J. Anat. (1974), **117**, 1, pp. 17–25
With 3 figures
Printed in Great Britain

Histogenesis of the anterior limb of the anterior commissure of the mouse brain

I. A quantitative study of changes in the glial population with age

R. R. STURROCK
Department of Anatomy, The University, Dundee

(*Accepted* 2 *October* 1973)

INTRODUCTION

Glia cell proliferation associated with myelination within the central nervous system was first described by Roback & Scherer (1935). Since then changes in cell number related to myelination in the whole brain have been studied by Uzman & Rumley (1958) and by Bass, Netsky & Young (1969) by estimating the total DNA content of the brain. Certain fibre tracts have also been examined, for example the corpus callosum (Hillebrand, 1966; Schonbach, Hu & Friede, 1968; Schmidt, 1969; Lucas, 1972), the pyramidal tract (Heider, 1967; Matthews & Duncan, 1971) and the optic nerve (Vaughn, 1969; Blunt, Baldwin & Wendell-Smith, 1972). These latter studies showed changes in glial population per unit area, and were not primarily concerned with the increase in total cell population which would accompany growth in length and cross-sectional area of the particular tract being studied.

The present work is an attempt to measure the increase in the total glia population within a well-defined fibre bundle of the mouse brain, before, during, and after the onset of myelination. The anterior limb of the anterior commissure was selected as it is a small, well-defined tract which can be easily identified in both horizontal and coronal sections, and unlike the commissure proper its fibre content eventually shows a uniform degree of myelination (Nakamuru, Omori & Omori, 1960).

In order to relate changes in nuclear population to the phase of myelination, particularly the fast phase described by McIlwain (1955), the percentage of myelinated fibres present at various ages was also recorded.

MATERIALS AND METHODS

Mouse embryos were obtained at one day intervals from 12 to 19 days of post-conceptional age. In the 12–16 day age group the whole embryo was fixed by immersion in Carnoy's solution. The 17–19 day embryos were decapitated and the head alone was fixed by immersion in Carnoy's solution. At least three embryos were available at each age.

Postnatal mice obtained at one day intervals from birth until 14 days postnatum and thereafter at 17, 21, 25, 30, 35, 45, 60 and 240 days postnatum were decapitated and their brains removed and fixed in Carnoy's solution. In addition, perfusion-fixed brains were obtained from postnatal mice aged 0, 2, 5, 8, 11, 14, 17, 21, 25, 30,

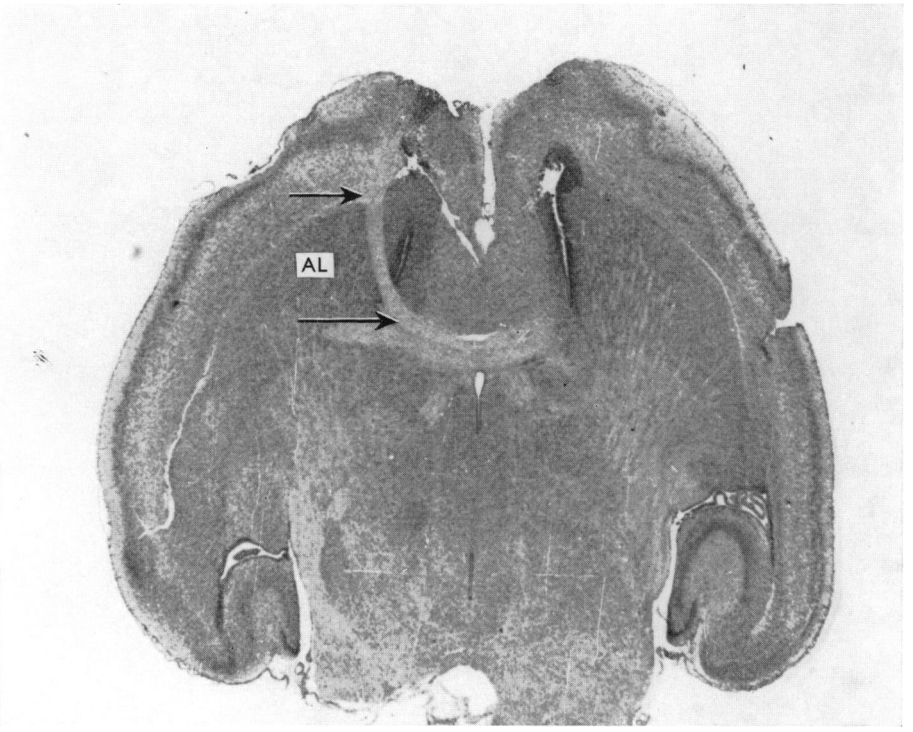

Fig. 1. Horizontal section of a 2 day postnatal mouse brain showing the anterior limb of the anterior commissure (AL between arrows). The anterior limb appears only on the left side due to the obliquity of the section.

35, 45 and 240 days, killed by perfusion with Bouin's solution via the ascending aorta after flushing out the vascular system with physiological saline.

At least five brains were available at the ages selected for perfusion-fixation before 17 days postnatum, three immersion-fixed and two perfusion-fixed. From 17 days postnatum onwards two immersed brains and two perfused brains were available. At other ages usually only one immersion-fixed brain was examined.

The three prenatal brains available at each age were serially sectioned at 6 μm, two coronally and one horizontally. Coronal and horizontal serial sections at 6 μm were also prepared from the postnatal material. All serial sections were stained with haematoxylin and eosin.

For electron microscopy mice aged 0, 2, 5, 8, 11, 14, 17, 21, 25, 32 and 240 days postnatum were fixed by perfusion with an ice-cold buffered solution of 2% paraformaldehyde and 3% gluteraldehyde through the ascending aorta and the brain subsequently removed.

Total glial cell estimations

Because of the inaccuracies introduced by applying Abercrombie's correction (1946) when the size of the nuclei varies with the types of glial cells present (Penfield, 1932), the stages of development, (Mitrova, 1967) and the method of fixation,

(Cammermeyer, 1960*a*, *b*; Blenk, 1969), the following procedure was developed for identifying with greater certainty the mid-point of nuclei lying within a particular section.

The extent of the anterior limb of the anterior commissure studied at each age is shown in Fig. 1.

Three consecutive coronal serial sections of the anterior limb were photographed at × 100 magnification on film, using a Zeiss Photomicroscope I with a green filter. This procedure was followed after every 8th section (48 μm) in animals younger than 8 days postnatum or 17th section (102 μm) in animals 8 days postnatum and older. The photographs were printed at × 8 magnification, giving a total magnification of × 800.

The photograph of the middle section of each set of three was placed under an acetate sheet and every nucleus and fragment of a nucleus was outlined on the sheet with a chinagraph pencil. The two other sections of each set were placed under the marked sheet in turn. As the thickness of the section approximated to the diameter of the nuclei, parts of most nuclei appeared in at least two of the three sections examined. The first and third photographs were orientated until the maximum number of nuclei corresponded to those outlined on the acetate sheet. The photographs, and when necessary the original sections, were carefully examined until all the nuclei whose centre point lay within the middle section had been identified. This method enabled the centre points for all shapes and orientations of nuclei to be identified.

From these results the average number of nuclear mid-points per section was found. This number was multiplied by the total number of sections containing parts of the anterior limb to give an estimate of the total number of nuclei in the whole anterior limb.

This procedure was repeated with each set of coronal serial sections at each age period.

Cell density estimations

The area of the anterior commissure in each middle section was found by measuring the area of the commissure with a Beck metric planimeter. This was converted into μm^2 and multiplied by six (the section thickness) to give the volume of the section of the commissure in μm^3.

The nuclear density per 10^6 μm^3 of the anterior limb of the anterior commissure was estimated at each age.

Distribution of nuclei

The number of nuclei in each group of nuclei was studied by examining horizontal sections at each age. Horizontal sections were preferred for this task as the rows of nuclei occurred parallel to the plane of section. The number of solitary nuclei, i.e. nuclei separated by at least 15 μm from other nuclei, and the average number of nuclei in a group at each age was recorded. A minimum of 400 nuclei was examined in each horizontal section.

Table 1. *Changes in the total number of glia, the glial density, and the cross-sectional area and length of the anterior limb with age*

Age (days)	Estimated total number of cells	Number of cells per 10^6 μm^3 (cell density)	Average cross-sectional area $\times 10^3$ μm^2	Average length (μm)
F.16	225	165	2·9	600
F.17	450	236	2·6	600
F.18	640	295	4·0	650
F.19	720	191	5·9	700
Newborn	820	144	10·2	700
1	1540	121	18·6	600
2	1690	123	20·7	740
3	1250	117	22·1	—
4	2100	132	25·2	—
5	1980	124	21·5	725
6	2600	120	29·4	—
7	2425	167	24·0	—
8	2440	159	25·5	840
9	3260	179	22·7	—
10	4570	223	25·3	—
11	4030	212	27·3	925
12	5230	223	27·8	—
13	6180	253	28·6	—
14	6730	269	27·2	1050
17	7470	231	33·2	1125
21	8320	260	33·8	1225
25	10110	231	45·1	1225
30	10900	256	41·8	1250
35	11450	248	45·7	1225
45	11180	254	41·2	1300
60	11960	230	51·08	1250
240	11030	276	39·6	1300

Assessment of myelination

The anterior limbs of the anterior commissures were removed by careful microdissection from brains perfused with mixed aldehydes. These tissue blocks were post-fixed for 1 hour in osmium tetroxide and flat embedded in Araldite blocks. Thin (1 μm) coronal sections were cut using a Spencer microtome with a glass knife. These sections were stained with 0·5% toluidine blue (Richardson, 1962) and examined microscopically. When the anterior limb of the commissure was identified the block was trimmed for electron microscopy. Ultra-thin sections were obtained using a Reichert ultramicrotome, mounted on grids, stained with uranyl acetate and lead citrate, and examined in an AEI 801 electron microscope.

The degree of myelination was assessed by counting myelinated and unmyelinated fibres in electron micrographs. A minimum of four areas of the anterior limb of the anterior commissure was photographed at $\times$10000 magnification. This procedure was carried out at 11, 14, 17, 25 and 32 days postnatum and in the adult commissure. (Myelination was absent prior to 11 days postnatum.)

The EM plates were printed at an enlargement of $\times$25000. Each micrograph was examined and all unmyelinated fibres completely within the micrograph were counted. Each part of an unmyelinated fibre along the lower edge and the left-hand

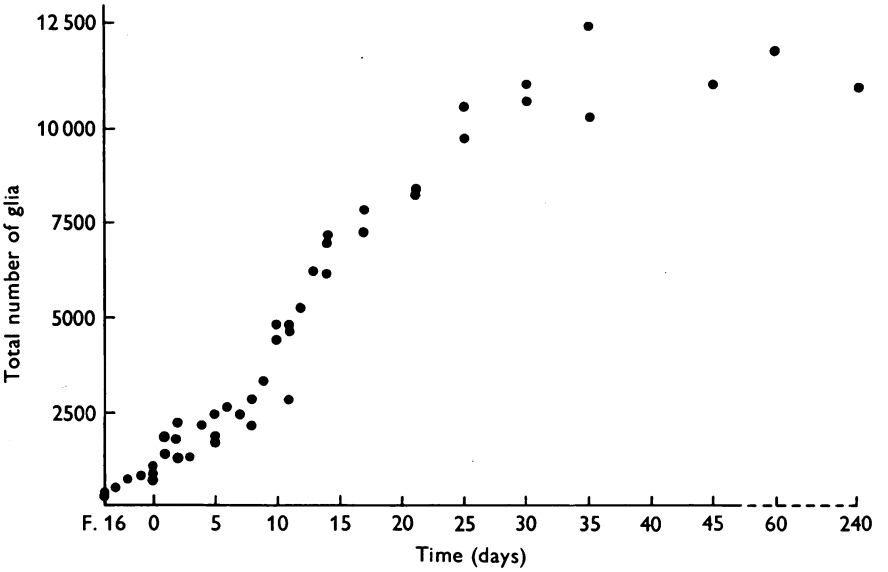

Fig. 2. Changes in the total glial population with time. F.16 refers to 16 days post-conception and 0 to birth which normally occurred on the 20th day post-conception.

edge of the picture was also counted. As each fibre was counted using a tally counter, it was pierced with a probe to prevent the same fibre being counted twice. Astrocytic and oligodendrocytic processes were not counted. This process was then repeated for the myelinated fibres.

RESULTS

Increase in total glia

The anterior limb of the commissure can first be distinguished at 15 days post-conception as a fibre tract devoid of nuclei. Nuclei first appear within the anterior limb of the anterior commissure at 16 days post-conception. The total nuclear population increases from 225 at 16 days post-conception to about 11000 at 30 days postnatum (Table 1 and Fig. 2), the most rapid increase occurring between 8 and 14 days postnatum (Fig. 2). The number of nuclei per unit volume (Table 1 and Fig. 3) changes markedly before birth rising from 165 nuclei per 10^6 μm^3 at 16 days post-conception to 295 nuclei per 10^6 μm^3 at 18 days post-conception. Nuclear density falls to 121 nuclei per 10^6 μm^3 at one day postnatum. This figure is maintained until 7 days postnatum when there is a progressive increase to 253 nuclei per 10^6 μm^3 at 13 days postnatum. Although there is subsequently a variation in the number of nuclei per unit volume the average nuclear density remains about 250 cells per 10^6 μm^3.

Distribution of glia

At birth 33% of nuclei occur in groups parallel to the nerve fibres, consisting mainly of pairs of nuclei with the longest row containing only four nuclei (Table 2). By 5 days postnatum 50% of cells are arranged in groups. Although most of these

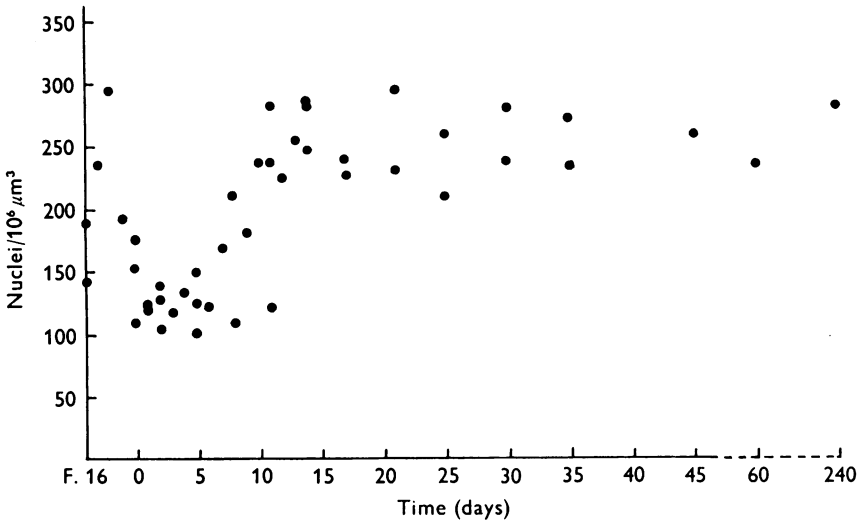

Fig. 3. Graph of the change in glial density with time.

Table 2. *Changes in the arrangement of glial rows with age*

Age (days postnatal)	% of cells arranged in rows	Average number of cells in each row	Maximum number of cells in any row
Newborn	33	2·8±0·9	4
1	36	2·6±0·8	4
2	44	2·7±1·0	5
5	51	3·0±1·7	8
8	67	2·9±1·5	8
11	63	2·4±0·6	4
14	77	3·4±1·6	7
17	76	3·1±2·0	10
21	76	3·3±1·9	10
25	77	4·3±3·8	16
30	75	3·6±3·2	16
45	78	4·6±3·1	12
60	77	4·1±3·8	15
240	74	4·4±2·6	11

groups still consist of pairs of cells, rows of up to 8 cells are present. By 14 days postnatum the nuclei in groups increase to 75% which is maintained into adult life. The number of cells in each row, however, increases until at 25 days postnatum rows of up to 16 cells can be found (Table 2).

Increase in length and cross-sectional area of the anterior limb

Between 16 days post-conception and 21 days postnatum the anterior limb of the anterior commissure doubles in length from 600 to 1225 μm (Table 1). In the adult they vary between 1250 and 1300 μm (Table 1).

Table 3. *Myelination of the anterior limb*

Age (days postnatal)	Total number of fibres counted	Number of myelinated fibres	% of myelinated fibres
11	1694	8	0·5
14	657	5	0·8
17	884	19	2·1
25	972	71	7·3
32	717	137	19·1
240	765	138	18·0

Between 17 days post-conception and 4 days postnatum the cross-sectional area increases from $2{\cdot}6 \times 10^3$ μm^2 to $25{\cdot}2 \times 10^3$ μm^2. This is followed by a small increase to $27{\cdot}2 \times 10^3$ μm^2 at 14 days postnatum. From 14 to 25 days postnatum the cross-sectional area increases from $27{\cdot}2 \times 10^3$ μm^2 to $42{\cdot}7 \times 10^3$ μm^2 (Table 1). The cross-sectional area thus increases from $2{\cdot}6 \times 10^3$ μm^2 to an average to $42{\cdot}7 \times 10^3$ μm^2 after 25 days postnatum (Table 1) which is equivalent to a fourfold increase in diameter.

Myelination

The percentage of myelinated fibres increases from 0·5 % at 11 days postnatum to the adult value of 18–19 % at 32 days postnatum (Table 3). The fast phase of myelination occurs between 14 and 22 days postnatum.

DISCUSSION

From these results it appears that the increase in total glial cells within the anterior limb of the anterior commissure falls into three main phases. In the first, which lasts from 16 days post-conception until 8 days postnatum, the total glia increases by about 200 nuclei per diem. The second phase, which lasts from 8 to 14 days postnatum, is a period of more rapid population growth of around 700 cells per diem. The final period extends from 14 to 30 days postnatum, during which cell numbers increase more slowly by about 275 cells per diem.

The onset of myelination occurs after 8 days postnatum and before 11 days postnatum, and continues to 32 days postnatum. The phase of most rapid increase in glia numbers begins before the onset of myelination and ceases before the end of fast myelination. These results suggest that earlier studies may have underestimated the duration of increase in total glia in the mouse. Smart & Leblond (1961) believed that although glia were produced throughout life the total number of glia became steady at 14 days postnatum, and Sakla (1965) stated that the glial population became fixed at 10 days postnatum. This study indicates that although the number of glia per unit area becomes constant at 14 days postnatum the total population continues to increase at a rate just sufficient to compensate for the increase in volume of the anterior limb of the commissure.

Most quantitative studies of glial production in relation to myelination have shown a rapid increase in glia preceding myelination (Roback & Scherer, 1935; Fleischhauer & Hillebrand, 1966; Hillebrand, 1966; Schonbach, Hu & Friede, 1968;

Schmidt, 1969; Matthews & Duncan, 1971; Lucas, 1972) which continues during the early stages of myelination. These authors found that as myelination continued there was a marked fall in glial density except in the corpus callosum of the rat (Schonbach *et al.* 1968) and rabbit (Lucas, 1972). Vaughn (1969) and Blunt *et al.* (1972) described a similar fall in glial density with myelination in the optic nerve of the rat and kitten respectively. Even when the total glial number increased, a fall in glial density was found in the pyramidal tract of the rat spinal cord (Matthews & Duncan, 1971). However, in this study, a post-myelination fall in glial density was not found in the anterior commissure. This may be peculiar to the anterior commissure; for example, Friede (1961) found that the glial population density of the anterior commissure exceeded that of all other tracts he studied in the human brain except the tractus solitarius.

In the anterior limb of the anterior commissure short rows of glial nuclei are found at all ages after birth. The percentage of cells occurring in rows rises from birth until 14 days postnatum. After 14 days postnatum the maximum number of cells found in a row increases greatly but the average number of cells per row shows a less marked increase, suggesting that as existing rows increase in number new shorter rows are being formed. Other workers have found that the time of appearance of rows varies greatly. They may precede myelination, as in the internal capsule and ventral funiculus of the spinal cord of the fetal sheep (Barlow, 1969), or occur during myelination, as in the human corpus callosum (Fleischhauer, 1967), or even be absent, as in the fasciculus cuneatus of the rat (Matthews & Duncan, 1971). In the anterior limb of the anterior commissure of the mouse, cells begin to form rows before myelination but the greatest increase in the number of cells per row occurs during the period of fast myelination.

SUMMARY

1. The changes in total glial population and glial density with age were studied in the anterior limb of the anterior commissure of the mouse from fetal to adult life.

2. The total glial population increases in three main phases. There is a phase when the population increases by about 200 cells per day from 16 days post-conception until 8 days postnatum. This is followed by a rapid increase of over 700 cells per day from 8 to 14 days postnatum during which time myelination begins. Finally there is a period when the cell population continues to increase at the rate of about 275 cells per day until the end of the first month of postnatal life.

3. Cell density fluctuates greatly before birth but stabilizes at about 120 cells/$10^6 \mu m^3$ between 1 and 6 days postnatum. Cell density then rises to around 250 cells/$10^6 \mu m^3$ by 13 days postnatum and remains at this level throughout life.

Thanks are due to Mrs Heather Reid and Mrs Sheila Ramsay for their patience in producing the serial sections used in this study; to Mrs Elspeth Lloyd-Davies for her invaluable assistance in production of the material for electron microscopy; and to Dr I. H. M. Smart for his advice, criticism, and encouragement during the preparation of this work.

This work was carried out with the assistance of a grant from the Medical Research Council.

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