J. Anat. (1977), **123**, 2, pp. 515–525
With 15 *figures*
Printed in Great Britain

The location of nuclei of different labelling intensities in autoradiographs of the anterior forebrain of postnatal mice injected with [^{3}H]thymidine on the eleventh and twelfth days post-conception

I. H. M. SMART AND M. SMART
Anatomy Department, The University, Dundee, Scotland

(*Accepted* 23 *April* 1976)

INTRODUCTION

The purpose of this study was to establish as precisely as possible the location of neuron nuclei of differing labelling intensities in autoradiographs of coronal sections of the anterior forebrain of postnatal mice which had been injected with [^{3}H]thymidine at 11 and 12 days of post-conceptional age, a time when the ependymal layer of the mouse is commencing the production of cortical neurons. The method used was to count the number of grains overlying neuron nuclei in an autoradiographed section and then to identify and mark each nucleus on a photocollage of the same section enlarged 175 times. The maps produced by this extremely tedious procedure were accurate and revealed interesting patterns of distribution of cells of differing labelling intensities which gave some new insights into forebrain histogenesis.

MATERIALS AND METHODS

The material consisted of the 22 day old offspring of two mother mice which had been injected intraperitoneally with 10 μc/g body weight of [^{3}H]thymidine during pregnancy. One mother was injected on the morning of the eleventh day, and the other on the morning of the twelfth day, post-conception. The mice were allowed to have their litters, and two offspring from each mother were killed 22 days after birth, 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 in turn kept for a further 24 hours in Bouin's fluid before further processing.

Each brain was serially sectioned and all the sections autoradiographed by the dipping technique, using the method for Ilford K_2 emulsion described by Rogers (1967). The slides were exposed for 28 days and the time of development was 10–11 minutes. With this dosage and exposure time, labelled nuclei with 35 or more overlying silver grains were seldom encountered. The majority of 'heavily labelled' nuclei had of the order of 30 overlying silver grains. The slides were post-stained with haematoxylin and eosin.

A section with a minimum number of artefacts was selected from the anterior forebrain at a level anterior to the anterior commissure. This corresponded approximately to the level of Plate 26 in Sidman, Angevine & Pierce's atlas (1971). A series

of overlapping photographs of one cerebral hemisphere was made and prints at a final magnification of 175 times were trimmed and mounted to give a photocollage more than a metre square in which it was easy to identify individual nuclei. The photographed hemisphere was then searched systematically for labelled cells and the silver grains overlying the nucleus, or touching the external perimeter of the nuclear membrane, were counted.

Labelled endothelial and pial cells were not recorded. No attempt was made to differentiate between labelled neurons and neuroglia during the recording process. Inspection of sections for this feature alone, however, revealed no distinct examples of labelled neuroglia. Labelling intensity was classified according to the number of grains per nucleus in the following manner:

Group				Not recorded
I	II	III	IV	
⩾25	20–24	16–19	10–15	<10

It will be seen that Group III spans four integers and Group IV, six; this was a procedural error discovered too late to alter. After counting the overlying grains each nucleus was identified on a 10 × 12 cm print from a duplicate set of unmounted photographs and 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. After recording the positions of all the appropriately labelled nuclei, 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 drawn in. The nuclei belonging to each labelling intensity group were marked on separate sheets. Thus for each section 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 photography. The resulting photographs are reproduced in Figures 2–9. Only one section showing the distribution of nuclei of differing labelling intensities was mapped at each age, as recordings for each map took over 50 hours to carry out. Each mapping session lasted for 15–30 minutes in order to make sure accuracy was not lost because of tiredness of the observer. The comprehensive maps were made by one author (M.S.). A second nearby section was mapped by the other author (I.H.M.S.), only the heavily labelled nuclei being recorded. About 20 sections were also searched without recordings being made, but none showed an obviously different pattern of distribution of heavily labelled cells. We are thus moderately certain that the distributions given in Figures 2–9 correspond with the actual distributions, at least at the level of the brain chosen for examination. However, there are factors which have not been taken into account. For example, the material from each age group was processed at different times, using different batches of thymidine and emulsion. Also, included in the 'weakly labelled' groups were undoubtedly some nuclei which were in fact strongly labelled but were only partly included in the section.

The method described is based on that used by Angevine & Sidman (1961) in their pioneer study of cortical histogenesis.

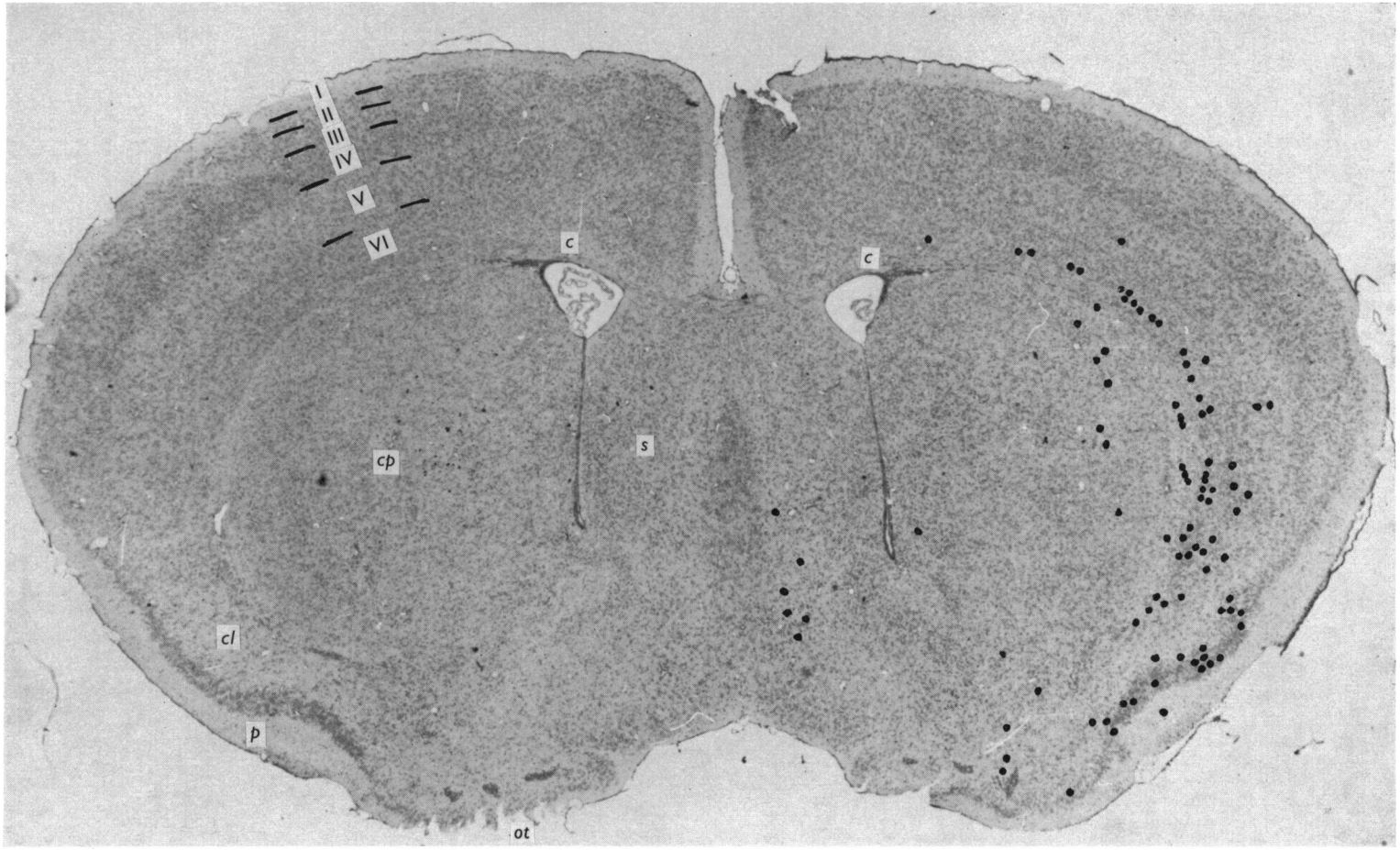
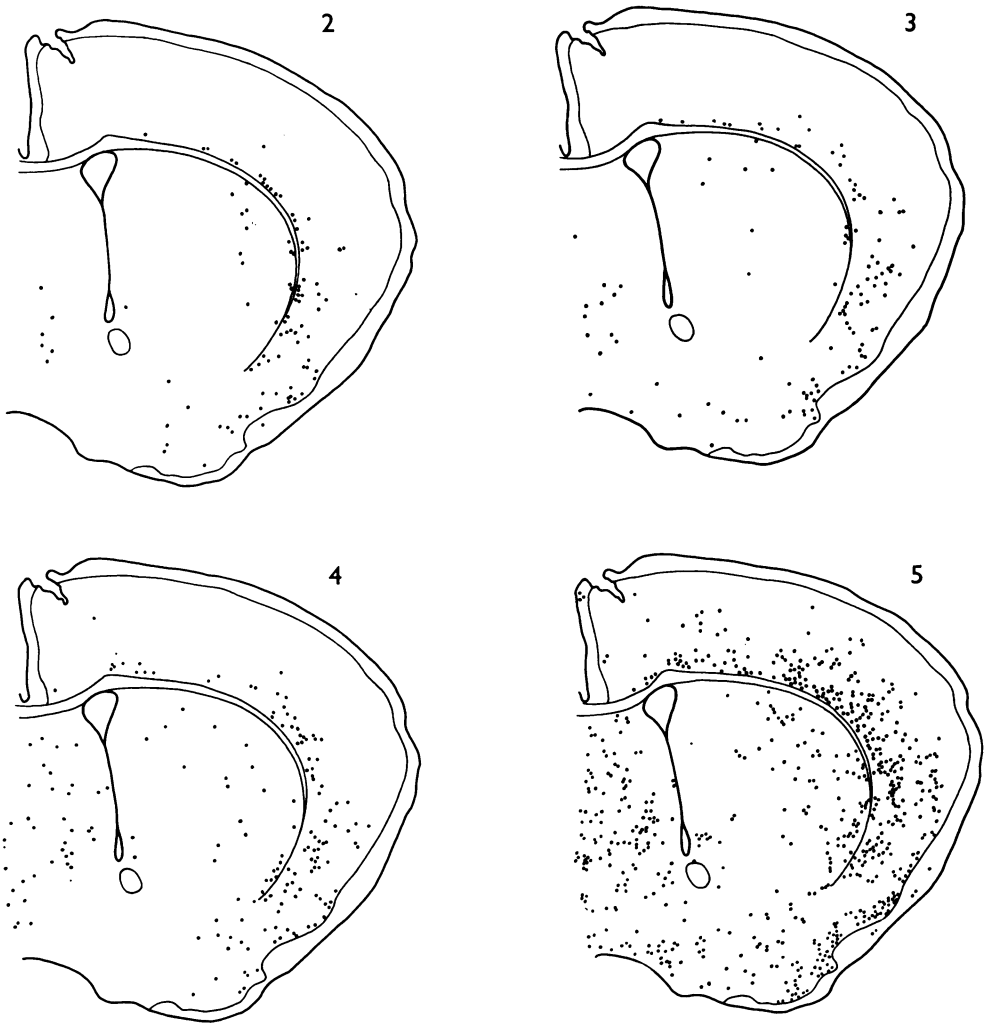


Fig. 1. Photograph of 6 μm haematoxylin and eosin stained coronal section through forebrain of 22 day old mouse injected with [^{3}H]thymidine at 11 days post-conceptional age. The hemisphere on the right-hand side is the one depicted in Figs. 2–5. The positions of heavily labelled nuclei (25 or more silver grains overlying each nucleus) are marked by black dots as in Fig. 2. On the left-hand side of the section the areas of the forebrain referred to in the text are indicated. *c*, corpus callosum (cingulum immediately above marker); *cl*, claustrum; *ot*, olfactory tubercle; *cp*, caudate-putamen nucleus; *p*, pyriform area; *s*, septum.Neocortical layers I–VI are indicated in the dorso-lateral quadrant of the hemisphere. × 35.



Figs. 2–5. Outlines of forebrain of 22 day old mouse injected with [^{3}H]thymidine at 11 days post-conception showing lateral ventricle, anterior limb of anterior commissure, corpus callosum, and boundary between molecular layer and deeper cortical layers. Dots indicate location of labelled nuclei. Fig. 2, location of nuclei with 25 or more overlying silver grains; Fig. 3, location of nuclei with 20–24 overlying silver grains; Fig. 4, location of nuclei with 16–19 overlying silver grains; Fig. 5, location of nuclei wih 10–15 overlying silver grains.

RESULTS

Mouse injected at 11 *days post-conception*

Heavily labelled nuclei at this age were most numerous in the deep parts of cortical layer VI along the external surface of the corpus callosum in its lateral and ventral regions (Figs. 1, 2). Traced dorsally, heavily labelled cells did not extend as far as the cingulum. Traced ventro-laterally such nuclei were found in more superficial parts of cortical layer VI until, at the junctional area between the neo- and pyriform

cortices, they were found in the claustrum and among the superficial cells of the pyriform area (Fig. 1). In the caudate/putamen nucleus, heavily labelled cells were found at the most lateral segment of its perimeter deep to the fibres of the corpus callosum. A few labelled cells were present in the medial part of the septum.

The distribution of each group of more weakly labelled cells in the cortex showed a progressive tendency to spread into more superficial cortical layers and move past the cingulum toward the mid-sagittal plane (Figs. 3–5). In the caudate/putamen nucleus weakly labelled cells increased in number in its lateral half. In the septal area weakly labelled cells tended to become more uniformly distributed.

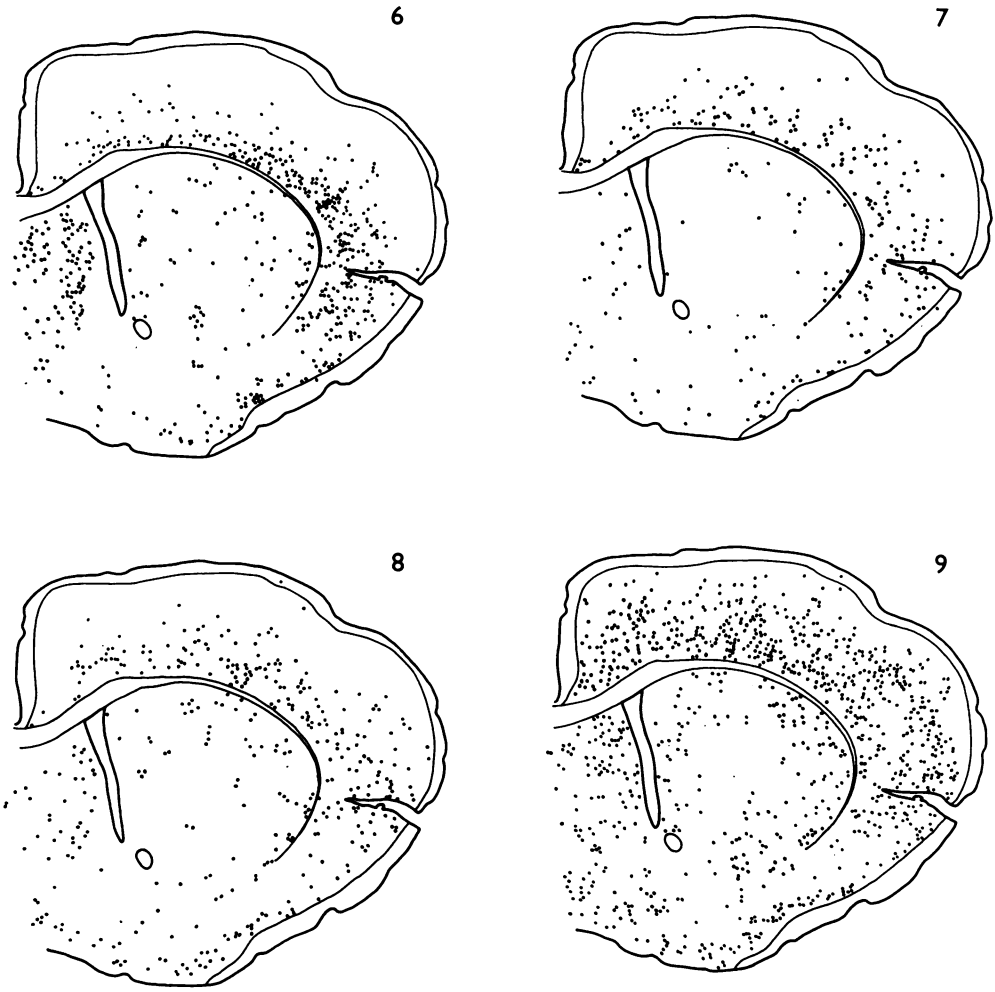
Mouse injected at 12 *days post-conception*

At this age heavily labelled nuclei were more numerous than at 11 days and were found scattered throughout the deeper two thirds of the cortex, their distribution extending past the cingulum as far as the longitudinal fissure (Fig. 6). At this dorsomedial extremity they tended to be restricted to cortical layer VI, but traced laterally they were found spreading into layers V and IV and eventually into the superficial pyriform cortex. In the caudate/putamen nucleus, heavily labelled cells were scattered without apparent pattern. In the septum, heavily labelled cells were numerous and fairly uniformly scattered.

The distribution of each group of more weakly labelled nuclei showed a progressive tendency to shift into more superficial cortical layers and to spread towards the mid-sagittal fissure, repeating the pattern found in the mouse injected at 11 days post-conception. In the caudate/putamen and septal nuclei weakly labelled cells were uniformly distributed.

DISCUSSION

These results, based on very accurate mapping of the positions of nuclei of varying labelling intensities, do not conform in certain important respects to the model of cortical histogenesis proposed by Angevine & Sidman (1961), Berry & Rogers (1965) and Shimada & Langman (1970). The central thesis of these authors is that the neocortex is built up as a result of a series of neuroblast migrations which form the deepest cortical layer first and the most superficial layer last, and that each layer is formed throughout its extent by one contemporaneous migration. Such is certainly the general impression that would be gained if all the labelled nuclei in Figures 2–5 were plotted on one diagram and compared with a similar diagram plotted from Figures 6–9 (comparing Figures 5–9 gives an adequate approximation to such diagrams). If, however, we compare Figures 2 and 5, in which the positions of the heavily labelled nuclei alone are plotted, a different picture emerges. Since the heavily labelled nuclei are likely to belong to cells born at or about the time of injection of [^{3}H]thymidine, their distribution is of particular significance. In Figure 2 the cells presumably born about the 11th day of gestation lie predominantly in the lateral aspect of the neocortex in the deepest part of cortical layer VI. More ventrally they tend to extend into the more superficial part of layer VI and reach layer V. In Figure 6, on the other hand, the cells presumably born on or shortly after the twelfth day, extend more dorsally, in which position they lie throughout layer VI except at the most dorsal 'leading edge' where they are restricted to the deeper part



Figs. 6–9. Outlines of forebrain of 22 day old mouse injected with [^{3}H]thymidine at 12 days post-conception showing the same features as in Figs. 2–5. Fig. 6, location of nuclei with 25 or more overlying silver grains; Fig. 7, location of nuclei with 20–24 overlying silver grains; Fig. 8, location of nuclei with 16–19 overlying silver grains; Fig. 9, location of nuclei with 10–15 overlying silver grains.

of layer VI. Traced laterally they have a more scattered distribution, extending from layer VI into layers V and IV, and in the most ventral extent of the neocortex into layers III and II. These results can be reconciled with the Angevine–Sidman–Berry–Rogers model if account is taken of the fact that the ventricular wall whence the neuroblasts are liberated is increasing in area during the early stages of cortical histogenesis. For example, between 11 and 12 days post-conception the area of the ventricular wall (excluding the part occupied by the ganglionic eminences) increases by about 40 % (Smart, unpublished data). Figures 10–13 attempt to relate such change in the area of the ventricular wall to neuroblast release. Figure 10 represents the successive boundaries of the lateral ventricular wall viewed obliquely from the

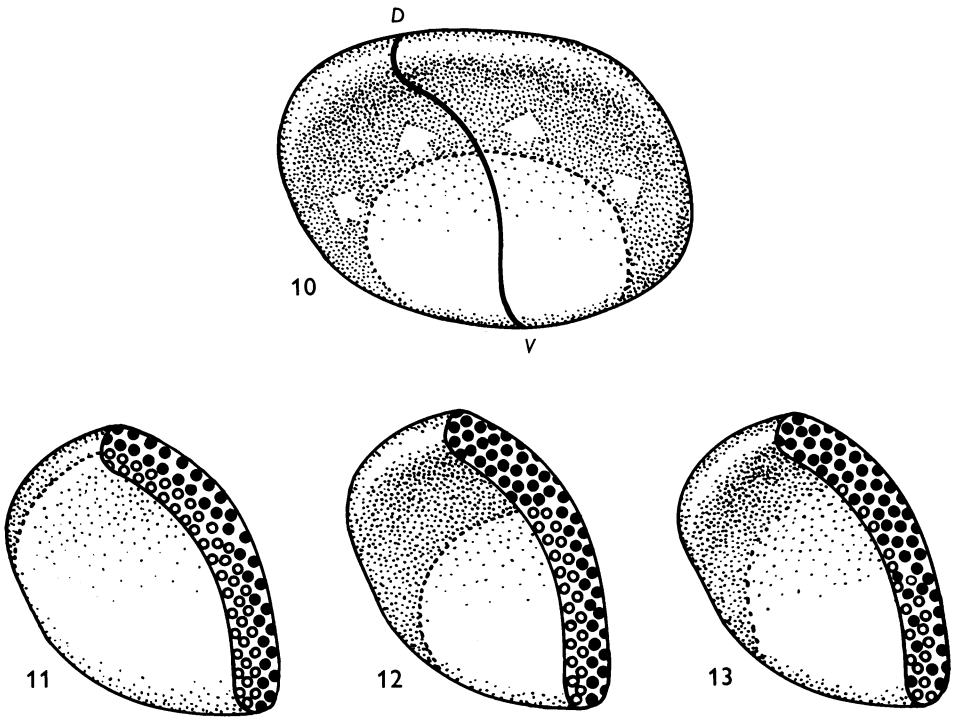


Fig. 10. Oblique view of a diagrammatic simplification of the lateral wall of the lateral ventricle seen from the medial side. The inner ellipse marks the 11 day post-conception boundary, the perimeter marks the 12 day post-conception boundary.

Figs. 11, 12 and 13. Diagrams of coronal sections through Fig. 10 showing three possible patterns of neuron distribution – see text for explanation. Open circles represent neurons born at 11 days post-conception and solid black roundels those born at 12 days post-conception.

medial side at 11 and 12 days post-conception. Figures 11–13 represent cross sections through the ventricular wall at the level of the plane *DV* in Figure 10, and depict three of the theoretically possible patterns of distribution that could be taken up by neuroblasts released on the 11th and 12th days. In Figure 11, the 11 day generation, originally liberated from the ependymal layer over the area of the inner ellipse, spreads with the growing ventricular wall to form a continuous layer of neurons. Through this the 12 day generation has migrated to lie externally. A sequence such as this is portrayed for each cortical layer by Berry & Rogers (1965).

In Figure 12 an alternative sequence is visualized in which the 11 day generation of neuroblasts remains over the segment of the ventricular wall which gave rise to them (the area represented by the inner ellipse in Fig. 10), and none of them follows the subsequent growth of the ventricular wall. The 12 day generation has, as before, migrated through the 11 day crop where this is present, but has remained adjacent to the ependyma where the 11 day generation is absent. Comparing the diagrams in Figures 2 and 6, it is clear that the maps correspond more closely to the model in Figure 12. There is a definite tendency for the earlier (11 day) generation of neurons to remain localized in the lateral and ventral aspects of the cortex (Figs. 1, 2),

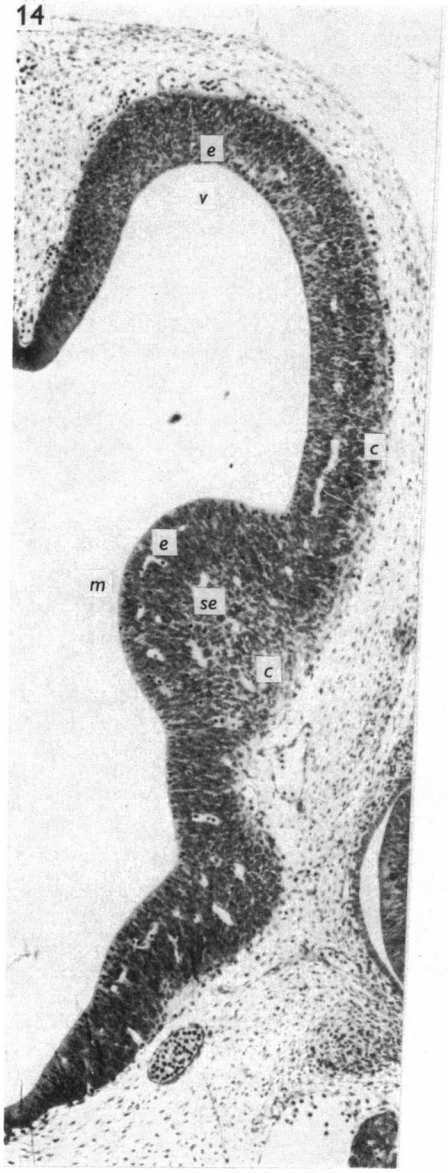
whereas the 12 day generation extends more dorsally as well as more superficially (Fig. 6). There is also a tendency for both the 11 and 12 day generations to migrate to more than one cortical layer, a tendency which increases latero-ventrally until there is considerable overlap between the two populations. Figure 13 depicts a model in which both dorsal extension of the ventricular wall and overlap of cell generations are taken into account.

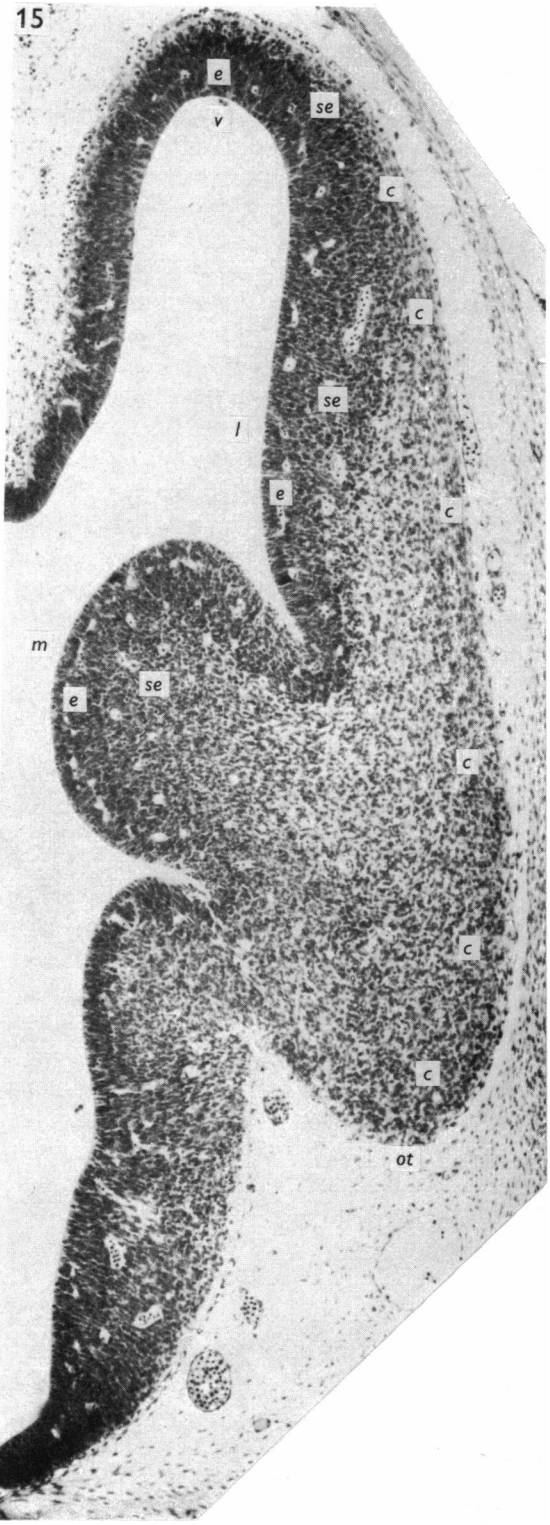
The distribution of more weakly labelled nuclei reveals intermediate patterns consistent with this model. In the neocortex of the (22 day old) mouse labelled at 11 days post-conception, distribution of each group of more weakly labelled cells is seen to spread more dorsally and into more superficial cortical layers (cf. Figs. 2 and 3, 3 and 4, 4 and 5) until the most weakly labelled group (Fig. 5) is distributed in a pattern similar to that of the most strongly labelled nuclei of the mouse injected at 12 days post-conception (Fig. 6). This would be expected, as the majority of weakly labelled nuclei in the mouse injected at 11 days (Fig. 5) belong to cells which have undergone one or more divisions after the time of injection and are therefore equivalent to the cells that would be taking up labelled thymidine from an injection given at 12 days. Thus it seems probable that the distribution of progressively more weakly labelled nuclei in the maps of the 12 day injected mouse (Figs. 7–9) give an idea of the destinations of neurons released during the subsequent day or two. If this is so then the same sequence is repeated; the later formed neurons move progressively into more dorsal and more superficial layers. Thus Figures 2–9 could be read as one continuous sequence showing the destinations of neurons born at successive intervals between the 11th and 13th days post-conception.

The observation that the dorsal neocortex is formed later than the lateral has already been made (Hicks & d'Amato, 1968; Smart, 1973). Also, a statistical study by Bisconte & Marty (1975) in which neurons were separated into generations according to the intensity of their [^{3}H]thymidine label has led them to the conclusion that 'there is a strong interpenetration of the different cell generations in a given layer', and that 'cortical neuronic production is a continuous phenomenon and there is no correspondence with the discontinuity of the cortical layers'. This view would certainly be supported by the present work so far as the inner two thirds of the cortex are concerned.

It is also of interest to examine the internal structure of the cerebral hemisphere at the times of injection of the labelled thymidine. From the location of immature neurons present at 11 and 12 days post-conception some inferences can be made about the origin of the labelled neurons in the postnatal brain. Figures 14 and 15 are coronal sections through the anterior part of the forebrain vesicle of 11 and 12 day post-conceptional mouse embryos respectively. At these times there is no corpus callosum or obvious caudate/putamen nucleus presenting a barrier to migrating cortical cells. The wall of the cerebral vesicle consists of ganglionic eminences and the smoothly curving roof of the lateral ventricle (Figs. 14, 15). The ependymal and

Figs. 14 and 15. Photographs of 6 μm coronal sections stained with haematoxylin and eosin through forebrain vesicle of developing mouse brain. *v*, roof of lateral ventricle; *l*, lateral ganglionic eminence; *m*, medial ganglionic eminence; *e*, ependymal layer; *se*, subependymal layer; *c*, presumptive cortical cells; *ot*, olfactory tubercle. Fig. 14, 11 days post-conception; Fig. 15, 12 days post-conception. Both × 70.


14
e
v
c
e
m
se
c


15
e
se
v
c
c
se
l
e
c
m
e
se
c
c
c
ot

subependymal layers lining the ventricles are overlain at each time by a layer of immature neurons, thickest over the medial eminence and tapering dorsally. Although there is some variation in population density, the immature neurons are not conspicuously separated into cortex and corpus striatum. Since at 11 days post-conception (Fig. 14) the immature neuron population is small, most of the neurons present at 12 days post-conception (Fig. 15) must have been 'born' during the previous 24 hours and their final destinations must correspond to the distribution of heavily labelled cells in Figure 1.

The lobular ventral margin of the cerebral vesicle in the 12 day post-conception mouse (Fig. 15, *ot*) is the future olfactory tubercle. Its cells, in all probability, have been produced by the adjacent medial ganglionic eminence and will eventually be incorporated into the cortex of the olfactory tubercle, pyriform cortex, and perhaps claustrum as well (cf. Figs. 15, 1 and 2). The neuroblasts in Figure 15 lying in the wall of the vesicle at the level of the lateral ganglionic eminence (which appears between the eleventh and twelfth day post-conception (cf. Figs. 14 and 15) are presumably those that come to lie in the lateral aspect of the caudate/putamen nucleus, the deeper layers of the lateral neocortex, and the transitional area where the neocortex merges into the pyriform cortex and claustrum (cf. Figs. 15, 1 and 2). There would seem to be no good reason for assuming that the ganglionic eminences are solely concerned with the production of striatal cells; initially at any rate it would seem possible that they are also contributing to cortical cell populations.

SUMMARY

The location of neuron nuclei of different labelling intensities in autoradiographs of the anterior forebrain of two 22 day old mice which had been injected with [^{3}H]thymidine at 11 and 12 days post-conception respectively was charted on photocollages of sections enlarged 175 times. The pattern of distribution of the heavily labelled nuclei, i.e. those nuclei belonging to cells most likely to have been born shortly after the time of [^{3}H]thymidine injection, indicated that the inner two thirds of the neocortex is laid down along a ventro-dorsal gradient, i.e. the lateral neocortex starts to form before the dorsal; and that cells born at a particular time lie in cortical layer VI at the dorsal edge of the gradient but become progressively more scattered through the cortex as the gradient is traced ventrally. Progressively more weakly labelled cells formed intermediate steps in this migration. A model of cortical growth fitting these findings is presented. Some inferences are also made about the possible role of the ganglionic eminences in providing cortical cells, at least during the initial stages of cortical histogenesis.

We are grateful to Dr S. M. Mann and Mrs Sheila Ramsay for their skilful production of the autoradiographs on which this work is based. Thanks are also due to Miss Joy Nicoll for typing the manuscript. This work was supported by a grant from the Medical Research Council.

REFERENCES

ANGEVINE, J. B. & SIDMAN, R. L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* **192**, 766–768.

BERRY, M. & ROGERS, A. W. (1965). The migration of neuroblasts in the developing cerebral cortex. *Journal of Anatomy* **99**, 691–709.

BISCONTE, J.-C. & MARTY, R. (1975). Étude quantitative du marquage radioautographique finale dans le cerveau de l'animal adulte; loi d'interpretation et concept de chronoarchitectonie corticale. *Experimental Brain Research* **22**, 37–56.

HICKS, S. P. & D'AMATO, C. J. (1968). Cell migrations to the isocortex of the rat. *Anatomical Record* **160**, 619–634.

ROGERS, A. W. (1967). *Techniques of Autoradiography*. Amsterdam: Elsevier.

SHIMADA, M. & LANGMAN, J. (1970). Cell proliferation, migration and differentiation in the cerebral cortex of the golden hamster. *Journal of Comparative Neurology* **139**, 227–244.

SIDMAN, R. L., ANGEVINE, J. B. & PIERCE, E. T. (1971). *Atlas of the Mouse Brain and Spinal Cord*. Cambridge, Mass.: Harvard University Press.

SMART, I. H. M. (1973). Proliferative characteristics of the ependymal layer during the early development of the mouse neocortex: a pilot study based on recording the number, location and plane of cleavage of mitotic figures. *Journal of Anatomy* **116**, 67–91.