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Bridging grafts and transient nerve growth factor infusions promote long-term central nervous system neuronal rescue and partial functional recovery

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ABSTRACT **Grafts of favorable axonal growth substrates were combined with transient nerve growth factor (NGF) infusions to promote morphological and functional recovery in the adult rat brain after lesions of the septohippocampal projection. Long-term septal cholinergic neuronal rescue and partial hippocampal reinnervation were achieved, resulting in partial functional recovery on a simple task assessing habituation but not on a more complex task assessing spatial reference memory. Control animals that received transient NGF infusions without axonal-growth-promoting grafts lacked behavioral recovery but also showed long-term septal neuronal rescue. These findings indicate that (*i*) partial recovery from central nervous system injury can be induced by both preventing host neuronal loss and promoting host axonal regrowth and (*ii*) long-term neuronal loss can be prevented with transient NGF infusions.**

Neurons of the adult mammalian central nervous system (CNS) possess a limited capacity for recovery after injury (1–4). Factors that account for this limitation probably include a minimal or absent increase in neurotrophic factor levels after injury, a lack of suitable guidance channels and substrates to promote and direct axonal regrowth after injury, and the presence of growth-inhibiting molecules on CNS myelin (5). In contrast, elevation in neurotrophic factor levels (6, 7) and production of substrate molecules that promote and guide axonal growth (8–10) contribute to functionally significant axonal regeneration in the peripheral nervous system.

Various substances have been grafted to the CNS to promote regeneration of host projections after injury, including peripheral nerve segments (1, 3), synthetic growth substrates (e.g., nitrocellulose, collagen gels, or amniotic membrane) (11, 12), and fetal brain grafts that are utilized as "bridges" for host axonal neurite growth rather than as replacements for host neurons (13, 14). In the latter instance, host fibers penetrate into and through the fetal graft to reach their host target, rather than forming synapses exclusively in the graft (13, 14). These methods for reconstructing host circuitry have resulted in partial reinnervation and electrophysiological responsiveness of host targets after axonal transection (14, 15). To date, however, behavioral recovery has not been demonstrated after regeneration of host neuronal projections.

One means of potentiating reconstruction and behavioral recovery after injury may be to combine neurotrophic factor administration to the brain with grafts of substrates that promote host axonal growth. This strategy predicts that neurotrophic factors can prevent host neuronal degeneration and augment the population of neurons that can contribute to a regenerative response, while bridging grafts provide a physical substrate for host axonal regrowth. The cholinergic septohippocampal projection in the rat offers a model for testing this hypothesis, since the loss of cholinergic neurons after axotomy is prevented by nerve growth factor (NGF) infusions (16–18), and neurons of this pathway regenerate when provided permissive substrates for growth (3, 13, 14, 19). Thus, we transected the septohippocampal projection in Fischer rats by bilateral aspiration of the fimbria–fornix pathway, which normally connects the septum to the hippocampus. Animals then immediately received infusions of NGF, to prevent the loss of host septal cholinergic neurons, and embryonic-day-18 fetal hippocampal grafts to the lesion cavity, to promote host septal axonal regrowth to the hippocampus by using the fetal graft as a bridge. Since the hippocampal grafts do not possess intrinsic projecting cholinergic neurons, cholinergic fibers reentering the hippocampus are host-derived. Animals received NGF infusions for 9 weeks, then the pumps were removed, and the animals were sacrificed 6 months later. Behavioral testing was conducted prior to sacrifice in both a simple [habituation chamber (20)] and a more complex spatial [Morris water maze (21)] memory paradigm. Control animals received fimbria–fornix lesions alone, lesions plus 9-week NGF infusions, lesions plus hippocampal grafts to the lesion cavity, or no lesions.

MATERIALS AND METHODS

Thirty-eight female Fischer rats (each weighing 180 g) were anesthetized deeply, and bilateral lesions of the fimbria–fornix were made with a microscopically guided aspiration pipet. Sixteen animals then received solid grafts of embryonic-day-18 fetal hippocampal neurons to the lesion cavity, one fetal hippocampus per side of the brain. In the same session, animals received intracerebroventricular infusion devices (Alzet miniosmotic pumps) through which mouse submaxillary gland-derived NGF at 100 μg/ml in artificial cerebrospinal fluid or artificial cerebrospinal fluid alone was infused at a rate of 0.25 μl/hr for a 9-week period. The infusion devices were then removed, and the animals were sacrificed 6 months later. Prior to sacrifice animals underwent behavioral testing (see below). After transcardial perfusion with 4% (wt/vol) paraformaldehyde, brains were sectioned in the coronal or sagittal planes at 40-μm intervals and examined for Nissl staining, acetylcholinesterase (AChE) histochemistry, or p75 NGF receptor (NGFr) immunocytochemistry. Sixteen unlesioned animals served as intact controls. For quantification of basal forebrain cholinergic neurons, every third section through the medial septal region was immunocytochemically labeled for NGFr, as described (22). Five septal sections were counted per animal at distances of 340, 420, 500, 580, and 660 μm rostral to the decussation of the anterior commissure, as described (14).

Morphometric Analysis—AChE Fiber Innervation. Labeling of AChE-positive fibers was used to assess the extent of fiber regrowth into the hippocampus by measuring (*i*) the

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Abbreviations: NGF, nerve growth factor; NGFr, NGF receptor; CNS, central nervous system; AChE, acetylcholinesterase.

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longitudinal (rostral to caudal) distance over which fiber labeling was present and (*ii*) the density of cholinergic reinnervation. Longitudinal distance measurements were obtained by measuring the distance from the most caudal section of hippocampus in which reinnervating cholinergic fibers were observed to the most rostral section of hippocampus. These 40-μm-thick sections were then compared to a standard anatomical atlas (23), as described (14). Fiber density measurements were performed on a Q-2 image analysis system (Olympus, New Hyde Park, NY). Three hippocampal sections were quantified per animal, with six sites sampled per section (three sites per side of the brain: dentate gyrus, CA1 subfield, and CA3 subfield); thus, fiber density was measured in 18 regions per animal. The first section chosen for sampling was the most rostral section containing a recognizable and complete dentate gyrus and CA1 and CA3 subfields. The second section consisted of the most caudally located region of hippocampal reinnervation. The third section was located midway between the most rostrally and caudally reinnervated hippocampal region. All fiber density measurements were performed on a video image of the section of interest, magnified $\times$200, and transmitted through the camera to the Q-2 image analysis system. Each analyzed region consisted of a rectangular field measuring 5×10^4 μm^2. The video image was edited by the operator of the system to maximize contrast between AChE-positive fibers and the unstained background, with results expressed as percent of field occupied by AChE-positive fibers. Thresholding and contrast values were kept constant for comparison of given hippocampal fields in the same animal to reduce potential bias from staining or density variations. Repeated practice by the operator resulted in reproducible AChE fiber density measurements in hippocampi of intact animals, with variance of no more than 23% between similar fields in different animals and <10% difference between repeated measures on the same field.

Table 1. Morphological effects of septohippocampal reconstruction

Group	*n*	NGFr$^+$ cells, % of neurons	Fiber growth distance, mm	Fiber growth density, % of normal fiber density
L	8	40 ± 5*†	0.40 ± 0.11‡§	16 ± 5¶‖
L/N	14	76 ± 4*	0.52 ± 0.14‡§	14 ± 3¶‖
L/G	10	44 ± 5*†	1.26 ± 0.25‡	30 ± 7¶
L/N/G	6	69 ± 3*	1.14 ± 0.15‡	28 ± 3¶
CTL	16	100 ± 8	3.25 ± 0.0	100 ± 1

Groups: L, lesion only; L/N, lesion + NGF; L/G, lesion + bridging graft; L/N/G, lesion + NGF + bridging graft; CTL, intact (control). *n* is the number of experimental subjects per group; NGFr$^+$ cells are the number of cholinergic neurons in basal forebrain medial septal nucleus (mean ± SEM), standardized to percentage of neurons in intact animals. ANOVA: $F(4,25) = 23.9$; $P < 0.0001$. *, Significant differences from intact group; †, significant differences of noninfused lesioned animals from NGF-infused lesioned animals (post hoc Fisher's least square difference). Fiber growth distance (mean ± SEM) is measured in millimeters of fiber growth within the hippocampus proper. ANOVA: $F(4,50) = 87.9$; $P < 0.0001$. ‡, Significant difference compared to intact animals; §, significant differences between groups that did not receive bridging grafts compared to those that did (post hoc Fisher's LSD). Cholinergic fiber density is expressed as the mean ± SEM. ANOVA: $F(4,47) = 206$; $P < 0.0001$. ¶, Significant difference compared to intact animals; ‖, significant differences between groups that did not receive bridging grafts compared to those that did (post hoc Fisher's LSD).

Behavioral Assessment: Habituation (20). Animals were placed into a 70 × 70 cm activity chamber and exploratory activity was measured, i.e., number of crossings in an evenly spaced 4 × 4 photoelectric grid. On day 1, animals were placed

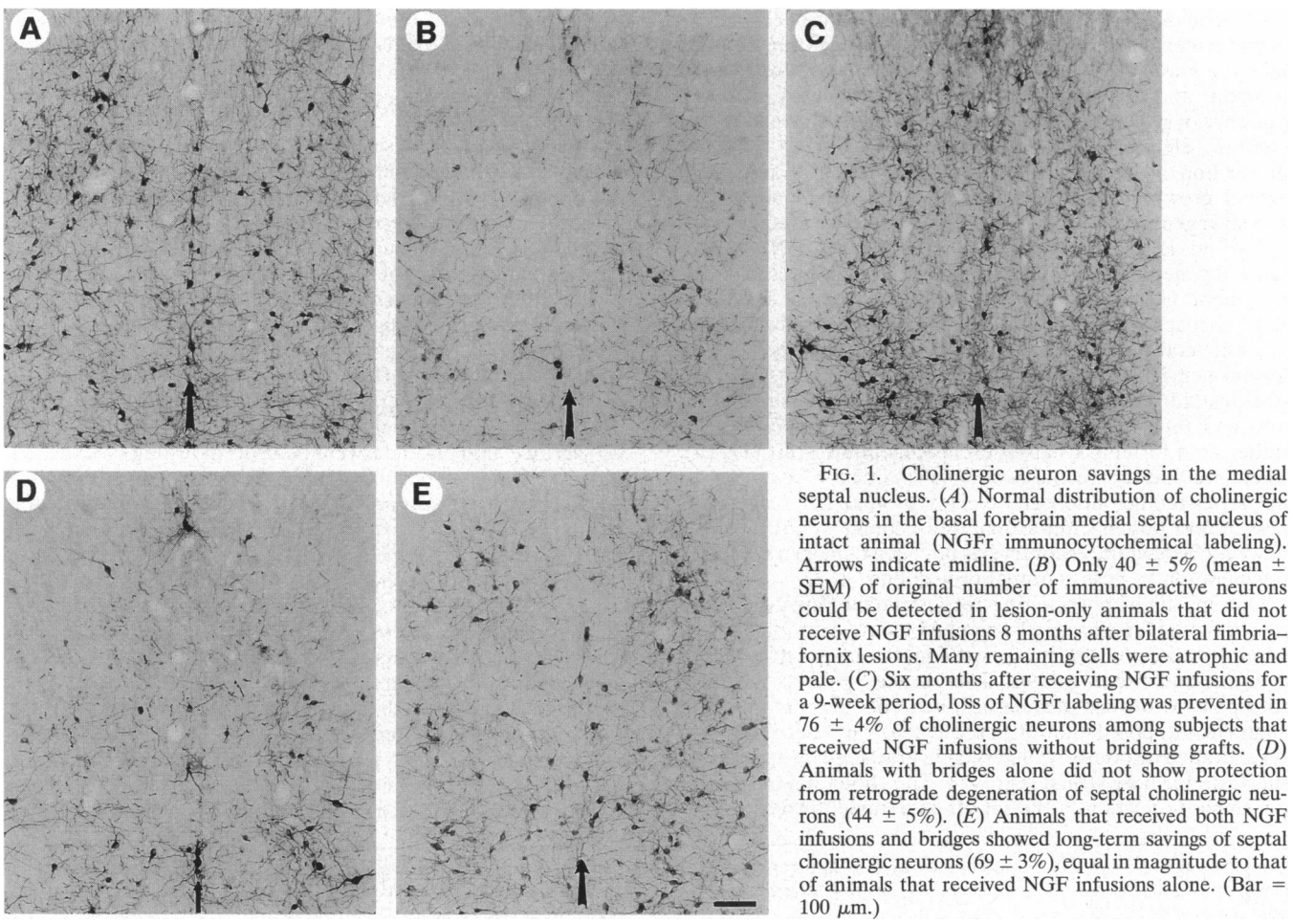

FIG. 1. Cholinergic neuron savings in the medial septal nucleus. (*A*) Normal distribution of cholinergic neurons in the basal forebrain medial septal nucleus of intact animal (NGFr immunocytochemical labeling). Arrows indicate midline. (*B*) Only 40 ± 5% (mean ± SEM) of original number of immunoreactive neurons could be detected in lesion-only animals that did not receive NGF infusions 8 months after bilateral fimbria–fornix lesions. Many remaining cells were atrophic and pale. (*C*) Six months after receiving NGF infusions for a 9-week period, loss of NGFr labeling was prevented in 76 ± 4% of cholinergic neurons among subjects that received NGF infusions without bridging grafts. (*D*) Animals with bridges alone did not show protection from retrograde degeneration of septal cholinergic neurons (44 ± 5%). (*E*) Animals that received both NGF infusions and bridges showed long-term savings of septal cholinergic neurons (69 ± 3%), equal in magnitude to that of animals that received NGF infusions alone. (Bar = 100 μm.)

in the activity chamber for 20 min (session 1), removed for 15 min, and then placed in the chamber again for a second 20-min period (session 2). On day 2, animals were placed in the chamber for a third and final 20-min exposure (session 3). For each group independently, total activity in the third period was compared to total activity in the first period to determine whether activity significantly diminished (i.e., whether habituation occurred) by using Student's *t* test.

Behavioral Assessment: Morris Water Maze (21). Subjects were tested for 15 days in the water maze, receiving four trials per day (each with a maximum duration of 90 sec) with a 10-sec rest between trials. Latency to locate the platform was measured. On days 1–5, a platform was clearly visible in the pool ("visible platform" test). On days 6–15, the top of the platform was located 2 cm below the water surface, and animals used spatial cues in the environment to locate the platform (non-visible platform test) (21). Overall group differences were determined by the analysis of variance.

RESULTS

Medial septal cholinergic neurons were quantified by specific labeling with the antibody to the p75 low-affinity NGFr, which colocalizes with basal forebrain cholinergic neurons (22). The transient (9-week) infusions of NGF were sufficient to provide long-term savings of 69–76% of cholinergic neurons ($P <$

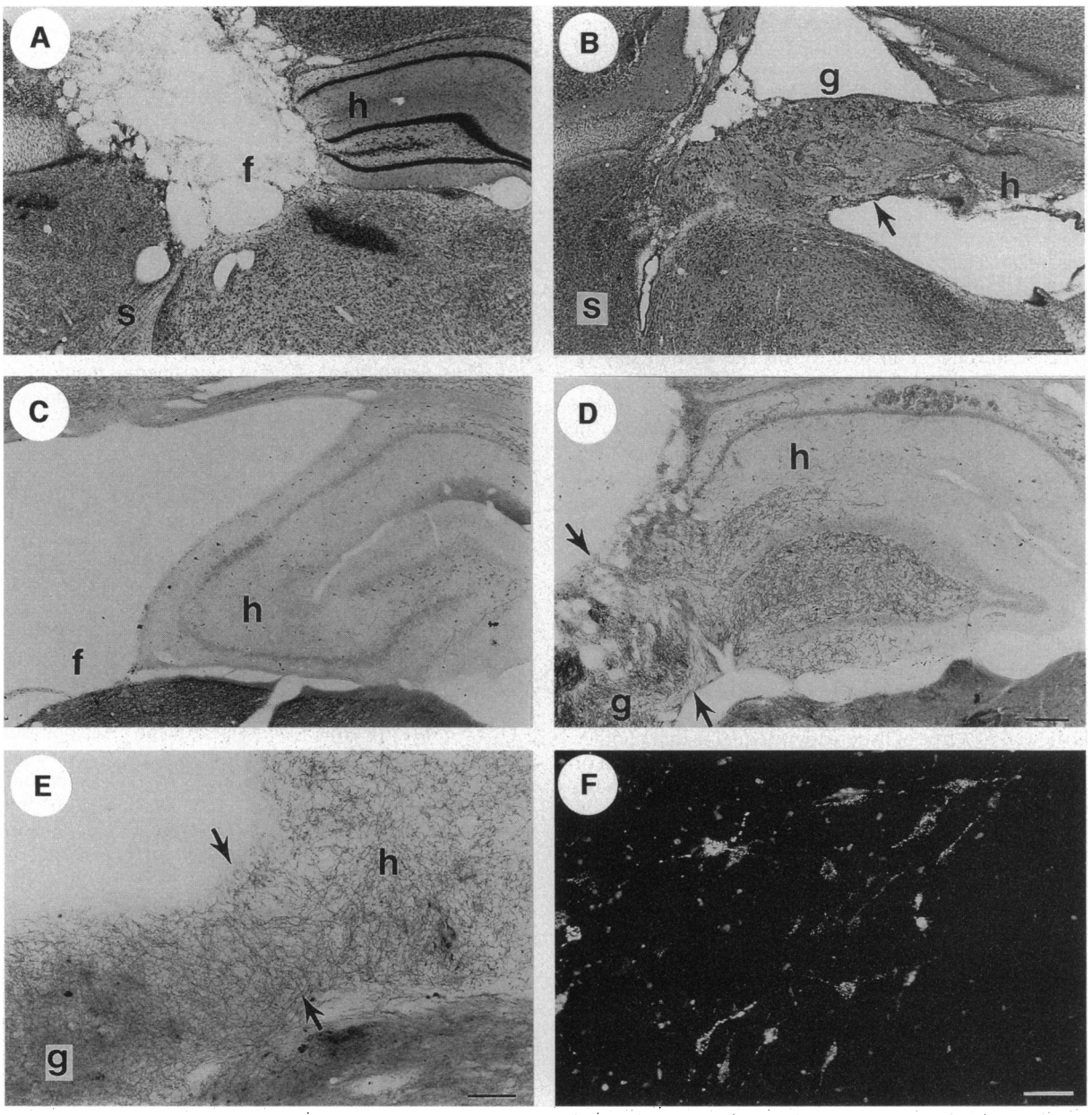

FIG. 2. Morphology of bridging grafts in the septohippocampal projection. (*A*) Example of a lesioned septohippocampal projection in a control subject not receiving a bridging graft. s, septum; f, fornix; h, hippocampus. (*B*) Graft occupies the fimbria–fornix lesion cavity, providing anatomical connectivity between host septum and hippocampus. The g and arrows indicate graft. (Nissl stain. Bar: *A* and *B*, 400 μm.) (*C*) Denervated hippocampus in subject without bridging graft. (*D*) Fibers stained for AChE partially reinnervate host hippocampus, passing freely between bridging graft, host septum, and host hippocampus. Arrows indicate interface between graft and host hippocampus. Cholinergic fibers were not contributed from the fetal hippocampal graft, since intrinsic cholinergic neurons of the hippocampus are few in number and do not project AChE-labeled fibers. (AChE staining. Bar: *C* and *D*, 200 μm.) (*E*) Example of graft–host interface in another animal, demonstrating free passage of cholinergic fibers. Arrows indicate graft–host transition (graft to left). (Bar = 80 μm.) (*F*) Host septum reinnervates host hippocampus, demonstrated by retrograde labeling of septal neurons with rhodamine microspheres injected into host hippocampus 5 days before sacrifice. (Bar = 200 μm.)

0.0001) in animals that received NGF, regardless of the presence of a bridging graft (Table 1 and Fig. 1). In contrast, only 40% of the original population of cholinergic neurons showed persistent p75 labeling in animals lacking NGF infusions.

AChE histochemistry revealed significant fiber growth into the denervated hippocampus only among animals possessing bridging grafts (Table 1 and Fig. 2). All animals with fetal bridges that spanned the lesion cavity showed significant cholinergic fiber growth into the hippocampus for distances of up to 2.8 mm ($P < 0.0001$) and a significantly elevated density of fibers within the hippocampus ($P < 0.0001$) compared to control subjects lacking bridges. The extent of fiber growth in animals that received NGF infusions with bridges did not differ from that in animals receiving bridges alone. Injections of retrogradely transported fluorescent rhodamine microspheres into the hippocampi of selected animals showed restoration of connectivity between host septum and hippocampus in grafted animals only (Fig. 2).

On behavioral testing, only lesioned animals that received combined NGF infusions with bridges (NGF–graft) showed significant recovery of habituation (Table 2). In the habituation task, repeated exposure to a novel environment results in diminished exploratory behavior, possibly reflecting retention of memory for previous exposures (20, 24, 25). This paradigm evaluates nonassociative learning without manipulation of motivational conditions, thereby assessing function without introducing artifact by manipulation of motivational parameters or the environment (24). NGF–graft animals showed a significant diminishment in activity on the second day of testing compared to their first exposure to the chamber, with a drop of 15.0 ± 5.6% in exploratory activity ($P < 0.05$; Table 2). Intact animals showed a similar degree of reduction in exploratory movement: 18.6 ± 10.3% ($P < 0.05$). All other groups failed to exhibit habituation on the second day of testing ($P > 0.05$). All groups of animals showed habituation between sessions 1 and 2 on the first day of testing, in which the duration between exposures to the activity chamber was very brief (15 min; Table 2). All groups of lesioned animals also showed motor hyperactivity relative to intact animals, yet the magnitude of activity reduction expressed proportionately in lesioned animals from sessions 1 to 2 was equal to that of intact animals. Thus, lesioned animals were capable of habituating, but only the NGF–graft animals continued to sustain their habituation after a prolonged 24-hr delay. Since NGF–graft animals showed a degree of hyperactivity equal to that of other lesioned groups during session 1 of testing, the mechanism of this improvement is unlikely to be attributable simply to a reduction in motor hyperactivity.

On a second behavioral task, the Morris water maze, the ability of subjects to locate a hidden platform by using visual cues assessed spatial reference memory (21). This task depends upon acquisition of a procedural memory component (recognition that escape from the pool is possible by climbing onto a hidden platform) as well as subsequent acquisition of a spatial memory component (finding the location of the hidden platform). Two-way analysis of variance over all 10 days of nonvisible platform testing demonstrated significant effects over groups and over time, but nonsignificance in the interaction term, groups × days [$F(36,270) = 1.17$; $P = 0.24$; Fig. 3]. Thus, a significant effect of experimental manipulations on the more demanding behavioral task was not evident.

Table 2. Habituation

Group	Session 1: Total activity count	% decrease in activity	
		Day 2	Day 1
Lesion	1032 ± 53*	7.7 ± 6.1	46.6 ± 7.0†
NGF	1270 ± 83*	1.9 ± 10.0	48.0 ± 7.9†
Graft	1170 ± 68*	5.1 ± 8.1	23.6 ± 17.3†
NGF–graft	1011 ± 108*	15.0 ± 5.6‡	38.0 ± 4.0†
Intact	648 ± 68	18.6 ± 10.3‡	54.5 ± 7.3†

*, Significant difference from intact animals ($P < 0.001$); †, significant reduction in activity on day 1, comparing second trial to first trial on day 1 (spaced 15 min apart) ($P < 0.01$); ‡, significant reduction in activity on day 2 compared to first trial on day 1 ($P < 0.05$). The magnitude in reduction of activity did not differ among the experimental groups ($P = 0.65$).

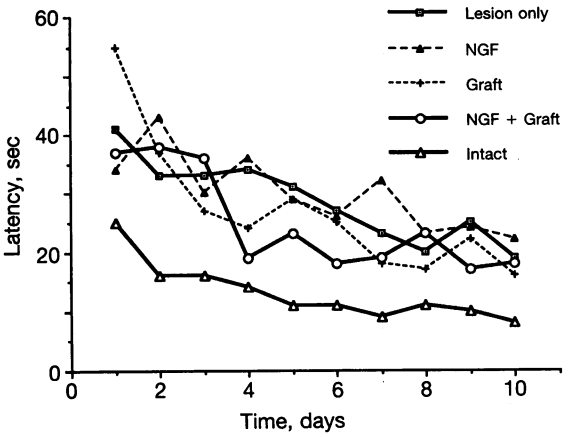

FIG. 3. Acquisition of spatial memory in Morris water maze (21). No overall benefit of experimental manipulations occurs on the water maze task [$F(36,270) = 1.17$; $P = 0.24$]. Prior testing in the presence of a visible platform for 5 days revealed no significant differences among groups ($P = 0.29$), indicating that subjects were equally motivated and motorically capable of performing the water maze task.

DISCUSSION

Recovery after injury in the CNS is normally limited by at least two factors: neuronal death and lack of functionally significant axonal regeneration from surviving neurons. The present study has shown that (*i*) partial functional recovery can be induced when NGF infusions are combined with grafts of substrates that promote host axonal regeneration and (*ii*) transient (9 week) trophic factor infusions protect the majority of basal forebrain cholinergic neurons from long-term degeneration.

The functional recovery achieved in this experiment extends the findings of previous studies that have shown axonal growth (1, 3, 13, 14, 26), synaptogenesis (26), and electrophysiological responsiveness (14, 15) in reconstructed host projections. The observed degree of behavioral recovery was partial, since function improved on a simple (habituation) (20) but not a complex (spatial reference memory) task (21). Recovery of habituation reflects hippocampal function, although whether the character of that function is mnemonic, attentional, or another cognitive element has not been proven (20, 24, 25, 27–32). The fact that cell savings and axonal regrowth were elicited from a projection to the hippocampus that is known to subserve mnemonic function, however, suggests that mnemonic dysfunctions were ameliorated. Recovery of more complex behaviors will require more extensive cell savings and/or more complete host reinnervation. Functional recovery may also be enhanced in this model by influencing hippocampal–septal projections, which form reciprocal inputs that modulate hippocampal function (33). The degree of functional recovery elicited in the present work demonstrates that attempts to promote functional recovery in injured host projections should be directed both at preventing neuronal degeneration and stimulating axonal reextension. Cell savings in the absence of target reinnervation and target reinnervation in the absence of cell savings do not elicit behavioral recovery in this paradigm.

The present study has also shown that transient (9-week) NGF infusions provide long-term (8-month) protection from

cholinergic neuronal degeneration after injury. This transient need for NGF infusion is consistent with the hypothesis that injury induces neurotrophic factor dependence in the adult brain but that intact adult cholinergic neurons do not require NGF for survival. Previous work has shown that excitotoxic ablation of the hippocampus does not cause basal forebrain cholinergic neuronal degeneration, despite loss of NGF-producing cells in the hippocampus (34). If basal forebrain neurons are subsequently axotomized, however, they degenerate (34). We have now shown that the dependence of degenerating cholinergic neurons on NGF is an injury-induced event that is temporary rather than permanent. The transient need for neurotrophic factors in the adult CNS after injury recapitulates a pattern seen during development, wherein specific neuronal populations transiently require target-derived neurotrophic factors for survival.

The finding that temporary NGF infusions prevent long-term neuronal loss is relevant to clinical trials of neurotrophic factors currently in progress. Junard *et al.* (35) reported that continuous NGF injections protected lesioned cholinergic neurons for 5 months, and we have shown (14) that limited 2-week NGF infusions did not prevent long-term (8 month) cholinergic degeneration. The present study shows that 9-week NGF infusions are protective for 8 months. However, delay in the start of NGF therapy for 3 weeks after injury reduces the degree of cholinergic neuronal rescue to 50% (36). Thus, neurotrophic factor support of degenerating CNS neurons should optimally begin immediately after injury, but intermediate dosing periods of NGF provide long-term (8 month) neural protection.

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