J. Anat. (1998) **193**, pp. 195–201, with 3 figures Printed in the United Kingdom

A strong myelin thickness-axon size correlation emerges in developing nerves despite independent growth of both parameters

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(*Accepted 21 April 1998*)

ABSTRACT

The axon determines whether or not it is myelinated by the Schwann cell. At maturity there is a positive correlation between sheath thickness and axon calibre. This correlation is initially very low or absent, but gradually strengthens during development. This increase could come about because the axon continuously controls Schwann cell myelinating activity, so that a given axon calibre is associated with a particular myelin sheath thickness, an interaction which would entail the Schwann cell continuously monitoring and responding to axon size. This seems unnecessarily complex. This theoretical study shows that the strong correlation between the 2 parameters within a given myelinated fibre population may come about in a much simpler way than outlined above. This is demonstrated by modelling the growth and myelination of a hypothetical population, utilising data from earlier studies on cervical ventral motoneuron axon development. The hypothesis tested shows that the only instructive interactions by the axon on the Schwann cell necessary for the strong correlation between the 2 parameters to emerge are for the initiation of myelination, its continuation and its termination. These could result from a single stimulus being switched on, persisting for a time and being switched off. Under this influence, the Schwann cell is assumed to proceed to form the myelin sheath at a constant rate which it itself inherently determines, in the absence of any quantitative influence exerted by the axon. This continues until the stimulus for myelination ceases to emanate from the axon. The validity of the hypothesis is demonstrated, because the resulting myelin-axon relationships correspond closely to those observed during development.

Key words: Peripheral nerve; myelinogenesis; axon-glial interactions.

INTRODUCTION

The axon determines whether or not it is myelinated (Weinberg & Spencer, 1975, 1976; Aguayo et al. 1976*a*, *b*). Morphometric studies on mature nerve fibre populations have demonstrated a positive correlation between axon calibre and myelin sheath thickness (Friede & Samorajski, 1967; Williams & Wendell-Smith, 1971; Hildebrand & Hahn, 1978; Fraher, 1989, 1992). This is also true when more complex parameters such as axonal surface area, internodal length and sheath volume are related to one another (Murray & Blakemore, 1980; Friede & Bischhausen, 1982; Smith et al. 1982; Friede et al. 1985; Jacobs, 1988). Developmental studies show that the correlation is initially very low or nonsignificant, but becomes stronger as maturation proceeds (Friede & Samorajski 1967, 1968; Williams & Wendell-Smith, 1971; Fraher, 1972, 1973, 1976, 1978*a*, *b*; Friede, 1972; Low, 1976; Schröder et al. 1978). These findings may be interpreted as indicating that the axon continuously controls sheath thickness throughout development in such a way that an axon of a given calibre tends to be surrounded by a myelin sheath of a specific thickness. Thus, the Schwann cell has been tacitly regarded as continuously monitoring the perimeter, surface area, or even the volume of the axon segment to which it is apposed, and responding

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by producing a myelin sheath of an appropriate thickness. Furthermore, the impression that the axon-Schwann cell interaction is essentially numerical and mechanical could be strengthened by the fact that the onset of peripheral myelination takes place in relation to a minimum axon calibre of about 1 μm (Friede, 1972). However, the wide variation in the calibre of axons undergoing incipient myelination casts doubt on the precision of any such relationship (Fraher, 1972). Nevertheless, axon size does seem to have some relationship to the onset and presence of myelination (Voyvidic, 1989). It is unclear, however, if axon size continues to influence sheath thickness once myelination is under way. Such a relationship as outlined above, involving continuous elaborate feedback interactions between Schwann cell and axon, is called into question by the following observations. Myelin-axon relationships may differ considerably between nerves, even when these are closely similar in type (Fraher, 1989), between levels along the same nerve, or between the same nerve on different sides (Fraher, 1992). This variability argues against a rigid universal quantitative association between axon size and sheath thickness. The present study examines how the correlation associated with a simple axon-myelin interaction changes during development.

It is self-evident that a statistically significant correlation between 2 variables does not necessarily entail a cause and effect relationship between them. It could arise because both are changing in parallel over time, whether by chance, or because both are responding to the same external stimulus, or as part of the general growth of an organism. The aim of this study is to examine the possibility that the degree of correlation observed between myelin sheath thickness and axon calibre could result from an axon-Schwann cell interaction which does not entail continuing control of sheath thickness by the axon during maturation. The hypothetical model examined is based on the following 5 conditions governing the growth and myelination of an axon population. First, each axon stimulates the surrounding Schwann cells to myelinate it. Secondly, the axons commence myelination at similar sizes but at different ages. Thirdly, both the axon and the myelin sheath grow over time. Fourthly, axons cease growth at different stages, so that the fibres of a given mature population vary in axon calibre and sheath thickness. Fifthly, axons and sheaths grow at rates which are independent of one another. The first 4 conditions operate universally in developing myelinated fibre populations (Fraher, 1972; Aguayo et al. 1976*a*, *b*; Fraher et al. 1988). Only the fifth is hypothetical.

MATERIALS AND METHODS

The study uses a hypothetical fibre population. The values used for axon calibre, myelin sheath thickness and the growth rates of both parameters were derived from earlier studies of axon growth and myelination of the same fibre population, namely, that of rat cervical ventral spinal nerve roots (Fraher, 1972, 1973; Fraher et al. 1988; Fraher & O'Sullivan, 1989). These roots are particularly suitable because they consist almost entirely of one class of axons, namely, those of ventral motoneurons, all of which eventually become myelinated. Their simple composition avoids the confusing effects of admixture with other types, such as preganglionic sympathetic axons, which mature at different rates and have different myelin-axon relationships (Fraher, 1974). In the system studied most axons commence myelination over a period of about 15 d (Fraher & O'Sullivan, 1989).

Axon growth and myelination were studied from birth (P1) to 18 d postnatum (P18), at which stage sheath thicknesses have reached values which are well over half those found at 300 d (Fraher et al. 1988). The axon calibre values at which myelination was deemed to commence, as well as the growth rates of axon calibre and myelin sheath thickness, were based on mean internodal values found in a previous study at P2, P7, P13 and P18 (Fraher, 1973). For each age the largest 10 fibres were selected on the basis of axon perimeter and overall means were calculated for both parameters (Table 1). It is reasonable to assume that individual fibres retain their size ranking during growth, so that, for example, the set comprised of the 10 largest fibres remains the same throughout development. Therefore, growth rates calculated using these give a good estimate of the growth of this fibre cohort. Growth rates and hypothetical populations were determined using SPSS and EXCEL software on an IBM compatible PC. Growth rates were calculated by first plotting the mean values against time for each of the ages given above. Of several types of curve fitted to the data, that for a straight line fitted at least as well as any other. This indicates that the daily rates of increase in axon perimeter and sheath thickness

Table 1. *Observed fibre population; 10 largest fibres**

Age	P2	P7	P13	P18
Axon perimeter	6.8	10.1	10.5	13.4
Lamellae	12.3	24.5	35.2	57.3

*Mean values for axon perimeter (μm) and myelin thickness (lamellae). Derived from Fraher (1973).

Table 2. *Proportions of axons commencing myelination between P1 and P15 estimated from Fraher & O'Sullivan (1989), and numbers of axons commencing myelination at each day in the hypothetical population.*

Days	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
Proportion	36	7	7	4	4	4	4	4	4	4	4	4	1	1	1
Axon number	200	40	40	20	20	20	20	20	20	20	20	20	5	5	5

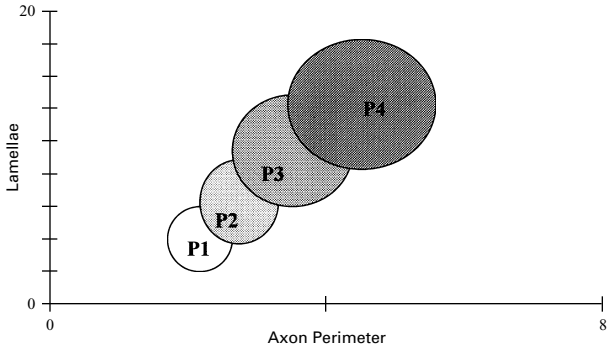

Fig. 1. Diagram illustrating the growth of one hypothetical cohort of myelinated fibres. This shows the profiles of scatterplots relating sheath thickness to axon perimeter for the *same* cohort of fibres at each of 4 consecutive days (P1 to P4). As the fibre parameters grow independently, the scatterplot shifts upwards and to the right.

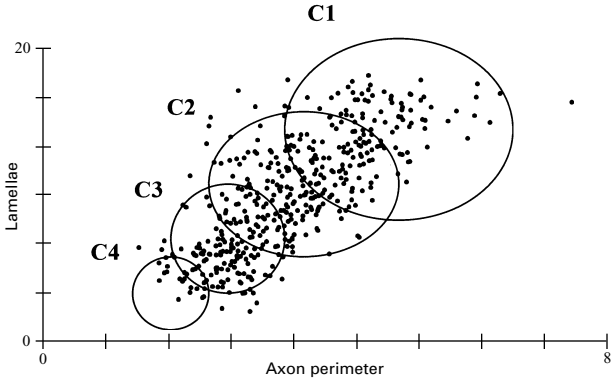

Fig. 2. Composition of the scatterplot at P4. It comprises all of those cohorts, C_1, C_2, C_3 and C_4, which *commenced* myelination at P1, P2, P3 and P4, respectively.

both remained the same over the period studied. At each age, the standard deviation of both parameters was calculated from previous data (Fraher, 1972, 1973).

The growth rates thus derived were used to describe the growth of a hypothetical population of cervical ventral motoneuron axons. Within this population, around 85% of all presumptively myelinated fibres come to possess a myelin sheath between P1 and P15 (Fraher & O'Sullivan, 1989). The proportion of all axons which begins to myelinate on each day over this period was calculated (Table 2). The hypothetical population of presumptively myelinated fibres was divided into 15 cohorts, one of which commenced myelination on each day from P1 to P15, inclusive. The number of axons was set according to proportions already determined (Table 2). The growth and myelination of the axons within each cohort were calculated as follows, according to the growth rates previously determined.

1. In accordance with earlier studies (Fraher, 1972, 1973) the mean axon perimeter and standard deviation at the beginning of myelination were set at 4.1 ($\pm$0.84) μm, and the distribution was assumed to be normal (Kaar & Fraher, 1985). Each axon was assigned a perimeter value, derived at random from that hypothetical cohort. Myelin sheath thickness distribution was also assumed to be normal, with a cohort mean of 5.6 lamellae and a standard deviation of 2.5 lamellae, derived from the same samples as used for the axon calibre data (Fraher, 1972, 1973). As for axon calibre, sheath thickness values were derived at random from this population, for an equal number of fibres. Since the basic hypothesis being tested assumed that both axon calibre and sheath thickness grow at rates which are independent of one another, there is no necessary correlation between the 2 parameters within each cohort. Accordingly, lamellar values were assigned at random to axon perimeter values. The resulting pairs of values were plotted for P1 (Fig. 1).

2. Axons and myelin sheaths within that cohort were then assumed to grow for 1 d, their mean values increasing according to the rates derived from the actual population (Table 1). Using these new mean values, and the standard deviations based on the observed population, a set of pairs of values for P2 was derived in the same way as for P1. These were then plotted to show the scatterplots for the same cohort of fibres at P2 (Fig. 1). The process was repeated for each day up to and including P18.

3. A similar procedure was carried out for each of the cohorts of axons which were assumed to commence myelination at P2 and at each subsequent day up to and including P15. Figure 1 illustrates the growth of such a cohort over 4 d. After P15 no new cohorts were added, since it was assumed that no new fibres entered the myelination process after that. Thus, the growth of each of 15 cohorts of fibres was described from the stage at which it commenced myelination until P18.

The population of fibres at any given day consisted of those cohorts which commenced myelination on and previous to that day. Thus the scatterplot for the fibre population at P4 consisted of the combined scatterplots for the 4 cohorts which commenced myelination from P1 to P4 inclusive, and which had been growing for 3, 2, 1 and 0 d, respectively. Figure 2 shows such a hypothetical scatterplot consisting of four cohorts superimposed.

RESULTS

Within the hypothetical population the cluster of points representing each cohort of fibres shifted upwards and to the right with time (Fig. 1), reflecting the concomitant but unrelated growth of axon perimeter and sheath thickness. In each case, there was generally no statistically significant correlation between the 2 parameters *within* any of the sets of point clusters (Table 3). The values of the correlation coefficient varied randomly and showed no trend. The actual fibre population at each age was represented by plotting fibre cohorts *together*. Thus, at P4 for example, the combined cohorts 1, 2, 3 and 4 comprised a scatterplot elongated upwards and to the right, representing the groups of fibres which at that stage were undergoing myelination (Fig. 2). At P15 and subsequently all 15 cohorts were present, and by P18 (Fig. 3*b*) cohort 15 has already been undergoing myelination for 3 d. A statistically significant correlation developed between the parameters as age advanced and, after some apparently random variation in the early stages, its strength increased progressively with time (Table 4). Comparison of the correlation coefficients (Table 4) and scattergrams

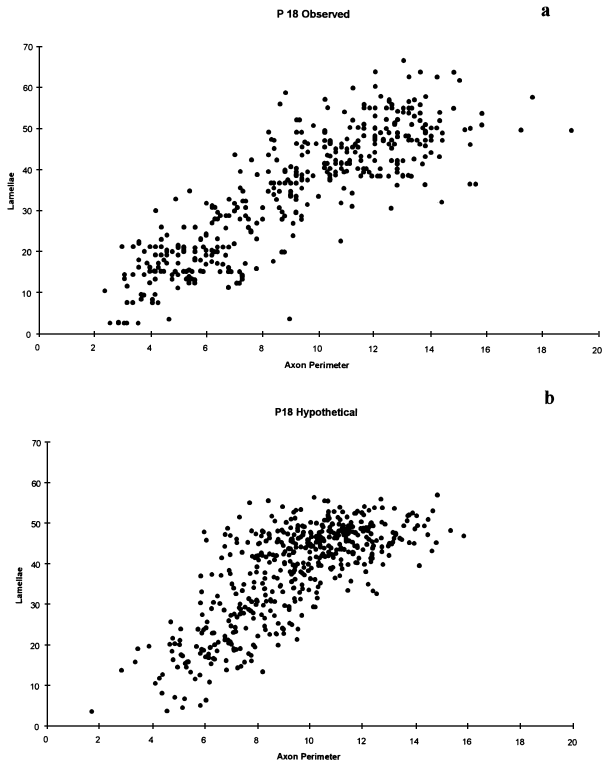

Fig. 3. Scatterplots relating myelin sheath thickness to axon perimeter at P18 for (*a*) the observed population, derived from Fraher (1972) and (*b*) the hypothetical population. Note the close similarity of the scatterplots. A well defined secondary cluster of points (representing gamma fibres) is present at the lower left of the observed plot (see text).

(Fig. 3) with corresponding values for the equivalent stages among the observed fibre populations, showed that both changed similarly with time. It is noteworthy that the hypothetical scattergram at P18 (Fig. 3*b*) included a dense cluster of points extending to the right from its upper end. Such a cluster was seen in scattergrams for mature roots (Fig. 3*a*) (Kaar &

Table 3. *Correlation coefficient* (*r*) *relating myelin sheath thickness to axon perimeter within each fibre cohort of the hypothetical population at P 18* (*Cohort 1 commenced myelination at P1, Cohort 2 at P2 etc.*)

Cohort number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
r	0.32	0.00	−0.28	−0.46	0.32	−0.11	−0.14	−0.22	−0.28	0.02	−0.12	−0.07	0.07	0.37	−0.09

* Statistically significantly different from zero ($P < 0.05$).

Table 4. *Correlation coefficients* (*r*) *relating myelin sheath thickness and axon perimeter*: *r observed from Fraher* (*1972*); *r hypothetical from present study*

Days	P1	P2	P3	P4	P5	P9	P12	P18
r observed	0.16	0.12*	0.00	0.19*	0.25*	0.66*	0.83*	0.88*
r hypothetical	0.06	−0.03	0.29*	0.20*	0.29*	0.60*	0.69*	0.74*

* Statistically significantly different from zero ($P < 0.05$).

Fraher, 1985). One minor difference between the hypothetical and observed scattergrams was the absence of a definite cluster respresenting gamma fibres in the former. This was because these had not been modelled.

DISCUSSION

Over the period studied, fitting of curves to the observed data shows that axon perimeter and sheath thickness growth are each best described by a straight line. Thus, each grows at a constant rate from P1 to P18. This finding more readily permits the interpretation that growth of the 2 parameters is independent than if their growth curves were similar and nonlinear. Were that to be so, and the growth rates of both varied in a similar and corresponding manner with time, the case for a continuous causal interaction of axon and Schwann cell would be strengthened.

The form of the hypothetical scattergram generated for P18 (Fig. 3*b*) corresponds to that found in mature ventral roots (Fig. 3*a*). However, the hypothetical distribution (Fig. 3*b*) lacks a well developed cluster of points representing gamma fibres, such as is present in the observed scatterplot (Fig. 3*a*). This is because the present study models alpha fibre development only. The absence of the gamma cluster accounts for the slightly lower values of the correlation coefficient for the hypothetical compared with the observed distribution (Table 4). To render the populations more truly comparable, the gamma fibre group may be removed from the latter (Cox, 1966; Kaar & Fraher, 1985) and the correlation coefficient recalculated. The resulting r value is 0.73, which is virtually indentical to that of the hypothetical population. Accordingly, the hypothetical myelin-axon relationships are good descriptors of those of the observed population. This clearly demonstrates the validity of the central hypothesis, namely, that a strong positive correlation between myelin sheath thickness and axon perimeter may gradually develop within a population of myelinating axons when both parameters grow independently of one another, in the absence of any continuing cause-effect relationship between them. The model accurately describes fibre growth and the development of the correlation between axon perimeter and myelin sheath thickness, in accordance with the findings of a large number of studies on myelinated fibre development (Aguayo et al. 1976*a*, *b*; Williams & Wendell-Smith, 1971; Fraher, 1972, 1973; Schröder et al. 1978; Fraher & Kaar, 1985; Kaar & Fraher, 1985). Growth of the 2 populations was modelled up to P18 only, since directly comparable observed data were available over this period. It seems likely that extending the period of modelling to older age groups would continue to yield a strong correlation on the continuing assumption of the absence of a cause-effect relationship of the kind proposed.

The proposed hypothesis entails a simple set of interactions between the axon and the Schwann cell. These may involve only a single axonal stimulus which triggers myelination. Myelination then continues as long as the stimulus persists and terminates as soon as it is switched off. Models where the myelinating Schwann cell is considered to continuously monitor axon calibre and match its activity in elaborating new myelin membrane in response to changing axon size values would entail far more complex feedback mechanisms of intercellular signalling, which the present study shows to be unnecessary.

The interactions involved in the proposed hypothesis are likely to include upregulation and downregulation of neuronal genes for the synthesis of intercellular messengers or surface marker molecules involved in initiating and/or terminating the myelinating activity of the Schwann cell. These events are likely to take place following the establishment of contact between the axon and its target developing muscle fibres. This, through retrograde axoplasmic flow may influence the perikaryon to initiate an axon growth spurt (O'Brien et al. 1998) and also to produce chemical messengers for myelination which pass distally along the axon. Consequent expression of the myelinogenic signal by the axon could trigger the cascade of events in the Schwann cell leading to myelin formation.

The nature of the myelinogenic stimulus remains unclear. Several reviews summarise the molecular biological profiles of developing axons and related glia (Salzer, 1995; Scherer & Salzer, 1996; Colello & Pott, 1997). Despite some evidence that myelin associated glycoprotein (MAG) and neural cell adhesion molecules (N-CAMs) could be implicated, no specific axonal signal which unequivocally induces Schwann cell myelination has yet been identified. It could be a soluble chemical messenger released by the axon which binds to the Schwann cell plasma membrane. Alternatively, the myelination trigger could consist in subtle changes in the type and pattern of cell adhesion molecules associated with the axolemma, such as the NCAMs, L1 and N-cadherin (Jessen et al. 1987*a*, *b*; Martini & Schachner, 1988; Low, 1989; Martini et al. 1992; Salzer, 1995). Whatever the nature of the stimulus, the Schwann cell's response includes mesaxonal elongation and

spiralisation, accompanied by upregulation of the expression of myelin sheath components, such as MAG, protein zero, and myelin basic protein (Jessen & Mirsky, 1991). This is followed by compaction to produce the early myelin sheath, and then elongation of the myelin spiral. The present findings support the hypothesis that these biochemical and cytological events in the Schwann cell occur at rates which are determined by the Schwann cell itself and which are not dependent on continuous instructive interactions with the axon. Cessation of myelination could take place through downregulation of the gene(s) responsible for production of the myelinogenic stimulus in the first place, or by upregulation of others responsible for the production of appropriate molecular signal(s) for cessation of myelination. The first of these possibilities is the simpler since it entails one interaction only, which continuously operates between the axon and the Schwann cell. On its withdrawal myelination ceases.

The similarities between the observed and hypothetical scattergrams extend as far as the presence of an extension of points to the right from the upper end of each cluster (Fig. 3*a*, *b*). This has been explained previously on the basis that sheath growth fails to keep up with increase in axon size. The present study shows that it may be accounted for simply by the large size of the initial cohort of myelinating fibres. This, by virtue of its size, has a large variance both of sheath thickness and axon perimeter. As a result the cluster extends further to the right than the smaller clusters commencing myelination at P4 and subsequently. The value of the proposed hypothesis is therefore further strengthened because it predicts this right-ward projection of the scattergram showing the mature myelin-axon relationship. The presence of this projection has been taken as evidence that the myelin-axon relationship is curvilinear (Hildebrand & Hahn, 1978; Biscoe et al. 1982). By showing that the projection can occur on the basis of independent linear growth of both parameters, the present study removes the need for this added complexity in the relationship. However, doubt has already been cast on this interpretation on another count, namely, that the statistical analysis of the relationship as curvilinear was inappropriate (Fraher et al. 1988). The data were bi- or multimodal and were analysed as a single entity. This was less than fully rigorous since correlation and regression analyses should properly be applied only to unimodal distributions. Thus, the 2 distinct components of bimodally distributed data for ventral rootlets, namely, those for alpha and gamma fibre groups, are best analysed separately. It has been shown that each is most appropriately described by a distinct linear relationship between the 2 parameters (Fraher & Kaar, 1985; Fraher et al. 1988).

If the proposed mechanism operates, then myelin sheath growth resulting from the inherent activity of the Schwann cell could be influenced by the specific, including developmental, conditions operating on the Schwann cells in any given nerve. These could include variations between nerves in numbers of Schwann cells per unit length of axon at the onset of myelination and in rates of elongation of the nerve, possibly in accordance with body growth in general (Vizoso & Young, 1948; Thomas & Young, 1949). These local conditions could modify myelinating activity in different ways so as to yield the different myelin-axon relationships which have been shown to exist between, for example, the third, fourth and sixth cranial nerves, between different levels along the phrenic nerve, or even between that nerve on the right and left sides (Fraher, 1989, 1992).

ACKNOWLEDGEMENTS

The work on which this study was based was supported by the Health Research Board of Ireland and the Wellcome Trust.

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