

1991

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Recommended Citation

Varon, Silvio; Hagg, Theo; Vahlsing, H. Lee; and Manthorpe, Marston (1991) "Peripheral Nerve Bridges for Central Nervous System Regeneration," *Cells and Materials*: Vol. 1 : No. 1 , Article 9.

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PERIPHERAL NERVE BRIDGES FOR CENTRAL NERVOUS SYSTEM REGENERATION

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(Received for publication July 6, 1990, and in revised form October 9, 1990)

Abstract

A neuronotrophic hypothesis for the adult central nervous system (CNS) has been increasingly supported by *in vivo* studies of Nerve Growth Factor (NGF) impacts on adult rat CNS cholinergic neurons. We have extended these investigations to NGF effects on cholinergic axonal regeneration in an adult rat septo-hippocampal model. A fimbria-fornix lesion deprives the dorsal hippocampal formation (HF) of its cholinergic afferents and provides a cavity into which sciatic nerve segments can be implanted to serve as regeneration bridges from septum to HF. Septal cholinergic fibers enter and course through fresh cell-containing nerve bridges to attain a maximal number by the end of one month, but enter and grow further into the HF much more slowly and to a limited depth. The importance of NGF for axonal regeneration in the nerve bridge was shown by the demonstration that an "acellular" nerve preparation deprived of its NGF-producing living cells fails to be invaded by the cholinergic fibers, but regains its competence if pre-incubated in NGF. The importance of NGF for intra-hippocampal regeneration was documented by the much greater cholinergic invasion of the HF induced by local (i.e., intra-hippocampal) NGF infusions. Advances in biomaterial science should provide further progress regarding: i) the continuous intracerebral delivery of NGF or other protein factors, and ii) the availability of surrogate materials as regeneration bridges.

KEY WORDS: basal lamina; cholinergic neurons; hippocampal formation; nerve bridge; Nerve Growth Factor; neurotrophic factors; Schwann cells; septum
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Introduction

The remarkable progress of neurobiology over the past decade is providing new opportunities for potential clinical advances in at least two areas of neuropathology: neurodegenerative diseases and neural regeneration. Both areas take advantage of a neuronotrophic hypothesis of the central nervous system (CNS) (Varon et al, 1984), which rests on three main propositions: (1) CNS neurons in the adult mammal continue to depend on, for their welfare, function and repair capabilities, a class of special proteins called neuronotrophic factors (NTFs); (2) CNS deficits may, therefore, reflect a relative inadequacy in the availability of endogenous NTF; and (3) administration of exogenous NTF can compensate for or supplement an insufficient endogenous supply and, thus, prevent or alleviate the resulting neuronal damage. In this article, we shall review recent information that has substantiated such a hypothesis, and will point out directions in which new biomaterials might provide advantages for future clinical applications.

CNS cholinergic neurons and Nerve Growth Factor

The breakthrough for testing the CNS neuronotrophic hypothesis came with the discovery that Nerve Growth Factor (NGF), a protein factor best known for its trophic activity on certain peripheral ganglionic neurons, can also affect central cholinergic neurons in the basal forebrain and the neostriatum of the rat (Whittemore and Seiger, 1987; Hefti et al, 1989). Among the responsive cholinergic CNS nerve cells, the medial septum and vertical diagonal band neurons project to their innervation territory, the hippocampal formation, largely through the fimbria-fornix tract. A complete fimbria-fornix transection (FFT)

axotomizes these neurons, thereby also interrupting the retrograde axonal pathway through which they receive endogenous NGF from their hippocampal territory. The resulting septal neuron damage (Gage et al, 1986; Hagg et al, 1988) is manifested by a progressive decline, over the next 2-3 weeks, in the number of septal neurons that can be recognized as cholinergic by their stainability for acetylcholinesterase (AChE) or choline acetyltransferase (ChAT), the acetylcholine regulating enzymes. This apparent loss of cholinergic septal neurons is largely prevented by intraventricular administration of exogenous NGF from lesion time onward (Hefti, 1986; Williams et al, 1986; Kromer, 1987), but will take place if the NGF treatment is interrupted (Montero and Hefti, 1988). The numerical decline of cholinergic septal neurons is not due to actual neuronal death but to a reduction of their cholinergic "markers" beyond current detection thresholds. Exogenous NGF administration starting after septal neuron disappearance causes their progressive and nearly complete reappearance even when the NGF treatment is delayed by as long as 3 months (Hagg et al, 1988, 1989a). Neuronal recovery upon delayed NGF treatment is not restricted to the cholinergic enzymes, and includes a reversal of the immunostainable NGF receptor and somal size reduction induced by axotomy (Hagg et al, 1989a).

A recent study (Sofroniew et al, 1990) has shown that septal cholinergic neurons, although shrunken, remain detectable with the persistence of only a very small compacted remnant of fimbria-fornix and hippocampal formation after excitotoxic lesions of the latter. As that study points out it could be that this remnant, or the reactive astrocytes in it, provided enough trophic support (NGF) for the septal neurons or that these neurons acquire another source of trophic factors. These, data, therefore, are not necessarily in conflict with the neuronotrophic hypothesis but will require further and more detailed analyses.

The ability of NGF to influence adult CNS cholinergic neurons has been shown to extend beyond the acute FFT model. Basal forebrain and striatal cholinergic neurons play a crucial role in the cognitive activities of the brain, and learning or memory deficiencies are accompanied by cholinergic deficits in the aged brain as well as the neurodegenerative Alzheimer's disease. Aged rats selected for learning impairment have been reported

to respond to intraventricular NGF treatments with a behavioral improvement as well as partial reversal of an age-induced atrophy in some of their cholinergic neurons (Fischer et al, 1987), and clinical trials for intraventricular NGF treatments in Alzheimer's patients are currently under consideration in several countries. Exogenous NGF administration can also upregulate cholinergic neurons in the absence of an experimental or pathological lesion (Hagg et al, 1989b; Cavicchioli et al 1989), drawing attention to the potential importance of local NGF concentrations for differential effects on different subsets of neuronal targets.

The septo-hippocampal model for adult CNS regeneration

The acknowledged failure of adult mammalian CNS neurons to regenerate their axonal projections after intracranial damage is no longer attributed to an intrinsic or acquired inability of the neurons themselves to regrow an axon, but rather to a "resistance" of the adult CNS tissue to be re-invaded by regenerating nerve fibers. Such a revised perspective owes a great deal to the work of Aguayo's group, showing that axons from a variety of adult neurons in the rat brain or spinal cord will readily grow inside a peripheral nerve "bridge" (known to favor axonal regeneration from peripheral neurons) but stop shortly after re-entering CNS tissue at the other end of the bridge (e.g., Aguayo 1985). Little, however, has been learned about the cellular and molecular components that underlie the regeneration-favorable and -unfavorable features of peripheral and central neural tissues, respectively.

The septo-hippocampal system has provided new opportunities to evaluate the CNS neuronotrophic hypothesis with regard to the roles of a neuronotrophic factor (here, NGF) on the axonal regeneration of adult CNS neurons (here, the septal cholinergic ones). Aspirative FFTs, applied bilaterally, generate a lesion cavity between the septum and the hippocampal formation on each side of the brain. The cavity on either side can be "bridged" by experimental materials to solicit axonal regrowth from the septal to the hippocampal regions. In additional experiments, exogenous NGF can be administered in different locations to modulate the number of septal cholinergic neurons involved, the extent of their axonal regeneration into the bridge, and/or the depth and distribution patterns

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of the regenerating cholinergic axons within the hippocampal re-innervation territory.

Figure 1 illustrates this septo-hippocampal model and its susceptibility to quantitative analyses (Hagg et al, 1990a). Part of the brain (Fig. 1,A) is used to provide coronal sections for the evaluation

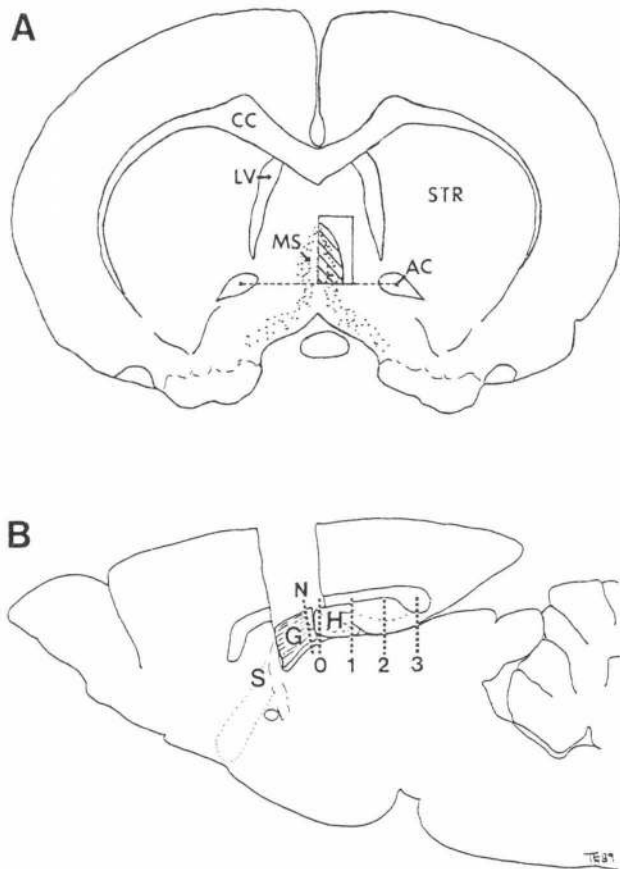


FIGURE 1 The septo-hippocampal model used for quantification of the effect of trophic factors on regeneration. A) Schematic diagram of a coronal section through the forebrain of an adult rat illustrating the medial septum (MS) area (hatched) that was counted on both sides for cholinergic neurons. (AC = anterior commissure, CC = corpus callosum, LV = lateral ventricle, STR = neostriatum,). B) Schematic sagittal representation of the rat brain illustrating the placement of sciatic nerve grafts (G) in a lesion gap between the septum (S) and the hippocampal formation (H). In sagittal sections determinations were made of the number of AChE-positive fibers intersecting imaginary lines through the hippocampal end of the nerve graft at -0.2 (N), and at 0.1 (0), 1, 2 and 3 mm from the rostral tip of the hippocampal formation.

of ChAT-immunostained septal cholinergic neurons. The rest of the brain yields sagittal sections (Fig. 1,B) to be stained for AChE-positive fibers emerging from the septum (S) into the grafted bridge tissue (G) and on to the hippocampal formation (HF). Counting intersections on eyepiece gridlines positioned perpendicularly to the axis of graft and HF allows a quantitative determination of cholinergic fibers that: a) extend through the experimental bridge (line N), b) enter the cholinergically denervated HF (line 0 = 0.1 mm into the hippocampal tissue), and c) advance to different hippocampal depths (lines 1,2,3 = 1,2, and 3 mm into the HF).

Figure 2 summarizes the time course of cholinergic axonal regeneration in adult rats that have received freshly collected adult peripheral (sciatic) nerve grafts in FFT-caused lesion cavities (but no deliberate administration of NGF)(Hagg et al, 1990a). AChE-positive fibers invade the nerve bridge after 1 week and reach a maximal number at the bridge end by about 1 month. Entry into the HF is achieved by a much smaller number of fibers (than are available at bridge exit) in the first few months but continues to increase with time to nearly match the number of bridge fibers by 6 months post-

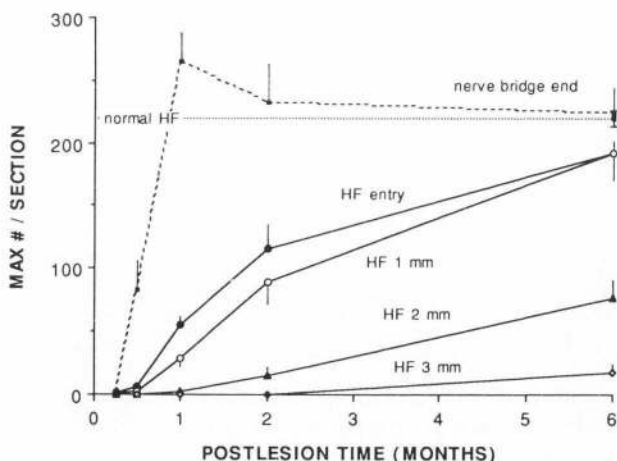


FIGURE 2 Time course of cholinergic axonal regeneration into sciatic nerve grafts and hippocampal formation. Presented are the maximal numbers (\pm SEM) of AChE-positive cholinergic fibers found at different times in single sagittal sections at the end of a sciatic nerve "bridge" and at sequential 1 mm distances into the hippocampal formation. Also presented is the maximal number of fibers found in normal animals at 0 and 1 mm.

lesion. Essentially all the entering fibers can advance 1mm into the HF, but very few of them penetrate further even after several months. In control animals, whose lesion cavity received gelfoam rather than a nerve graft, entry into the HF by cholinergic fibers (not shown) was as delayed and as modest as the reaching of the 2mm intra-HF line by nerve bridge derived fibers. Such results have confirmed for the septo-hippocampal cholinergic system: i) the competence of adult peripheral nerve tissue to serve as a CNS regeneration bridge, and ii) the resistance of adult central nervous tissue to be re-innervated. Such a resistance, however, appears to vary in space and/or time with regard to the regenerating fibers made available by the nerve bridge.

NGF roles in septal cholinergic axonal regeneration

The quantitative observations reviewed thus far encourage the speculation that NGF, the neurotrophic factor for the septal cholinergic neurons, may control cholinergic regeneration by its availability: a) in the septal tissue itself, to dictate which individual neurons are capable of engaging in a regeneration process, b) in the bridge, to recruit and support regenerating axons across the gap, and c) in the hippocampal formation, to sustain further growth into the innervation territory (for a review see Hagg 1990c).

Animals implanted with a nerve bridge but not otherwise supplied with exogenous NGF exhibited the expected FFT-induced reduction in detectable septal cholinergic neurons, suggesting that cholinergic axonal regeneration into the bridge originated from septal neurons spared by the lesion (Hagg et al, 1990a). Intraventricular administration of NGF to nerve-implanted rats (data not shown), while protecting from damage all septal cholinergic neurons, caused a marked reduction rather than an increase in the number of cholinergic fibers entering the nerve bridge. Intraventricular NGF is known to induce the appearance of cholinergic sprouting in the dorsal septum (Williams et al, 1986; Hagg et al, 1989b), suggestive of an attempt to regrow fibers toward the interrupted fimbria-fornix. In our hands, the cholinergic sprouting induced by intraventricular NGF, whether in bridge-free or nerve-implanted rats, appears with a gradient toward the infused ventricle. The reduced number of fibers entering the

nerve bridge concurrently with the periventricular sprouting suggests a tropic as well as a trophic effect of NGF on cholinergic axonal regeneration into CNS tissue, favoring regrowth toward the regions where NGF concentrations would predominate -- as similarly noted in vitro (e.g., Gundersen 1985).

Freshly dissected peripheral nerve contains living Schwann and other cells which are known to produce NGF in vitro (Varon et al, 1981; Muir et al, 1989) and in vivo (Heumann et al, 1987). It also contains a mechano-chemical scaffold, co-axially oriented along the Schwann cell chains and composed of basal lamina "tubular" spaces initially occupied by the now degenerating distal segments of the nerve axons. An "acellular" nerve preparation can be obtained (Manthorpe et al, 1989; Hagg et al, 1991) by: i) transecting a sciatic nerve and leaving it in situ for 6 weeks, to eliminate degenerating axons and myelin distal to the lesion, ii) collecting the distal nerve segment and repeatedly freeze-thawing 1cm pieces of it, to lyse all nerve-associated cells, and iii) culturing the cell-free pieces in vitro in the presence of peritoneal macrophages, to eliminate residual cell debris. The final preparation no longer contains living cells to act as local sources of NGF but retains the apparently intact scaffold of laminin-rich basal lamina tubular spaces (Figure 3). When used as a bridge in the septo-hippocampal model, acellular nerve did not promote entry and elongation of cholinergic fibers (Manthorpe et al, 1989; Hagg et al, 1991). However, acellular nerve that was incubated for 24 hr with NGF before being grafted as a potential bridge revealed nearly as many fibers at 1 month as a freshly dissected cellular nerve. Such results confirmed that: i) performance of a nerve graft as a regeneration bridge requires local availability of endogenous or exogenous NGF, and ii) a cell-free nerve scaffold remains regeneration-competent when accompanied by the appropriate neurotrophic factor.

Safe intraparenchymal infusions have recently become possible by use of a modified infusion device which prevents cytotoxic substances leaching off the osmotic minipump from mixing with the infusion fluid and causing necrotic lesions in the infused tissue (Vahlsing et al, 1989). As illustrated in Figure 4 one month long infusions of NGF directly into the hippocampal formation (Hagg et al, 1990b), 2mm away from the lesion gap, massively increased the number of cholinergic fibers entering the HF from a competent nerve bridge as well as the number

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of fibers advancing 2 and 3mm into the hippocampal tissue -- almost reaching the extent of cholinergic reinnervation that would be seen in untreated HF several months later. Thus, the apparent resistance of hippocampal tissue to extensive penetration by

regenerating cholinergic fibers (cfr. 2 and 3mm locations in Fig. 2), like that generated in a nerve bridge deprived of its cellular NGF sources, can also be overcome by an increased availability of local NGF.

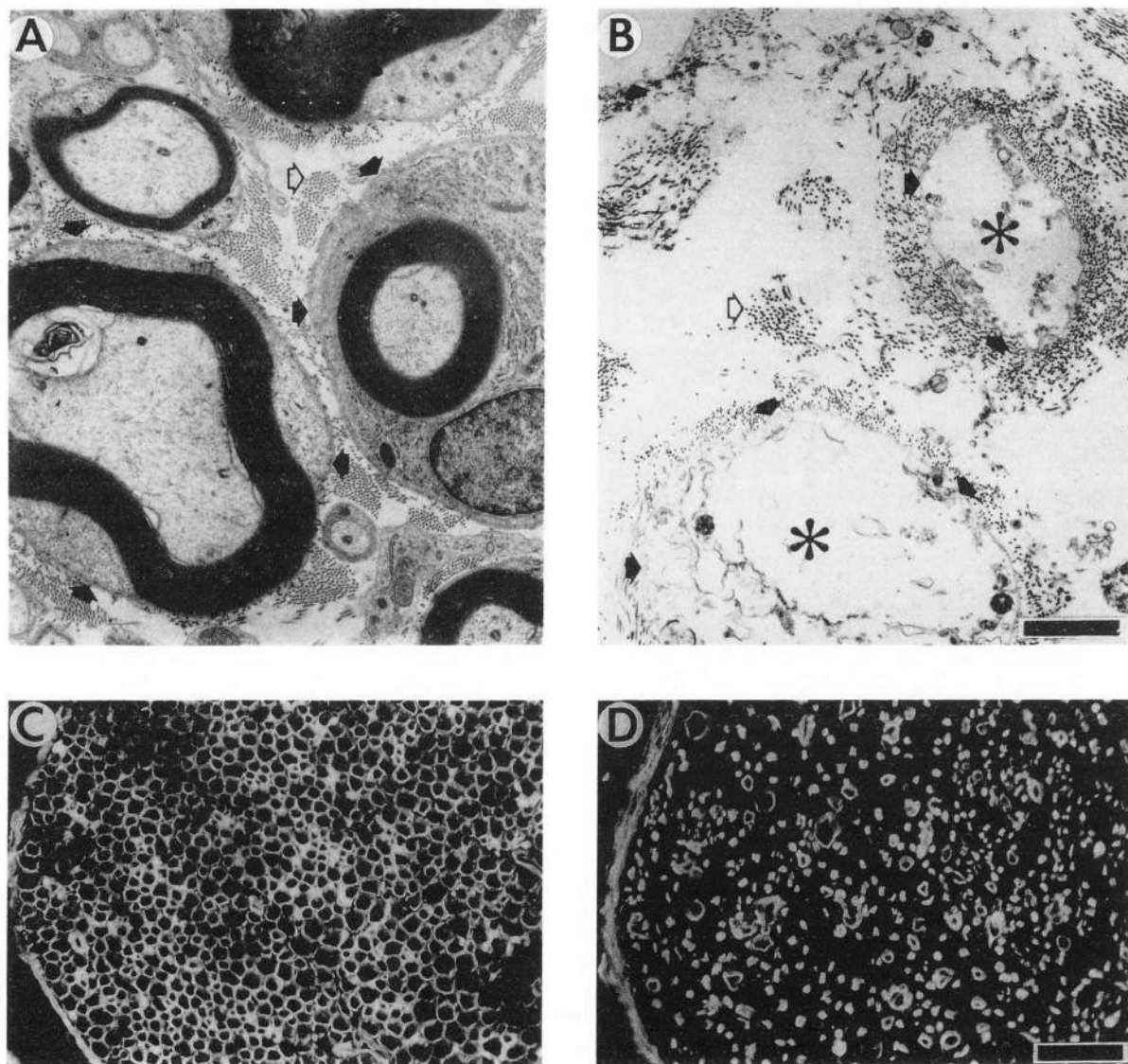


FIGURE 3 Morphology of normal and acellular nerve. **A)** Electron micrograph of a cross-section through a normal fresh cellular and **B)** acellular peripheral nerve showing the presence of endoneurial collagen (open arrows) and basal lamina (solid arrows) in both nerves and the virtual absence of cellular debris in most basal lamina tubes (asterisks). Bar = 2 μm .

C) Anti-laminin stained (fluorescence) cross section of a fresh cellular and **D)** acellular peripheral nerve showing the preservation of smaller diameter laminin-rich basal lamina tubes in the latter. The density of tube-shaped laminin-positive structures in the acellular nerve (2600/mm²) is nearly as high as that in the cellular one (3000/mm²). Bar = 100 μm .

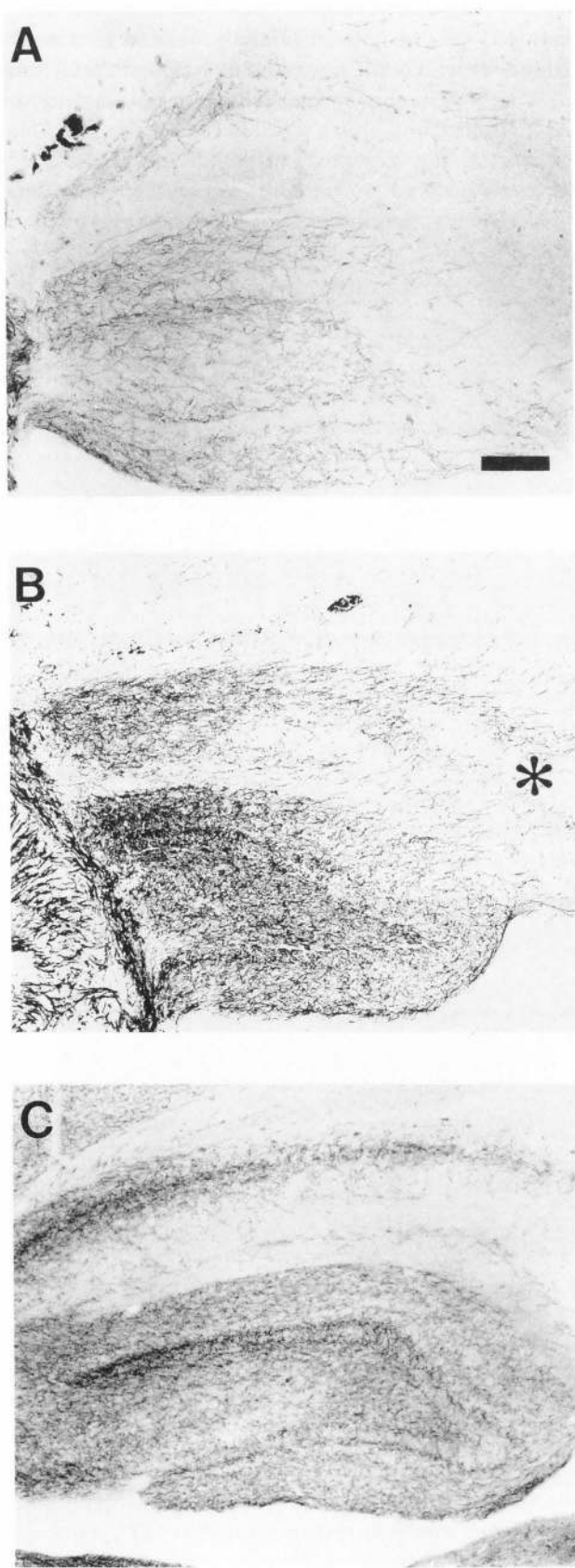


FIGURE 4 Infusion of nerve growth factor into the hippocampal formation promotes its cholinergic axonal reinnervation. **A)** One month after fimbria-fornix transection and sciatic nerve graft, but no NGF treatment, many fibers had regrown into the dorsal hippocampal formation. **B)** Nerve growth factor infusion for one month into the dorsal hippocampal formation (infusion site = asterisk), in addition to a nerve graft, dramatically increased hippocampal re-innervation by AChE-positive fibers, almost reaching the extent of reinnervation seen after 6 months without NGF treatment (**C**). Bar = 250 μ m.

Potential contributions by biomaterials

Two aspects of the studies just reviewed would greatly benefit from the development of suitable biomaterials if they are to progress toward potential clinical applications : a) the continuous delivery of the putative therapeutic agent, and b) the availability of surrogate materials for regeneration bridges.

NGF delivery into CNS tissue.

The support that NGF provides to basal forebrain and striatal cholinergic neurons in adult and aged rats may yield crucial benefits for the learning disabilities that characterize Alzheimer's disease. Similarly, local administration of exogenous trophic factors directly into re-innervation territories may prove necessary for a substantial regeneration of injured CNS axons.

Peripheral administration of trophic proteins are likely to be both ineffective and undesirable : ineffective, because of the inability of such molecules to cross the blood-brain barrier in order to reach the CNS parenchyma, and undesirable, because of i) their competence to affect important peripheral neurons and cause unwanted side effects and ii) their immunogenicity that may lead to peripheral production of antibodies and potentially severe immune reactions.

Central administration is demonstrably effective at much lower doses than would be required peripherally and can be further improved by judicious selection of the delivery sites. Continuous infusions driven by an osmotic minipump through a cannula permanently implanted intraventricularly or intraparenchymally have obvious drawbacks for widespread adoption, mainly because of the risks for mechanical trauma and infection involved in: i) the permanent implantation of the cannula and ii) the required periodic refilling of the infusion reservoir.

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Hence, the need for new ways to achieve CNS-located, long-lasting, slow-delivery sources of NGF or other relevant biological agents.

Polymer science offers various avenues toward solutions of this problem. Polymer pellets could contain the intended trophic agent itself, and act as local slow-delivery depots. More interestingly, polymers are under study (e.g., Jaeger et al, 1990) which could contain natural or bio-engineered cell sources of the desired agent and regulate its release according to the pore size of the polymer. Eventually, polymer-delivered agents could even be designed to stimulate production of the desired trophic factor by normal host cells, for example local astroglial cells (Manthorpe et al, 1990).

Surrogate regeneration bridges.

The septo-hippocampal model has shown that a freshly dissected nerve bridge can be resolved into two components: i) the living cells acting as sources of a required neuronotrophic factor such as NGF, which can be replaced by their own trophic product, and ii) a mechano-chemical scaffold providing the needed geometry and extracellular matrix, which remains available in acellular nerve preparations. Further investigations are likely to identify which extracellular matrix elements may be removed without consequence and, conversely, which of them remain critical for the scaffold competence -- thereby further advancing the reduction of a competent regeneration scaffold to its minimal terms. It would then become possible to construct an equivalent scaffold incorporating the essential features and, perhaps, even the separately needed trophic factors.

Such a task will gain considerably from the use of another experimental system, i.e. the "chamber" model (Lundborg et al, 1982) already well investigated for peripheral nerve regeneration. In this chamber model, the proximal and distal stumps of a transected sciatic nerve are inserted into the opposite ends of a silicone tube so as to leave an interstump gap (the "chamber") of the desired length. Through this chamber space, a new nerve structure will regenerate allowing the proximal axons to regrow through the distal nerve segment to the appropriate peripheral innervation territories. The sequence leading to this de novo regenerate includes the following steps (Williams et al, 1983; Varon and Williams, 1986): (1) fluid exudate from the two nerve stumps, rich in neuronotrophic

factors, fills the chamber (1 day); (2) fibrin polymerization from the exudate constructs a non-cellular matrix of coaxial fibrin-fibronectin fibers across the chamber (1 week); (3) fibronectin-promoted immigration of fibroblastic, Schwann and endothelial cells from both stumps converts the acellular matrix into a vascularized, cellular "nerve-like" structure including longitudinal laminin-coated spaces (3-4 weeks); and (4) the cell-conditioned, laminin-rich structure promotes the regeneration of axons out of the proximal stump into and through the chamber-bridging structure, progressively followed by axon myelination (3-6 week period). Experimental manipulations of the chamber fluid and matrix should, eventually, permit the construction of an artificial "surrogate" chamber bridge capable of substituting for the naturally developing one. Examples in this directions are the reported uses of: i) biodegradable polymers replacing the silicone tube (Molander et al, 1982; Seckel et al, 1984), ii) laminin-containing gel as a starting chamber matrix (Madison et al, 1987), iii) a matrix of preformed collagenous fibers (Rizvi et al, 1990), and iv) nitrocellulose-bound or polymer-released basic Fibroblast Growth Factor, a potent angiogenic protein (Danielsen et al, 1988; Aebischer et al, 1989); among others. Ultimately, information from both the "constructionist" approach with the chamber model and the "reductionist" approach with the septo-hippocampal model will define optimal surrogate biomaterials that would serve as competent bridges for the regeneration of optic nerve and spinal cord, two CNS structures whose damage contributes vastly to human pathologic traumata.

Acknowledgements

Work reviewed here has been partially supported by NINDS grants NS-16349 and NS-25011, and by NSF grants BNS-81-8847 and BNS-88-08285.

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Discussion with Reviewer

E.C. Azmitia: Is NGF the only player or could laminin also have a regeneration promoting role?

Authors: The data presented here show that NGF is a necessary player but not necessarily a sufficient one. In the bridge, the laminin-lined "tunnels" of the nerve scaffold may also be crucial for axonal advance and guidance -- even though they do not work without an NGF source. In the hippocampal tissue, there are no organized "laminin tracks" as in the bridge, but laminin-like materials are present within neurons (Neuron, 3:721-732) and may have a role to play. We conclude that any molecules (laminin or others) needed for cholinergic regeneration beside adequate NGF must be already available in the hippocampal CNS tissue. It may be that laminin injections could help the transition from nerve bridge to CNS tissue. Injections of laminin into adult brain have promoted extension of 5-HT-positive fibers from fetal transplants (*J. Chem. Neuroanat.*, 1:133-146).