J. Anat. (1972), **113**, 1, pp. 109–129
With 12 *figures*
Printed in Great Britain

Proliferative characteristics of the ependymal layer during the early development of the mouse diencephalon, as revealed by recording the number, location, and plane of cleavage of mitotic figures

I. H. M. SMART

Anatomy Department, The University, Dundee, Scotland

(*Accepted* 8 *August* 1972)

INTRODUCTION

In a previous study (Smart, 1972) limiting factors affecting the proliferative capacity of the ependymal layer during early development of the neural tube were discussed. The inquiry stemmed from the supposition that the efficiency of the central nervous system is partly a function of the number of nerve cells available to it and that its evolution will therefore be associated with improvements in cell production. Two major limitations on cell production were distinguished and these were set forth in the form of the following two 'rules'.

(1) *In order to produce the maximum number of nerve cells in a given number of generations differentiation must be delayed.* This stems from the fact that nerve cells themselves do not divide but originate from a population of undifferentiated ependymal cells which form a discrete generative compartment present only for a short time at the beginning of the life span of the animal. Loss of cells from this compartment by differentiation consequently represents a loss of cell-producing power.

(2) *Given the pseudostratified nature of the ependymal layer, proliferation without a corresponding increase in area of the central canal will lead to a disproportion between cell number and canal surface such that cell production will decrease unless the apical migration of mitotic figures characteristic of the layer is discontinued and nuclei are free to go into mitosis away from the central canal surface.*

These rules were found to be heuristically useful as they provided a basis for making the same simple observations in different parts of the neural tube and for interpreting the resulting patterns.

In the initial study in this series (Smart, 1972) the proliferative pattern of the developing spinal cord during the period of neuron production between 10 and 14 days of post-conceptional age was examined by recording the number, location and plane of cleavage of mitotic figures, and the results obtained were presented as illustrating the operation of these rules. This communication describes a similar study carried out on the developing mouse diencephalon. The generation of the larger neuron population of this segment of the neural tube was found to illustrate more clearly the operation of the limiting factors which have been proposed and the adaptations of the epithelium to overcome them.

MATERIALS AND METHODS

The material consisted of mouse embryos taken at 1 day intervals between 10 and 15 days post-conception. They were the same embryos used in the study of the early development of the spinal cord referred to in the introduction (Smart, 1972). These ages span the period of maximum nerve cell production by the thalamic portion of the diencephalon. The embryos were fixed in Carnoy's solution, embedded in paraffin wax, serially sectioned at 6 μm in the transverse plane, and stained with haematoxylin and eosin. All embryos were killed between 10 a.m. and noon. At least two embryos were available for each age group.

The procedures carried out were, with certain modifications described below, the same as were used in the study of cell proliferation in the spinal cord already reported (Smart, 1972). The same microscope, lens system, and ocular micrometer were also used, a division of the latter being calibrated at 43 μm. An undetected error in the previous paper (Smart, 1972) gave this measurement as 63 μm.

The surface and area indices were calculated in the same way as in the spinal cord except that only 5 alternate serial sections instead of 20 were examined in each of two animals at each age group because of the greatly increased time required to make the counts. However, as in the spinal cord, the variations in the counts from section to section were small and the increased accuracy to be obtained by counting more sections would have been slight. Alternate sections were used in order to avoid counting the same mitotic figures twice, i.e. those figures which might be lying half in one section and half in another.

Surface index

The surface index (the number of mitotic figures occurring per unit length of the surface of the third ventricle) at each age period was determined by lining up the scale of an ocular micrometer parallel to the surface of the ventricle and recording the number of mitotic figures lying opposite each division of the micrometer scale. The scale was moved progressively along the ventricular wall and the area covered included the hypothalamic part of the diencephalon. When the counts had been performed on 5 alternate serial sections the results for corresponding divisions in each section were averaged. As the eventual length attained by the wall of the third ventricle was of the order of 30 divisions of the ocular micrometer this made for long tables of figures. To simplify the presentation of the data the results of individual divisions were gathered into groups of 5 and the average for the group was calculated. The final figures in Fig. 2 and Table 2 therefore give the average number of mitotic figures per unit length of the central canal within each 5 division group. The summing procedure was begun ventrally, which meant that the dorsal part of the alar lamina usually did not correspond to a complete 5 division unit. The linear index for such incomplete divisions was calculated proportionately. The 5 division grouping corresponded fairly well with the pattern of the counts and did not obscure the transition from one pattern to another.

Area index

The area index, or average number of mitotic figures per unit area of the ependymal layer, was calculated by repeating the procedure for the linear counts except that this

time the number of non-surface mitotic figures occurring within the ependymal layer adjacent to each division of the micrometer scale was recorded. These counts were made on the same 5 sections as were used for estimating the corresponding linear index. The results of the surface and deep counts for each division were then pooled, and the total divided by 5 to provide an average. A section from the middle of each series was then projected with a projecting microscope on to paper at a standard magnification (one division of the micrometer scale corresponding to 2 cm on the projection paper), and the outlines of the ventricular surface and ependymal layer were drawn in. The surface of the ventricle was marked off in 2 cm divisions corresponding to those of the micrometer, and the ependymal layer was converted into a series of columns based on these divisions. The area of each column was estimated using a planimeter. By dividing the area of each column into the average number of mitotic figures occurring within it an estimate of the number of figures per unit area was obtained. The results for every 5 divisions were pooled and averaged as in the linear counts.

In the 10 day specimen, however, the total length of the diencephalic wall was only 13 micrometer divisions and in this case it was more convenient to sum the counts from the ventral 8 and dorsal 5 divisions as the pattern was fairly uniform within each group. In the later stages it was difficult to define the boundary of the ependymal layer (Fig. 7) and the line drawn round the periphery of the layer passed through the transitional zone marked in Fig. 6.

Distribution of non-surface mitotic figures and orientation of planes of cleavage

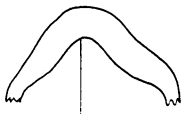
While the counts of non-surface mitotic figures for calculation of the area indices described above were being made, the positions of the figures were estimated and plotted on an outline diagram of the section, as in Figs. 1–6. Prophases and other figures of doubtful orientation were recorded as dots. Metaphases, anaphases, and telophases in which the plane of division could be determined were recorded as dashes, the orientation of the dash corresponding to the estimated orientation of the plane of cleavage. The figures from only 3 sections are depicted in Fig. 1 in order not to overcrowd the diagrams.

A second more critical estimate of the pattern of distribution and orientation of non-surface figures was made by searching 10 sections at each age period under a $\times 100$ oil immersion lens. The location of a figure was recorded by counting the number of ependymal nuclei which intervened between it and the surface of the ventricle: the layer of nuclei at the surface of the ventricle was designated layer 0, the next layer 1 and so on. In addition to the location, the stage of mitosis and, where possible, the orientation of the plane of cleavage were recorded. These counts were restricted to the proliferatively active dorsal (or alar lamina) half of the diencephalon.

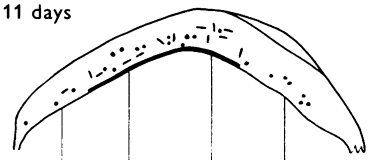
Orientation of surface figures

A check on the orientation of the plane of cleavage of surface figures was made by counting at each age period the orientation of 100 mitotic figures whose orientation could be distinguished. The orientations were put into vertical, oblique and horizontal catagories according to the angle made by the plane of cleavage with the

10 days

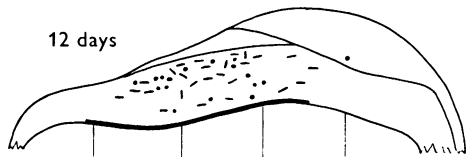


11 days



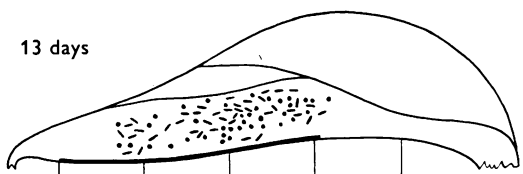
23	73	Total surface figures	37	96	99	79	59
–	–	Total non-surface figures	2	11	22	15	4
0·9	1·8	Surface index	2·1	3·7	4·4	3·2	2·4
0·19	0·30	Area index	0·58	0·59	0·52	0·35	0·34

12 days



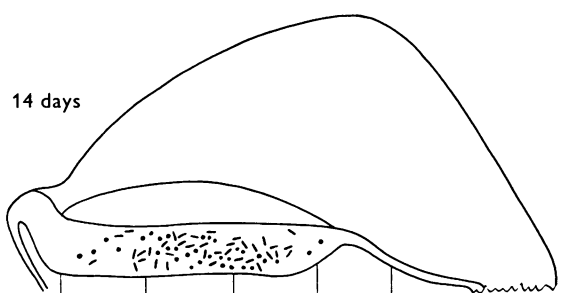
Total surface figures	65	88	89	34	18
Total non-surface figures	–	18	31	4	–
Surface index	3·6	3·5	3·6	1·4	0·7
Area index	0·31	0·37	0·35	0·11	0·06

13 days



Total surface figures	37	69	83	59	55	18
Total non-surface figures	–	22	61	46	4	–
Surface index	3·0	2·7	3·3	2·4	2·2	0·7
Area index	0·43	0·37	0·47	0·36	0·21	0·05

14 days



Total surface figures	59	68	60	27	15
Total non-surface figures	42	67	55	4	–
Surface index	2·4	2·7	2·4	1·1	0·6
Area index	0·32	0·38	0·30	0·13	0·18

surface of the central canal using the criteria established in a previous study of a simpler epithelium (Smart, 1970).

These procedures were carried out in two embryos at each age period. The level of the diencephalon at which the counts was made lay just caudal to the pituitary complex. As the results were similar in each pair of embryos only one set of counts is given for each age period. The counts made at this level form the first series reported in the Results. A second set of counts was made at different levels along the caudo-cephalic axis of the diencephalon in one embryo of 13 days of post-conceptional age. The procedures were carried out on runs of 5 serial sections separated by a gap of 15 sections, i.e. 30 μm of tissue were sampled in every 120 μm. These counts form the second series reported in the Results.

RESULTS

Series 1: studies made on sections taken at a level just caudal to the pituitary complex

10 days post-conception

Histology. There was little variation in the thickness of the epithelium forming the diencephalic walls, although there was some slight tapering towards the dorsal extremity (Fig. 1). The epithelium measured about 90 μm in thickness and accommodated about six layers of closely packed elongated nuclei arranged with their long axes at right angles to the ventricular lumen. No sign of differentiation was present (Fig. 1).

Surface index. At 10 days post-conception the length of the ventricular wall was relatively short, measuring only 12 divisions of the ocular micrometer. The individual surface indices fell into two groups formed by the ventral 7 divisions and the dorsal 5. Within each group the values obtained for the linear index were relatively constant. In this case, an exception was made to the general rule by pooling the total counts from the ventral 7 and deriving the surface index by dividing by 35. The index was low compared to subsequent values. The values for the ventral 7/12 were on average twice that of the dorsal 5/12 (Fig. 1).

Fig. 1. Outlines of transverse hemisections through developing mouse diencephalon between 10 and 14 days of post-conceptional age. The outlines are taken at levels just caudal to the region of the pituitary complex. The surface of the third ventricle lies inferiorly and the dorsal and ventral aspects of the diencephalon lie to the left and right respectively. The boundary of the ependymal layer is shown. The compartment overlying the dorsal part of the ependymal layer from 12 days onward is composed of differentiating cells as in Fig. 6. The thick dark line along the ventricular surface at 11, 12 and 13 days marks the region where 'subsurface' prophase accumulations are found. At these ages prophases in the layer of nuclei immediately beneath the nuclear layer at the surface of the ventricle are too numerous to be depicted individually. The markings within the ependymal layer show the estimated locations of other non-surface mitotic figures. The figures are those which could be identified in the first 3 of the 5 serial sections used in the counts. The dashes are orientated to indicate the plane of cleavage of those figures in which this feature could be distinguished. The dots indicate the positions of other mitotic figures. The lines of figures below each outline refer to the counts made on five serial sections at each age period. The vertical lines relate each group of figures to the segment of the ependymal layer from which they are derived. Each segment corresponds to 5 divisions of the ocular micrometer used in the counts. The most dorsal segment in each case, however, usually comprises less than 5 micrometer divisions.

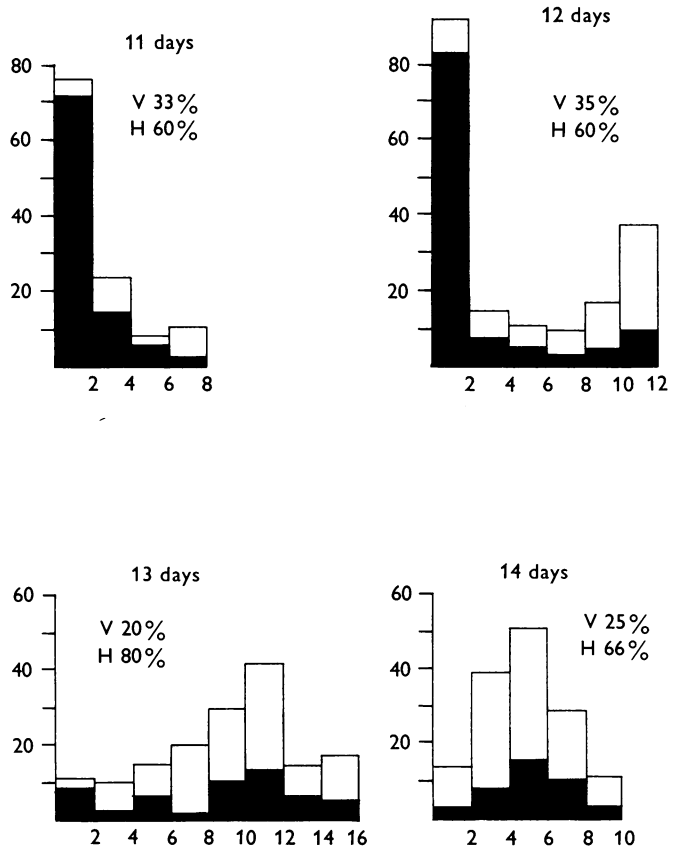


Fig. 2. Histograms depicting the results of counts of the location of non-surface mitotic figures in the dorsal half of the ependymal layer of the diencephalon in mouse embryos of 11 to 14 days post-conceptional age. The abscissa gives the number of nuclei separating the figures from the ventricular surface, and the ordinate the number of figures at each location. The stippled part of each column corresponds to the proportion of figures recorded as being in prophase. The inset percentages in each histogram record the percentage of the figures, in which the cleavage plane could be recognized, which were partitioning vertically (V) and horizontally (H) with respect to the ventricular surface. Note that at 14 days the proportion of prophases among the mitotic figures in the first two subsurface layers has decreased.

Area index. The area index at this stage is derived entirely from surface mitotic figures and, as with the linear index, is higher ventrally than dorsally.

Mitotic distribution. Virtually all mitotic figures were located at the ventricular surface. A few early prophases were found in the subsurface layer, i.e. the layer of nuclei immediately deep to the layer at the surface of the ventricle.

Mitotic orientation. Surface figures were orientated to give a plane of cleavage at right angles to the ventricular surface in over 95 % of cases. *Surface figures at all subsequent age periods showed a similar incidence of vertical cleavage.*

11 days post-conception

Histology. The ependymal layer measured about 110 μm in thickness and accommodated about eight layers of radially arranged nuclei. There was a thickening of the ventral one-third of the diencephalic wall, formed by differentiating cells which were characterized by rounded, less densely packed nuclei, superimposed on an ependymal layer of undiminished thickness (Fig. 1).

Surface index. There was a substantial rise in the value of the surface index throughout the length of the ventricular wall (Fig. 1). The values were greatest in the middle segment of the diencephalic curvature.

Area index. Dorsally the value of the area index increased by a factor of about 3 and ventrally by 2 (Fig. 1). This served to reverse the situation at 10 days and made the dorsal three-fifths of the diencephalic wall the most active mitotically. Although a certain number of non-surface mitotic figures were present the increase in the area index stemmed from an increase in the number of surface figures and the related relatively low degree of pseudo-stratification.

Mitotic distribution. Non-surface mitotic figures were most numerous in the middle and ventral parts of the diencephalic wall where, as counted under the $\times 50$ lens, they constituted about 20 % of the total number of figures (Fig. 1). The majority of these figures occurred in the first and second nuclear layers away from the ventricular surface and in this location over 75 % were prophase figures (Figs. 2, 4).

Orientation of cleavage planes. About 60 % of the non-surface mitotic figures in which the cleavage planes could be distinguished were partitioning in a plane parallel to the ventricular surface.

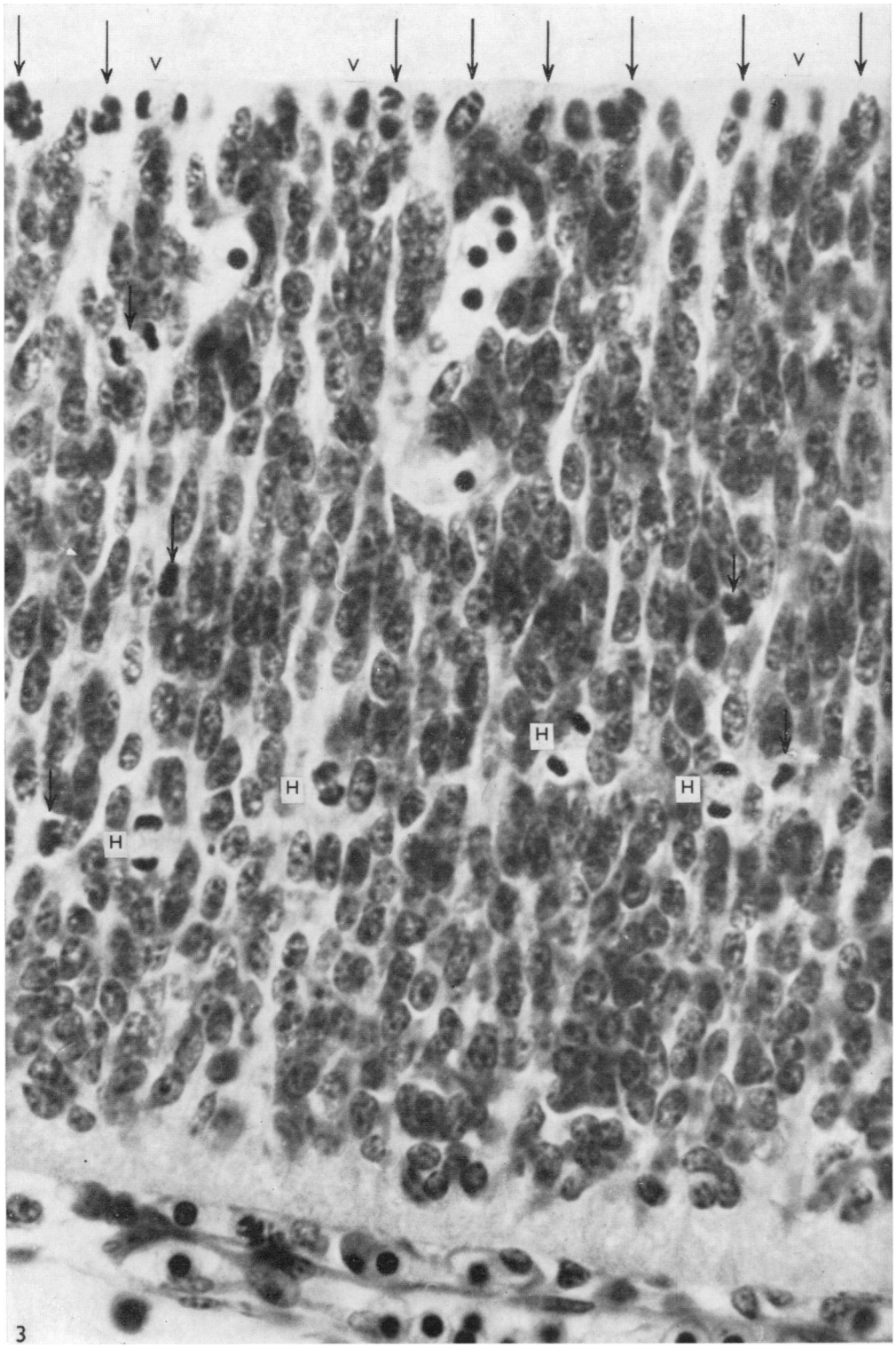
12 days post-conception

Histology. The diencephalic wall showed variations in its structure throughout its cephalo-caudal axis. At the level at which the counts were made the ependymal layer remained about the same thickness (110 μm) as at 11 days in the ventral third, but dorsal to this it increased to about 150 μm in thickness and 12 nuclei deep (Fig. 3) tapering slightly again towards the dorsal extremity. The area of differentiating cells overlying the ependymal layer in the ventral third had increased in thickness and extent (Fig. 1).

Surface index. The value of the surface index in the ventral two-fifths of the diencephalon underlying the area of incipient differentiation showed a marked decrease. Dorsally the value remained uniformly high at 3·5 to 3·6.

Area index. There was a general decrease, most marked ventrally, from the high values obtained for the area index at 11 days. The decrease was due principally to an increase in the depth of the ependymal layer. There was also a slight decrease in the number of surface mitotic figures but this was more than offset by a rise in the number of non-surface figures.

Mitotic distribution. Non-surface figures were most numerous at this age in the dorsal half of the diencephalon, where they constituted an average of from 20 to 40 % of the total number of mitotic figures (Fig. 1) and in some localized areas even more (Fig. 3). Figures were most numerous in the nuclear layers near the surface of the ventricle (Fig. 2). There was also a rise in the number of figures at the periphery of the ependymal layer; these were separated from the ventricular surface by some


V
V
V
H
H
H
H
3

8–12 intervening nuclei. About 80 % of the figures near the ventricular surface were in prophase and of the peripheral figures only 20 % were in prophase (Fig. 2).

Mitotic orientation. Some 60 % of the non-surface mitotic figures in which the planes of cleavage could be identified were cleaving in a plane parallel to the ventricular surface.

13 days post-conception

Histology. At the level at which the counts illustrated in Fig. 1 were made the ependymal layer in its ventral half had decreased in thickness, but in the dorsal half had reached its maximum thickness of 200 μm or about 16 nuclear layers. The outer 50 μm of nuclei showed less dense packing of its radially arranged nuclei (Fig. 6). This transitional layer gradually blended with a 50 μm thick zone of rounder nuclei, which became progressively more loosely packed towards the periphery (Fig. 6). This area of incipient differentiation extended ventrally to blend with the more massive area of longer established differentiation of the hypothalamic region, and dorsally to decrease progressively in depth and finally taper into an unmodified ependymal layer.

Surface index. The value of the surface index in the ventral half decreased progressively towards the floor plate. Dorsally the value remained close to an average of 3·0, which represented a slight decline from 12 days post-conception.

Area index. The values for the area index were generally slightly higher than those obtained at 12 days. This was due to an increase in the number of non-surface mitotic figures.

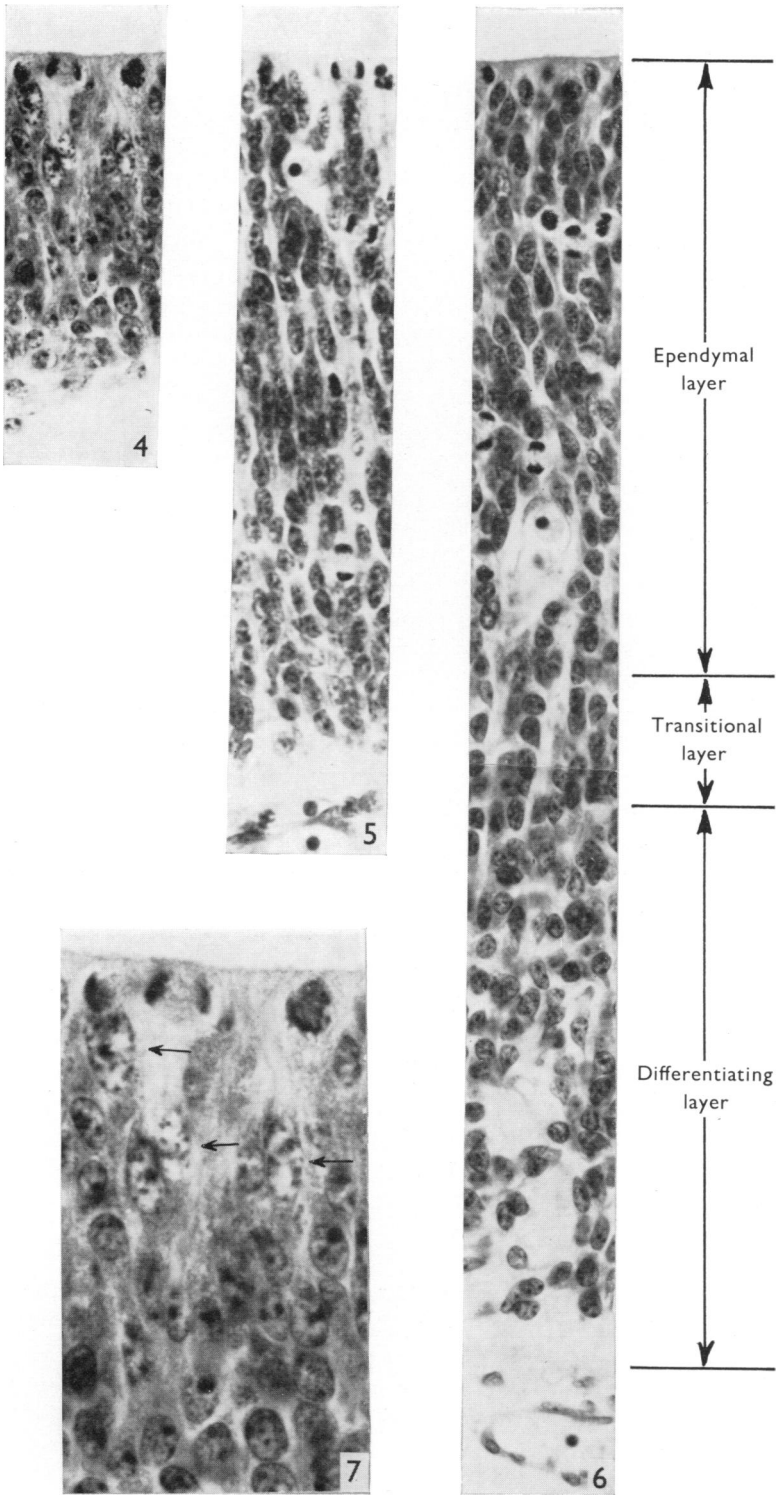
Mitotic distribution. In the middle regions of the diencephalic wall the number of non-surface mitotic figures had increased to about 40 % of the total. This was associated with a slight decline in the number of surface figures. The majority of the non-surface figures at this stage lay towards the periphery of the ependymal layer (Fig. 2). Prophases constituted 80 % of the sub-surface mitotic figures and only 20–30 % of those in the periphery.

Mitotic orientation. About 80 % of the non-surface mitotic figures, the orientation of which could be determined, were cleaving in a plane parallel to the ventricular surface.

14 days post-conception

Histology. The ventral third of the ependymal layer had been reduced to a couple of layers of widely spaced nuclei. Dorsally the perimeter was more difficult to define, but the layer appeared to have diminished in thickness to about 10–12 nuclei in depth, about the same as at 12 days. At its periphery the layer blended with 50 μm or so of rounder, more loosely packed, nuclei which in turn merged with a more superficial layer of more mature-looking neuron nuclei.

Fig. 3. Photomicrograph of 6 μm thick transverse section through dorsal part of thalamus of 12-day post-conception mouse embryo, stained with haematoxylin and eosin. The apical surface of the neural epithelium bordering the lumen of the third ventricle lies at the upper part of the picture and the basal surface at the lower. Note the vertical planes of cleavage of the mitotic figures (V) at the ventricular lumen and the predominance of horizontal cleavage planes in mitotic figures (H) located at the outer surface of the layer. Other mitotic figures are marked by arrows (× 800).


4
5
6
7
Ependymal layer
Transitional layer
Differentiating layer

Surface index. There was a further general decline in the value of the surface index, which followed the pattern described at 13 days post-conception.

Area index. The area index in the dorsal divisions remained high, chiefly due to an increase in the number of non-surface figures which were, at this stage, almost as frequent as the surface figures. Ventrally the area index was low where the ependymal layer had declined.

Mitotic distribution. In certain regions the counts of non-surface figures were slightly more numerous than those at the surface. The majority of non-surface figures were found in the middle zone of a somewhat diminished ependymal layer. Prophases constituted 20–30 % of the mitotic figures at all levels (Fig. 2).

Mitotic orientation. About 60 % of non-surface figures whose planes of cleavage could be determined were partitioning parallel to the ventricular surface.

15 days post-conception

The ependymal layer had further decreased in thickness and in one specimen fusion of the ventricular walls to form the interthalamic adhesion had occurred. A few mitotic figures were distinguished along the line of fusion. The linear indices along the separated ventral and dorsal parts of the lumen of the third ventricle were below 0·5 and were associated with decreased depth of the ependymal layer.

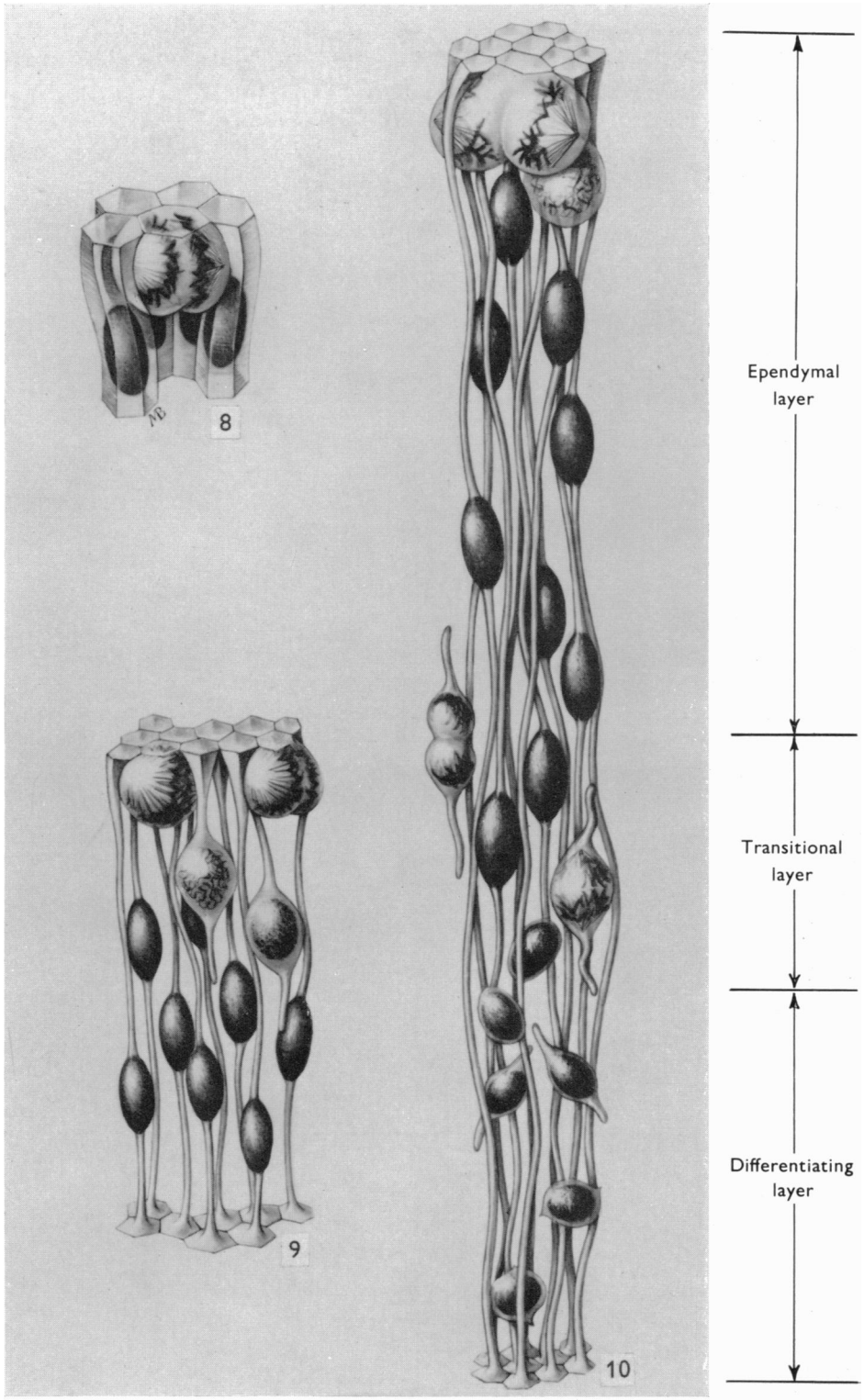
Series 2: studies on sections taken at different levels of a 13 day post-conception embryo

Histology. At 13 days the variations in the histological picture along the cephalo-caudal axis were well seen. At the mesencephalic junction the ependymal layer related to the thalamic portion of the diencephalon was made up of 20 layers of closely packed nuclei with little overlying differentiation. Traced cephalad the layer became progressively thinner and the overlying differentiation greater, until at the region of the interventricular foramen the ependymal layer was 4–6 nuclei deep and underlay a region of rounder, more dispersed, differentiating nuclei twice as deep. In contrast, the ventral or hypothalamic portion of the diencephalon showed a reversal of this pattern, as the ependymal layer associated with the pituitary stalk and anterior hypothalamus was thicker, and exhibited less overlying differentiation than in the dorsal hypothalamus.

Surface index. In Tables 1 and 2 the total number of mitotic figures per unit length of third ventricular surface and the corresponding surface indices are given. The figures portray a decline in the incidence of surface mitosis in a dorso-ventral direction at all levels of the diencephalon, the exceptions being the extreme dorsal lip of the alar lamina, where figures were invariably less frequent than the adjacent

---

Figs. 4–7. Photomicrographs of 6 μm sections through the wall of the thalamus during different stages of its early development. The sections are stained with haematoxylin and eosin and orientated with the apical or ventricular poles of the ependymal cells uppermost. Figs. 4–6 are taken through the thalamic wall at 11, 12 and 13 days of post-conceptional age respectively ($\times$ 600), and thus illustrate the increase in thickness of the ependymal layer. Fig. 7 is a portion of Fig. 4 enlarged to $\times$ 1200 in order to show three early prophases (marked by arrows) occurring in the subsurface layers of an area of the ependymal layer, which is only about 8 cells deep.


8
9
10
Ependymal layer
Transitional layer
Differentiating layer

maximum value, and the ventro-cephalic area where mitotic activity around the pituitary stalk disturbed the pattern. Traced in a caudo-cephalic axis the surface index showed a slight decline in its average value; this decline became more noticeable in the most cephalic levels.

Area index. In Tables 3 and 4 are shown both the total number of mitotic figures occurring within the ependymal layer away from the ventricular surface, and the corresponding area indices. The number of non-surface figures was high caudally and declined towards the cephalic end of the series. In the dorso-ventral axis they were found to be most numerous at level 3, about the middle of the ventricular wall. The patterns of the surface and area indices were therefore similar. The effect of including the non-surface figures was to bring out more clearly the decline in overall mitotic activity along the caudo-cephalic axis.

Mitotic distribution and orientation. Non-surface mitotic figures, as previously noticed, were most numerous along the periphery of the ependymal layer, the majority (about 80 %) cleaving in a plane horizontal to the surface of the third ventricle.

DISCUSSION

The results demonstrate a dorso-ventral gradient in mitotic activity in the diencephalon (Fig. 1) similar to that found in the spinal cord (Smart, 1972). The delayed differentiation in the dorsal or thalamic part of the diencephalon is consistent with the generation of the larger cell populations of the thalamus, as compared to the hypothalamus. The study of a sequence of sections along the long axis of the neural tube, as in the 13 day specimen (Tables 1–4) reveals a caudo-cephalic gradient in which the anterior part of the thalamus commences to differentiate before its caudal regions, which eventually form the most massive and populous of the thalamic nuclei. Both these gradients are consistent with operation of the first rule stated in the Introduction. The continuing accumulation of ependymal precursor cells associated with delayed differentiation, particularly in the dorso-caudal thalamic regions, leads to increasing pseudostratification and this in turn is associated with the appearance of non-ventricular mitotic figures (Fig. 1 and Table 3), as would be expected under the second rule.

The gradient can be divided into stages similar to those suggested for ependymal proliferation in the spinal segment of the neural tube (Fig. 11, and Smart, 1972). In Stage 1 proliferation occurs without differentiation. In Stage 2 differentiation

---

Figs. 8–10. Diagrammatic reconstructions of epithelium of thalamic wall at different stages of development. All figures are drawn about the same scale. For reasons of clear illustration Figs. 9 and 10 are drawn with much looser packing of the component cells than is actually the case, as can be seen by comparing Figs. 6 and 10. Fig. 8 shows a simple columnar epithelium with one cell in mitosis. This cell shows the characteristic migration of the nucleus to the apical pole and the rounding up of the cytoplasm. Fig. 9 depicts an 11-day epithelium (cf. Fig. 4) with elongated columnar cells and apical mitotic figures. In addition, two prophase nuclei are shown awaiting a vacancy at the ventricular surface (cf. Fig. 7). Fig. 10 depicts an epithelium of 12–13 days and corresponds approximately with Figs. 5 and 6. Surface and subsurface figures as in Fig. 9 are shown at the apices of extremely elongated columnar cells. In addition a pair of mitotic figures cleaving horizontally, as in Fig. 3, are shown at the periphery of the ependymal layer. The boundary of the cytoplasm of these cells during mitosis is not known, and therefore the peripheries of the cells shown here are speculative.

Tables 1–4. *Results of counts of surface and non-surface mitotic figures in the ependymal layer of a mouse embryo of 13 days post-conceptional age.*

(In each table the vertical columns A-F give the counts obtained from sets of 5 consecutive serial sections taken at different cephalo-caudal levels. Column A, for example, comes from cross-sections just anterior to the mesencephalic flexure, and column F from cross-sections taken at the posterior margin of the interventricular foramen. The intrusion of this feature is indicated by the letters *fm* in column F line 1. The columns are separated by 15 sections 6 μm in thickness, and therefore represent samplings of the diencephalic wall taken every 15 × 6 μm. The horizontal lines 1–6 denote corresponding dorso-ventral levels in each set of sections. Line 1 is at the tip of the alar lamina and line 6 at the ventral extremity of the third ventricular wall. Each line corresponds to 5 divisions of the ocular micrometer used in the counts. In line 1, however, fewer than 5 divisions are represented as the length of the ventricular wall did not correspond to exact multiples of 5. The figures in heavy type denote regions in Stage 1 of the proliferative sequence, those in italic type regions in Stage 2, and those in small dark type regions in Stage 3.

Table 1. *Total number of mitotic figures occurring at the surface of the third ventricle in 5 consecutive serial sections*

	A	B	C	D	E	F
1	**64**	**37**	**33**	**69**	22	*fm*
2	**120**	*69*	*83*	*86*	35	11
3	*85*	*83*	*78*	*68*	*85*	31
4	*63*	*59*	*64*	*63*	71	33
5	26	55	26	49	24	28
6	6	18	7	21	**56**	**65**

Table 2. *Variations in surface index in different parts of the wall of the third ventricle computed from data in Table 1*

	A	B	C	D	E	F
1	**3·2**	**3·0**	**2·2**	**2·8**	0·9	*fm*
2	**4·8**	*2·7*	*3·3*	*3·4*	1·4	0·7
3	*3·4*	*3·3*	*3·1*	*2·7*	*3·4*	1·2
4	*2·1*	*2·4*	*2·5*	*2·5*	2·8	1·3
5	1·1	2·2	1·0	1·8	2·0	1·1
6	0·2	0·7	0·3	0·6	**2·2**	**2·6**

Table 3. *Total number of non-surface mitotic figures occurring within the ependymal layer in 5 consecutive serial sections*

	A	B	C	D	E	F
1	**3**	—	—	**1**	—	*fm*
2	**43**	*22*	*27*	*45*	—	—
3	*82*	*61*	*35*	*15*	*5*	—
4	*63*	*46*	*37*	*3*	11	—
5	1	4	—	—	1	—
6	—	—	—	—	—	—

Table 4. *Variations in area index in different parts of the ependymal layer of the third ventricle computed from data in Tables 1 and 3*

	A	B	C	D	E	F
1	**0·54**	**0·43**	**0·37**	**0·45**	0·13	*fm*
2	**0·79**	*0·37*	*0·47*	*0·54*	0·16	0·28
3	*0·59*	*0·47*	*0·40*	*0·32*	*0·38*	0·22
4	*0·39*	*0·36*	*0·36*	*0·23*	0·30	0·17
5	0·09	0·21	0·10	0·17	0·17	0·25
6	0·02	0·05	0·03	0·07	**0·16**	**0·33**

commences but is balanced by proliferation and the precursor pool consequently remains undiminished. In Stage 3 the rate of differentiation exceeds the rate of proliferation and the precursor pool shrinks. Provided the duration of the generative cycle and the mitotic index remain constant, cell productivity is greatest in Stage 1 and least in Stage 3, and is consequently increased by delayed progression through the sequence. Stage 1 therefore persists longest in the dorso-caudal thalamus (Tables 1–4) and as the dorsal extremity of the compartment migrates further dorsally with continuing proliferation, its ventral extremity commences to produce differentiating cells and passes into Stage 2. The boundary between Stages 1 and 2 therefore follows the dorso-caudal extension of the dorsal lip (Fig. 12). The ventral extremity of the Stage 2 compartment, on the other hand, remains relatively stationary after 11 days, and probably marks the boundary between the definitive thalamic and hypothalamic parts of the diencephalon. The variation in the types allotted to the figures in Tables 1–4 gives an idea of the two-dimensional shape of the compartments and demonstrates that the dorso-ventral and cephalo-caudal gradients are in fact sections through different planes of one gradient.

In the Stage 1 compartment mitotic figures lay either at the ventricular surface or in the immediately adjacent or subsurface layers, and were rare in the more superficial layers (Figs. 2, 4 and 7). The majority of these subsurface figures were prophases. They were first found at 11 days when the epithelium of the Stage 1 compartment was only 8 nuclei or so in depth (Fig. 7). At this time the surface index increased from the fairly low values of 0·9–1·8 found at 10 days (Fig. 1) to maximum values of 3·7 and 4·4 (Fig. 1). Although the maximum number of mitotic figures ever observed in one division of the ocular micrometer was 7, this represented extreme crowding and superimposition of nuclei within the 43 μm dimension of the scale, and a value for the surface index of 4·4 represents the highest *average* value obtained in this study. Taken with the relatively low degree of pseudostratification it also gives the highest area index (0·59) recorded in the series. The area index is a crude indication of the mitotic index, i.e. number of mitotic figures per hundred nuclei. The mitotic index itself unfortunately cannot be calculated because extreme nuclear crowding in the ependymal layer makes it impracticable to distinguish the boundaries of so many superimposed nuclei, and they therefore cannot be counted. Although a high area index indicates that a high proportion of the cell nuclei are in the visible stages of mitosis, it does not necessarily mean a high rate of cell production, as this is also dependent on the duration of the generative cycle, particularly, in this situation, on the duration of the mitotic figures at the ventricular surface. As discussed elsewhere (Smart, 1972), the effect of the retention of nuclear migration to the cell apex by the mitotic cells of the neural epithelium is to create a disproportion between the available ventricular surface and the number of mitotic nuclei striving to gain access to it. Consequently the appearance of subsurface prophases may indicate either an increase in the incidence of mitosis or an increase in the duration of the mitotic figure, the former increasing the number of nuclei competing for room at the surface, and the latter increasing the time during which a mitotic figure blocks off ventricular space to other incipiently-mitotic nuclei. Although there is evidence that the entire generative cycle of ependymal cells, including the mitotic duration, increases during development (Jelínek, 1959; Källén, 1961; Fujita, 1963), it is likely

that at 11 days the generative cycle is still relatively short and that the appearance of subsurface prophases in an area of low pseudostratification does reflect a true increase in the mitotic rate.

After 11 days, the Stage 1 compartment of the alar lamina increased in degree of pseudostratification in its ventral part (Fig. 1), and although the surface index remained unchanged and subsurface prophases persisted, the area index consequently decreased slightly. As there was unlikely to have been a decrease in the duration of mitosis or of the generative cycle, this can be taken to indicate a decrease in the rate of cell production relative to the 11 day period. Also from 11 days onward the Stage 1 compartment progressively decreased its dorso-ventral extent and virtually disappeared by 14 days.

The Stage 2 compartment first appeared in the ventral or hypothalamic portion of the diencephalic wall at 11 days (Fig. 1). One day later in this area it had begun to change to Stage 3, and by 14 days the ependymal layer in the ventral third of the diencephalon had virtually disappeared as a site of proliferative activity. This marks a fairly rapid progression through the sequence, consistent with the relatively small neuron population of the hypothalamus. An exception was the area around the pituitary stalk, where slight mitotic activity continued until 14 days (Tables 1–4). This region is in fact worthy of more detailed study, as it has an interesting subpattern of its own, based on non-congestive proliferation and surface figures. To follow this up at the moment would, however, be a diversion from the main investigation.

In the dorsal two-thirds or thalamic part of the diencephalon the Stage 2 compartment appeared at 12 days, increased in size by 13 days, started to decline into Stage 3 at 14 days, and had virtually ended proliferative activity by 15 days (Fig. 12). Subsurface prophases were present throughout the compartment at 11, 12 and 13 days (Figs. 1, 2), indicating that the number of mitotic figures migrating to the cell apices was exceeding the available ventricular surface. Also during this interval increasing numbers of mitotic figures were observed scattered throughout the layer, in some areas exceeding the number at the adjacent ventricular surface. These figures served to maintain the value of the area index in the face of increasing pseudostratification (Fig. 1, Tables 3 and 4).

Compared with the previous findings in the developing spinal cord (Smart, 1972), the Stage 1 and 2 compartments in the thalamus are larger, particularly the latter (Fig. 11). The values of the surface and area indices in the thalamus in some areas also exceed anything found in the spinal cord, and in general between 11 and 14 days maintain the highest levels reached by the spinal cord during its much shorter period of maximum proliferative activity. Congestive phenomena characterized by subsurface prophases and other non-surface mitotic figures are also more conspicuous in the thalamus.

In the spinal cord the posterior horns, which formed the most populous part of the system, stemmed from the rapid proliferation of ependymal cells to form a crowded highly-pseudostratified Stage 1 compartment, followed by a very rapid differentiation, i.e. short progressions through Stages 2 and 3. The ependymal layer of the thalamic diencephalon, on the other hand, depends for the production of its neuron pool on the generation over a longer period of a large Stage 1 compartment

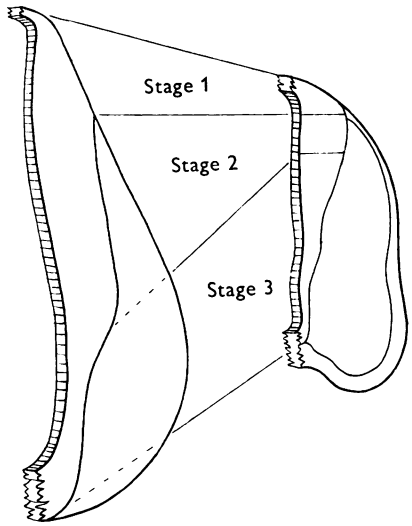


Fig. 11. Outline drawing of sections of developing spinal cord and thalamus of about 13 days of post-conceptional age to show relative sizes of the three proliferative compartments defined in the text. The former is taken from the region of the cervical enlargement as in Smart (1972) and the latter from Series 1 of the present investigation. The magnification of both drawings is the same.

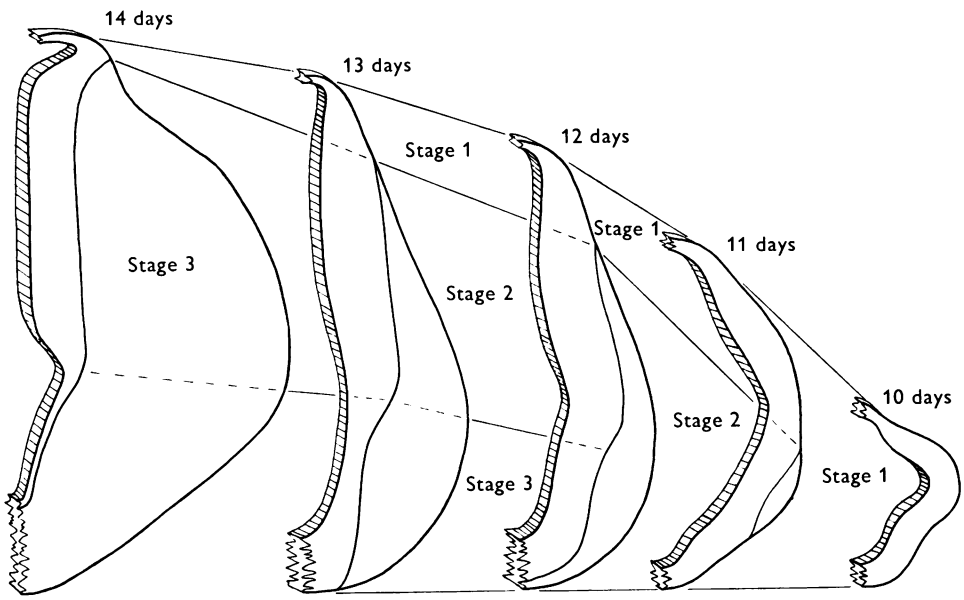


Fig. 12. Outline drawings of thalamus taken from Fig. 1 arranged to show the change in size of the proliferative compartments at different ages. The comparison is only approximate, as it is not possible to control exactly the cephalo-caudal level of section or the plane of section relative to the cephalo-caudal axis.

followed by a long period in Stage 2 (Fig. 12), and on the evolution of a large non-migrating population of mitotic cells which counteracts the choking effect of pseudostratification.

The distribution of mitotic figures within the ependymal layer at the various stages of development has some features of interest which indicate changes in the histological organization of the layer. As already noted, in the Stage 1 and 2 compartments subsurface prophase accumulations were apparent between 11 and 13 days (Figs. 1, 2 and 7). In the Stage 2 compartment, however, the majority of non-surface figures were located in the more peripheral parts of the ependymal layer (Figs. 1–3, 5 and 6). These figures were found in all stages of mitosis (Fig. 2), and where cleavage planes could be distinguished, up to 80 % were orientated to give horizontal partitioning. Histologically the lateral boundary of the Stage 2 ependymal compartment showed an ill-defined transitional zone characterized by looser packing of nuclei and a tendency for the long axes of the nuclei to have a less uniform arrangement than the underlying radially arranged nuclei of the unmodified layer (Figs. 6, 10). This arrangement gave way to an outer zone of rounder, more widely spaced nuclei of differentiating cells.

It can therefore be inferred that in the Stage 2 compartment two populations of mitotically active ependymal cell are present, one continuing the migratory cycle and reproducing the congestive phenomena inherent in this procedure, and another which has abandoned nuclear migration at the time of mitosis and shows a tendency for its mitotic figures to collect at the interface between the true ependymal layer and a more loosely organized transitional zone. The architecture of this transitional zone can only be guessed at, but presumably the more random orientation of nuclei reflects loss of contact with the surfaces of the epithelium by the cells. The predominantly horizontal cleavage planes in this area at 12 and 13 days (Figs. 5, 6) are the reverse of the situation at the surface. The factors which maintain this 90° difference in cleavage plane between the figures at opposite surfaces of the epithelium are unknown.

At 14 days there appeared to be a further change in the character of the layer. It was slightly thinner, there was a decrease in the number of surface figures (Fig. 1), and subsurface prophases were more or less absent. The non-surface mitotic figures on the other hand continued to maintain their numbers and tended to congregate towards the middle of the layer (Figs. 1, 2).

It would be interesting to know if the duration of the generative cycle in the migrating and non-migrating mitotic cells was the same. It is possible that the increase in length of the cycle during development described by Jelínek (1959), Källén (1961), and Fujita (1962) applies only to the apically migrating population, and the non-migrating cells, once released from the ventricular excursion, may be able to proliferate more rapidly. However, a decrease in the percentage of mitotic figures in a cell population need not necessarily mean a decrease in cell production. For example, although the surface mitotic figures were declining at 14 days, if this were accompanied by a decrease in the duration of the generative cycle it could, paradoxically, be associated with an increase in cell production. More data on the length of the generative cycle is required before these relative rates of production can be established. Nevertheless it would appear likely that at 14 days we are witnessing

a decline in the productivity of the apically attached, surface-migrating nuclei, and the transfer of the major source of cell production to the middle regions of the ependymal layer.

Finally, it is of some interest to inquire into the nature of the mechanism controlling these somewhat complex events in the ependymal layer. How is the size and duration of each compartment controlled and varied in different parts of the central nervous system? The three compartments into which the ependymal layer can be divided on the basis of its proliferative characteristics provide a model somewhat resembling Wolpert's 'French Flag'. Wolpert (1968) used the three compartment construction of the French tricolour as the paradigm for pattern formation, in the sense that it is the simplest pattern for which a solution, if found, can be expanded to give a more general explanation of pattern control. Basically the mechanisms suggested for generation and regulation of pattern depend on the balanced production and destruction of postulated substances which are capable of maintaining cells in a particular state. Applied to the ependymal tricolour the pattern could be explained by either one of the following mechanisms: (1) A diffusable substance is present which forms a concentration gradient through the tissue which the cells use to 'compute' their position in the system. The cells in the system respond to specific ranges of concentration of the substance by a specific change of state; for example there is a range of concentration which induces and maintains Stage 1, another controlling Stage 2, and so on. Or (2) the cells of Stage 1 produce a substance which, when it reaches a sufficient concentration, initiates a change into Stage 2. Stage 2 cells in turn produce a substance which in sufficient concentration initiates Stage 3. In each case the size and duration of the compartment is regulated by a balance between the rate of production and removal of the operative substance. The substances concerned may act through the medium of surface exoenzymes as postulated in the model proposed by Heinmets (1968), or through morphologically distinguishable low resistance junctions which are equivalent to the tight junctions of electron microscopists (Furshpan & Potter, 1968*a*). The latter structures are common in embryonic tissues, and Furshpan & Potter (1968*a*) present a great deal of circumstantial evidence suggesting that they may serve as pathways for the short range control of intercellular activity. According to a statement in their review (Furshpan & Potter, 1968*b*), neural tissue is an interesting example of a tissue in which the cells are coupled by low resistance junctions during the neural tube stage of development but largely uncoupled once development is over.

The formulation of the 'French Flag Problem' has resulted in considerable discussion about the basic mechanism of pattern formation, and Wolpert (1969) and Goodwin & Cohen (1969) have since attempted to formulate a generalized mechanism, the features of which should be common to all pattern-forming systems. These discussions of the fundamentals of pattern formation are difficult to follow but seem to command the respect of other theoreticians. Meanwhile, the fact that the present heuristically initiated investigation has tripped over someone else's paradigm is of sufficient interest for the investigator to pause a while in wonder. Such encounters need not be productive but they are rare enough to be worth further probing.

SUMMARY

Cell production by the ependymal layer of the diencephalon of mouse embryos of 10 to 15 days of post-conceptional age was studied. The number of mitotic figures per unit length of the central canal surface and per unit area of the ependymal layer was recorded. A particular search was made in the ependymal layer for mitotic figures which were located away from the ventricular surface. The location, stage of mitosis and, where discernible, the plane of cleavage of these figures were recorded. The most interesting results were obtained from the dorsal or thalamic portion of the diencephalon. In this area at 10 days there was no evidence of differentiation and only moderate mitotic activity. At 11 days mitotic activity increased, as indicated by tripling of the value for the surface and area indices. Although at this stage the epithelium was only about 8 nuclei in depth, prophase figures appeared in the subsurface nuclear layers, a finding which was interpreted as indicating a high mitotic rate. At 12 and 13 days the layer increased in thickness and, in addition to persisting subsurface prophases, figures at all stages of mitosis appeared at all levels in the ependymal layer. Most of these tended to locate themselves at the periphery of the layer and to undergo division in a plane parallel to the ventricular surface. At 14 days the layer decreased slightly in thickness, and mitotic figures were more uniformly distributed, with less regularly orientated cleavage planes and very few subsurface prophases. This sequence was interpreted as a progressive breakdown in the pseudostratified structure of the ependymal layer. The effect of the appearance of non-surface figures was to maintain the proportion of cells in mitosis; this would otherwise have dropped as the nuclear population increased with increasing pseudostratification.

Proliferation continued longest, and congestive phenomena were greatest, in the dorso-caudal part of the thalamus, which is the area giving rise to the largest and most populous of the thalamic nuclei.

These findings are taken to support the two rules proposed in the introduction.

Thanks are due to Mrs Heather Reid and Miss Sheila Stewart for skilled technical assistance. I am grateful to Miss Mary Benstead of the Department of Medical Art for preparing the illustrations and figures, particularly Figs. 8–10. The work was carried out with the assistance of a grant from the Medical Research Council.

REFERENCES

FUJITA, S. (1963). The matrix cell and cytogenesis in the developing central nervous system. *Journal of Comparative Neurology* **120**, 37–42.

FURSHPAN, E. J. & POTTER, D. D. (1968*a*). Low resistance junctions between cells in embryos and tissue cu'ture. In *Current Topics in Developmental Biology* (Eds. A. A. Moscona and A. Monroy), **3**, 95–127.

FURSHPAN, E. J. & POTTER, D. D. (1968*b*). Low resistance junctions between cells in embryos and tissue culture. In *Current Topics in Developmental Biology* (Eds. A. A. Moscona and A. Monroy), **3**, 113.

GOODWIN, B. C. & COHEN, M. H. (1969). A phase-shift model for the spatial and temporal organization of developing systems. *Journal of Theoretical Biology* **25**, 49–107.

HEINMETS, F. (1968). Computer analysis of cellular interactions. In *Current Topics in Developmental Biology* (Eds. A. A. Moscona and A. Monroy) **3**, 129–156.

JELÍNEK, R. (1959). Proliferace v centrálním nervovém kuřecích zárodků. I. Doba trvání mitosy v germinální zoně míchy od 2. do 6. dne zárodečného vývoje. *Československ Morfologie* **7**, 163–173 (cited by Källén, 1961).

KÄLLÈN, G. (1961). Studies in cell proliferation in the brain of chick embryos with special reference to the mesencephalon. *Zeitschrift für Anatomie and Entwicklungsgeschichte* **122**, 388–401.

SMART, I. H. M. (1970). Changes in location and orientation of mitotic figures in mouse oesophageal epithelium during the development of stratification. *Journal of Anatomy* **106**, 15–21.

SMART, I. H. M. (1972). Proliferative characteristics of the ependymal layer during the early development of the spinal cord in the mouse. *Journal of Anatomy* **111**, 365–380.

WOLPERT, L. (1968). French flag problem: a contribution to the discussion on pattern development and regulation. In *Towards a Theoretical Biology, I, an I.U.B.S. Symposium* (Ed. C. H. Waddington). Edinburgh: Edinburgh University Press.

WOLPERT, L. (1969). Positional information and the spatial patterns of cellular differentiation. *Journal of Theoretical Biology* **25**, 1–47.