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CNS-Derived Neural Progenitor Cells for Gene Transfer of Nerve Growth Factor to the Adult Rat Brain: Complete Rescue of Axotomized Cholinergic Neurons after Transplantation into the Septum

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A CNS-derived conditionally immortalized temperature-sensitive neural progenitor (CINP) cell line was used to generate NGF-secreting cells suitable for intracerebral transplantation. The cells were transduced by repeated retroviral infection, using a vector containing the mouse *NGF* cDNA under the control of the LTR promoter. Subcloning at the permissive temperature (33°C) identified a highly NGF-secreting clone (NGF-CINP), which contained multiple copies of the transgene and released NGF at a rate of 2 ng/hr/10^5 cells *in vitro*, both at 33 and 37°C, which was approximately 1 order of magnitude higher than what was possible to achieve in the heterogeneously infected cell cultures. After transplantation to the brain, the NGF-CINPs differentiated into cells with a predominant glia-like morphology and migrated for a distance of 1–1.5 mm from the implantation site into the surrounding host tissue, without any signs of overgrowth and tumor formation. Grafts of NGF-CINP cells implanted into the septum of adult rats with complete fimbria-fornix lesion blocked over 90% of the cholinergic cell loss in the medial septum and grafts placed in the intact striatum induced accumulation of low-affinity NGF receptor positive fibers around the implantation site. Expression of the NGF transgene *in vivo* was demonstrated by RT-PCR at 2 weeks after grafting. It is concluded that the immortalized neural progenitors have a number of advantageous properties that make them highly useful experimental tools for gene transfer to the adult CNS.

[Key words: neurotrophin, immortalization, stem cell, cholinergic system, retroviral vectors, grafting, gene therapy, striatum, septum]

Knowledge about the *in vivo* actions of neurotrophic factors in the brain so far has been mostly obtained from studies using intracerebral injections or infusions by means of osmotic minipumps. Such infusion techniques, however, have clear limitations as potential therapeutic procedures due to the limited duration of the active protein stored in the infusion devices, as well as the diffuse, nonlocalized delivery obtained after infusion or injection into the cerebrospinal fluid. Infusion into the cerebrospinal fluid, moreover, necessitates the use of very high amounts of neurotrophic factors to obtain sufficiently high intraparenchymal concentrations to produce the desired biological effects, which raises concerns as regards the specificity of action and the possibility of unpredictable nonspecific effects. Alternatively, systemically delivered nerve growth factor (NGF) may be made to cross the blood–brain barrier (Friden et al., 1993); factors delivered in this way would reach all parts of the CNS, as well as other tissues of the body, which may confound the interpretation of the effects obtained and may also induce unwanted side effects.

Cell-based gene transfer has emerged as an interesting alternative approach for long-term intracerebral delivery of therapeutically active substances at physiological levels in a localized manner. Studies on genetically transduced cells for transplantation to the brain were initially carried out with immortalized fibroblastic cell lines or cell lines of tumor origin. This approach, however, has been limited by the fact that such cells may overgrow and produce tumors *in vivo* after grafting (Horellou et al., 1990a,b; Gage et al., 1991; Gage and Fisher, 1993). More recently therefore the preferred choice has been the use of primary cells, such as cultured fibroblasts or myoblasts, that appear to survive well in the brain, and do not produce tumors (Fisher et al., 1991; Jiao et al., 1992, 1993; Kawaja et al., 1992; Dekker et al., 1994). Cells of non-neural origin, however, do not integrate into the host brain tissue but remain relatively isolated as a tissue mass at the site of implantation. We and others have argued therefore that the cells to be used for transplantation and gene transfer into the CNS should ideally be CNS derived. Such cells may become better integrated into the cellular architecture of the host brain and thus be able to interact more closely with the receptive neuronal elements of the host (McKay 1992; Björklund, 1994; Martinez-Serrano et al., 1994a,b; Snyder, 1994).

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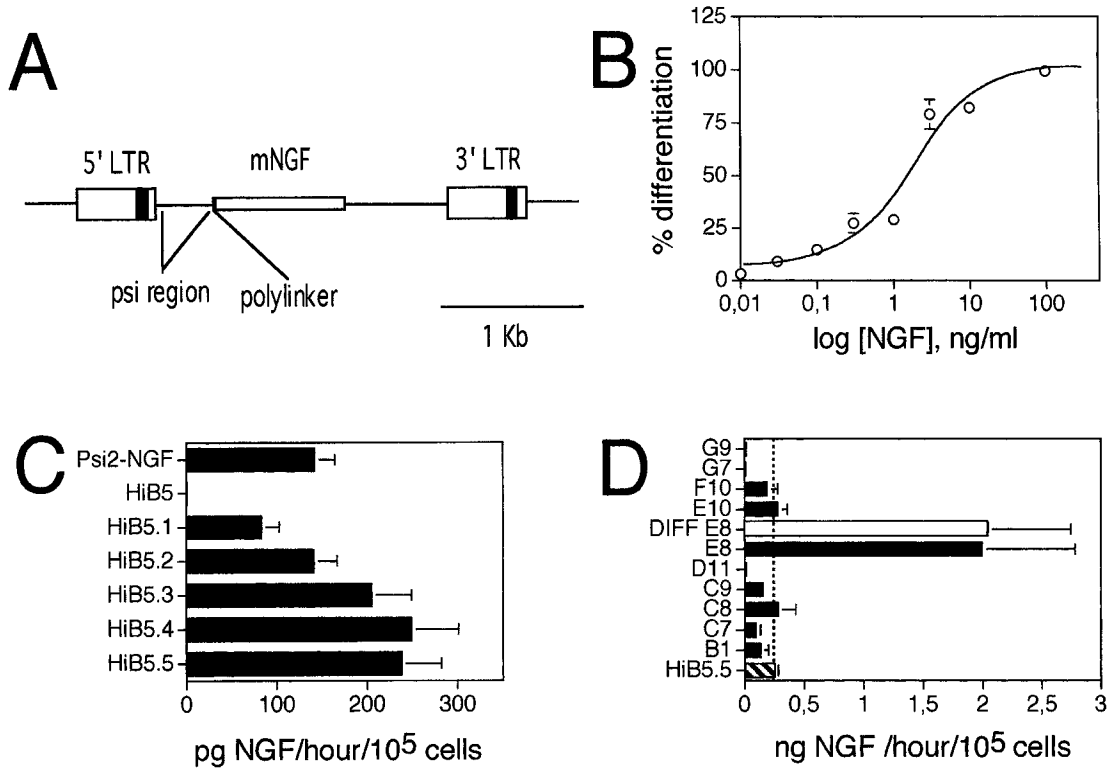

Figure 1. Production of NGF-CINPs. *A,* Schematic drawing showing the internal organization of the different elements in the vector. *B,* Dose–response curve for the NGF-induced differentiation of primed PC12 cells, used to estimate the rate of NGF release at every step in the preparation of the NGF-CINPs. The curve gives the pooled data of 19 independent assays; error bars (SEM) are indicated only when they exceed the size of the symbols. *C,* Repeated infection of HiB5 cells with retroviruses from the packaging cell line Psi2-NGF increases the NGF production in the infected culture. The HiB5 cell cultures were infected every second day for 10 d (*HiB5.1-5*). *D,* NGF release rates in different subclones isolated from the parental heterogeneous culture, HiB5.5 (*hatched bar*). For the clone with the highest rate of release (*E8*), the values at both the permissive temperature, 33°C, and after 7 d at the nonpermissive temperature, 37°C, in defined media (*open bar, DIFF E8*) are shown.

In the present study, we report the use of an established brain-derived neural progenitor cell line, HiB5, for targeted delivery of NGF to the brain. These conditionally immortalized cells, which express a temperature-sensitive mutant allele of the large T-antigene from SV40 (Renfranz et al., 1991), can be proliferated at the permissive temperature (33°C), but they stop growing and start to differentiate at the nonpermissive temperature of the brain, which makes them well suited for transplantation purposes (Frederiksen et al., 1988; Jat and Sharp, 1989; Renfranz et al., 1991; McKay, 1992). For gene transduction experiments, the conditionally immortalized cells have a particular advantage, compared to neural primary cell cultures, in that they can be produced in unlimited amounts and exposed to different selection and subcloning procedures, which will greatly facilitate the generation of optimal cells in terms of synthesis and release of the desired gene product. Indeed, recent studies have shown that both *lac-Z* and tyrosine hydroxylase can be stably expressed in immortalized cell lines, both *in vitro* and after grafting to the brain *in vivo* (Snyder et al., 1992; Onifer et al., 1993; Anton et al., 1994).

In the present experiments we have used a modified murine retroviral vector containing the mNGF cDNA, followed by subcloning of the infected HiB5 cells, to generate a NGF-transduced clonal cell line that survives well in the brain, produces high amounts of biologically active NGF *in vitro*, and maintains biologically significant transgene expression and NGF secretion also after grafting to the brain.

A preliminary report of this work has been presented elsewhere (Martinez-Serrano et al., 1994a,b).

Materials and Methods

Vector and packaging cell line. A single gene retroviral vector based on the Moloney Murine Leukemia Virus was prepared from pMoMuLV-TH by the addition of a polylkinker (Horellou et al., unpublished observations) and pSP6-NGF (generous gift from Dr. E. Dicou; see Dicou, 1989). Briefly, following described procedures (Sambrook et al., 1989) we excised the region between the Psi site and the 3′LTR in the retroviral vector (SphI-NsiI fragment), replacing it with the SmaI-PstI fragment of pSP6-NGF containing the full length cDNA for mouse Nerve Growth Factor (mNGF) (nucleotides 67–1029 in Scott et al., 1983). The region of interest for the production of retroviruses carrying the NGF cDNA consists mainly in 5′LTR-Psi site-NGF-3′LTR, using the viral polyadenylation signal in the 3′LTR (Fig. 1).

The vector was transfected into the packaging Psi2 cell line (Mann et al., 1983; Miller, 1990) (kindly provided by Dr. R. C. Mulligan) by calcium phosphate precipitation; since the vector does not contain any selection gene we cotransfected as well a plasmid carrying the neomycin resistance (Neo^r) gene (pUC-SVNeo, a generous gift from Dr. G. Lutfalla) in a ratio 10:1 NGF:Neo^r. After selection in the presence of 600 μg/ml of the neomycin analog G418, we isolated 77 neomycin-resistant clones. Based on their growth rate, 26 were further characterized, looking for the best producer clone; since it is not straightforward to measure the viral titer without a selection gene in the retroviral vector, we chose the clone with the highest expression and release of NGF using three independent approaches: bioassay on PC12 cells, NGF double-site immunoassay (ELISA), and RT-PCR using random hexameres for reverse transcription and a set of primers to amplify a 0.5 kb length fragment from the 5′LTR to a site inside the mNGF cDNA. Three optimal clones were found, and one of them was chosen and expanded

on the basis of their growth rate, being designated as the Psi2-NGF cell line.

Cell culture. Psi2 fibroblasts and HiB5 cells, as well as their derivatives, were grown in DMEM supplemented with 10% heat inactivated fetal calf serum, 10,000 units/ml of penicillin and streptomycin, and 2 mM glutamine. For proliferation of the CINP cells, the cultures were placed in an incubator set at 33°C, while for differentiation, the cultures were incubated at 37°C (used also for the Psi2 and PC12 cell lines). When the CINP cell cultures were shifted to 37°C, the culture medium was changed to a defined serum-free medium based on DMEM:F12: hormone mixture, as described in Reynolds et al. (1992).

Infection of the target cell line. Conditioned media containing the retrovirus was collected at the time of passing the Psi2-NGF cells and stored frozen until use. For the infections, an aliquot was slowly thawed, 8 μg/ml polybrene was added, and the medium was centrifuged to remove any cellular debris. Infection was carried out by incubation of the HiB5 cells in this medium for 2 hr; after that time, fresh medium was replaced, without rinsing. For repeated infection (five times), this protocol was followed every second day, passing the cells in between the infections, with a one-in-five split. After each infection step, an aliquot of the cells was expanded as an individual cell line for further analyses. These cell lines were designated as HiB5.1-NGF to HiB5.5-NGF. The overall procedure was done in triplicate, starting from three different batches of HiB5.

Isolation of NGF- and control-CINPs for transplantation. The HiB5.5-NGF cell lines were pooled, and clone isolation was done by plating a low number of cells per well in a multiwell plate. Briefly, the cells were seeded in 96-well plates, so that theoretically one to five cells were distributed into each well. The wells were examined after a 2 hr attachment period, and those with a unique cell were selected. These wells were then monitored every 12 hr until the first division occurred, and then, every day, until colonies of 50 to 100 cells were obtained. At each examination step, special care was taken to recheck for the presence of a unique cell or clone in each well. Then, the cells were detached and allowed to grow again in the same well; once confluent, the cells were expanded in 24-well plates, then in 6-well plates, and, finally, serially expanded and frozen as individual clones, designated by the letter and number code of their position in the plate. A total of 11 clones survived the entire procedure.

All of these clones were analyzed for the net rate of release of NGF. The day before reaching confluence, the media were aspirated, the cells rinsed, and fresh medium was added. The next day, all of the media was collected and frozen, and the total number of cells in each flask counted. The total amount of NGF present in the media was determined by a quantitative NGF bioassay (see below). Following NGF analysis, two of the clones were chosen for transplantation, corresponding to the one showing the highest release (E8) and one of the NGF-negative clones (D11) (Fig. 1*D*).

The absence of a helper virus coding for NGF in the E8 cell line was tested by multiple mock infection (three times) of the parental HiB5 cell line with culture supernatant from the E8 clone. The "infected" HiB5 did not release any NGF to the medium after those infections, when 1:10 or 1:100 dilutions were tested in the NGF bioassay (see below); other pieces of evidence obtained by Northern blot of total RNA or RT-PCR from this E8 clone, rule out as well the presence of rearranged viral genomes that could result in the propagation of helper, infective viruses coding for NGF.

Quantitative NGF bioassay on primed PC12 cells. Measurements of the net rate of NGF release from the cells were done with a quantitative NGF bioassay (Green, 1977; Rukenstein and Greene, 1983; Greene and Rukenstein, 1989). This NGF determination has the advantage of detecting only bioactive NGF, while other techniques can detect mutated, nonactive NGF forms as well.

Briefly, PC12 cells were "primed" by exposing them for 1 week to a saturating concentration of NGF (50 ng/ml) to induce their differentiation. The cells were then rinsed at room temperature three times with complete culture media, and then resuspended. After five more washes of the cells at 4°C with complete media by centrifugation, the cells were aliquoted and stored frozen in liquid nitrogen. For the bioassay, the primed cells were thawed and seeded in a 24-well plate, allowing 2 hr for attachment. Thereafter, the regular culture medium (without NGF) was replaced by 1:50 and 1:100 dilutions of the conditioned medium containing unknown amounts of NGF. After 24 hr, the bioassay was scored against a NGF dose–response curve (generated under identical conditions) determining the percentage of differentiated clumps of cells at each dilution of the samples (purified mouse b-NGF was kindly provided by Regeneron Pharmaceuticals Inc.) (a differentiated clump was scored as such when showing processes longer than the diameter of the clump). After interpolation in the curve, and correction for dilutions and cell numbers, the net release rate was obtained. The standard curve shown in Figure 1 is the average of 19 independent assays, and each medium coming from the cell lines was tested a minimum of 10 times. In selected cases, the accuracy of this bioassay was checked against a double site immunoassay (ELISA; performed according to Houlgatte et al., 1989), always finding a good correlation between the two methods.

Animals, lesions, and transplantation surgery. Young adult female Sprague–Dawley rats, weighing 225–250 gm at the time of surgery, were used (BK Universal, Stockholm, Sweden). The animals were fed a standard animal diet and water ad libitum. All procedures were carried out according to the regulations of the Ethical Committee for Experimental Research in Animals of the University of Lund.

For the experiment in nonlesioned animals, cells were implanted bilaterally into the striatum at the following coordinates: (1) A +0.6, L ±2.5; V −4.5, and (2) A +1.8, L ±2.5; V −4.5 (TB = 0). The animals received control-CINPs on the left side and NGF-CINPs on the right side. To check the potential risk for immunological rejection of the grafted cells, some of the rats were given daily intraperitoneal injections of 10 mg/kg Cyclosporin A (Sandimmune, Sandoz).

In the fimbria-fornix (FF) lesion experiment, two groups of rats received grafts of either NGF-CINP cells (n = 6) or control-CINP cells (n = 5) into the septum at the following coordinates: A +0.5, L −0.6; V −7.0 (TB = −1.0). Immediately after grafting, a complete fimbria-fornix transection (including the overlying cingulate cortex) ipsilateral to the transplant was done by aspiration (Gage et al., 1986). These animals were grafted and lesioned on the right side, leaving the left side intact to be used as a reference in the histochemical analyses.

For transplantation, cells obtained from the two subclones, the NGF-positive E8-clone (called NGF-CINP cells) and the NGF-negative-D11 clone (control-CINP cells) were prelabeled *in vitro,* either with ^{3}H-thymidine (at 0.25 μCi/ml for 24 hr before grafting) or with BrdU (10 μM, for 2 hr, 16 hr before grafting; used only for transplants in striatum). Immediately before surgery, the cells were resuspended by trypsinization, collected by centrifugation (10 min, 300 rpm), and washed in HBSS (GIBCO). The suspension was prepared in HBSS at a cell density of 10^5 cells/μl and used within 2 hr. One microliter of the cell suspension was injected stereotaxically using a 10 μl Hamilton syringe at the coordinates above (10^5 cells per site) at a speed of 0.5 μl every 30 sec. After an additional 2 min the cannula was withdrawn.

Immunocytochemistry and histochemistry. The rats were perfused transcardially after 1 or 2 weeks with ice-cold 4% phosphate-buffered paraformaldehyde. Following postfixation in the same fixative, first overnight, and then in 20% sucrose for 24 hr, the brains were sectioned in a freezing microtome at 30 μm. Eight series were taken through the striatum and five through the septal region. Complete series were stained with a monoclonal anti-BrdU antibody (Amersham), a monoclonal anti-low-affinity NGF-receptor (192-IgG, courtesy of Dr. E. Johnson), or double stained for BrdU and vimentin (Dako). The monoclonal antibody anti-LNGFR (192IgG) (Taniuchi et al., 1986) was obtained from a hybridoma cell culture supernatant. Immunostaining was performed as described elsewhere (Fischer et al., 1994) using biotinylated secondary antibodies (Dako) and developed with diaminobenzidine (DAB, Sigma) after incubation with avidin-biotin complexes (Dako). BrdU staining followed the protocol suggested by the supplier. In the double-staining procedure, the anti-BrdU antibody was applied first, using nickel intensification of the DAB reaction product, followed by anti-vimentin-DAB; in this procedure, the BrdU-labeled nuclei stain black and the vimentin-positive cytoplasm brownish.

For ^{3}H-thymidine autoradiography, slides were dipped in K5 Ilford autoradiographic emulsion, developed after 5 weeks exposure, lightly counterstained with cresyl violet and mounted.

In situ *hybridization histochemistry (ISHH). In situ* hybridization was carried out on fixed cultures, using a digoxigenin (dig)-labeled oligonucleotide, NGFas45 (depicted in Fig. 3*A*). The sequence of this oligonucleotide is 5′ GAG TGT CTG AAG AGG TGG GTG GAG GCT GGG TGC TGA ACA GCA 3′, which is the antisense sequence predicted to hybridize at nucleotides 416–460 in the mNGF cDNA sequence (Scott et al., 1983). The specificity of this probe for the transcript transcribed from the vector was checked by Northern blot hybridization (Fig. 3*C*). The oligonucleotide was dig-dUTP tailed in its 3′ end, following the recommendations of the supplier (Boehringer–

Mannheim). ISHH was carried out as follows; the cells in culture were rinsed once with PBS, and fixed for 30 min in 4% buffered formaldehyde, then rinsed three times with PBS, dehydrated, dried, and stored frozen until used. After thawing the plates, the cells were prehybridized for 2 hr at 42°C in hybridization buffer (Campbell et al., 1992) and hybridized in the presence of 16 ng/ml of the dig-labeled probe overnight at 42°C. The wells were then washed to remove nonspecific binding as follows: 1 hr, room temperature (RT), 2× SSC; 1 hr, RT, 1× SSC; 45 min, 42°C 0.5× SSC; and 1 hr, RT 0.5× SSC. Detection of the dig-labeled oligonucleotides was done by immunostaining with an alkaline phosphatase conjugated monoclonal antibody (Boehringer–Mannheim), and developed with NBT and BCIP, following supplier's suggested procedures.

Total RNA extraction from cultures or brain tissue. For cell cultures, the cells were detached by trypsinization and collected by centrifugation. The sediment was immediately frozen and stored at −20°C no longer than 2 weeks. For brain tissue, the rats were decapitated under deep anesthesia, the brain removed and frozen under crushed dry ice. The dissection of the regions of interest was done onto a dry-ice cooled plate, keeping the tissue frozen during the whole procedure. After dissection was complete, the pieces were homogenized in a glass–glass homogenizer, at 4°C, with complete culture medium (1:4 ratio weight: volume), aliquoted, and frozen in dry ice. Samples were stored at −20°C.

For RNA extraction, the acid guanidium method of Chomczynski and Sacchi (1987) was used, thawing the cells or tissue directly with the denaturing solution in the first step of the extraction. An additional acid phenol-chloroform extraction was included at the end of the extraction, to eliminate contaminating DNA that would otherwise give false positive signals in subsequent analysis.

Southern blot analysis of genomic DNA. High molecular weight genomic DNA was isolated from cultured cells, digested with appropriate restriction enzymes, electrophoresed, and transferred to nylon filters (Hybond-N, Amersham) following established procedures (Sambrook et al., 1989; Honma et al., 1994). The filter was hybridized with a 5′-^{32}P-labeled NGFas45 antisense oligonucleotide probe.

Northern blot hybridization of RNA. After RNA isolation, 10 μg of total RNA for each sample was electrophoresed through a 1% agarose-formaldehyde gel, and transferred to nylon membranes (Hybond-N, Amersham), following established procedures (Sambrook et al., 1989). The blots were probed with 5′-^{32}P-labeled antisense oligonucleotides against the mNGF sequence in the retroviral transcript or the junction between the retroviral Psi region and the polylinker in the vector (POLYas36, depicted in Fig. 2*A*). The sequence of POLYas36 is as follows: 5′ CCA TAG ATC TGG TCG ACC TGC AGC AGA CAA GAC GCG 3′, complementary to a stretch of 13 nucleotides of the retroviral genome and 23 nucleotides of the polylinker sequence. Washings after hybridization were as follows: 4×, 30 min, 37°C and 4×, 30 min, 55°C, in 1× SSC containing 0.5% SDS. Probes against retroviral sequences (LTR, for instance) resulted in a complex pattern of hybridization, since retroviral sequences were endogenously represented in the host cell genome (not shown in Fig. 2*C*).

Amplification of RNA. Samples of total RNA isolated from dissected regions from transplanted brains were subjected to *in vitro* reverse transcription using murine Moloney reverse transcriptase (MLV-RT) and amplified in the polymerase chain reaction (PCR) to detect the presence of the transcript coming from the vector in the grafted cells. Briefly, 1 μg of total RNA was reverse transcribed in a volume of 20 μl containing 200 units of MLV-RT, 1 mM deoxynucleotide triphosphate, 1 mM $MgCl_2$, and 5 pmol/μl of random hexameres as primers. Five microliters of the reverse transcription products were then subjected to PCR amplification without any manipulation, in a final volume of 20 μl containing 1.25 units of Taq polymerase, 1.5 mM $MgCl_2$, 250 μM deoxynucleotide triphosphate, and 0.2 pmol/μl of each primer. The samples were denatured at 95°C for 3 min and then subjected to 35 cycles with the following temperature profile: 30 sec, 55°C; 90 sec, 72°C; 25 sec, 95°C. After the reaction was complete, 50% of the sample was run in a 1.5% agarose gel, and visualized either directly by Ethidium Bromide fluorescence or Southern blotted and hybridized with the POLYas36 probe described above. The primers used were, for upstream, sense sequence, 5′ TCG GTT TGG GAC CGA AGC C 3′ (MLVse19) and downstream, antisense, 5′ GCT TGC TCC GGT GAG TCC T 3′ (NGFas19). The position of the primers and probes is depicted in Figure 2*A*. Note that with the primers used, the RT-PCR will be specific for the retroviral NGF transcript and will not detect the endogenous rat NGF.

To obtain an indication of the expression levels of the vector in the grafted cells *in vivo,* the grafted tissue samples were compared with a standard curve made by mixing known amounts of NGF-CINP cells in culture with striatal tissue pieces of the same approximate weight as the graft samples, but dissected from a nongrafted animal. These standards were processed in parallel to the samples to be analyzed through the complete procedure (from the homogenization and RNA extraction to autoradiography of the amplified products).

Morphometric analysis. The number and volume of cholinergic neurons in the medial septum of FF-lesioned and grafted rats were calculated using stereological procedures (optical fractionator and Cavalieri's volume method; Gundersen et al., 1988), essentially as described elsewhere (Fischer et al., 1994). Medial septum was defined as extending from the genu of the corpus callosum rostrally to the crossing of the anterior commissure caudally. Only the neurons in the region above the level crossing the anterior commissure in both hemispheres were analyzed. LNGFR-positive cells were identified at 40× magnification in an Olympus BH-2 microscope interfaced to a video color camera (Hitachi) and an Amiga 2000 computer both connected to an RGB color monitor (Trinitron, SONY). The GRID software (Intractivision, Silkeborg, Denmark) commanding an X-Y step motor on the microscope stage, was used to sample the sections in an unbiased but systematic fashion.

Results

Production of NGF-secreting conditionally immortalized neural progenitor (CINP) cells

The mNGF cDNA was transduced into the target HiB5 cell line using repeated infections with conditioned media collected from a Psi2-NGF virus-producing cell line (constructed as described in Experimental Procedures). Since the current Moloney Murine Leukemia Virus vector (MMLVv-NGF) does not contain any selection gene in its construct (Fig. 1*A*), we designed a five-step infection protocol to increase the percentage of infected cells. Infection was performed every second day to allow for recovery and passage of the cells after each infection step and to keep the HiB5 cells continuously growing to achieve maximum efficiency of infection (Miller et al., 1990).

The increase in the number of transfected cells in the culture was monitored after each infection step by measuring the NGF release rate into the medium using an *in vitro* bioassay for NGF (Fig. 1*B,C*). The NGF release data indicate that the percentage of NGF-transduced cells in the HiB5 population reached a plateau after four to five infections. Interestingly, the three to five time infected HiB5 cells, even though they were not homogeneously infected, released more NGF than the Psi2-NGF viral packaging cell line, which is a fibroblastic clone. *In situ* hybridization histochemistry (ISHH) using specific probes (see below) showed that the infection reached a maximum of approximately 50% clearly NGF-positive cells in the HiB5.5-NGF cell line obtained after five successive infections (Fig. 3).

To further increase the *in vitro* efficiency of the transduced cells in terms of NGF release, the HiB5.5-NGF cell line was subcloned by limiting dilution. As shown in Figure 1*D*, 11 clones were chosen on the basis of their growth rate during the isolation procedure. Analysis of NGF release into the culture medium identified three negative clones and eight positive ones. The highest secreting clone, E8, showed a net NGF release of about 2 ng NGF/hr/10^5 cells, which is 10 times higher than the parental heterogeneous HiB5.5-NGF cell line. The spectrum of release rates of the isolated clones seems to be representative of the subpopulations originally present in the HiB5.5-NGF culture, since the average NGF release from the 11 subclones was similar to that of the heterogeneously infected cell line.

To further characterize the NGF-producing E8 subclone, we

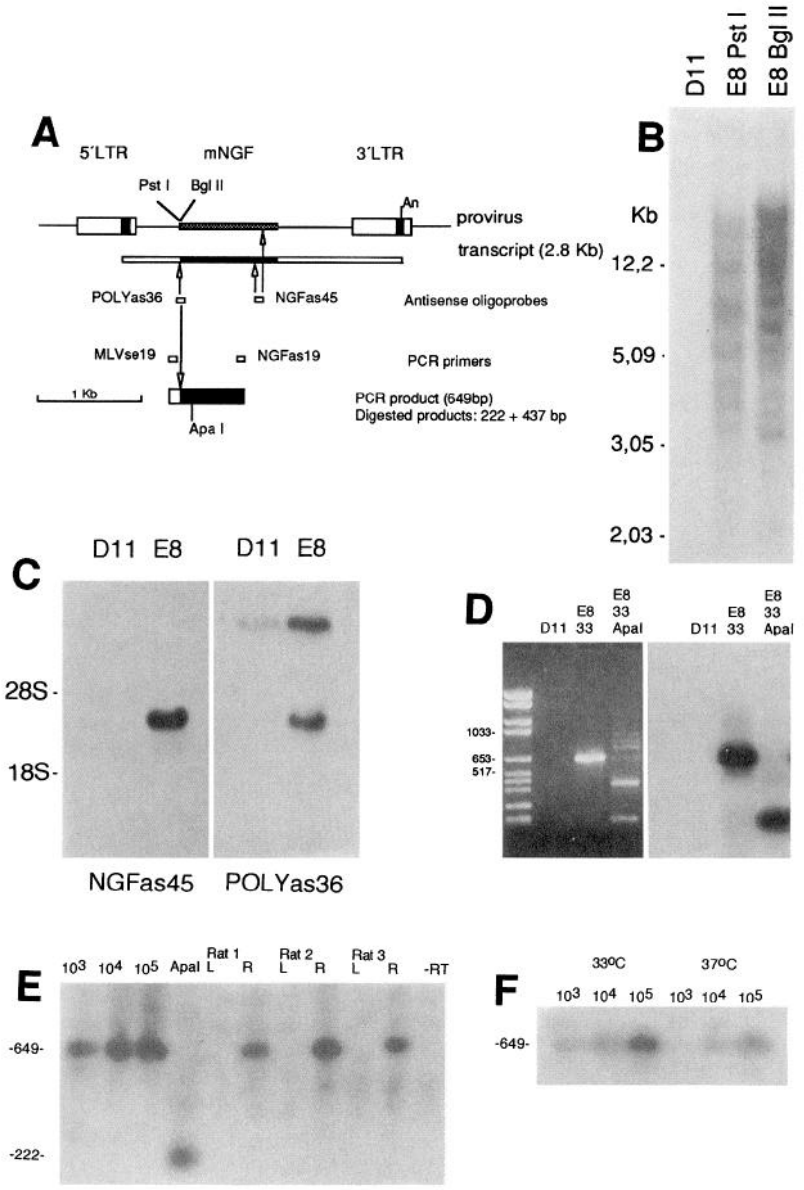

Figure 2. Retrovirus structure, integration, and expression. *A,* Vector map showing the target regions for the antisense oligonucleotides used in subsequent hybridizations, as well as the primers used for RT-PCR, and the characteristics of the amplified product. *B,* Southern hybridization of digested genomic DNA. Samples (10 μg per lane) were digested with the indicated enzymes, blotted, and hybridized with ^{32}P-labeled NGFas45. The hybridization pattern suggests the presence of around 10 copies of the integrated provirus in the genome of the E8 clone, whereas the negative (*D11*) clone is not infected. *C,* Northern hybridization of total RNA (10 μg per lane) from the negative (*D11*) and the positive (*E8*) clones, hybridized with either NGFas45 (*left*) or POLYas36 (*right*), showing that both probes detect a unique transcript coming from the NGF retrovirus only in the positive clone (*E8*). POLYas36 detects in addition a longer mRNA species transcribed from the immortalizing retrovirus that is present in both clones. *D,* Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of the retroviral NGF transcript. *Left,* ethidium bromide fluorescence, and *right,* autoradiogram of the corresponding Southern blot, hybridized, with the POLYas36 probe, detecting a major PCR product in the NGF producing E8 cells. Molecular weight markers are type VI (Boehringer–Mannheim). Cells (10^5) were mixed with 50 mg of rat brain tissue and subjected to total RNA extraction. RNA (1 μg) was then reverse transcribed, and one-fourth of the reaction amplified with PCR. Half of the PCR products were loaded on the gel; considering the yield of the RNA preparation, the detected PCR product comes from approximately 200 cells. The gel shows a product of the predicted size (649 nucleotides) that can be cleaved in two subfragments by Apa I, one of them (at 222 nucleotides) is recognized by the labeled probe, as detailed in *A*. *E,* RT-PCR amplification of total RNA extracted from frozen dissected tissue from three transplanted animals (*L,* left striatum, control-CINPs; *R,* right striatum, NGF-CINPs). The rats were grafted with 10^5 cells in the striatum and sacrificed 2 weeks later. Total RNA was prepared and subjected to RT-PCR. Standards were generated (as in *D*), by mixing known amounts of cells, grown in culture at 33°C (10^3, 10^4, or 10^5 cells), with equivalent pieces of striatal tissue from nongrafted animals. Hybridization of the Southern blots with the POLYas36 probe (see *A*) visualizes a band at 649, corresponding to the amplified NGF transcript in the grafted (*right*) striatum of all three grafted rats. Additional controls show the digestion of the PCR product with ApaI and a lane without reverse transcriptase (−RT), in which RNA from the 10^5 cells sample was amplified without reverse transcriptase to check for any DNA contamination of the RNA samples. *F,* RNA amplification of the NGF retroviral transcript from known amounts of cells growing at 33°C or growth arrested at 37°C; a difference of approximately 1 order of magnitude in expression is evident as a result of the temperature shift.

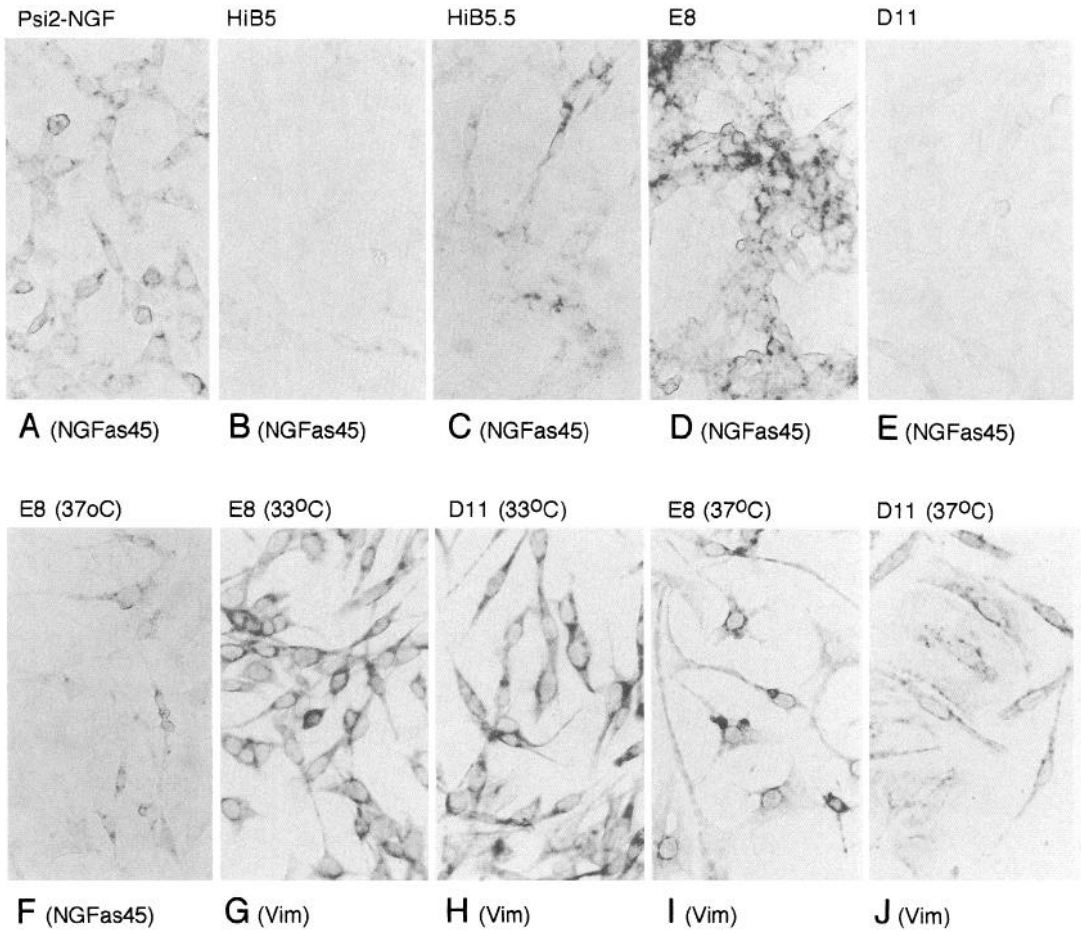

Figure 3. *In vitro* characterization of the NGF-CINPs and control-CINPs under different culture conditions. *In situ* hybridization histochemistry (*A–F*) (probe *NGFas45*) or immunocytochemistry (vimentin) (*G–J*) of different cell lines used in this study: Psi2-NGF (*A*), the parental HiB5 cell line (*B*), the heterogeneous HiB5.5 (*C*), and the subclones D11 and E8 (control- and NGF-CINPs, respectively). For the E8 and D11 subclones, examples of the cultures growing at 33°C or 37°C are given. *A–E* are cultures grown at 33°C.

studied some molecular aspects of the transfection, expression, and differentiation of these cells. The number of copies of the integrated provirus in the E8 clone, as determined from the hybridization pattern on Southern blots (Fig. 2*B*), appeared to be more than 10 integration sites per cell, probably as a result of the multiple, repeated infection procedure used to generate the parental cell line, HiB5.5-NGF. By contrast, the negative clone, D11, showed no signs of provirus integration. The structure of the transcribed message appeared not to be altered, judging from the positive hybridization of antisense probes against two different regions in the mRNA, the transcript size in Northern hybridization (Fig. 2*C*), and the correct amplification of RNA with an appropriate set of primers. Moreover, the PCR product was restriction endonuclease digested at the predicted site (Fig. 2*D*).

The expression of the MMLVv-NGF transgene was studied in culture with ISHH. The NGFas45 probe, shown to be specific for this transcript (Fig. 2*C*), recognizes the cells transfected with the NGF construct, i.e., the Psi2-NGF, HiB5.5-NGF, and the E8 subclone (Fig. 3). Using this probe, clear-cut expression of the NGF transgene was detected in approximately 50% of the cells in the heterogeneous HiB5.5-NGF line (Fig. 3*C*), and in close to 100% of the E8 subclone (Fig. 3*D*). This signal was completely absent in the parental HiB5 cells (Fig. 3*B*) as well as the NGF-negative clone D11 (Fig. 3*E*), which is consistent with the NGF secretion data.

The HiB5 cells have earlier been shown to be able to differentiate to a certain extent *in vitro* at the nonpermissive temperature (37–39°C), particularly after culture in defined media or under influence of agents promoting neuronal differentiation (Frederiksen et al., 1988; Refranz et al., 1991; McKay, 1992). Since cell lines may drift or change their characteristics after extensive *in vitro* culture, we performed a basic characterization of two of the subclones: the NGF-expressing E8 cells (referred to as the NGF-CINP cells), and the NGF-negative D11 clone (referred to as the control-CINP cells). After 1 week at 37°C in DMEM/F12/hormone mixture (see Experimental Procedures), the cells of both the NGF-CINP and the control-CINP subclones greatly attenuated their division rate. In contrast to the bipolar, spindle-like morphology characteristic for the continuously dividing CINPs at 33°C (Fig. 3*G,H*), both types of CINP cells changed their morphology at 37°C towards a multipolar shape. This morphological change was most pronounced for the NGF-CINPs, some of which displayed more elaborate cellular shapes with long processes and round cell bodies (Fig. 3*I,J*).

Consistent with previous *in vitro* studies on the HiB5 cell line, the two CINP clones were vimentin positive (Fig. 3*G–J*), but

negative for both GFAP and MAP-2 at both 33 and 37°C (data not shown), suggesting that the cells remained relatively immature also at the nonpermissive temperature *in vitro*.

Possible changes in expression of the NGF transgene and in release of the NGF protein were studied after 1 week in culture at the nonpermissive temperature. The NGF-CINPs (but not the control-CINPs) showed a clear signal with the NGF probe at 37°C, similar to that obtained in the cultures grown at 33°C (Fig. 3*D–F*). Measurement of NGF secretion showed that the release rate was indistinguishable for the NGF-CINPs at both temperatures (Fig. 1*D*). Interestingly, RT-PCR amplification of RNA from cells grown at 33°C or 37°C revealed downregulation of the message (Fig. 2*F*). This could, at least in part, be ascribed to a lower overall synthesis of total RNA in the differentiating, nondividing cells, which in our hands amounts to no more than one-third of the synthesis seen in growing cultures. The apparent discrepancy between the level of RNA expression and rate of protein release suggests that the limiting step for NGF production in the NGF-CINP cell is the protein synthesis and release rather than the level of RNA expression. In any case, these data indicate that NGF transgene expression and NGF release remained high also at body temperature, an important prerequisite for the use of the NGF-CINPs in grafting experiments.

Morphological features of the CINP cells after grafting to the brain

In a first series of experiments, the two types of CINP cells were grafted bilaterally into the striatum (10^5 cells per site) in adult intact rats. This characterization was done in parallel to the naive HiB5 cell line, not finding any notable difference between the genetically modified cells and the parental cell line. Prior to grafting, the cells were labeled with bromodeoxyuridine (BrdU) or ^{3}H-thymidine to allow for identification of the cells *in vivo*. These brains were analyzed 1–4 weeks after transplantation. Large numbers of labeled cells were detected, the vast majority of which had a glia-like morphology (as judged by the size and shape in the Nissl-stained sections (Fig. 4*E,F*) and were found scattered in the host brain parenchyma surrounding the injection site (Fig. 4). No distortion of the host tissue was observed; the site of injection was usually marked by a small graft deposit (asterisks in Fig. 4), and the cells were never seen to form large clumps or cell aggregates. The survival and microscopic appearance of the labeled CINP cells were similar, regardless of whether the animals had been immunosuppressed (with daily injections of 10 mg/kg cyclosporin) or not. Careful analysis of double-stained sections (stained for BrdU in combination with vimentin) indicated that all grafted cells located outside the actual injection site were vimentin negative (Fig. 4*C,D*). Some of the BrdU-labeled grafted cells that were located among the reactive astrocytes at the site of injection, however, were vimentin positive.

The BrdU- or ^{3}H-thymidine–labeled cells were distributed over an area extending up to about 1–1.5 mm from the striatal implantation site. The majority of cells occurred in gray matter areas (i.e., avoiding the myelinated bundles of the internal capsule) (Fig. 4*D*) and they were never seen to pass into or through the corpus callosum (except for cells that had leaked back along the needle track). The results were similar with both labels, but ^{3}H-thymidine clearly labeled a larger number of cells throughout the host striatum (compare Fig. 4*A,C*). Since both labeling protocols were equally efficient in labeling the cells *in vitro* prior to grafting (close to 100%), this quantitative difference is likely to be due to incomplete visualization of BrdU in the brain tissue sections in the current staining procedure (probably due to the use of cross-linking fixative for animal perfusion).

Expression of the MMLVv-NGF transgene in vivo

Expression of NGF-mRNA *in vivo* was studied by RT-PCR in dissected tissue samples from animals grafted either with control- or NGF-CINPs in the striatum, 2 weeks after surgery. In all three rats analyzed in this way there was clear expression of the transgene in the NGF-CINP grafted (right) striatum, but not in the control-grafted (left) striatum (Fig. 2*E*). (With the primers used, only the retroviral mouse NGF transcript, but not the endogenous rat NGF, is amplified.) To assess for any possible downregulation or adjustment of expression due to cessation of cell division and maturation of the cells at the nonpermissive body temperature, we compared the standard curve generated from cells of growing cultures (at 33°C) to a curve generated from the same number of growth-arrested cells (cultured at 37°C). As shown in the autoradiogram in Figure 2*F*, there seemed to be 1 order of magnitude difference in the amount of amplified product between the two growth conditions *in vitro*. These PCR data thus indicate that NGF transgene expression *in vivo* might be close to the maximum that can be expected from NGF-CINP cells at the nonpermissive temperature; there is little indication therefore of *in vivo*-induced downregulation of the expression of the transgene during the 2 weeks postgrafting period studied here (Fig. 2*E,F*).

In vivo *neurotrophic effects*

Striatum. In the first experiment, where the two types of CINP cells were grafted to the intact striatum, there was a marked accumulation of fibers staining for the low-affinity NGF receptor (LNGFR) around the site of implantation of the NGF-CINP cells (Fig. 5). No such fibers were present around the control-CINP cell grafts. A few weakly LNGFR-positive cell bodies were detectable in the host striatal parenchyma around the NGF-CINP cells, but not the control-CINP cell grafts.

Septum. In the second experiment, CINP cells of both types were labeled with ^{3}H-thymidine *in vitro* and grafted into the septum unilaterally in rats subjected (in the same surgical session) to an ipsilateral complete fimbria-fornix (FF) lesion. At 2 weeks after transplantation, autoradiographically labeled cells were found scattered throughout the medial and lateral septum, including the subependymal zone boarding onto the lateral ventricle (Fig. 6*A*). Few cells were seen to have crossed over the midline into the contralateral septum. As in the grafts in striatum (Fig. 4), only tiny cell clusters were seen at the injection site. No sign of overgrowth and tumor formation was observed in any specimen.

As reported earlier (Gage et al., 1986; Williams et al., 1986; Fischer and Björklund, 1991), a complete aspirative lesion of the FF-pathways results within 2 weeks in a 60–70% loss of LNGFR-positive cholinergic neurons in the medial septal nucleus on the side of the lesion. This axotomy-induced degeneration can be almost completely blocked by intraventricular infusions of exogenous NGF (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Fischer and Björklund, 1991; Tuszynski and Gage, 1991). In animals with transplants of control-CINP cells (10^5 cells implanted into the medial part of the lateral septal nucleus), the number of LNGFR-positive neurons on the side of the FF lesion was reduced to 30% of the contralateral control side (Fig. 6*B,C*). In the animals grafted with NGF-CINP cells (Fig. 6*D,E*), by

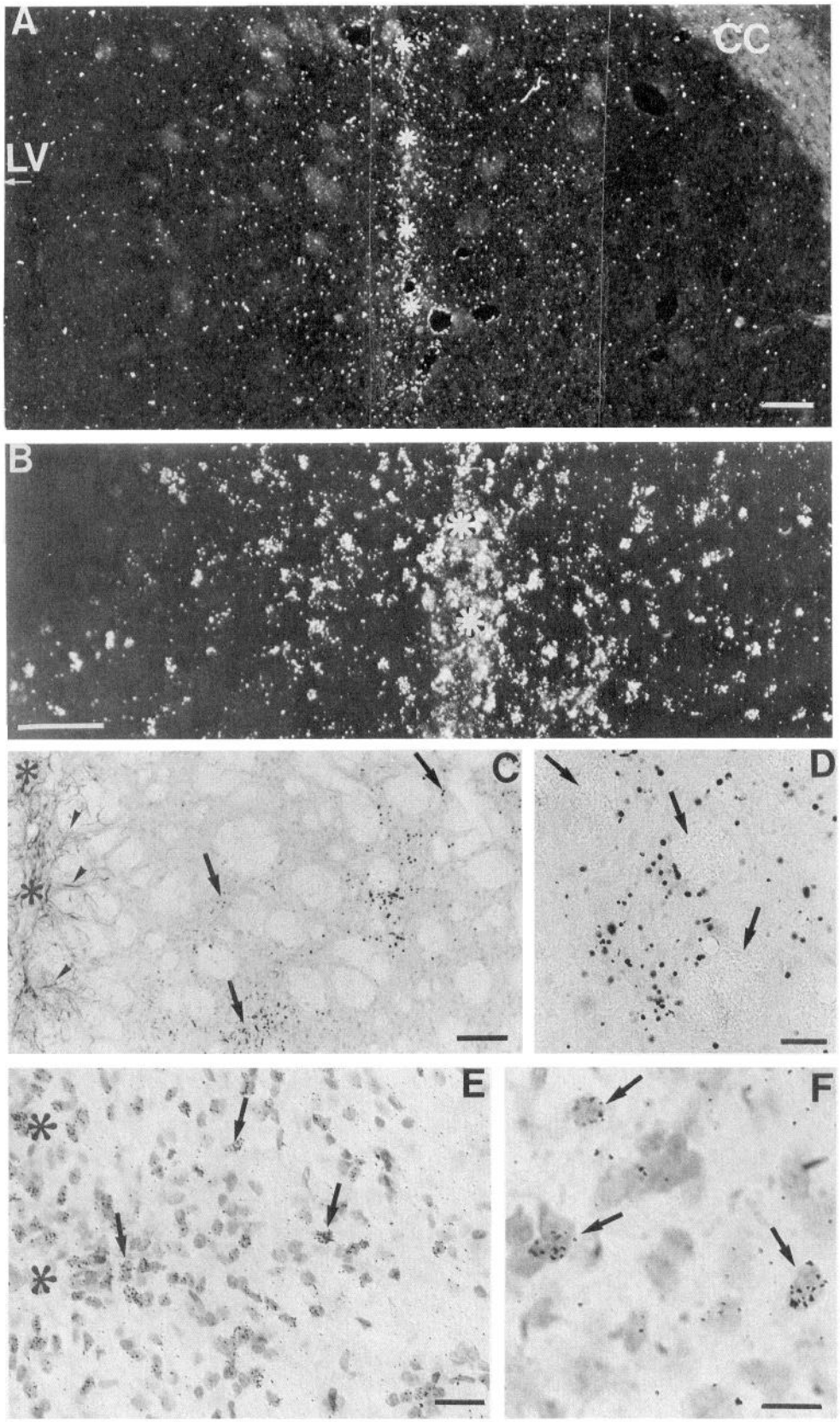

Figure 4. Transplantation into the striatum. The parental cell line HiB5 (*A*) or the NGF-CINP cells (*B–F*) were grafted into the head of the caudate-putamen and the animals were sacrificed after 1 (*B, C,* and *D*), 2 (*A*), or 4 weeks' survival (*E* and *F*). *A* and *B,* Dark-field low- and high-power pictures, respectively, of ^{3}H-thymidine labeled transplanted cells, showing extensive migration of the labeled cells through the entire dorsal striatum. At low magnification (*A*) the labeled cells appear as *white dots;* at higher power (*B*) the cells are identified by the clustered grains in the autoradiographic emulsion. *C,* Double immunocytochemistry for vimentin (*arrowheads*) and BrdU (*arrows*), revealing migration of the cells away from the injection site (*asterisks*); note that the BrdU-labeled cells are vimentin negative. *D,* Higher power picture of the BrdU-labeled cells at some distance from the injection site, showing a clear preferential distribution of stained nuclei in the gray matter (Nomarski optics; the *arrows* point to transected fiber tracts). *E* and *F,* Morphology of transplanted cells in the striatum; the photomicrographs show two examples of cresyl violet-stained sections combined with emulsion autoradiography to identify the ^{3}H-thymidine–labeled cells by the grains in the emulsion; the pictures include the transplantation site (*asterisks in E*), as well as individual cells that had migrated away from the injected deposit (*arrows in E and F*); the field in *F* was at a distance of 200 μm from the injection site. *CC,* corpus callosum; *LV,* lateral ventricle; *asterisks* indicate the injection site. Scale bars: *A* and *C,* 100 μm; *B* and *D,* 50 μm; *E,* 20 μm; *F,* 10 μm.

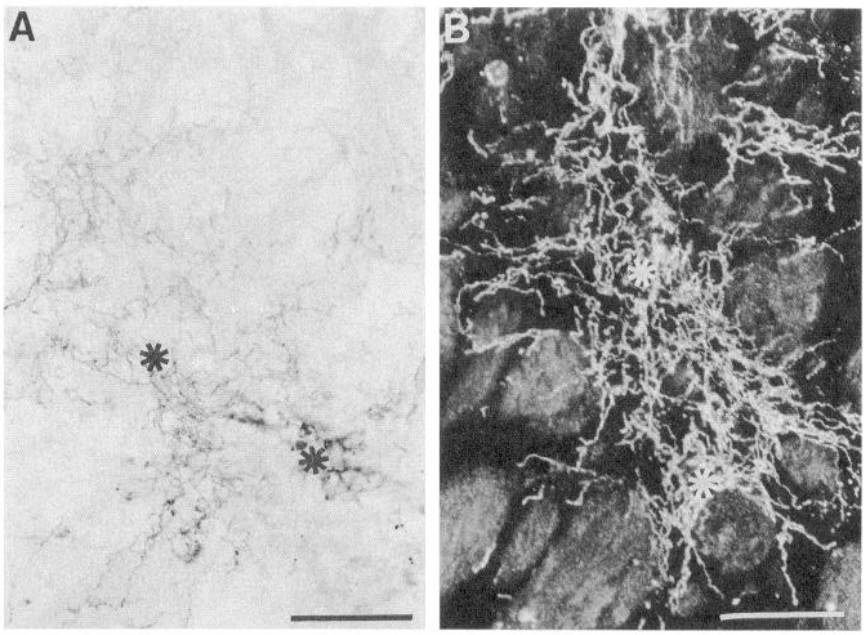

Figure 5. *A*, Bright-field and *B*, dark-field microphotographs showing NGF-induced accumulation of LNGFR-immunostained positive fibers after grafting NGF-CINPs into the striatum, 1 week survival. *Asterisks* denote injection track. Scale bars, 100 μm.

contrast, this effect of the FF-lesion was almost completely blocked (93% of control side; Fig. 7, left). Cell-size measurements indicated that the rescued neurons were similar in size to those on the contralateral intact side (Fig. 7, right).

Discussion

The NGF-CINP cells reported here are among the highest NGF-producing cells obtained so far. Their *in vitro* secretion rate is about 2 ng/hr/10^5 cells, which is about 1 order of magnitude higher than that previously reported for NGF-transduced fibroblastic cell lines (0.05–0.16 ng/hr/10^5 cells; Rosenberg et al., 1988; Schumacher et al., 1991). By comparison, repeated retroviral infection of primary cultures of rat skin fibroblasts, combined with neomycin selection, has yielded cells with a net NGF release of 0.15 ng/h/10^5 cells (Kawaja and Gage, 1991; Kawaja et al., 1992; using a vector containing mouse NGF) and about 2 ng/hr/10^5 cells (Dekker et al., 1994; using a vector containing human NGF). Information on the rate of NGF release is lacking in other reports where mouse NGF has been expressed in NIH-3T3 cells (Ernfors et al., 1989; Strömberg et al., 1990). Rough calculations indicate that the NGF-CINPs studied here have a production rate of 1.5×10^6 NGF molecules per cell per day, which is close to the antibody production in a good hybridoma (Bebbington and Henkschel, 1985).

The clonal nature of the NGF-CINPs may offer clear advantages over heterogeneously infected cell lines or cell cultures; not only because of the high and consistent rate of infection, but, more importantly, because in heterogeneously infected cultures the cells do not all express the vector with the same efficiency. This variability may depend not only on the number of insertion sites, but also on how much the cell has been disturbed by the insertion of the retrovirus, as well as on *cis*-acting sequences in the host genome that influence expression of the retrovirus (Varmus, 1990; Levine and Friedmann, 1991; Miller, 1992; Ray and Gage, 1992). There are two other features of the NGF-CINPs that may positively affect the expression from the retroviral vector: first, the vector was designed to be monocistronic, without internal promoters, since the presence of internal *cis*-regulatory sequences in retroviral vectors has been described to inhibit expression in an unpredictable way, both *in vitro* and *in vivo* (Xu et al., 1989; Osborne, 1991; Palmer et al., 1991; Soriano et al., 1991; Boris-Lawrie and Temin, 1993). Secondly, in our case, the viral LTR promoter may be compared to a housekeeping promoter *in vitro,* since the parent HiB5 cells were immortalized with the double mutant tsA58-U19 allele of the SV40 large T-antigen driven by the MMLV LTR. Since the procedure selects for cells that continuously divide under the influence of the T-antigen, this will ensure that the LTR promoter is not shut down, at least not during clone isolation at the permissive temperature (33°C).

Expression of the NGF transgene in vivo

In vitro, the level of NGF secretion from the NGF-CINP cells was unchanged after 1 week of culture at the nonpermissive temperature (37°C) when the nondividing cells start to differentiate. Consistent with this, both *in situ* hybridization histochemistry and RT-PCR amplification demonstrated maintained expression of the NGF transcript at 37°C, but from the PCR data it appeared that mRNA expression was reduced *in vitro* by approximately 1 order of magnitude compared to the cultures maintained at 33°C. This could, at least in part, be ascribed to a lower overall RNA synthesis in the differentiating, nondividing cells. This apparent discrepancy between the changes in NGF production and mRNA expression may readily be explained by assuming that the NGF synthesis and/or secretion rate is higher at 37 than at 33°C. In any case, this observation is interesting, since it suggests that the rate-limiting step for the secretion of NGF from the NGF-CINPs is not the level of mRNA expression but, rather, the rate of protein synthesis or secretion, at least under the present culture conditions *in vitro.*

NGF expression 2 weeks after transplantation of the NGF-CINP cells to the brain was demonstrated, first, by RT-PCR showing the expression of the NGF-mRNA transcript in the grafted striatum receiving NGF-CINP, but not control-CINP cells; and, secondly, by the *in vivo* observation of neurotrophic effects in the intact striatum (induction of LNGFR-immunoreactive fibers around the site of implantation) and in the FF-lesion model (rescue of axotomized cholinergic neurons in the medial

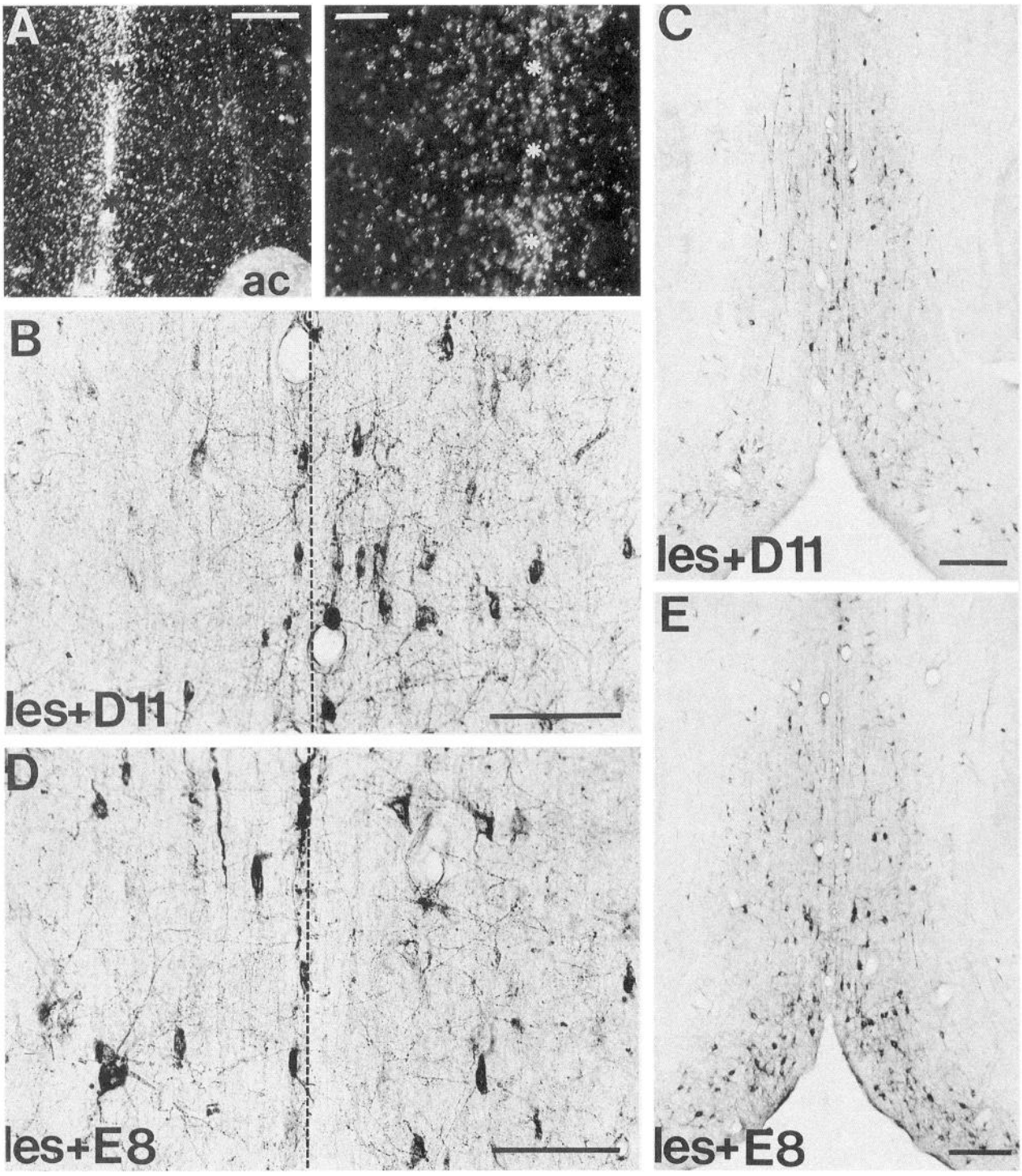

Figure 6. Cell rescue in the FF-lesion model. Rats were unilaterally grafted into the septum with either control- or NGF-CINPs, and a complete ipsilateral aspiration of the fimbria-fornix pathways was done in the same surgical session. The contralateral intact side served as reference in the stereological measurements. *A*, Low-power (*left*) and high-power (*right*) dark-field autoradiograms showing the ^{3}H-thymidine–labeled NGF-CINPs in the septum. High- (*B* and *D*) or low- (*C* and *E*) magnification microphotographs of LNGFR-immunostained cholinergic neurons in the medial septum of lesioned animals grafted with control-CINP (*les+D11*) or NGF-CINP cells (*les+E8*) (midline is denoted with a *dashed line*, in *B* and *D*). In *B–E*, the grafted and lesioned side is to the left in the pictures. Scale bars: *A* (*right*), *B* and *D*, 50 μm; *A* (*left*), 200 μm; *C* and *E*, 100 μm.

septum). Further evidence for stable long-term transgene expression *in vivo* (up to 10 weeks postgrafting, the longest time studied so far) has been obtained in a parallel study where the NGF-CINP cells have been grafted to the nucleus basalis in young and aged intact rats (Martinez-Serrano et al., 1994a,b). Indeed, increased tissue levels of biologically active NGF were detected (at 4 and 10 weeks' survival) by the PC12 bioassay in the grafted nucleus basalis region in animals receiving NGF-CINP (but not control-CINP) cells (Martinez-Serrano et al., 1994b).

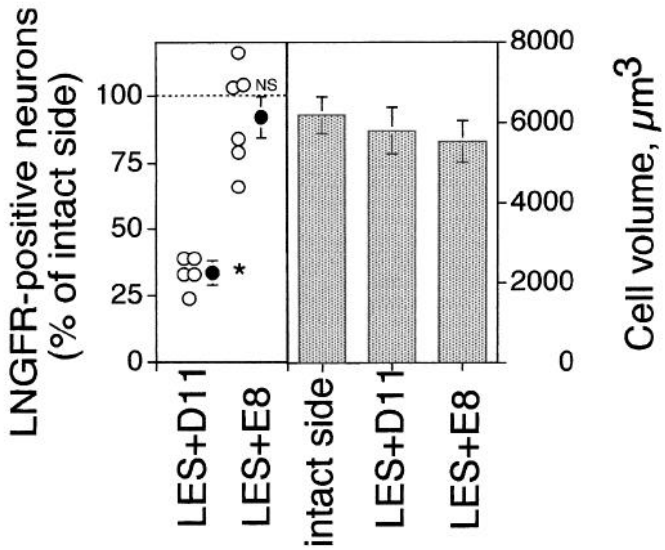

Figure 7. Rescue of cholinergic neurons in the medial septum by NGF-CINP cells 2 weeks after an aspirative FF lesion. The number and size of the LNGFR-positive neurons in the medial septum were determined using stereological procedures in animals grafted with control-CINP cells (*LES+D11*) or NGF-CINP cells (*LES+E8*), 2 weeks after surgery. *Cell numbers* are expressed as percentage of the intact side. *Open symbols* refer to individual animals, whereas the *dark circles* represent group means. The 93% of LNGFR-positive neurons in the NGF-grafted rats when compared to the intact sides in the same animals is considered as maximal or complete rescue in similar experiments infusing NGF with minipumps; $*p < 0.01$, control versus intact ($p = 0.007$) or control versus NGF ($p = 0.0039$); *NS,* nonsignificantly different, NGF versus intact ($p = 0.39$; one-tail, Student's t test).

Although the PCR analysis performed on the graft-containing brain regions only allowed for rough estimations of the level of transgene expression, our data indicate that the level of expression seen after transplantation to the brain is close to that obtained from the same number of cells taken from cultures maintained at 37°C *in vitro* (mixed with an equivalent amount of nongrafted brain tissue). Any *in vivo* decrease in expression seen when comparing samples from the individual animals to standards obtained with cells from growing cultures (at 33°C) therefore appears to be associated with the transition of the cells from a proliferative to a growth-arrested state (i.e., from 33°C to body temperature) (Fig. 2*E,F*).

Efficiency of NGF secretion in the FF-lesion model

Quantitative analyses of the survival of the HiB5 cells after grafting to the striatum (using *in vitro* ^{3}H-thymidine prelabeling) has shown that the cells increase about twofold in number within the first few days after grafting and remain stable (at a total number of 2×10^5 cells) from 1 week to at least 6 months postgrafting (Lundberg et al., 1994). Based on the *in vitro* release data therefore the expected NGF secretion rate from each graft ($1–2 \times 10^5$ cells) can be estimated to be in the range of 2–4 ng/hr, i.e., approximately 50–100 ng/d. In the FF-lesion model, the minimum effective dose for rescue of > 90% of the axotomized cholinergic neurons in the medial septum is around 100–300 ng/d when NGF is infused by minipump into the lateral ventricle (Williams et al., 1986, 1989; Gage et al., 1988; Fischer and Björklund, 1991; Hagg et al., 1992). The latter authors reported 95% septal cell survival with a dose of 130 ng/d, and 53% survival with a dose of 4 ng/d. Similarly, Knusel et al. (1992) obtained 82% septal cell survival with a dose of 50 ng/d, and 92% survival with a dose of 700 ng/d. In a biochemical study, Williams et al. (1989) studied the effect of graded doses of NGF, infused intraventricularly, on septal choline acetyltransferase levels in FF-lesioned rats. From their dose–response curve they could derive a minimal effective dose (ED_{50}) equivalent to 120 ng/d, which is quite consistent with the cell rescue data. Assuming that intraventricularly infused NGF is diluted 20–30-fold in the CSF (see Discussion in Dekker et al., 1994), a dose in the range of 5–15 ng/d would be expected to be sufficient to induce a significant response in the FF-lesion model when NGF is delivered directly into the septum, as done here. This estimate seems consistent with the present data, as well as with the results of previous cell transplantation studies using the FF-lesion model. Thus, Kawaja et al. (1992) obtained 75% septal cholinergic cell survival with NGF-producing primary fibroblasts with an estimated *in vitro* secretion rate of 12 ng/d, Hoffman et al. (1993) and Winn et al. (1994) reported 85–88% cell survival with NGF-secreting fibroblasts implanted in a polymer capsule that had a release rate of 5–24 ng/d. With the present NGF-CINP cells, we obtained > 90% cell survival with grafts with an estimated *in vitro* release rate of 50 ng NGF/d. By contrast, Whittemore et al. (1991), using a conditionally immortalized NGF-secreting neuroblastoma cell line, observed marginal cell rescue with grafts whose estimated release rate was 1.2 ng/d. The results of Rosenberg et al. (1988), on the other hand, are somewhat at variance with this picture, since they reported 92% survival of medial septal neurons with grafts that had an estimated *in vitro* NGF secretion of only 5 ng/d. However, the cells used in the Rosenberg et al. study are a continuously growing fibroblastic cell line (208F) which is likely to increase markedly in size (and, hence, increase their NGF production) during the first weeks after grafting (see Discussion in Kawaja et al., 1992).

From available published data it is thus clear that the new CNS-derived NGF-CINP cells represent the first nontumorgenic NGF-producing cell that has allowed sufficiently efficient intracerebral NGF secretion after transplantation to induce complete protection in the rat FF-lesion model (undistinguishable from that obtained after infusion of microgram doses of NGF with minipumps). The accumulation of LNGFR-immunoreactive fibers around the site of implantation of NGF-CINP cells in the striatum, on the other hand, is similar to that observed around striatal transplants of NGF-producing primary fibroblasts (Kawaja et al., 1991) and around intrastriatal implants of collagen gels containing NGF-secreting NIH-3T3 cells (Ernfors et al., 1989).

Concluding remarks

On the basis of a conditionally immortalized temperature-sensitive neural progenitor cell line, HiB5, derived from the embryonic rat CNS, we have obtained a highly NGF-secreting clonal cell line with properties suitable for grafting to the intact or lesioned adult rat brain. By retroviral infection, using a MMLV vector containing the mouse NGF gene under control of the LTR promoter, a NGF-secreting cell was isolated by subcloning at the permissive temperature of 33°C. This NGF-secreting clone (called NGF-CINPs) contained multiple copies of the gene and secreted biologically active NGF at a rate of 2 ng/hr/10^5 cells *in vitro.* Transplantation to the brain of adult recipients shows that the NGF-CINP cells exhibit a number of advantageous properties: they survive well, differentiate into cells with a predominant glia-like morphology, and migrate for a dis-

tance of about 1–1.5 mm from the site of implantation to become structurally integrated with the surrounding host brain tissue, without any signs of overgrowth and tumor formation. After grafting to the striatum or septum, the NGF-CINP cells continued to express the NGF transgene and secrete NGF at a level sufficient to induce complete (> 90%) rescue of axotomized septal cholinergic neurons in rats with complete transection of the fimbria-fornix pathways. Thus, CNS-derived conditionally immortalized neural progenitors, CINPs, may represent a highly versatile cellular vehicle for gene transfer to circumscribed targets within the intact or lesioned adult CNS. This approach should be useful not only for the intracerebral expression of neurotrophic factors, as shown here, but also for *ex vivo* gene transfer of neurotransmitter-related enzymes, as shown in the recent study of Anton et al. (1994).

Morphological studies (Renfranz at al., 1991; Lundberg et al., 1994) indicate that the HiB5 cells behave as genuine neural progenitor cells after implantation into the brain and that they can generate both neurons and glia in the developing CNS, and almost exclusively glial cells (in part, at least, astrocytes) in the mature brain. The CINP cells therefore offer the possibility, in adult intact or lesioned animals, to insert new mature glial cells in defined brain regions expressing selected transgenes, thus creating cellular chimeras that are regionally confined to circumscribed areas of the CNS. Indeed, since the transgene is not targeted to endogenous cells of the host brain, but rather expressed in the implanted cells, this gene transfer approach should minimize any possible risk of interference with endogenous host brain functions. This system should provide a highly useful experimental tool to study the therapeutic potential of long-term localized neurotrophin delivery in, for example, animal models of neurodegenerative diseases and stroke. Indeed, in a parallel study we have found that grafts of NGF-CINP cells in the nc. basalis and septum can, over a 1 month period, reverse age-dependent spatial memory impairments and cholinergic neuron atrophy in cognitively impaired aged rats (Martinez-Serrano et al., 1994a,b).

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