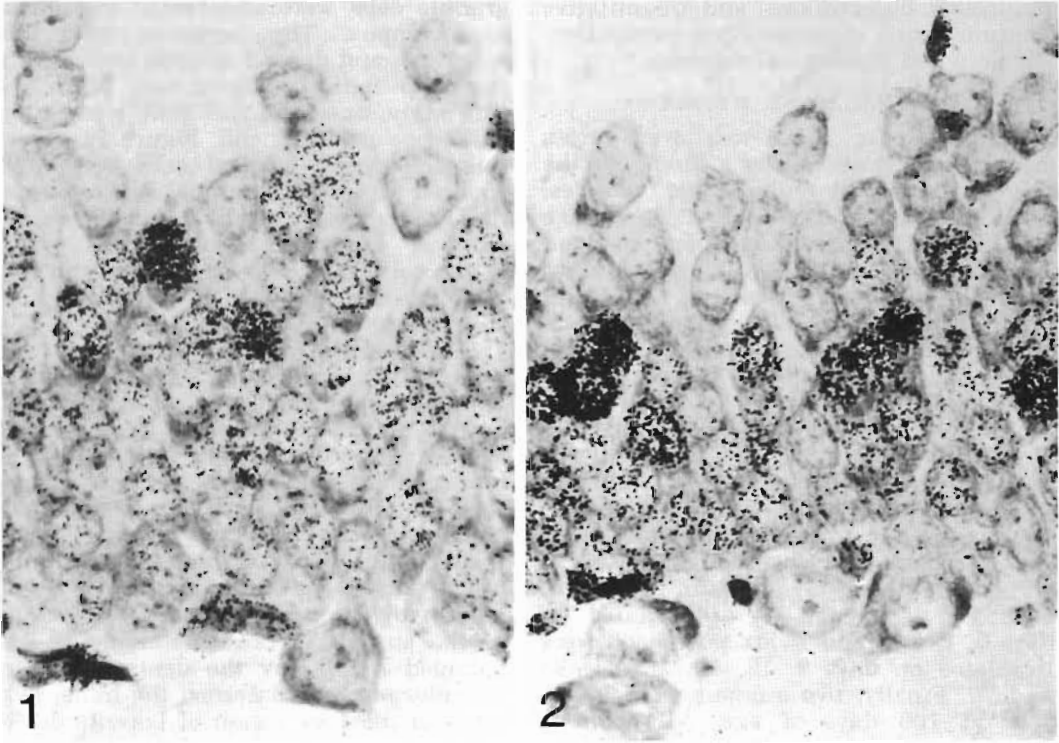


Fig. 1 Autoradiogram of labeled granule cells in the ectal arm of the dentate gyrus in a rat that received four successive injections of ^3H -thymidine on postnatal days 0, 1, 2 and 3. Cresyl-violet, $\times 640$.

Fig. 2 As in figure 1, from a rat injected on days 4, 5, 6 and 7.

Dentate gyrus

The results in figure 5A are combined percentages based on counts of 300 mature labelled or nonlabelled granule cells in each arm (ectal and endal) of the dentate granular layer. The ectal arm faces the cerebral hemispheres; the endal arm faces the thalamus. Close to 85% of the granule cells were labelled when injections began a few hours after birth and were then repeated on days 1, 2 and 3. This indicates that most or all of the 15% of the cells that could not be labelled were formed before birth. These unlabelled cells were located in the outermost portion of the granular layer at the interface of the molecular layer. These cells tended to be more loosely packed than the underlying labelled cells. Among the latter, a gradient in labelling intensity was evident: those situated near the prenatally formed elements being most heavily labelled, those situated at the

base of the granular layer were most lightly labelled (figs. 1-4). Since 60% of the granule cells could be relabelled when the injections were given on days 4-7, 25% of the population was considered to have stopped dividing and started to differentiate on days 0-3. Within the remainder of the population, 20% formed on days 4-7, 13% on days 8-11, 14% on days 12-15, and 13% after 16 days. With successively delayed cumulative labelling, the rows of heavily labelled cells were progressively shifted basally (figs. 1-4)

In figure 5B, the data are percentages based on counts of 100 labelled or nonlabelled cells (no cells were excluded) in each arm of the dentate molecular layer. Of this population at most 4% is formed prenatally and only 13% is added during the first week. The bulk of the population (64%) is formed between days 8 and 16. However, 19% of the cells have not yet formed at 16 days. It is important to note

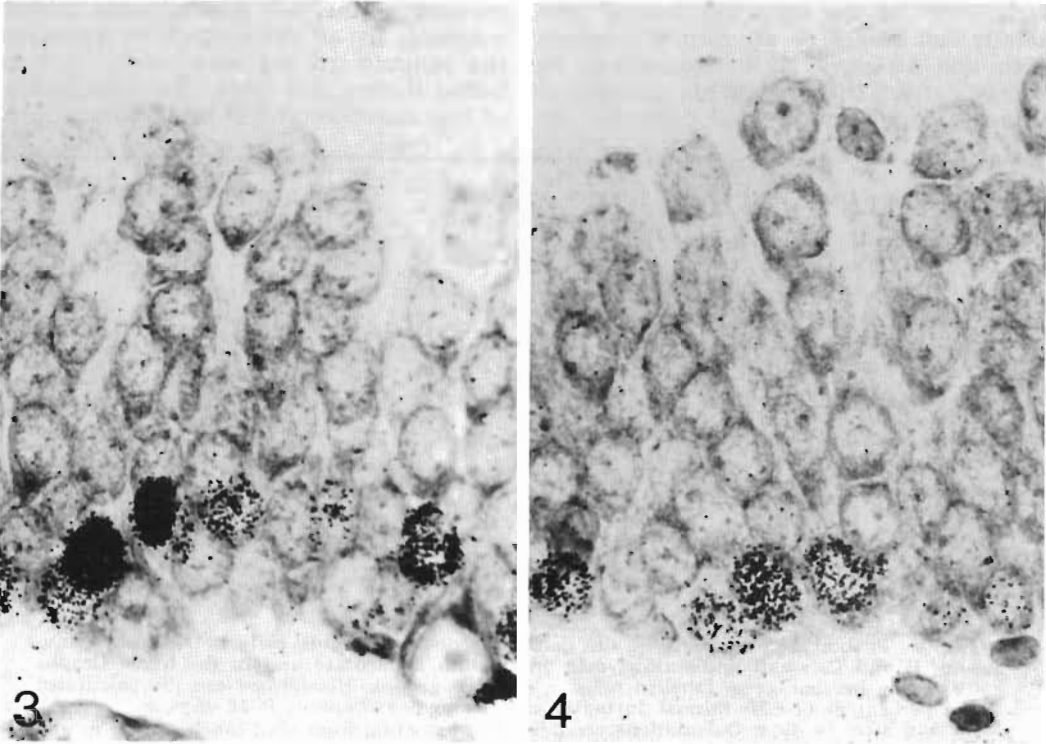


Fig. 3 As in figure 1, from a rat injected on days 12, 13, 14 and 15.

Fig. 4 As in figure 1, from a rat injected on days 16, 17, 18 and 19. Note the progressive basal shift in the position of labelled cells with progressive delay in labelling.

that the few large neurons that are present in the dentate molecular layer were not labelled with any of the injection schedules, supporting the claim of Hine and Das ('74) that these neurons are of prenatal origin. In figure 5C, the data are percentages based on counts of 100 labelled or nonlabelled cells in the hilus of the dentate gyrus. The pyramidal cells of CA4, those that lie within the hilus, were excluded from the sample area. In this population, 30% of the cells are formed prenatally and 70% are formed postnatally. Fifty-four percent are formed between four and 15 days with 16% still to be formed after 16 days. The scattered large cells of the hilus (polymorph cells and the pyramidal cells of CA4) are never labelled, confirming the prenatal origin of these neurons (Angevine, '65; Hine and Das, '74).

Ammon's horn

Pyramidal cells and the large neurons

in the stratum oriens, stratum radiatum and stratum lacunosum-moleculare are not labelled during any of the injection periods, again supporting the conclusion (Angevine, '65; Hine and Das, '74) that these cells are of prenatal origin. However, a large proportion of the small cells of Ammon's horn were labelled. Figure 6A shows the labelling pattern in the stratum oriens. Percentages are based on a total of 100 cells counted in area CA1. A total of 86% of the cells are formed postnatally. Again a large part of the population (47%) is formed between eight and 16 days with 14% forming after 16 days. The same procedure was used to analyze cyto-genesis in the stratum radiatum and the stratum lacunosum-moleculare of CA1 (fig. 6B). Of this population 13% is formed prenatally; 87% is formed postnatally, with 46% being added between eight and 10 days.

Figure 6C summarizes cell proliferation in the fimbria. According to these

data, 27% of the cells are formed prenatally and only 41% are formed between birth and 16 days; 32% are still to be formed after 16 days. This system is unique in that many small cells are un-

labelled in the 0-3 day injection period; whereas, in all other systems examined the majority of the small cells were labelled during this time. The implications of this observation will be discussed later.

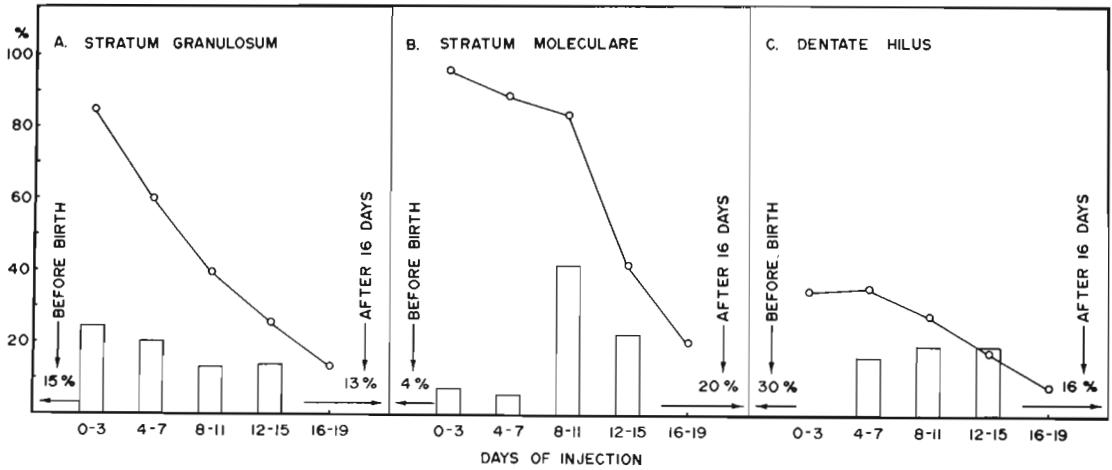


Fig. 5 Postnatal cytogenesis in the dentate gyrus. A, granule cells in stratum granulosum, B and C, small unidentified cells in stratum moleculare and in the hilus. Graphs refer to the percentage of labelled cells in the five groups. Histograms are the calculated mean percentage of cells formed during blocks of four days between 0-16 days and prior to birth and after 16 days. Calculations are based on deducting from total labelled cells in one period those that could be re-labelled in the next period. Note that cytogenesis is slightly higher in the granular layer during the first week than in the second week, that in the molecular layer cell labelling is highest during the second week, and that in the dentate hilus it is relatively uniform between 4-15 days. Relatively small differences were seen in different regions in cytogenesis after day 16.

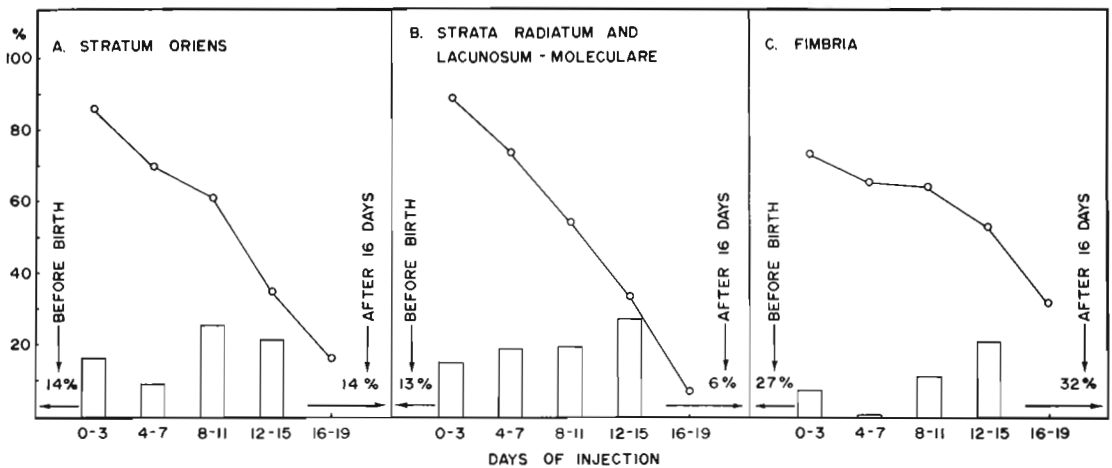


Fig. 6 Postnatal cytogenesis in Ammon's horn. Graph, percentage of labelled cells; histograms, percentage of cells formed during a four-day period. Note the higher level of cytogenesis during the second week of life in all regions and the large amount of cytogenesis still to be completed in the fimbria after 16 days.

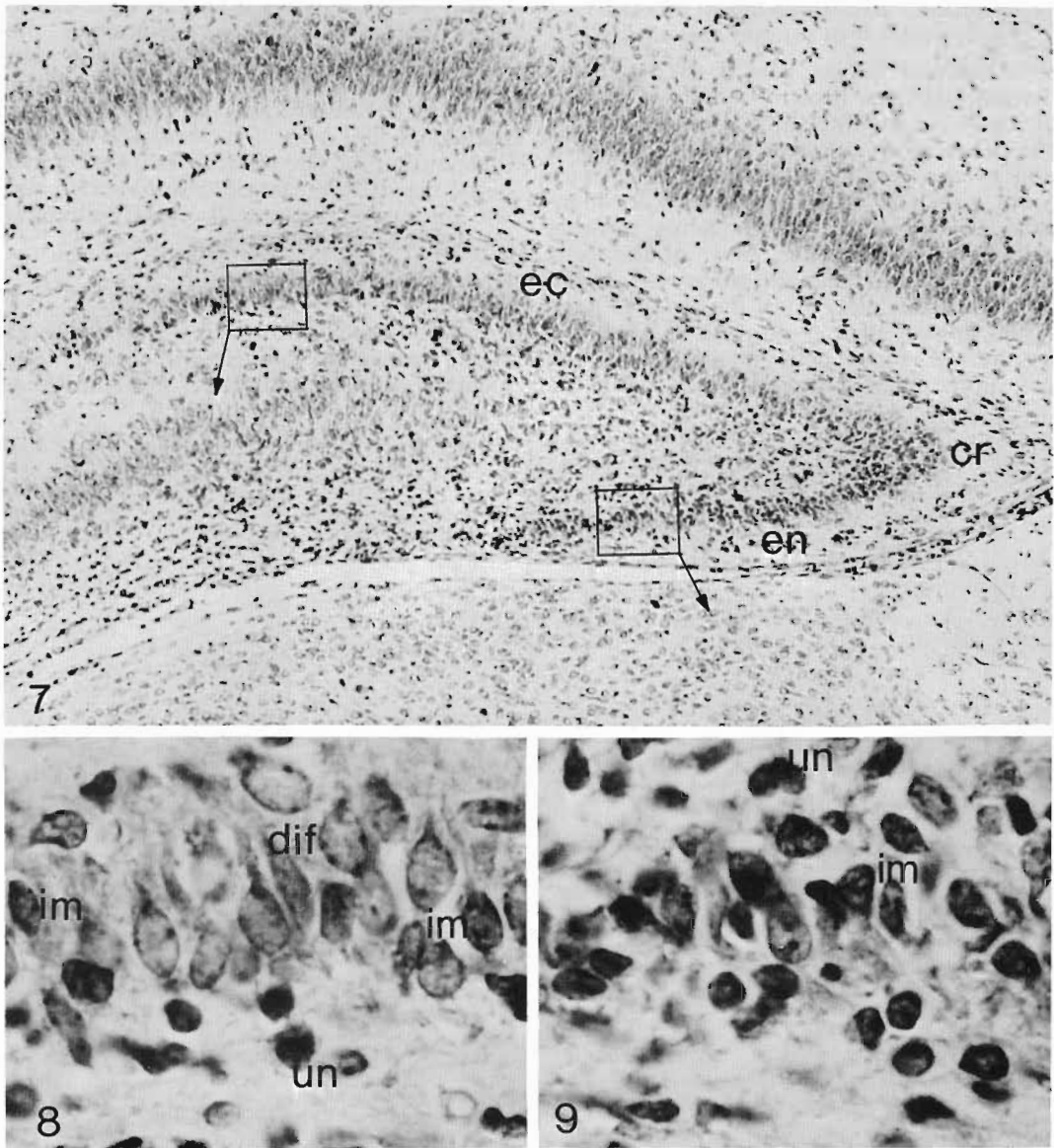


Fig. 7 The dentate gyrus of a newborn rat with the clearly delineated ectal arm (ec), crest (cr) and the formative, short endal arm (en). The enclosed area of the ectal arm shown enlarged in figure 8, the endal arm in figure 9. Cresyl-violet, $\times 157.25$.

Fig. 8 Cell types in the ectal arm. Differentiated (but still small) granule cells (dif) are located superficially, the spindle-shaped immature differentiating cells (im) are oriented at a right angle to the base of the granular layer but may stain as darkly as the morphologically undifferentiated precursor cells (un). $\times 952$.

Fig. 9 In the less developed endal arm, differentiated granule cells are rare and the layer is composed mostly of immature granule cells (im) and morphologically undifferentiated precursor cells (un). $\times 952$.

Cell proliferation and onset of cell differentiation: Examined with low-level X-irradiation in infants and adults

In normal infant rats (0–3 days) the dentate granular layer is composed mainly of an ectal arm forming a crest with a rudimentary endal arm (fig. 7). The far end of the ectal arm contains cells with large, pale and round nuclei and are identical in appearance with adult granule cells (fig. 7). However, most of the cells in the granular layer are somewhat smaller. Still other cells stain darkly and have either spherical or pyramidal shapes. The smaller of these cells is similar to the primitive cells scattered throughout the

hilus (figs. 8, 9). The somewhat larger darkly staining cells sometimes have prominent apical cones directed toward the granular layer (figs. 8, 11). These two types of cells form the base of the mature areas in the ectal arm, the crest, and the reduced endal arm. The arrangement and distribution of the undifferentiated small cells, the differentiating granule cells and the mature granule cells rapidly changes during the first few weeks of life.

A note on the use of X-irradiation

In this study two attempts were made. First, we tried to identify and quantify the location and magnitude of cell prolifera-

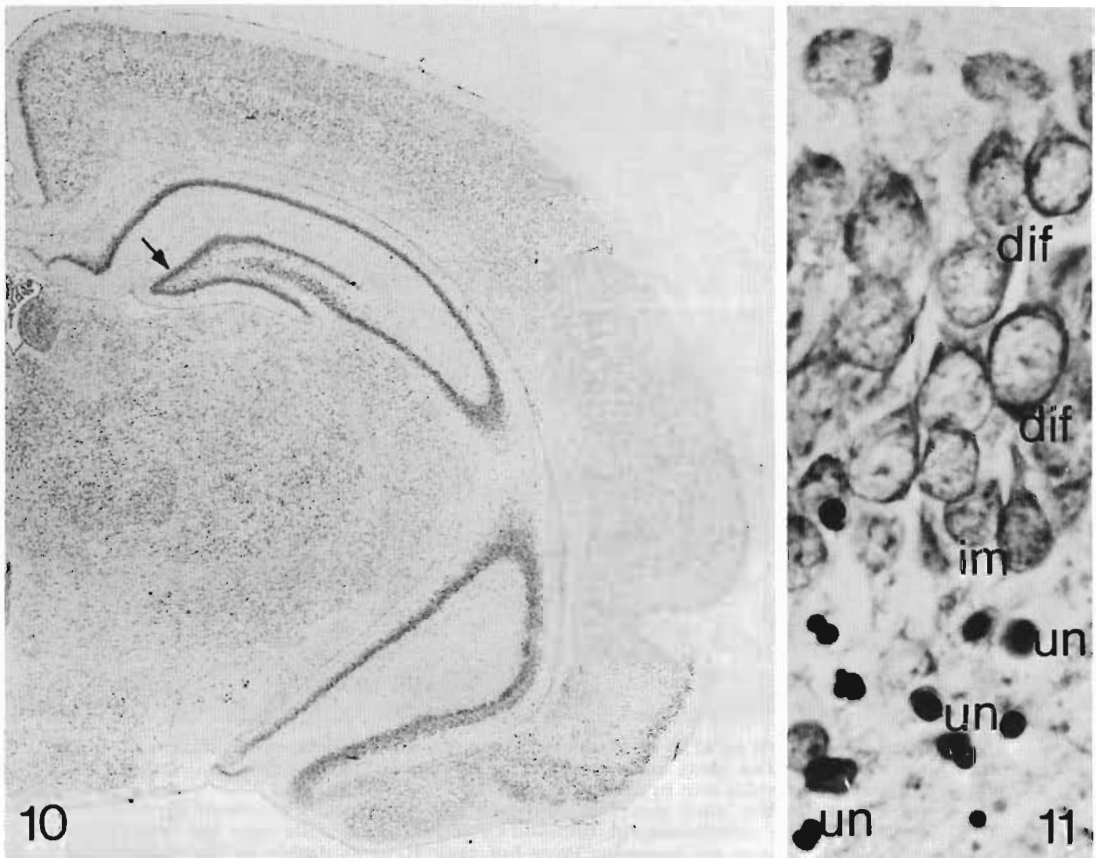


Fig. 10 The brain of a 15-day old rat six hours after exposure to 200r X-ray. This coronal level was used for the cell counts described in the text. Arrow points to the portion of the granular layer enlarged in figure 11. Cresyl-violet, $\times 15$.

Fig. 11 A portion of the granular layer in the ectal arm of the irradiated hippocampus. The differentiated (dif) and immature (im) granule cells are not affected, while the undifferentiated precursor cells (un) located in the subgranular zone became pyknotic. $\times 1,040$.

tion in the dentate region as a function of age. Second, we sought to distinguish undifferentiated from differentiating primitive cells and determine their distribution at different ages. In earlier studies the location of sites of cell proliferation in the hippocampus was studied with short-survival autoradiography (Altman and Das, '66; Altman, '66). But this procedure is not well suited for multiple injections and tends to label only a small proportion of the multiplying cells, i.e., those entering the synthetic phase of the cell cycle. Therefore, we employed another technique—the radiation of the hippocampal region with low-level X-ray.

It is well established that multiplying cells of the nervous system are extremely radiosensitive and die when exposed to 150–200r of X-ray (Hicks, '58; Hicks and D'Amato, '66). In agreement with this, the primitive proliferating cells in the re-

gion of the hippocampus are killed when this region is irradiated in infant rats (Altman et al., '68). In other studies in which the cerebellum was irradiated, it was noted (Altman and Nicholson, '71; Bayer and Altman, unpubl. obs.) that similar doses of X-ray kill a very large proportion of the cells of the proliferative zone of the external germinal layer but virtually all the descending cells in the molecular layer are spared. This indicated that differentiating primitive cells, like mature cells, become radioresistant. Consequently, a study of the brains of animals killed shortly after exposure to a low dose of X-irradiation (200r) at various times during hippocampal development should shed light not only on the process of cell acquisition, but also give clues as to the nature of morphological development of the hippocampus.

Within the dentate gyrus of the irradi-

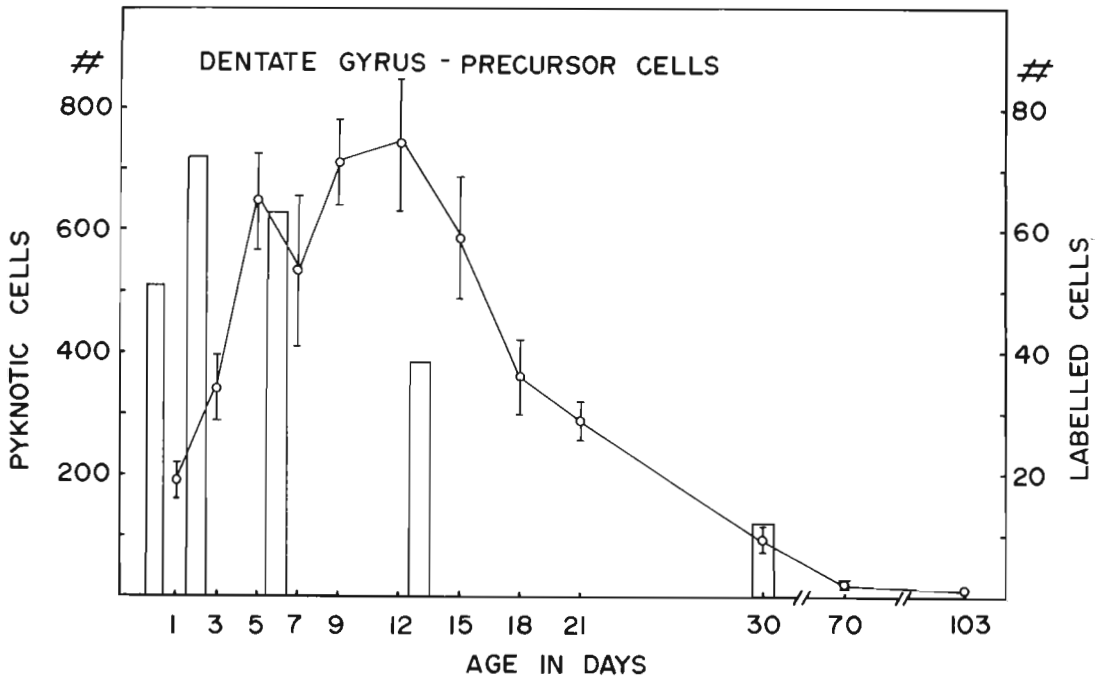


Fig. 12 Concentration of primitive precursor cells in the dentate gyrus as determined with the autoradiographic and X-irradiation techniques. Graph shows the number of pyknotic cells six hours after exposure to 200r X-ray (each point represents the mean from six animals with standard deviation). Histograms show the number of labelled cells six hours after injection with ^3H -thymidine ($10 \mu\text{Ci/gbw}$) in animals 0, 2, 6, 13 and 30 days of age. Note that the scale for the pyknotic cells is ten times that of the labeled cells. There is also a shift in the time of maximal labelling (2 days) and the time of maximal pyknosis (12 days). See text for further details.

ated rats (fig. 10), pyknotic cells are largely limited to the region of undifferentiated small cells in the hilus and comparatively few are found within the granular layer, even though many of the cells there are obviously still immature (fig. 11). No mature granule cells were ever observed becoming pyknotic. Since the immature granule cells and mature granule cells are not affected by the X-ray, counts of these populations from irradiated animals are essentially the same as those of the non-irradiated animals. Therefore, a separate group of non-irradiated animals was not deemed necessary to establish numbers in these populations.

Radiation-induced cell pyknosis

Figure 12 shows the change in numbers of radiosensitive undifferentiated cells from days 1–103 along with the numbers of labelled cells six hours after a single injection of ^3H -thymidine. The pyknotic, or undifferentiated radiosensitive cells increase rapidly during the first five days of life, remain at high levels during the second week, then begin to decline to low levels by the third week. The decline continues at a slower rate on into the adult period, and some remnants of the undifferentiated cell population are still present at 103 days of age. The analysis of variance showed significant differences between age groups ($F = 64.351$, $df = 10/53$, $p \leq 0.001$). The Duncan test was used to determine differences between group means. There were significant differences ($p < 0.01$) between groups on days 1–3, 3–5, 7–9, 12–15, 15–18 and 21–30. The number of labelled cells increased more rapidly and declined sooner than the pyknotic cells, but followed the same basic pattern. The maximal level in cell labelling was reached at two days; decline was already evident at 13 days and continued on to 30 days. The location of the undifferentiated radiosensitive cells and the labelled cells also followed the same pattern during the observed period. These cells were randomly scattered throughout the hilus at 0–3 days. The zone of scattered pyknosis and labelled cells gradually narrowed during the next few days to be located in the spongy material basal to the granular layer. By 18 days and beyond, pyknotic cells and/or labelled cells were found only in a very narrow zone

immediately beneath the granular layer. Small undifferentiated cells can be seen in this zone, and many times we have observed darkening of the nucleus similar to what is described by Altman and Nicholson ('71) in the cerebellum as indicating the beginning of cell death after a low dose of X-irradiation. Thus, the proliferating cells in the hilus are radiosensitive. However, there are always many more pyknotic cells than there are labelled cells (fig. 12). This indicates that X-irradiation is more efficient in tagging proliferating cells than is autoradiography. This and other possible explanations for the discrepancies between pyknosis and autoradiography will be discussed later.

Immature granule cells

The numbers of immature radioresistant granule cells follow a similar pattern to that of the histologically assessed undifferentiated cells (fig. 13A). The sampling area for this cell group was restricted to the granular layer although some radioresistant cells already differentiating into granule cells may have been located in the hilus region. Therefore, these numbers reflect a conservative estimate of the actual number of cells in the population. The analysis of variance showed groups differed significantly ($F = 187.407$, $df = 10/53$, $p \leq 0.001$). A Duncan test of the differences between group means showed significant ($p \leq 0.01$) differences between days 1–3, 3–5, 7–12, 12–15, 15–18, 18–21, 21–30 and 30–70.

From 0–3 days, immature granule cells are located beneath the mature granule cells in the ectal arm and make up a large portion of both the crest and the endal arm. From day 5 on, mature granule cells are easily distinguishable in the crest and endal arm while the immature granule cells are located immediately below these cells (the subgranular zone) throughout the entire granular layer. This same pattern is essentially maintained into adulthood, the zone of immature cells becoming thinner. However, some cells of this type are present at 103 days of age indicating that the granular layer is still acquiring new granule cells in the adult.

Mature granule cells

Figure 13A shows the number of mature granule cells present in homologous

sections of the hippocampus from 1–103 days of age. After a gradual increase during the first week, the differentiated granule cell population accumulates 60% of its total between seven and 21 days (fig.

13B). There is a more gradual rise between 21 and 30 days, and an asymptotic level is reached by 70 days, followed by a slight decline (7%) at 103 days. An analysis of variance showed that the age groups dif-

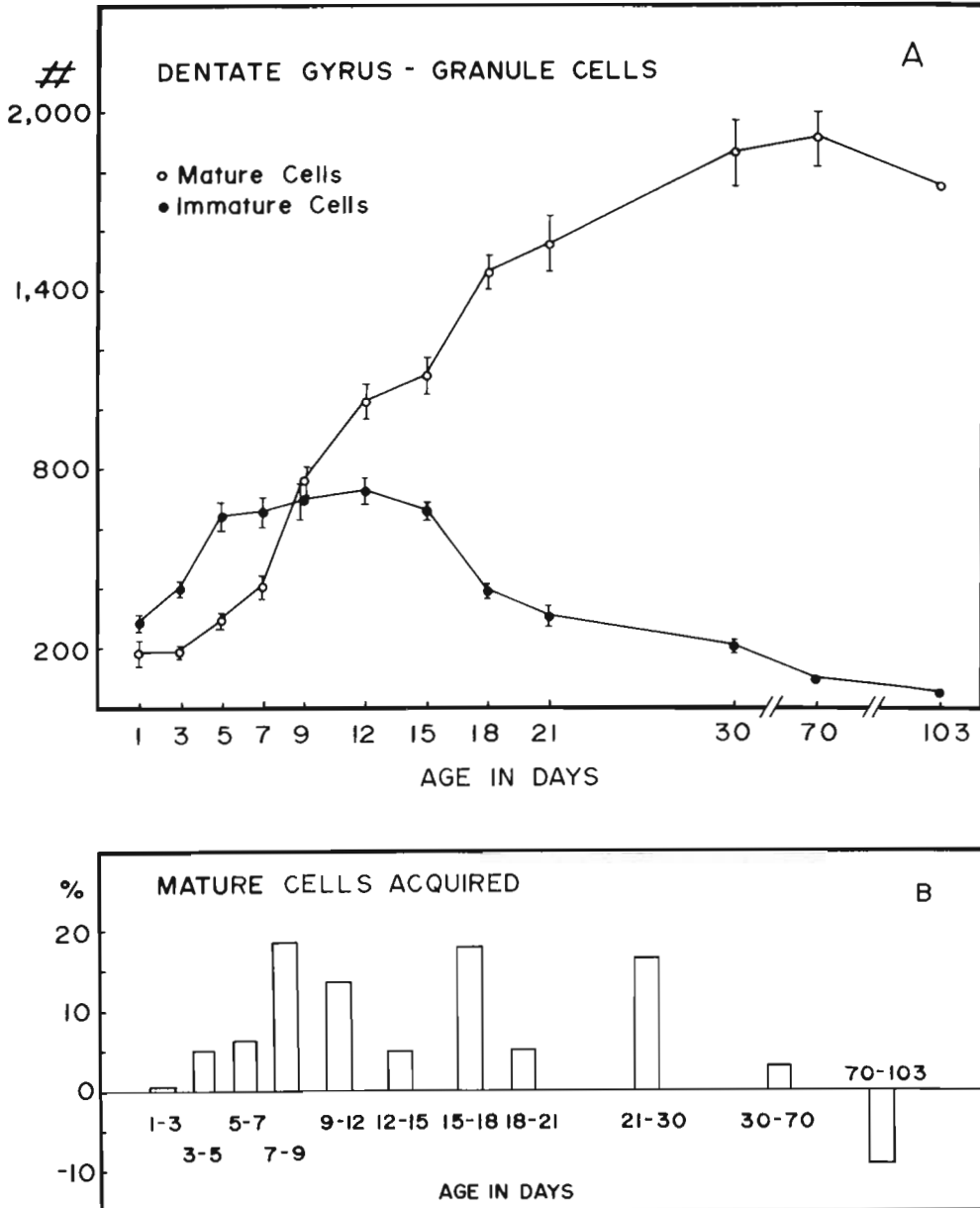


Fig. 13. A. Radioresistant mature and immature granule cells in the dorsal dentate gyrus at various postnatal ages (each point is the mean of six animals with standard deviation). Immature cells decline by the third week but a few are seen in adults. B. The percentage of granule cells that differentiate over blocks of three days up to 21 days, and over variable periods thereafter. There appears to be a spurt in cell maturation between 15–18 days. There is a decline in cell number between 70–103 days.

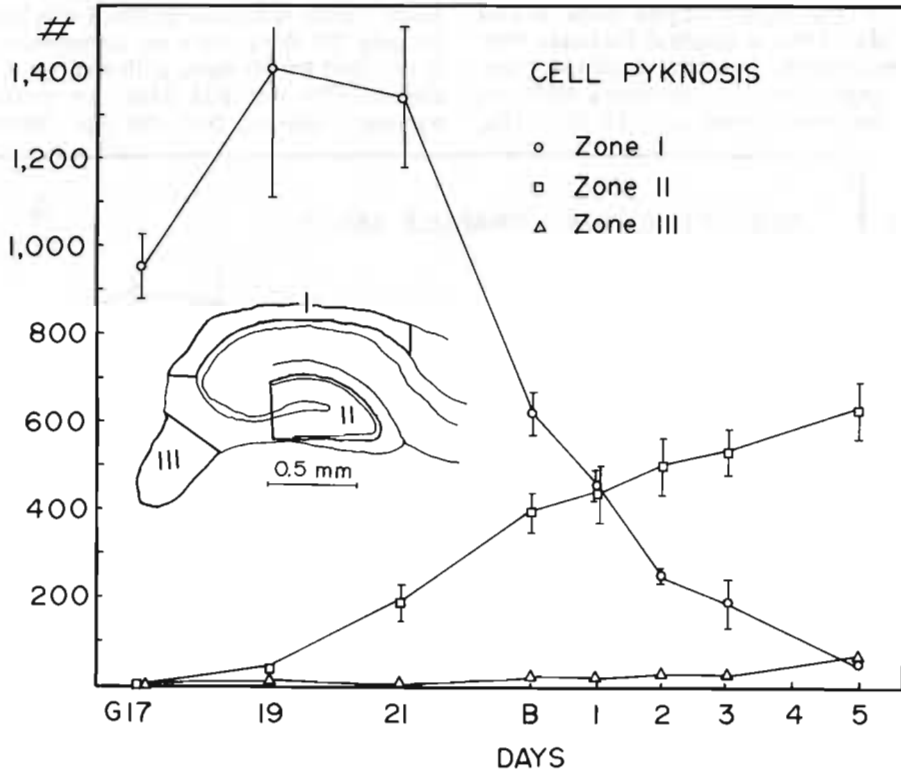


Fig. 14 Regional differences in the time course of cell pyknosis in the hippocampus from gestation (G) day 17 to day 5 after birth (B). The parcellation of three zones is shown in inset. Each point represents the mean of six animals with standard deviation. The absolute sizes of the three zones (I, stratum oriens; II, dentate gyrus; III, fimbria) were different. The numerical data reveal the trends in concentration of radiosensitive cells in the three distinct regions.

ferred significantly ($F = 576.795$, $df = 10/53$, $p \leq 0.001$). The Duncan test performed on the group means showed significant differences ($p \leq 0.01$) between means on days 5–7, 7–9, 9–12, 15–18 and 21–30. Since only two animals were studied at 103 days of age, these data were not included in the analysis of variance. However, the slight declining trend noted in this group has also been observed by Altman and Das ('65b). Very early on, the mature granule cells are located in the far end of the ectal arm of the granular layer. As early as day 5, mature granule cells are located throughout the layer. After this, the granular layer simply increases in depth to reach adult levels.

Morphogenesis of the hippocampus: Examined with low-level X-irradiation during the perinatal period

At birth, both Ammon's horn and den-

tate gyrus—even though immature—have recognizable adult morphology. This is to be expected since cytogenesis of the Ammonic pyramidal cells is entirely prenatal and begins also prenatally in the dentate granule cells. Consequently, it was necessary to examine the hippocampus from the late prenatal stages onward. We used homologous sagittal sections from the brains of X-irradiated animals ranging in age from 17 days gestation to the fifth postnatal day and examined them both qualitatively and quantitatively.

Qualitative description

Gestation day 17. Figures 16–21 show photomicrographs of homologous sagittal sections of the hippocampus at various times during its development. On gestation day 17 (fig. 16), the hippocampus is a tongue-like extension of cortex overlying the thalamus. There is a noticeable swell-

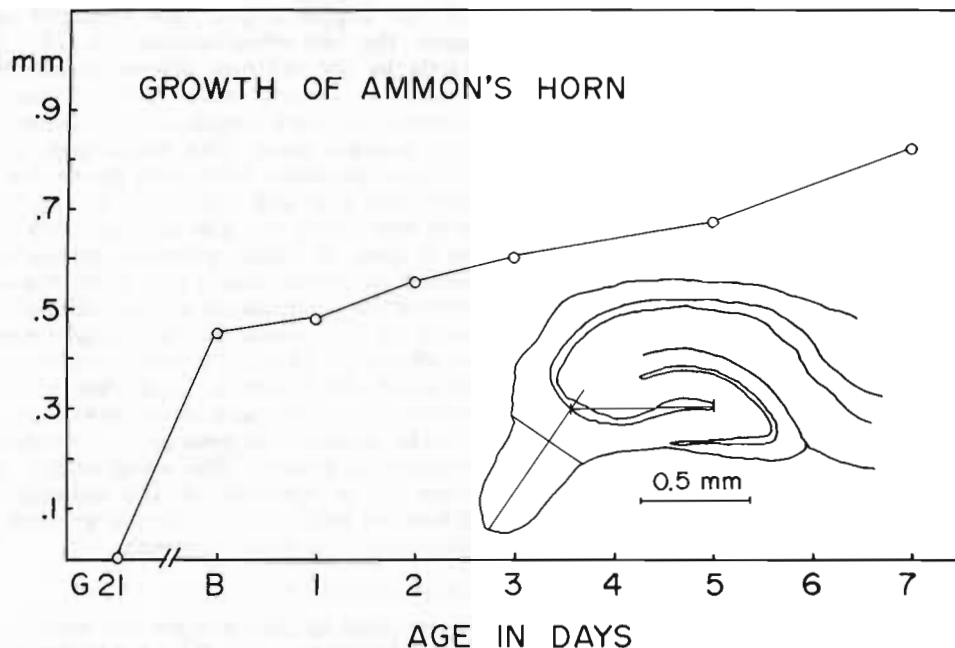


Fig. 15 Sagittal length changes in the CA₃-CA₄ region of the stratum pyramidale measured according to the indicated diagram. Means are based on six animals for each age. The rapid growth of Ammon's horn is essentially a late prenatal and early postnatal feature.

ing at the rostral end of this cortical lip. A thin but recognizable ependymal layer bordering the endal wall of the lateral ventricle forms its dorsal roof. A subependymal zone lies immediately below this layer where dense pyknotic cell clusters are scattered among unaffected small, undifferentiated cells; the pyknotic cells become more sparse in the rostral portion. Just beneath the subependymal zone is a region of loosely packed larger cells, containing few pyknotic cells and becoming thicker as it extends rostrally. Situated between the large cells and the ventral pial surface, a thin sheet of more closely packed small cells is seen, which also has only scattered pyknosis. This sheet may be the future site of the dentate gyrus. The fimbria is not yet clearly recognizable, suggesting that the outgrowth of pyramidal cell axons has not yet begun.

Gestation day 19. Within the time space of 48 hours (fig. 17), the alignment of the pyramidal cells of Ammon's horn and the formation of both the fimbria and the hippocampal fissure first become apparent. The ependymal layer has become thicker and there is still a zone of dense pyknotic cells in the also thickened subependymal

layer surrounding the primitive Ammon's horn. No subependymal zone and few pyknotic cells are found in the fimbria. The alignment of Ammon's horn pyramidal cells is most noticeable near the subicular end of the hippocampus, and becomes less distinct toward the rostral end. It is in this region that a large mass of loosely organized cells covers the edge of Ammon's horn and extends posteriorly under the pyramidal layer, where it is separated from this area by the hippocampal fissure. There are few pyknotic cells within this mass. It is noteworthy that this cell aggregation extends to the ventral pial surface just posterior to the outgrowth of the fimbria. This cell mass may be the primitive dentate gyrus which was only a thin sheet on the seventeenth gestation day. With this technique, no statement can be made about the cellular composition of the primordial dentate gyrus.

Gestation day 21. A thick ependymal layer still surrounds the hippocampus (fig. 18). The subependymal zone is beginning to thin out, but there are still dense clusters or strings of pyknotic cells stretching from this zone into what is probably the stratum oriens of Ammon's horn. The

alignment of the pyramidal cells is quite distinct, except rostrally. There, the pyramidal cell layer gradually merges into a mass of loosely packed small cells curving posteriorly. This group of cells is separated from the pyramidal layer above by a longer and more distinct hippocampal fissure. Pyknotic cells are now commonly seen within this cell mass and are especially dense just superficial to the ventral pial surface posterior to the fimbria. For the first time, a layer of radioresistant cells, in the dorsal aspect of this region is beginning to form what is probably the ectal arm of the dentate granular layer. The fimbria has grown larger, but there is no subependymal zone and very few pyknotic cells are found there.

Day of birth and postnatal day one. Drastic changes have taken place since the twenty-first gestation day (fig. 19). The ependymal zone has become much thinner (1–2 cells deep) and the subependymal zone has disappeared, although the scattered pyknotic cells in the stratum oriens may be its remnants. The pyramidal layer has decreased in depth, and it curves posteriorly invading the dentate hilus to form the typical adult morphology. The thin dentate granular layer also has the beginnings of adult morphology, being composed of an ectal arm, a crest and a short endal arm. There are many pyknotic cells in the molecular layer and also within the dentate hilus. Relatively few pyknotic cells are noticed in the granular layer itself. The most dense cluster of pyknotic cells is located in a juxtapietal position at the rostral end of the endal arm where they are interspersed with the small undifferentiated cells characteristic of the hilus. The former site may be the major source of granule cell precursors.

Postnatal day 3. The hippocampus has changed little in the past 48 hours (fig. 20). Pyknosis in the stratum oriens of Ammon's horn is much more sparse. The pyramidal layer has extended further into the hilus. The molecular layer has only few pyknotic cells but there are still many within the hilus. The endal arm of the dentate gyrus extends further rostrally, and there is still the prominent zone of densely clustered pyknotic cells in the juxtapietal proliferative matrix.

Postnatal day 5. The gross morphology

of the hippocampus has changed little since the last observations (fig. 21). Pyknosis in the stratum oriens of Ammon's horn has become even more sparse; the pyramidal layer continues to grow into the dentate hilus. The endal arm of the dentate granular layer has grown further rostrally and has separated the proliferative zone from the pial surface. Now there is a zone of dense pyknosis immediately below the endal arm. Essentially this same pattern is maintained into adulthood. The scattered pyknosis in the hilus becomes confined to narrowing zones immediately beneath the granular layer. Small, undifferentiated cells and some pyknotic cells can be seen in this zone in the X-irradiated brains of adults. This subgranular zone may be a remnant of the subpial proliferative matrix for extended granule cell production on into adulthood.

Quantitative results

In order to characterize the time course of proliferation in each region of the hippocampus quantitatively, pyknotic cells from three different zones of the hippocampus were counted in matched sagittal sections from the brains of animals ranging in age from gestation day 17 to postnatal day 5. Zone I was the stratum oriens extending from the subiculum to the beginning of the curvature of the Ammon's horn pyramidal layer. Zone II was formed by drawing an imaginary vertical line from the most rostral extension of the ectal arm to the ventral pia posterior to the outgrowth of the fimbria. This included the hilus of the dentate gyrus and the juxtapietal zone of dense pyknosis. Zone III was the fimbria.

Figure 14 shows the numbers of pyknotic cells in each of these three zones. Zone I increases from gestation day 17 to reach high levels on both gestation day 19 and 21. There is a drastic reduction in pyknosis by the day of birth and the sharp decline continues to day 5. An analysis of variance showed age-groups differed significantly ($F = 92.952$, $df = 7/37$, $p \leq 0.001$). The Newman-Keuls test on the differences between group means showed significant differences ($p \leq 0.05$) between groups on gestation days 17–19, gestation day 21–day 0, 0–1, 1–2, and 2–5. Zone II is not distinguishable at gestation day 17, but from gestation day

19 on, it increases rapidly each day of the observed period. The analysis of variance showed groups differed significantly ($F = 90.781$, $df=6/32$, $p \leq 0.001$). The Newman-Keuls test on the differences between group means showed significant differences ($p \leq 0.05$) between groups on gestation days 19–21, gestation day 21–day 0, 0–2, 1–3 and 3–5. In contrast to the other two zones, pyknosis in the fimbria remains low throughout the observation period. However, the analysis of variance showed groups differed significantly ($F = 59.532$, $df=6/32$, $p \leq 0.001$). The Newman-Keuls test performed on the differences between the group means showed significant differences ($p \leq 0.05$) between groups on gestation day 21–day 1 and 3–5.

It is well established that the pyramidal cells are formed during prenatal hippocampal development. But their morphological alignment as a thin layer of cells extends to the first few days of postnatal life. The reduction of the depth in the pyramidal layer is coupled with the rapid extension of its length. Instead of growing rostrally, the pyramidal layer sharply curves posteriorly to invaginate the still developing dentate gyrus. In order to quantify the degree of Ammon's horn growth into the hilus of the dentate gyrus, the sagittal length of the CA₃–CA₄ region was measured from a point above the center of the fimbria to the termination of CA₄ in the hilus at various times during hippocampal development (fig. 15, inset). Figure 15 shows that the rapid growth of Ammon's horn into the dentate gyrus is essentially a postnatal feature since the posterior curvature of Ammon's horn is not apparent until the day of birth. An analysis of variance showed groups differed significantly ($F = 85.93$, $df = 5/29$, $p \leq 0.001$). The t-test performed on the differences between group means showed significant ($p \leq 0.05$) changes from day 0–2, 1–3, 2–5, 3–5 and 5–7.

DISCUSSION

Postnatal cytogenesis in the hippocampus using progressively delayed comprehensive labelling

This technique requires more injections of ³H-thymidine and larger numbers of animals than the more common method of counting intensely labelled cells after

single injections of the radiochemical. But its use allowed us to estimate with some accuracy the proportion of dentate granule cells formed postnatally and the time course of their acquisition. It also brought evidence of more widespread postnatal cytogenesis in other components of the hippocampus than was hitherto assumed.

Although many previous studies have reported labelled granule cells after postnatal injections of ³H-thymidine in the mouse (Angevine, '65) and rat (Altman and Das, '65a,b, '66; Altman, '63, '66), no numerical estimate of the amount of postnatal neurogenesis has to date been determined. The estimate of 85% obtained in this study concurs with our other study (Bayer et al., '73) where eight exposures to focal X-irradiation (150–200r) of the hippocampus from postnatal days 2–15 led to a comparable permanent reduction in the granule cell population. Evidently all the postnatally forming granule cells, and only these, are eliminated when the hippocampus is exposed to multiple doses of low-level X-ray during its postnatal development.

Both Angevine ('65) and Hine and Das ('74) state that neurogenesis is completed during prenatal life in the molecular layer of the dentate gyrus and in Ammon's horn. They referred to the large or medium-sized neurons and did not deal with small cells. In agreement with their report we did not find any large cells labelled with postnatal injections; however, the majority of the small cells were labelled. Although many of these small cells are neuroglia, at least some of them may be neurons. Both Cajal ('11) and Lorente de Nó ('34) described small neurons in these regions, especially in the dentate molecular layer and in the Ammonic stratum lacunosum-moleculare. In view of these findings, it is possible that neurogenesis in these regions continues into the postnatal period. This must be confirmed with a technique involving both autoradiography and electron microscopy to determine the identity of the postnatally-formed cells.

The fimbria was the only area examined where many small cells (27%) were unlabelled during 0–3 days injection period. However, the conclusion that one might draw from this, namely, that over one-

fourth of the glia in this fiber tract is of prenatal origin is contradicted by the observation that very few cells are present in this region in the immature brain. That glial proliferation in the fimbria is a late postnatal event is suggested by our observation that at least 30% of these cells are formed after day 16. It is also what one would expect when two additional facts are considered. First, that the rat fornix stains only moderately for myelin at 21 days and, second, that "myelination gliosis" (Roback and Scherer, '35) usually precedes myelination by only a few days (Bensted et al., '57; DeRobertis et al., '58). To resolve this paradox we assume that four successive injections on days 0-3 proved insufficient to keep tagged the multiplying cell population of this late maturing region. We have chosen the technique of four successive cumulative injections for economic reasons, as it is sufficient to label all the postnatally forming granule cells. Experiments are now in progress where five injections of ^3H -thymidine (3 days apart) are given from 0-12 days. Here, we hope to remove the problem of excessively diluted labelling of late-forming cells and to further clarify the degree of postnatal development in these cell populations.

At this junction we may point out two additional drawbacks of the cumulative injection schedule that we selected for this study. First, we can only follow the development of these cell populations from birth to 16 days, and second, we cannot describe changes within any of the injection periods. In most of the systems analyzed, some or all of particular cell populations were formed before birth and over 10% of the cells were formed after 16 days. The time course for the formation of this portion of the population would be worth knowing. Given the extensive cytogenesis during the first week in the dentate granule cell population and during the second week in the cells of the dentate molecular layer, it would be advantageous if we could follow their development over a shorter time period by using an overlapping cumulative procedure.

Postnatal cytogenesis in the dentate gyrus examined with low-level X-irradiation

Primitive undifferentiated radiosensitive

cells. It is much less time consuming and more economical than autoradiography to use low-level X-irradiation for tagging proliferative cells in the developing brain. X-irradiation can also be used in some species where autoradiography is not applicable. For example, the blood brain barrier in the cat (Das and Altman, '71) excludes the labelling of proliferative cells with systematically injected radiochemical. However, the two techniques yield different results both quantitatively and qualitatively. When both pyknosis and short-survival autoradiography data are compared, there are always more pyknotic cells than there are labelled cells (fig. 12). This may be due to the following. First, the autoradiographic technique is much more selective in that only cells in the synthetic phase of their cycle incorporate label. In contrast, the high yield of pyknotic cells with a single dose of 150-200r in nonsynchronized mitotic populations (an estimated 90% in the proliferative zone of the external germinal layer of the cerebellum) indicates that multiplying cells in virtually all mitotic phases are killed by the dose used. Second, not only mitotic cells but also postmitotic, undifferentiated migratory cells in the developing brain are radiosensitive (Hicks and D'Amato, '66; Altman et al., '68). The granule cell precursors that move from the proliferative compartment (whether it be the juxtapial zone or elsewhere) retain their radiosensitivity for some time longer than they can be labelled. Since cells respond to X-irradiation during more stages of their proliferation and remain radiosensitive for some time thereafter, X-irradiation will give a numerically higher yield than autoradiography. There is also the possibility that cells killed by X-irradiation become fragmented, thus increasing the number of small, densely-stained particles counted as pyknotic cells. This can be eliminated if one is careful to exclude small fragments from the counts.

Neither pyknosis nor short survival autoradiography can predict the fate of the marked cells. Consequently, the data obtained from both of these techniques can only be interpreted along with long-survival autoradiography. Since long-survival autoradiography shows that there is a high degree of postnatal cytogenesis in both the dentate hilus and the dentate

molecular layer, only some of the pyknotic or labelled cells are destined to become granule cells. Both labelling and pyknosis levels drop around the end of the second week of life — after much of cytogenesis is completed in each of these three populations.

Granule cell maturation patterns. The data presented here follows similar patterns to that reported by Altman and Das ('65b). The slight discrepancy reflects different criteria used for mature and immature granule cells. From the long survival autoradiography data we know that the majority (60%) of the dentate granule cell precursors stop dividing and begin to differentiate by the end of the first postnatal week. The number of immature cells remains high during the second postnatal week, then gradually drops off. The mature granule cells continue to increase in numbers for up to two months of age as the maturation of the differentiating cells adds to this compartment. The differences in the maximal levels of labelling, immature cells and mature cells reflects the time lag in the succession of events from undifferentiated precursor cells → differentiating granule cells → mature granule cells.

Paradoxically, in adult brains there are immature and proliferating cells at the base of the granular layer and this is coupled with a slight decline in granule cell number, as previously reported by Altman and Das ('65b). Immature granule cells and proliferating cells may provide replacements for those that die but apparently not in sufficient quantity to prevent a net cell loss. This question cannot be resolved without further quantitative studies.

Morphogenesis of the hippocampus and the problem of cell migration

We hoped to accomplish two objectives in this part of the study. First, we wished to get some understanding of the manner in which adult morphology is achieved. Second, in view of the fact that previous investigators have postulated that granule cell precursors migrate into the dentate gyrus (Angevine, '65; Altman and Das, '66), we wanted to see if a migratory stream of cells could be followed during dentate morphological development. We will address ourselves to each of these questions in turn.

Time course of hippocampal morphological development. On day 17 of gestation, the hippocampus is an undeveloped structure, and no hint of its future morphology is evident. There are some larger cells just beneath the subependymal zone which are probably differentiating, but none of these resemble pyramidal cells. Many pyramidal cell precursors incorporate label on days 17 and 18 of gestation (Hine and Das, '74). Soon thereafter, the precursors line up and begin to differentiate into pyramidal cells (gestation days 19 and 21). At birth, not only is the cytogenesis of the pyramidal cell population complete, but so also is the active process of pyramidal cell alignment. During early postnatal life, the pyramidal cell somatas are pushed further apart reflecting — among other things — the processes of dendritic growth and gliogenesis. This volumetric expansion continues on into the adult period (Bayer et al., '73).

The situation within the dentate gyrus is somewhat different. Although some granule cell precursors incorporate label at the same time as do those of the pyramidal cells, the cytogenesis of the dentate granule cell population can never be considered "complete" since both undifferentiated precursors and differentiating elements are present in the adult. The dentate granule layer has the basic outlines of adult morphology near the end of the first postnatal week when 40% of its population is still to be formed. From shortly after gestation day 21 to the end of the first week of life, Ammon's horn is rapidly pushing into an initially ball-like mass of undifferentiated cells (the dentate gyrus at gestation day 21). This growing force may be responsible for shaping the dentate gyrus into a sharply folded leaf of cortex covering the leading edge of Ammon's horn. It is interesting to note that the granular layer is exactly opposite to the pyramidal layer in that it first extends in length — starting with the ectal arm, crest, then endal arm — then increases in depth. Consequently, it is possible that the V-shaped dentate gyrus results from the distortion of an initially round undifferentiated mass of cells by the invagination of Ammon's horn rather than by being actively formed by the migration of dentate granule cell precursors to produce a sharply folded configuration. In

this case, the process of morphogenesis is completed before and may not depend on the completion of cytogenesis.

The problem of migration. Granule cell precursors have been postulated to proliferate in the neuroepithelium of the lateral ventricle, then migrate along fiber tracts either leading into or out of the hippocampus to reach the dentate gyrus. Altman and Das ('66) suggested that the precursors migrate into the dentate gyrus via the fimbria. But the few cells present and the sparse pyknosis in the fimbria (figs. 14, 16-21) during pre- and early postnatal development would indicate that the precursors do not take this route. Angevine ('65) suggested that the precursors migrate via the stratum oriens. Indeed, pyknosis in the stratum oriens declines during the late prenatal and early postnatal period while that of the dentate gyrus is increasing (fig. 14). Since the number of pyknotic cells in the stratum oriens declines most sharply between gestation day 21 and the day of birth, one would expect a "spurt" of pyknosis in the dentate gyrus soon thereafter if migrating cells take this route. On the contrary, dentate pyknotic cells increase steadily from gestation day 19 (before the rapid decline in the stratum oriens) to postnatal day 5. Since a considerable amount of cytogenesis in Ammon's horn extends into postnatal periods, the pyknotic cells in the stratum oriens may be proliferating rather than migrating cells. In fact, Altman and Das ('65a,b, '66) have observed labelled cells here shortly after ^3H -thymidine injections in the early postnatal period. In both man (Humphrey, '66) and the sheep (Godina and Barasa, '64) the anlage of the dentate gyrus is stated to be a region of undifferentiated cells near the outgrowth of the fimbria. A similar region can be seen in the rat (the juxtapial zone of primitive cells), and Angevine ('65) found labelled cells here shortly after perinatal ^3H -thymidine injections in the mouse. From this region, cells move a relatively short distance to form the granular layer of the dentate gyrus. Although we cannot say with certainty that dentate granule cell precursors are situated near the anlage of the dentate gyrus from the outset of its mor-

phogenesis, neither do we have to postulate long migratory pathways for the precursor cells. The solution of this problem awaits the development of refined techniques that will allow us to follow the fate of undifferentiated cells more accurately.

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PLATE 1

EXPLANATION OF FIGURES

Pyknotic cells in the hippocampus at different ages. Animals were killed six hours after exposure to 200r X-ray. Cresyl-violet, $\times 101$.

- 16 Gestation day 17. The core of the hippocampus (H) has few pyknotic cells. In its thick germinal matrix bordering the lateral ventricle (V) pyknotic cells are scarce in the ependymal layer. Pyknotic cells are abundant in the subependymal layer (single arrows) and in the transitional layer (double arrows). (Note the rupturing of the wall of the dorsal, juxtacortical aspect of the lateral ventricle and the sloughing of pyknotic cells into the lumen. This is not an artifact but due to the circumstance that at this stage of development the neuroepithelial cells forming the wall of the ventricle are also killed.)
- 17 Gestation day 19. At this age the fimbria (F), hippocampal fissure (arrows), and the alignment of pyramidal cells in Ammon's horn (dashed line) have become evident. The primordium of the dentate gyrus (DP) is also recognizable. A few scattered pyknotic cells are present in the latter region.

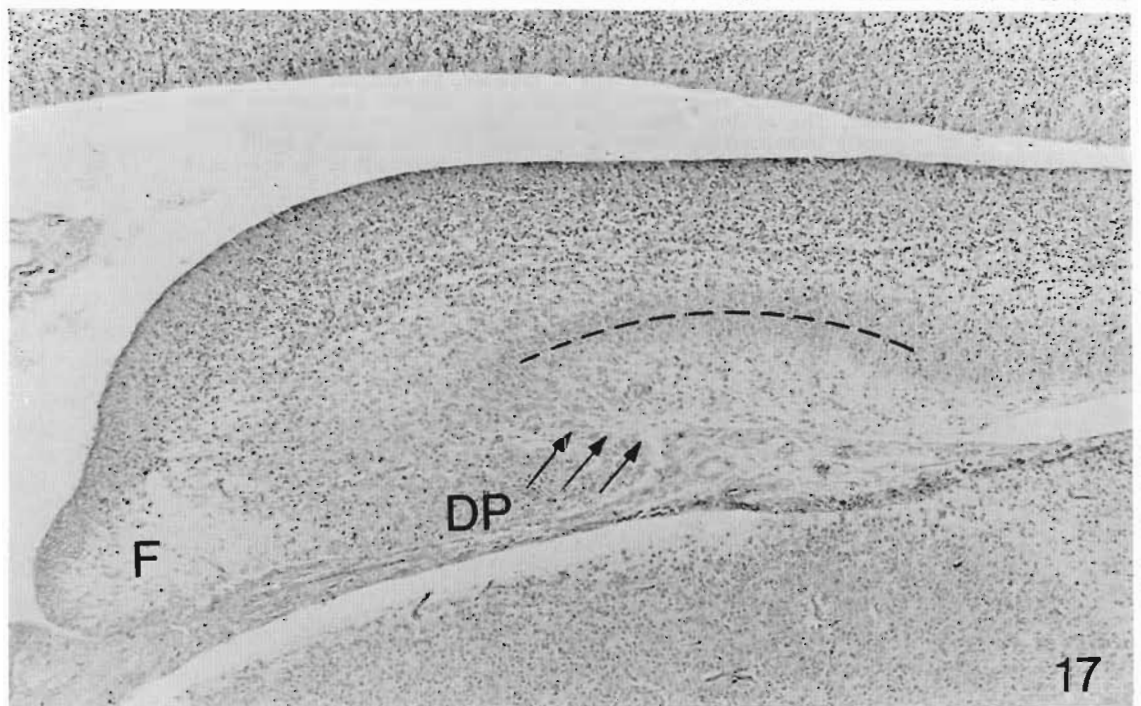


PLATE 2

EXPLANATION OF FIGURES

Pyknotic cells in the hippocampus at different ages. Animals were killed six hours after exposure to 200r X-ray. Cresyl-violet, $\times 101$.

- 18 Gestation day 21. The germinal matrix has become thinner and contains few pyknotic cells. Pyknosis is dense in the transitional zone (alveus?) and in the stratum oriens (SO). It follows the curvature of the stratum pyramidale (SP) and can be traced into the hilus of dentate gyrus. The ectal arm (EC) of the granular layer is beginning to form. In the dentate hilus pyknosis extends to the pial surface (arrows).
- 19 Postnatal day 1. Only remnants of the germinal matrix remain and pyknosis in the stratum oriens is reduced. In contrast cell pyknosis in the juxtapial zone of the dentate gyrus (arrows) has become more dense. The stratum pyramidale has become much thinner and has greatly increased its length by curving into the dentate gyrus. The dentate granular layer has also increased in length to be composed of an ectal arm (EC), crest (cr) and endal arm (EN).

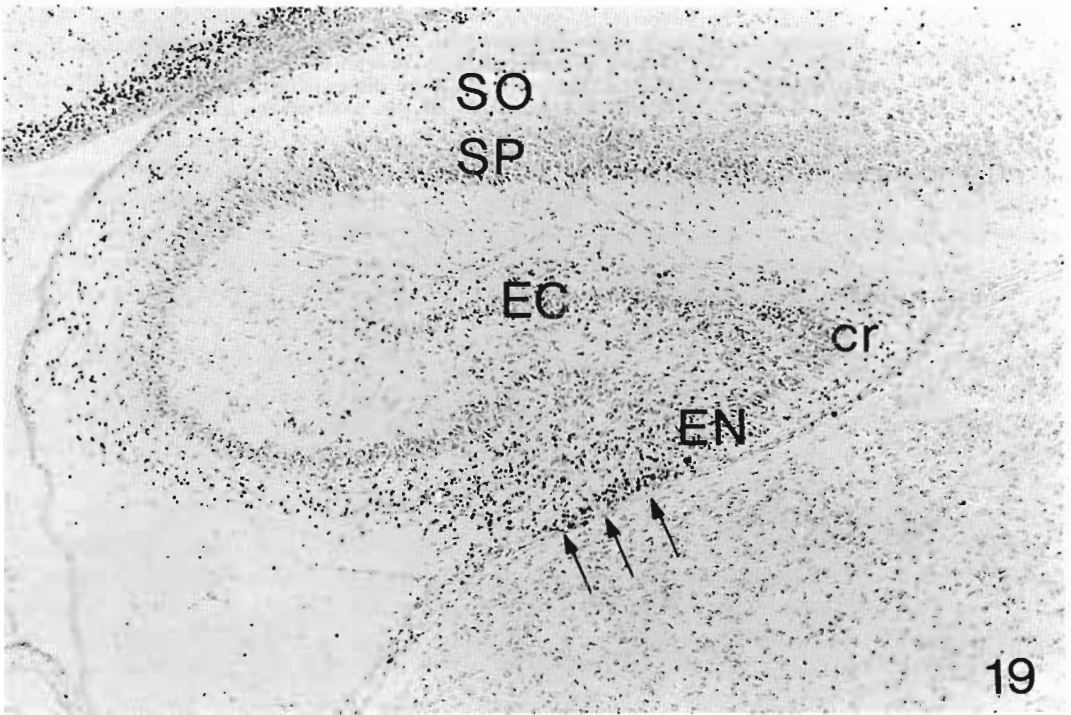
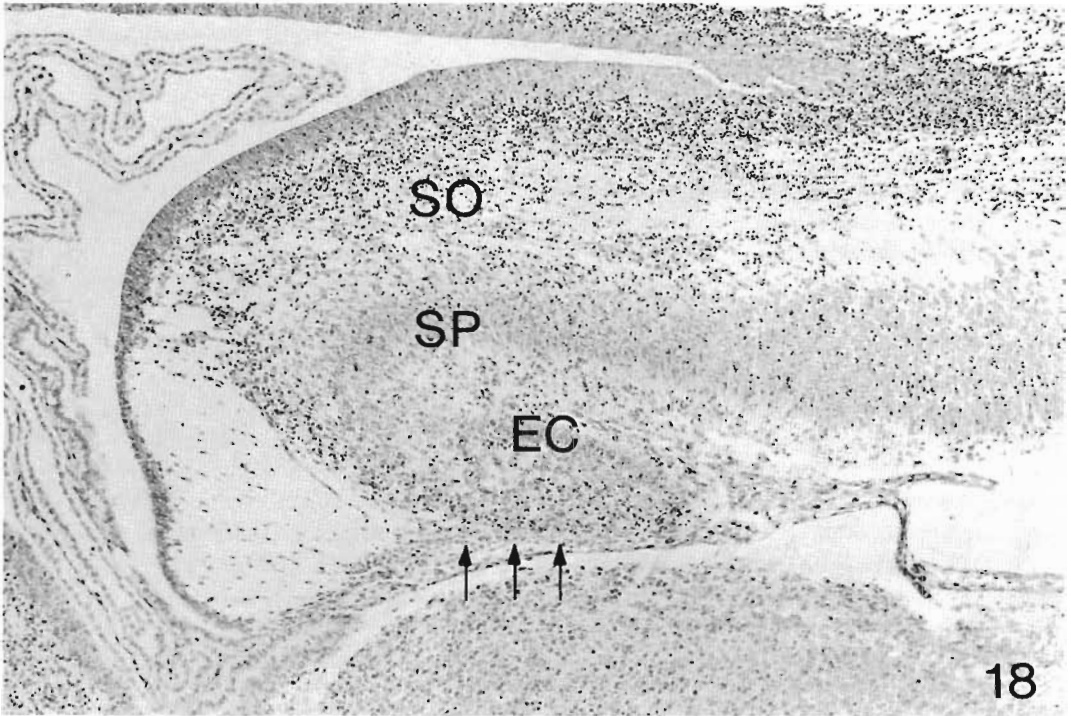


PLATE 3

EXPLANATION OF FIGURES

Pyknotic cells in the hippocampus at different ages. Animals were killed six hours after exposure to 200r X-ray. Cresyl-violet, $\times 101$.

- 20 Postnatal day 3. Cell pyknosis in stratum oriens (SO) is reduced with respect to day 1 but it remains high in the juxtapial zone (arrows) of the dentate gyrus near the endal arm and throughout the hilus, following the curvature of the granular layer.
- 21 Postnatal day 5. Only a few pyknotic cells are present in the stratum oriens (SO). The cluster of pyknotic cells previously in a juxtapial position is separated from the pial surface by the growth of the endal arm. Pyknotic cells are still clustered at the base of the granular layer.

