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The aged monkey basal forebrain: Rescue and sprouting of axotomized basal forebrain neurons after grafts of encapsulated cells secreting human nerve growth factor

(neurotrophins/Alzheimer disease/regeneration)

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ABSTRACT **Six Rhesus monkeys between 24 and 29 years of age received unilateral transections of the fornix. Three monkeys then received intraventricular transplants of polymer-encapsulated baby hamster kidney (BHK) fibroblasts that had been genetically modified to secrete human nerve growth factor (hNGF). The remaining three monkeys received identical grafts except the cells were not modified to secrete hNGF. Monkeys receiving the fornix transection and control grafts displayed extensive reductions in the number of choline acetyltransferase- (57–75%) and p75 NGF receptor- (53%) immunoreactive medial septal neurons ipsilateral to the lesion/implant. In contrast, monkeys receiving transplants of encapsulated hNGF-secreting cells display only a modest loss of choline acetyltransferase- (0–36%) and p75 NGF receptor- (7–22.4%) immunoreactive septal neurons. Additionally, all monkeys receiving the hNGF-secreting implants, but none receiving control implants, displayed robust sprouting of cholinergic fibers within the septum ipsilateral to the transplant. Just prior to sacrifice, the capsules were retrieved and found to contain viable BHK cells releasing biologically relevant levels of hNGF. These data demonstrate that hNGF can provide trophic and tropic influences to aged primate basal forebrain neurons undergoing lesion-induced degeneration, supporting the contention that hNGF may prevent the degeneration of basal forebrain neurons in Alzheimer disease.**

In the central nervous system, converging lines of evidence indicate that nerve growth factor (NGF) provides trophic and tropic influences for cholinergic neurons of the basal forebrain. For instance, injections of radiolabeled NGF into the hippocampus or cerebral cortex results in the specific retrograde transport to cholinergic neurons within the septal diagonal band complex and nucleus basalis, respectively (1, 2). NGF enhances cholinergic tone in normal and developing rats (3) and cholinergic basal forebrain (CBF) neurons are the only cells in the brain shown to express both the low-affinity p75 NGF receptor (NGFr) and the high-affinity trkA receptor (4, 5). NGF supports the viability of CBF neurons *in vitro* (3) and prevents the degeneration of axotomized CBF neurons *in vivo* (6–8). Furthermore, the atrophy of CBF neurons and the cognitive deficits displayed by aged rats can be reversed with intraventricular administration of NGF (9).

CBF neurons consistently degenerate in Alzheimer disease (AD; refs. 10 and 11). The cholinergic deficit in AD occurs early in the disease process and correlates with the severity and duration of the disease (11, 12). Furthermore, numerous studies in experimental animals and humans have demonstrated that an intact forebrain CBF system is necessary for normal cognitive function. The ability of NGF to augment the function of intact CBF neurons and prevent the degeneration of injured CBF neurons has led to the concept that NGF may be a useful treatment strategy for the treatment of AD. Although one patient with AD has recently been treated with NGF (13), studies demonstrating the efficacy of NGF in young and aged nonhuman primates have been recommended prior to the initiation of widespread clinical trials (14). Toward this end, intraventricular infusion of NGF has recently been demonstrated to prevent the degeneration of CBF neurons after unilateral fornix transection in young adult nonhuman primates (15, 16). We have recently demonstrated that grafts of polymer-encapsulated NGF-secreting cells can rescue degenerating basal forebrain neurons in nonhuman primates as well (17). However, AD is a disease of the elderly and no data exist that demonstrate the ability of NGF to provide trophic influences for degenerating CBF neurons in the aged primate brain. The present study demonstrates that grafts of polymer-encapsulated cells that have been genetically modified to secrete human NGF (hNGF) can prevent the degeneration of axotomized CBF neurons in aged monkeys. Furthermore, these implant induce the sprouting of cholinergic fibers proximal to the implants.

MATERIALS AND METHODS

Subjects. Six female Rhesus monkeys (*Macaca mulatta*) between 24 and 29 years of age were employed in this study. All monkeys were housed one per cage with food and water available ad libitum. Care exceeded that recommended by the National Institutes of Health.

Surgical Procedures. Unilateral transections of the left fornix were performed using an open microsurgical approach (18). A surgical drill was used to create a parasagittal bone flap (size = 1.5 cm × 4.0 cm) that exposed the frontal superior sagittal sinus. The dura was retracted and a self-retaining retractor used to expose the interhemispheric fissure. The corpus callosum was longitudinally incised exposing medial subcortical structures from the septum and head of the caudate rostrally through the foramen of Monro caudally. At the level of the foramen of Monro, the fornix was easily visualized as a discrete white fiber bundle. The fornix was initially transected using a ball dissector then the cut ends of the fornix were suctioned to ensure completeness of the lesion.

Immediately after the transection of the fornix, individual baby hamster kidney (BHK) cell-containing capsules were manually placed within the ipsilateral lateral ventricle with

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Abbreviations: NGF, nerve growth factor; hNGF, human NGF; NGFr, NGF receptor; ChAT, choline acetyltransferase; CBF, cholinergic basal forebrain; AD, Alzheimer disease; ir, immunoreactive; AChE, acetylcholinesterase.

fine forceps between the head of the caudate and the septum. The methods for transfecting BHK cells with the hNGF construct and encapsulating the cells within polymer capsules have been detailed (17, 19, 20). A total of five capsules were implanted in each animal oriented in a row in the rostrocaudal direction. The capsules abutted the caudate and septum, remained upright, and did not need to be secured further. Three animals received BHK-hNGF capsules and three received mock-transfected BHK-control cell-loaded capsules. The bone flap was then sutured back in place and the galea and skin were sutured using routine methods.

Histology. All monkeys were sacrificed 3–4 weeks after the lesion/implant. Just prior to sacrifice, all monkeys were anesthetized, the bone flap was removed, and the capsules were retrieved for histological and neurochemical analyses. The monkeys were then perfused transcardially with 0.9% saline and the brains were fixed with a 4% Zamboni's fixitive. The brains were then cryoprotected in 30% (wt/vol) sucrose/0.1 M sodium phosphate-buffered saline. Frozen sections (40 μm) were then cut on a sliding knife microtome and processed for the histochemical visualization of acetylcholinesterase (AChE) (21) and the immunohistochemical visualization of choline acetyltransferase (ChAT; antibody 1:10,000 dilution), low-affinity NGFr (p75 NGFr; antibody 1:80,000 dilution), dopamine β-hydroxylase (antibody 1:2000 dilution), and β-amyloid (antibody 1:1000 dilution) as described (4, 18, 22, 23).

Data Analysis. Counts of cholinergic neurons within the medial septum were performed manually. The number of ChAT- and p75 NGFr-immunoreactive (ir) neurons within the medial septum were quantified bilaterally from a minimum of six sections matched for level per animal. The number of cholinergic neurons was compared across groups using a two-tailed Student's *t* test with a Bonferroni correction factor for multiple comparisons.

RESULTS

Postoperatively, all monkeys receiving NGF-secreting transplants appeared lethargic relative to control grafted monkeys and this behavioral change was sustained for the duration of the experiment. All monkeys displayed numerous β-amyloid-ir plaque-like structures within the temporal and parietal neocortex, the amygdala, and hippocampus (Fig. 1 *A* and *B*). NGF administration had no effect on the number or distribution of amyloid plaques in comparison to control animals (data not shown). All monkeys also displayed complete unilateral lesions of the left fornix. Nissl and immunostained sections through the lesion site revealed that the left fornix was transected in all animals at the level of the caudal foramen of Monro. This lesion resulted in a comprehensive loss of AChE-containing fibers within the ipsilateral hippocampus (Fig. 1*D*) relative to the intact contralateral side (Fig. 1*C*). The implants appeared to be well tolerated *in vivo*, as Nissl-stained sections revealed minimal gliosis proximal to the capsules. A capsule in one monkey appeared to have penetrated the ventricle and its ventral aspect was lodged within the basal forebrain.

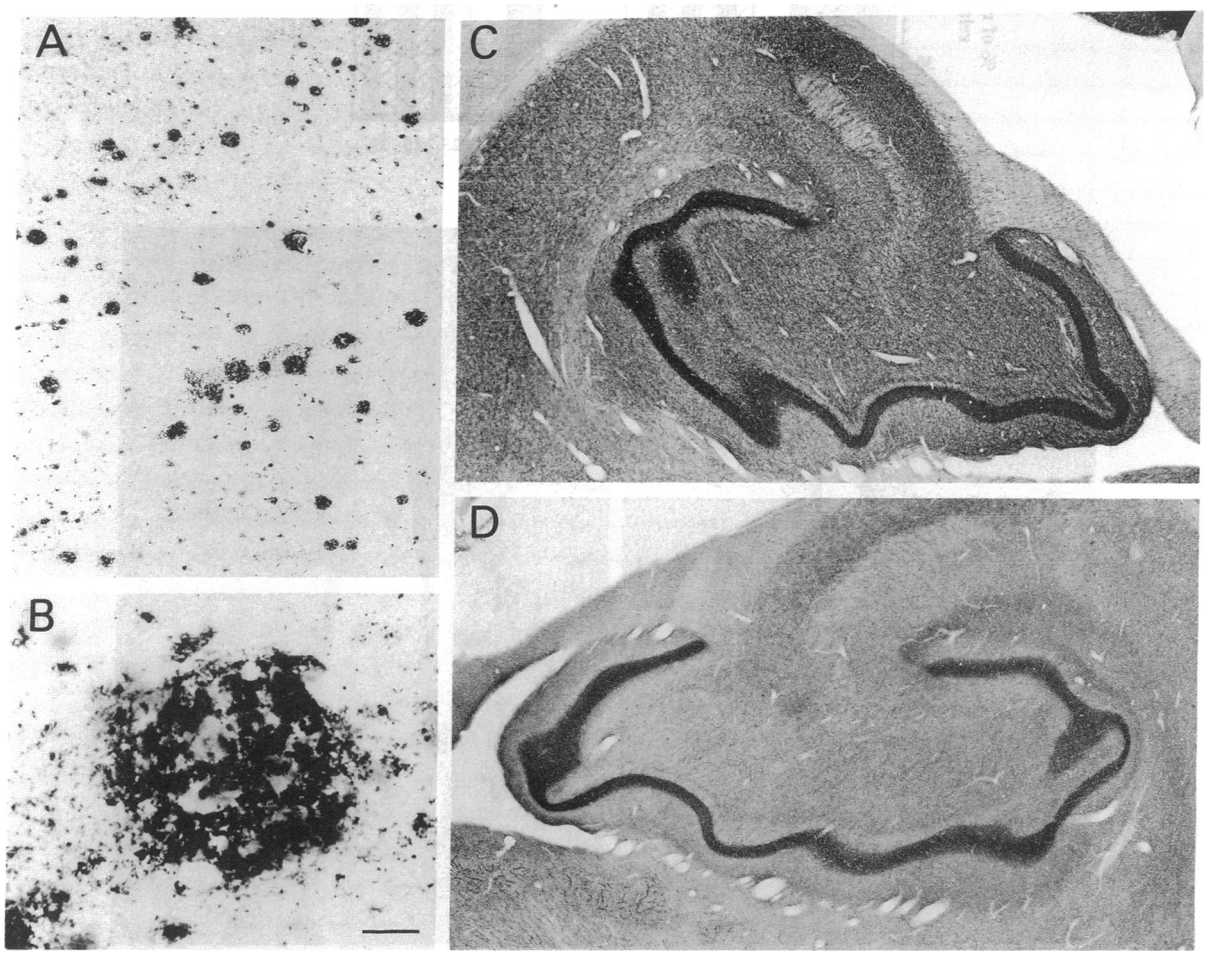

FIG. 1. Low (*A*) and high (*B*) power photomicrographs through the temporal neocortex of a 26-year-old monkey, demonstrating the presence and morphology of numerous β-amyloid-ir senile plaques. (*C*) The pattern of AChE-staining within the hippocampus of a 25-year-old monkey contralateral to the fornix transection. (*D*) The same section as illustrated in *B* illustrates a comprehensive loss of AChE-containing fibers ipsilateral to the fornix transection. (Bar in *B*: *A*, 200 μm; *B*, 50 μm; *C* and *D*, 500 μm.)

Monkeys receiving BHK-control grafts displayed a significant reduction (53%) of p75 NGFr-ir neurons within the medial septum ipsilateral to the transplant (Fig. 2 *A* and *B*). Many remaining neurons within the septum appeared atrophic relative to p75 NGFr-ir septal neurons on the contralateral side. The lesion-induced degeneration of septal neurons was significantly attenuated ($P < 0.001$) in monkeys receiving grafts of polymer-encapsulated BHK-hNGF cells as monkeys displayed only a 7–23% reduction in p75 NGFr-ir neurons within the medial septum ipsilateral to the lesion relative to the contralateral side (Fig. 2 *A* and *C*). The loss of ChAT-ir neurons within axotomized aged medial septal neurons was also prevented by the BHK-hNGF implants. Fornix-lesioned monkeys receiving BHK-control transplants displayed a 57–75% reduction in the number of ChAT-ir septal neurons. In contrast, lesioned monkeys receiving BHK-hNGF implants displayed a 0–37% reduction in ChAT-ir septal neurons (Fig. 2*A*). Furthermore, septal neurons receiving BHK-hNGF implants appeared to be of normal size ipsilateral to the lesion/implant relative to the atrophic appearance of many cholinergic cells in monkeys receiving BHK-control transplants.

In addition to maintaining the viability and/or expression of the cholinergic markers in septal neurons, BHK-hNGF grafts induced a robust sprouting of cholinergic fibers within the ipsilateral septal region (Fig. 3). All monkeys receiving BHK-hNGF grafts displayed a plexus of p75-NGFr-ir-containing fibers throughout the dorsoventral extent of the septum proximal to the grafts that ramified against the ependymal lining of the lateral ventricle (Fig. 3 *A*, *C*, and *D*).

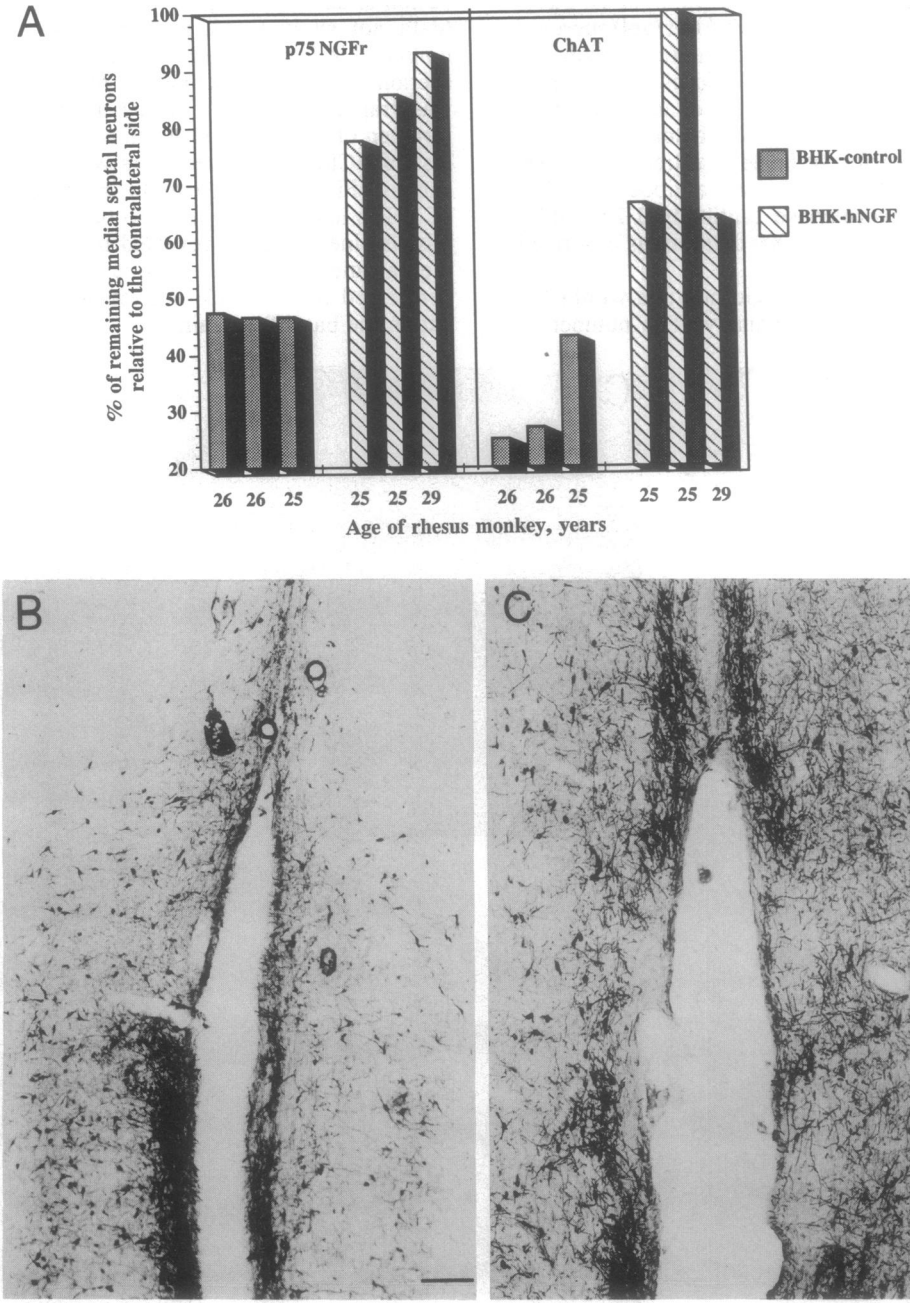

FIG. 2. (*A*) Quantification of the loss of p75 NGFr-ir and ChAT-ir neurons in BHK-control and BHK-hNGF-grafted aged monkeys. (*B*) p75 NGFr-ir-stained section through the medial septum of a monkey receiving a BHK-control graft. Note the extensive loss of neurons ipsilateral (right) to the lesion relative to the intact (left) side. (*C*) Monkeys receiving BHK-hNGF grafts displayed a symmetrical pattern of p75 NGFr-ir staining within the medial septum with minimal neuronal loss. (Bar in *B*: *B* and *C*, 100 μm.)

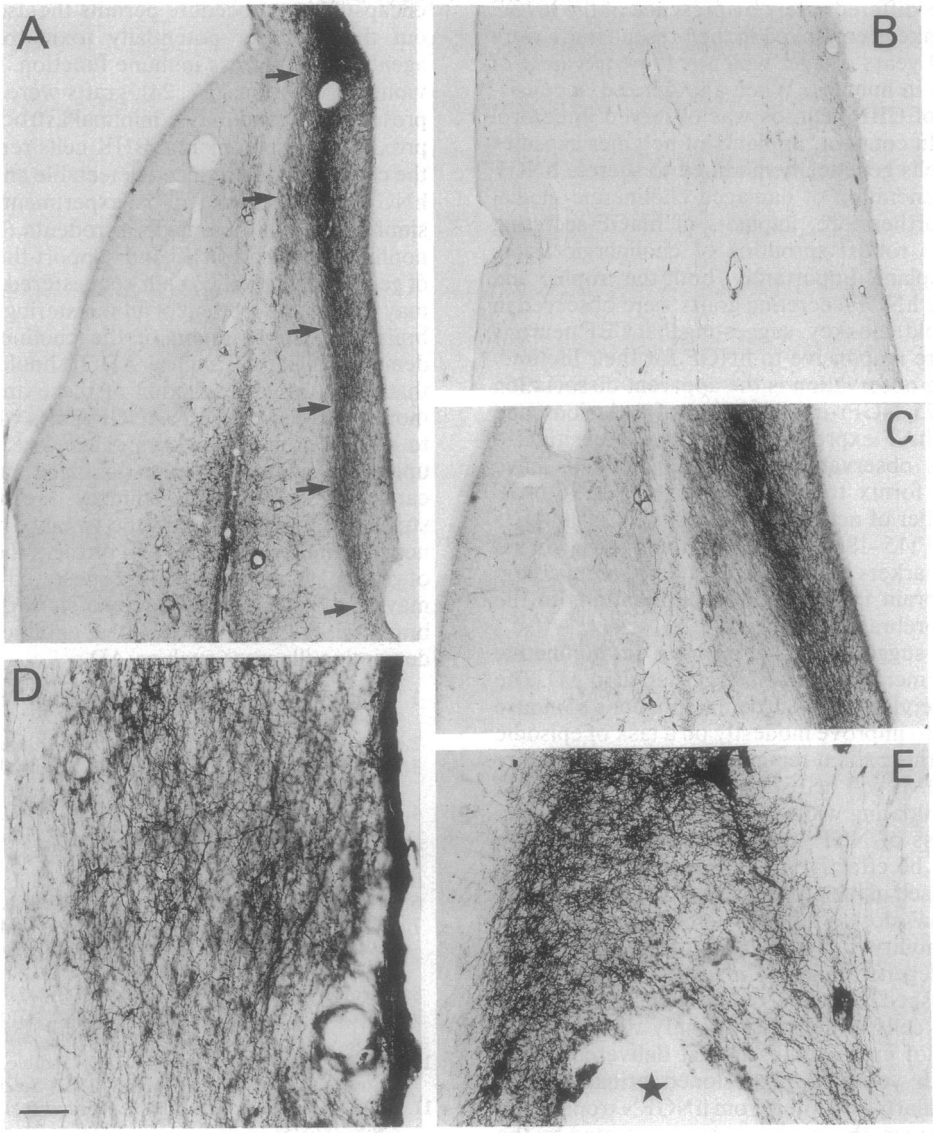

FIG. 3. (*A*) Dense unilateral p75 NGFr-ir sprouting response (arrows) within the septum proximal to a BHK-hNGF graft in a 29-year-old monkey. (*B*) No such staining pattern is observed within the septum of a 25-year-old monkey receiving a BHK-control graft. (*C* and *D*) Morphology of the p75 NGFr-ir sprouting response that can be resolved into individual fibers that ramify against the ependyma of the ventricle. (*E*) In one animal, the capsule penetrated into the underlying parenchyma (star). A dense network of p75 NGFr-ir fibers was also in this location adjacent to the implant. (Bar in *D*: *A*, 500 μm; *B* and *C*, 250 μm; *D* and *E*, 50 μm.)

In contrast, none of the BHK-control grafted monkeys displayed a cholinergic sprouting response (Fig. 3*B*). These fibers were also ChAT-ir and AChE-positive, confirming the cholinergic nature of this sprouting response. In one case, a BHK-hNGF capsule appeared to have penetrated the lateral ventricle ventrally and was lodged within the parenchyma of the rostral basal forebrain. A focal plexus of p75 NGFr-ir fibers was observed proximal to this implant site (Fig. 3*E*). While a few fibers originating from the posterior septum and from the posterolateral vertical limb of the diagonal band appeared to contribute to this fiber plexus, the cells of origin for the bulk of this fiber bundle remains to be established. What is clear is that these fibers are not derived from the sympathetic nervous system since they were thin, varicose, and not immunoreactive for dopamine β-hydroxylase. Interestingly, an occasional p75 NGFr-ir neuron was observed within the collection of p75 NGFr-ir fibers but these cells were too few in number to contribute significantly to the overall fiber plexus.

Prior to implantation, analysis by ELISA indicated that the BHK-hNGF grafted monkeys received capsules producing a total of 44.65 ± 0.95 ng of NGF per 24 h per animal. At the time of their retrieval just prior to sacrifice, numerous BHK cells were observed within the capsules, which now produced hNGF at a rate of 9.6 ng per 24 h per animal. This level of NGF production was biologically relevant, as media obtained from these capsules after grafting for 1 month in aged monkeys induced a robust differentiation of PC12A cells *in vitro* in a manner similar to that seen with exposure to 50 ng of hNGF (data not shown).

DISCUSSION

To our knowledge, this study is the first demonstration that NGF can provide trophic and tropic influences to degenerating CBF neurons in the aged primate brain and extends previous observations in young adult monkeys that infusions of hNGF (15, 16) or grafts of polymer-encapsulated hNGF-secreting cells (17) sustain the viability and induce the sprouting of degenerating CBF neurons. The trophic and tropic effects presently observed can be attributed to implant-derived hNGF with a high degree of certainty since the treatment strategies in monkeys receiving BHK-hNGF and

BHK-control grafts differed solely by presence of the hNGF construct. The monkeys employed in the present study were between 25 and 29 years of age, which is the equivalent of 75–87 years of age in humans. When axotomized, a consistent degeneration of CBF neurons was observed in control grafted monkeys. In contrast, implants of polymer capsules containing BHK cells genetically modified to secrete hNGF prevented the degeneration of damaged cholinergic medial septal neurons. Furthermore, implants of hNGF-secreting capsules induced a robust sprouting of cholinergic fibers proximal to the implant. Importantly, both the trophic and tropic effects of the hNGF-secreting grafts were observed in the oldest (29 year old) monkey, suggesting that CBF neurons in aged primates are responsive to hNGF for their lifetime. Another interesting observation is the apparent dissociation of ChAT-ir and p75 NGFr-ir expression after lesions and transplants with ChAT expression being more sensitive to axotomy. Previous observations in young monkeys have demonstrated that fornix transections reduce, and hNGF increases, the number of neurons expressing ChAT and p75 NGFr-ir in parallel (15–18). The present data suggest that these cholinergic markers may be differentially regulated in the aged primate brain in response to pertubations in the cholinergic basal forebrain system.

NGF has been suggested as a primary or adjunctive therapy for the treatment of the cholinergic deficit in AD. The first AD patient receiving intraventricular infusions of mouse NGF was reported to improve modestly on a task of episodic memory, although this patient remained unchanged on other cognitive tasks (13). It will be useful to evaluate a series of variables prior to initiating widespread clinical trials assessing the effectiveness of NGF treatment. One variable, the ability of hNGF to be effective in aged primates, has been successfully addressed in the present study. However, issues such as the optimal dose of hNGF required for clinical benefits and the ability for hNGF to provide long-term trophic support to degenerating CBF neurons in primates still remain to be established. The level of hNGF delivered by the encapsulated BHK cells in the present study was generally similar at the time of implantation as that delivered in our previous work with young fornix-lesioned primates (17). These monkeys similarly benefited from hNGF's trophic and tropic effects. However, the levels of hNGF secreted by the capsules at the time of sacrifice was five times lower in the present study. If continuous NGF support is required to achieve the trophic effects presently observed, this suggests that relatively low levels (ng/day) of hNGF may suffice in supporting degenerating basal forebrain neurons and the effective dose of hNGF needed for CBF neuronal sparing/sprouting may be lower than those used in past primate (15, 16) and clinical (13) studies. The ability of hNGF to prevent age-related cognitive decline in monkeys would also be an important line of evidence supporting the use of hNGF for the treatment of AD. However, the demonstration that hNGF can enhance cognitive performance in aged primates may not be essential prior to its testing in AD patients. NGF may provide a protective but not symptomatic benefit in AD. A neuroprotective effect can only be discerned after long-term treatment and evaluation and practical issues may make such a study prohibitive in aged nonhuman primates.

Since hNGF does not readily cross the blood-brain barrier and long-term hNGF treatment is likely to be required for the treatment of AD patients, methods need to be devised for the chronic delivery of this neurotrophin. The present study demonstrates the feasibility of grafting encapsulated cells that have been genetically modified to secrete hNGF in the aged primate brain. Pores in the polymer capsule are sufficiently large to allow hNGF and nutrients bidirectional passage but sufficiently small to prevent immune cells from entering and destroying the grafted BHK cells. Indeed, this encapsulation procedure permits the use of xenografts without the need for potentially toxic treatments employing agents that suppress immune function. In concert with previous observations (17, 24), grafts were well tolerated in the present study and only a minimal astrocytosis was observed proximal to the implants. BHK cells remained viable within the capsules and produced detectable and biological levels of hNGF for the duration of the experiment. These data confirm similar observations made in rodents (24) and young adult nonhuman primates (17) and support the concept that grafts of genetically modified cells sequestered in a polymer capsule may be a viable method of administering hNGF to the human brain for the treatment of the cholinergic deficit seen in dementing illnesses such as AD. It should be noted, however, that daily visual inspection of these animals indicated that monkeys receiving BHK-hNGF grafts were lethargic relative to control grafted monkeys. Therefore, the effects of NGF upon the functional status of aged primates need to be carefully evaluated. In summary, the present data demonstrate that hNGF supports the viability of degenerating CBF neurons in aged primates and furthers the concept that grafts of genetically modified cells sequestered in a polymer capsule may be a viable method of administering hNGF to the human brain for the treatment of the cholinergic deficit seen in dementing illnesses such as AD.

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