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The development of alpha and gamma motoneuron fibres in the rat

I. A comparative ultrastructural study of their central and peripheral axon growth

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INTRODUCTION

This paper and the next in the series (Fraher & Kaar, 1985) investigate the maturation of alpha and gamma ventral motoneuron fibres in the fifth lumbar spinal nerve of the rat. In the most proximal part of the ventral rootlet, close to the transitional zone between the central and peripheral parts of the nervous system, five distinct morphological stages of axon maturation can be identified, based on axon ensheathment. At first axons are *bare* and closely apposed to one another. Three to four days before birth they become *segregated* by a matrix of fine processes derived from transitional Schwann cells (Fraher & Rossiter, 1983). They then become *promyelin* in form. Up to this stage their calibre distribution is unimodal. This subsequently becomes bimodal as the internodes immediately central and peripheral to the transitional zone become myelinated. At first these internodes are separated by an axon segment of variable length which is enveloped by astrocyte processes. The fibres are then at the *presumptive transitional node* stage (Fraher & Kaar, 1984). The length of the gap between the two then gradually decreases to a value close to that of typical nodes. The fibres are then at the *definitive transitional node* stage.

The growth of alpha and gamma axons is examined in this paper over a number of intervals from the segregated stage up to maturity. Previous studies on the development of bimodal axon calibre distribution (Skoglund & Romero, 1965; Sturrock, 1975; Arees, 1978; Berthold & Carlstedt, 1982; Berthold, Nilsson & Rydmark, 1983) were restricted to the myelinated stage. Studies on axon calibre at the onset of myelination (Friede & Samorajski, 1967; Fraher, 1972; Hildebrand & Hahn, 1978; Schröder, Bohl & Brodda, 1978; Friede & Bischausen, 1980) have been restricted to selected populations of large (alpha) fibres.

The study also compares the growth of both axon groups centrally and peripherally, in the *intramedullary segment* immediately central to the transitional zone and in the most proximal part of the ventral rootlet where they are myelinated by the *transitional Schwann cell.* Previous studies which compared central and peripheral axon segments (Fraher, 1976, 1978*a*, *b*) did not do so for both groups separately.

MATERIALS AND METHODS

The material used in this study was the same as that used in the morphological study of transitional node development (Fraher & Kaar, 1984), in which the methods

of preparation, embedding, sectioning, staining and microscopic examination of tissues, as well as the manner in which magnifications were calculated, are described.

Ten animals were studied at each of the following ages: 20 days fetal, 1, 3, 6, 12, 20 and 300 days postnatum. At each age, a number of ventral motoneuron axon bundles were sectioned by alternate sequential series of thin and semithin sections over the intramedullary segment, the transitional zone and the ventral rootlet. Individual myelinated fibres possessing presumptive or definitive transitional nodes were traced on serial sections over distances of about 40 μm on either side of the central–peripheral transition at 3 days and subsequently. They were examined ultrastructurally at intervals of 5 μm. Only transversely sectioned fibres (Fraher, 1972) were studied. The circumference of each axon was measured at each abnodal level, using a Reichert-MOP AMO3 semiautomatic image analyser. All data were processed using an IBM 4341 computer. Graphs were produced using a Tectronix 4051 Graphics Display Unit and a Tectronix 4662 Flat-Bed plotter. The mean central and peripheral circumference of each myelinated axon was found. Percentage frequency histograms were set up to show the pooled distributions of these values for each class of myelinated axon at each age (Fig. 3). Circumference distributions were also found for segregated and promyelin axons at each age (Fig. 2). These were based on measurements of the bundles as a whole. The distribution of all axon circumferences at each age was also plotted (Fig. 1).

Most distributions were unimodal up to 3 days and a normal curve was fitted to the overall distribution up to that age (Fig. 1). Subsequently, most distributions were bimodal. These distributions were assumed to correspond to a mixture of random samples from two normally distributed populations, each with its own mean and variance. This assumption was plausible on the basis of probability plots, as described by Cox (1966). The model for the relative frequency density function underlying the data was:

$$f(x) = p\,\frac{1}{\sqrt{2\Pi}}\cdot\frac{1}{\sigma_1}\,e^{-\frac{1}{2}(x-\mu_1)^2/\sigma_1} + (1-p)\,\frac{1}{\sqrt{2\Pi}}\cdot\frac{1}{\sigma_2}\,e^{-\frac{1}{2}(x-\mu_2)^2/\sigma_2},$$

where p is the proportion of small fibres and (μ_1, σ_1) and (μ_2, σ_2) represent the mean and standard deviation for the populations of gamma and alpha fibres, respectively.

Estimates of the mean and standard deviation of each normal distribution contributing to the bimodal distribution, as well as of the mixing proportion where the two distributions overlapped, were obtained by an iterative method, based on the EM algorithm (Dempster, Laird & Rubin, 1977). The method was as follows: each observation was first provisionally allocated to the alpha or gamma fibre population. This gave an estimate of the mean and standard deviation of that population. These statistics in turn were used to estimate the probability of each observation coming from one or other population. This information was then used to provide a revised estimate of the mean and standard deviation. This procedure was repeated until all estimates stabilised and final estimates of the basic parameters of each population were obtained. The *dividing circumference* separating the two populations was taken as that circumference value at which an axon had an equal probability of coming from either the alpha or the gamma population of axons, Where a bimodal distribution occurred, almost all fibres could be assigned to either the alpha or the gamma group. A normal curve was fitted to each component distribution. For each of these, the mean axon circumference and its standard deviation are given in Table 1. These

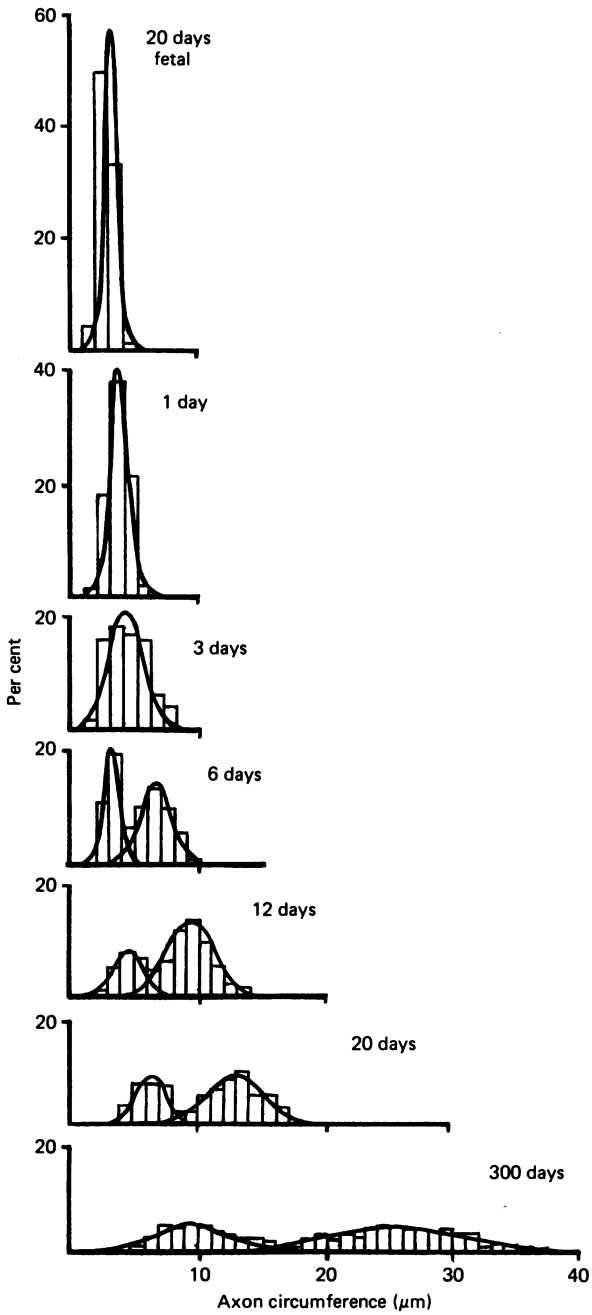

Fig. 1. Percentage frequency histograms, with superimposed normal curves fitted by computer, for the circumference of all axons examined at each age studied.

Table 1. *Data for axon circumference (μm) in pooled animals at the ages indicated. Mean values ($\bar{x}$), standard error of the mean* (S.E.M.) *and the number of axons examined at each stage (n) are given for central and peripheral levels of fibres in the classes indicated. In the case of myelinated fibres, the value used for each axon was itself the mean of several measurements at different levels along the axon*

Fibre class	Age (days)	Central			Peripheral		
		$\bar{x}$	S.E.M.	n	$\bar{x}$	S.E.M.	n
Segregated	20 (fetal)	2·9	0·02	612	2·9	0·02	643
	1	3·6	0·05	481	3·5	0·05	373
	3	3·6	0·12	222	3·7	0·10	232
	6	3·2	0·08	57	3·0	0·08	56
		Fibres possessing presumptive node					
Promyelin	1	4·2	0·14	28	4·3	0·12	31
	3	4·1	0·10	150	4·6	0·09	161
	6	4·0	0·28	86	4·2	0·26	92
	12	3·6	0·13	30	3·4	0·09	33
Myelinated	3	6·2	0·09	120	6·3	0·08	165
	6	6·1	0·13	83	7·3	0·12	94
	12	5·1	0·19	48	5·4	0·16	50
		Fibres possessing definitive node					
Gamma	12	4·5	1·0	177	4·9	1·4	36
	20	6·2	1·2	131	7·2	2·8	76
	300	9·7	2·6	106	11·6	3·9	77
Alpha	6	6·6	1·3	242	6·8	1·1	95
	12	9·4	1·8	456	9·7	1·6	208
	20	13·0	2·4	258	14·0	2·7	99
	300	25·7	5·5	184	30·7	8·7	144

values in turn were used to estimate and plot the growth rates of alpha and gamma axons over the period studied (Fig. 5).

OBSERVATIONS

Overall axon circumference distribution

Up to 1 day postnatum, the overall axon circumference distribution was unimodal in each animal and in all animals together (Fig. 1). A single normal curve accurately characterised each distribution. At 3 days, though one animal had a bimodal distribution, the remaining individual distributions as well as the pooled distribution were unimodal, though broader than at 1 day. At 6 days and at each subsequent age, axon circumference had a bimodal distribution, each component of which was accurately characterised by a normal curve. There was little overlap between the curves for alpha and gamma fibres. With age the two groups progressively separated from one another. At all stages, gamma axons made up between 30 and 40 % of the total.

Axon circumference in the various fibre classes

The circumference frequency distribution of *segregated* axons was narrow and high peaked in all cases (Fig. 2). The mean was at its greatest between 1 and 3 days (Table 1). At each stage, central and peripheral circumferences did not differ

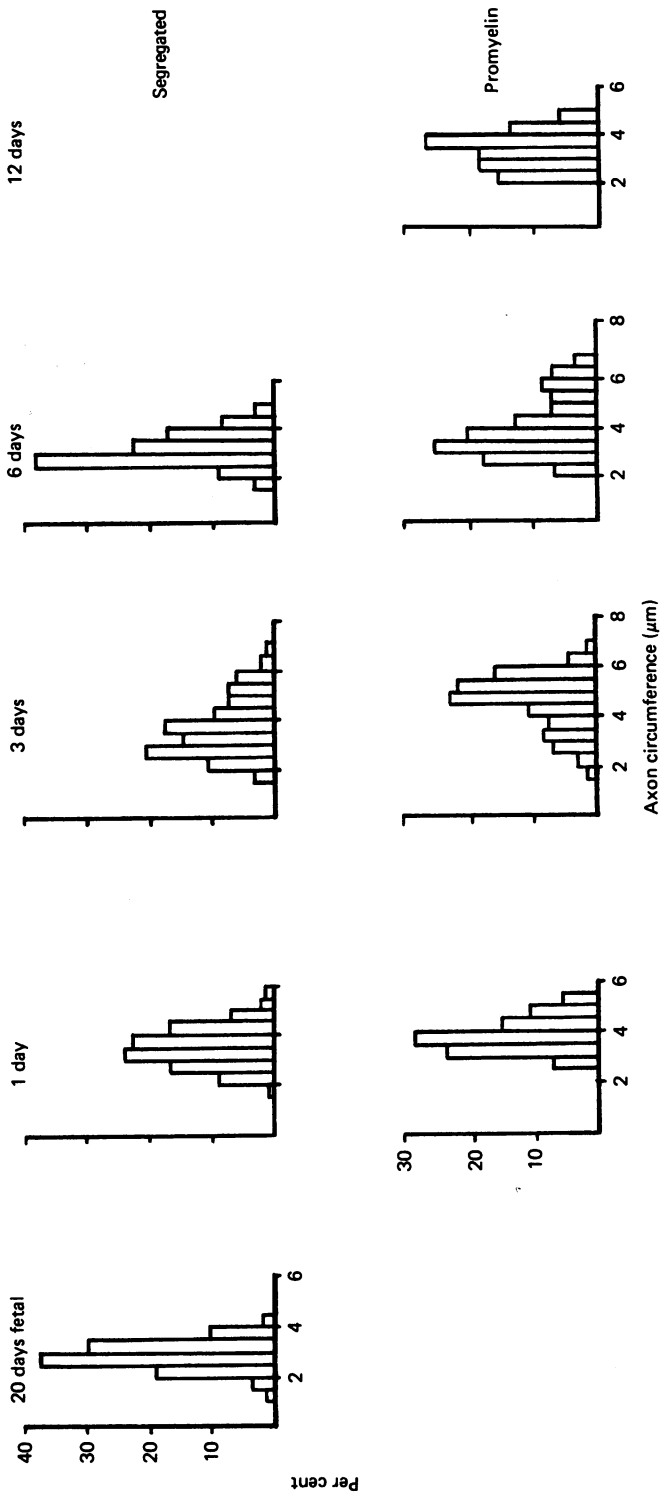

Fig. 2. Percentage frequency histograms for the circumferences of all segregated and promyelin axons studied at the ages indicated.

Table 2. *Percentage frequencies of segregated axons, of axons possessing presumptive and definitive transitional nodes at each age. At* 6 *and* 12 *days the percentage of all fibres which were* alpha *in type and which possessed* presumptive *transitional nodes are given in parentheses*

Age (days)	Segregated	Presumptive node		Definitive node	
		Promyelin	Myelinated	Small	Large
20 (fetal)	100	—	—	—	—
1	94·6	5·4	—	—	—
3	40·4	29·1	30·5	—	—
6	14·7	21·1 (6·8)	29·5 (27·1)	—	33·4
12	—	8·7	10·4 (0·09)	15·7	65·2
20	—	—	—	34·0	66·0
300	—	—	—	36·0	64·0

significantly from one another. *Promyelin* axons (Table 1; Fig. 2) had a unimodal circumference distribution. This was similar centrally and peripherally. Mean circumference reached a maximum peripherally at 3 days. At each age it was significantly greater than that of segregated axons. *Myelinated* axons possessing *presumptive* transitional nodes occurred from 3 to 12 days (Fig. 3). Their mean circumference reached a maximum at 6 days (Table 1). At 12 days it was statistically significantly smaller than at previous stages. *Myelinated* axons possessing *definitive* transitional nodes were first seen at 6 days. Alpha and gamma axons each had a unimodal circumference distribution at each age (Fig. 3). The range of each distribution increased progressively with time. The central distribution closely resembled the peripheral distribution in form at each age but tended to be shifted to the left relative to it. Mean values were correspondingly less for central than for peripheral segments of both alpha and gamma axons (Table 1).

Age changes in the proportions of axons in the various classes

Age changes in the proportions of the various classes of axon are shown in Table 2 and Figure 4. Segregated axons made up 100% of the total at 20 days fetal. With maturation their frequency decreased and none were present after 6 days. Promyelin axons first appeared at 1 day and persisted until 12 days. At 3 days about 60% of axons possessed a presumptive transitional node and were either promyelin or myelinated. At 6 days a similar proportion was myelinated and possessed either a presumptive or a definitive node. Subsequent to this just over 60% of all axons were alpha and myelinated in type and possessed definitive nodes. The proportion of segregated axons at 3 days was similar to that made up by small myelinated axons at 20 and 300 days.

Axon growth

Total axon counts showed that all prospective alpha and gamma axons were present at 20 days fetal, though they could not be separated from one another on the basis of their circumferences. The calibre of both groups of axons had increased by 6 days and continued to increase progressively after that (Fig. 5*a*). The relative increase in circumference over the entire period was greater for alpha than for

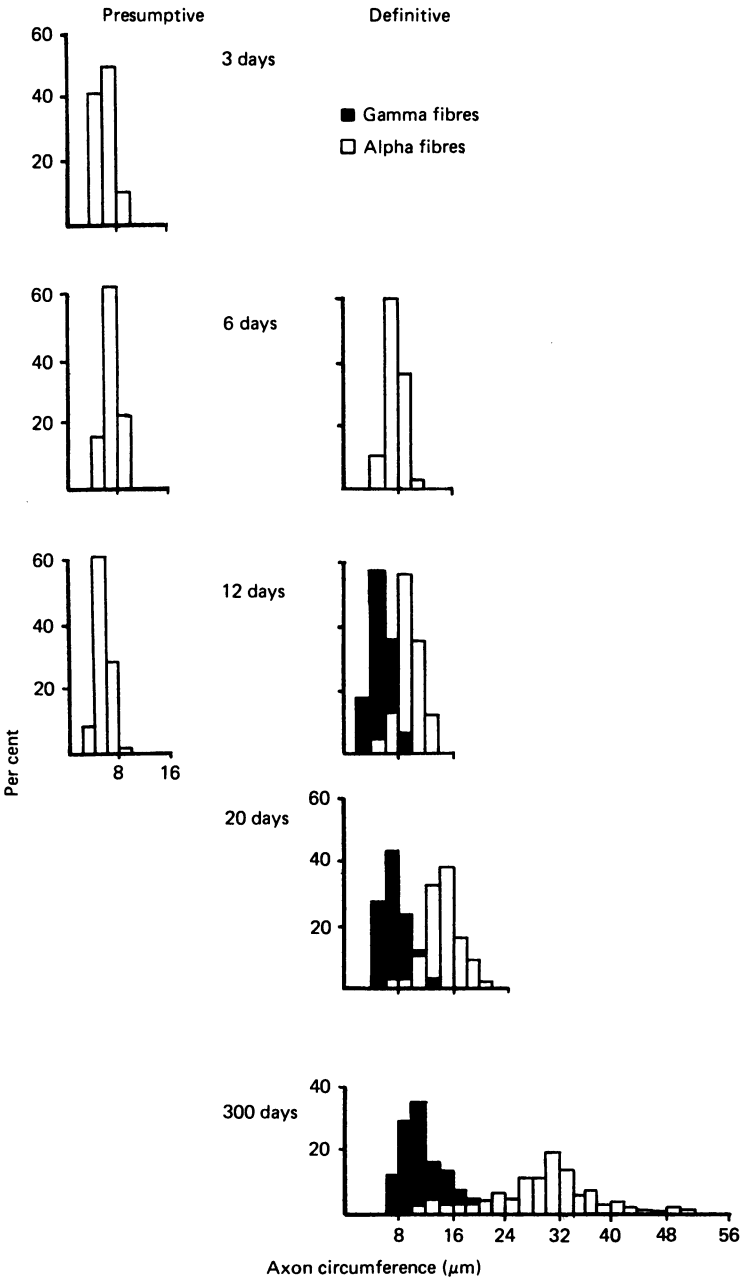

Fig. 3. Percentage frequency histograms for the circumferences of all myelinated axons possessing presumptive or definitive transitional nodes.

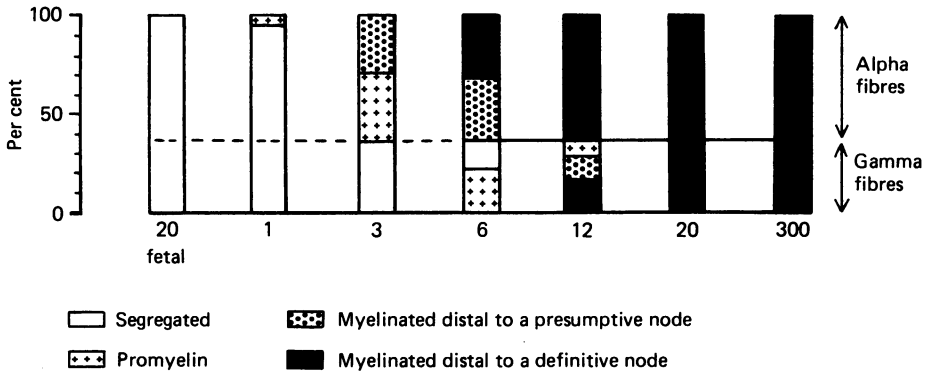

Fig. 4. To illustrate age changes in the proportions of the various classes of alpha and gamma axon ensheathed by the transitional Schwann cell.

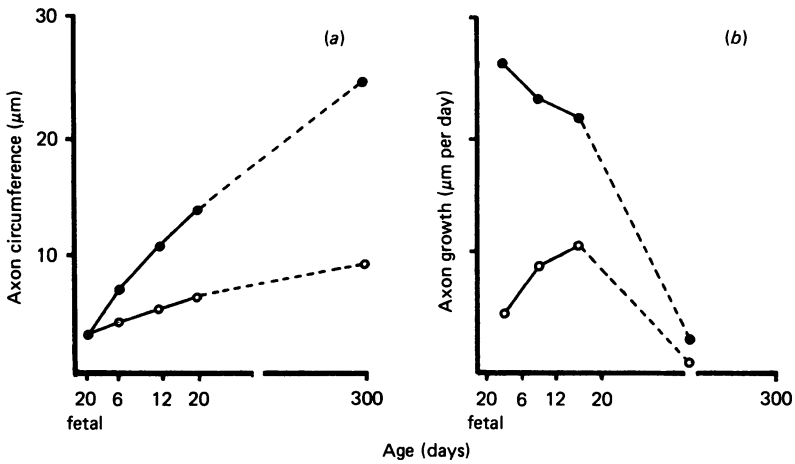

Fig. 5(*a*, *b*). o—o, Gamma fibres; ●—●, alpha fibres. (*a*) Age changes in the mean circumference of peripheral segments of alpha and gamma axons. (*b*) The growth of axon circumference (microns added per day) of alpha and gamma axons over the intervals shown. (Based on values for central circumference.)

gamma axons. At 20 days fetal the proportion of the 300 day circumference reached was 30% for gamma but only 11% for alpha axons. The daily rate of increase in axon circumference was slightly greater at peripheral than at central levels at each age. The growth in calibre of alpha fibres was maximal between 20 days fetal and 6 days postnatum (Fig. 5*b*). By contrast, the growth rate of gamma fibres was low between those ages and reached its highest values between 6 and 20 days. The period of maximum growth in calibre was accompanied by myelination of the great majority of axons in each size class. Most alpha axons began to be myelinated between 20 days fetal and 6 days postnatum and most gamma axons between 6 and 12 days postnatum.

DISCUSSION

Axon growth

Rapid axon growth takes place up to 3 weeks after birth. Between 20 days fetal and 6 days postnatum this is accompanied by the differentiation of alpha and gamma axon populations, manifested first in the broadening of the unimodal distribution and

then in the transformation of this to a bimodal form between 3 and 6 days. (This may occur towards the earlier part of that interval, since one 3 days old animal had a bimodal distribution.) The proportions of both axon groups are relatively fixed by 6 days. Alpha axons make up 60–70 % of the total at all subsequent stages. The appearance of the bimodal distribution is likely to be due to a spurt of growth involving presumptive alpha axons, the growth rate of which is most rapid over the 20 days fetal to 6 days postnatum interval. At the same time, most of these become myelinated (Fraher & Kaar, 1985) with the result that the two components of the 6 day calibre distribution are further distinguished from one another morphologically; the alpha axons are myelinated but the gamma axons are not. The most rapid phase of growth of gamma axons occurs somewhat later.

At ages before alpha and gamma classes of axon can be statistically distinguished from one another, inferences may be drawn regarding their growth by examining changes in the proportions of axons in the various ensheathment classes (Table 2; Fig. 4). It is likely that *promyelin axons at* 1 *day* are presumptive alpha axons, since their mean calibres are greater than those of the remaining segregated axons. Moreover, alpha axons would be expected to differentiate before gamma axons. The axons which are *myelinated and possess presumptive transitional nodes at* 3 *days* are also likely to be prospective alpha axons for three reasons. Firstly, their mean circumference is considerably greater than that of all other axons at that age. Secondly, it is significantly greater than that of myelinated gamma axons when these are first observed, i.e. at 12 days, even though this is at a much later stage (Table 2). Thirdly their circumference distribution is towards the right of that for myelinated gamma 12 day axons, despite the age difference (Fig. 3). They form a similar proportion (approximately 30 %) to that made up by all axons which possess definitive transitional nodes at 6 days (Table 2), which for similar reasons are probably alpha axons. It is likely that between 3 and 6 days the former category of axon matures to form the latter. *Promyelin axons at* 3 *days* are also likely to be presumptive alpha axons since the combined proportion which they make up with myelinated axons possessing presumptive transitional nodes is similar to that eventually made up by all alpha myelinated axons. Also, their mean circumference is larger than that of segregated axons at the same age. They constitute a similar proportion (approximately 30 %) to that made up by all axons which are myelinated and which possess presumptive transitional nodes at 6 days (Table 2). It is likely that between 3 and 6 days the former category of axons matures to form the latter.

Presumptive alpha and gamma axons can therefore be distinguished from one another from shortly after birth. Between 1 and 3 days postnatum the great majority of presumptive alpha axons become promyelin or myelinated in form, while most presumptive gamma axons remain segregated. At 6 days most myelinated axons are likely to be prospective alpha axons. About half possess presumptive and half possess definitive nodes. All possess definitive nodes at 12 days. The separation of future alpha and gamma axons is therefore well advanced when the mean diameter of gamma axons is about 1 μm and that of alpha axons is about 2 μm, before myelination of gamma axons has begun. After this they follow separate maturation tracks. These calibres are considerably smaller than the diameter of 3 μm proposed by Berthold, Nilsson & Rydmark (1983) for separation of the two axon categories in the cat.

Myelinated gamma axons possessing definitive transitional nodes are first seen at 12 days. Those axons possessing presumptive transitional nodes at this stage have a

similar calibre distribution and are also likely to be gamma axons. By 20 days, all gamma axons have definitive nodes. It is likely that the delay in myelination of gamma axons results from selective prolongation of the segregated stage of their development, since most or all of them are probably still segregated at 3 days and about 40% of them remain so at 6 days (Table 2, Fig. 4). Since axons begin to be segregated in the lumbar roots at 18 or 19 days fetal, when this process is taking place in the proximal segments of cervical roots (Fraher & Rossiter, 1983), the segregated phase is about twice as long for gamma as for alpha axons. Later stages of gamma axon development do not appear to be selectively prolonged. Approximately 40% of gamma axons pass from the promyelin to the myelinated definitive nodal stage between 6 and 12 days, while approximately 50% of alpha axons pass through the same stages between 3 and 12 days. There is thus a rough similarity between the time intervals involved for at least a proportion of the two groups.

Circumference growth rates for presumptive alpha and gamma axon groups as identified above can be examined separately for each group as a whole over the intervals from 20 days fetal to 3 days postnatum and from 3 to 6 days postnatum. The rates for *alpha* axons (0·52 to 0·53 μm/day, respectively) are very similar to that for the whole period (0·53 μm/day), suggesting that these grow at a similar rapid rate over the period. *Gamma* axons on the other hand grow more rapidly over the first interval (0·07 μm/day) than over the second (0·01 μm/day). Thus, their growth is arrested in the period immediately preceding the growth spurt which is related to the onset of their myelination.

Once alpha and gamma classes of myelinated axon have been established and possess definitive nodes, both increase progressively in calibre with time (Table 1). This is not the case with those axon classes which eventually disappear through further differentiation. All three (segregated axons; promyelin and myelinated axons possessing presumptive nodes) reach a maximum circumference one or two stages before they disappear. This may be because presumptive alpha axons differentiate out of each class first, so that towards the later part of its existence it contains only presumptive gamma axons.

Onset of myelination in alpha and gamma fibres

The following observations from the present study indicate that myelination commences in relation to a *smaller* axon circumference in gamma than in alpha fibres. Firstly, promyelin axons at 1 and 3 days probably represent gamma axons (Table 1). Secondly, axons myelinated by the transitional Schwann cell, which possess presumptive transitional nodes, are likely to be mainly alpha axons at 3 and 6 days and gamma axons at 12 days. The mean circumference in the 3 and 6 day groups are statistically significantly *greater* than in the 12 day group. Thirdly, when definitive transitional nodes are first observed in relation to them, the overall mean abnodal circumference of alpha axons (7·5 μm) is significantly greater than that of gamma axons (5·1 μm). Maturing alpha axons therefore appear to be less myelinogenic than gamma axons, inducing myelination only when they have achieved an absolutely greater calibre than gamma axons.

Relationship of axons and muscle development

The development of a bimodal axon calibre distribution takes place in association with the onset of myelination. A similar association is found in the chick optic nerve (Arees, 1978), the anterior commissure of the mouse (Sturrock, 1975) and the cat

first sacral dorsal root (Berthold & Carlstedt, 1982). The rapid growth of the alpha axon population observed in the present study may in turn depend upon the establishment of functioning neuromuscular junctions. This process does not take place simultaneously for all fibres within a given muscle. Muscle cells and neuromuscular junctions are found at various stages of development over a considerable period, but differentiation is generally complete at 4 days in the rat (Kelly & Zacks, 1969; Koenig, 1973). The maximum growth rate of alpha axons is closely related in time to this and so may be at least partly dependent upon it. The delay in maturation of gamma axons is associated with a delay in growth of their circumferences. This appears to be suspended for a time and occurs as a burst between 6 and 20 days. Its timing may also be related to the formation of their terminal connections. In the rat hindlimb, while neuromuscular junctions are seen initially at the polar region of intrafusal muscle fibres at birth, they are not fully differentiated until approximately 12 days after birth (Zelená, 1964).

SUMMARY

The growth of alpha and gamma motoneuron axons was studied from late fetal life to maturity in the fifth lumbar ventral spinal root of the rat. Overall axon circumference distribution is unimodal up to three days postnatum and bimodal subsequently. Alpha and gamma fibre categories can be distinguished from one another before the bimodal distribution appears, since their degrees of segregation differ. By 3 days postnatum, the great majority of presumptive alpha axons are either promyelin or myelinated in form. The two populations follow separate maturation tracks subsequently, alpha axons making up between 60 and 70% of the total. Alpha axons grow most rapidly during the first week, gamma axons during the second and third weeks postnatum. The onset of myelination in both groups coincides with the most rapid period of growth. The delayed rapid growth of gamma fibres is due to a prolongation of their segregated stage; subsequent stages have a similar duration to those of alpha fibres. The periods of most rapid growth in both alpha and gamma fibres may be related to the formation of their respective terminal connections in the muscles. Myelination commences in relation to a smaller axon circumference in gamma than in alpha fibres. The former are therefore more myelinogenic than the latter.

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