Pioneer growth cones in virgin mesenchyme: an electron-microscope study in the developing chick wing

By L. K. AL-GHAITH AND J. H. LEWIS

From the Department of Biology as Applied to Medicine, The Middlesex Hospital Medical School, London and the Department of Anatomy, King’s College, London

SUMMARY

Axons grow out along predictable routes to specific destinations. An EM study of the early development of one of the main chick wing nerves - the interosseous nerve - helps to show how they do it. The growing tip of the nerve appears frayed, consisting of nerve fibres occurring singly or in small bundles, taking slightly divergent paths. Most of these pioneer nerve fibres have the characteristic appearance of growth cones. They are not seen to advance along fibrils of the intercellular matrix (as one might expect from some tissue culture experiments), but instead are generally in close contact over their whole surface either with mesenchyme cells or with other nerve fibres. The same is true of the axons at more proximal levels of the developing nerve: they too are never naked, but always in contact either with other axons or with mesenchyme cells. Later nerve fibres follow the pioneers: their growth cones travel out in contact with the pre-existing axons, within the primitive perineurium formed by the enveloping mesenchyme cells, and most often close to the periphery of the fascicle.

INTRODUCTION

Axons grow out along predictable routes to specific destinations. How do they do it? What guides them, and how do the advancing growth cones interact with the surrounding tissues? In the central nervous system, recent observations (Singer, Nordlander & Eggar, 1979; Silver & Sidman, 1980; Katz, Lasek & Nauta, 1980) have suggested that axons grow down preformed channels between the neuroepithelial cells. But in the peripheral nervous system, this idea is scarcely applicable. The mesenchyme of a limb bud, for example, consists of a loose meshwork of cells; the interstices between them extend in all directions, and do not appear to form oriented channels defining a direction of outgrowth for axons. The growth cones must feel their way forward by reference to some less obvious cues.

There have been many studies of the outgrowth of neurites in artificial conditions (reviewed by Johnston & Wessells, 1980). They have been watched crawling along the filaments of a spider’s web, through plasma clots, over the

1 Author’s address (for reprints): Department of Anatomy, King’s College, London, WC2R 2LS.
surface of tissue-culture dishes sputtered with stripes of palladium, and so on (Harrison, 1910, 1935; Dunn, 1973; Letourneau, 1975). The general conclusion has been that growth cones prefer to advance over the surfaces to which they adhere more strongly, and that they will follow the direction of any sort of groove or fibre that they may cling to (Weiss, 1955). They have, for example, been seen to be guided by the orientation of the collagen in oriented collagen gels (Ebendal, 1976).

But the question is, what guides growth cones \textit{in vivo}? Are they perhaps crawling along oriented collagen fibres or along some other fibrillar component of the extracellular matrix in the embryonic limb mesenchyme; or might they rather be feeling their way along the surfaces of mesenchymal cells? There have been several studies of the later stages of development of peripheral nerves, and especially of their Schwann cells and connective tissue sheaths (reviewed by Webster, 1975). At these stages, axons already present can, and probably do, serve as fibrous guides for subsequent growth cones (Wessells \textit{et al.} 1980). But the first growth cones to enter a given region of mesenchyme have no such precedent to follow; and there have been remarkably few studies at the electron-microscope level of pioneer or pathfinder growth cones in normal peripheral tissues. The old observations of Ramon y Cajal (1909), Speidel (1933) and others, valuable as they are, do not disclose the ultrastructural relationships. Tennyson (1970) has, however, described the appearance in transmission EM of growth cones of the centrally directed processes of dorsal root ganglion cells in the rabbit embryo; Roberts (1976) has made a scanning EM study of growth cones in the skin of a \textit{Xenopus} embryo; and Prestige & Wilson (1980) have given a transmission EM account of the development of a limb spinal nerve in \textit{Xenopus}. In these studies, the growth cones generally had a part of their surface closely applied to other cells or to basal lamina, and the rest of their surface apparently naked and exposed to the extracellular medium.

In many target organs such as the skin, the growth cones are not guided along precisely defined paths, but advance haphazardly, to form a loose network of nerve fibres. The guidance problem is rather different for a deep nerve trunk of a limb. Here, the pioneer growth cones must follow a specific route through the mesenchyme, reproducible from embryo to embryo within an accuracy of a few tens of micrometres. In this paper, we examine by transmission EM the advancing tip of the interosseous nerve in the forearm of the embryonic chick wing. We find no sign of an association between the pioneer growth cones and fibrils of the extracellular matrix; instead, we find that almost every growth cone is in close contact with the membranes of other cells, over practically all of its surface.

\textbf{MATERIALS AND METHODS}

White Leghorn chick embryos were used, and staged according to Hamburger & Hamilton (1951).
Embryos were fixed at room temperature either by immersion in half-strength Karnovsky fixative at pH 7-4 (for stages 27 and 29) or by immersion combined with perfusion with 5% glutaraldehyde in cacodylate buffer (0-075 M) at pH 7-4. The perfusion was done by injecting fixative into the beating heart with a glass micropipette. The wing buds were then post fixed with OsO₄ and embedded in Araldite in the standard way. Batches of sections were cut at known intervals, measured by means of the micrometer advance of the microtome knife. Thick sections were stained with toluidine blue to identify the interosseous nerve, and accompanying thin sections were stained with lead citrate/uranyl acetate and examined by transmission EM. The systematic analysis is based on photographic montages of the developing nerve and its immediate surroundings.

To clarify the gross anatomy of the pattern of innervation, a number of normal embryos were fixed and silver-stained as whole mounts by a modified Bodian method, as described elsewhere (Lewis, 1978; Lewis, Chevallier, Kieny & Wolpert, 1981).

**RESULTS**

The interosseous nerve is one of the two main deep mixed nerves innervating the ventral part of the forearm in the chick wing. The EM observations to be reported here are based on five normal specimens: one fixed at stage 26, one at stage 27, one at stage 28, and two at stage 29. In the period from stage 26 to stage 29, the growing tip of the nerve advances from the region of the elbow to the region of the wrist (Roncali, 1970). Its location and gross structure can be seen (Fig. 1) in silver-stained whole mounts. The nerve tapers towards its tip, but does not taper precisely to a point. Instead, the growing end has a slightly frayed appearance, implying that the pioneer growth cones are taking slightly divergent routes. At high magnification (Fig. 2), the individual silver-stained axons in the whole mount are visible under the light microscope, and at the growing end of the nerve they can be followed as they diverge and go their separate ways, either singly or in small bundles. A few terminal expansions resembling growth cones can be seen, but for most of the growing axons no such structure is apparent. (This probably represents a limitation of the staining procedure; reduced silver methods are believed to stain chiefly neurofilaments, and it is known that there are relatively few of these in growth cones (Johnston & Wessells, 1980).) The extent of the ‘fraying’ of the growing tip of the nerve is greatest when it is passing the elbow and again when it is approaching and passing the wrist; the wrist and the elbow are regions where most of the limb nerves divide into several definitive branches. In the segment of forearm that lies between, very few branches develop, and the ‘fraying’ is only just perceptible in a whole mount viewed under a dissecting microscope.

Electron microscopy reveals the same structure, and confirms that the whole mount stain conveys a generally truthful impression. To examine the growing tip of the nerve in the EM, we cut transverse sections of the limb, starting at
Fig. 1. Ventral views of chick wing buds silver-stained as whole mounts, to show the growing end of the interosseous nerve (arrow). (A) Stage 26/27. The pioneer fibres have advanced just beyond the elbow; a number of other nerve branches besides the interosseous nerve will diverge from this broad mass of fibres. (B) Stage 28. The pioneer fibres of the interosseous and median nerves are arriving at the wrist; note the 'frayed ends' of the fascicles. (C) Stage 29. The interosseous nerve fans out as it passes through the region of the wrist; again, a number of distinct nerve branches will ultimately be formed in this region. Scale bar represents 200 μm in each case.
about the level of the elbow, and collecting batches at measured intervals. The decreasing diameter of the nerve gave warning that we were approaching its tip, and accordingly we reduced the interval between one batch of sections and the next, so as to get thin sections of the tip region spaced about 5 \( \mu \text{m} \) apart.

Proximally, the axons were grouped together to form a single well-defined nerve. With increasing maturity, the bundle did, however, become more compact than it was at the outset. Towards the growing tip, an increasing proportion of the axons in the nerve had the appearance of growth cones (Fig. 3): they were filled with small irregular anastomosing membranous vesicles; they were larger than the proximal parts of axons, being typically between 1 and 3 \( \mu \text{m} \) wide, as against a typical axon diameter of 1 \( \mu \text{m} \) or less; and they were irregular in shape, whereas ordinary axons were generally more or less round. Occasionally, the growth cones were seen to contain clusters of round dense-cored vesicles of diameter about 60 nm (Fig. 4), similar to those observed in growth cones of cultured dorsal root ganglion cells (Yamada, Spooner & Wessells, 1971). Growth cones could be distinguished from mesenchymal cell processes by the absence of ribosomes.

At its tip, the nerve split up into several smaller fascicles. Towards the distal ends of these, almost all the nerve fibres had the appearance of growth cones, travelling alone (Fig. 5) or in small bundles (Fig. 6), making their way separately through the mesenchyme. These are the pioneers. The observation that we wish
Fig. 3. Two growth cones (G) at the perimeter of a nerve fascicle, at stage 29. They are in contact both with other axons (A), and with mesenchyme cells (M) that surround the fascicle. × 22000.

To emphasize is that they, and indeed all the other growth cones and axons at more proximal levels of the nerve, were almost always in close contact with the membranes of other cells over most if not all of their surfaces. Thus isolated growth cones are closely enwrapped by mesenchymal cells, and do not pass naked through the extracellular matrix. Likewise, the growth cones in small bundles (typically containing up to about half a dozen members) cohere closely, and again have mesenchyme cells closely applied to those parts of their surfaces which are not in contact with other growth cones. The separation of the membranes along the surfaces of contact is of the order of 10 to 20 nm, and no obvious junctional specializations are visible. In a few cases, a single mesenchymal cell process could be seen to wrap all the way around an isolated growth cone (Fig. 7). This phenomenon should probably not be considered as a beginning of myelination, since true myelination will not start until about 6 days later on in development. In our material, we had no way to distinguish future Schwann cells from other mesenchyme cells.

To make the foregoing statements more quantitative, we counted all the growth cones we could see in our EM photomontages of the interosseous nerve and its immediate surroundings, and classified them as either (A) isolated pioneers amid the mesenchyme or (B) members of small bundles of pioneer
growth cones or (C) members of an established nerve fascicle, and so associated with the more proximal parts of other axons. Each growth cone was examined to see whether the whole of its surface was in contact with other cells, and if not, what fraction was naked. The results were as follows. A total of 271 growth cones was seen. Of these, 214 were within an established nerve fascicle, that is, in contact over at least part of their surface with axons which did not have the appearance of growth cones. The remaining 57 represent pioneer growth cones. Of these, 24 were members of pioneer bundles, and thus in contact with other growth cones over at least part of their surface; while 33 were isolated pioneers. It was only in this last category, of isolated pioneers, that we ever saw any naked regions of the growth cone surface. Of the isolated pioneers 14 had no part of their surface naked. The other 19 had usually only a small part naked, and in most cases even this appeared to be an artifact of fixation – detached fragments of cell membrane often seemed to be adhering to the ‘naked’ regions, and adjacent cells had a slightly torn or damaged appearance (see Fig. 5). Taking an average over all the isolated growth cones, we find that the mean fraction of the surface that appeared naked in our sections was 0·16. Allowing for artifacts, the fraction of the pioneer growth cone surface that was naked in the living tissue was probably much less than this. It is striking, furthermore, that none of the 24 growth cones belonging to pioneer bundles had any part of their surface naked:

Fig. 4. A cluster of dense-cored vesicles (arrow) in a growth cone belonging to a three-fibre fascicle (stage 29). × 22000.
Fig. 5. A large isolated pioneer growth cone (G) at stage 29. Most of the cytoplasm is filled with typical masses of irregular membranous vesicles: the two regions empty of this material (long arrows) may be parts of filopodia. More than 70% of the growth cone surface is in close contact with surrounding mesenchyme cells. The material indicated by the short arrow may be a torn-off fragment of a cell adhering to the growth cone membrane. Note the pinocytotic (coated?) vesicles (asterisks) in mesenchymal cells adjacent to the growth cone, similar to the coated vesicles observed by Singer et al. (1979) in analogous circumstances in the regenerating newt spinal cord.
in every case, there was a sort of primitive perineurium formed of mesenchymal cells closely surrounding the nerve fibres.

In the more proximal segments of the nerve, cell contact relationships were essentially the same. The fascicle was again surrounded by a primitive perineurium of mesenchymal cells, making close contact with the peripheral axons. Deeper within the fascicle, the axons were in close contact with one another, or with the surfaces of the occasional non-neural cells that lay amidst them. Growth cones could be seen within the fascicle. They lay often but not always at its periphery, just beneath the primitive perineurium so that they were commonly in contact not only with precursor axons, but also with mesenchymal cells; no part of their surface was naked.

DISCUSSION

Since growth cones are almost everywhere in close contact with surrounding cells, it appears most likely that they are guided in their choice of path by cell-surface affinities, rather than by oriented fibrils of the extracellular matrix. If the growth cones require extracellular material as a substratum for their advance, it seems that that extracellular material must be clinging closely to cell surfaces.
At the early stages we have examined, and with our fixation and staining procedures, there is indeed very little fibrous material to be seen in the spaces between the mesenchyme cells. Moreover, other studies of the extracellular matrix in developing limb buds (Fallon & Kelley, 1980), using specialized staining techniques, do not reveal any obvious oriented tracks formed of extracellular material that might serve to guide pioneer axons. We must, however, be somewhat hesitant in our conclusions, since we could not study the filopodia which probably extend from the growth cones and may be important in pulling them forward (Bray & Bunge, 1973; Yamada et al. 1971; Roberts, 1976). Such growth-cone filopodia cannot easily be distinguished from the numerous filopodia of mesenchyme cells.

The 'frayed' appearance of the growing end of the nerve implies that the pioneer growth cones follow paths somewhat less precisely defined than the eventual path of the mature nerve. The path of the nerve represents a consensus or compromise choice, in which slight aberrations in the guidance of individual pioneer growth cones are averaged out. It seems that the more proximal parts of the nerve fibres zip up together behind the divergent pioneer growth cones (as described for neurons in culture by Nakai, 1960), and that the growth cones of fibres that enter the limb later follow in contact with the axons of the fascicle that has already been established (Speidel, 1933; Harrison, 1935; Wessells et al.)
159 EM study of chick wing nerve

The observations suggest that the nerve fibres will rather make contact with the surfaces of mesenchyme cells than remain naked, and will rather make contact with the surfaces of other nerve fibres than with the surfaces of mesenchyme cells. Thus the axons tend to become compacted together into a more or less round fascicle, surrounded by a perineurial sheath of mesenchyme cells.

It is noteworthy that a perineurial sheath of sorts is present from the very beginning of the development of our peripheral nerve. Unfortunately, we could not distinguish future Schwann cells from mesodermally derived mesenchyme, and so cannot make any statements about their possible role in guiding or enfolding the pioneer nerve fibres.

We should like to thank Professor Lewis Wolpert for advice and encouragement, and Kuwait University and the M.R.C. for financial support.

REFERENCES


(Received 10 August 1981, revised 27 October 1981)