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Maintaining the Neuronal Phenotype After Injury in the Adult CNS

Neurotrophic Factors, Axonal Growth Substrates, and Gene Therapy

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Abstract

Multiple genetic and epigenetic events determine neuronal phenotype during nervous system development. After the mature mammalian neuronal phenotype has been determined it is usually static for the remainder of life, unless an injury or degenerative event occurs. Injured neurons may suffer one of three potential fates: death, persistent atrophy, or recovery. The ability of an injured adult neuron to recover from injury in adulthood may be determined by events that also influence neuronal phenotype during development, including expression of growth-related genes and responsiveness to survival and growth signals in the environment. The latter signals include neurotrophic factors and substrate molecules that promote neurite growth. Several adult CNS regions exhibit neurotrophic-factor responsiveness, including the basal forebrain, entorhinal cortex, hippocampus, thalamus, brainstem, and spinal cord. The specificity of neurotrophic-factor responsiveness in these regions parallels patterns observed during development. In addition, neurons of several CNS regions extend neurites after injury when presented with growth-promoting substrates. When *both* neurotrophic factors and growth-promoting substrates are provided to adult rats that have undergone bilateral fimbria–fornix lesions, then partial morphological and behavioral recovery can be induced. Gene therapy is one useful tool for providing these substances. Thus, the mature CNS remains robustly responsive to signals that shape nervous system development, and is highly plastic when stimulated by appropriate cues.

Index Entries: Neuronal phenotype; adult CNS; neurotrophic factors; axonal growth substrate; gene therapy.

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Introduction

During development the neuronal phenotype is determined both by intrinsic cellular events, such as differential gene expression, and by extrinsic environmental cues, including neurotrophic factors and extracellular matrix-associated molecules (Singer et al., 1979; Purves et al., 1985; Thomas and Capecchi, 1990; Lee et al., 1992; Garbe et al., 1993; Hamelin et al., 1993; Mansuy et al., 1993; Nedivi et al., 1993; Sasaki and Hogan, 1994; Stipp et al., 1994; Turner et al., 1994; Vanselow et al., 1994; Zimmerman et al., 1994). Similar mechanisms contribute to developmental cell migration and neurite projection patterns (Letourneau, 1978, 1983; Goodman et al., 1984; Dodd and Jessell, 1988; Mounard, 1988; Edgar, 1989; Gordon-Weeks, 1989; Nottebohm, 1989). These events continue throughout nervous system development into postnatal time periods, and in some species into adulthood (Nottebohm, 1989).

After CNS injury, neurons degenerate and adopt one of three potential fates: death, persistent atrophy, or recovery. Which of these fates a neuron assumes depends on several factors, including:

1. The nature of the injury (Lieberman, 1971; Torvik, 1976);
2. The distance of injury from the cell body (Torvik, 1976; Grafstein, 1978);
3. The number of "sustaining" collaterals possessed by the damaged cell (Lieberman, 1971; Torvik, 1976); and
4. The neurotrophic factor-dependent state of the injured neuron (Hefti, 1986; O'Brien et al., 1990; Sofroniew et al., 1990; Tuszynski et al., 1990).

For example, the majority of basal forebrain medial septal cholinergic neurons degenerate and eventually die after axotomy (O'Brien et al., 1990; Tuszynski et al., 1990), whereas cholinergic neurons of the nucleus basalis of Meynert enter a state of prolonged atrophy (Sofroniew et al., 1983). Cholinergic hypoglossal neurons recover from injury (Armstrong et al., 1991). All of these cholinergic neuronal groups express the low-affinity nerve growth factor (NGF) receptor and are presumably NGF-responsive to varying degrees, yet display markedly different fates after injury.

Even if neurons recover from injury, functional restoration requires re-extension of damaged or lost axons. The ability of a mature neuron to reextend axons depends on several factors, including:

1. Ability to re-express growth-related genes (Thomas and Capecchi, 1990; Lee et al., 1992; Garbe et al., 1993; Hamelin et al., 1993; Mansuy et al., 1993; Nedivi et al., 1993; Sasaki and Hogan, 1994; Stipp et al., 1994; Turner et al., 1994; Vanselow et al., 1994; Zimmerman et al., 1994);
2. Availability of substrate molecules to which growing neurites may attach and extend (Richardson et al., 1980; Taniuchi et al., 1988; Bunge et al., 1989);
3. Availability of neurotropic factors that promote and guide axon growth (Letourneau, 1978); and
4. Myelin-associated growth inhibiting molecules that impair neurite extension (Schwab, 1990).

Inducing recovery of the injured CNS requires delivery of substances that promote both neuronal survival and axonal regrowth. Neurotrophic factors are protein molecules, usually target-derived and biologically available in limited quantities (Thoenen and Barde, 1980; Levi-Montalcini, 1987; Sorensen and Zimmer, 1988), that promote neuronal survival and neurite outgrowth (Letourneau, 1978; Hefti, 1986; Williams et al., 1986; Kromer, 1987). Several neurotrophic factors that influence survival and function of CNS neurons have now been identified, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophins-4/5 (NT-4/5), basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), and glial-derived neurotrophic factor (GDNF) (Table 1). The delivery of neurotrophic factors to regions of CNS injury in the adult can promote neuronal rescue (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Hagg et al., 1992; Knüsel et al., 1992; Sendtner et al., 1992; Clatterbuck et al., 1993; Cotman et al., 1993; Widmer et al., 1993).

Table 1
Neurotrophic Factors

Name	Location	Responsive neurons
NGF	PNS	Sympathetic
		Sensory (dorsal root)
	CNS	Basal forebrain cholinergic
BDNF	PNS	Dorsal root ganglion
		Nodose ganglion
	CNS	Subst. nigra
		Retinal ganglion
		Basal forebrain cholinergic (mild)
NT-3	PNS	Sympathetic ganglia
		Sensory (dorsal root)
		Nodose ganglion
	CNS	Locus ceruleus (noradrenergic)
		Motor neurons
NT-4/5	PNS	Trigeminal ganglion
		Motor neurons
	CNS	Motor neurons
bFGF	PNS	Ciliary ganglion
	CNS	Basal forebrain cholinergic (mild)
		Entorhinal cortex
		Retinal ganglion
		Motor neurons
CNTF	PNS	Ciliary ganglion
		Sympathetic ganglia
		Sensory (dorsal root)
	CNS	Motor neurons
		Basal forebrain cholinergic
		Thalamic neurons
GDNF	CNS	Ventral Mesencephalon

However, rescued neurons will not re-extend axons to appropriate targets unless substrate molecules are available to which neurites can attach and grow (Richardson et al., 1980; Kromer et al., 1981; Wendt et al., 1983; Carter et al., 1989; Tuszynski et al., 1990). One approach to optimizing the recovery of the injured adult CNS therefore is to deliver neurotrophic factors to a region of injury together with substrates that support and promote axonal regrowth. Combining intracerebroventricular infusions of neurotrophic factors with grafts to the injured brain of fetal growth substrates is one way of addressing this problem (Tuszynski et al., 1990). Another is to genetically modify host cells to produce and secrete neurotrophic factors, then graft these cells embedded within a conducive growth substrate (such as collagen) to an area of host injury (Rosenberg et al., 1988; Kawaja et al., 1992).

Promoting Recovery of the Adult Brain

Neurotrophic Factor Infusions Combined with Grafts of Growth-Promoting Substrates

The septohippocampal projection has been a frequently utilized model in studies of neuronal regeneration because it is well characterized anatomically and functionally, and demonstrates clear neurotrophic-factor responsiveness (Wyss et al., 1979; Hefti, 1986; Williams et al., 1986; Kromer, 1987; Swanson et al., 1987). Cholinergic neurons with cell bodies in the medial septum (MS) and vertical limb of the diagonal (VDB) band project to the hippocampus through the fimbria–fornix fiber bundle (Milner and Amaral, 1984; Amaral and Kurz, 1985; Swanson et al., 1987). Following transection of this pathway, cholinergic cell bodies in the MS and VDB undergo retrograde degeneration and eventual death (Armstrong et al., 1987; O'Brien et al., 1990; Tuszynski et al., 1990). If NGF is infused into the brain at the time of the fimbria–fornix lesion, however, this retrograde cholinergic neuronal degeneration can be entirely prevented (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Barnett et al., 1990). First demonstrated in the adult rat brain, these findings were subsequently extended to the adult primate brain (Fig. 1) (Koliatsos et al., 1990; Tuszynski et al., 1990, 1991). In the intact brain, NGF is continually produced by hippocampal neurons that are targets of basal forebrain cholinergic innervation (Ayer LeLievre et al., 1983; Seiler and Schwab, 1984; Sheldon and Reichardt, 1986), providing a means for target

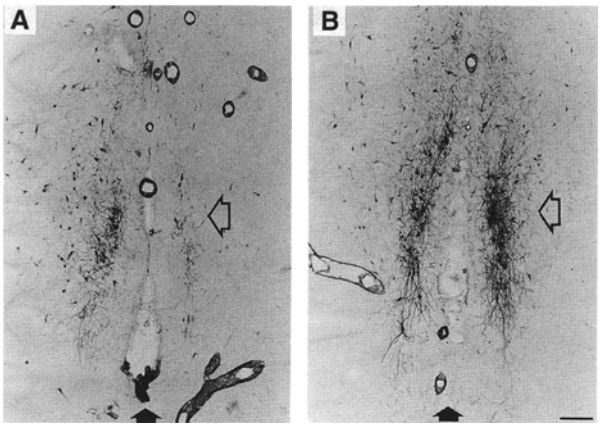

Fig. 1. Cholinergic neuronal degeneration is prevented by NGF infusion into the adult primate brain. **(A)** Control subjects with unilateral, right-sided fornix transections show loss of NGF receptor immunolabeling in the right medial septal nucleus. Open arrow indicates region of lost neuronal labeling; closed arrow indicates midline. The left, intact medial septum shows a typical distribution of cholinergic cell bodies. **(B)** NGF infusions prevent cholinergic neuronal degeneration in the right medial septum, ipsilateral to the fornix transection (open arrow). Scale bar = 100 μm.

neurons to regulate their cholinergic synaptic input. The regulation of synaptic input by limiting trophic factor availability is therefore a mechanism thought to be operative both during development (Purves and Lichtman, 1985) and in adulthood.

Although cholinergic neuronal degeneration in the medial septum after fimbria–fornix transection can be prevented by NGF infusions, the spared cholinergic neurons remain disconnected from their natural hippocampal target. The length of time that these isolated cholinergic neurons continue to require NGF for survival is unclear: Is NGF required for survival only of *injured* cholinergic neurons, or is it required continuously for the survival of *intact* neurons as well? To address this question, Sofroniew and coworkers destroyed the source of NGF to intact cholinergic neurons by injecting excitotoxins into the hippocampus (Sofroniew et al., 1990, 1993; Svendsen et al., 1994). Cholinergic axons were uninjured by this lesion. When medial septal cholinergic neurons were examined from 2 wk to more than 1 yr later, they had become atrophic, but total neuronal numbers had not been reduced. These findings indicated that NGF was not required for the survival of *intact* neurons, although it was required for the maintenance of normal neuronal phenotype. Subsequent transection of the fimbria–fornix some time after hippocampal destruction by excitotoxins *did* result in cholinergic neuronal degeneration and probable cell death, suggesting that *injured* neurons are NGF-dependent for survival (Sofroniew et al., 1990).

To further address whether medial septal cholinergic neurons continually or transiently require NGF for survival in adulthood, we performed bilateral fimbria–fornix lesions in rats and infused either NGF or artificial CSF intracerebroventricularly for 9 wk (Tuszynski et al., in press). Infusions were then discontinued, and animals were allowed to survive an additional 6 mo without further NGF treatment. We hypothesized that if medial septal neurons were only transiently NGF-dependent after injury, then the 9-wk infusion period would be sufficient to support them to recovery and NGF beyond this period would not be required. Indeed, we found long-term survival of 76 ± 4% (± SEM) of cholinergic neurons in NGF-treated subjects, compared to 40 ± 5% in untreated animals ($p < 0.0001$; Fig. 2). These results suggest that the dependence of septal neurons on NGF is transient after injury. Neurotrophic-factor dependence is one example of a shared plastic characteristic between the adult and developing nervous system.

Having shown that basal forebrain cholinergic neurons require NGF only transiently after injury, we next sought to determine whether reinnervation of host targets could be achieved by combining NGF infusions with grafts of neurite growth-promoting substrates to fimbria–fornix lesion cavities (Tuszynski et al., in press). Rats received bilateral lesions of the fimbria–fornix, then grafts into the lesion cavity of embryonic d 18 (E18) fetal hippocampus. The fetal grafts were placed in the lesion

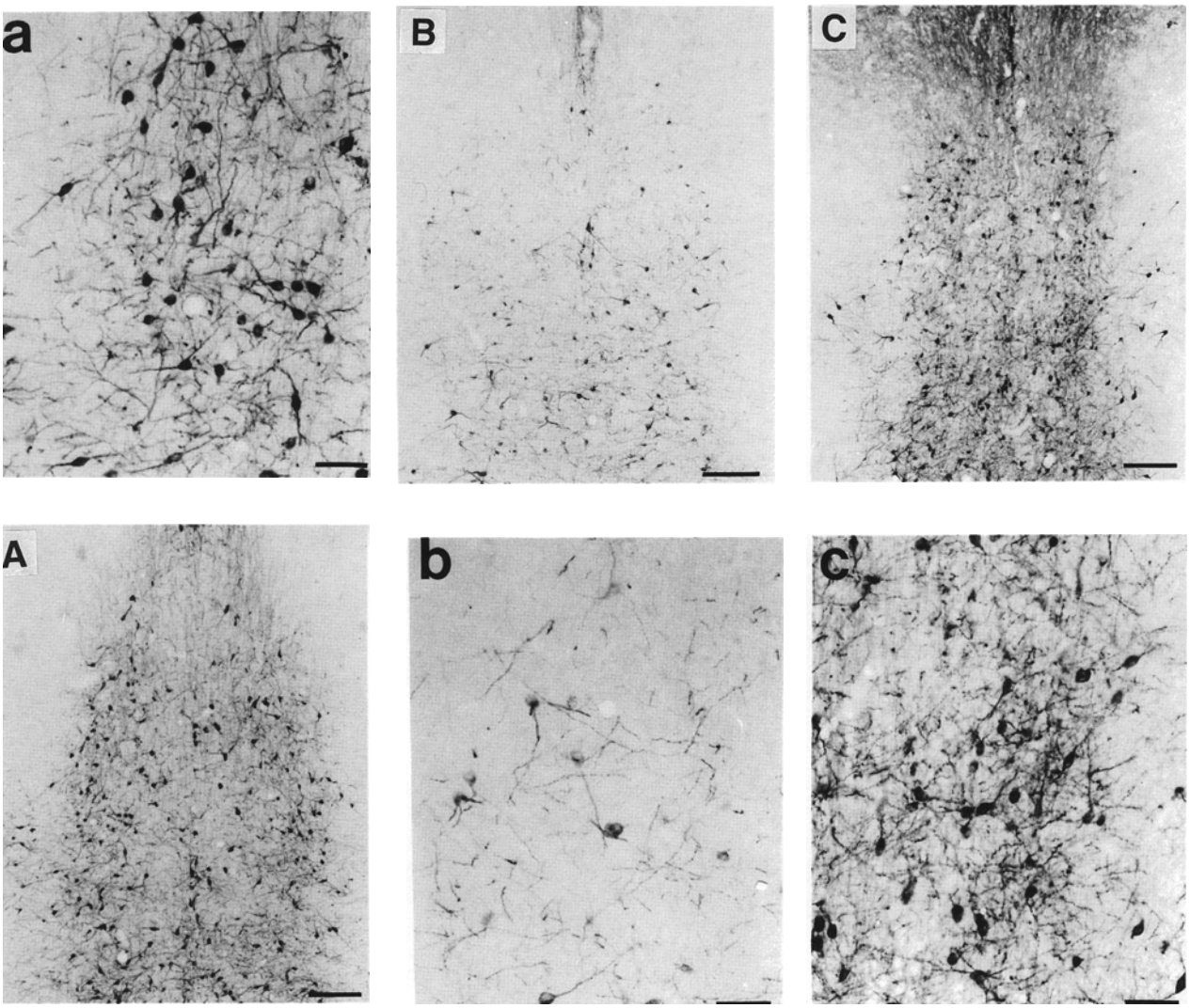

Fig. 2. Transient NGF infusions prevent long-term cholinergic neuronal degeneration. **(A,a)** Normal distribution of medial septal cholinergic neurons in the intact rat brain at low and high magnification. Immunocytochemical label for the p75 low-affinity NGF receptor. **(B,b)** After bilateral fimbria–fornix transections, medial septal cholinergic neurons undergo retrograde degeneration that persists up to 8 mo after the lesion. **(C,c)** Subjects that receive bilateral fimbria–fornix transections and transient 9-wk NGF infusions show long-term rescue of medial septal cholinergic neurons. Six months after NGF infusions are discontinued, a high proportion of neurons remain labeled for the p75 receptor. Scale bars (A,B,C) = 62 μm; (a,b,c) = 25 μm.

cavity to act as a "bridge" over which host neurons could regenerate from the host medial septum to the host hippocampus (Fig. 3). Fetal tissue was chosen as a bridge because it is a conducive environment for the growth of axons, containing factors that promote neurite adhesion and extension (Kromer et al., 1981; Tuszynski et al., 1990). Fetal hippocampal tissue does not contain an appreciable population of intrinsic cholinergic neurons (Swanson et al., 1987; Butcher et al., 1989; Oh et al., 1992), so any cholinergic fibers penetrating the graft and entering the host hippocampus would be host-derived (Kromer et al., 1981; Tuszynski et al., 1990). Concurrently with graft placement, animals received

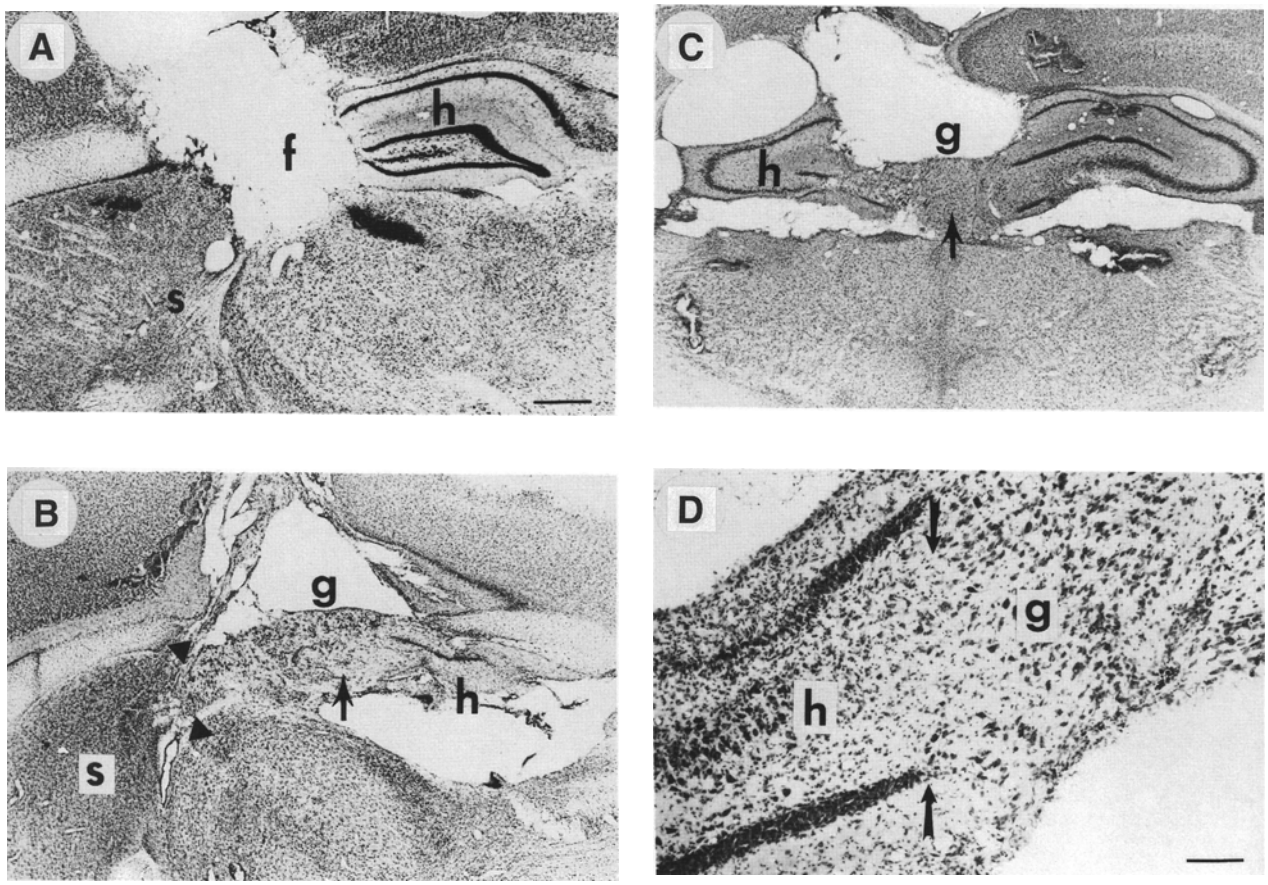

Fig. 3. Fetal hippocampal grafts bridge fimbria–fornix lesion cavities. **(A)** Sagittal view of the lesioned septohippocampal projection. The region of the fimbria–fornix (f) has been surgically aspirated, disconnecting the hippocampus (h) from its septal (s) cholinergic input. **(B)** Eight months after fimbria–fornix aspiration, the previous lesion cavity is completely occupied by a bridging graft (g and arrow). Arrowheads indicate area of excellent fusion of host with graft at septal/graft interface. Sagittal view. **(C)** A coronal view demonstrates graft (g and arrow) bridging to both hippocampi (h). Scale bar (A–C) = 100 μm. **(D)** High magnification view of host/graft interface (arrows) at hippocampal pole of graft. There is excellent graft integration, without evidence of glial scar. Scale bar = 25 μm.

intracerebroventricular infusions of NGF (100 μg/mL) for 9 wk. NGF infusions were then discontinued and animals survived an additional 6 mo. Prior to sacrifice, animals were tested on two behavioral tasks. The first task assessed a simple form of mnemonic function, habituation (Corey, 1978; Brennan et al., 1984; Markowska et al., 1989), and the second task assessed a more complex form of memory, acquisition of spatial reference memory in the Morris water maze (Morris et al., 1984). Four control groups were compared to the experimental subjects:

1. Unlesioned animals;
2. Lesioned animals that received infusions of artificial CSF;
3. Lesioned animals that received infusions of NGF but not bridging grafts; and
4. Lesioned animals that received fetal bridging grafts but not NGF infusions.

After completion of behavioral testing, the hippocampi were injected with a retrogradely transported fluorescent tracer to determine whether connectivity with the host medial septum was re-established. Animals were then sacrificed and the brains examined for com-

pleteness of the fimbria–fornix lesion, savings of host medial septal cholinergic neurons, and degree of host hippocampal reinnervation.

On behavioral testing, intact animals showed habituation. For this task, animals were placed into an activity chamber and their locomotor activity was measured by counting the number of interruptions of a 4 × 4 grid of photoelectric beams (Markowska et al., 1989). After three exposures to the chamber on 2 separate days (20 min/exposure), activity among intact animals on the final trial decreased by 25 ± 10% (± SEM; $p < 0.05$) compared to the first trial, reflecting habituation (Corey, 1978; Brennan et al., 1984; Markowska et al., 1989). Animals that received both NGF infusions and bridging grafts also showed a significant degree of habituation, with a decrease in activity of 17 ± 6% ($p < 0.05$). None of the other groups of lesioned animals showed significant habituation. Thus, the combination of NGF infusions and bridging grafts induced recovery of a simple mnemonic task (Tuszynski et al., in press).

In contrast, none of the lesioned groups showed significant behavioral recovery on the more complex mnemonic task, acquisition of spatial reference memory in the Morris water maze. There was a trend favoring recovery in the NGF/graft group, and on individual days of testing the performance of the NGF/graft group did not differ significantly from intact animals, unlike the other lesioned groups. However, two-way analysis of variance of combined data from all 10 d of testing demonstrated significant effects over groups and over time, but nonsignificance in the interaction term, groups × days ($F[36,270] = 1.17$, $p = .24$) (Swanson et al., 1987). Thus, combined NGF infusions and bridging grafts induced recovery of a simple (habituation) but not a more complex (spatial reference memory) behavior function.

Histological analysis of the same experimental animals revealed that all recipients of 9-wk NGF infusions showed long-term savings of medial septal cholinergic neurons, regardless of the presence of a bridging graft (*see* Fig. 2). Animals without NGF infusions showed no long-term savings of cholinergic neurons. Animals with fetal grafts but not NGF infusions showed no rescue of medial septal cholinergic neurons, indicating that graft-derived trophic factors could not support degenerating host cholinergic neurons. On the other hand, only subjects that received fetal bridging grafts showed hippocampal reinnervation by host cholinergic fibers ($p < 0.001$; Fig. 4). Injections of retrogradely transported fluorescent dyes into host hippocampi resulted in labeling of medial septal neurons only among recipients of bridging grafts, confirming re-establishment of connectivity between the host septum and hippocampus. Animals that received both NGF infusions *and* bridging grafts did not show more hippocampal reinnervation at the light microscopic level than animals that received bridging grafts *only*. The latter finding is not surprising: NGF was infused intracerebroventricularly rather than into the hippocampal target and therefore did not provide directional guidance to regenerating host cholinergic fibers (Letourneau, 1978; Hagg et al., 1990).

Thus, only animals that received NGF infusions *and* fetal bridging grafts showed both significant savings of host cholinergic cell bodies and reinnervation of the host hippocampus. Further, only animals that received both NGF infusions and bridging grafts showed functional recovery on a simple mnemonic task. These results indicate that functional recovery requires both reinnervation of host targets and prevention of host neuronal loss; remediation of either element alone will not restore function. More extensive functional recovery will require either heightened degrees of host neuronal savings, more extensive reinnervation of host targets, or both. With the availability of several neurotrophic factors that influence diverse neuronal populations in the adult CNS, it may be possible to combine neurotrophic factor infusions with delivery of axonal growth substrates to restore host neural circuitry after injury to diverse brain regions.

This study gave rise to another observation: Aberrant host sprouting responses after fimbria–fornix lesions can be modified by reconstructing host circuitry (Fig. 5). Previous

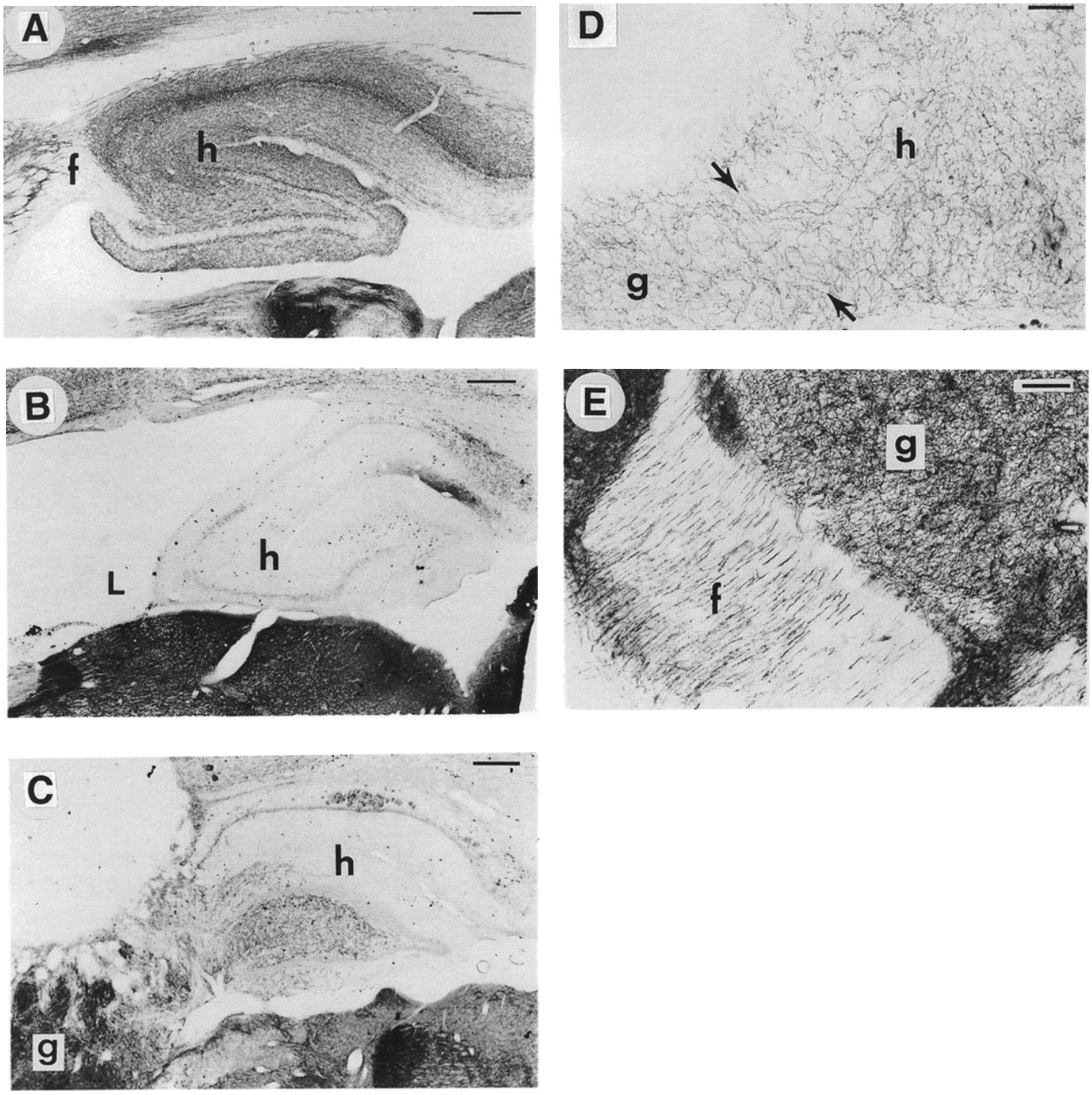

Fig. 4. The adult host hippocampus is reinnervated by host septal cholinergic fibers after grafting of bridging substrates. **(A)** Normal appearance of the unlesioned hippocampus (h) in sagittal section of acetylcholinesterase-stained section, showing cholinergic fibers in all hippocampal subfields. Hippocampal cholinergic innervation originates in the basal forebrain and is relayed through the fornix (f) **(B)** Eight months after fimbria–fornix lesions, the lesioned hippocampus contains no cholinergic fibers. L, area of lesioned fimbria–fornix. **(C)** In the presence of a bridging graft (g) there is substantial hippocampal reinnervation primarily in the dentate gyrus. Scale bar: (A–C) = 60 μm. **(D)** High magnification of graft/host interface at hippocampal pole of graft (arrows) illustrates free exchange of cholinergic fibers from graft to host target region. **(E)** Similarly, high magnification rate of graft/host interface at septal pole of graft illustrates free passage of fibers from host fimbrial remnant **(F)** to graft. Scale bar: (D,E) = 20 μm.

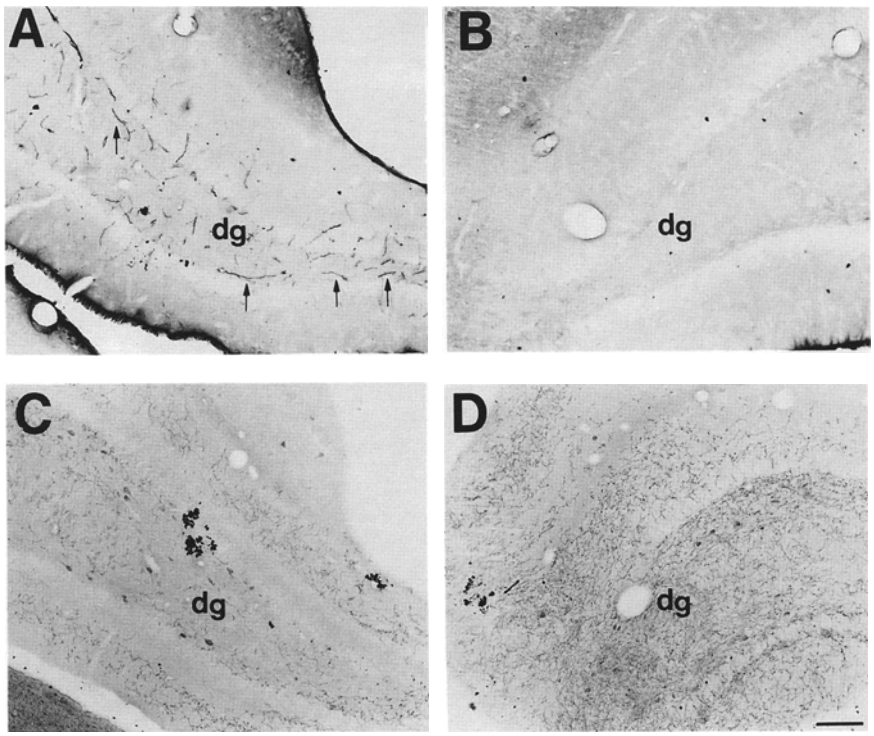

Fig. 5. Aberrant host sprouting responses can be modified by regenerating host inputs. **(A)** Tyrosine hydroxylase (TH) immunocytochemical label shows sprouting of sympathetic neurites (arrows) into the dentate gyrus (dg) of the hippocampus following fimbria–fornix transection. Cholinergic denervation of the same hippocampus is present **(C)**. In contrast, sympathetic (TH-labeled) fibers are not present **(B)** in a hippocampus that *is* reinnervated by host cholinergic fibers **(D)**. All sections are from the same animal; a small bridge connected the left hippocampus to the septum, and a large bridge connected the right hippocampus to the septum. (B,D) are stained for acetylcholinesterase. Scale bar = 40 μm.

investigators have reported that fimbria–fornix lesions result in sprouting of sympathetic neurites into the hippocampus (Soffran et al., 1989). This response occurs because hippocampal neurons normally produce NGF that is taken up by cholinergic terminals in the hippocampus and retrogradely transported to cell bodies in the medial septum; following cholinergic axotomy, NGF levels transiently rise in the hippocampus because NGF is not removed by cholinergic terminals. Sympathetic neurons are NGF-responsive and sprout into the hippocampus in response to the elevated levels of NGF. In the experiment reported earlier, animals with host cholinergic reinnervation of the hippocampus did not show sprouting of tyrosine hydroxylase-immunolabeled, sympathetic neurites into the hippocampus (Fig. 5). In contrast, animals lacking cholinergic reinnervation of the hippocampus did show sympathetic sprouting. Thus, reconstruction of normal host projections can alter aberrant host sprouting responses, indicating another means by which host responses can be modulated in the adult brain after injury.

Promoting Recovery of the Adult Brain: Gene Therapy

The ideal system for delivering substances to the CNS would bypass or penetrate the blood–brain barrier and would be regionally specific, chronically active, regulatable, and well tolerated. Intracerebroventricular infusions have been a useful model for studying

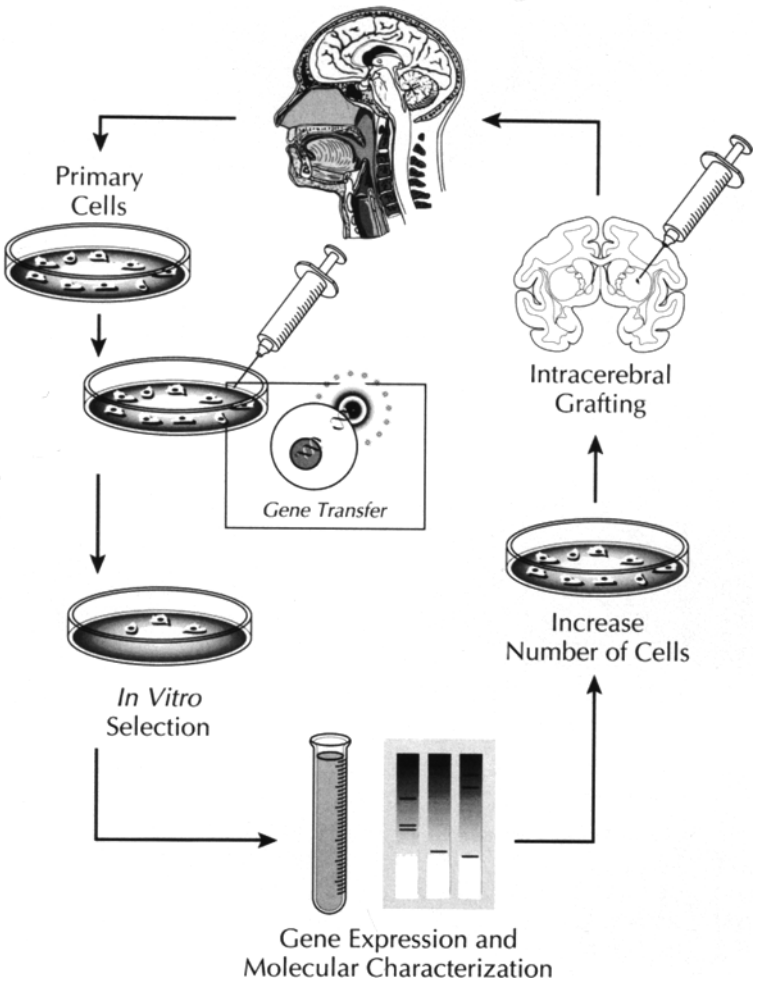

Fig. 6. Schematic view of gene therapy. A donor animal or human provides primary cells. New genes are transferred to the host cells in vitro, and cells that successfully incorporate the transgene are selected. Expression of the complete gene product is assessed in vitro, and clones or plates that produce the highest amounts of the desired gene product and are selected for subsequent increase in cell number. When sufficient numbers of cells have been grown, they are harvested for in vivo grafting.

neurotrophic-factor responsiveness in the brain, but have potential drawbacks, including broad effects of neurotrophic factors on nontargeted CNS structures. For example, intracerebroventricular infusions of NGF not only protect cholinergic neurons from degeneration but also induce sprouting of sympathetic neurites that invest cerebral blood vessels (Saffran et al., 1989), cause weight loss (Williams, 1991), and promote sprouting of spinal cord sensory neurites (Tuszynski et al., 1994b). These diverse NGF effects, a consequence of the broad delivery of NGF after infusion into the ventricular system of the brain, could limit clinical utility. Further, large molecules the size of NGF may penetrate CNS parenchyma for distances of only 1 mm after intracerebroventricular infusions (Lapchak et al., 1993), rendering them incapable of reaching targeted regions. Gene therapy holds promise as an alternative means of delivering molecules to the CNS in a well-localized, long-term, and chronic manner (Fig. 6) (Gage et al., 1987).

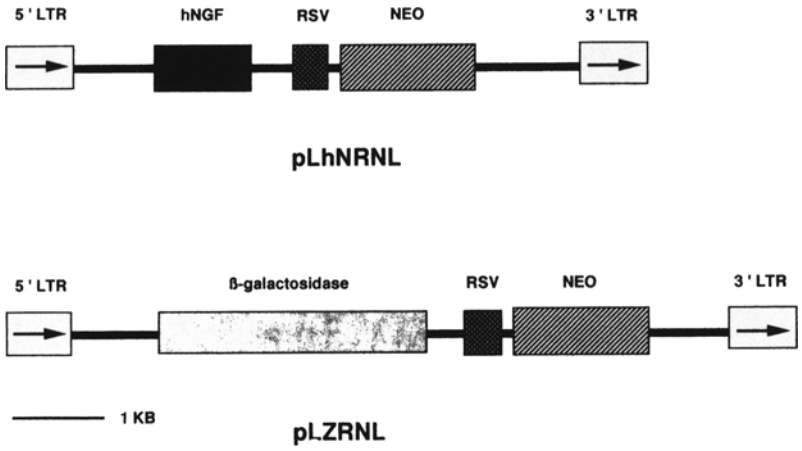

Fig. 7. Retroviral expression vector. The 5' long terminal repeat (LTR) region of the wild-type Moloney murine leukemia virus contains a constitutive promoter that expresses the first transgene of interest, in this case beta-galactosidase. This is followed by a second promoter derived from Rous sarcoma virus (RSV) that constitutively expresses the *neo* gene. Cells that express *neo* are resistant to neomycin added to the culture medium, permitting selection in vitro of cells that incorporate the transgene. The retroviral vector is concluded by the wild-type 3' LTR.

To explore the potential utility of gene therapy in models of neuronal degeneration, we genetically modified primary rat fibroblasts to produce and secrete nerve growth factor (Rosenberg et al., 1988). Replication-incompetent retroviral vectors derived from wild-type Moloney murine leukemia virus were constructed containing the cDNA for the active beta-component of murine NGF (Fig. 7). Constitutive NGF expression occurred under the influence of a long-terminal repeat (LTR) promoter from the wild-type retrovirus, whereas a second internal promoter from Rous sarcoma virus (RSV) constitutively expressed a second gene, *neo*. Cells containing the *neo* gene become resistant to neomycin added to the culture medium, thereby allowing for in vitro selection of cells containing the transgene. Primary skin fibroblasts were chosen as target cells for gene modification in vitro since they are readily obtainable by skin biopsy, divide in culture, and survive grafting to the brain (Rosenberg et al., 1988). In vitro, genetically modified fibroblasts secrete an average of 15 ng NGF/10^6 cells/h, a rate comparable to that attained with intracerebroventricular pumps. These NGF-secreting fibroblasts were then grafted to the brains of rats that had undergone unilateral fimbria–fornix transections. Two weeks after grafting, retrograde degeneration of cholinergic neurons was prevented in 100% of basal forebrain cholinergic neurons in recipients of NGF-secreting grafts, whereas control fibroblast grafts genetically modified to secrete *E. coli* beta-galactosidase did not prevent cholinergic degeneration (Rosenberg et al., 1988). Thus, gene therapy prevented retrograde neuronal loss in the brains of adult rats, and was subsequently shown to sustain this effect for at least 2 mo after in vivo grafting (Kawaja et al., 1992). Parallel results have been reported by other investigators using animal models of cholinergic and dopaminergic neuronal degeneration (correlative animal models of Alzheimer diseases and Parkinson disease, respectively) (Ernfors et al., 1989; Horella et al., 1990a,b,c, 1991; Strömberg et al., 1990; Le Gal La Salle et al., 1993).

We then sought to determine whether gene therapy could also prevent neuronal degeneration in the adult primate brain. Adult cynomolgous or rhesus monkeys underwent unilateral fornix transections and received grafts of their own (autologous) fibroblasts, genetically modified to secrete human NGF, into the basal

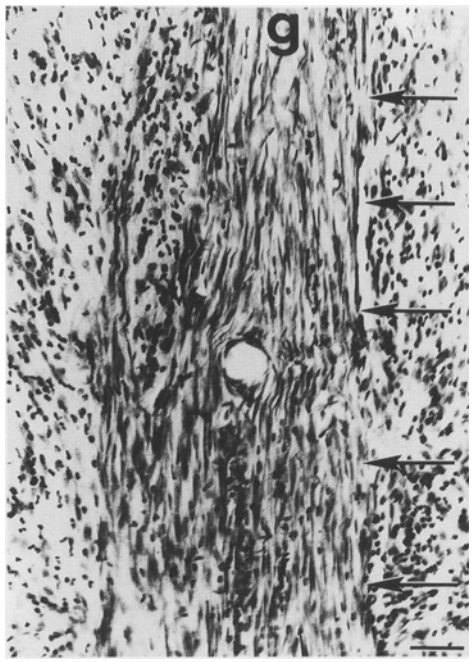

Fig. 8. Primary fibroblast graft in the adult primate brain. One month after grafting, cells with typical fibroblast morphology are present in the medial septal nucleus. g, graft. Arrows indicate right margin of graft. Scale bar = 40 μm.

forebrain septal nucleus (Fig. 8). Grafts were placed adjacent to cholinergic cell bodies in the medial septum. Control subjects received grafts of autologous fibroblasts genetically modified to produce beta-galactosidase. One month later, retrograde cholinergic neuronal degeneration was significantly prevented in animals that received NGF-secreting grafts compared to control grafts (67.8 ± 8.4% vs 27.2 ± 5.8%, $p < 0.02$; Fig. 9). The beneficial effect of grafts correlated strongly with graft size and distance from host cholinergic cell bodies: Smaller grafts located more than 2.8 mm from the host target did not prevent host cholinergic degeneration, whereas larger grafts closely located to host neurons prevented the degeneration of up to 92% of cells. Smaller grafts located adjacent to host neurons had intermediate effects, preventing degeneration in 65% of neurons compared to the contralateral, unlesioned side.

Interestingly, NGF-secreting grafts in the host septal nucleus appeared to induce expression of NGF receptor and choline acetyltransferase immunocytochemical labeling in neuronal cell bodies that do not normally express these substances (Fig. 10). The latter observation raises the possibility that neurotrophic factors in the adult brain either upregulate expression of otherwise quiescent genes in cells or else induce neuronal migration. If true, the former effect would indicate that neurotrophic-factors can alter the adult neuronal phenotype. Such a phenomenon has been observed in in vitro developmental models, but not in the adult in vivo.

Gene therapy can be combined with grafts of neurite growth-promoting substrates to promote reconstruction of lesioned host projections (Kawaja et al., 1992; Eagle et al., 1993). In rat models, fibroblasts genetically modified to produce NGF have been embedded in collagen matrices and grafted to the lesioned septo-hippocampal projection (Kawaja et al., 1992; Eagle et al., 1993). In theory, NGF secreted by the genetically modified cells would promote host septal cholinergic neuronal rescue and attract lesioned cholinergic neurites into the grafts, whereas collagen would provide a conducive substrate for neurite growth. In these experiments, host septal neuronal rescue and partial hippocampal reinnervation was observed, corroborating results of previous studies combining NGF infusions with fetal hippocampal bridging grafts.

In the intact spinal cord, grafts of fibroblasts genetically modified to produce NGF induced the robust ingrowth of sensory neurites (Tuszynski et al., 1994b). After grafting to the lesioned spinal cord, NGF-secreting fibroblasts embedded in collagen matrices promoted ingrowth of motor as well as sensory neurites, indicating that motor neurites can re-express neurotrophic-factor responsiveness after injury (Tuszynski et al., 1993). Both sensory and motor spinal cord neurons express NGF receptors during development (Raivich et al., 1985; Richardson et al., 1986; Taniuchi et al., 1986; Buck et al., 1987; Yan et al., 1988; Ernfors et al., 1989; Ruit et al., 1992), thus developmental patterns

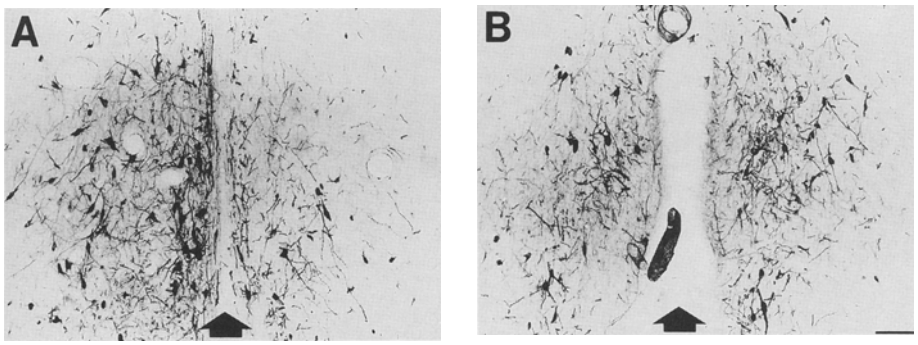

Fig. 9. Fibroblasts genetically modified to produce NGF prevent cholinergic neuronal degeneration in the adult primate brain. **(A)** In control monkeys with grafts of β-galactosidase producing fibroblasts, right-sided fornix transections result in retrograde cholinergic neuronal degeneration in the medial septum. Arrow indicates midline. **(B)** In contrast, cholinergic neuronal degeneration is prevented in monkeys with autologous grafts of fibroblasts genetically modified to produce NGF. Scale bar = 40 μm.

of neurotrophic-factor responsiveness are recapitulated in the adult. Adult spinal cord sensory neurites also sprout in response to grafts of fibroblasts genetically modified to produce the neurotrophic factors NT-3 and basic FGF (Tuszynski et al., 1993), further recapitulating developmental patterns (Gospodarowicz et al., 1987; Baird et al., 1988; Eckenstein et al., 1991; Ferguson et al., 1991; Sweetnam et al., 1991; Yan et al., 1991).

The ability of somatic gene transfer to prevent neuronal degeneration provides an alternative to intracerebroventricular drug administration for the treatment of neurodegenerative or traumatic disease (Tuszynski et al., 1994a). By avoiding systemic exposure to neurotrophic factors, gene therapy can target desired neuronal populations without eliciting potentially deleterious responses from other neurotrophic-factor-responsive neurons. Significant issues that remain to be addressed before gene therapy is of practical clinical use include:

1. Documentation of long-term transgene expression. We have shown persistent responsiveness of the adult primate brain to grafts of NGF-secreting cells for at least 6 mo in vivo (Tuszynski et al., 1994c), but direct evidence of transgene expression for 6 mo to 1 yr in vivo should be established.
2. Regulation of gene expression. Most transgene vectors utilize constitutive gene promoters, but the ability to regulate gene expression would be desirable. Use of steroid response elements or enhancers/suppressors of gene expression may be helpful in this regard.
3. Transgene "escape." In some cases it may be useful to turn off transgene expression completely, or to destroy genetically modified cells in vivo. One means of doing this should be to incorporate apoptotic or killer genes (e.g., thymidine kinase) into cells (Ezzeddine et al., 1991; Barba et al., 1993; Tuszynski et al., 1994c).
4. Transfection of primary CNS cells. In some circumstances it may be desirable to directly introduce genes into nondividing CNS cells. Although retroviral vectors target dividing cells (Miller, 1990), other vectors, such as adenovirus and herpes virus, can transfect nondividing cells and are currently a subject of active study (Geller and Freese, 1990; Federoff et al., 1992; Le Gal La Salle et al., 1993).

Summary

The adult CNS maintains responsiveness to several factors that influence the nervous system during development, including neurotrophic factors and neurite growth substrates. During both development and in the adult

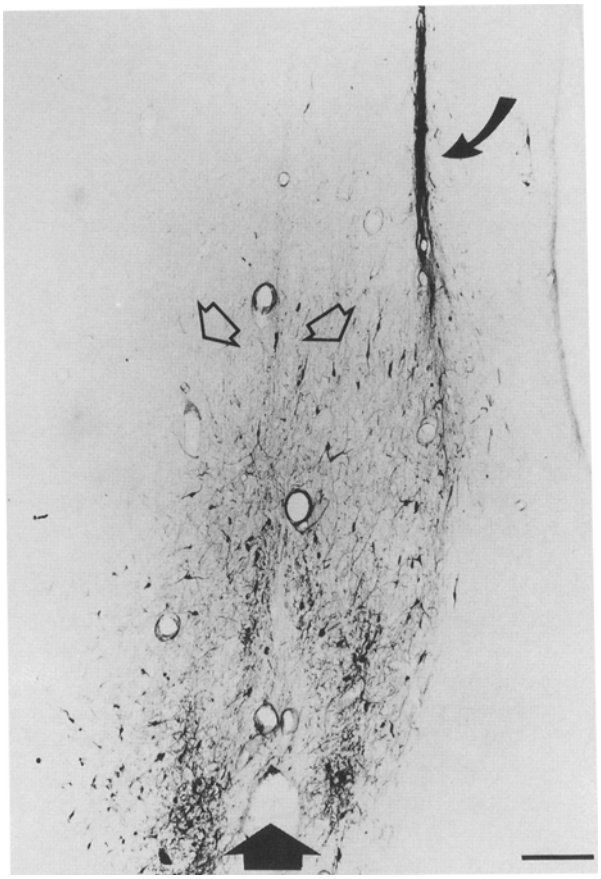

Fig. 10. Does NGF induce alteration of the adult neuronal phenotype? Adjacent to an NGF-secreting fibroblast graft (curved arrow) in the right medial septum of the adult primate are neurons labeled for the low-affinity NGF receptor (right open arrow). Such cell bodies are not visible in the same septal region on the contralateral side of the brain (left open arrow).

brain, neurotrophic factors prevent neuronal degeneration, and conducive neurite growth substrates, such as collagen and laminin promote neurite extension. The pattern of plasticity of the adult CNS is neither random nor unpredictable; rather, it appears to be regionally predictable based on patterns displayed during development, allowing one to design strategies to promote nervous system recovery from injury. When neurotrophic factor infusions are *combined* with grafts of neurite growth-promoting substrates, limited morphological and functional recovery can be elicited from the injured adult brain. Gene therapy offers a useful means of providing both neurotrophic-factor support and conducive growth substrates to the injured CNS in a highly localized, stable, and well-tolerated manner.

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