

Changes in axonal numbers in developing human trochlear nerve

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INTRODUCTION

Over-production of cells, with subsequent death and disappearance of those in excess of requirements, is a rather general phenomenon during embryonic and fetal development, and is known to occur in at least some parts of the nervous system. (Prestige, 1974). Reier & Hughes (1972) also have presented convincing evidence of the degeneration of both myelinated and unmyelinated nerve fibres in the sciatic nerve of young rats and mice, and thought that the true frequency of degeneration might be rather higher than the proportion of fibres actually observed (1-2%). Aguayo, Terry & Bray (1973) have made complete counts of axons and Schwann cells present in the cervical sympathetic trunk in late fetal, newborn, young and adult rats and have found a striking reduction in the axonal populations (from about 16000 two days before birth to 5470 eight days later) while Schwann cells progressively increased in numbers during the two weeks after birth.

Gamble & Breathnach (1965) and Gamble (1966) opined that similar changes occurred during the development of human fetal nerves, but were unable to produce direct evidence. The trochlear nerves of three human fetuses have now been examined by electron microscopy. Total axon counts, and some estimates of the numbers of Schwann cells present, have been made and compared with the axon counts of the adult nerve published by Zaki (1960). Changes similar to those recorded for developing rats have been found.

MATERIALS AND METHODS

Parts of the trochlear nerve were dissected from human fetuses of 9.2, 10 and 24 cm crown-rump length obtained at hysterotomy and believed to be normal in development. Their ages, from the menstrual histories, were estimated to be 10, 13 and 26 weeks respectively.

The cerebrum and tentorium cerebelli were removed and the brain stem was bathed in chilled 5% glutaraldehyde in phosphate buffer at pH 7.3. In the 10 cm crown-rump length specimen the roof of the orbit was removed (and its contents similarly bathed) to expose the intraorbital part of the trochlear nerve. Short lengths of the trochlear nerve from just proximal to the point where it pierced the dura mater (and, in the 10 cm crown-rump length specimen, from just proximal to the entry into the superior oblique muscle) were then removed for further fixation (about 4 hours) in buffered glutaraldehyde. After several rinses in chilled 10% glucose, similarly buffered, the tissues were further fixed in buffered 1% osmium tetroxide for 1 hour. After dehydration and embedding in Araldite, thick sections (about 1 μm) were stained with a mixture of Azur II and methylene blue (Richardson,

Jarett & Fink, 1960) and examined by light microscopy. Thin sections were mounted on uncoated grids, stained with uranyl acetate, and examined in an A.E.I. EM 6 B electron microscope. Montages were made from thin sections mounted on Formvar covered 'one hole' grids (to avoid difficulties with grid bars), and these were examined in an A.E.I. E.M.M.A. electron microscope.

Serial sections were often obtained, although the exact sequence was not always certain on the grid. Preservation of tissues was generally good. The total cross sectional area of each nerve within its cellular sheath was measured by planimetry. By similarly measuring the area occupied by endoneurial space the area occupied by nervous tissue was obtained.

RESULTS

Intracranial trochlear nerve of fetus of 9.2 cm crown-rump length

In a complete cross section of this nerve (examined at a final magnification of $\times 9000$) 4022 axons arranged in 18 large bundles were counted. They occupied an area of $870 \mu\text{m}^2$: the endoneurial space occupied a further $180 \mu\text{m}^2$ of the nerve. Axon diameters ranged from < 0.1 to $1.4 \mu\text{m}$, and no hint of myelin formation was seen. Preservation of axons was generally very good, but some exhibited incomplete plasma membranes, and sometimes, in addition, they were unusually electron-lucent (Fig. 1). No direct evidence of axon degeneration was found, but these atypical profiles may represent local axon degeneration, or perhaps axonal growth cones or locally inadequate preservation. Nerve bundles examined in near serial sections sometimes showed quite marked variations in the calibre of the axons at different levels along their lengths. Such sections also showed, on occasion, that axons present at one level might be absent from another, so that the axon count quoted above (4022) might have been slightly less, or greater, at other levels nearby.

Besides the main trunk of the trochlear nerve described above, there was also present a small and extremely compact bundle of unmyelinated axons lying separately, though fused to and outside the trochlear perineurial (or nerve root) sheath. The preservation of this bundle was not good and its constituent axons could not be counted or measured with any degree of accuracy.

A typical cross section of the nerve contained 20 Schwann cell nuclei, so that one nucleus was present in most of the 18 large nerve bundles, albeit associated with several processes of cytoplasm from the same or another Schwann cell. Clearly the 'true' number of Schwann cells present at any level cannot be computed; there is likely to be considerable overlap of the processes of more proximal and more distal cells. The ratio of axons to Schwann cell nuclei is about 200:1; perhaps a more useful fact is that there was one nucleus/ $43 \mu\text{m}^2$ of nervous tissue in cross section.

Intracranial trochlear nerve of 10 cm crown-rump length specimen

In a complete cross section of this nerve (examined at a final magnification of $\times 7500$) 5975 axons, arranged in 475 bundles, were counted. They occupied an area of $5550 \mu\text{m}^2$: another $2050 \mu\text{m}^2$ constituted the endoneurial space of the nerve. Axon diameters ranged from < 0.1 to $2.1 \mu\text{m}$. Seventy axons were singly invaginated by processes of Schwann cell cytoplasm, but the remainder occurred in more complex bundles of up to 60 axons which were associated with several processes of Schwann cell cytoplasm. No certain evidence of axonal degeneration was recognized, but electron-lucent profiles, incompletely invested by plasma membrane as in the smaller specimen, were occasionally seen.

A typical cross section of the nerve contained 150 Schwann cell nuclei together with many more non-nucleated processes of Schwann cell cytoplasm. Few of the 475 nerve bundles contained more than one nucleated Schwann cell, and the great majority contained none. None of the Schwann cell processes ensheathing a single axon contained a nucleus. The ratio of axons to Schwann cell nuclei is about 40:1; this corresponds with one nucleus/ $37 \mu\text{m}^2$ of nervous tissue in cross section. The whole cross section of the nerve was not of uniform appearance, however. Approximately one third of the area was rather sharply demarcated from the rest by slightly increased electron densities in axoplasm and Schwann cell cytoplasm, associated with unusually numerous and irregularly shaped Schwann cell nuclei.

A small nerve fascicle, surrounded by one or two layers of flattened fibroblast-like cells and a little collagen, was found lying just outside the perineurial (or root) sheath. In a typical cross section it consisted of about 600 unmyelinated axons and about 15 processes of Schwann cell cytoplasm. Axon diameters ranged from < 0.1 to $0.3 \mu\text{m}$ (but a few appeared swollen and electron-lucent). The axons were mostly arranged in large bundles, the Schwann cell processes being for the most part of rather simple configuration.

Intraorbital trochlear nerve of fetus of 10 cm crown-rump

A complete cross section of this part of the nerve (examined at a final magnification of $\times 7500$) contained 5300 axons arranged in eight bundles. They occupied an area of $4200 \mu\text{m}^2$; another $500 \mu\text{m}^2$ constituted the endoneurial space of the nerve. Axonal diameters ranged from < 0.1 to $3.1 \mu\text{m}$, the largest profiles being associated with unusual electron lucency and probably not completely normal (Figs. 2A-C).

The complete cross section of the nerve contained 35 Schwann cell nuclei, together with a larger number of non-nucleated processes of Schwann cell cytoplasm. The nerve bundles were extremely large and usually contained several nucleated as well as several non-nucleated processes of Schwann cell cytoplasm. The gross ratio of axons to Schwann cell nuclei was about 150:1; this corresponds with one nucleus/ $120 \mu\text{m}^2$ of nervous tissue in cross section. In some sections, a singly invested axon was seen; its absence in other serials suggests that it had separated from a large bundle at a more proximal level. In this specimen no separate bundle or fascicle of axons was found outside the perineurial sheath of the nerve.

Intracranial trochlear nerve of fetus of 24 cm crown-rump length

In a complete cross section of this nerve 3098 axons were counted in a montage made from 15 prints at an overall magnification of $\times 3000$. They occupied an area of $12200 \mu\text{m}^2$, while another $6100 \mu\text{m}^2$ constituted the endoneurial space. 750 axons (about 25%) were myelinated, 640 axons (about 20%) were singly invaginated and the remaining 1698 (about 55%) were communally invested by Schwann cells. The smallest axons were not identifiable with certainty at this magnification, so further counts were made from prints at final magnifications of $\times 9000$ and $\times 15000$. Of the 1240 axons so counted, 24% (299) were myelinated, 15% (188) were singly invaginated unmyelinated and 61% (753) were multiply wrapped unmyelinated. It is clear that many of the 'singletons' counted at $\times 3000$ magnification consisted of one large and two or three tiny axons enwrapped together. It is also clear that rather fewer 'singletons' in the earlier count were in fact the nodal regions of myelinated axons. Assuming that there were 760 myelinated axons present, and that the percentages obtained by the later counting were correct (or nearly so) then about

480 singleton unmyelinated axons and about 1930 multiply enwrapped unmyelinated axons accompanied them, to give a grand total of around 3170.

Myelinated axons ranged from 0.7 to 2.5 μm in diameter, and as many as 21 lamellae of myelin were counted on the largest fibres. Some evidence of degeneration was very occasionally found in the form of irregularity in the myelin or, more rarely still, as debris, of which two small clumps were found.

Unmyelinated axons ranged from 0.3 to 2.5 μm in diameter when singly invaginated, and from < 0.1 to 1.9 μm in diameter when communally invested. As in the smaller specimens, a few unmyelinated axons exhibited incomplete axon membranes and were sometimes very electron-lucent. The internal structures of myelinated and unmyelinated axons were similar.

The complete cross section of the nerve contained 240 Schwann cell nuclei, of which 82 were associated with myelinated axons, giving a ratio of 9:1. The remaining nuclei could not be allocated with certainty to singly or multiply invested axons in this montage at $\times 3000$ magnification. Of the 188 singly invested axons counted in micrographs at higher magnifications, 19 were associated with Schwann cell nuclei to give a ratio of about 10:1. The 753 multiply invested axons were associated with 40 nuclei, giving a ratio of about 19:1. The overall density of Schwann cell nuclei (240 in 12200 μm^2) may be expressed as one nucleus/51 μm^2 of nervous tissue in cross section.

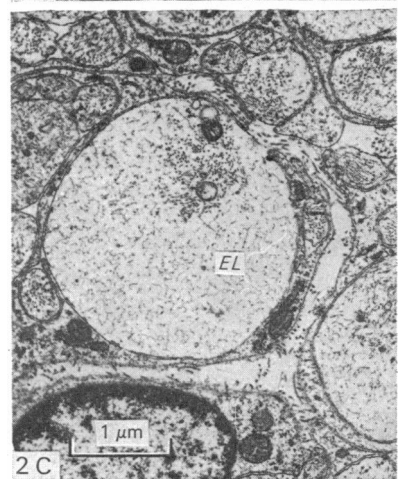
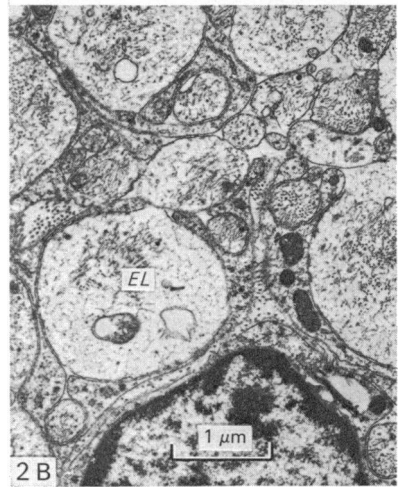
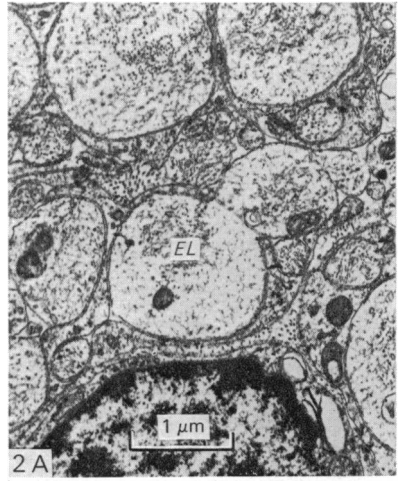
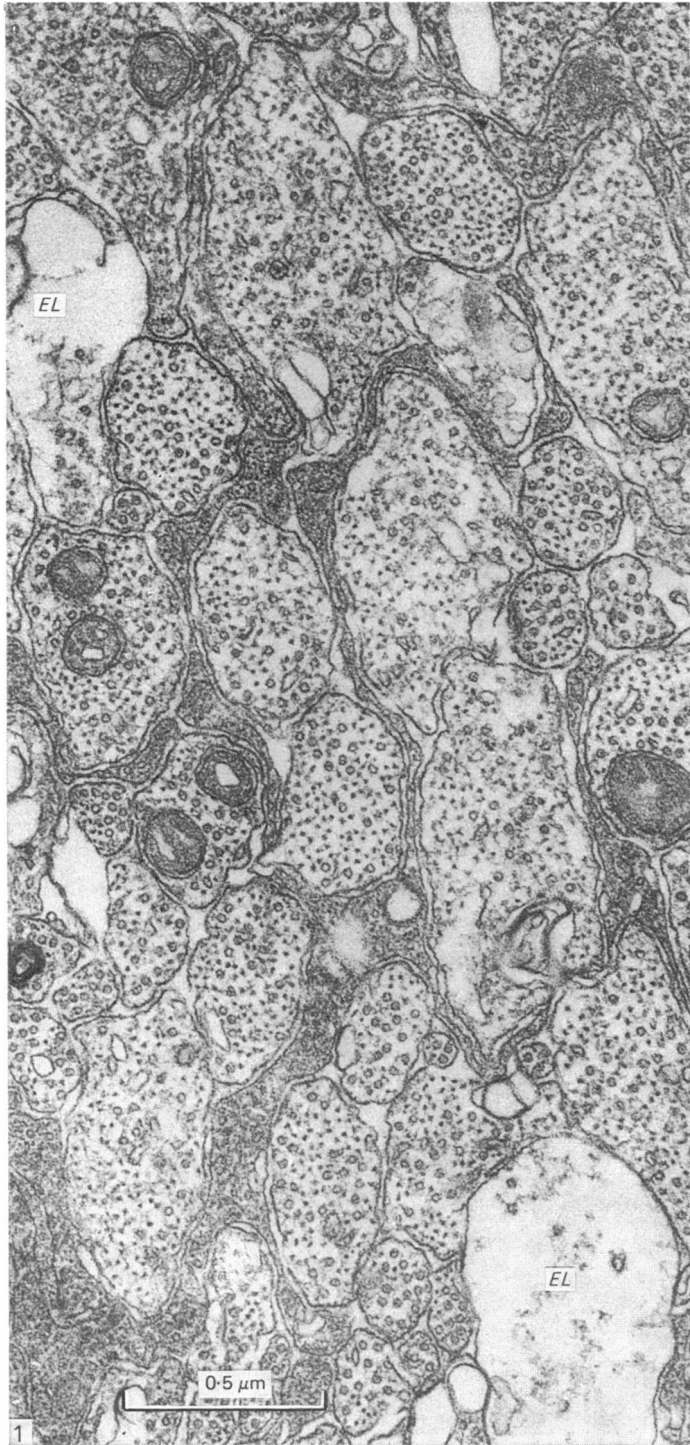
A small nerve bundle of unmyelinated axons was found embedded in the perineurial (or root) sheath of this specimen. It consisted of about 170 axons with diameters ranging from < 0.1 to 1.0 μm ; for the most part they were rather simply invested by three processes of Schwann cell cytoplasm. In the general disposition of its axons and Schwann cells it resembled the younger specimens much more nearly than other bundles of unmyelinated axons in the main trunk of the nerve.

DISCUSSION

The number of axons present in the specimens of fetal trochlear nerve described in this study conforms with the pattern established in the developing nerves of rat and mouse, i.e. an early over-production is followed by quite considerable loss. It is difficult to guess how many unmyelinated axons are present in the adult trochlear nerve in the subarachnoid part of its extent, so one cannot tell how complete was the axonal count recently made by Zaki (1960), who found 2400 myelinated axons by light microscopy. (The earlier literature, cited by Björkman & Wohlfart (1936), shows widely disparate results, with counts of 1200 and 2147 compared with their own estimate of 3400 axons at the point where the nerve joins the brain stem). If no unmyelinated axons were present in the adult nerve, then the sequence would be about 4000 (9.2 cm crown-rump length specimen), 6000 (10 cm crown-rump length specimen), 3200 (24 cm crown-rump length specimen) and 2400 (adult). This fall in

Fig. 1. From trochlear nerve of 9.2 cm crown-rump length specimen. Slender sheets of Schwann cell cytoplasm extend and branch among unmyelinated axons. Electron-lucent profiles are also present (*EL*). $\times 52000$.

Fig. 2 (A-C). From intraorbital part of trochlear nerve of 10 cm crown-rump length specimen. Electron micrographs have been made from three near-serial sections, and show slender cytoplasmic processes of Schwann cells separating and wrapping unmyelinated axons. The electron-lucent profiles (*EL*) shown in A, B and C contain a group of microfilaments, while the increasingly large remainder of the profile is filled with flocculent material and scattered vesicles, tubules and possibly, mitochondria. $\times 13000$.



numbers is not as great as that found by Aguayo *et al.* (1973), but the trochlear nerve is probably less subject to branching (at least in the intracranial part of its length) than is the cervical sympathetic trunk, and variations attributable to such a cause can presumably be ignored. One may wish that more human fetal specimens of the trochlear nerve had been available for study, but they are difficult to obtain in a state suitable for electron microscopy. It would also be interesting to see, with electron microscopy, whether or not unmyelinated axons are present in the adult trochlear nerve, but suitable adult specimens have not so far been obtainable.

The drop in axon numbers from intracranial to intraorbital parts of the same nerve in the 10 cm crown-rump length specimen is associated with markedly less maturity (fewer, much larger, Schwann cell/axon complexes) in the distal part of the nerve. It is most easily explicable as being due to the failure of new axons, present proximally, to have grown sufficiently far to enter the distal part. There is indirect evidence (e.g. Gamble, Fenton & Allsopp, 1978) that the innervation of one of the extrinsic ocular muscles is a long-continuing process, and it was suggested by Gamble (1966) that the development of a peripheral nerve involves, at some stages, the simultaneous presence of axons which will survive and mature, of axons which will undergo degeneration, of axons undergoing degeneration, and of axons newly invading the nerve.

In his study of the trochlear nerve from several human adults, Zaki (1960) reported around 1000 more fibres in the post-cavernous part of the nerve than had been counted in the part lying in the subarachnoid space. He concluded that there had been an accession of proprioceptive fibres from the ophthalmic division of the trigeminal nerve. Although it is impossible to be certain, it seems likely that Zaki's figures derive from the subarachnoid part of one nerve and from one (possibly the same nerve) or more nerves distal to the cavernous sinus. Swenson (1949) found an increase of only about 150 nerve fibres between the pre- and post-cavernous parts of the trochlear nerve, and it may be that interconnexions between the ophthalmic nerve and the nerves to extrinsic ocular muscles are variable, as Sunderland & Hughes (1946) seem to suggest.

The rather fragmentary and inconclusive evidence of degeneration obtained in this study was not wholly unexpected, for similar results had been obtained in earlier studies of human fetal peripheral nerve (Gamble & Breathnach, 1965; Gamble, 1966). Reier & Hughes (1972) obtained results suggesting that only a very small number of axons might exhibit signs of degenerative change at any one time, perhaps because the process of degeneration was extremely rapid. Even this evidence, however, is not wholly satisfactory for, as Ochoa has recently emphasized (1976), there may be difficulty in distinguishing between degenerating unmyelinated axons and growth cones. The axonal counts of this study, taken with those reported by Aguayo *et al.* (1973) in the growing rat, thus constitute the best evidence of the axonal loss (and a measure of its magnitude) which occurs during nerve development.

Reier & Hughes (1972) also reported, and illustrated, some degeneration of myelinated nerve fibres and although, again, only a very small proportion was involved (about 1–2% at any one time), still some trace of their passing might be expected to persist. However, bands of von Büngner are not a feature of normal adult peripheral nerve so that Schwann cells of degenerated myelinated axons must have been removed.

Despite such loss of Schwann cells as may occur in association with known losses of axons, still the population of Schwann cells increases progressively as development

proceeds, although the density of their packing decreases slightly. The increase in numbers, presumably, continues until all those axons which are going to myelinate have achieved one-to-one relationships with Schwann cells, and thereafter growth of axons is accompanied by elongation of the myelinating Schwann cells (Thomas & Young, 1949).

It is interesting that the 24 cm crown-rump length specimen exhibited very similar ratios of Schwann cell nuclei to axons in both the myelinated (1:9) and the singly invaginated unmyelinated (1:10) nerve fibres. This strongly suggests that the latter category of axons has been singled out by Schwann cells and the axons are at the stage immediately prior to myelin formation. In the 10 cm crown-rump length specimen, however, not one of the 70 singly invaginated axons was found associated with a nucleated Schwann cell process. This suggests equally strongly that these axons had not been singled out by Schwann cells but, rather, were invested by outlying cytoplasmic processes of Schwann cells of much more complex form which, elsewhere in the section, ensheathed groups of axons in common: that is to say, in this specimen myelination was not imminent.

The bundles of small unmyelinated axons lying on or in the sheaths of all three nerves in their intracranial parts have not been identified with certainty. They may comprise a branch of the *nervi tentori*, a meningeal nerve arising from the ophthalmic nerve where this lies in contact with the trochlear nerve in the wall of the cavernous sinus (e.g. Pearson, 1943). Alternatively, they may be sympathetic fibres from the internal carotid plexus passing centrally to innervate vessels of the arachnoid mater and pia mater close to the trochlear nerve.

SUMMARY

Complete axonal counts have been made in the intracranial parts of trochlear nerves from human fetuses of 9.2, 10 and 24 cm crown-rump length. A count was also made in the intraorbital part of the nerve from the 10 cm specimen. Schwann cell nuclei were also counted in typical cross sections, but do not necessarily reflect very accurately the Schwann cell contents of the nerves.

Axonal numbers conform to the propositions (1) that they do not all grow out at once, (2) do not all survive and (3) that degeneration may occur before or after myelination has begun.

It seems inevitable that some loss of Schwann cells occurs in relation to the degeneration of myelinated axons, but there is no evidence for or against such a loss in relation to the degeneration of unmyelinated axons. Overall, however, Schwann cell numbers tend to increase as the number of myelinated axons increases.

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