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LONG-DISTANCE REGULATION OF REGENERATING FROG AXONS

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SUMMARY

Following a nerve crush, damaged frog motor axons regenerate to reinnervate their denervated muscle fibre targets. The axons do this by growing down their old nerve tubes to their former synaptic sites. It is the naked basal lamina of the nerve tube that appears to direct the regenerating axons by providing a substratum over which the axons will preferentially grow. Another possible mechanism for directing the regenerating axons to the end-plates is the release of molecular signals by the cells of the nerve tube or by the denervated muscle fibres. This paper provides evidence that chemical signals do direct the regeneration process. Such signals, released several millimetres from a growing nerve tip, cause it to change direction and bend towards the source. Both the cells of the nerve tube and the denervated muscle fibres release diffusible substances and thereby establish a gradient that affects the regenerating axons.

INTRODUCTION

When a muscle nerve is cut, the distal portions of the axons degenerate leaving behind the cells of the nerve tube and the denervated muscle fibres. To reinnervate their former targets the damaged axons regenerate and grow down the old nerve tube (Letinsky, Fischbeck & McMahan, 1976). This consists of perineurial cells, fibroblasts, Schwann cells and the basal lamina tubes of the Schwann cells.

The role of substances within the peripheral nerve stump in directing the regenerating axons has been extensively discussed since the concept was proposed by Cajal (1928). Recently several experiments have provided strong evidence that supports this idea. The nature of the various signals, however, as well as their means of action and their relative roles remain unknown (Lundborg *et al.* 1982).

A major question is whether the signals used for directing reinnervation of muscle act locally or over long distances. Local signals would be those associated with the surfaces of cells or their basal laminae that could only influence axons coming into direct contact with the surface molecules. Long-distance signals would be those released by cells directing the axons along a concentration gradient.

The nerve tube and the denervated target are obvious candidates for the source of the factors, since axons can grow across gaps between the central and distal nerve stumps. In particular the Schwann cells of the nerve tube have been proposed as the

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most likely source of these signals (Cajal, 1928; Bunge, 1980; Guth, 1969; Haftek & Thomas, 1968; Saito & Zacks, 1969; Varon & Bunge, 1978; Wood, 1976). Experiments on tissue cultures have further demonstrated that Schwann cells can release factors that foster the development of neurones and promote neurite outgrowth (Ard, Bunge & Bunge, 1985; Bard, Edgar & Thoenen, 1982; Berg, 1984; Davis, Manthorpe, Engvall & Varon, 1985; Manthorpe *et al.* 1983; Matthew & Sandrock, 1985; Richardson & Ebendal, 1982; Varon & Adler, 1981). The evidence that Schwann cells can influence axon regeneration is also strengthened by *in vitro* experiments showing that cells of the denervated distal stump can promote outgrowth of regenerating axons over a distance of millimetres (Longo, Manthorpe & Varon, 1982; Lundborg *et al.* 1982; Politis, Ederle & Spencer, 1982; Williams, Powell, Lundborg & Varon, 1984).

Another possible source of an axon growth-directing factor is the denervated muscle fibre target. *In vivo* and *in vitro* experiments have shown that denervated muscle fibres provide factors that cause axons to grow and arborize (Angant-Petit, Mallart & Faille, 1982; Brown & Ironton, 1977, 1978; Brown, Holland & Hopkins, 1981; Fischbach, 1974; Gurney, 1984; Letinsky *et al.* 1976; Lewis, Chevalier, Kieny & Wolpert, 1981; Nurcombe, Hill, Eagleson & Bennett, 1984). The results from these experiments would require that the signal from the denervated muscle should act on the axons over a long distance.

This paper summarizes a series of experiments which was aimed at examining the roles played by the Schwann cells of the nerve tube, their basal laminae, and the denervated muscle fibres in directing axonal regeneration. Using a new preparation, experiments were also aimed at testing for the release of long-distance axon growth-directing signals from these same types of cells. The new preparation has the advantage over intact preparations that the influence of a particular cell type on the pattern of axonal outgrowth can be studied in the absence of other possible contributing or competing cells. The findings demonstrate that both nerve tube cells and denervated muscle fibres release diffusible signals that direct the pattern of outgrowth of regenerating axons and that the signals act over distances of millimetres.

MATERIALS AND METHODS

The experiments were performed on two types of preparation. One set was done on the thin paired cutaneous pectoris muscles which lie just beneath the skin of the thorax of the frog (*Rana pipiens*). The cells of the nerve tube and muscle fibres were first destroyed *in situ* in the frog by freezing. Reinnervation of the old synaptic sites was examined under the electron microscope in the absence of these cells or after allowing regeneration of the muscle fibres (McMahan, Edgington & Kuffler, 1980; Kuffler, 1986*b*). Muscle fibres regenerated from satellite cells in their unfrozen end. To block muscle fibre regeneration the cutaneous pectoris muscles were X-irradiated (Sanes, Marshall & McMahan, 1978).

The second series of experiments was done in the area normally occupied by the cutaneous pectoris muscles. Each cutaneous pectoris muscle was completely removed after cutting its nerve at the point of entry to the muscle. Target cells to be tested, nerve satellite cells and denervated muscle fibres were placed on top of the pectoral muscle that underlies the cutaneous pectoris muscle (Fig. 1).

The target cells were placed at various distances from the end of the central nerve stump and in different locations within the area formerly occupied by the cutaneous pectoris muscle. They adhered to the underlying muscle within a few minutes. Within 3 weeks of removing the cutaneous pectoris muscle a thin connective tissue sheet had formed in its place. The target cells became embedded in the sheet. The transparent connective tissue sheet with the regenerating axons and target cells could then be dissected out. The preparations were examined after periods of 4 weeks to 13 months. The axons were filled with horseradish peroxidase (Kuffler, 1986*a*) or stained with nitroblue tetrazolium (modified from Letinsky & Decino, 1980).

Camera lucida drawings were made of the stained regenerated axons in the transparent connective tissue sheet. The drawings were analysed to determine a single 'directional value' for the orientation of the regenerating axons. This is defined as the mean trajectory of all the regenerating axons with respect to their orientation to the target cells, or, in control preparations (with no target), with respect to the orientation of the central nerve stump. Directional values (DV) approaching zero indicate an increasingly random pattern of axonal outgrowth. Directional values approaching 1·00 indicate an increasingly directed pattern of outgrowth (see Kuffler, 1986*a*, for further details of the analysis).

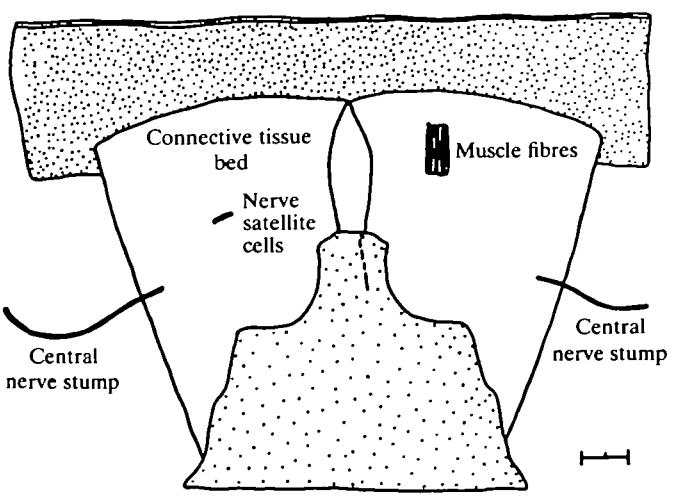

Fig. 1. Schematic diagram of the preparation used to study the influence of various targets on the outgrowth of axons from the cutaneous pectoris nerve. The entire cutaneous pectoris muscle was removed leaving only the central nervous stump intact and a connective tissue bed developed in this place. A 1 mm length of nerve that had entered the muscle stripped of its perineurium (left) or 2 mm lengths of non-synaptic regions of muscle fibres (right) were placed in the area formerly occupied by the cutaneous pectoris muscle and the abdominal skin of the frog was sutured closed. Scale bar, 2 mm.

REINNERVATION IN THE PRESENCE AND ABSENCE OF CELLS OF THE NERVE TUBE AND MUSCLE FIBRES

When the nerve innervating the cutaneous pectoris muscle of the frog is crushed, leaving the cells of the nerve tube and the muscle fibres intact, more than 95 % of the original synaptic sites of the muscle fibres become reinnervated. In further experiments the cells of the nerve tube (Schwann cells, fibroblasts and perineurial cells) and the muscle fibres were killed and the muscle fibres then allowed to regenerate. Under these conditions fewer synaptic sites became reinnervated. The regenerating axons grew through the naked basal lamina tubes of the original Schwann cells, formed numerous branches and contacted the muscle fibres precisely at the original synaptic sites. After 6 weeks, 87 % of the original synaptic sites had been reinnervated (Kuffler, 1986*b*). When the cells of the nerve tube remained but the muscle fibres were removed, only about 50 % of the original synaptic sites became reinnervated (Sanes *et al.* 1978). In the absence of cells of both the nerve tube and the muscle fibre the axons branched less and reinnervated fewer (only 6 %) old synaptic sites (Kuffler, 1986*b*).

These results show (1) that the original cells of the nerve tube need not be intact for restoration of the normal pattern of innervation and (2) that the naked basal lamina has associated with it factors that direct the regenerating axons to the original synaptic sites. However, the more frequent reinnervation of old synaptic sites when the cells of the nerve tube or muscle fibres are present indicates that both cell types contribute to directing or facilitating the regeneration process, although apparently not to the same extent (Kuffler, 1986*b*).

AXONAL OUTGROWTH IN THE ABSENCE OF TARGETS

After removing the entire cutaneous pectoris muscle, the pattern of axonal outgrowth from the central nerve stump was studied. In 23 control preparations the mean directional value was $0{\cdot}18 \pm 0{\cdot}03$ S.E.M. This low value indicates a relatively random pattern of outgrowth and suggests the absence of signals to direct the axonal outgrowth. The positive value, however, indicates that the axons tend to grow more in the direction of orientation of the central stump than in the opposite direction. An example of one such preparation is shown in Fig. 2.

INFLUENCE OF THE SATELLITE CELLS OF THE NERVE TUBE

A 1 mm length of cutaneous pectoris muscle nerve was dissected free, stripped of its perineurium and used as a target. It is estimated that this length of nerve contains from 50 to 100 Schwann cells in their basal lamina sheaths, some epineurial cells and fibroblasts.

In 49 preparations with a 1 mm length of isolated muscle nerve tube as the target a strong influence on the outgrowth of regenerating axons was found. The mean directional value for these preparations was $0{\cdot}79 \pm 0{\cdot}03$ S.E.M. This influence was

exerted when the cells of the nerve tube and the central stump were separated by up to 8·4 mm. An example of this influence is seen in Fig. 3.

Preparations were found in which no contact had been established between the regenerating axons and the nerve tube target. Three such preparations, with the central nerve stump and the target separated by 4·4, 4·5 and 8·4 mm, had a mean directional value of 0·83 ± 0·02 S.E.M. This indicates that the regenerating axons can establish trajectories towards the cells of the nerve tube even before any of the axons have made contact with it.

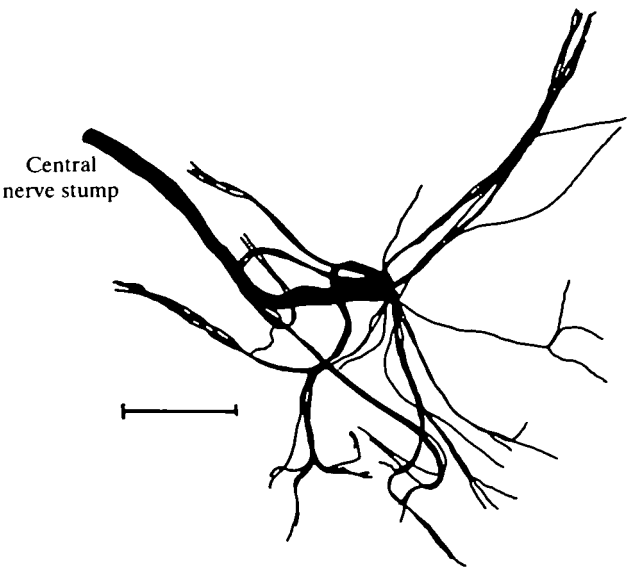

Fig. 2. *Camera lucida* drawing of a control preparation with no target cells for the regenerating axons. In the absence of any target cells the axons grow out of the central stump in a relatively random manner. The mean directional value for the axons is −0·01. Scale bar, 0·5 mm.

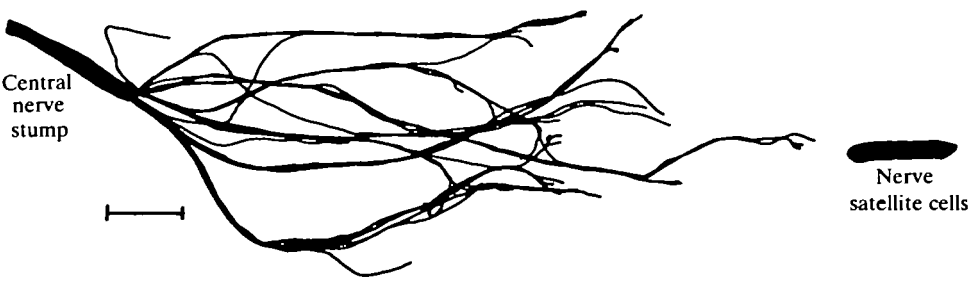

Fig. 3. *Camera lucida* drawing of an experimental preparation with a nerve satellite cell target placed 4·5 mm from the central stump in the area formerly occupied by the cutaneous pectoris muscle. Most of the axons have a trajectory towards the nerve satellite cell target although none of the axons has yet contacted the target cells. The mean directional value for the axons is 0·89. Scale bar, 0·5 mm.

Degenerating axons in the nerve tube do not significantly influence axonal outgrowth. Experiments were made using nerve tube targets that had been denervated for 3 months, by which time all axonal fragments had disappeared. The directional value for these preparations was $0{\cdot}66 \pm 0{\cdot}06$ S.E.M. ($N = 10$) which is slightly lower than the values from preparations in which the nerve tubes were used as targets with no prior denervation (Kuffler, 1986*a*).

The influence of cells of a cutaneous sensory nerve was also examined. Targets of these nerve tubes of 1 mm length had a mean directional value of $0{\cdot}76 \pm 0{\cdot}03$ S.E.M. ($N = 42$), which is not statistically different from the results with targets from muscle nerve ($0{\cdot}78 \pm 0{\cdot}03$ S.E.M.) (Kuffler, 1986*a*).

MUSCLE FIBRES AS TARGETS

Denervated muscle fibres were also examined for their ability to direct the outgrowth of regenerating axons. To avoid possible effects of the cells of the nerve tube and the old synaptic sites, non-synaptic regions of muscle fibres were cut out and used as targets. Muscle fibres cut in lengths of 1·5–2 mm survived for 6–8 weeks as targets.

Regenerating axons grew with trajectories towards the muscle fibres. The mean directional value for 23 muscle fibre targets was $0{\cdot}84 \pm 0{\cdot}05$ S.E.M. When the axons reached the muscle fibres they established functional innervation with them. Stimulation of the nerve caused muscle contractions. The influence of the muscle fibres on regenerating axons was exerted over distances of up to 8·6 mm. An example of one preparation is seen in Fig. 4.

THE NEED FOR LIVE CELLS AS TARGETS

How do the cells of the nerve tube and the muscle fibres direct the regenerating axons? Some possible mechanisms are that the target cells: (a) organize the connective tissue bed over which the axons grow, giving rise to some form of mechanically directing properties; (b) release a signal that becomes attached to the connective tissue bed and is recognized by the axons as they come in contact with it; and (c) release a diffusible signal that forms a gradient up which the axons grow.

An experiment was designed to examine the first two possibilities. Nerve tube targets were placed normally. Reinnervation of the area was delayed by crushing the cutaneous pectoris nerve several millimetres from its cut central stump at 5-day intervals. At the time that the axons would normally have established a trajectory towards the target cells, the target cells were removed surgically or killed *in situ* by freezing. The axons were then allowed to reinnervate the area. No influence on the regenerating axons was found from the former location of the live target cells. Control preparations in which reinnervation was delayed but the target cells were not killed or removed showed directed outgrowth towards the target cells.

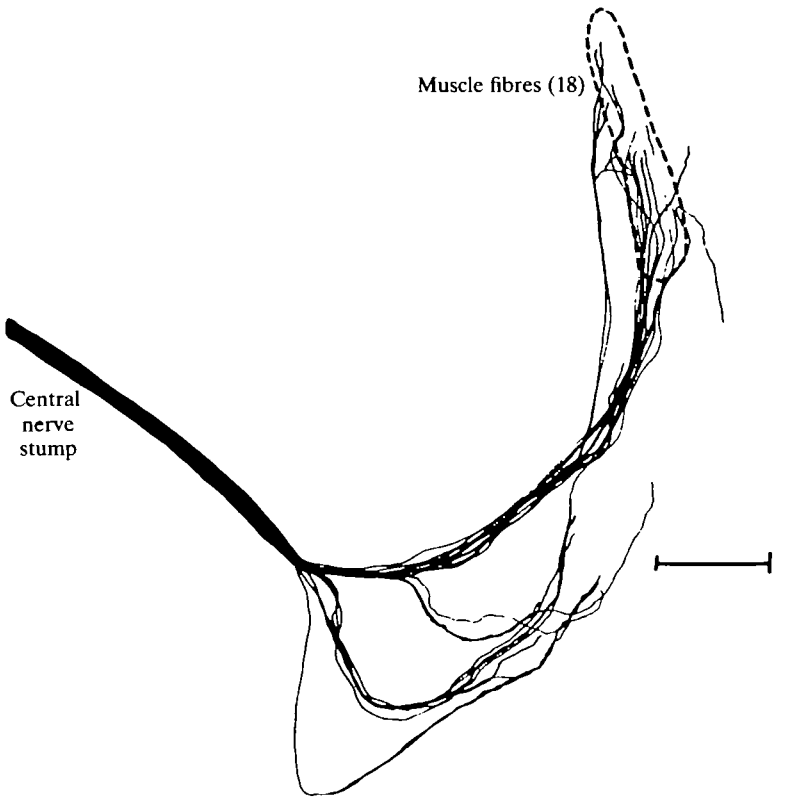

Fig. 4. *Camera lucida* drawing of a preparation with a target of non-synaptic regions of muscle fibres 2·1 mm from the central nerve stump. Examination in the electron microscope showed the target to consist of 18 muscle fibres. The mean directional value of the axons is 1·00. Scale bar, 0·5 mm.

These results indicate that live target cells must be present for directed outgrowth to occur and that no long-lasting changes of the connective tissue bed are caused by the initial presence of the target cells. The mechanism for directing the regenerating axons may therefore be *via* a diffusible signal that establishes a gradient along which the axons grow. Experiments have been designed to test for the release of such diffusible signals from the cells of the nerve tube.

To isolate the target cells from the developing underlying connective tissue bed, and to prevent any cell migration away from the site of implantation, 1 mm lengths of nerve tube can be packaged in filters. Small strips of Millipore filter material (hydrophilic Durapore filters with low protein retention) with a pore size of 0·22 μm can be folded around the cells of the nerve tube and sealed at the edges. Preliminary results show that the target cells within such filters can influence axonal outgrowth. At the same time it was clear that these cells were much less effective than cells that are not in a filter. Several possible explanations for the reduced influence of the cells in the filters are (a) plugging of the holes of the filters by connective tissue and other cells, thus reducing the amount of the signal reaching the outside environment, or (b) the environment of the filters is not optimal for the healthy survival of the cells.

DISCUSSION

Re-establishment of functional innervation after nerve damage requires that the regenerating axons find their way back to their original targets. The information used by the regenerating axons may come from a number of sources. Mechanical guidance is perhaps the simplest method and requires that the axons enter a guide from the point of damage if appropriate sorting out of the axons to the correct targets is to occur.

The presence of specific substances in the Schwann cell basal lamina that makes it particularly attractive to axonal outgrowth has also been proposed (Ide *et al.* 1982; Vracko, 1974). This idea is supported by the finding that regenerating axons grow along the cellular side of a naked Schwann cell basal lamina rather than along its interstitial side (Ide *et al.* 1982). The presence of a polarity in the chemical composition of the basal lamina has now been demonstrated (Tohyama & Ide, 1984; Tohyama, 1985; Yokota, Tohyama & Ide, 1983) and this may explain some of the selective pattern of outgrowth. Strong evidence indicates that this attractive substance may be extracellular matrix glycoprotein (Davis *et al.* 1985; Madison *et al.* 1985; Manthorpe *et al.* 1983; Timple *et al.* 1980; van Evercooren *et al.* 1982), fibronectin (Akers, Mosher & Lilien, 1981; Williams & Varon, 1985) or a heparan sulphate proteoglycan complexed with laminin (Davis *et al.* 1985).

A factor associated with synaptic basal lamina of the neuromuscular junction is recognized by regenerating axons and brings about the differentiation of nerve terminals at old synaptic sites (Sanes *et al.* 1978). This factor is now partially purified (Nitkin *et al.* 1983; Wallace *et al.* 1985). It is not known whether it plays a role beyond directing the differentiation of the axons at a particular site on the basal lamina that comes into contact with it.

The release of specific signals recognized by particular axons could provide a more reliable method of ensuring correct reinnervation. Release of directing signals from the cells of the nerve tube is particularly important when gaps exist. The nerve tube, however, is only a pathway towards the denervated target and therefore the release of signals, acting over long distances from the denervated target of the axons, would provide the precision in the reinnervation of targets.

In this paper evidence has been presented for the presence of signals, acting over long distances, that direct axons to a nerve tube as well as to denervated muscle fibre targets. The finding that cells of the nerve tube inside filters can influence the outgrowth of regenerating axons suggests that the signal may be acting by means of a gradient that is recognized by the regenerating axons.

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