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Growth patterns in the lateral wall of the mouse telencephalon: I. Autoradiographic studies of the histogenesis of the isocortex and adjacent areas

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INTRODUCTION

In a previous pilot study of neuron production in the anterior mouse telencephalon, using autoradiography after pulse-labelling with tritiated thymidine (Smart & Smart, 1977), the locations of neurons labelled during fetal life were recorded on enlarged photocollages of the adult brain. The procedure, although tedious, provided a permanent display of the distributions of successive cell generations against their histological background. Since completing the pilot work, further contemplation of the photocollages has led to progress in the analysis of the distribution patterns of neuron generations in relation to regional boundaries and to the histological texture of the tissue. In the present study, the pilot work has been repeated and extended to cover the prenatal period of neuron production up to 18 days post-conception. Major attention has been directed to working out the events in the isocortical segment of the lateral telencephalic wall. The study has confirmed the observations of others that (1) neurons forming the classical six horizontal layers are born in an inside-first outside-last sequence (Angevine & Sidman, 1961; Berry & Rogers, 1965; Hicks & d'Amato, 1968; Shimada & Langman, 1970; Fernández & Bravo, 1974; Rakic, 1974) and (2) the process follows a ventrodorsal gradient so that a given generation of neurons occupies progressively deeper cortical layers as its destination is traced from the ventrolateral to the dorsomedial aspect of the isocortical arc (Hicks & d'Amato, 1968; Fernández & Bravo, 1974; Bisconte & Marty, 1975*b*). Analysis of our present results showed that if the isocortical arc was divided into radial units of convenient width each unit had the same history of distribution of successive cell generations. The ventrodorsal gradient was found to be consistent with radial units entering and completing the inside-first outside-last sequence progressively later as the isocortical arc was followed from ventral to dorsal.

Also in our pilot study (Smart & Smart, 1977) the location of cells with four different intensities of labelling had been recorded. Further analysis has suggested that this procedure can be used to identify the distributions of the first and second generation of neurons born after administration of tritiated thymidine. The recording of different labelling strengths was therefore continued in the present study. The relative numbers of first and second generation cells indicated that neuron birth reached a peak during the middle of the period of isocortical cell production and that this peak occurred earlier in the ventral isocortex than in the dorsal.

The results were consistent with a wave of differentiation passing across the

isocorticogenic segment of the periventricular generative layers so that neuron birth begins, peaks and declines progressively later as the isocorticogenic part of the ventricular layer is followed from ventrolateral to dorsomedial. The repetitive history of the distributions of successive cell generations within radial units accords with the radial structure of the area in the embryo and with the radial orientation of functional columns in the adult.

MATERIALS AND METHODS

The material consisted of the 22 days old offspring of mother mice each of which had received one injection of tritiated thymidine on one predetermined day during pregnancy. Conception dates were calculated by leaving a female mouse with a male overnight; the following morning, if a vaginal plug was present, was taken to mark the beginning of the first day of pregnancy (E1). The injection, of 10 μCi/gm body weight, was given intraperitoneally to one mouse on each morning between E11 and E18, inclusive, in the form of an aqueous solution of [methyl H^3] thymidine obtained from the Radiochemical Centre, Amersham, England.

The mice were allowed to have their litters, and two offspring from each mother were killed, 22 days after birth and under ether anaesthesia, by intracardiac perfusion of saline followed by Bouin's fluid. The heads with skull cap removed were left fixing for a further 4 hours before removal of the brain, which was kept for a further 24 hours in Bouin's fluid before further processing.

Each brain was serially sectioned at 6 μm in the coronal plane. The sections were pre-stained with haematoxylin and eosin and then autoradiographed by the dipping technique using the method for Ilford K_2 emulsion described by Rogers (1978). The slides were exposed for 28 days at 4 °C and the time of development was 10–11 minutes. With this dosage, duration of exposure and developing time, labelled nuclei with more than 35 overlying silver grains were seldom encountered. Between sets of slides processed at different times, however, there were slight variations in the size of the silver grains and in the amount of background. Because two sets of sections were available at each time interval, those showing the greater uniformity were used.

The investigation was carried out on sections selected from the anterior forebrain at a level just anterior to the decussation of the anterior commissure. This corresponded approximately to the level of Plate 26 in Sidman, Angevine & Pierce's *Atlas of the Mouse Brain* (1971). In some cases, however, the level taken was slightly rostral if the sections at the preferred level were damaged or affected by artefact.

In each section a series of overlapping photographs of one cerebral hemisphere was made and prints at a final magnification of × 175 were trimmed and mounted to give a photocollage more than a metre square in which it was easy to identify individual nuclei. The slide of the photographed hemisphere was then subjected to a systematic search under oil immersion for labelled cells whose locations were recorded as described below. The whole hemisphere including the septum, caudate-putamen nucleus and the entire cortical arc was surveyed in order to be sure of spanning critical boundaries.

Labelled nuclei belonging to, or suspected of belonging to, endothelial and pial cells were not recorded. No attempt was made to differentiate between labelled neurons and neuroglia during the recording process. Labelling intensity was assessed by counting only those silver grains which overlay the cell nucleus or lay external to

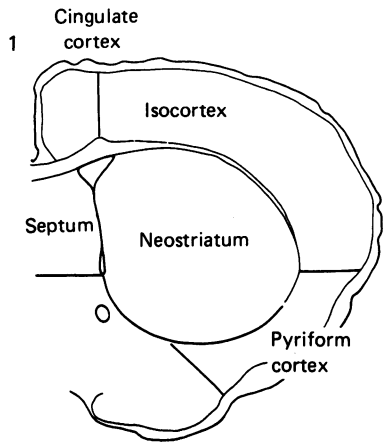
the nucleus while appearing to touch its perimeter. Cells were classified according to the number of grains per nucleus in the following manner:

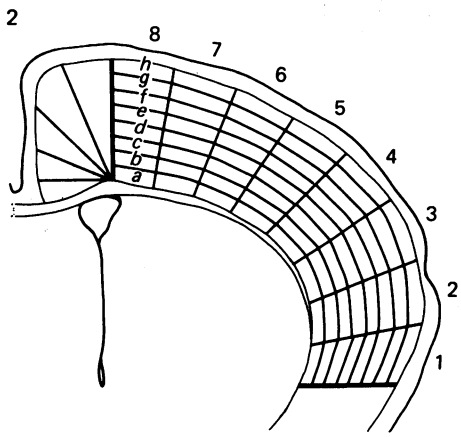
Group				*Not recorded*
I	II	III	IV	
> 25	21–25	16–20	10–15	< 10

These four labelling intensities were chosen in an attempt to define samples of the distributions of the first two cell generations after tritiated thymidine injection.

After counting the overlying grains, each nucleus was identified on a 10 × 12 cm print from a duplicate set of unmounted photographs and was marked in an appropriate colour. When all the nuclei in a 10 × 12 cm print had been examined, the results were transferred to the photocollage. When the positions of all the appropriately labelled nuclei had been recorded, the photocollage was covered with a clear acetate sheet and the outlines of the hemisphere, ventricles, corpus callosum, anterior commissure and deep boundary of the molecular layer of the cortex were drawn in. The nuclei belonging to each category of labelling intensity were marked. In this way, from each photocollage, four acetate sheets were produced, each recording the location of a set of nuclei with comparable labelling intensities. The sheets were then placed in a Plan Variograph (a standard machine used by geographers for altering the scale of maps) and retraced at a reduced magnification suitable for photocopy. The resulting photographs are reproduced in Figures 5–12.

The results were tabulated by dividing up the forebrain into major areas which were in turn subdivided further as depicted in Figures 1 and 2. The isocortex was demarcated from the cingulate cortex by drawing a line, parallel to the median plane, from the apex of the cingulum to the dorsal surface of the hemisphere. The line corresponded to the boundary between areas 6 and 8 in the map of the mouse cortex published by Caviness (1975). A second line, running at right angles to the median plane grazing the ventral margin of the lateral ventricle, was deemed to separate the isocortex from the pyriform cortex and claustrum. It traversed insular areas 13 and 14 in Caviness' map. Ventrally, the pyriform cortex was demarcated by dropping a line from the inferior border of the lateral ventricle at an angle of 45° to the line forming the ventral boundary of the isocortex. The pyriform cortex was considered to be the main cortical area lying within this 45° angle. Its deep boundary was formed by the corpus callosum or an extension of its curvature. It was also found convenient to delineate neostriatal and septal compartments. The former lay within the curvature of the corpus callosum, bounded ventrally by a continuation of this curvature to meet the ventral edge of the lateral ventricle on a course passing just medial to the anterior commissure; the septal area, for our purposes, lay between the corpus callosum and a line joining the ventral edge of the lateral ventricle to the mid-line (Fig. 1). Histograms were then constructed depicting the numbers of nuclei of each of the four labelling intensities within the neostriatal and septal compartments. The results from these non-cortical areas were used chiefly as cross checks on the progress of events in the cortical arc. The cingulate cortex was subdivided further by drawing a line from the apex of the cingulum at right angles to the boundary line already drawn between the cingulate and isocortex. Further lines were drawn subdividing the right angle so formed into four equal angles. The wedges of cingulate tissue were numbered 1–5 in clockwise direction (Fig. 2). The isocortical arc was subdivided into eight columns, roughly equal in area, by joining





3

	8	7	6	5	4	3	2	1
h	0	0	0	1	0	1	2	6
g	0	1	3	0	0	2	4	6
f	6	3	3	4	7	26	10	8
e	8	7	10	16	15	9	3	3
d	10	8	4	14	3	7	5	1
c	8	9	14	26	7	9	2	0
b	18	18	13	10	5	3	2	2
a	5	1	3	2	1	0	2	2

4

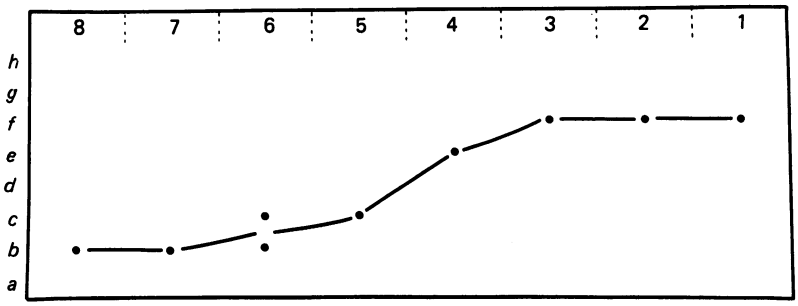


Fig. 1. Diagram of coronal section through mouse brain, immediately rostral to anterior commissure, showing subdivisions used.

Fig. 2. Part of diagram in Fig. 1 enlarged to show isocortical quadrant divided into 8 columns with equal bases; each column divided into 8 boxes by joining up equally spaced points along each side. Cingulate area has been divided into 5 wedge shaped areas.

Fig. 3. Table prepared by counting the number of Group I (heavily labelled) nuclei in isocortical boxes of E13 autoradiograph and entering the number into corresponding box in Table. The figures enclosed in ellipses denote the location of boxes with highest number of Group I cells in each column. Joined together they give the 'radial isochrone'.

Fig. 4. The radial isochrone extracted from Table in Fig. 3. and displayed alone.

up points equidistantly spaced along the external surface of the corpus callosum and along the boundary between cortical layers I and II. These were numbered 1–8 in an anticlockwise direction (Fig. 2). Histograms depicting the number of labelled cells in each radial unit on each day of ^{3}H-thymidine injection were then prepared. The units were further subdivided into eight boxes labelled *a* to *h* from deep to superficial by joining up equally spaced points on the sides (Fig. 2). The number of labelled nuclei in each box was counted and recorded in the corresponding place in a 8×8 Table as in Fig. 3. The Tables were particularly useful in providing a means of extracting isochrones* from the crude data. The most economical and informative procedure was to locate in each radial unit the level of the box containing the maximum number of labelled cells. When the levels so determined in all eight columns were joined up this gave the 'radial isochrone' (Figs. 3, 4). This isochrone when extracted from the Table and displayed alone (Fig. 4) depicted the level in each radial unit receiving most of the cells born at each generation after tritiated thymidine injection. Because the purpose of the radial isochrone was to depict the mean level of maximum concentration of labelled cells, then, if two boxes in the same radial unit both had the highest number of cells or if a second box had only one less than the highest number, the isochrone was drawn to run between them, as in Figure 4.

No attempt was made to perform counts of the identity of the labelled cells because of the difficulty of differentiating smaller neurons from neuroglial cells in 6 μm haematoxylin and eosin-stained sections, particularly when covered by a heavy label. However, in view of the continuous turnover of neuroglia, most neuroglial cells formed during the corticohistogenetic period can be expected to have dissipated their initial heavy label by subsequent cell division. In one trial collage at E15, an attempt was made to mark the location of labelled perineuronal satellite nuclei and other smaller 'glia type' nuclei but this was abandoned because the increase in recording time required to make the extra decisions was too great; the number of nuclei falling into this category was in this particular case less than 5 % of the total. Although the data presented are derived from only one section from one animal at each day of development, the distributions were, nevertheless, felt to be representative. For example, the distributions were sufficiently consistent so that with growing familiarity it became easy to identify the day of labelling if handed any autoradiographed section from a series with the experimental number hidden. Also the E11 and E12 distributions presented in this paper are similar to those in photocollages produced by the same method in a previous pilot study (Smart & Smart, 1977). Various other trial collages and differential labelling counts were also consistent with the distributions and ratios displayed in the material to be described in our results.

RESULTS

Isocortex

The changing distribution of the most heavily labelled (Group I) nuclei in the isocortical quadrant from E11 to E18 can be seen in Figures 5 *a*–12 *a*, respectively. Their areas of densest distribution were, at E11, in the deep ventrolateral isocortex; from E12 to E13, in an oblique band running from deep, medially to superficial, laterally; from E14 to E15, in a band across the isocortical quadrant; and finally, at E16, in the outer dorsal cortex. After E16, heavily labelled neurons were still found

* The term 'isochrone' introduced by Bisconte & Marty (1975*a*) denotes the area of maximum density of distribution of a particular cell generation.

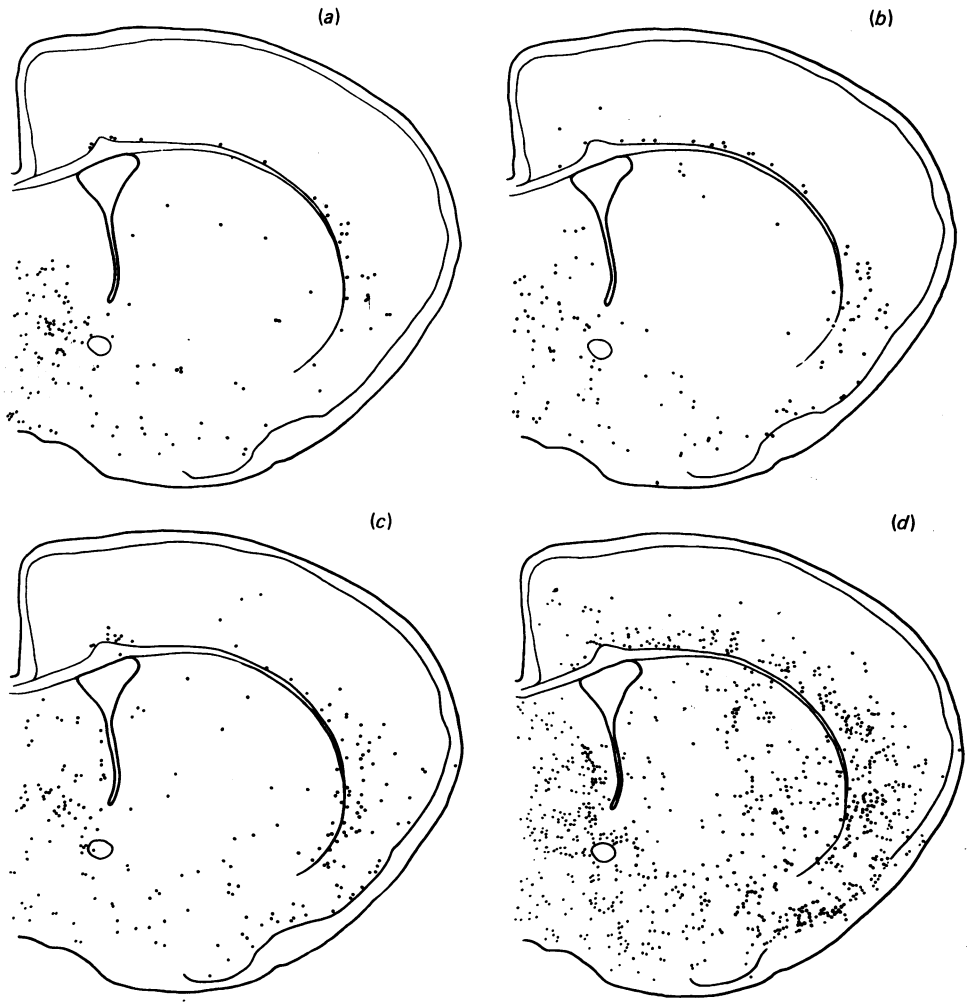


Fig. 5 (*a–d*). Cross section of anterior forebrain of adult mouse injected with tritiated thymidine at E11, showing location of nuclei of different labelling intensities: (*a*) Group I, > 25 grains per nucleus; (*b*) Group II, 21–25 grains per nucleus; (*c*) Group III, 16–20 grains per nucleus; (*d*) Group IV, 10–15 grains per nucleus.

located principally in the outer cortical layers but in diminished numbers (Figs. 11, 12). A synoptic view of the outward movement is given by the Group I radial isochrones in Figure 13.

The isochrones did not correspond to the conventional six circumferential layers. For example, at E13 (Figs. 7*a*, 13) the Group I isochrone crossed the boundary between layers IV and V as it was followed from dorsal to ventral. However, when the events in corresponding individual radial units were followed, the history of neuron acquisition and distribution was similar in each (Figs. 13, 14). In Figure 14, the distributions in two columns from opposite ends of the isocortex are set out. Labelled cells appeared first deep in layer VI, then had a more general distribution throughout layers VI and V and finally were virtually confined to the outer third of the cortex with the area of greatest density of distribution rising progressively

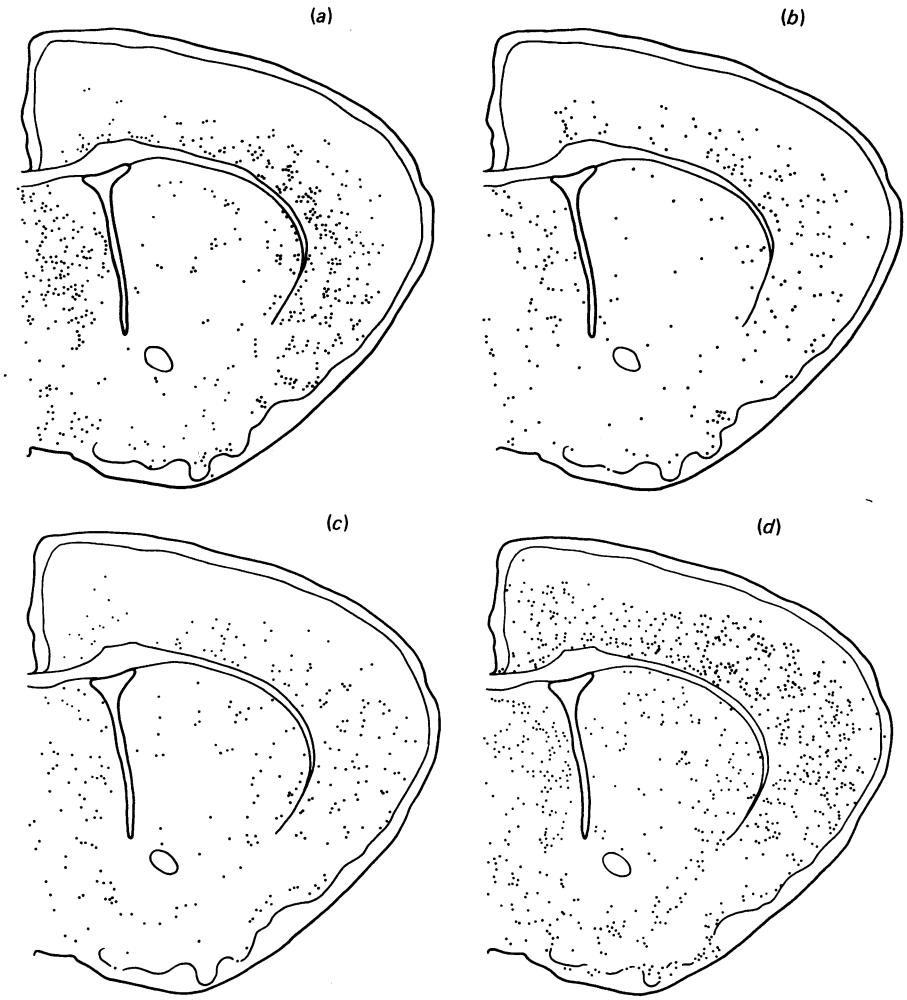


Fig. 6(*a–d*). Cross section of anterior forebrain of adult mouse injected with tritiated thymidine at E12 showing distribution of nuclei of labelling intensities I–IV, as in Fig. 5.

through layers IV, III and II. There was considerable overlap between the distributions at each day. This spread in the distribution of a single generation was widest in layers VI and V and least within the more superficial layers (Fig. 14). The history of the columns at the ventrolateral end of the quadrant started and finished ahead of those at the dorsomedial end (Fig. 14).

Study of the radial isochrones of the most weakly labelled cells recorded (Group IV) revealed a similar progression (Figs. 5*d*–12*d*, 13). The position of each Group IV isochrone lay more superficially in the cortex than the Group I isochrone of the same day.

The isochrones of the Group II and III nuclei are not depicted but were found to correspond more closely to those of Group I. (Cf., in Figs. 5–10, distributions *b* and *c* with *a* and *d*).

The ratio of the number of nuclei of each labelling intensity in the entire isocortical arc is given in Figure 16. Group IV nuclei exceeded Group I nuclei at E11, 12, and 14

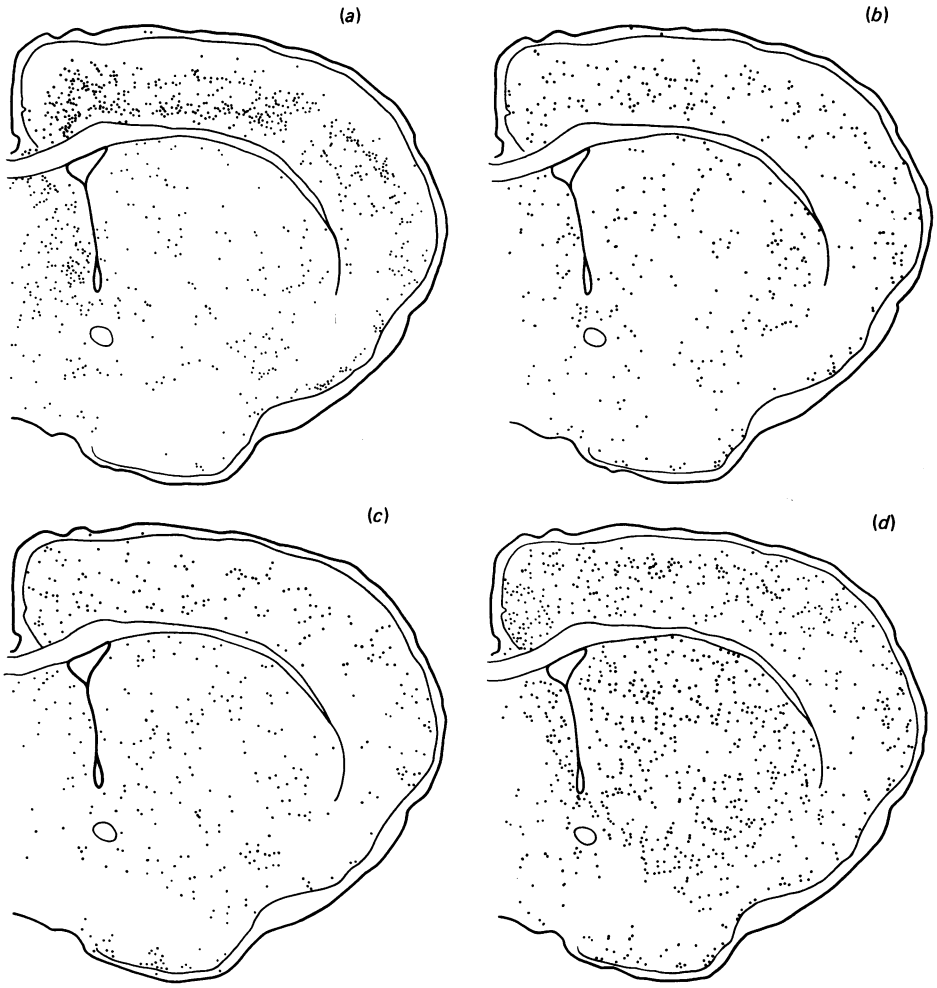


Fig. 7 (*a–d*). Cross section of anterior forebrain of adult mouse injected with tritiated thymidine at E13 showing distribution of labelling intensities I–IV as in Fig. 5.

but this ratio was reversed at E13, E15 and E16. When these ratios were examined in each radial unit of the isocortical arc (Fig. 17), a similar sequence was found in each unit. An interesting point, however, is that the units of the dorsolateral end of the isocortical quadrant had their maximum increase of Group I cells at E12 while in the dorsomedial columns this happened at E13 (compare, for example, in Figure 17 units 1 and 2 and 7 and 8 at E12 and E13).

Cingulate cortex

In this area, as delineated and subdivided in Figures 1 and 2, there was a general movement of Group I cells from deep to superficial between E12 and E15. Each of the wedge-shaped subdivisions seemed to have a similar history and the most ventral segment appeared to start and complete the sequence ahead of the most dorsal (Fig. 15). The number of nuclei of each labelling intensity in the entire cingulate cortex is given in Figure 16.

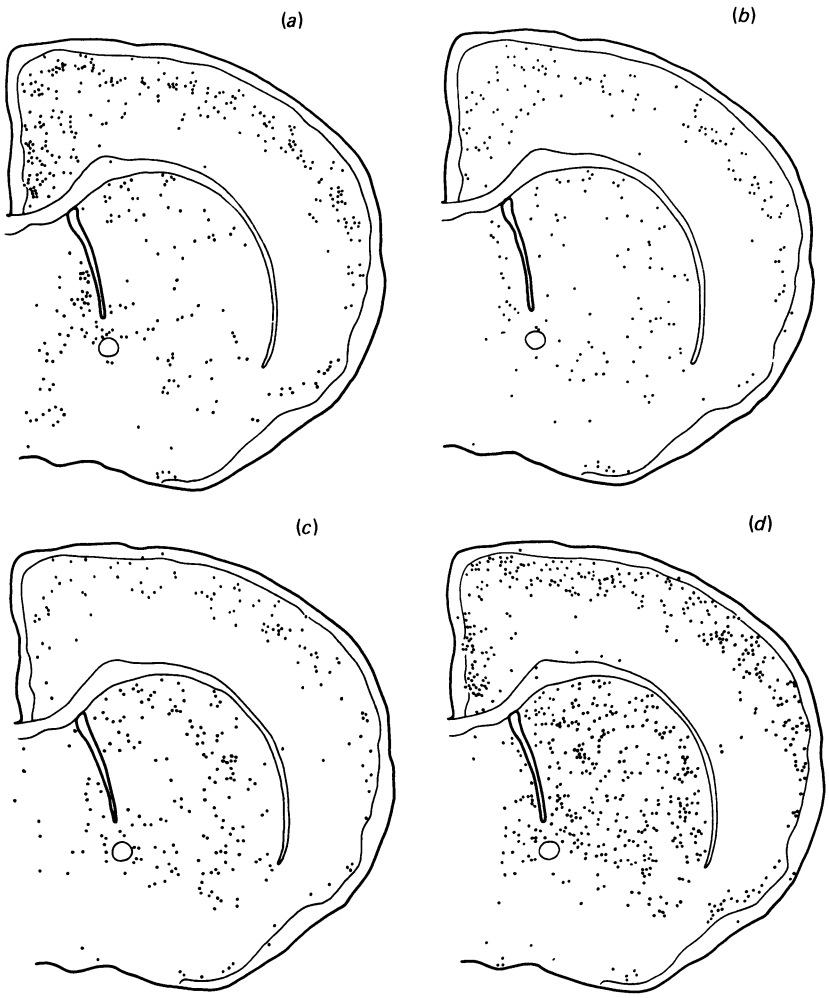


Fig 8 (*a–d*). Cross section of anterior forebrain of adult mouse injected with tritiated thymidine at E14 showing distribution of nuclei of labelling intensities I–IV as in Fig. 5.

Pyriform cortex

This subdivision in our delineation included the claustrum. Group I nuclei were found distributed throughout this area at E11 and E12. From E13 to E15 they lay principally within the pyriform cortex. Few neurons appeared to be produced for this sector after E14.

Neostriatum

Group I nuclei at E11 and E12 tended to be more numerous laterally towards the corpus callosum but might be found in any part of the neostriatal area (Figs. 5*a*, 6*a*). Between E13 and E15 they appeared to be uniformly distributed (Figs. 7*a*, 8*a*, 9*a*) and at E16 to E17 (Figs. 10*a*, 11*a*) they tended to be more numerous medially but, as at E11 and E12, might be found in any part of the neostriatal area.

The ratio of the number of nuclei of different labelling intensities (Fig. 16) showed

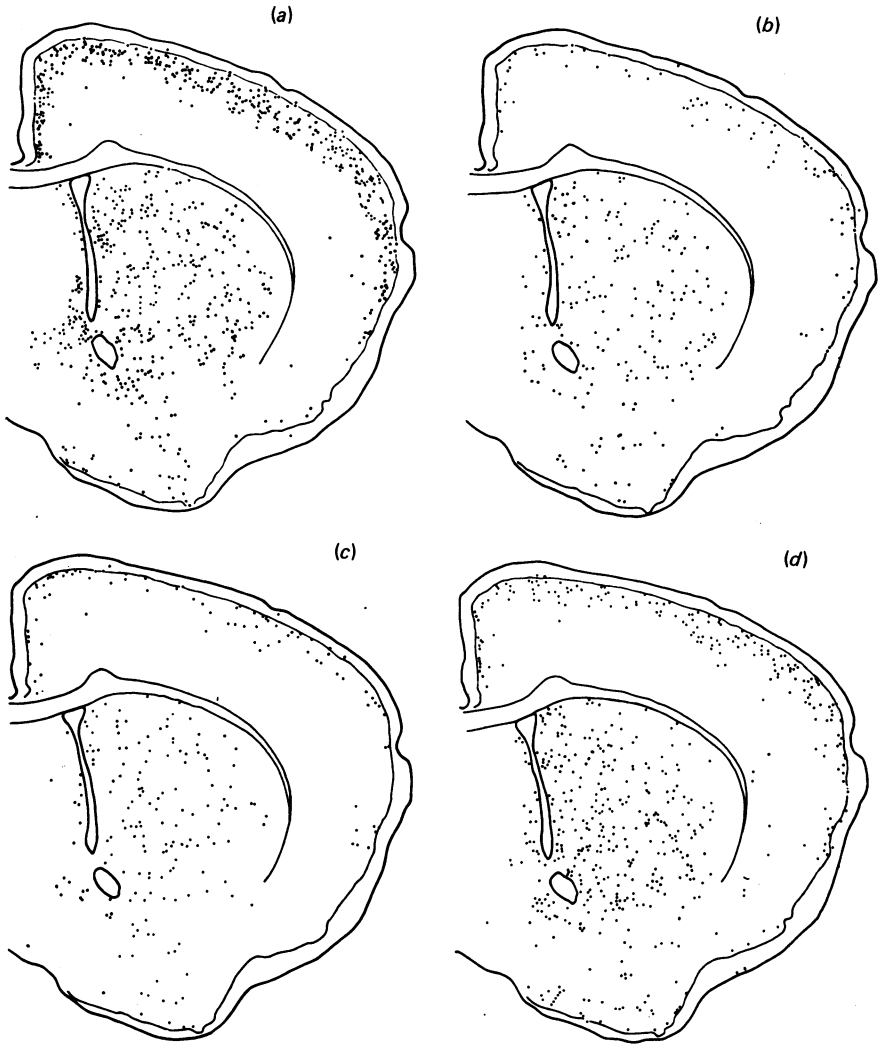


Fig. 9 (*a–d*). Cross sections of anterior forebrain of adult mouse injected with tritiated thymidine at E15 showing distribution of nuclei of labelling intensities I–IV as in Fig. 5.

a preponderance of Group IV nuclei except at E15 and E16 when the numbers became roughly equal.

Septal area

The distributions in this area were not analysed beyond establishing histograms of the differential counts (Fig. 16) which indicated that the major period of cell production lies between E12 and E13.

DISCUSSION

Interpretive caveats

This type of study is affected by certain unavoidable variables and unknowns which require to be taken into account when developing an interpretive strategy.

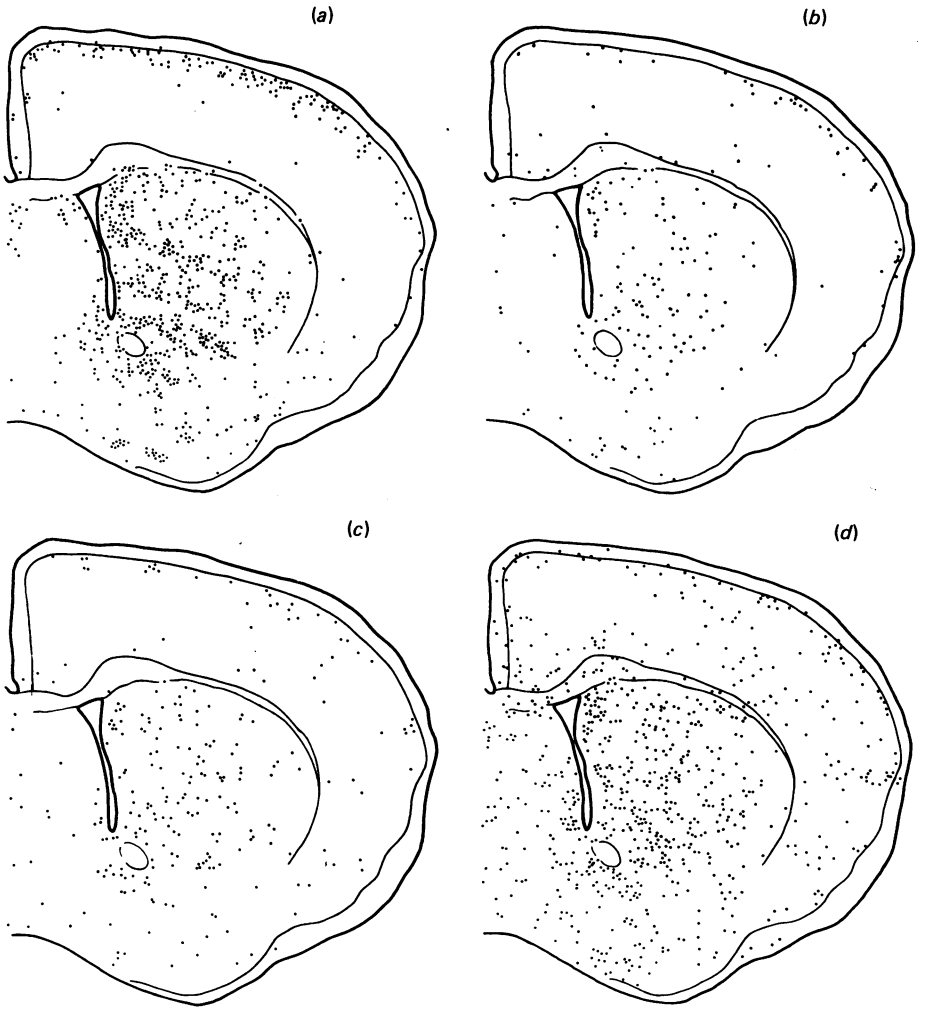


Fig. 10 (*a–d*). Cross sections of anterior forebrain of adult mouse injected with tritiated thymidine at E16 showing distribution of nuclei of labelling intensities I–IV as in Fig. 5.

Actual versus putative stages of development

Although it is desirable to have injections of ^{3}H-thymidine spaced out at known intervals of development in order to follow the changes in distribution of neurons liberated at successive equal time intervals, it is not possible, in fact, to be sure that this has occurred because of an inability to ascertain the exact time of conception and commencement of development. The age at injection was calculated by counting the morning at which a vaginal plug was found as the beginning of the first embryonic day. Different mice, however, may have mated at different times during the previous night, the stage of ovulation at which mating occurred may have varied and, according to Theiler (1972), there may be a spread of as much as 20 hours in the stage of development between embryos of the same mother due to asynchronous fertilisation of different ova by the sperm from one mating. Thus, it is possible for all the variables in a mouse injected at, say, E11 to have been long and in another mouse injected at

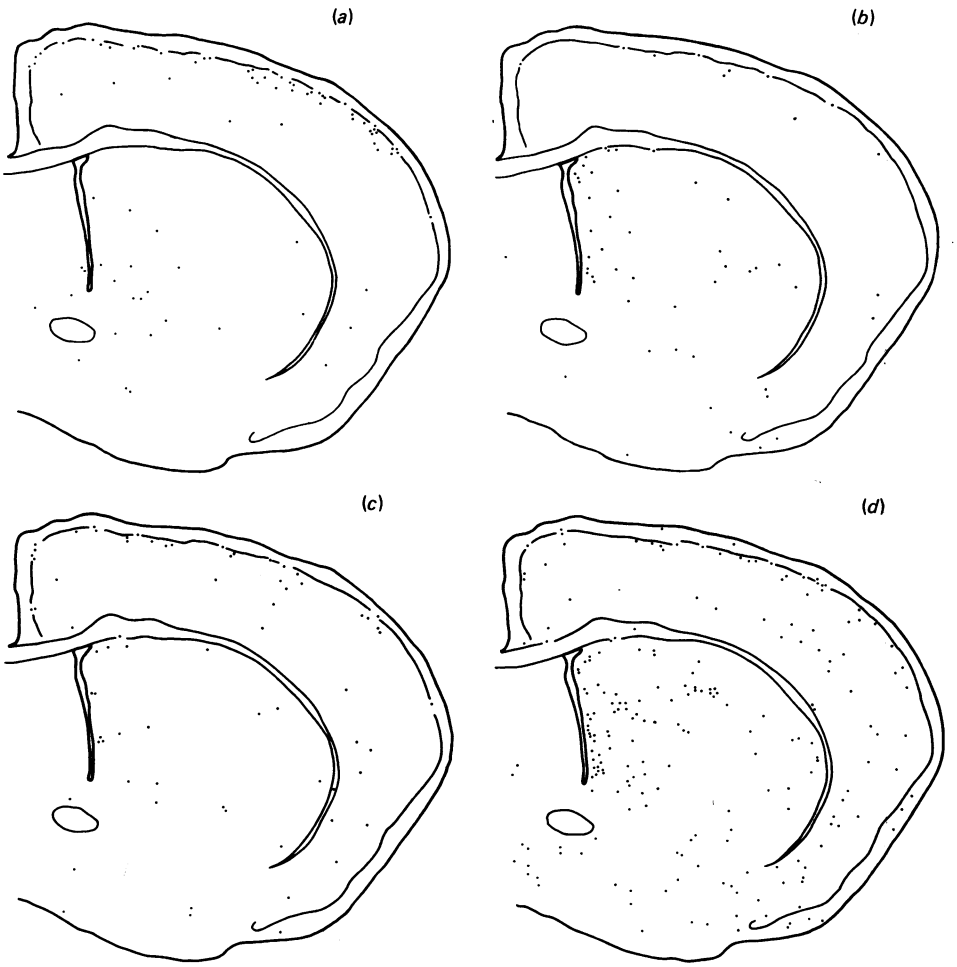


Fig. 11 (*a–d*). Cross sections of anterior forebrain of adult mouse injected with tritiated thymidine at E17 showing distribution of nuclei of labelling intensities I–IV as in Fig. 5.

E12 to have been short. In such cases the injection could be received by embryos at similar stages of development in each mouse. However, it is unlikely that all variables would combine to bring about the worst possible case in every mouse injected and, in fact, the distributions of heavily labelled cells on successive days differ from each other sufficiently to suggest that the embryonic stages at which the ^{3}H-thymidine pulses were received were separated by substantial, if not always equal, time periods.

Cycle time variation

A related variable is the length of the cycle time of ventricular layer cells; this is known to increase during the course of prenatal development from less than 10 hours to over 20 hours (see review by Korr, 1980), so that even if injections happen to be separated by equal developmental time intervals the number of cycles between injections will be fewer towards the end of cortical histogenesis than at the beginning. Conversely the inability to fix reliably the stage of development prevents injections being made to coincide with periods of known cycle time.

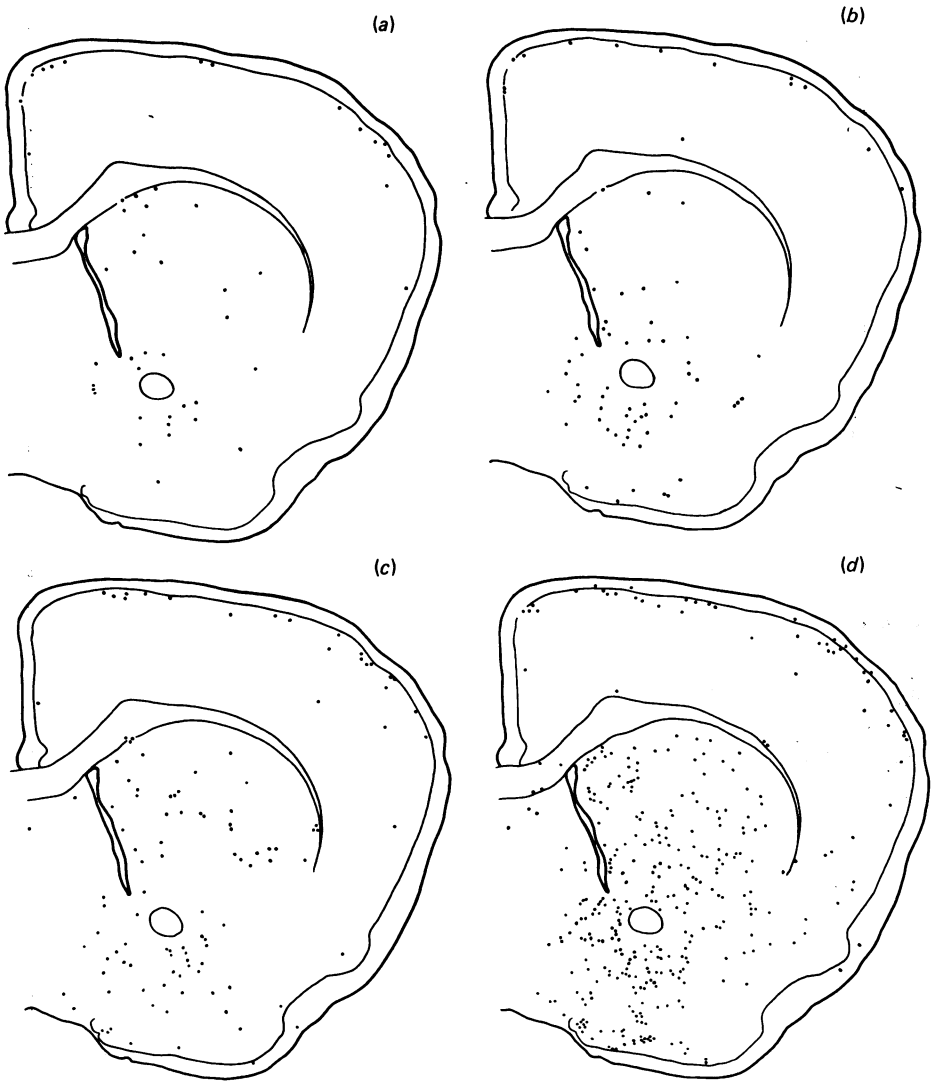


Fig. 12 (*a–d*). Cross sections of anterior forebrain of adult mouse injected with tritiated thymidine at E18 showing distribution of nuclei of labelling intensities I–IV as in Fig. 5.

Autoradiographic variables

The technical variables inherent in autoradiography are also well known and have been comprehensively reviewed many times (e.g. Sidman, 1970; Bisconte & Marty, 1974; Rogers, 1978). These require the exercise of particular caution in comparing counts of nuclei of different labelling intensities made in autoradiographs of different animals processed in different batches because even minor variations in animal response, technical procedure and emulsion characteristics can produce changes in grain number and size. This makes it desirable to extract the maximum amount of information from a single autoradiographed section where these factors will be minimal and, when making comparisons between sections on different slides or from different animals, to look for data which are the least subject to the effects of

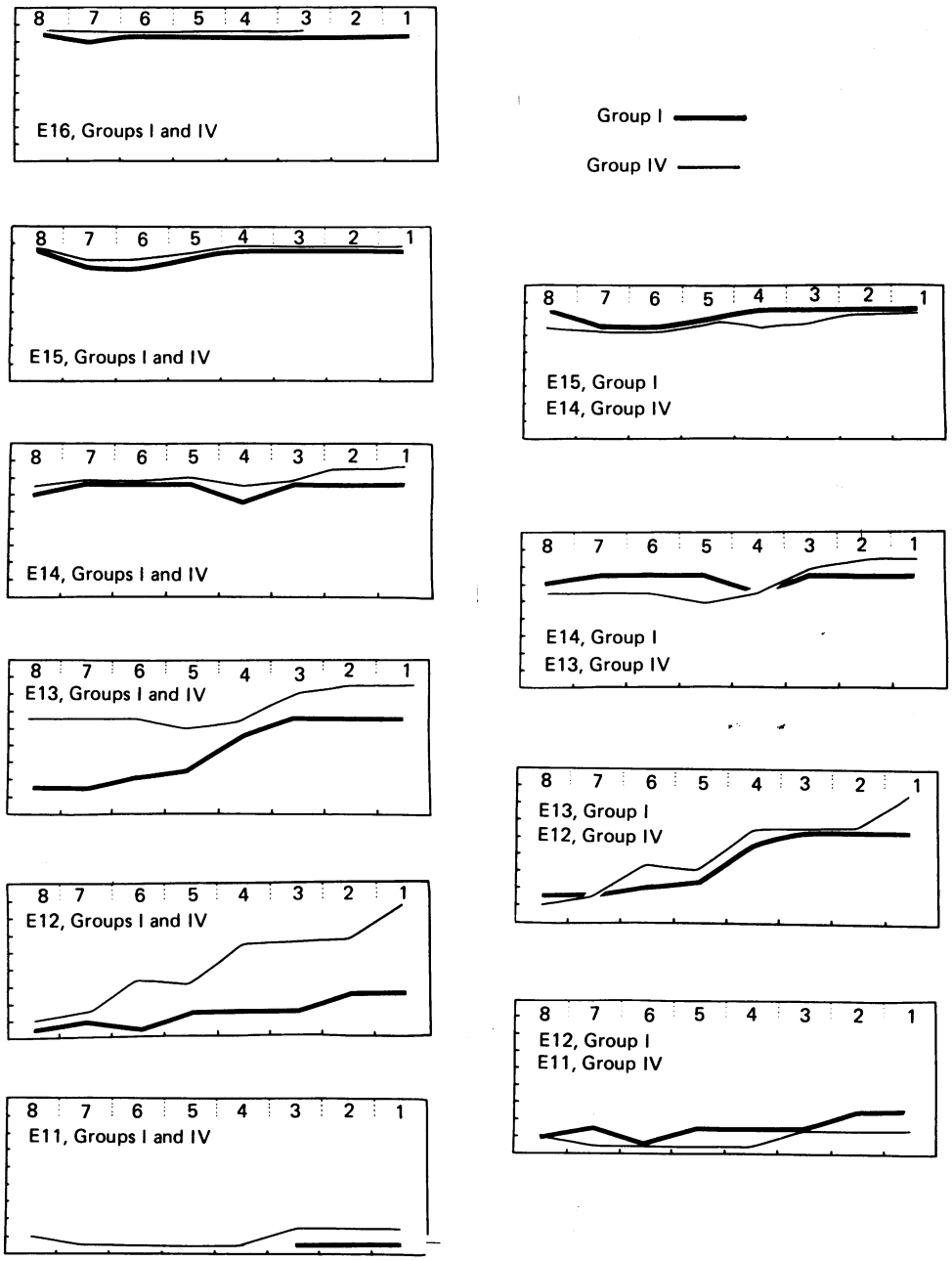
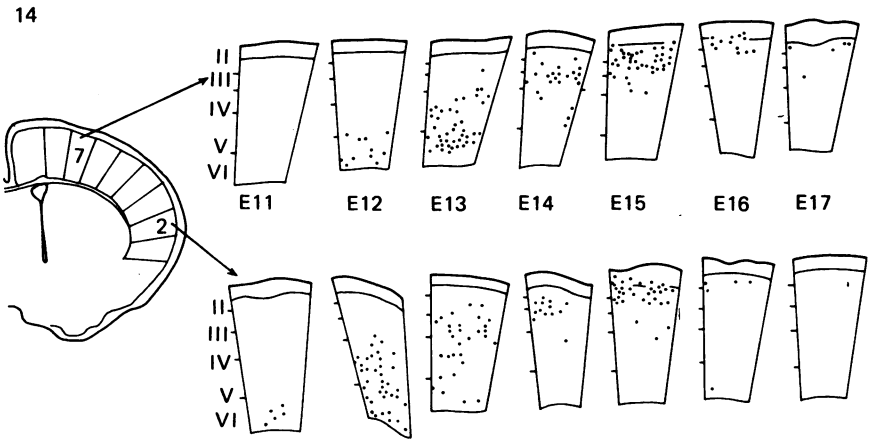


Fig. 13. Diagrams of radial isochrones constructed as in Figs. 3 and 4 from distributions in Figs. 5*a* to 10*a* and Figs. 5*d* to 10*d*. Sequence to be read from bottom to top. Left hand side of page: Groups I and IV isochrones at stated embryonic ages of tritiated thymidine injection. Right hand side of page: Group IV (second generation) isochrones of one day combined with Group I (first generation) isochrone of the mouse labelled on the succeeding day.



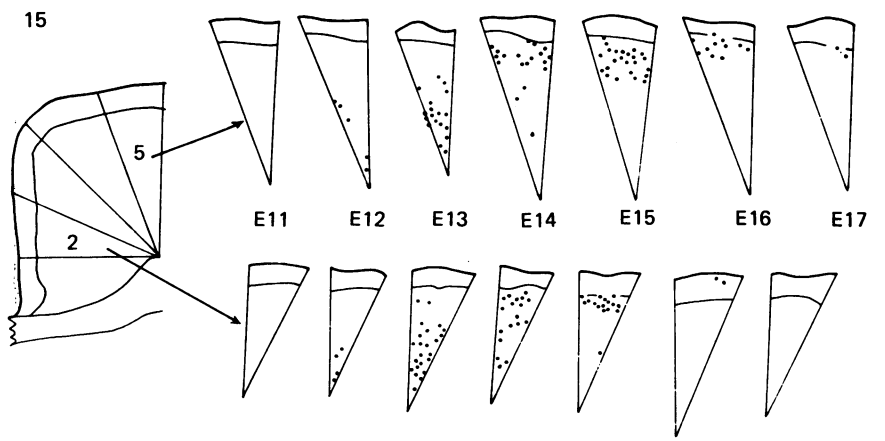


Fig. 14. Location of heavily labelled (Group I) nuclei in indicated columns at each extremity of isocortical quadrant.

Fig. 15. Location of heavily labelled (Group I) nuclei in indicated wedges of cingulate cortex.

technical variations. Two types of data are particularly useful in this respect: positional information and ratios of cells of different labelling intensities. The former is the most accurate component of our data and if a representative sample of the first generation of labelled cells can be identified in each autoradiograph its distribution will provide a reliable feature for comparison between different animals because it will depend on the distribution of labelled cells and not directly on their number. The ratio of cells of different labelling intensities also provides an index which is less likely to vary for reasons of technique. For example, autoradiographs of tissue from two similarly labelled animals subjected to *slight* variations in processing procedure could be expected to have different values for the mean number of grains per nucleus but to retain, nevertheless, the same ratio between groups of similar labelling intensity due to an approximately proportional addition of cells to each column of the histogram.

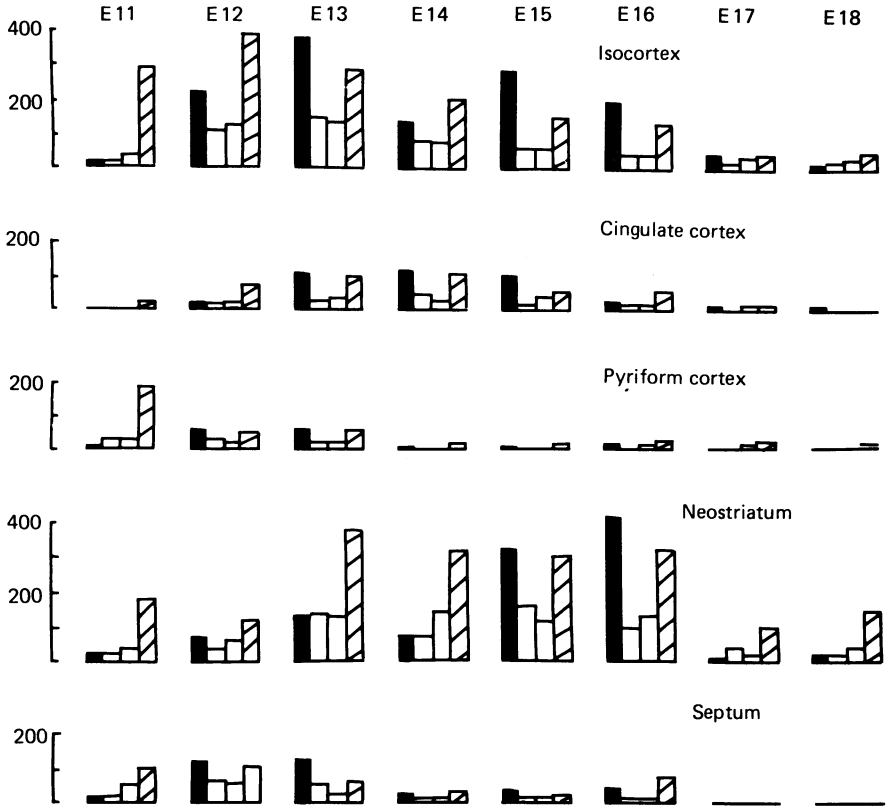


Fig. 16. Histograms showing the number of nuclei of each labelling intensity in the different areas of the anterior forebrain indicated in Fig. 1. The columns in each histogram represent, from left to right, respectively, the numbers of Groups I–IV nuclei at each embryonic day of labelling. Group I columns are in solid black and Group IV columns are cross hatched.

Treatment of differential labelling counts: identification of the first generation

The interpretation of differential counts in terms of the cell generations they represent has additional difficulties because the grain number per nucleus is affected by other factors than dilution of label by successive cell divisions and variations in processing procedures. In the system we are dealing with here, cells leave the proliferative compartment, differentiate into neurons and do not divide again. Thus, the first generation of neurons to be born after the passage of each tritiated thymidine pulse can be identified positively as those bearing the heaviest label and, once this label is defined, the desired sample of one generation will be obtained. Our pilot counts indicated that the maximum number of silver grains overlying any nucleus in the autoradiographs of all the animals chosen for study was seldom much in excess of 30 and never less than 25 and so our Group I, with more than 25 grains per nucleus, can be interpreted as providing a reliable sample of first generation cells and its distribution interpreted accordingly.

Identification of the second generation: operational difficulties

More weakly labelled neurons are a group with mixed origins. The majority can be expected to belong to cells in which an original maximum label has been diluted by successive mitoses. Some, however, will belong to cells in which S-phase was

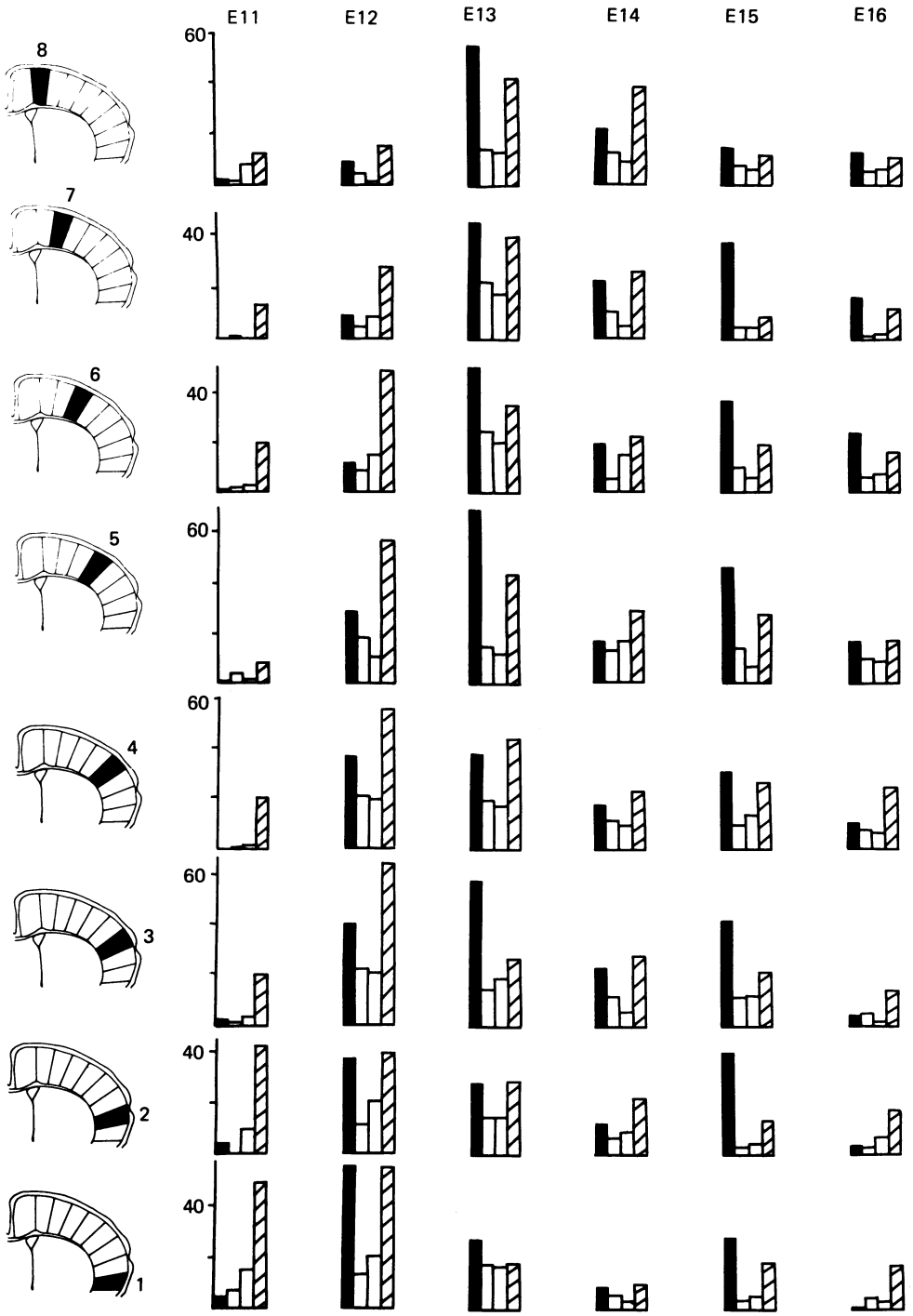


Fig. 17. Histograms showing total number of nuclei of each labelling intensity in each of the eight columns into which the isocortical quadrant was divided. Group I columns in solid black. Group IV columns cross hatched. Read across, the histograms give the history of the indicated columns from E11 to E16. Read from bottom to top, the histograms give the distribution of nuclei of different labelling intensity across the cortical quadrant at the stated embryonic ages of tritiated thymidine injection.

beginning or ending at the time of tritiated thymidine injection and which, therefore, were not exposed to circulating tracer for the full time of its availability and were thus, although more weakly labelled, fairly close in birth date to the strongly labelled cells. Others will appertain to cells lying deep in the histological section or only partially included in it and which, consequently, will be exerting less than their actual labelling strength on the overlying emulsion. The effects will operate on nuclei of all labelling intensities and will relegate a proportion of more heavily labelled cells to lower groups.

The intrinsic character of the histogram of grains overlying a single cell generation is difficult to establish. In a technically perfect autoradiograph of an 'ideal' population of one generation, uniformly labelled, with every nucleus optimally sectioned to give beta particles an equal opportunity of affecting the superimposed emulsion, the number of silver grains overlying the nuclei would vary because of the randomness of beta particle release by the tritiated thymidine molecules and would give the Poisson histogram characteristic of radioactive decay. A Poisson distribution is in fact obtained when the grains over nucleus-sized areas are counted in an autoradiograph of a uniformly distributed source such as tritium bound to polymer molecules and spread evenly on a slide (England & Rogers, 1970). However, when the tritium label is incorporated into nuclear DNA the distribution of grains over nuclei in standard tissue autoradiographs is no longer Poisson and indeed can vary considerably from this type (Bisconte & Marty, 1974). This is probably a geometrical effect produced by suboptimal sectioning of cell nuclei (Todd, unpublished observations). Thus, differential grain counts provide a 'global histogram' composed of the merged individual histograms of several generations each of which has been distorted from the ideal Poisson distribution by the technical, temporal and geometric factors mentioned above. Bisconte & Marty (1974, 1975*a*) have analysed the effects of these factors and have devised a method of statistical analysis which enables the proportion of cells contributed by all participating generations to be extracted from differential counts; their procedures provide this information as estimates of the statistical mean proportion of cells of each generation in chosen areas, e.g. superficial, middle, and deep cortex. In this study, we are interested in the distributions of only the first two cell generations after tritiated thymidine injection and information about the distribution of second generation cells sufficient for our purposes may be gained by a simpler non-parametric approach.

Identification of the second generation: operational decisions

It is assumed, for the following two reasons, that a sample of second generation cells will be present among the Group IV nuclei: (1) the silver grain count per nucleus of the second generation should be half that of the first, and this condition is fulfilled by our Group IV category which, with a count of 10–15 grains per nucleus, registers about half that of Group I; (2) the distribution of second generation cells should be positionally more advanced than that of the first, and this condition is also fulfilled by the Group IV nuclei (e.g. compare Figs. 5*a* and 5*d*). The distributions of Group II and III nuclei are similar to that of Group I nuclei with a small number of cells scattered towards the Group IV distribution (Cf. Figs. 5*a–d*) suggesting that Group II and III nuclei are mainly of first generation cells with a few strongly registering members of the second generation. Group IV nuclei, therefore, can be expected to belong principally to second generation cells with a number of undervalued first generation cells and some strongly registering cells of the third generation. Thus, to obtain an approximation to the distribution of second generation

cells a proportion of nuclei with a distribution pattern similar to Group I must be subtracted to compensate for undervalued first generation cells. A similar operation must be carried out to remove third generation nuclei which can be assumed to have a more advanced distribution. Second generation cells can, therefore, be expected to have a distribution corresponding to that of a 'narrowed' Group IV. Although this reduction is difficult to accomplish by statistical procedures it can be assumed that the regions of maximum density of distribution of Group IV nuclei will contain the greatest proportion of second generation cells. The Group IV radial isochrone links the levels of maximum density of weakly labelled cells in each radial unit and could be expected to retain this property were it possible to identify and remove all non-second generation cells with a Group IV label. The Group IV radial isochrone will therefore be used as an index of the movement of second generation cells up each radial unit. The distributions of Group I and IV nuclei will now be discussed separately assuming that their radial isochrones contain representative samples of the distribution of first and second generation neurons born after each pulse of ^{3}H-thymidine. The information that can be gathered from the changing ratio between the two groups will then be considered.

Distribution of Group I nuclei

Isocortex

The most strongly labelled (Group I) nuclei can be assigned with some confidence to cells which were born shortly after the administration of each pulse of tritiated thymidine. Thus, in the isocortical quadrant as demarcated in Figure 1, cells born at the beginning of neuron production are more numerous in the deeper layers of the lateral isocortex (Figs. 5*a*, 6*a*, 13). Those born in the middle period of cell production (about E13) are found dispersed in an oblique band across the middle isocortex (Figs. 7*a*, 13) while in the final period of major cell production cells are produced principally for the dorsal part of the layer II (Figs. 10*a*, 14). These findings are in agreement with the descriptions given by Hicks & d'Amato (1968) of the dispositions of cell generations in the rat isocortex. Thus, the most striking feature of the successive distributions of first generation isocortical cells is their lack of conformity with the six horizontal layers into which the adult isocortex is customarily divided. The two patterns, however, can be reconciled if the isocortical quadrant is divided up into suitably narrow radially orientated units and the successive distributions of labelled cells in corresponding units followed. Each radial unit can be seen to have a similar 'inside first-outside last' distribution of cell generations (Fig. 17). The non-conformity between the isochrones and the classical cortical layering results from radial units commencing their acquisition of neurons progressively later as the isocortical quadrant is followed from ventrolateral to dorsomedial. Thus, the obliquity of tne E13 isochrone in Figure 13 indicates that the ventrolateral units, being further advanced in development, are receiving the majority of their neurons at a higher level that the dorsomedial, which commenced their history some hours later. The developmental succession, therefore, leads us to regard the isocortex as a series of radial units, formed in sequence from lateral to medial, whose component cells eventually come into register, that is, neuroblasts at similar levels in each unit differentiate into similar types of neuron to produce the horizontal layers of cytoarchitectonic similarity emphasised by the classical description. On the other hand, the adult isocortex is known to be organised in columns or slabs, each composed of radially arranged neuron assemblies synapsing

with each other along the length of the column but not directly with neurons of adjacent columns (Mountcastle, 1957, 1979; Hubel & Wiesel, 1962, 1972). The suggestion has already been made (Angevine & Sidman, 1961) that the inside first-outside last sequence of cell acquisition, observed generally in the isocortex, also applies to individual functional columns. Our maps, however, demonstrate that there is considerable scatter in the distribution of a single generation and consequent overlap between the distribution of succeeding generations. The relatively wide radial units into which we have arbitrarily divided the isocortical quadrant would contain many functional cell columns. The scatter and overlap of cell generations observed in our radial units could, therefore, result from the neurons of individual functional columns being stacked out of chronological sequence of birth or from neighbouring columns of chronologically stacked neurons developing asynchronously. Because it is of some importance to ascertain the way these fundamental structural units of the cortex are built up, the ambiguity presented by scatter and interpenetration of cell generations is treated further in the last paper of this series (Todd & Smart, 1982).

Finally, although neuron production for the isocortex decreases after E16, it does not cease, as the presence of strongly and weakly labelled cells at E17 and E18 demonstrates (Figs. 11 *a–d*, 12 *a–d*). These late-formed cells predominate in the outer cortical layers and their location is consistent with their being the last arrivals in the retrograde accumulation of cortical cells. There is, thus, no evidence of a terminal injection of 'microneurons' into all levels of the cortex. Altman & Das (1966), the main protagonists of the special status of microneurons as a generalised final addition to an established neuron population, also noticed the lack of this phenomenon in the isocortex.

Cingulate cortex

The area medial to the apex of the cingulum is mainly composed of cingulate cortex. The majority of the first born neurons in this area tend to lie in the deeper, ventral part of the cingulate quadrant (Figs. 6 *a*, 15), the cells from the middle period are more widely distributed throughout the middle layers (Figs. 7 *a*, 15) and, in the terminal phase of neuron acquisition, neurons accrue principally in the more superficial and dorsal part of the cingulate area (Figs. 9 *a*, 10 *a*, 15). This changing distribution (which has been confirmed in other sections) is consistent with the presence of a ventrodorsal differentiation gradient on the medial side of the hemisphere, similar to but more compressed in time and space than that pertaining to the isocortex on the lateral side (Cf. Figs. 14 and 15). The position of the cingulum, at this particular coronal level at least, appears to coincide with a 'watershed' area between the growth patterns of the lateral and medial walls of the developing cerebral vesicle, each wall giving rise to a different type of cortex.

Pyriform cortex

The major feature of interest in our results is that the isochrones of this area appear to be continuous with those of the ventral isocortex (Cf. Figs. 5 *a*–8 *a*) and this would indicate that the neurons of the claustrum-pyriform area are produced within a common gradient of neuron release from the ventricular layer. This observation has also been made by Fernández & Bravo (1974) from autoradiographic studies in the rabbit. The site in the ventricular layer giving rise to cells of the claustrum-pyriform area has yet to be identified.

Distribution of Group IV nuclei

Isocortex

Group IV nuclei are taken to be composed mainly of neurons derived from precursor cells which had undergone a second division after taking up labelled thymidine, the 'true' distribution of these second generation cells being more closely approximated by the Group IV radial isochrone than by their crude distribution for the reasons entered into at the beginning of this discussion. The Group IV isochrones follow the same pattern of change as those of Group I but at each day lie higher in the cortex, as would be expected of later born cells (Fig. 13). The distance between the Group I and Group IV isochrones of a given day represents the amount by which the radial units have 'filled up' during one cycle time. The distance increases up to E13 (Fig. 13) and, thereafter, is less. This could indicate that neuron production reaches a peak at E13 and then declines. The greatest distance is found separating the Group I and IV isochrones of E12 in the ventrolateral half of the isocortical arc and the same isochrones at E13 in the dorsomedial half of the arc. This would suggest that the lateral cortex receives a major injection of neurons born between the first and second generations at E12 while a similar major injection into the medial cortex occurs later between the two generations represented at E13. This is consistent with the ventrodorsal progression of histogenesis across the isocortical quadrant discussed in the previous section.

Comparison of the isochrones of Group IV neurons of one day (the supposed second generation of that day) with those of the Group I (first generation) neurons of the next seriatum from E11 to E16 should indicate by their relative positions whether the second generation cells of the first day were born before or after or contemporaneously with the first generation of the next day. The cycle times of the ventricular cells of the mouse at about the same coronal level as our study were estimated by Hoshina, Matsuzama & Murakami (1973) to increase during development linearly from 7 hours at E10 to 26 hours at E17. It would be expected, if our injections happened to have been separated by 24 hour intervals of development, that the Group IV isochrones would shift progressively towards the Group I isochrones of the succeeding day until at E16 and E17 the two were coincident. This was found to be more or less the case except that the E12 Group IV isochrone was slightly in advance of that of the succeeding day (Fig. 13). Such an occurrence would indicate a cycle time of over 24 hours or a shorter period than the putative 24 hours of development between the E12 and E13 pulses of tritiated thymidine. The latter is more likely; all our distributions could be juggled to fit the cycle time estimates of Hoshina *et al.* (1973) if, in our material, variations between the actual and putative periods of development of up to 10 hours were allowed. In view of the uncertainty in determining the exact stage of development at which the injections were made this is not an unreasonable variation to invoke.

Ratio of Group I to Group IV nuclei

If, as has been assumed, the Group I and Group IV nuclei are composed of samples of neurons born after the first and second divisions, respectively, of precursor cells which were in S-phase at the time of administration of the pulse of tritiated thymidine, then their ratio will give an index of the relative numbers of neurons differentiating out from the precursor pool at each generation. More weak than strong labels would indicate more cells born at the second generation, and *vice*

versa. Although this ratio is also an internal comparison within one section, it may still be affected by variations in autoradiographic technique. For example, a general increase in background grain count, or a proportional increase in overlying silver grains as a result of technical factors, will be caught preferentially by Group I because this is not a closed class like Groups II to IV but has an open end to include any number of silver grains per nucleus in excess of 25. Variations in the grain counts introduced by differing background in these sections, however, were low and such variations as there were would have amounted in all cases to a difference of less than one grain per nucleus. Further, a purely technical cause for a change in the ratio of cells of different labelling intensities would be expected to affect all areas of the section equally. Here the comparison of differential counts between the subdivisions established in Figure 1 reveals that changes in the ratio of the numbers of Group I to IV nuclei vary from region to region as depicted by the histograms in Figure 16. These caveats notwithstanding, the assumption will be made that major changes in the ratio of Group I to Group IV nuclei are due to changes in the relative number of first and second generation cells. In the expression of the ratios, the crude numbers of the Group IV nuclei were used so that comparison could be made with other areas of the anterior telencephalon where a 'narrowed' Group IV distribution cannot be obtained by the method used in the isocortex. However, in the isocortex, the ratios obtained by comparing the numbers in the boxes determining the Group I and IV isochrones gave similar results.

Isocortex

The set of histograms in Figure 16 displays some interesting changes in the ratio of first and second generation cells produced for the isocortical quadrant on different days. At both E11 and E12, the second generation cells exceed the first in number, which is consistent with a system producing increasing numbers of neurons at successive generations over this period. At E13, however, the ratio reverses, suggesting that fewer neurons are produced at the second E13 generation than at the first. At E14, the ratio changes again to suggest another increase in cell production and at both E15 and E16 the ratio reverts to the E13 situation in which it appears that fewer cells are produced at the second generation on these days. The E13 ratio, at least, is unlikely to be artifactual because the counts in the adjacent neostriatum give the opposite ratio (Cf. the E13 histograms of isocortex and neostriatum in Figure 16). The absolute numbers of first generation cells at each day also follow this pattern; joining the summits of the Group I columns in the isocortical histograms in Figure 16 gives a pleasing biphasic curve. The histological evidence based on the changes in magnitude of the cortical neuron compartment is also consistent with peaks of neuron production at those times (Smart & McSherry, 1982). Possibly analogous peaks have been described in human cortical histogenesis by Poliakov (1935–1938) and Sidman & Rakic (1973). This is a phenomenon of some interest and deserves further stringent investigation.

It is also useful to examine the histograms showing the changing ratios of first and second generation cells in each of the eight isocortical radial units (Fig. 17). Each individual radial unit has a history similar to that of the composite histogram for the entire isocortical quadrant given in Figure 16, particularly in the presence of two reversals of the ratio of first to second generation cells in each column. It is of interest to note that the first reversal is manifest at E12 in radial units 1 and 2, but occurs in the other units at E13. Also at E12 the absolute number of Group I nuclei

decreases progressively from columns 1–8 (Figs. 6*a*, 17) while at E13 the reverse tends to happen; first generation cells tend to increase as the cortical arc is followed dorsomedially (Figs. 7*a*, 17). This suggests that the first peak of neuron production has reached the ventrolateral isocortex at E12 but does not reach the dorsomedial isocortex until E13.

Non-isocortical areas

The differential counts of labelled cells in the septal area and cingulate and pyriform cortices are set out in Figure 16.

For each area, neuron production appears to be monophasic and to peak at different times. In the neostriatum, neuron production appears to peak at E15 and E16 and then to decline rapidly, although, as in the isocortex, cells continue to be produced after the main surge of cell production has passed. The main point of interest, as far as this study is concerned, is that different areas exhibit different patterns of change in the ratio of cells of different labelling intensities in the same autoradiographed section; and this can be adduced as evidence that those variations are not due to technical factors affecting the whole section.

Miscellaneous observations

Molecular layer

The status of isocortical layer I (molecular layer) deserves special comment. In histological studies of the early development of the isocortex, Marin-Padilla, (1971, 1972, 1978) has described the cortical plate as forming within the intermediate layer, splitting the latter into a deep and a superficial lamina. The deep lamina becomes the deeper part of adult layer VI and the superficial lamina furnishes the cell population for layer I. In support of Marin-Padilla's interpretation, both Rickmann, Chronwall & Wolff (1977) and Raedler, Raedler & Feldhaus (1980) have produced autoradiographs of the lateral telencephalic wall of the rat embryo, labelled a few days previously with tritiated thymidine, in which labelled cells were present both deep and superficial to a cortical plate composed entirely of unlabelled nuclei. However, the fate of all these superficial cells in the adult is still a matter for discussion (Rickmann *et al.* 1977). In our maps, occasional heavily labelled neuron-like nuclei were found in layer I at all ages of tritiated thymidine injections. Because layer I is sparsely occupied by nuclei, a single section is insufficient to form an opinion. A special count was made of the number of heavily labelled nuclei in the isocortical section of layer I between E11 and E18 in 10 alternate serial sections at each stage. The total counts were: E11, 1; E12, 2; E13, 2; E14, 1; E15, 20; E16, 8; E17, 2; E18, 1. These results are more in accord with the inside first-outside last hypothesis of Angevine & Sidman (1961). However, the origin of the cortical plate as a later intrusion into the middle regions of a pre-existing neuron population is an observable fact confirmed by all authors who have looked. We, thus, believe that both hypotheses may be true; the molecular layer neurons may be supplied in some part by early formed, possibly short lived (Duckett & Pearse, 1968; Sas & Sanides, 1970) cells from the outer part of the early intermediate layer and in greater part by later formed cells from the dominating 'inside first-outside last' sequence.

Rostrocaudal variations

Although the distributions of labelled cells at only one coronal level have been described we also had at our disposal photocollage maps made at three additional

coronal levels of the telencephalon at each embryonic day of tritiated thymidine injection between E11 and E16 in which the distributions of Group I nuclei had been recorded. The general pattern of distribution of labelled cells was the same at each level and fitted the pattern of global growth suggested for the isocortex in a previous communication (Smart, 1973). However, we have noted rostrocaudal differences in timing and minor, though consistent, regional variations of the general pattern which remain to be reported.

The advantages of photocollage mapping

The procedures we have used are extremely time-consuming. Once produced, however, the photocollage maps provide a permanent record of the distribution of labelled cells against the background of a large scale photograph of the ambient tissue. This makes it easier to relate the distributions of labelled cells to histological boundaries and to obtain a 'feel' for the character of the tissue. In our opinion, a set of photocollage maps is a useful, if not essential, control against which to calibrate maps produced by automated data-gathering methods. For example, the most interesting theoretical treatment of cell production in the central nervous system in recent years has been the series of studies by Bisconte & Marty (1974, 1975*a*, 1975*b*). These workers used a sophisticated, semi-automatic grain counting and plotting apparatus with computer aided statistical analysis of the recorded data. However, in their study, the transits of the sections were made by the scanner at spaced intervals along lines parallel to the mid-sagittal plane. The transit lines thus crossed cortical layers and more importantly, cortical columns at varying angles out of phase with the natural orientation of the tissue structure and this obscured the radial distributions which were eventually apparent from the study of photocollages. Automated grain counting and data analysis will be essential for gathering and digesting the considerable number of data required for identifying and quantitating the mosaic of subpatterns that go to produce the definitive isocortex of even a small experimental animal such as the mouse. For ease in subsequent data analysis, the natural lines of the tissue must be defined and the recording machine instructed to follow them. The need for such a preliminary orientation in studies of the histogenesis of neural tissue has been comprehensively emphasised by Nieuwenhuys (1974).

SUMMARY

The distribution of cells of different labelling intensities in the anterior forebrain of adult mice injected with tritiated thymidine at daily intervals during prenatal life was determined by mapping the location of labelled cells on enlarged photographs of autoradiographed sections. The isocortical arc was subdivided into an arbitrary number of radially orientated units. Each radial unit was found to have a similar sequence of arrival and distribution of labelled cells; the ventrolateral units, however, entered and completed the sequence ahead of dorsomedial units indicating the presence of a wave of differentiation spreading in this direction across the generative layers giving rise to cortical neurons. An attempt was made to identify (from differential grain counts) comparable samples of first and second generation cells produced after each pulse of labelled thymidine. The changing ratio between the two generations suggested that there may be two peaks in neuron birth during the generative period.

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