J. Anat. (1975), **119**, 1, pp. 67–75
With 1 *figure*
Printed in Great Britain

A quantitative electron microscopic study of myelination in the anterior limb of the anterior commissure of the mouse brain

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(*Accepted* 8 *August* 1974)

INTRODUCTION

In a previous study of gliogenesis within the anterior limb of the anterior commissure of the mouse brain (Sturrock, 1974*a*) an increase was noted in cross sectional area of this tract during development. This raised the question of how much of this increase in cross sectional area was due to an increase in the number of axons, how much to an increase in diameter of axons already present, and how much to myelination. The aim of the present work was to assess the relative contribution of these factors to the increase in diameter of the anterior limb from its appearance at 16 days post-conception.

An attempt was also made to find evidence of 'physiological' demyelination between 25 and 45 days postnatum, when there is a large increase in the number of pyknotic cells in the anterior limb (Sturrock, 1974*c*). Such demyelination has been observed in the cat (Hildebrand, 1971).

MATERIALS AND METHODS

The materials used in this study consisted of anterior limbs of anterior commissures from the brains of fetal mice aged 16, 17, 18 and 19 days post-conception, from newborn mice, and from mice aged 2, 5, 8, 11, 14, 17, 21, 25, 32, 35, 45 and 240 days postnatum.

The brains were fixed by vascular perfusion with a mixture of 2 % paraformaldehyde and 3 % glutaraldehyde for twenty minutes after washing out with physiological saline for thirty seconds. The anterior limbs were removed, processed and embedded in Spurr's resin as previously described (Sturrock, 1974*b*).

One complete anterior commissure was removed from a 240 day mouse and, after post-fixation in buffered osmium tetroxide, the surrounding tissue was carefully removed. This commissure was placed on a slide and covered with a coverslip. The anterior limb was slightly crushed so as partially to separate fibre bundles and photographed at $\times 25$ magnification.

Light microscopy

Coronal and horizontal 1 μm sections were obtained for light microscopy using a Spencer microtome, and stained with 0·5 % toluidine blue.

The area of anterior limb examined electron microscopically was found by photo-

graphing adjacent 1 μm toluidine blue sections, printing the photomicrographs at $\times 800$, and measuring the area, using a Beck metric planimeter.

The horizontal sections were examined at $\times 1000$ magnification in an attempt to measure directly internodal length. This unfortunately was unsuccessful as few fibres could be observed for any length owing to the entwining pattern followed by the fibre bundles (Fig. 1).

Electron microscopy

Sections prepared for electron microscopy, using either an LKB or Reichert ultramicrotome, were stained with uranyl acetate and lead citrate and examined in an AEI Corinth electron microscope.

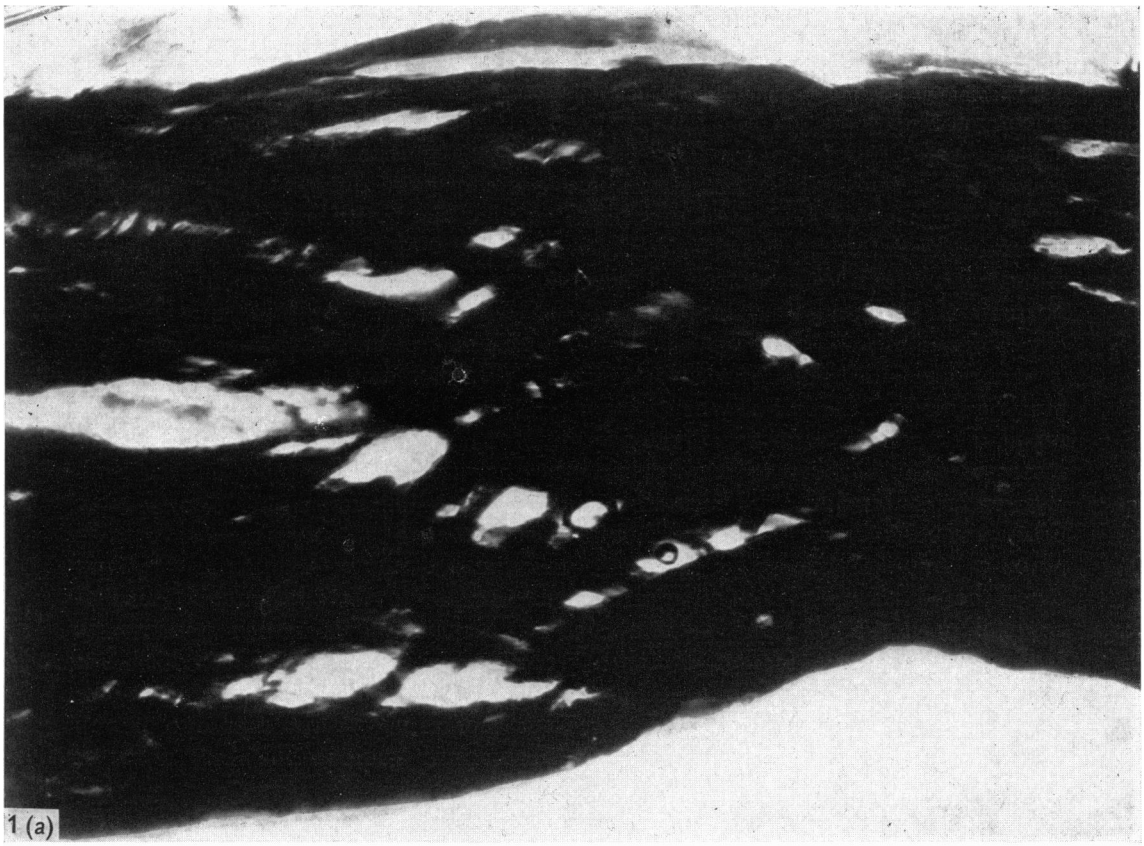
In fetal mice and mice up to 8 days postnatum, photographs were taken at $\times 15000$ magnification. To ensure randomness of sampling the first photograph was taken at the top left hand corner of the grid space and the following photographs were taken at regular intervals (2 complete turns of the microscope stage control) until the top right hand corner was reached. Micrographs which were badly scored, or which contained much stain precipitate, were discarded. Anterior limbs of animals aged 11 to 17 days postnatum were photographed in a similar manner at a magnification of $\times 6000$, and a further series of micrographs at $\times 15000$ was taken to include the maximum number of myelinated fibres, which were scarce at these ages. In animals 21 days postnatum and older, photographs were taken at regular intervals at a magnification of $\times 6000$, and after every third photograph a further photograph was taken at $\times 15000$.

All photomicrographs were printed at a further magnification of $\times 2$.

Estimation of the total number of axons at each age. In animals aged up to 8 days postnatum, the total number of axons was estimated by counting at least 1500 axons. The fibres were counted by placing a piece of card 4×4 cm in size, with a central aperture 2×2 cm, over four areas of each photomicrograph. These areas were selected by placing the outer edge of the cardboard square over the corner of the micrograph, so that two adjacent sides and one corner of each coincided. All axons entirely within the aperture of the square, and each part of an axon in contact with the lower and left hand edges of the square, were counted. Parts of axons in contact with the upper and right hand edges were not recorded. When at least 1500 axons had been counted the area containing them was estimated and the total number of axons at each age was calculated by extrapolation, using the known area of the anterior limb at that age.

From 11 days postnatum the total number of axons was estimated by counting the total number of axons in every fourth micrograph at $\times 12000$ magnification. As before, all parts of axons at the lower and left hand edges were counted, and those at the upper and right hand edges were not recorded. (As the square micrographs were printed on rectangular photographic paper, the edge with a large unexposed area was always considered to be the lower edge to avoid bias in counting.) Between 1600 and 3500 axons were counted at each age.

Estimation of the total number of myelinated axons at each age. From 11 days postnatum all the $\times 12000$ micrographs were examined and every unmyelinated axon surrounded by a glial process (promyelin fibre), and every myelinated axon,



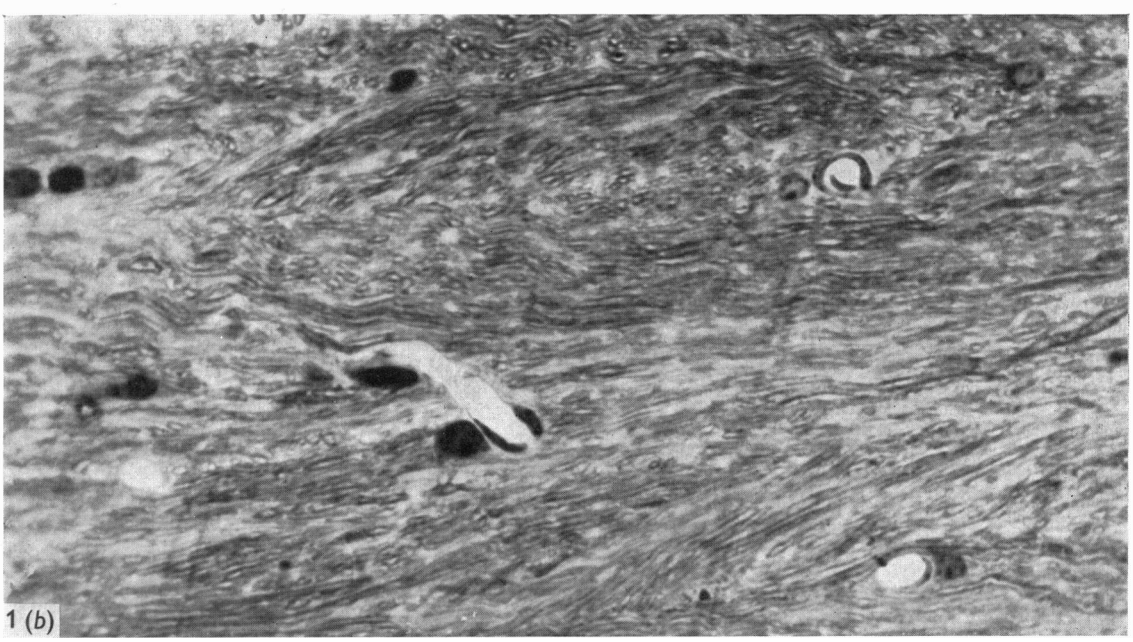


Fig. 1. (*a*) Crushed preparation of the anterior limb of the anterior commissure showing the rope-like entwining of the fibre bundles. × 125.

(*b*) Horizontal 1 μm section of the anterior limb stained with toluidine blue. Longitudinally and transversely sectioned sheaths can be seen. × 1000.

was counted in an identical manner to that used to estimate the total number of axons.

From these results the total number of promyelin fibres and myelinated axons was estimated. The percentage of each was calculated.

Diameter of unmyelinated axons. At all ages the diameters of unmyelinated axons were estimated by covering the ×30000 micrographs with a sheet of centimetre graph paper. The graph paper was pierced with a probe where every second centimetre line crossed (i.e. at 2 cm intervals) in a squared pattern. This procedure was continued until 25 unmyelinated axons had been pierced. The area of these axons was found using a planimeter, and the diameter calculated on the assumption of an axon being circular in cross section. This was preferable to direct measurement of diameters, as few axons were completely circular in cross section. Unmyelinated axonal diameters at successive ages were compared statistically using Student's '*t*' test.

Diameter of myelinated axons. The diameter of myelinated axons was found using a similar method to that described above. At the same time, the inner and outer diameters of the myelin sheath were estimated with the aid of a planimeter. The number of lamellae was recorded in each case by counting the major dense lines on the micrographs with the aid of a binocular dissecting microscope. Between 19 and 39 myelinated fibres were examined at each age.

Statistical comparisons of myelinated axonal diameter at successive ages, and comparisons of unmyelinated and myelinated axonal diameter at the same age, were carried out using Student's '*t*' test.

Examination of the myelin sheath. From 11 days postnatum, fifty myelin sheaths were examined to find the position of the inner tongue processes in relation to the outer tongue process. Each sheath was divided into four quadrants and the inner tongue was taken as the fixed point. If the inner tongue was considered as 12 o'clock, the first quadrant extended from 12 o'clock to 3 o'clock, the second quadrant from 3 o'clock to 6 o'clock, the third quadrant from 6 o'clock to 9 o'clock, and the fourth quadrant from 9 o'clock to 12 o'clock. This refers of course to sheaths with a right hand spiral. In cases of sheaths with left hand spirals the positions of the quadrants were reversed.

RESULTS

Internodal length. No estimate of internodal length was possible because of the 'rope-like' twisting of the fibre bundles in the anterior limb (Fig. 1).

Changes in the total number of axons. There was little difference in the total number of axons at 16 and 17 days post-conception (Table 1). The total number of axons increased steadily thereafter until 11 days postnatum. Although there was an increase in total axons after 11 days postnatum it seemed probable that changes after this age were due to individual variation as the total number of axons in animals aged 21, 25, 32, 45 and 240 days postnatum was slightly less than at 11 days postnatum, while at 14, 17 and 35 days it was greater.

Changes in promyelinated and myelinated axons. Promyelinated axons were first observed in very small numbers at 8 days postnatum. By 11 days postnatum the percentage of promyelinated axons was 0·17 % (Table 2). After 14 days postnatum

Table 1. *Changes in the estimated total number of axons, promyelinated axons, and myelinated axons with age*

Age	Total axons	Promyelinated axons	Myelinated axons
16 d.p.c.*	46000	—	—
17 d.p.c.	48700	—	—
18 d.p.c.	72400	—	—
19 d.p.c.	87500	—	—
Birth	140700	—	—
2D†	138100	—	—
5D	176000	—	—
8D	212700	Too few to estimate	—
11D	286500	500	400
14D	312500	600	400
17D	334700	3000	4500
21D	284800	4600	28000
25D	281300	2000	45000
32D	253000	2300	55200
35D	301300	2900	68600
45D	276100	900	67800
240D	271100	—	78700

* d.p.c. = days post-conception.
† D = days postnatum.

Table 2. *Percentage of promyelinated and myelinated axons at different ages and estimated daily increase in the percentage of myelinated axons*

Age	% promyelin fibres	% myelination	Estimated daily % increase in myelination
11D	0·17	0·14	0·05
14D	0·19	0·12	—
17D	0·90	1·4	0·43
21D	1·60	9·8	2·10
25D	0·71	16·0	1·55
32D	0·93	21·8	0·83
35D	0·96	22·8	0·33
45D	0·33	24·6	0·18
240D	—	29·0	0·02

the percentage of promyelinated axons rose to a maximum of 1·6% at 21 days postnatum. After 21 days postnatum the percentage of promyelinated axons fell to 0·7% at 25 days postnatum. There was a slight increase at 32 and 35 days postnatum, but after 35 days postnatum the percentage fell rapidly, until by 240 days postnatum no promyelin fibres were observed.

Myelinated fibres were first found at 11 days postnatum. There was little change in the percentage of myelinated fibres between 11 and 14 days postnatum, but after this time the percentage of myelinated fibres increased, the most rapid increase occurring between 17 and 21 days postnatum. After 21 days postnatum the estimated daily percentage increase (Table 2) fell steadily, but there was a continued,

Table 3. *Mean diameter of unmyelinated and myelinated axons and mean number of myelin lamellae at different ages*

Age	Mean diameter of unmyelinated axons	Mean diameter of myelinated axons	Mean number of myelin lamellae
16 d.p.c.	0·33 ± 0·12	—	—
17 d.p.c.	0·38 ± 0·10	—	—
18 d.p.c.	0·30 ± 0·09	—	—
19 d.p.c.	0·30 ± 0·08	—	—
Birth	0·28 ± 0·07	—	—
2D	0·28 ± 0·07	—	—
5D	0·25 ± 0·06	—	—
8D	0·28 ± 0·08	—	—
11D	0·24 ± 0·06	0·64 ± 0·16	3·6 ± 1·6
14D	0·25 ± 0·06	0·57 ± 0·14	2·4 ± 1·1
17D	0·22 ± 0·07	0·56 ± 0·15	4·2 ± 1·0
21D	0·26 ± 0·11	0·50 ± 0·10	6·3 ± 2·4
25D	0·24 ± 0·09	0·52 ± 0·12	6·4 ± 2·3
32D	0·27 ± 0·07	0·48 ± 0·15	5·9 ± 2·5
35D	0·26 ± 0·13	0·47 ± 0·16	5·8 ± 2·2
45D	0·27 ± 0·08	0·50 ± 0·18	6·3 ± 2·2
240D	0·24 ± 0·07	0·53 ± 0·19	7·1 ± 3·4

Table 4. *Percentage of axons of each diameter at different ages*

Age	1·1 μm	1·0 μm	0·9 μm	0·8 μm	0·7 μm	0·6 μm	0·5 μm	0·4 μm	0·3 μm	0·2 μm
11D	—	5·3	5·3	15·8	15·8	26·3	15·8	15·8	—	—
14D	—	—	—	15·0	10·0	30·0	30·0	10·0	5·0	—
17D	—	—	4·8	4·8	23·8	14·3	33·3	9·5	9·5	—
21D	—	—	—	—	10·7	17·9	50·0	17·9	3·6	—
25D	—	—	—	5·4	10·8	29·7	32·4	16·2	5·4	—
32D	—	—	2·9	2·9	8·8	20·6	14·7	26·5	17·6	5·9
35D	—	—	2·6	5·1	7·7	10·3	23·1	30·8	15·4	5·1
45D	3·3	—	3·3	3·3	13·3	6·7	30·0	26·7	13·3	—
Adult	—	3·6	3·6	10·7	3·6	17·9	32·1	14·3	10·7	3·6

though small, increase in myelination even after 45 days postnatum. There was no evidence of degenerating myelin sheaths at any age.

There was a correlation ($r = 0{\cdot}75$) between the daily percentage increase in myelinated axons and the percentage of promyelinated axons present. This correlation was significant at the 5 % level.

Diameter of unmyelinated axons. The mean diameter of unmyelinated axons (0·27 μm) showed no significant variation throughout development, except at 17 days post-conception, and this was probably due to individual variation.

Diameter of myelinated axons. The mean diameter of myelinated axons (0·53 μm) showed no significant change at any age from the first appearance of myelinated axons at 11 days postnatum (Table 3). At all ages the myelinated axons had a modal diameter between 0·4 and 0·6 μm (Table 4). Although a few large myelinated axons ($\geqslant$ 0·8 μm diameter) were found between 11 and 17 days postnatum the main increase in myelinated axons of large diameter occurred from 25 days postnatum.

Table 5. *Mean number of myelin lamellae around axons of each diameter at different ages*

Age	1·1 μm	1·0 μm	0·9 μm	0·8 μm	0·7 μm	0·6 μm	0·5 μm	0·4 μm	0·3 μm	0·2 μm
11D	—	5·0	4·0	4·0	5·0	4·2	2·0	2·0	—	—
14D	—	—	—	1·7	1·5	2·7	2·0	3·5	5·0	—
17D	—	—	5·0	5·0	4·2	4·7	4·4	3·5	3·0	—
21D	—	—	—	—	10·0	6·0	5·7	6·6	3·0	—
25D	—	—	—	10·1	7·0	5·9	6·8	5·3	5·5	—
32D	—	—	12·0	12·0	8·3	6·4	5·4	5·2	4·2	3·0
35D	—	—	11·0	7·0	7·7	5·5	6·2	5·6	4·3	3·0
45D	7·0	—	9·0	10·0	6·8	5·0	6·8	6·3	3·8	—
Adult	—	17·0	13·0	7·7	12·0	7·0	6·4	5·0	5·3	4·0

Table 6. *Percentage of changes in configuration of inner and outer myelin tongues with age*

Age	1st quadrant	2nd quadrant	3rd quadrant	4th quadrant
11D	38·5	15·4	19·2	26·9
14D	44·0	8·0	24·0	24·0
17D	48·4	9·7	29·0	12·9
21D	56·0	12·0	22·0	10·0
25D	70·0	8·0	12·0	10·0
32D	72·0	14·0	6·0	8·0
35D	70·0	14·0	8·0	8·0
45D	74·0	10·0	10·0	6·0
240D	82·0	0	10·0	8·0

Small axons ($\leqslant$ 0·3 μm diameter) mainly myelinated from 32 days postnatum, although some small myelinated fibres of 0·3 μm diameter were found earlier.

At all ages the difference in diameter between unmyelinated and myelinated axons was highly significant ($P < 0{\cdot}001$).

Number of myelin lamellae. The mean number of myelin lamellae (Table 3) showed a marked variation during the early stages of myelination from 11 to 21 days postnatum, but thereafter became fairly steady, no statistically significant variation being found after 21 days postnatum.

After 17 days postnatum there was a tendency for fibres of larger diameter to have more lamellae (Table 5). The correlation between axonal diameter and the number of lamellae was significant at the 5 % level at 21, 25 and 45 days postnatum, and at the 0·1 % level at 32, 35 and 240 days postnatum.

Relative position of inner and outer myelin tongues. At all ages from the appearance of myelin sheaths at 11 days postnatum, more sheaths were arranged with their inner and outer tongues in the same quadrant than in any other single quadrant (Table 6). With increasing age the percentage of sheaths arranged in this configuration rose from 38·5 % at 11 days postnatum to 70 % at 25 days postnatum and to 82 % at 240 days postnatum.

DISCUSSION

The increase in cross sectional area previously described in the anterior limb of the anterior commissure (Sturrock, 1974*a*) can be seen to fall into two phases when correlated with the present data. The first phase, between 17 days post-conception and 11 days postnatum, is due solely to an increase in the number of axons, the mean axonal diameter remaining constant. There is probably no increase in the number of axons after 11 days postnatum, the increase in cross sectional area of the tract after this time being due to the formation of myelin sheaths and the increase in diameter of myelinating axons.

The changes in the percentage myelination with age differed from those observed in a pilot study of myelination in the anterior limb (Sturrock, 1974*a*). These differences were probably the result of the much larger samples examined in the present study. For example, in the pilot study myelinated axons were counted on only four micrographs at an initial magnification of $\times 10000$, while in the present study a minimum of twelve photomicrographs at an initial magnification of $\times 6000$ was used at each age, an increase in area examined of more than ten times. The greatest difference found in the present study was the continuation of myelination, albeit slowly, after 32 days postnatum.

The finding of a constant diameter for unmyelinated axons throughout the period of study is in agreement with the results of Fleischhauer & Wartenberg (1967) from their study of the cat corpus callosum, as is the finding that the mean diameter of myelinated axons also tended to remain constant, but at a significantly higher level than that of unmyelinated axons. Matthews & Duncan (1971), on the other hand, found an increase in the mean diameter of unmyelinated axons in the posterior columns of the rat spinal cord prior to myelination, suggesting a different pattern of myelination in tracts which myelinate completely from those which are only partially myelinated.

Myelination in the anterior limb usually began with axons of 0·4–0·6 μm diameter, although a few larger and smaller myelinating axons were found. Axons of 0·2–0·3 μm diameter myelinated mainly from 32 days postnatum, while myelinated axons of 0·8–1·1 μm mainly increased in number from 25 days postnatum.

As unmyelinated axons showed no increase in diameter with age, it appears probable that the larger myelinated axons were not recently myelinated large axons, but axons which were already myelinated and had increased in diameter. This seems to be borne out by the fact that after 25 days postnatum the mean number of lamellae around axons of 0·7 μm diameter and above always exceeded that around smaller axons.

Peters (1964) found that, in optic nerves of mature rats, mice and toads, the inner and outer tongues of the myelin sheath lay in the same quadrant in over 74% of cases. Even in developing animals this configuration tended to be the most common. A similar pattern emerged in this study, and even at 11 days postnatum more myelinated axons had the inner and outer tongues in the same quadrant than seems likely to have occurred by chance. Peters (1964) and Fraher (1972) suggested the reason for this configuration was that myelination occurs in bursts, rather than as a continuous process.

There appeared to be no evidence of damaged and degenerating myelin sheaths during myelination, as described by Hildebrand (1971), and as had been expected from the results of a previous study (Sturrock, 1974*c*) which showed a great increase in pyknotic glia in the anterior limb from 25 to 35 days postnatum. The fall in the average number of myelin lamellae during this period was not statistically significant, and could in any case be explained by the myelination of small axons occurring during this period.

SUMMARY

An electron microscopic study of myelination was carried out in the anterior limb of the anterior commissure of the mouse brain. The total number of axons increased from 48700 at 17 days post-conception to 286500 at 11 days postnatum. The first evidence of myelination was the presence of a few promyelin fibres at 8 days postnatum. Myelinated axons were first found at 11 days postnatum. The most rapid increase in myelinated fibres occurred between 17 and 21 days postnatum, but myelination continued to increase even after 45 days postnatum.

There was no change in mean diameter (0·27 μm) of unmyelinated axons after 18 days post-conception. The mean diameter of myelinated axons (0·53 μm) also showed no variation with age.

The modal diameter of myelinated axons lay between 0·4 and 0·6 μm. Small fibres (0·2–0·3 μm) myelinated around 32–35 days postnatum. The greatest increase in large myelinated axons ($\geqslant$ 0·8 μm) occurred after 25 days postnatum.

At all ages sheaths with outer and inner tongues in the same quadrant predominated and by 240 days postnatum 80 % of sheaths showed this configuration.

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