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SUBSTRATE INTERACTIONS AFFECTING MOTOR GROWTH CONE GUIDANCE DURING DEVELOPMENT AND REGENERATION

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SUMMARY

Most serious injuries of spinal nerves or roots in man and other higher vertebrates lead to permanent loss of control of skeletal muscles. In some cases this may be due to a failure of motor axons to regenerate, although even when functional neuromuscular connections are re-established, coordinated use of body and limb muscles may be absent. In both mammals and lower vertebrates, damaged motor axons usually regrow and reform functional connections with muscles, although these connections are often inappropriate. The selectivity of reinnervation is improved by maintaining alignment of the severed ends of the nerve. Thus, factors operating near the lesion site may direct regenerating motor axons into fascicles in the distal nerve stump that lead to inappropriate muscles. The identity of some of these factors is suggested by recent studies of developing systems which have shown that motor axons are directed in their growth. (a) The filopodia of their growth cones sample a limited region of the periphery. If motor growth cones extend too far from their normal pathways they establish connections with inappropriate muscles. (b) Motor growth cones normally extend into regions of embryos rich in the extracellular matrix molecule laminin, and avoid regions containing fibronectin. Moreover, motor growth cones extend on laminin but not on fibronectin substrates *in vitro*. In peripheral nerves, these two molecules are differentially distributed; laminin is expressed by Schwann cells in the endoneurium whereas fibronectin is expressed by fibroblasts primarily in the perineurium. These studies suggest that regenerating motor growth cones may be directed to appropriate muscles if their original fascicles within the distal nerve stump are within filopodial reach but may not be able to escape the fibronectin-rich perineurial sheath once directed into an inappropriate fascicle.

PERIPHERAL NERVE INJURIES

Following lesions that damage spinal roots, spinal nerves or proximal regions of the limbs, axons almost always regenerate through the injury site although functionally appropriate peripheral connections are usually not re-established. When nerve lesions interrupt axons within a nerve, the degree of retrograde damage varies according to the severity of the injury and axons can retract for a considerable distance proximal to the trauma site. At the injury site fibroblasts respond rapidly, proliferate and deposit new and usually poorly structured collagen (Kline & Hudson,

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1981). Distal to the injury site, axons and myelin degenerate although Schwann cells survive, proliferate and can remyelinate regenerating axons. Remyelination is probably triggered by contact of Schwann cells with the axons and the extracellular matrix (Bunge & Bunge, 1978) and may require soluble factors produced by fibroblasts (Moya, Bunge & Bunge, 1980; Bunge, 1983).

In injuries where the nerve is completely severed, fibroblasts produce new connective (scar) tissue that ultimately links the severed ends of the nerve (Thomas, 1966; Jurecka, Ammerer & Lassmann, 1975; Liu, 1981; Lundborg & Hansson, 1981). As the growth cones of regenerating axons enter this region of new connective tissue they interact with a complex mixture of extracellular substrate and cell-surface molecules and their growth may be obstructed. It has been suggested (Kline & Hudson, 1981) that disorganized connective tissue may misdirect regenerating axons in the region of scar formation and may force axons to branch. Some branches may grow into epineurial tissues (Hubbard, 1972; Hudson & Hunter, 1976; Morris, Hudson & Weddell, 1972*a*,*b*,*c*,*d*) and distal extrafascicular pathways (Kline & Hudson, 1981) or they may fail to achieve the diameter and degree of remyelination required to conduct impulses properly. These observations suggest that the permanent loss of function after severe nerve injury may be due to misdirected growth of axons into inappropriate fascicles in the distal nerve trunk (Kline & Hudson, 1981) and/or a failure of axons to regain function after axotomy. In contrast, the excellent anatomical and functional recovery after distal cutaneous nerve crush (Devor & Govrin-Lippmann, 1979; Horsch, 1979) is thought to be due to the Schwann cell basal laminae that remain intact in the damaged region thus guiding regenerating axons into appropriate fascicles leading to their original targets (Thomas, 1974; Richardson, Aguayo & McGuinness, 1983).

REGENERATING MOTOR AXONS

Selective versus *non-selective reinnervation*

These theories explaining the differences between functional and dysfunctional recovery after peripheral nerve injury have been supported by our recent studies of regenerating motor axons. We examined the selectivity of reinnervation of limb muscles by the regenerating axons of bullfrog spinal cord motoneurones. Individual motoneurones were identified by intracellular recording and by retrograde transport of horseradish peroxidase (HRP) applied to peripheral lesions of their axons. Using the criteria of behaviour, peripheral connectivity patterns, the pattern of anatomical positions of cell bodies (Fig. 1), and the pattern of sensorimotor synaptic connections (Fig. 2), we found (Westerfield & Powell, 1983) that normal patterns of motor innervation were re-established after crushing the ventral root, whereas abnormal reinnervation patterns were observed after cutting the root.

Degeneration and regeneration of peripheral axons

Our results suggested that adult spinal cord motoneurones can regenerate, selectively reinnervate their peripheral targets and reform functional connections.

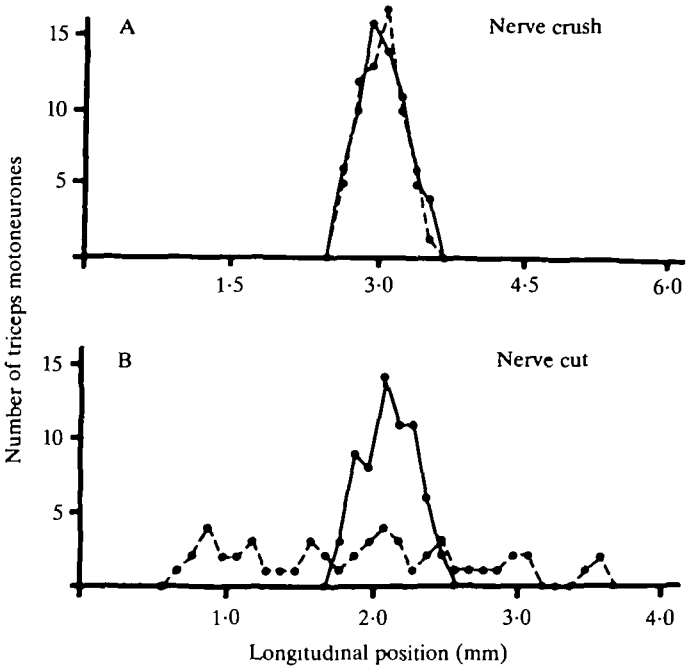

Fig. 1. *Triceps motoneurones are located normally after crushing, but not after cutting* the ventral root. The longitudinal positions within the bullfrog brachial spinal cord of motoneurones (number of labelled motoneurones per transverse section, 50 μm thickness) labelled by retrograde transport of horseradish peroxidase (HRP) injected into the triceps muscles on the control (solid curves) and regenerated (broken curves) sides is shown. The second ventral root was crushed in one animal (A) and cut in the other (B) 7 months before the HRP injection. Motoneurones located in the centre of the brachial spinal cord (the location normally occupied by triceps motoneurones) selectively reinnervated the triceps muscles after crushing the ventral root, whereas inappropriate motoneurones located throughout the brachial region reinnervated triceps muscles after cutting the ventral root. (From Westerfield & Powell, 1983.)

An alternative explanation for the normal innervation patterns following nerve crush is that the motor axons are not actually severed by crushing and simply recover from local damage rather than regenerating their distal processes. This possibility was excluded by subsequent experiments which showed that (a) degenerating axonal profiles are observed in the distal stumps following both procedures (Fig. 3), (b) the number of myelinated axons in individual muscle nerves drops by a similar amount during the first few days following cuts or crushes of the ventral root and then recovers from both procedures with a similar time course (Fig. 4), and (c) functional recovery from either procedure is observed first in proximal muscles as one would expect if axons regrow into the limb (Fig. 5)

Proximity of the lesion to the cell bodies

The difference in the selectivity of reinnervation following cut or crush injuries could be due to the severity of the motoneurones' reaction to axotomy. Perhaps crushing the root is a milder procedure, with respect to retrograde changes, that

leaves the neurone with a better ability to regenerate. This idea was tested by cutting the spinal nerve distal to the ventral root in four animals, thus moving the site of the lesion farther from the cell body and reducing the retrograde reaction. Even when the nerve was cut far from the spinal cord, however, motoneurones failed to reinnervate their peripheral targets selectively.

Role of sensory innervation of the motoneurones

Since sensory innervation was intact after the ventral root had been crushed, we wondered if motoneurones might recognize their original target muscle because they

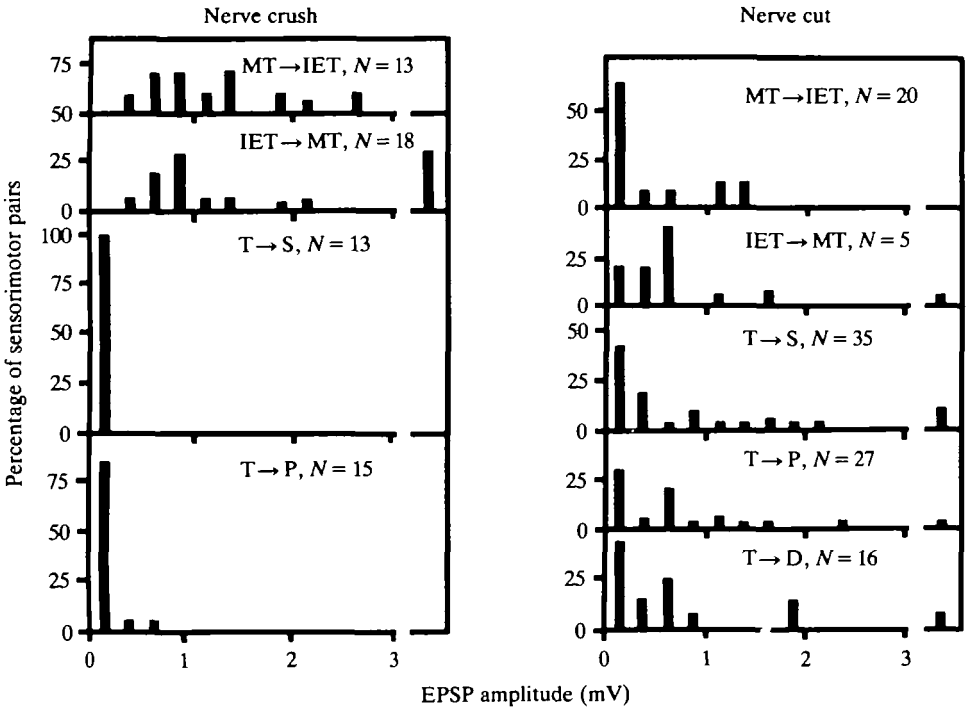

Fig. 2. Triceps sensory neurones project specifically to triceps motoneurones after crushing but not after cutting the ventral root. Normally, triceps sensory neurones project specifically to triceps motoneurones and produce strong, large-amplitude, excitatory postsynaptic potentials (EPSPs). This normal pattern of innervation is observed following recovery from a crushed second ventral root (left panel), but not after cutting the root (right panel). The percentage of EPSPs of a given amplitude recorded in motoneurones (identified by antidromic activation of the motor axon in muscle nerves in the limb) upon stimulation of triceps sensory neurones is plotted for several different sensorimotor pairs. It is possible that the projection of triceps sensory neurones onto inappropriate motoneurones after cutting the root was due to sprouting of the sensory axons, although this seems unlikely since the EPSPs were of normal amplitude and were found only in motoneurones located in the region normally occupied by triceps motoneurones. Data were obtained from six frogs that recovered from crushed roots and five frogs that recovered from cut roots. MT, medial triceps; IET, internal and external triceps; T, triceps; S, subscapularis; P, pectoralis; D, deltoideus. (See Frank & Westerfield, 1982, for methods.)

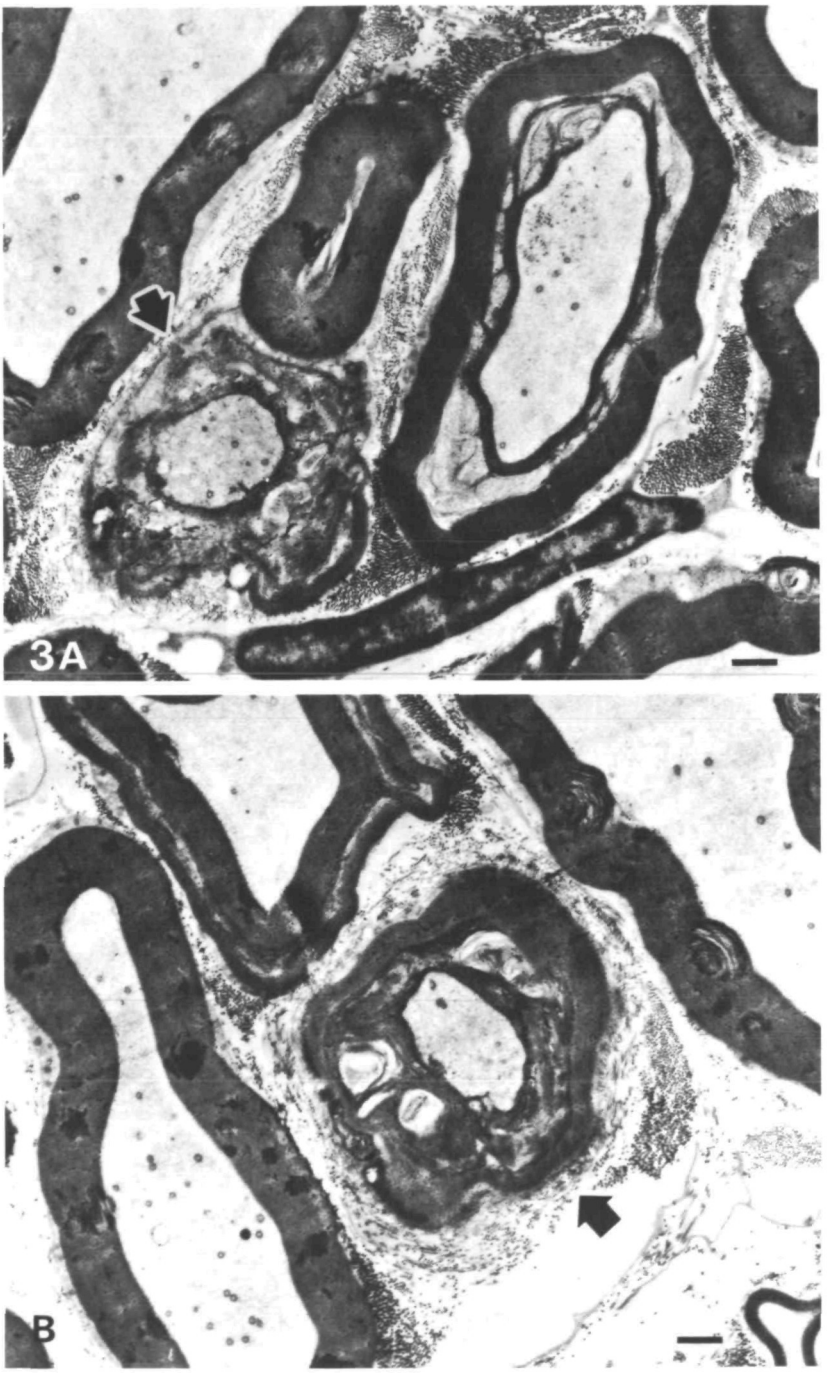

Fig. 3. Distal axon stumps degenerate after crushing or cutting the ventral root. Electron micrographs of transverse sections through the second ventral root distal to the lesion site showed the presence of degenerating axons (arrows) within 2 days of crushing (A) or cutting (B) the root. Scale bars, 1·0 μm.

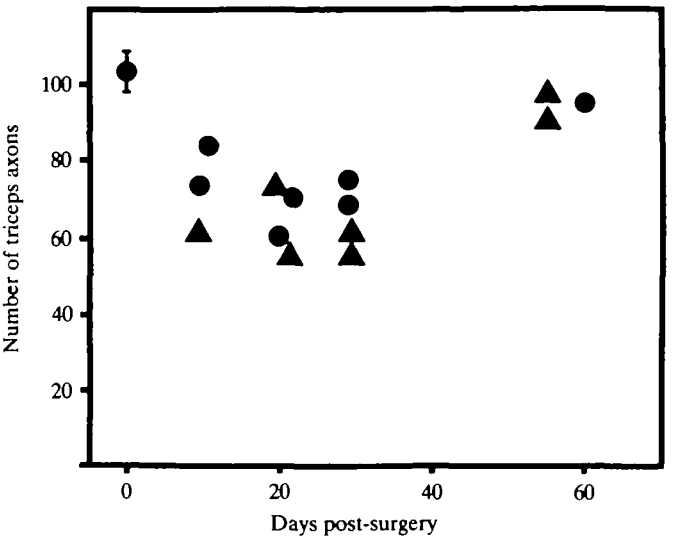

Fig. 4. Axons in muscle nerves degenerate and regenerate with similar time courses after crushing or cutting the ventral root. The rates of degeneration and regeneration were determined by counting the number of myelinated axons in the nerve innervating the medial triceps muscle at various times after lesioning the ventral root. A decrease in the number of myelinated axons was obvious within 10 days after either cutting (circles) or crushing (triangles) the ventral root. The number of axons dropped by about 40% and remained at that level for approximately a month. There were no significant differences in either the time course or the amount of degeneration created by the two techniques. After a month, there was a recovery in the number of axons present in the medial branch of the triceps nerve, and normal numbers of axons were observed after 2 months. This time course is consistent with behavioural observations previously reported (Westerfield & Powell, 1983).

received direct synaptic input from sensory neurones innervating that particular muscle. To test this idea, synaptic input from the limb to motoneurones was eliminated by removing the dorsal root at the time the ventral root was crushed. In all cases (four animals) normal patterns of peripheral innervation by motor axons and normal anatomical distributions of motoneurones were observed in animals that recovered from crushing of the ventral root after removing the dorsal root. Thus, selective motor reinnervation occurs even in the absence of sensory input from the limb.

Delaying regeneration after the crush

Another possible explanation for selective reinnervation after crushing the ventral root is that after the root is cut it might take longer for the regenerating axons to cross the lesion site, allowing more time for degeneration in the severed distal stump. In contrast, during recovery from a crush injury the motor axons might grow quickly across the lesion and, thus, interact with factors in the distal stump that have not yet dissipated and that guide them into the proper fascicle in the peripheral nerve leading to their original targets. Although the time course of degeneration, as measured by counting axons in peripheral nerves (Fig. 4), showed that there was no great

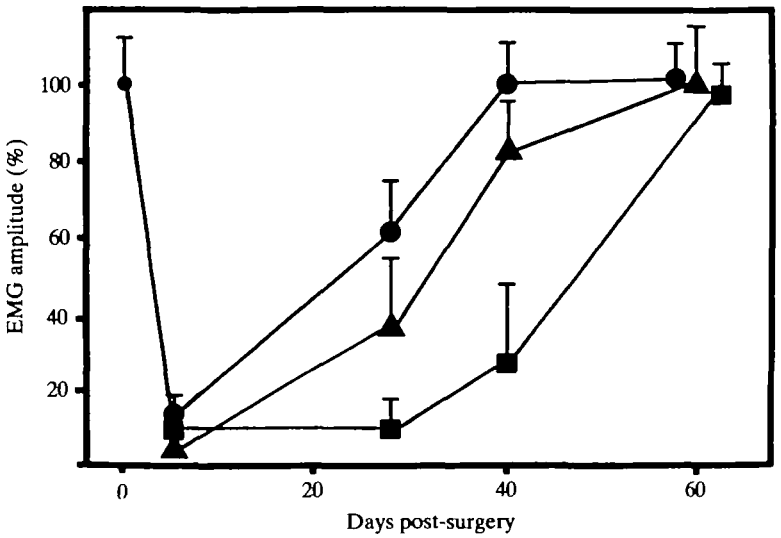

Fig. 5. Function recovers first in proximal muscles after crushing the ventral root. The time course of functional recovery after crushing the ventral root was determined by electromyographic (EMG) recordings obtained from limb muscles in response to stimulation of the second spinal nerve. Proximal muscles recovered function sooner than muscles located more distally on the limb. The relative amplitudes of the peak negativity of EMG recordings obtained in three different muscles at various times before (day 0) and after the ventral root was crushed are plotted. As shown, the time couse of functional recovery is similar for different arm muscles but occurs later in the hand muscle, flexor digitorum longus (squares), than in the upper arm muscle, medial triceps (triangles) or in the shoulder muscle, latissimus dorsi (circles). This delay is consistent with the idea that additional time is required for functional recovery due to the longer distance that regenerating motor axons must grow to reach muscles in the hand. Each point is the average value obtained from 2–4 frogs. Bars show ±S.E. Similar results were obtained from frogs recovering from cut ventral roots.

difference between cutting and crushing the root, there could still have been a significant delay before growth cones entered the distal stump after cutting the root.

To examine this possibility, regeneration of the motor axons after crushing the ventral root was delayed by repeating the crush. In four animals, the ventral root was crushed. After 2 or 4 weeks, the root was crushed again near the site of the original lesion. The effectiveness of this procedure was indicated by a corresponding 2- to 4-week delay in the return of movement in the limbs of these animals when compared to animals receiving only a single crush. Analysis of the reinnervation patterns demonstrated the re-establishment of normal connections, despite the second lesion. Thus, motoneurones selectively reinnervated their original target limb muscles even when regeneration of their axons was delayed.

Substrate guidance of regenerating axons

These experiments demonstrated that following either cut or crush injuries of peripheral nerves, motoneurones are axotomized and the distal parts of their axons

degenerate and then regenerate to re-establish functional neuromuscular connections. Moreover, the dramatic difference in the accuracy of reinnervation after cutting or crushing the nerve supports the notion that permanent dysfunction following peripheral nerve injury is due to factors operating at the injury site. This interpretation was supported by experiments with mammals (Brushart & Mesulam, 1980; Brushart, Tarlov & Mesulam, 1980; Brushart, Henry & Mesulam, 1981) where the selectivity of reinnervation was improved when individual fascicles within the severed nerve were realigned. Specifically, the accuracy of reinnervation may be determined by the interactions of the growth cones of regenerating axons with substrate molecules and cells in the injured region and these interactions may be different during recovery from the two procedures. Following crush injuries, growth cones enter their original fascicles in the distal nerve stump whereas following cut injuries the regenerating axons are more likely to encounter inappropriate fascicles. The substrate interactions of growth cones are probably crucial in determining the direction, rate of growth and the ultimate success or failure of selective reinnervation of regenerating axons. Thus, to understand how selective reinnervation can occur it is necessary to know (a) the composition and distribution of substrate molecules in the distal nerve stump and in the region connecting the severed ends, and (b) how regenerating growth cones interact with these various substrates. Experiments investigating these two issues will be reviewed in the following sections.

DEVELOPING MOTOR AXONS

Much of what we know about how growth cones extend and interact with various substrates has come from studies of developing systems. In vertebrates, most observations have been made during development of the visual and motor systems (Westerfield & Eisen, 1987). Studies of neuronal pathfinding have demonstrated that in most systems growth cones extend towards their targets in a directed fashion and appear to interact in cell-specific ways with substrates and cells in their environments. This evidence is most clearly documented for developing motor axons.

Directed growth of motor axons

Early theories of the development of limb innervation (Lamb, 1976; McGrath & Bennett, 1979; Pettigrew, Lindeman & Bennett, 1979; Bennett, Davey & Uebel, 1980) proposed that motor axons initially project diffusely into the limb bud with inappropriate projections being eliminated by withdrawal of collateral sprouts (Pettigrew *et al.* 1979) or selective cell death (Lamb, 1977; McGrath & Bennett, 1979). Subsequent observations in the chick (Hollyday, 1980, 1983; Landmesser, 1980) demonstrated that motor axons grow along stereotyped and appropriate pathways throughout development. This analysis has been carried to the level of individual, identified motor growth cones by our recent studies of the developing zebrafish (Eisen, Myers & Westerfield, 1986; Myers, Eisen & Westerfield, 1986). The muscles on each side of each body segment of the zebrafish are innervated by three uniquely identifiable motoneurones (Westerfield, McMurray & Eisen, 1986).

Each motoneurone innervates a cell-specific, contiguous subset of muscle fibres in a stereotyped region of its own segment that does not overlap the regions innervated by the other two motoneurones. By watching the fluorescently labelled growth cones of these motoneurones in developing embryos, we demonstrated that each growth cone follows a cell-specific pathway and extends directly towards the region appropriate for its adult function.

Motor growth cones sample a limited region of the periphery

During development of the chick hindlimb, motor growth cones also follow stereotyped pathways from the spinal cord to the plexus region at the base of the limb bud where they then follow divergent pathways into the limb nerves (Tosney & Landmesser, 1985*a*). This pathfinding may be due to recognition of factors in the environment, since growth cones that are forced to leave the spinal cord at abnormal positions (by moving their cell bodies) can navigate along novel pathways and, yet, still find their appropriate targets. However, this navigation is apparently limited, since displacing the cell bodies of motoneurones too far from their normal positions within the spinal cord prevents motor growth cones from innervating appropriate muscles (Lance-Jones & Landmesser, 1980). One limitation on pathfinding has been suggested by observations of individually labelled axons during normal development (Tosney & Landmesser, 1985*b*); motor growth cones extending into the limb bud have filopodia that are approximately 20–50 μm long, whereas the nerve plexus region extends for several hundred micrometres at the base of the limb bud. The range of pathway choices of a given growth cone may be limited to the region of the embryo that the filopodia can reach.

Motor growth cones interact with laminin during development

What are the molecular substrates with which motor growth cones interact? One candidate is the extracellular matrix molecule laminin. The distributions of laminin and another important matrix molecule, fibronectin, have been examined in embryos using antibodies that specifically bind to these molecules. In some cases, it has been possible to correlate the distributions of these immunoreactivities with the locations of nerve growth. In the chick (Tosney, Watanabe, Landmesser & Rutishauser, 1986; Rogers, Edson, Letourneau & McLoon, 1986), laminin is distributed throughout the region where the nerve roots develop. Specifically, the space between the neural tube and the base of the limb bud, the region where the nerve plexus forms, is rich in laminin although it is also found in many other parts of the embryo. In contrast, fibronectin is found primarily between the somites, areas that border the region of nerve outgrowth (Thiery, Duband & Delouvee, 1982; Rogers *et al.* 1986).

Our studies of the zebrafish embryo (Frost & Westerfield, 1986) have demonstrated a remarkable correlation between the distributions of substrate molecules and the outgrowth of motor growth cones. At the time that the pioneering motoneurones first extend growth cones into the periphery, laminin immunoreactivity is uniformly distributed on the medial surfaces of the myotomes, the surfaces along which the growth cones extend. In contrast, fibronectin staining is concentrated on the lateral

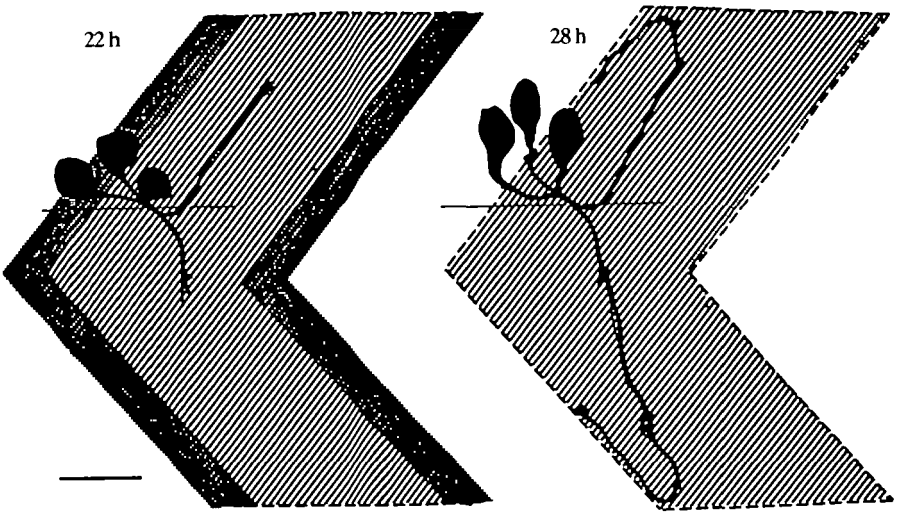

Fig. 6. Laminin and fibronectin distributions change during early development of the zebrafish embryo. The distributions of laminin (cross-hatched areas) and fibronectin (stippled areas) are drawn on outlines of zebrafish body segments as viewed from the side with dorsal to the top and rostral to the left. The segment boundaries are shown as dotted lines and the cell bodies and axons of the three identified primary motoneurones (Westerfield, McMurray & Eisen, 1986) are shown as solid lines. The ventral edge of the spinal cord is drawn as a solid line. At 19 h after fertilization of the egg, the growth cones of the motoneurones have extended from the spinal cord and are growing dorsally and ventrally on the medial surface of the segment. At this time, laminin immunoreactivity is found uniformly distributed over the medial surface of the segment while fibronectin immunoreactivity is concentrated in the clefts between adjacent segments. By 28 h after fertilization, the motor growth cones have reached the ventral edge of the segment and have turned into the clefts. Fibronectin immunoreactivity is gone by this developmental stage although laminin staining is still present. The motoneurones were taken from Myers, Eisen & Westerfield (1986). Scale bar, 20 μm.

surfaces of the somites and in the clefts between the adjacent segments, regions that these growth cones initially avoid. A few hours later in development, the fibronectin immunoreactivity diminishes when the growth cones turn laterally and extend into these regions (Fig. 6).

These observations suggest that motor growth cones and, perhaps, all neuronal growth cones (Rogers *et al.* 1986) interact with laminin, but not fibronectin, during outgrowth. Moreover, laminin itself may induce or mediate axonal growth in the embryo since it is associated with the 'neurite outgrowth-promoting factors' found in conditioned media (Lander, Fujii & Reichardt, 1985) and it promotes neurite outgrowth from a variety of cultured neurones even in the absence of nerve growth factor (Timpl & Martin, 1982; Baron-Van Evercooren *et al.* 1982; Rogers *et al.* 1983; Manthorpe *et al.* 1983; Edgar, Timpl & Thoenen, 1984).

Motor growth cones prefer growing on laminin and avoid fibronectin

The relationship between the spatial and temporal pattern of motor axon outgrowth and the distributions of laminin and fibronectin immunoreactivities in

embryos suggests opposing roles for these two molecules as substrates for neurite outgrowth. This idea is supported by *in vitro* observations of the interactions between zebrafish neuronal growth cones and defined substrates (Frost & Westerfield, 1986). Explants of embryonic zebrafish contain neurones, including motoneurones identified by their morphology and their expression of acetylcholinesterase activity (Hanneman & Westerfield, 1984). The neurones extend growth cones on laminin-coated tissue culture dishes, but not on fibronectin. The neurones respond to increased concentrations of laminin by growing longer neurites. This effect can be blocked in a dose-dependent manner by addition of polyclonal laminin antibodies. In contrast, increased concentrations of fibronectin decrease the length and number of neurites. These experiments suggest that laminin is permissive for and may support motor axon growth whereas fibronectin may inhibit motor growth cone extension.

In the zebrafish embryo, then, several different interactions with substrates are important for motor growth cone guidance during normal development. Laminin, which promotes neurite outgrowth *in vitro*, is found in areas *in vivo* where motor growth cones extend and, hence, may define regions that are permissive for growth. However, fibronectin, which inhibits motor axon outgrowth *in vitro*, borders these areas and may function to limit growth to particular regions. Thus, the spatial and temporal differences in the distributions of these molecules may explain how the growth of motor axons is guided and confined to particular regions of the developing embryo, although there is no suggestion that these molecules are involved in the pathway choices made by individual motor growth cones. Presumably some other, as yet unknown, factor or property of the system is required to explain cell-specific pathfinding.

DISTRIBUTIONS OF LAMININ AND FIBRONECTIN IN PERIPHERAL NERVES

Since laminin and fibronectin may provide substrates for motor growth cone guidance during normal development, they may also function during regeneration. In peripheral nerves, laminin immunoreactivity is restricted to regions of the endoneurium known to contain basal lamina, particularly the basal lamina of each ensheathing Schwann cell (Cornbrooks *et al.* 1983; Palm & Furcht, 1983; Bignami, Chi & Dahl, 1984*b*). Laminin is probably produced by Schwann cells since these cells are known to synthesize laminin in tissue culture (Brockes, Fields & Raff, 1979; Baron-Van Evercooren *et al.* 1982; Cornbrooks *et al.* 1983). In contrast, the epineurium, surrounding the outside of nerves, and the perineurium, surrounding axonal fascicles, are rich in fibronectin immunoreactivity (Cornbrooks *et al.* 1983; Palm & Furcht, 1983) which appears in a distinctly lamellar pattern. Fibronectin is probably produced mainly by fibroblasts (Cornbrooks *et al.* 1983) which constitute up to 25 % of the cells in the endoneurium (Peters, Palay & Webster, 1976; Thomas, 1974), although certain Schwannoma cell lines have laminin on their surfaces and release fibronectin into the medium (Palm & Furcht, 1983). There is also some overlap in the distributions of laminin and fibronectin in peripheral nerves. Laminin

is found in the innermost layers of the perineurium and some fibronectin immunoreactivity is located in the endoneurium where it is associated with fibroblasts and the surfaces of capillaries and blood vessels (Cornbrooks *et al.* 1983; Palm & Furcht, 1983).

SUBSTRATE INTERACTIONS DURING REGENERATION

These overlapping but distinct distributions of laminin and fibronectin in peripheral nerves and their effects on motor axon growth may indicate distinct functions for these substrate molecules during regeneration. The localization of laminin, but very little fibronectin, associated with axons within the endoneurium is consistent with the notion that laminin may provide a substrate for the guidance of regenerating growth cones. Indeed, regenerating rat sciatic nerve axons grow on a substratum of intensely laminin-positive Schwann cells (Bignami, Chi & Dahl, 1984*a*). Fibronectin, however, is a major constituent of the perineurial sheath where it may mediate the Schwann–neurone–connective tissue interactions that are required for remyelination (Bunge & Bunge, 1978; Bunge *et al.* 1983) and where it may function to contain regenerating growth cones within a given fascicle. During the reaction to injury, fibroblasts proliferate, invade the injured region and lay down new collagen and, importantly, fibronectin which may then act as a barrier to growth cone extension. Axons that succeed in growing through the lesion enter the distal stump where they stimulate Schwann cell proliferation and laminin production, thus promoting continued neurite extension within the laminin-rich endoneurium.

CONCLUSIONS

Functional recovery following peripheral nerve injury depends upon the interactions of growth cones with factors operating at the injury site. Since motor growth cones sample a limited region of their environments, the accuracy of reinnervation will depend largely upon realignment of the severed ends of the nerve. Regenerating motor growth cones will enter their original fascicles in the peripheral nerve stump only if these fascicles are within filopodial reach. Moreover, correct fascicles will be reached only if interactions between the growth cones and disorganized collagen and fibronectin deposited at the injury site by fibroblasts can be prevented, since these interactions will alter the extent and direction of axonal elongation. Thus, to explain the basis of selective reinnervation and to improve the chances of functional recovery following nerve injury, it is not enough to understand how axons regenerate, how they remyelinate or even what makes them regenerate faster, but rather we must learn how the growth cones of regenerating axons interact with cellular and extracellular components at the injury site. In particular, it will be important to learn how new laminin and fibronectin are produced and laid down at the lesion site and how these processes can be influenced following nerve injury.

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