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Polymer-encapsulated cells genetically modified to secrete human nerve growth factor promote the survival of axotomized septal cholinergic neurons

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ABSTRACT **Effective treatments for neurodegenerative disorders are limited by our inability to alter the progression of the diseases. A number of proteins have specific neuroprotective activities *in vitro*; however, the delivery of these factors into the central nervous system over the long term at therapeutic levels has been difficult to achieve. BHK cells engineered to express and release human nerve growth factor were encapsulated in an immunoisolation polymeric device and transplanted into both fimbria–fornix-lesioned rat brains and naive controls. In the lesioned rat brain, chronic delivery of human nerve growth factor by the encapsulated BHK cells provided nearly complete protection of axotomized medial septal cholinergic neurons. Human nerve growth factor continued to be released by encapsulated cells upon removal from the aspirative site after 3 weeks or from normal rat striatum after 3 and 6 months *in vivo*. Long-term encapsulated cell survival was confirmed by histologic analysis. This encapsulated xenogeneic system may provide therapeutically effective amounts of a number of neurotrophic factors, alone or in combination, to virtually any site within the body.**

Cholinergic afferents from neurons in the medial septum and vertical limb of the diagonal band project to the hippocampus via the fimbria, dorsal fornix, and supracallosal stria. The survival of septal cholinergic neurons is dependent upon retrograde signals (1) of internalized nerve growth factor (NGF) from pyramidal and granule neurons in the hippocampus (2). Transections of the fimbria–fornix produce a marked loss of cholinergic neurons in the medial septum as assessed by choline acetyltransferase (ChAT) immunocytochemistry (3, 4), providing a useful paradigm for evaluating treatments that prevent or attenuate the loss of the cholinergic neurons. Several convergent lines of evidence indicate that administration of NGF has potent and beneficial effects on cholinergic neurons after axotomy. For instance, chronic intraventricular administration of NGF prevents the loss of cholinergic neurons (5–8); NGF infusions alone (9) or in combination with fetal hippocampal grafts (10) stimulate the regeneration of transected cholinergic neurons, and infusions of NGF stimulate the expression of ChAT (11) and p75 NGF receptor mRNA (12). NGF has also been shown to upregulate ChAT and $p75^{NGFR}$ expression and increase the size of basal forebrain cholinergic neurons in developing and adult rats (13). Accordingly, the use of NGF may represent a useful primary or adjunct treatment strategy for Alzheimer disease and/or other diseases characterized by cholinergic dysfunctions.

Transplantation of cells that have been genetically engineered to release NGF provides a means of site-specific delivery. Previous studies demonstrated that immortalized cell lines (14) and primary cells (15) that express NGF rescued axotomized septal cholinergic neurons. A recent study demonstrated that immortalized, allogeneic fibroblasts encapsulated within a polymer membrane effectively prevented the loss of cholinergic neurons after fimbrial transections in rats (16). In the absence of an encapsulating device, the allografts of immortalized rat fibroblasts secreting mouse NGF resulted in various degrees of tumor formation. Polymer encapsulation prevents cells from entering or leaving the capsule, while the semipermeable nature of the membrane permits entry of nutrients and diffusion of macromolecules into the surrounding host tissue. Thus, unmatched human, or even animal, cells could be used without immunosuppression. Delivery of proteins from capsules placed directly in the central nervous system would overcome difficulties associated with the blood–brain barrier. The use of these types of polymeric devices would also enable retrieval of the encapsulated cells. The results presented here suggest that implantation of capsules containing cells producing neurotrophic factors may provide a safe and practical means of chronically delivering compounds to specific regions of the central nervous system as treatments for a variety of progressive neurodegenerative disorders.

MATERIALS AND METHODS

BHK-hNGF Cell Line Production. The human NGF (hNGF) gene with the rat insulin intron, as described by Hoyle *et al.* (17), was inserted between the *Bam*HI and *Sma* I sites of pNUT (18) to be driven by the metallothionein I promoter. The pNUT-hNGF construct (Fig. 1) was introduced into BHK cells by using a standard calcium phosphate-mediated transfection method. BHK cells were grown in Dulbecco's modified Eagle's medium/10% fetal bovine serum/antibiotic/antimycotic/L-glutamine (GIBCO) in 5% CO_2/95% air and at 37°C. Transfected BHK cells were selected in medium containing 200 μM methotrexate (Sigma) for 3–4 weeks, and resistant cells were maintained as a polyclonal population either with or without 200 μM methotrexate.

NGF-Induced Neurite Outgrowth. Conditioned medium (CM) from unencapsulated BHK-control and BHK-hNGF cells was passed through a 0.2-μm filter and added to cultures of a PC-12 cell subclone, PC-12A (19), grown on 6- or 24-well plates at a density of 200,000 cells per ml to test for the presence of hNGF. Encapsulated cells in the polymeric devices were also tested for their ability to release bioactive hNGF by placing the devices in individual wells of a 24-well plate and allowing them to equilibrate for 1–2 days in serum-

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Abbreviations: NGF, nerve growth factor; hNGF, human NGF; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; CM, conditioned medium.

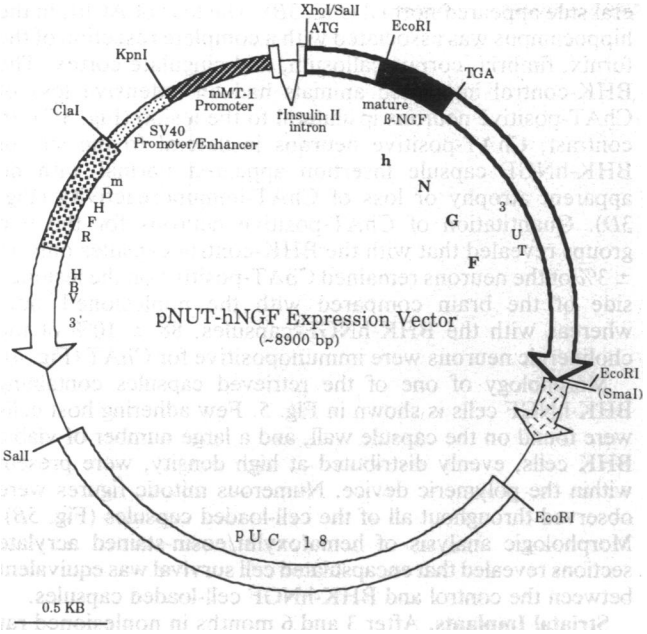

FIG. 1. pNUT-hNGF expression vector. The 2.51-kb hNGF fragment was subcloned into a dehydrofolate reductase-based pNUT expression vector downstream from the mouse metallothionein I promoter (mMT-I) and the rat insulin II intron. This construct was introduced into BHK cells (see text). SV40, simian virus 40; HBV, hepatitis B virus; rInsulin, rat insulin II intron A; UT, untranslated region.

free-defined PC-1 medium (Hycor, Portland, ME); then the medium was removed and replaced with 1 ml of fresh PC-1 for an additional 24 hr. This CM was collected, placed on the PC-12A cells, and evaluated. Neurite processes that were equal to or greater than three times the length of the cell-body diameter were scored as positive. In addition, the rate of neurite induction and the stability of the neurites was examined.

NGF ELISA. Quantitation of hNGF released from both encapsulated and unencapsulated BHK-hNGF cells was performed by a two-site enzyme immunoassay. The protocol was a modification of that described by Boehringer Mannheim using Nunc-Immuno Maxisorp ELISA plates. After color development (30 min), the samples were analyzed on a plate reader and measured against recombinant mouse NGF protein standards.

Encapsulation Procedure. Preformed asymmetric hollow fibers were cast from poly(acrylonitrile/vinyl chloride, PAN-PVC) according to Cabasso (20) and dried by standard glycerol techniques. These fibers exhibited cross-sectional dimensions of 600 ± 20-μm i.d. and 720 ± 25-μm o.d. Devices 7 ± 0.5 mm in length had a septal fixture at the proximal end for cellular loading access and were sealed at the distal end. BHK cells were prepared as a single-cell suspension and infused into the septal port at a density of 10^4 cells per μl after mixing 1:1 with physiologic collagen. After infusing 2–2.5 μl of the cellular suspension, the septum was removed, and the access port was sealed. BHK cell-loaded devices were maintained in PC-1 medium 4–5 days before implantation. After 3 or 4 days, the capsules were rinsed in Hanks' balanced salt solution and placed in 1 ml of fresh PC-1 medium overnight to be analyzed for hNGF by ELISA.

Animals. Adult male Lewis rats (Harlan Breeders, Indianapolis) weighing 300–350 g were housed in groups of three in a temperature- and humidity-controlled colony room maintained on a 12-hr light/dark cycle with lights on at 0700 hr. Food and water were available ad libitum. Before surgery, rats were anesthetized and positioned in a Kopf stereotaxic instrument (21). A sagittal incision was made in the scalp, and a craniotomy was performed extending 2.0 mm posterior and 3.0 mm lateral from Bregma. An aspirative device with a 20-gauge tip was mounted on the stereotaxic frame, and the medial parietal cortex, cingulate cortex, corpus callosum, dorsal hippocampus, dorsal thalamus, and fimbria–fornix were aspirated by placing the suction tip 1.40 mm posterior to Bregma and lowering it immediately lateral to the sagittal sinus to a depth of 5.0 mm. The tip was moved laterally in 0.5-mm increments by repeatedly retracting and inserting the aspirative device until a position of 3.0 mm lateral to Bregma was attained. After the aspiration, the rats were unilaterally implanted within the previously prepared cavity with either BHK-hNGF (n = 8) or BHK-control cell-loaded capsules (n = 6) using an 18-gauge Teflon catheter mounted to the stereotaxic frame (21). Stereotaxic implant coordinates were as follows: (−) 0.5 mm to Bregma, 1.0 mm lateral, and 7.5 mm below the cortical surface (22). A second set of control (n = 2) and hNGF-releasing BHK capsules (n = 4) were also stereotaxically implanted into the striatum of naive rats as described (21). Three of the cell-loaded capsules were retrieved from the striatum at 3 months, and the remaining three were retrieved at 6 months.

Histology. Three weeks after the aspirative surgery, animals were transcardially perfused with 20 ml of 0.9% NaCl at 20°C. Then, the following solutions in 50 mM phosphate-buffered saline, pH 7.4, were perfused at 4°C: 120 ml of 0.1% glutaraldehyde, 500 ml of 0.1% glutaraldehyde/4% (wt/vol) paraformaldehyde, 300 ml of 4% paraformaldehyde, and finally 300 ml of 10% (wt/vol) sucrose. Brains were removed, placed in 25% buffered sucrose, pH 7.4, and refrigerated for 24–48 hr. Tissue was cut at 20-μm intervals on a cryostat and mounted onto polylysine-coated slides. Every third section throughout the septum was collected and processed for ChAT immunoreactivity, as described (21), with the primary antibody (goat antiserum to ChAT; Chemicon) at a dilution of 1:1000. Adjacent sections were stained with hematoxylin/eosin. To verify the extent of the lesion produced after aspirations of the fimbria–fornix, every 10th section was taken throughout the hippocampus and stained for acetylcholinesterase (AChE) (23). For quantification of cholinergic cell loss, ChAT-positive neurons were counted in the medial septum and vertical limb of the diagonal band at a magnification of ×10. ChAT-positive neurons on the midline were excluded from this analysis. Representative sections (three per brain) located ≈0.7, 0.5, and 0.2 mm anterior to Bregma from each animal were used for this analysis.

After the collection of CM from the retrieved cell-loaded capsules, removed from either naive rat striatum or the fimbria–fornix aspirative sites, the capsules were fixed in 4% paraformaldehyde/phosphate-buffered saline overnight, processed for glycol methacrylate embedding, as described (24), sectioned, and stained with hematoxylin/eosin.

RESULTS

BHK-hNGF Cells. Transfection of the expression vector pNUT-hNGF (Fig. 1), into BHK cells, and subsequent selection with 200 μM methotrexate resulted in a stable polyclonal population of cells that secrete high levels of hNGF for at least 25 passages *in vitro*, in the absence of drug selection, and for 6 months *in vivo*. As measured by ELISA, these cells secrete 275–325 pg of hNGF per 10^3 cells per hr. No hNGF was detectable in medium from control BHK cells. CM from BHK-hNGF cells induced a marked outgrowth of neurite processes from PC-12A cells. Addition of pure mouse βNGF at 50 ng/ml to identically plated cells elicited neurite outgrowth that was indistinguishable from hNGF in CM in terms of the speed of neuritogenesis and the stability of the neurites over a 4-day-exposure period.

Fimbria–Fornix Lesions and Implants. To assess the efficacy of hNGF, cell-loaded capsules were unilaterally implanted into the fimbria–fornix region of rats that had undergone neuronal axotomy by aspiration (Fig. 2). Before implantation (3–4 days after the loading procedure), hNGF levels from CM were determined for all devices (n = 8 hNGF; n = 6 controls). ELISA values of hNGF levels averaged 27.2 ± 2.5 ng per capsule per 24 hr, whereas the BHK-control levels were equivalent to medium controls. After implantation of the BHK cell-loaded devices into the aspirative site, the animals rapidly recovered and revealed no signs of systemic toxicity.

The BHK cell-loaded devices were retrieved from the fimbria–fornix region 3 weeks later with little to no host tissue adhering to the capsules. The level of hNGF produced by the retrieved capsules was 5.2 ± 0.9 ng per capsule per 24 hr (n = 4). The BHK-control capsules produced no detectable hNGF. The BHK-hNGF cell-loaded devices were left *in situ* in four of the animals for fixation to observe the host tissue response.

Histological sections taken through the hippocampus and septum of all animals were examined by histochemistry for AChE in the hippocampus and by immunohistochemistry for ChAT in the septum. Fig. 3*A* shows a nearly complete loss of AChE staining in CA3, CA2, and the dentate gyrus of the ipsilateral denervated hippocampus, whereas the contralateral side appeared normal (Fig. 3*B*). The loss of AChE in the hippocampus was associated with a complete resection of the fornix, fimbria, corpus callosum, and cingulate cortex. The BHK-control implanted animals had an extensive loss of ChAT-positive neurons ipsilateral to the lesion (Fig. 3*C*). In contrast, ChAT-positive neurons ipsilateral to the site of BHK-hNGF capsule insertion appeared normal with no apparent atrophy or loss of ChAT-immunoreactivity (Fig. 3*D*). Quantitation of ChAT-positive neurons for the two groups revealed that with the BHK-control capsules only 14 ± 3% of the neurons remained ChAT-positive on the lesioned side of the brain compared with the nonlesioned side, whereas with the BHK-hNGF capsules, 88 ± 10% of the cholinergic neurons were immunopositive for ChAT (Fig. 4).

Morphology of one of the retrieved capsules containing BHK-hNGF cells is shown in Fig. 5. Few adhering host cells were found on the capsule wall, and a large number of viable BHK cells, evenly distributed at high density, were present within the polymeric device. Numerous mitotic figures were observed throughout all of the cell-loaded capsules (Fig. 5*B*). Morphologic analysis of hematoxylin/eosin-stained acrylate sections revealed that encapsulated cell survival was equivalent between the control and BHK-hNGF cell-loaded capsules.

Striatal Implants. After 3 and 6 months in nonlesioned rat striatum, CM from control and BHK-hNGF cell-loaded cap-

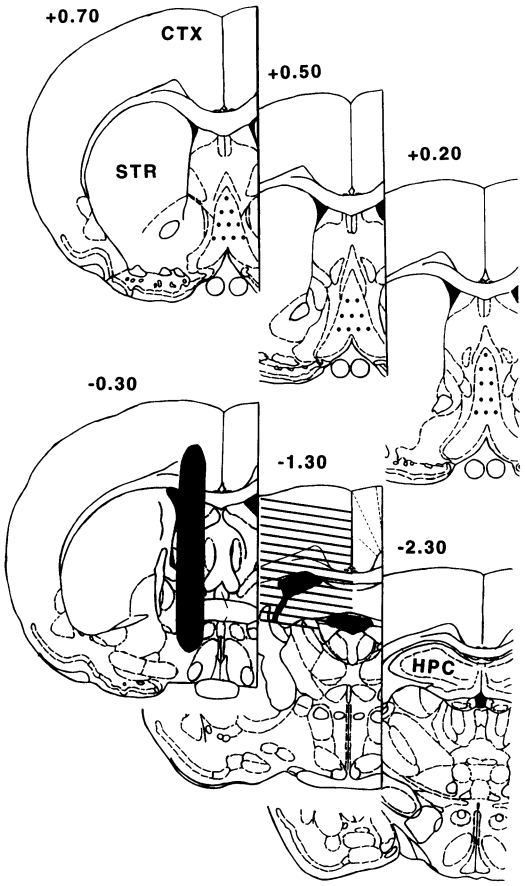

FIG. 2. Schematic illustration of rat brain sections where the histological analysis (*Upper*), and BHK capsule implantation and surgical aspiration (*Lower*) were conducted. The solid dots indicate the regions of the medial septum/vertical limb of the diagonal band in which cholinergic neurons were counted. The lower panels depict the implantation site of the capsules (*Left*). The approximate aspiration site of the fimbria–fornix is depicted in the *Middle* by the hatched solid lines. Numbers at top refer to the distance (mm) anterior (+) and posterior (−) to Bregma. CTX, cortex; STR, striatum; HPC, hippocampus.

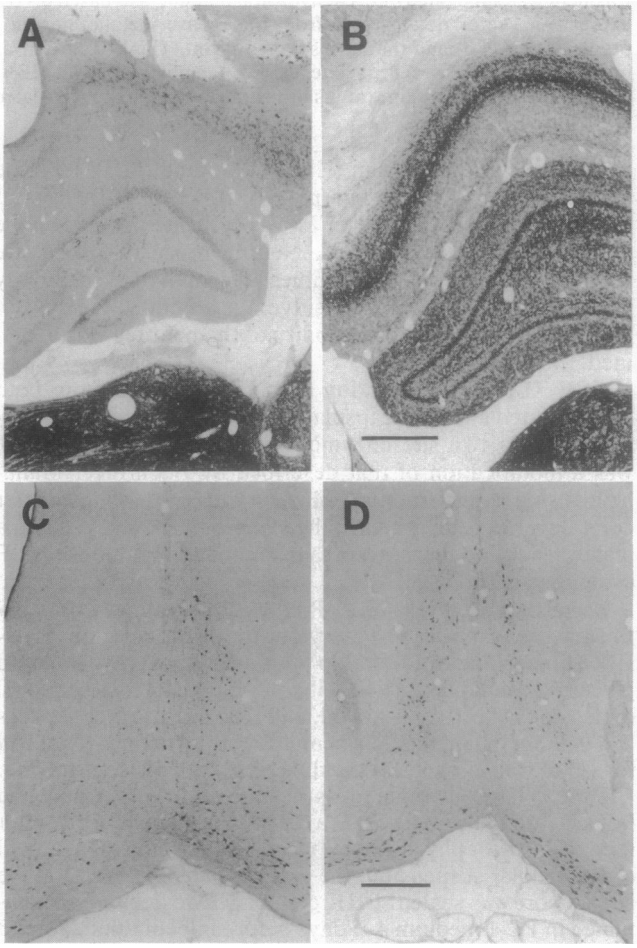

FIG. 3. Histological analysis of fimbria–fornix lesions. (*Upper*) AChE-stained sections of the dorsal hippocampus ipsilateral (*A*) and contralateral (*B*) to a unilateral aspiration of the fimbria–fornix. Note the nearly complete loss of staining in CA3, CA2, and dentate gyrus of the hippocampus. (*Lower*) ChAT immunohistochemistry of brain sections through the medial septum after implantation of BHK-control (*C*) and BHK-hNGF (*D*). Note the marked sparing and normal appearance of cholinergic neurons ipsilateral to NGF treatment. (Bars = 500 μm.)

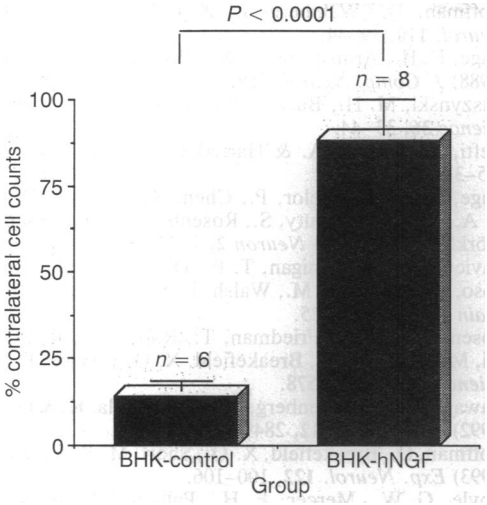

FIG. 4. Cell counts of ChAT-positive neurons in the medial septum of rats after unilateral aspirations of the fimbria–fornix. Animals that received the hNGF-releasing capsules showed a significant sparing of cholinergic neurons [$F(1,12) = 38.413$, $P < 0.0001$, one-way ANOVA]. Data are presented as means ± SEMs.

sules was assessed in the PC-12A cell bioassay. CM from BHK-hNGF cells produced a robust outgrowth of neurites from PC-12A cells within 24 hr, but no activity was detected in control samples. ELISA quantitation of the CM samples from the encapsulated BHK-hNGF cells revealed 17.1 and 13.1 ng of hNGF per capsule per 24 hr for capsules retrieved from two rats after 3 months, whereas capsules retrieved from two rats after 6 months released 21.6 and 2.8 ng of hNGF per capsule per 24 hr. In contrast, no hNGF was measured by ELISA from the capsules containing control BHK cells at either 3 or 6 months. Morphologic observations revealed BHK cell survival at the lumenal surface of the devices; however, cellular debris had accumulated within the central core of the capsules.

DISCUSSION

The present studies describe a BHK polyclonal cell line that maintains hNGF secretion for at least 25 passages *in vitro* without continued drug selection and from cell-loaded capsules maintained for 3 and 6 months *in vivo*. Previous transplantation studies using rat fibroblasts genetically modified to secrete mouse NGF demonstrated markedly lower levels of NGF secretion *in vitro* and no data from cells maintained *in vivo* for >1 month. In an initial study (14), NGF-transfected rat fibroblasts were reported to secrete 0.5 pg per 10^3 cells per hr. More recently, studies using similarly prepared rat fibroblast cell lines have reported NGF levels of 1–2 pg per 10^3 cells per hr (15, 16). In the current study, ELISA measurements from the BHK-hNGF cells maintained *in vitro* have demonstrated the capacity to secrete >300 pg per 10^3 cells per hr. The hNGF levels from serum-free-defined CM measured by ELISA after capsular retrieval after 3 and 6 months in rat striatum revealed that the encapsulated cells continue to release nanogram quantities of hNGF per day. The PC-12A *in vitro* bioassay confirmed that hNGF was biologically active.

Delivery of growth factor(s) to central nervous system structures remains a challenge in the development of potential therapies for chronic degenerative disease(s). Fimbria–fornix aspirative lesions have been widely used as a model for evaluating the neuroprotective effects of NGF (4–8). Miniosmotic pumps used to release mouse or human recombinant NGF into parenchyma or the ventricular space resulted in significant cholinergic neuronal sparing in rats (6–8, 25) and nonhuman primates (26–29) concurrent with aspirative axotomy. These findings indicated that NGF therapy in a fimbria–fornix aspirative model was sufficient to prevent cholinergic cell loss.

Others have shown that immortalized cell allografts secreting NGF (15, 16) or expressing tyrosine hydroxylase (30) form tumors or are rejected in the absence of host immunosuppression (15, 31). In the present studies, a single hNGF-releasing capsule protected up to 90% of cholinergic septal neurons from atrophy and exhibited 6-month survival with hNGF release from xenotransplants. In the absence of an immuno-isolatory polymeric membrane, BHK cells injected into rat striatum are rejected by host-cell immune elements within 25 days (data not shown). Although it has been reported that primary cells genetically modified using retroviral vectors survive up to 8 weeks (32) and 6 months (33) in rat striatum, the biological significance of transgene expres-

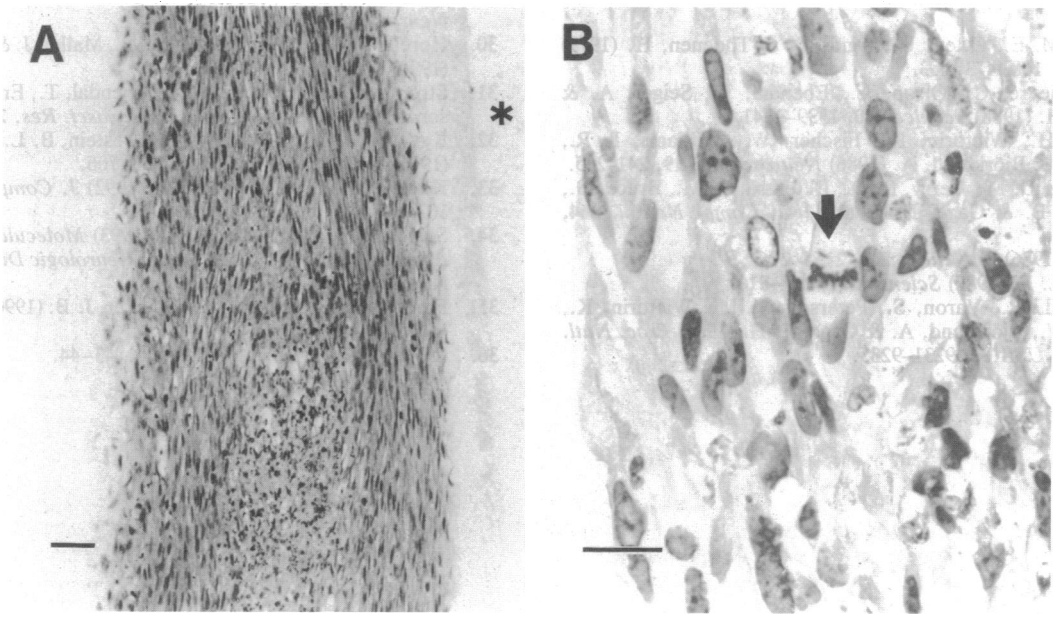

FIG. 5. Longitudinal sections stained with hematoxylin/eosin of a BHK-hNGF cell-loaded device after retrieval from the fimbria–fornix. Note the abundance of viable cells within the capsule wall (*) and mitotic figures (arrow). [Bars = 50 μm (*A*) and 20 μm (*B*).]

sion in those studies remains unclear. Retroviral expression systems generally rely on one or a few transgenes per cell, which are often subject to inactivation or the negative influence of cytokines after transplantation (34). In contrast, *in vitro* methotrexate selection allows only high-copy-number transformants to survive, and the metallothionein promoter in the context of the pNUT construct results in high constitutive levels of expression that can be further induced by zinc or cadmium salts (18).

The use of immortalized cell lines engineered for central nervous system delivery of neuroactive substances, such as trophic molecules, has several advantages over primary tissues. The limited availability of fetal and adrenal tissues may preclude their widespread clinical use. Additionally, transplant survival is essential. Adrenal tissue autografts have provided limited efficacy in Parkinson disease; however, graft survival has, in general, been poor (35). Cell lines, in contrast, offer the ability to establish cell banks that are free of adventitious agents, can be clonal, and can be genetically engineered to secrete desired molecules. Furthermore, the potential for tumor formation in allografts and a report that transplants of engineered cells in nonhuman primates resulted in cancer from transmittable Moloney murine leukemia virus (36) provide additional safety incentives that can be provided by polymer encapsulation technology, as well as the ability to easily retrieve the implanted cells.

In summary, the results suggest that this encapsulated xenogeneic system may allow the release of therapeutically effective amounts of neurotrophic factors, alone or in combination, to many sites within the central nervous system. The sustained release of hNGF observed from a retrieved capsule for at least 6 months in the present study is relevant for chronic neurodegenerative disorders such as Alzheimer disease. Additionally, the implantation of encapsulated, genetically engineered cells may provide a safe and practical therapeutic approach for the chronic delivery of factors for a number of other human neurodegenerative disorders.

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