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Promoting and Directing Axon Outgrowth

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Abstract

Establishment of appropriate neuronal connections during development and regeneration requires the extension of processes that must then grow in the correct direction, find and recognize their targets, and make synapses with them. During development, embryonic neurons gradually establish central and peripheral connections in an evolving cellular environment in which neurotrophic factors are provided by supporting and target cells that promote neuronal survival, differentiation, and process outgrowth. Some cells also release neurotropic factors that direct the outgrowth of neuronal processes toward their targets. Following development the neurotrophic requirements of some adult neurons change so that, although they respond to neurotrophic factors, they no longer require exogenous neurotrophins to survive or to extend processes. Within the central nervous system (CNS), the ability of neurons to extend processes is eventually lost because of a change in their cellular environment from outgrowth permissive to inhibitory. Thus, neuronal connections that are lost in the adult CNS are rarely reestablished. In contrast, the environment of the adult peripheral nervous system fosters process outgrowth and synapse formation. This article discusses the neurotrophic requirements of embryonic and adult neurons, as well as the importance of neurotropic factors in directing the outgrowth of regenerating adult axons.

Index Entries: Neurotrophins; neurotropic factors; development; regeneration; process outgrowth.

Introduction

Establishment of a functional nervous system requires survival, maturation, and differentiation of a large number of neuron populations. The developing embryonic neurons must also extend processes considerable distances along stereotyped pathways to reach their targets, then recognize the targets, and establish appropriate contacts with them. Many of these developmental processes are promoted or influenced by neurotrophic factors released from supporting cells of the neurons and their processes as well as their targets. Non-neurotrophic factors are also important, and may act alone or in conjunction with the neurotrophins. A number of neurotrophins have been isolated, some of which belong to a common gene family and others not. Although some of these neurotrophins distinctly influence different populations of neurons, several act on the same populations.

Establishment of correct neuronal connections, however, requires more than just neurotrophins. Another requisite is a cellular environment that is at least permissive to, and at best fosters, process outgrowth. The central nervous system (CNS) provides such a permissive environment for process growth during development. However, it becomes inhibitory at the end of the period of development.

An additional requirement for development of appropriate neuronal connections is the navigation of elongating axons toward the correct targets. Targeting may be brought about by factors that are

immobilized on specific pathways that are recognized by particular populations of outgrowing processes and direct them along these pathways to their targets. An alternative means of targeting process outgrowth is by (long-distance-acting) diffusible tropic factors released by the target cells. Such factors would establish a concentration gradient radiating away from the target cells up which the appropriate neuronal processes grow. Such diffusible neurotropic factors could act alone or in conjunction with substrate-bound factors.

Most studies on neurotrophic factors have been carried out on embryonic neurons addressing the important goal of understanding their roles in development. It has generally been assumed that embryonic and adult neurons are similar, and therefore, these same neurotrophic factors will play similar roles in both the intact and regenerating adult nervous system. New evidence suggests that although some of the neurotrophins may act similarly in the embryonic and adult nervous systems, distinct differences between adult and embryonic neurons are also apparent. Therefore, it is important to examine the influence of neurotrophins on adult, as well as embryonic neurons.

A number of neurotrophic factors have been isolated and some are fully characterized, but their relative roles during development and in the adult nervous system are not clear. Although a great deal is also known about a variety of immobilized factors that promote axon outgrowth, little is known about the factors that direct axons toward a target. There is now solid evidence that immobilized factors can exist in concentration gradients that could function to direct axon outgrowth. Virtually nothing, however, is known about (long-distance-acting) diffusible neurotropic factors or the mechanisms by which they could influence the growth cone to direct its path of growth.

This article addresses some of the varied influences neurons experience during their development and extension of processes. The primary focus is on the mechanisms that promote the reestablishment of neuronal connections in the adult.

A Changing CNS Environmental Scene: From Promoting to Inhibiting Process Outgrowth

Strong support for the hypothesis that conditions in the glial environment of the CNS play an important role in preventing successful axonal elongation in the adult CNS comes from experiments using transplants containing either central glia or peripheral Schwann cells as conduits of axon growth. In these experiments, injured CNS neurons that would not normally regenerate were provided with a peripheral nervous system environment *(1–3)*. The neurons extended processes many centimeters through the peripheral nervous system conduits, indicating that the neurons themselves were still capable of extending processes. However, when these regenerating processes encountered the cells of the CNS, their growth ceased. These results indicate the presence of an inhibitory environment in the CNS that blocks the ingrowth of the regenerating axons.

Neurite outgrowth-inhibiting factors in the CNS have now been clearly demonstrated and characterized. In vitro studies show that oligodendrocytes and their myelin are nonpermissive substrates for neurite growth of sympathetic and sensory neurons *(4)*. Two minor membrane proteins (mol wt 35 and 250 kDa) have now been found to be responsible for this inhibitory action *(5)*. Monoclonal antibodies were raised against these neurite inhibitors (NI-35 and NI-250) that neutralize the inhibitory activity *(6)*. In the presence of these antibodies (two clones called IN-1 and IN-2), neurites overgrew the oligodendrocytes and were able to sprout and elongate even on a substrate of CNS myelin. These antibodies have also been delivered in vivo to young rats with complete bilateral lesions of the cortico-spinal tract. Normally, there is a complete absence of cortico-spinal regeneration after the first postnatal week in rats. In the IN-1-treated rats, massive sprouting occurred at the lesion site, and fine axons and fascicles were observed 7–11 mm caudal to the lesion within 2–3 wk *(7)*. Thus, the appearance of growth inhibitory factors following neonatal development seems to be the major impediment to regeneration in the adult CNS *(7,8)*. Such inhibitory factors have not been found in the PNS.

Neurotrophic Factors: Promoting Neuronal Survival and Process Outgrowth from Adult and Embryonic Neurons

Nerve Growth Factor

Most embryonic dorsal root ganglion (DRG) neurons appear to require neurotrophic factors for sur-

vival, growth, and extension of processes *(9)*. Nerve growth factor (NGF) represents the prototype of target-derived, retrogradely transported neurotrophins, and supports the survival and differentiation of embryonic chick sensory neurons *(10)*. NGF is synthesized and released from target tissues of sympathetic neurons and the cholinergic basal forebrain neurons in the CNS. In the periphery, the target tissues are typically nonneuronal cells, whereas in the CNS, the targets are neurons *(11)*. During development, a retrograde flow of NGF is established, with the transport of NGF from the target into the nerve terminal and up the axon to the cell body *(12)*. Those neurons that establish this flow survive the period of naturally occurring neuronal cell death, whereas those that do not rapidly degenerate. Elimination of the supply of NGF by administration of antibodies against NGF during embryogenesis results in the loss of up to 85% of DRG neurons *(13)*. Conversely, if the supply of NGF is augmented, some of the neurons that would normally die are rescued *(14)*, and DRG neurons that normally die as a result of axotomy during neonatal development are almost completely spared *(15)*. Once the retrograde flow of NGF is established, it must continue for the lifetime of the neuron in order to maintain its functional differentiated state *(9)*.

Following nerve section, the levels of NGF mRNA and NGF increase in the distal part of the sciatic nerve *(16–18)*. In cultures of cells dissociated from sciatic nerve, NGF mRNA has been detected in Schwann cells and fibroblasts *(19)*. In the absence of their targets as a source of NGF, these Schwann cells in the distal part of the sectioned nerve might provide the necessary NGF for the survival of the neurons whose axons were severed and, therefore, facilitate the outgrowth of their regenerating axons. In addition to acting directly on neurons, another role for locally released neurotrophins is to influence nonneuronal cells in regions of axon regeneration. Thus, although NGF acts directly on isolated DRG neurons, it also appears to promote process outgrowth from sympathetic neurons by inducing Schwann cells to release factors that promote process outgrowth *(20)*.

Although it was initially accepted that all embryonic DRG neurons require NGF, it has been found that, in spite of the elimination of NGF by prenatal exposure to antibodies against NGF, some embryonic DRG neurons do survive *(13,21–23)*. These results indicate that the dependence on NGF is not universal and suggest that some of these DRG neurons rely on other neurotrophic factors.

NGF was only the first of a family of neurotrophins to be discovered. Five additional neurotrophins have now been isolated that are structurally related to NGF, namely:

1. Brain-derived nerve growth factor (BDNF) *(24,25)*;
2. Neurotrophin-3 (NT-3) *(26–30)*;
3. Neurotrophin-4 (NT-4) *(31)*;
4. Neurotrophin-5 (NT-5) *(32)*; and
5. The non-NGF-related neurotrophin, ciliary neurotrophic factor (CNTF) *(33,34)*.

All these neurotrophins are capable of promoting survival and differentiation of many, but not all, types of embryonic sensory neurons, whereas only some promote process outgrowth.

Brain-Derived Neurotrophic Factor

BDNF, which was first isolated from pig brain *(24)*, has a 50% homology of amino acid sequence to NGF *(25)*. Its synthesis occurs predominantly in the CNS, particularly in cortical and hippocampal neurons *(35–37)*. Despite the chemical similarities of NGF and BDNF, the two trophic factors have different effects on different target neurons. Whereas NGF is required for the survival of sympathetic neurons, BDNF does not show any effect on them *(38,39)*. BDNF supports the in vitro survival of a subpopulation of sensory neurons in the DRG of embryonic (E10) chicks that are not responsive to NGF *(38,40,41)*. Although BDNF promotes both survival and neurite outgrowth of embryonic chick and rat retinal ganglion cells and nodose ganglion neurons, NGF does not *(42–45)*. Still other neuronal populations, such as the cholinergic neurons in the basal forebrain, respond to both NGF and BDNF *(46,47)*.

In the PNS, lesion of rat sciatic nerve leads to a very marked increase in BDNF mRNA, which is higher than that of the increase in NGF mRNA *(48)*. The mRNA coding for BDNF is present in both fibroblasts and Schwann cells, and these cells produce both NGF and molecules with a BDNF-like activity *(49)*. Conditioned medium from Schwann cells and fibroblasts supports both NGF- and BDNF-responsive sensory neurons, and stimulates neurite growth from BDNF-responsive retinal explants *(49)*. Both the NGF- and BDNF-like activities in the CM are abolished by antibodies raised against NGF, suggesting that the molecules are immunologically related *(49)*.

Neurotrophin-3

NT-3 is found in the CNS, where it is localized to neurons in the neocortex and hippocampus, but not in cholinergic nuclei, whereas in the periphery, NT-3 is found in muscle *(27,28,50,51)*. NT-3 serves as a survival factor for cultured sympathetic and peripheral sensory neurons. In vivo experiments show that NT-3 genes are expressed during embryonic development and that NT-3 enhances the survival of motoneurons *(51,52)*. The influence of NT-3 is additive to that of BDNF, although the influence of NT-3 is substantially less than that of BDNF *(26)*. However, the importance of NT-3 as a motoneuron neurotrophin awaits further clarification.

Neurotrophin-4

NT has been detected in Xenopus ovary and has been shown to stimulate neurite outgrowth in cultured peripheral sensory neurons *(31)*. No specific developmental role has yet been established.

Neurotrophin-5

The distribution of NT-5 mRNA in organs innervated by sensory and sympathetic neurons raises the possibility that it could serve as a target-derived trophic factor for peripheral neurons *(32)*. Support for this comes from demonstrations that NT-5 is a potent survival factor for chick embryonic DRG neurons, and also supports survival of, and process outgrowth from, sympathetic ganglion neurons.

Ciliary Neurotrophic Factor

Northern blot, *in situ* hybridization, and immunocytochemical studies have shown that peripheral nerve is the richest tissue source of CNTF *(30,53)*. It appears that the myelin-related Schwann cells of intact peripheral nerve are the principal cellular source of CNTF *(48,54)*. CNTF influences the survival of populations of neurons not responsive to BDNF: certain sensory neurons, sympathetic neurons *(55)*, and embryonic chick and rat motoneurons *(56,57)*. In vivo during development, motoneurons also require CNTF for survival, and CNTF can rescue chick embryonic motor neurons from naturally occurring cell death during development *(58,59)*. In addition, CNTF applied to the proximal stump of a lesioned neonatal rat facial nerve almost completely prevents the large loss of motor neurons in the facial nucleus that normally results from such a lesion *(59)*.

These observations, plus the finding that developing motoneurons innervating skeletal muscle require the presence of their targets to develop appropriately, initially made it appear likely that muscles and Schwann cells would release CNTF to motor axons to maintain their viability *(60,61)*. However, this now seems unlikely, since the gene for CNTF is not expressed during development *(53)* and although CNTF induces sprouting from intact motor axons in vivo, the CNTF gene is not expressed in muscle *(53)*.

The results of studies in the adult nervous system also make the role of peripherally derived CNTF in promoting regeneration unlikely. CNTF is present in innervated sciatic nerve *(30,53)*. However, its synthesis is downregulated, with its concentration decreasing in the distal nerve following denervation, and it is only upregulated in remyelinating Schwann cells following reinnervation *(54,61–63)*. The role CNTF plays remains to be further clarified.

Changing Cellular Requirements for Embryonic and Adult Neurons

During development, most embryonic neurons appear to require some form of neurotrophic factor for their survival and maturation. In contrast to embryonic sensory neurons, not all adult sensory neurons require exogenous neurotrophins for their survival or to promote process outgrowth. DRG neurons, from both adult rat and frog, survive in a defined medium and extend processes in the absence of exogenous neurotrophins *(64,65)*. The adult rat DRG neurons respond, however, to NGF and BDNF by increasing their process outgrowth *(64)*. Similarly, adult frog DRG neurons increase their process lengths in response to NGF, but not as extensively as to an unidentified, but NGF-related, neurotrophin released from peripheral nerve *(65)*.

Developing motoneurons are also critically dependent on their muscle targets for survival and differentiation *(66)*. In vitro, neuronal survival and promotion of process outgrowth from cultured chick and rat embryonic motoneurons depend on factors released from cultured muscles *(67–74)*. Cultured avian motoneuron survival is also enhanced by muscle-derived factors that are distinct from several previously purified growth factors *(75)*. A factor has also been purified from postnatal rat muscle that promotes motoneuron differentiation and survival both in vitro and in vivo *(76)*. However, no

bona fide target-derived motoneuron neurotrophic factor has yet been identified.

In contrast to embryonic motoneurons, adult motoneurons can survive for more than 4 wk in a defined medium and extend processes *(77)*. Similar to embryonic neurons, however, these neurons respond to factors released from cocultured adult muscle fibers by increasing the length of processes (Brösamle and Kuffler, unpublished observations).

A possible explanation for the survival of these adult neurons, in the absence of exogenous neurotrophins, is that, although they remain sensitive to exogenous neurotrophins, the neurons synthesize their own neurotrophins. Support for this hypothesis comes from the finding that BDNF synthesis occurs in cortical and hippocampal neurons *(35–37)*, and that mRNA for BDNF is present in embryonic DRGs *(28,50)*. Thus, the neurotrophin may act as an autocrine factor rather than as a "classical" target-derived neurotrophic factor *(67)*. The synthesis of neurotrophins by other neurons must be further examined.

Navigation of Process Outgrowth—Neurotropic Factors

Most studies to date have focused on neurotrophic factors that promote neuron survival and process outgrowth, whereas few have looked for factors that direct process outgrowth, especially those of adult neurons. Factors that direct process outgrowth are referred to as neurotropic factors. Extensive evidence from in vivo and in vitro experiments indicates the presence of both local and long-distance acting cues that direct the outgrowth of neuronal growth cones. These molecular cues may be immobilized, existing as part of the substrate over which the growth cones grow, or they may be diffusible factors that establish diffusion gradients up which the growth cones navigate.

Immobilized Neurotropic Factors

Adult peripheral nervous system sensory and motor axons regenerate long distances along their former nerve pathways consisting of Schwann cells, fibroblasts, connective tissue, and extracellular matrix. These cellular and noncellular elements influence the regenerating axons by providing a substrate pathway that is preferential to the growth cones to the surrounding substrates. Especially important in this role is the extracellular matrix molecule laminin, which promotes the outgrowth of processes from a broad range of neurons in vitro *(78,79)*. Consistent with this role of promoting axon regeneration in the adult peripheral nervous system are results showing that in vivo laminin is upregulated in the peripheral nerve pathway following denervation and is downregulated following reinnervation *(78)*. This relatively simple mechanism of both promoting and directing axon regeneration, by providing a preferential substrate along which growth cones navigate, relies on the existence of a preexisting pathway. Specificity in promoting process outgrowth from axons of some neuron populations and not others could be accomplished by differential sensitivities of the growth cones of various neurons to laminin.

An alternative mechanism has been proposed for providing pathway specificity by which the target promotes the outgrowth of some axons while simultaneously inhibiting the outgrowth of the growth cones of other axons. This could result from the establishment of gradients of immobilized factors on cell surfaces or in the extracellular matrix. Such gradients would influence different populations of growth cones depending on whether their growth was promoted or inhibited up a particular concentration gradient. A fine-tuning of this promoting and inhibiting of process outgrowth could be accomplished by adjusting the steepness of the gradient that must be "read" by the specific populations of growth cone. Thus, a mixed population of processes could be promoted to grow along a particular gradient pathway. Then the elongation of some of the processes could be inhibited when their growth cones reached a certain concentration of the factor, whereas the other growth cones would continue to elongate. Such concentration gradients would offer a dynamic mechanism for directing process outgrowth.

One of the major challenges in testing the role of immobilized or diffusible gradients of process directing factors is establishing such gradients in vitro. Not only must a gradient be established, but it must be of the correct steepness. The most compelling evidence for the influence of immobilized neurotropic molecules on growth cones comes from the in vitro experiments of Baier and Bonhoeffer *(80)* and Snow and Letourneau *(81)*. Demonstration of a neurotropic influence in vivo, however, remains elusive.

The experiments of Baier and Bonhoeffer *(80)* took advantage of what is known about the projec-

tions of chick optic ganglion neurons. Once reaching the optic tectum, the ganglion cells from the anterior retina project to the posterior part of the tectum, whereas, the posterior ganglion cells project to the anterior tectum. This pattern of projection strongly suggests that the ganglion cell axons find their targets by detecting directional information in the form of gradients of guidance molecules in the tectum.

In a bioassay, retinal ganglion cells were cultured on cells from different tectal regions. Although axons from the posterior retina grew on membrane from both the anterior or posterior tectum, when provided a choice, they preferred to grow on the anterior tectal cells, their normal target *(82)*. This choice results, not from the presence of an attractive substrate, but because the posterior cells express high levels of a repellent activity, which is the result of a 33-kDa cell-surface glycoprotein *(83)*. The repellent activity of the protein is present in a gradient along the entire anterior-posterior axis, with the lowest expression in the anterior tectum where the posterior retinal axons normally project *(84)*.

To test the physiological relevance of this gradient, Baier and Bonhoeffer *(80)* developed a system for creating narrow lanes of the repellent protein in a smooth gradient. They found that the axons of the posterior retina grew up the gradient, but in a sufficiently steep gradient, the growth cones stopped growing forward, turned around, and grew down the gradient. If lateral growth was constrained by growing the neurons on a narrow path of the protein without enough width to allow turning, the growth cones stopped growing entirely at the same concentration at which they would have turned.

The principal determinant of the turning/stopping response was the slope of the gradient—the fractional change in the repellent concentration across the growth cone diameter—rather than the absolute concentration of the repellent. When the concentration changed by <1% across the diameter of the growth cone, no stopping was observed, whereas a change of 5% or more caused complete inhibition of axon extension.

Whether this inhibitory mechanism plays a similar role during development remains to be examined. This could be tested by blocking the repellent molecule with antibodies in vivo. Interestingly, anterior retinal ganglion cells are not sensitive to the repellent. However, these experiments clearly demonstrate that distinct molecular gradients along an axis can direct the outgrowth of particular populations of neurons.

Immobilized gradients are an effective mechanism for promoting and directing axon growth, and may be important during development. However, they lack an important element required to facilitate regeneration, particularly in the adult animal, especially in the face of traumatic injury that often results in a discontinuity of the axon pathway. Under these circumstances, the axons must be directed across the gap and into the distal nerve tube, or directly to a distant target.

Diffusible Neurotropic Factors

It is accepted that growth cones can be steered by diffusible extracellular gradients of ions and transmitters *(85)*, and it is highly likely that diffusible tropic factors in various regions of the central and peripheral nervous system play a crucial role following neuronal injury. However, no neurotropic factor has been identified that is accepted to play this role physiologically. Further, little is known of how a gradient might be "read" by growth cones.

The best demonstration of a diffusible neurotropic factor that can direct growth cone elongation along a diffusible gradient comes from in vitro experiments using NGF *(86,87)*. When NGF was released from a pipet to establish a local concentration gradient, the growth cones of chick sensory neurons turned and grew toward the pipet *(87)*. Although the response was clear and dramatic, its biological significance is unclear since NGF is not available to these neurons at the time they innervate their targets *(88)*.

In vivo studies of the sequential steps in regeneration of motor axons to their skeletal muscle fibers in frogs and mammals have shown that diffusible factors are involved in this process *(16,60,89–92)*. One example of directed axon outgrowth comes from in vivo experiments in the adult frog *(90,92)*. For these experiments, a novel preparation was developed in which all the original targets that might influence the outgrowth of the regenerating axons, such as original nerve pathways and denervated muscle fibers, were removed. This allowed the testing of the influence of specific targets on the pattern of axon outgrowth without competition from other potential targets. The targets tested were normal target cells of regenerating axons, skeletal muscle fibers, and pieces of peripheral nerve.

For this in vivo preparation, the cutaneous pectoris muscle was completely removed while leaving the central nerve stump in its original posi-

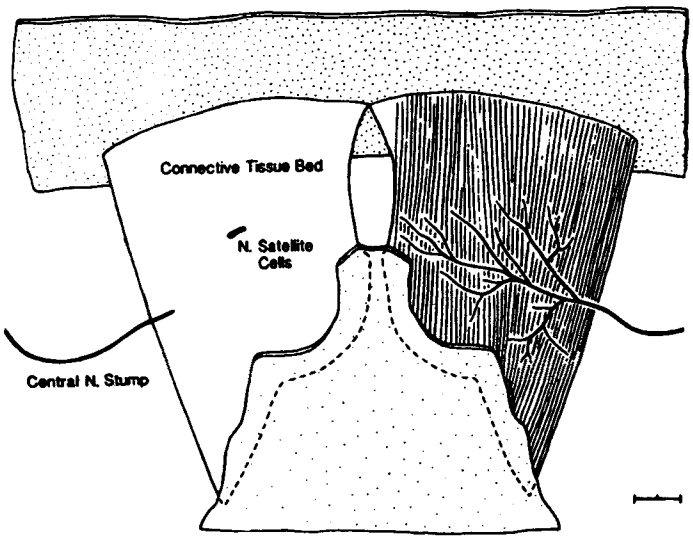

Fig. 1. Schematic diagram of the preparation used to study the influence of various targets on the outgrowth of axons from the cutaneous pectoris nerve. Right shows the intact cutaneous pectoris muscle with its axons innervating the central region of the muscle fibers. Left side shows the experimental preparation in which the entire muscle is removed leaving only the central nerve stump intact. A 1-mm length of nerve that had entered the muscle was stripped of its perineurium and placed in the area formerly occupied by the muscle, and the abdominal skin of the frog was sutured closed. Calibration bar = 2 mm.

tion (Fig. 1). As part of the normal repair process of the frog, a connective tissue sheet developed in place of the muscle. Axons growing out of the central nerve stump grew out on the connective tissue sheet, and the pattern of axon outgrowth was assessed. It is important to stress that in the adult frog, as with most adult mammals, the motoneurons survive following nerve section, and it is not necessary to promote axon outgrowth from the sectioned peripheral nerve.

In the absence of any targets for the regenerating axons, they grew out in a random manner (Fig. 2). In contrast, when either a piece of peripheral nerve or muscle fibers was placed in the periphery, the axon outgrowth was highly directed. The regenerating axons grew out of the end of the central stump and established trajectories toward both pieces of nerve (Fig. 3) and muscle fibers (Fig. 4), even though they were up to 12 mm away from the end of the central nerve stump. The axons established trajectories toward the targets even before any of them had made contact with the target cells.

These results show that the normal targets of the motor axons direct the outgrowth of regenerating axons and that their influence is effective over distances of many millimeters. Further, the highly directed axon outgrowth occurred regardless of

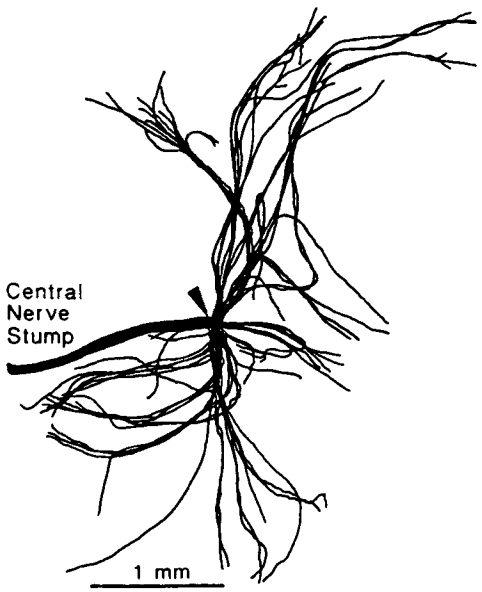

Fig. 2. Camera lucida drawing of a control preparation with no cell target. Arrowhead indicates end of the original central stump. The axons grew out in a random manner. Calibration bar = 1 mm.

where the target cells were placed in the field surrounding the central stump. Thus, it is possible to eliminate the presence of some general coordinate guidance system in the frog that is responsible for

Fig. 3. Light micrograph of axons stained with HRP coursing across the connective tissue bed of an experimental preparation in which the piece of target nerve was placed 1.9 mm from the end of the central nerve stump. The axons make a greater than a 90° turn to course toward the target, converge at its end, and enter the nerve tube. Calibration bar = 0.5 mm.

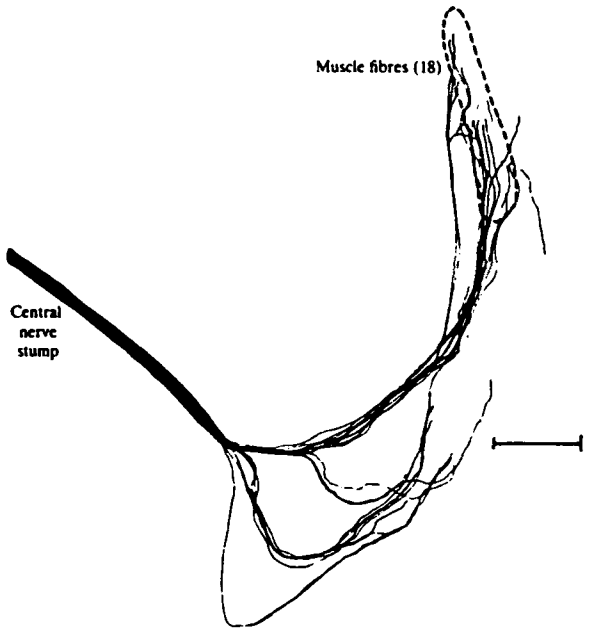

Fig. 4. Camera lucida drawing of a preparation with a target of nonsynaptic region of muscle fibers 2.1 mm from the end of the central nerve stump. All the axons have trajectories toward the 18 muscle fibers.

establishing the axon trajectories to a fixed location where their normal targets would be located.

The results of these experiments indicate that regenerating axons take trajectories toward their distant targets, but they do not address the underlying mechanism that establishes these trajectories. The results suggest that diffusible factors may be involved, but it is also possible that cells associated with the targets or other invading cells, such a macrophages, are responsible for directing the outgrowth. To determine whether diffusible factors were involved, the target cells were packaged in "ravioli," specially designed Millipore filters with a 0.22-μm pore size. These filters eliminated the possibility of migration of cells away from the targets, as well as contact between the cells inside the ravioli and those outside.

Even when pieces of peripheral nerve and muscle fibers were packaged in the filters, the regenerating axons developed trajectories toward the targets (Fig. 5). These results indicate that the cells inside the ravioli release diffusible factors that are involved in directing axon regeneration over distances of many millimeters. If the directed axon outgrowth results directly from a response to a diffusion gradient, then the growth cones must be acutely sensitive to the neurotropic factor to respond over such distances.

Although these experiments show that diffusible molecules are responsible for axon guidance, they do not eliminate the possible involvement of other cell types. Thus, however unlikely, other cells, such as macrophages, may be responding to the diffusible factor and by some unknown mechanism direct axon outgrowth. Further experiments are required to show that the axons are responding directly to the diffusible factor by growing up a concentration gradient of the factor.

Recent experiments have examined the influence of peripheral nerve and dissociated muscle fibers on cocultured adult sensory and motor neurons.

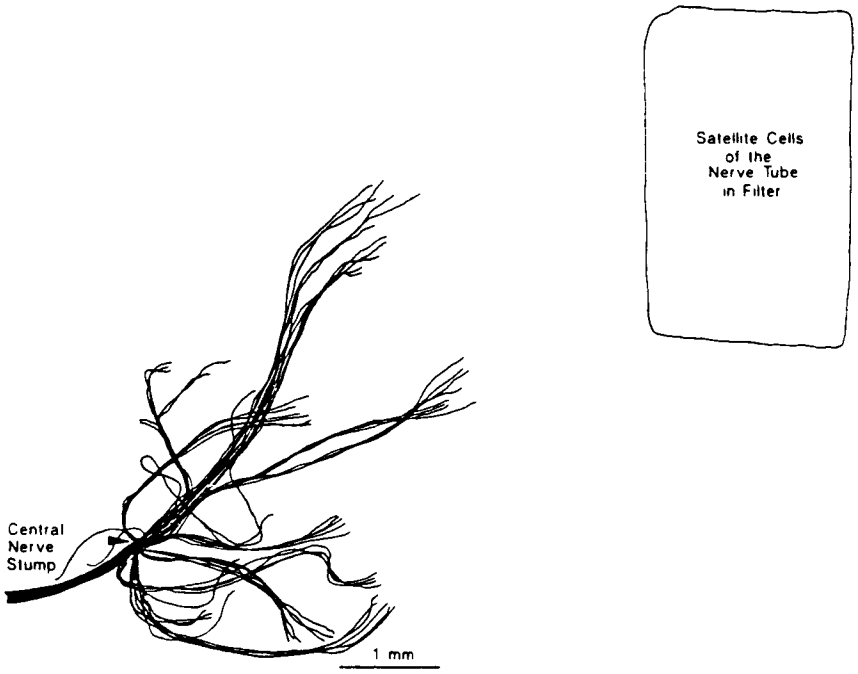

Fig. 5. Preparation in which a 1-mm length of nerve was placed inside a filter with a 0.22-μm pore size at a distance of 4.4 mm from the end of the central nerve stump. Most of the axons have trajectories toward the target cells in the filter. Arrowhead indicates the end of the central nerve stump.

Peripheral nerve was found to promote process outgrowth from both adult sensory and motor neurons in vitro *(64)*. Muscle fibers, however, promote process outgrowth from motor, but not sensory neurons (in preparation). Medium, conditioned by peripheral nerve and muscle fibers, must now be tested for its ability to direct the growth of sensory and motor neuron processes in vitro. If an influence is found in vitro, it will be possible to determine the steepness of the gradient the growth cone requires to "read" the gradient. These experiments will also allow the determination of the sensitivity of the growth cones to the tropic factor. Future experiments must examine whether medium containing the neurotrophic factor direct process outgrowth in vivo.

Conclusion

Establishment and maintenance of a functional nervous system require an interplay among neurons, their supporting tissues, and their targets. A number of neurotrophic factors have been isolated and characterized that are critical for the survival of neurons and to promote process outgrowth. Some of these factors arise in the supporting cells of the neurons and their processes, whereas others are target-derived. Although some specificity exists in the influence of these neurotrophins, there is also a great deal of overlap. The relative roles of these factors, whether they act separately, in a temporal sequence, or synergistically during development and in the adult animal remain to be clarified. In addition, the presently recognized neurotrophins are most likely only the beginning of a much longer list of trophic factors having more defined roles.

There is an increasing number of examples of neurotropic guidance for a variety of axon types in which the factors originate in the axon pathways and the axon targets. This article has focused on only two examples, one using an immobilized and the other a diffusible neurotropic factor. These studies show that neurotropic guidance plays an important role during development as well as for repair of neuronal connections in the adult. It is clearly difficult, however, to demonstrate the existence of neurotropic guidance. Axonal targets might secrete factors, such as NGF, that might act as neurotropic factors in vitro without playing a role in long-distance guidance during development if, for example, they are not present at the appropriate time or do not affect the developing axons.

A simplistic view attributing axon guidance to single factors acting alone is appealing but unrea-

sonable. The roles of a variety axon guidance factors are already apparent, such as extracellular matrix molecules and neurotropic factors. When acting independently, these factors may have limited axon guidance specificity and act on a broad range of axon types. It is now necessary to examine whether these factors might act in concert with one another to guide axons along a given path. Such interactions might allow use of a limited number of factors, but provide for a large number of combinations, each of which provides guidance specificity to different populations of axons. Such studies will require the isolation and characterization of the various neurotropic factors and examination of their roles in vivo during development, but especially in the adult, in which these factors might eventually be used to facilitate repair of nerve injury. Clearly our understanding of neurotropic factors is only in its beginning stages.

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