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Genetically Modified NT2N Human Neuronal Cells Mediate Long-Term Gene Expression as CNS Grafts In Vivo and Improve Functional Cognitive Outcome Following Experimental Traumatic Brain Injury

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Abstract. Human Ntera-2 (NT2) cells can be differentiated in vitro into well-characterized populations of NT2N neurons that engraft and mature when transplanted into the adult CNS of rodents and humans. They have shown promise as treatments for neurologic disease, trauma, and ischemic stroke. Although these features suggest that NT2N neurons would be an excellent platform for ex vivo gene therapy in the CNS, stable gene expression has been surprisingly difficult to achieve in these cells. In this report we demonstrate stable, efficient, and nontoxic gene transfer into undifferentiated NT2 cells using a pseudotyped lentiviral vector encoding the human elongation factor 1-α promoter and the reporter gene *eGFP*. Expression of eGFP was maintained when the NT2 cells were differentiated into NT2N neurons after treatment with retinoic acid. When transplanted into the striatum of adult nude mice, transduced NT2N neurons survived, engrafted, and continued to express the reporter gene for long-term time points in vivo. Furthermore, transplantation of NT2N neurons genetically modified to express nerve growth factor significantly attenuated cognitive dysfunction following traumatic brain injury in mice. These results demonstrate that defined populations of genetically modified human NT2N neurons are a practical and effective platform for stable ex vivo gene delivery into the CNS.

Key Words: Lentiviral vectors; Nerve growth factor (NGF); Neural progenitor cells; Transplantation; Traumatic brain injury.

INTRODUCTION

The Ntera-2 (NT2) cell line is a unique human cell line that terminally differentiates exclusively into postmitotic neurons (designated as NT2N neurons) when exposed to retinoic acid in vitro and has been widely used for experiments in developmental neurobiology (1–5). NT2N neurons have particular advantages for studies of neural transplantation. NT2 cells are differentiated into >95% NT2N neurons in vitro before transplantation (2, 6, 7), generating a uniform population of human cells whose neuronal fate is already specified, but which nonetheless respond to the local environment by assuming cellular morphologies and processes appropriate to the region into which they are engrafted (7). In contrast, neural stem cells can give rise to multiple lineages, but they rely on local cues in the recipient nervous system to specify their phenotype. In many regions of the post-natal CNS, transplanted neural stem cells differentiate predominantly into glia (8–10).

The NT2N neurons are an unlimited source of human cells that can be produced under good manufacturing practice conditions suitable for human clinical trials. They thus represent a potentially better alternative to other sources currently under investigation for CNS transplantation, including human embryonic stem cells, xenografts, and fetal mesencephalic cells (11, 12). Grafts of differentiated NT2N neurons in the CNS are stable and appear to be safe since they have not been found to dedifferentiate or to form tumors in vivo for at least 1 year post-transplantation in mice or humans (13–15). The grafted neurons develop a mature neuronal phenotype in vivo, based on axonal and dendritic polarity, synaptophysin expression, region-specific process outgrowth, and evidence of synaptic vesicle formation, suggesting that the grafts may contain functional neurons (6, 7, 16).

Purified NT2N neurons have been used to study the ability of neuronal CNS grafts to restore function in animal models of neurodegenerative disease and trauma (17–21). Particularly promising results were obtained in a model of ischemia (22). Based on this work and other preclinical studies, a phase I human clinical trial was initiated in which 12 patients with basal ganglia stroke were

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transplanted with NT2N neurons. Evidence for survival of the grafts has been obtained by imaging techniques at 1 year post-transplant (23) and in 1 postmortem case at 27 months post-transplant (15). Some of the patients showed improvement of motor function outcome scores (14), suggesting the possibility that the transplant may mediate this effect, but this was a phase I safety trial with too few patients to establish efficacy. However, all of the rodent and human studies to date have relied on the endogenous properties of the NT2N neurons for any therapeutic effect.

The combination of these factors—the ability to specify the neuronal fate of these cells before transplantation, the fact that grafts of NT2N neurons develop markers of mature CNS neurons in vivo, and the fact that the grafts are stable and nontumorigenic—strongly suggests that NT2N neurons could represent an excellent platform for therapeutic gene transfer into the CNS. However, previous attempts to genetically modify NT2 cells by transfection or viral vector transduction (2, 24–26) have met with only limited success, i.e. low efficiency of gene transfer, low levels of expression or only transient expression. Consequently, no attempts to transplant genetically modified NT2N neurons have been reported.

In this study we demonstrate efficient, stable, and nontoxic transduction of undifferentiated NT2 cells, using a self-inactivating lentiviral vector encoding the *eGFP* reporter gene (27). A constitutively active eukaryotic promoter (human elongation factor 1-α) resulted in stable *eGFP* reporter gene expression both in undifferentiated NT2 cells and also after the transduced cells were differentiated into NT2N neurons in vitro. Furthermore, the genetically modified neurons continued to express the reporter gene at long post-transplantation time points in the adult nude mouse brain.

Neural transplantation is one potential strategy for restoring cognitive and motor function following traumatic brain injury (TBI). Potential therapeutic effects of neural grafts may result from either functional replacement of dying neurons, or from support of endogenous injured cells by trophic factors secreted from the neural graft, or both. To distinguish between these mechanisms, we used a lentiviral vector to introduce the coding sequence for nerve growth factor (NGF) into NT2N neurons. Previous work from our lab and others has indicated that administration of NGF may attenuate cell death and improve behavioural dysfunction when administered 24 hours (h) following brain injury in rats. However, the severe side effects of neurotrophin infusion have led us to investigate methods for regional delivery of NGF. We hypothesized that using NT2N neurons to deliver the growth factor locally would provide an advantage for long-term neurotrophin delivery into selectively vulnerable regions of the injured brain.

In this report, transduced or untransduced neurons were engrafted into the injured CNS of mice that had been subjected to experimental TBI. Transplanted and control mice were tested for their ability to learn in the Morris Water Maze. We found that transduced NT2N neurons expressing NGF significantly improved learning in brain-injured mice at 1 month post-injury.

These results show that genetically modified NT2N neurons represent a practical new method for ex vivo gene delivery in the CNS. Homogeneous populations of these genetically modified human neurons should be of great utility for introducing therapeutic genes into the adult CNS.

MATERIALS AND METHODS

Virus Production

HEK 293T cells were plated on poly-D-lysine-coated 10-cm plates. One day later, the cells were transfected with 5 μg of pMD.G (encoding the VSV-G envelope glycoprotein [28]), 15 μg of pCMVΔR8.2 (encoding viral structural, enzyme, and accessory genes [28]) and 20 μg of the transfer plasmid (SIN-EF-nLacZ, gift of Dr. Lung-Ji Chang, University of Florida [27]), using the CalPhos Transfection Kit (Clontech, Palo Alto, CA). The SIN-EF-NGF-IRES-eGFP transfer plasmid was constructed by ligating the following fragments into pTYlinker (gift of L. Chang): 1) the human EF1α promoter (1.5 kb *Not*I fragment from pTY-EF-eGFP, gift of L. Chang); 2) the human *NGF*-β coding sequence from plasmids N8D8 and N8B9 (ATCC), amplified with primers 5′-TCAATGAGCTAGCATGTCCAT GTTGT-3′ and 5′-GATCAAGCTAGCTCAGGCTCTTCT-3′ to introduce *Nhe*I restriction sites; 3) the *XhoI-Xba*I fragment of pIRES (Clontech) containing the 638 bp internal ribosome entry site (IRES); and 4) the *Xba*I-*Eco*RI fragment encoding the 787 bp humanized *eGFP* coding sequence from pTY-EF-eGFP.

After transfection the cells were rinsed and the medium was replaced with OptiMem Reduced Serum Medium (Gibco BRL, Gaithersburg MD) 12 to 16 h later. Viral supernatants were collected every 24 h for 3 to 5 days, centrifuged at 1,000 rpm for 5 min at 4°C, and passed through a 0.45 μm filter to remove any remaining producer cells. Unconcentrated vector stocks were used for transduction and titering immediately after collection. Titering of marker vectors was performed on human 293T cells in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO) and titers were determined by counting the number of eGFP-positive cells 48 h later, or by reacting the cells with X-gal according to standard methods 48 h after transduction. No polybrene was used for transduction of undifferentiated NT2 cells.

Maintenance and Differentiation of NT2 Cells

Undifferentiated NT2 cells were maintained in growth medium (OptiMem with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate) and passaged twice weekly at a ratio of 1:4. For transplantation, NT2 cells were differentiated into NT2N neurons as described (29). Briefly, 2.3×10^6 cells were exposed to 10 μM retinoic acid (Sigma) for 5 weeks in growth medium, then the cells were changed to DMEM with high glucose, 5% serum, and mitotic inhibitors

(10 μM uridine, 10 μM 5-fluoro-2′deoxyuridine, 1 μM cytosine arabinoside, all from Sigma) for 10 to 13 days. NT2N neurons are purified from remaining undifferentiated epithelial-like cells by serial replating and are collected for transplantation at the replate III stage when they are approximately 95% pure (29). For in vitro differentiation experiments, NT2 cells were plated on a monolayer of rat astrocytes and cultured for up to 4 weeks.

Immunochemistry

Cells on coverslips were fixed in fresh 4% paraformaldehyde (pH 7.4) for 10 min, rinsed, permeabilized with 0.2% Triton X-100 for 10 min, rinsed, blocked in 5% donkey serum in PBS for 1 h, then exposed to the primary antibody 3 h at room temperature (RT) or overnight at 4°C. For immunofluorescence analysis, frozen sections were hydrated in 0.1 M Tris pH 7.4 for 5 min at RT and blocked in 0.1 M Tris with 2% IgG-free donor horse serum (DHS) for 5 min at RT. Sections were exposed to primary antibody overnight at 4°C. After a 5-min 0.1 M Tris wash, and a 5-min blocking step in 2% DHS, secondary antibody was applied for 1 h at RT. Sections were then washed in 0.1 M Tris at RT with gentle agitation twice for 20 min each. Primary antibodies were rabbit anti-eGFP (IgG fraction 1:200, Molecular Probes, Eugene, OR) and rat anti-human neurofilament (HO14, used neat; [4]). Secondary antibodies for immunofluorescence were Texas Red-coupled donkey anti-rat (1:400) and FITC-coupled donkey anti-rabbit (1:100) purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Slides were coverslipped in Vectashield fluorescence mounting media containing DAPI (Vector Laboratories, Burlingame, CA). For immunohistochemistry, sections were deparaffinized in xylene (2 × 5 min), hydrated through graded alcohols, incubated in methanol with 5% H_2O_2 to block endogenous peroxidase activity, washed in running H_2O (10 min) and 0.1 M Tris pH 7.6 (5 min), blocked in 2% DHS (5 min), incubated with undiluted HO14 primary antibody overnight at 4°C. Sections were rinsed in 0.1 M Tris (5 min), blocked in 2% DHS (5 min), and incubated with the secondary antibody (biotinylated anti-rat IgG, 1:1,000, Vector Labs) for 1 h at RT. Sections were washed and blocked as above and incubated with ABC reagent (1 h at RT, Vector Labs), washed and developed with diaminobenzidine solution, rinsed and counterstained with Harris' hematoxylin. Digital photomicrographs were acquired with a Zeiss Axiophot microscope, a Toshiba 3CCD camera, and ImagePro software.

Transplants in Nude Mice

Surgeries were performed as described (30). Ten to 20,000 NT2N neurons in 1 μl culture medium were transplanted into the striatum of adult female nude mice using stereotaxic coordinates (DV: 2.5 mm, ML: 2 mm, AP: on bregma) (31). Transplants were bilateral, with untransduced neurons on the right and transduced neurons on the left. Mice were killed at 1 week, 1 month, or 22 weeks post-transplant. Mice were deeply anesthetized with ketamine and xylazine and perfused with PBS followed by 4% paraformaldehyde (pH 7.4) for paraffin embedding, or perfused with PBS and brains were immersion-fixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose, and frozen for cryosectioning. Brains were unilaterally notched before embedding to ensure that right and left sides were identifiable. To obtain control sections for anti-eGFP immunohistochemistry and in situ hybridization, mice were injected (directly transduced) with a CMV-eGFP encoding lentiviral vector. For all injections, a 30-gauge blunt or beveled needle was attached to a 10-μl Hamilton syringe. Injections were performed at 1 μl/min.

In Situ Hybridization

For detection of *eGFP* mRNA or human *NGF* mRNA, the coding sequences were cloned into pBluescript II KS+ (Stratagene, La Jolla, CA) to generate antisense and control sense *eGFP* riboprobes (731 bp each) or 841-bp *NGF* antisense and 760-bp control sense *NGF* riboprobes. Nonradioactive in situ hybridization histochemistry was performed as described (32), except that paraffin-embedded tissue sections were first deparaffinized in 100% xylene (3 × 5 min) and 100% ethanol (1 × 1 min).

Assays for NGF Concentration and Bioactivity

PC12 survival and neurite regeneration assays were performed as described (33). Plates were coated with rat tail collagen (Collaborative Biomedical Products, Bedford, MA). Conditioned medium was obtained by incubating serum-free RPMI with 293T cells that had been transfected with the lentiviral transfer plasmid or control untransfected cells. For survival studies, naive PC12 cells were washed 5 times to remove serum and plated in serum-free RPMI medium alone or containing either 50 ng/ml recombinant human β-NGF (Chemicon, Temecula, CA) or 50% conditioned medium. For neurite regeneration assays, PC12 cells were primed by culturing in serum-containing medium with NGF for 1 week, then shearing off the processes by trituration and replating in serum-free medium alone or supplemented with 50% conditioned medium from NGF-transfected or untransfected cells. Cells were examined at 72 h after treatment. The amount NGF secreted from transduced NT2 cells was determined with the NGF Emax ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions. Conditioned media was diluted until the absorbance readings were in the linear range of the assay.

Controlled Cortical Impact (CCI) Brain Injury

All the animal procedures were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Veteran's Administration Medical Center of Philadelphia. A total of 32 adult male C57Bl/6 mice (19–24 g) were used for this study. Mice were anesthetized with intraperitoneal administration of sodium pentobarbital (65 mg/kg, Nembutal Sodium Solution, Abbott Laboratories, North Chicago, IL), and placed in a stereotactic frame. As described by our laboratory (34), a midline scalp incision was made to expose the skull and then, by trephination, a 5-mm rounded bone flap was removed from the center of the left parietal bone without damaging the underlying dura mater. Forty-five minutes after induction of the anesthesia the mice were subjected to controlled cortical impact (CCI) brain injury. Sham-injured control animals received the same anesthesia and the same surgical procedure (craniectomy), but were not subjected to brain injury.

This model of injury uses a 3-mm impounder driven by a pneumatic piston, rigidly mounted at an angle of 20° from the vertical plane and applied perpendicularly to the exposed dura mater over the left parieto-temporal cortex between bregma and lambda, at a velocity of 4.8 to 5.0 m/s and a 1-mm depth of deformation (35). After the procedure the craniotomy was covered with a cranioplasty and the scalp sutured. During surgery and recovery the mice were placed over a heating pad and maintained at 37°C.

Transplantation into Brain-Injured Mice

All cells for these experiments were grown, differentiated, harvested, and frozen in aliquots (in 95% serum + 5% DMSO) at the same time in order to create a uniform population for later transplantation. For transplantation, individual aliquots of NGF-expressing and control-untransduced NT2N neurons were thawed at 35°C, added to 5 ml DMEM to dilute the DMSO, collected by centrifugation, washed, and resuspended in serum-free DMEM. One μl of the cell suspension was added to 9 μl of trypan blue for cell counting and assessment of viability (range, 71.2%–80.8%). Yields were typically 15,000 to 20,000 live cells in 2 μl. Cells were kept on ice during the injection procedure for a total of less than 1.5 h. Separate aliquots of serum-free DMEM were used for the vehicle controls.

Twenty-four hours after CCI brain injury, all animals were subjected to cell transplantation or injection of the same medium in which the NT2N neurons were resuspended (vehicle controls). Animals were divided into 4 groups: 1) sham-injured animals (surgery without injury) injected with medium; 2) CCI-injured animals injected with medium; 3) CCI-injured mice engrafted with untransduced NT2N neurons; or 4) CCI-injured mice engrafted with NGF-transduced NT2N neurons. A randomized set of animals from each of the 4 experimental groups were injected on a given day and the vials of cells and media were coded so that the surgeon (L.L.) was blinded to the treatment status of each animal. The injection was performed at the following stereotaxic coordinates: anterior-posterior (AP), bregma + 0.7 mm; medial-lateral (ML), 0.2 mm over the injured hemisphere; dorso-ventral (DV), 3.7 mm from the skull (31) with a 10-μl Hamilton syringe fitted with a 30-gauge beveled needle. These coordinates target the medial septal nucleus, which is located ventral and rostral to the cortical injury cavity and the hippocampus and contains the cell bodies of cholinergic septo-hippocampal projection neurons that are normally sustained by retrograde NGF trophic support from the hippocampus (Fig. 4). Each animal was injected with 2 microliters of cell suspension or medium at 1 μl/min using a microprocessor-controlled pump (UltraMicroPump II, World Precision Instruments, Inc, Sarasota, FL). After the injection the syringe was left in place for 5 min and then gently retracted to avoid a negative pressure-driven dispersion of the cells or vehicle upward along the needle tract. This paradigm has been used successfully in our laboratory previously for transplantation of NT2N cells (18, 19). At the end of the procedure the scalp was sutured and mice were allowed to recover over a heating pad maintained at 37°C degrees. To prevent rejection of the human NT2N cells, all animals, regardless of treatment status, received daily intraperitoneal injections of cyclosporin A (CsA, 10 mg/kg) beginning at 1 h post-injury for the duration of the study.

Behavioral Analysis

Evaluation of cognitive function was performed using the Morris Water Maze. Our Morris Water Maze is a white circular pool (1-meter diameter) filled with water (18°C–20°C) that is made opaque by adding nontoxic water-soluble white paint. The task requires that the animals learn to locate a submerged platform placed 0.5 cm under the surface of the water using external visual cues after being randomly placed at 1 of 4 sites in the pool. Latencies to reach and climb onto the platform were recorded for each trial, with a maximal allowed time of 60 seconds. Mice were evaluated for post-injury learning at 4 weeks post-transplantation with 8 trials/day for 3 days. At this time point, CCI-injured mice show significant learning deficits when compared with sham-injured animals (unpublished data). Injured and sham-injured mice do not differ in their swim speeds or paths in this model of TBI (35). Since this experiment was designed to test learning (acquisition) and not working memory, analysis of path length and duration of time spent in each quadrant were not measured in this study.

Statistical Analysis

Learning latencies are presented as mean ± standard error of the mean. The comparison between groups was performed using a multivariate ANOVA followed by a Tukey-Kramer multiple comparison test. A probability value less than 0.05 is considered statistically significant.

RESULTS

Stable Transduction of NT2 Cells by a Lentiviral Vector

Undifferentiated NT2 cells were exposed to an HIV-based, self-inactivating (SIN) lentiviral vector pseudotyped with the vesicular stomatitis virus surface glycoprotein (VSV-G). The human elongation factor 1α promoter (EF1α) was used to express enhanced GFP (SIN-EF-eGFP) or NGF (Fig. 1A). Strong eGFP fluorescence was observed in the cells exposed to the SIN-EF-eGFP lentiviral vector (Fig. 1B), indicating highly efficient transduction.

Generation of Transduced NT2N Neurons In Vitro

To determine if eGFP expression driven by the EF1α promoter was maintained in differentiated NT2N neurons, transduced or untransduced NT2 cells were differentiated in vitro on an astrocyte monolayer. This method allows differentiation to be followed in vitro over time, although the neurons cannot be harvested for transplantation. After 7 days of coculture, the transduced NT2 cells extended processes that were eGFP-immunoreactive and also immunoreactive with a human-specific anti-neurofilament antibody, consistent with nascent NT2N neurons (Fig. 2A–D). In the transduced neurons, eGFP-immunoreactivity was detected in the cell bodies and throughout the processes (Fig. 2B, C, E, F). Control untransduced cells (Fig. 2G) extended processes that reacted with the human neurofilament antibody (Fig. 2H) but were not

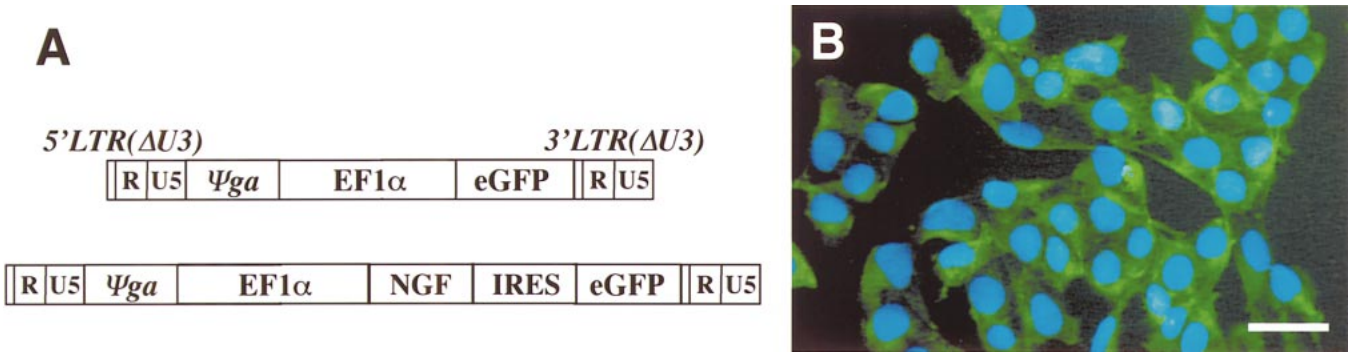

Fig. 1. Transduction of undifferentiated NT2 cells. **A:** Schematic representation of the self-inactivating (SIN) transfer vectors used in this study. Upper: SIN-EF-eGFP, Lower: SIN-EF-NGF-IRES-eGFP. **B:** Expression of eGFP (green) in cells transduced with SIN-EF-eGFP. Nuclei were stained with DAPI (blue). Scale bar, 20 μm.

eGFP-positive. These results indicate that the EF1α promoter remained active after the cells were differentiated into neurons and can be used to drive constitutive expression of foreign genes in NT2N neurons in vitro.

Transduced neurons were followed over time to determine the duration of eGFP expression in vitro. After 2 or 4 weeks of differentiation, the NT2N neurons were stained with an anti-human neurofilament antibody (huNF-M, HO14) and an anti-eGFP antibody (Fig. 2I–K). After 4 weeks in culture, HO-14-positive processes were substantially longer than at 1 week (compare Fig. 2I to 2C) and the morphology of transduced neurons was more complex, often with multiple branched processes (Fig. 2I–K, insets). We compared the percentages of eGFP-expressing cells before and after differentiation to examine the possibility of down-regulation of eGFP expression in the neurons. In the starting population of transduced undifferentiated NT2 cells, 29.1 ± 3.2 expressed eGFP (mean ± SEM, n = 735 cells counted). After differentiation, the percentage of eGFP expressing cells (as a percentage of HO-14-positive neurons) was 18.2 ± 4.0 at 1 week (n = 183), 21.3 ± 3.5% at 2 weeks (n = 177) and 18.8 ± 3.5% at 4 weeks (n = 151). These values are not significantly different from each other (1-way ANOVA, p = 0.1858). These data indicate that eGFP expression was not toxic to the neurons, was stable for up to 4 weeks in vitro and did not interfere with neuronal differentiation by this method. Furthermore, no colocalization of eGFP immunoreactivity was observed with the astrocyte marker GFAP (Fig. 2L–N), indicating minimal transfer of eGFP protein from the neurons to the underlying astrocytes, no production of infectious virus by the transduced neurons, and no differentiation of the transduced NT2 cells into astrocytes.

Transduced NT2N Cells Survive and Mediate Marker Gene Expression in the Adult CNS

To determine whether the gene-modified NT2N neurons survive and integrate into the CNS architecture in the same way as unmodified NT2N neurons, and whether they maintain expression of the transferred gene, untransduced or SIN-EF-eGFP-transduced NT2 cells were differentiated in vitro into NT2N neurons as described (29) and stereotactically injected into the striatum of adult nude mice. For these experiments, an unselected (nonclonal) population of transduced cells was used to balance individual differences in expression and neurogenic potential and for a better comparison to unselected untransduced cells. We used a highly transduced population of NT2 cells (76% eGFP+, n = 348 cells counted) to generate neurons for transplantation to maximize our ability to detect the transduced neurons in vivo. Neurons were generated for transplantation using retinoic acid treatment as described (29). Transplants were bilateral, with untransduced neurons on the right and transduced neurons on the left. Groups of 3 mice were killed at 1, 4, or 22 weeks post-transplant.

Expression of eGFP by NT2N neurons was analyzed in grafts in paraffin-embedded brains at 1 week and 1 month post-transplantion by in situ hybridization. While both transduced and untransduced grafts were human neurofilament-immunoreactive (Fig. 3A, C), only the transduced grafts hybridized with the *eGFP* antisense riboprobe (Fig. 3B, D). Neither the transduced nor the untransduced grafts hybridized with the control *eGFP* sense riboprobe (data not shown). To examine long-term eGFP expression in vivo, brains harvested at 22 weeks post-transplantation were frozen and cryosections were examined for eGFP and neurofilament expression. Grafts contained within the striatum were consolidated and showed no evidence of migration beyond the implantation site. No expansion of graft size was observed, thus there was no evidence of tumor formation.

Green fluorescence from eGFP expression in transduced grafts of the NT2N neurons was observable after ethanol fixation and direct examination by fluorescence microscopy of frozen sections (data not shown). To colocalize cellular eGFP expression in the graft with huNF-M, sections were reacted with both an anti-eGFP

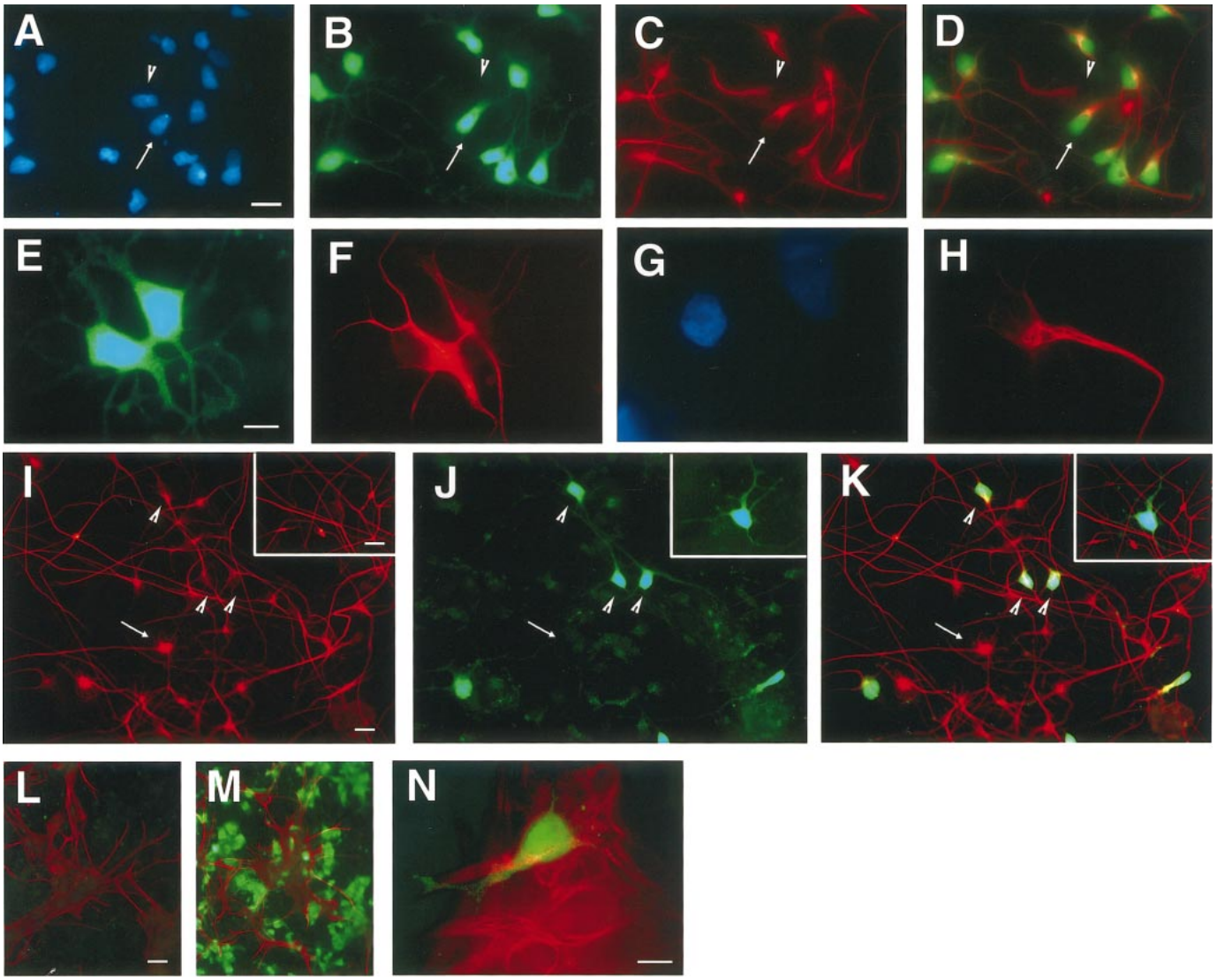

Fig. 2. Reporter gene expression was maintained after neuronal differentiation in vitro. NT2N neurons were generated by culturing a population of transduced undifferentiated NT2 cells on a monolayer of rat astrocytes and analyzed by immunofluorescence. **A–D:** The arrowhead marks an untransduced NT2N neuron; the arrow marks an eGFP-expressing NT2N neuron. **A:** All cellular nuclei in a field of transduced and untransduced NT2N neurons and astrocytes were marked by DAPI (blue). **B:** Transduced neurons expressed eGFP in the cell bodies and processes of NT2N neurons (green). **C:** Processes of both transduced and untransduced neurons were immunoreactive with anti-neurofilament antibody (HO14, red). **D:** Colocalization of neurofilament immunoreactivity and eGFP-immunoreactivity in a transduced neuron. Scale bar in A applies to panels A–D, 20 μm. **E–F:** High-power photomicrograph of 2 transduced neurons expressing eGFP (anti-eGFP, in green, panel **E**) and huNF-M (HO14, in red, panel **F**). **G–H:** High-power photomicrograph of a control untransduced neuron. The nucleus was stained with DAPI (blue, panel **G**) and the cell body and processes were immunoreactive with HO14 (red, panel H) but eGFP was not expressed. **H:** Image was created by merging the image from the rhodamine filter set (HO14) with the image from the FITC filter set, which in this case showed no positive eGFP staining. Scale bar in E applies to panels **E–H,** 10 μm. **I–K:** Cocultures of NT2N neurons on astrocyte monolayers 4 weeks after plating. **I:** Coculture stained with HO-14 (red) showed extensive process outgrowth (compare to panel C at 1 week after plating). **J:** Coculture stained with anti-eGFP showed eGFP throughout the cell bodies and apparent bipolar processes (green). **K:** Merged image of panels I and J showed colocalization of the neurofilament and eGFP immunoreactivity in the cell body and processes. **I–K, insets:** A highly branched transduced neuron at 4 weeks after plating. Scale bars in panel I and its inset apply to panels I–K and insets, 20 μm. **L–N:** Cocultures of NT2N neurons on astrocyte monolayers stained with anti-GFAP (red) and anti-eGFP (green). **L:** Coculture of untransduced neurons with GFAP-immunoreactive astrocytes (red). **M:** Coculture of transduced neurons expressing eGFP (green) with GFAP-immunoreactive astrocytes (red). Scale bar in L applies to panels L and M, 40 μm. **N:** Higher power photomicrograph of a transduced neuron expressing eGFP (green) on a monolayer of astrocytes (GFAP, red). No colocalization of eGFP with GFAP in astrocytes was observed. Scale bar in N, 10 μm.

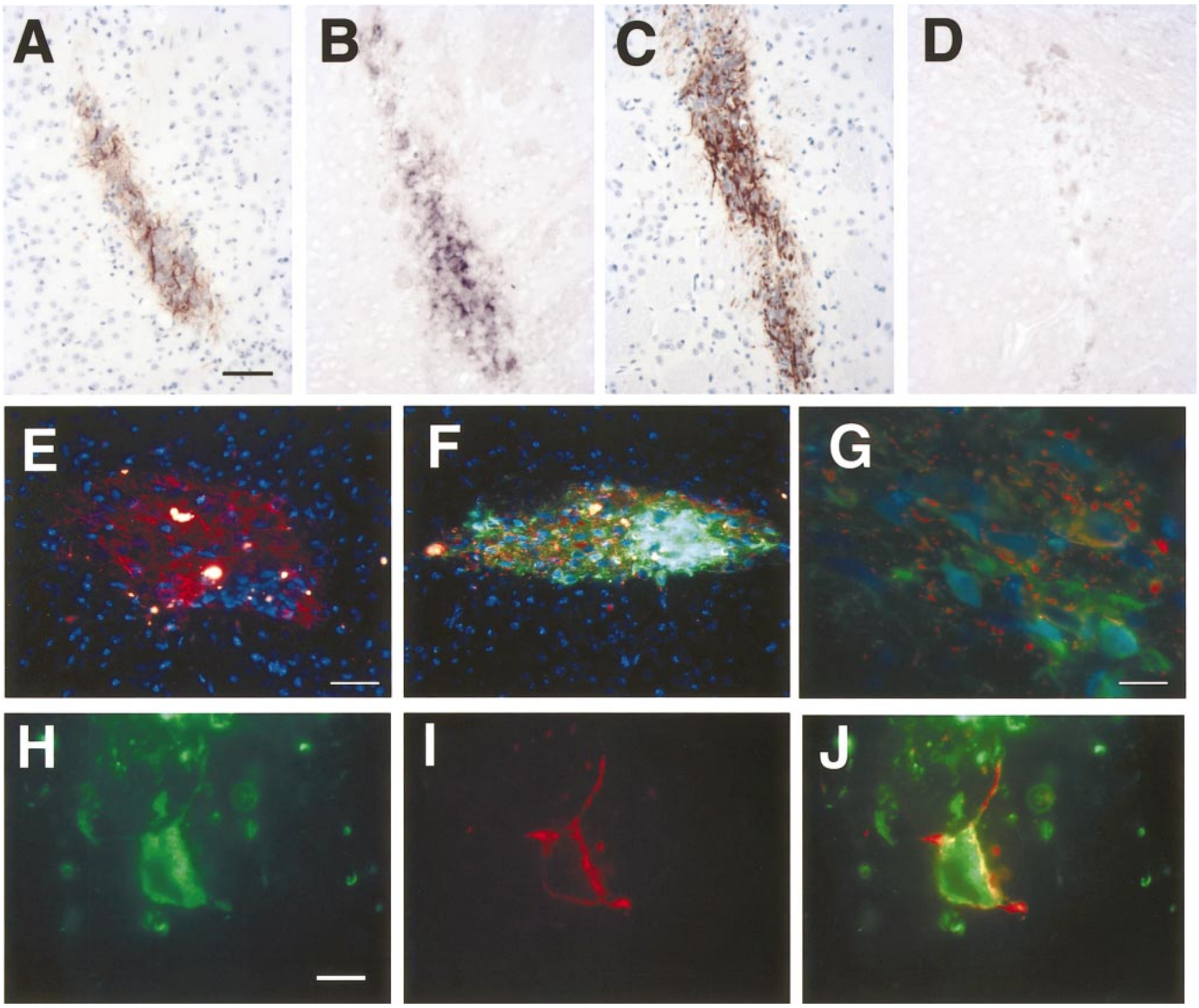

Fig. 3. eGFP expression in transduced grafts in vivo. **A–D:** Immunohistochemistry and in situ hybridization of NT2N neuronal grafts at 1 week post-transplant. Transduced NT2N neuronal grafts were detected with both anti-neurofilament antibody HO14 (**A**) and an *eGFP* antisense riboprobe (**B**), whereas the untransduced NT2N neuronal grafts were detected with HO14 antibody (**C**) but not the *eGFP* antisense riboprobe (**D**). **A, C:** Nuclei were counterstained with hematoxylin (blue). Scale bar in A applies to panels A–D, 50 μm. **E–G:** Immunofluorescent detection of eGFP and huNF-M in NT2N neuronal grafts at 22 weeks post-transplant. A bright autofluorescent red and green punctate pattern was present along the needle tracts in both transduced and untransduced grafts, probably representing residual blood/hemosiderin from the injection. The merged red and green autofluorescent signal appears as yellow in panels E and F and does not colocalize with cellular eGFP staining. **E:** An untransduced graft expressed huNF-M (HO14, red) but not green fluorescent protein. **F:** Transduced grafts expressed human neurofilament (HO14, red) and the transferred reporter gene, eGFP (anti-eGFP, green). Scale bar in E and F, 50 μm. **G:** The majority of the huNF-M-positive (red) NT2N neurons in the center of a transduced graft continued to express eGFP (green). **E–G:** Nuclei were counterstained with DAPI (blue). Scale bar in G, 25 μm. **H–J:** High-power photomicrograph of a transduced NT2N neuron in vivo expressing eGFP (green, panel H) and huNF-M (red, panel **I**). In the merged image (panel **J**), staining was colocalized in the cell body and in the processes (yellow). Scale bar in H applies to panels H–J, 10 μm.

antibody and an anti-neurofilament antibody (HO14). Untransduced grafts were not eGFP-positive but did show neurofilament expression surrounding a cluster of nuclei that indicated the presence of NT2N neurons at the graft site (Fig. 3E). In the transduced grafts, eGFP-immunoreactive cell bodies and huNF-M-immunoreactive filaments were both confined to the region of the graft (Fig. 3F). We examined neurofilament-positive NT2N neurons

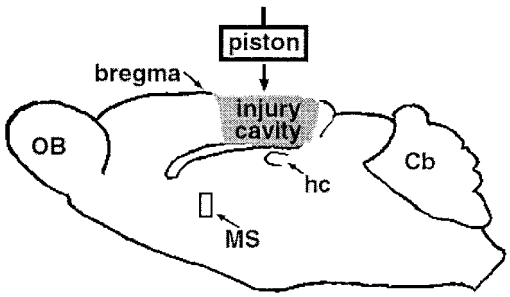

Fig. 4. Schematic representation of a sagittal section of the mouse brain at approximately 0.25 mm lateral to the midline. The location of the cortical injury cavity is shown in grey and the target area for the transplant (medial septum) is marked with a box. The maximal extent of the injury cavity is shown in this schematic, however the true center of the injury cavity is located approximately 2 mm lateral to this section, overlying the hippocampus. Abbreviations: MS, medial septum; OB, olfactory bulb; Cb, cerebellum; hc, hippocampus.

in the center of the striatal grafts to determine whether down-regulation of eGFP expression had occurred. In a majority of the cells, eGFP expression was visible (Fig. 3G), corresponding to the approximately 75% of cells that were eGFP-positive prior to injection. Isolated cells near the edge of the transduced NT2N grafts showed expression of eGFP immunoreactivity in the cell body and the processes of these transplanted neurons (Fig. 3H) with huNF-M in the cytoskeleton and the processes (Fig. 3I) and clear colocalization of the 2 markers of transduced neurons in vivo (Fig. 3J). Transduced and untransduced grafts were approximately the same size at 1 week and 5.5 months post-transplantation (compare Fig. 3A and C to 3E and F), and there was no evidence of necrosis near the grafts.

Therapeutic Efficacy of NGF-Expressing NT2N Neurons in Brain-Injured Mice

To test whether transduced NT2N neurons can contribute to functional recovery after an injury, we transplanted NGF-expressing or control NT2N neurons into the CNS of brain-injured mice (Fig. 4).

To express NGF in NT2N neurons, we constructed a lentiviral transfer plasmid encoding human *NGF*-β driven by the same EF1-α promoter (Fig. 1A). The bioactivity of NGF encoded by this construct was tested using a PC12 survival assay (Fig. 5A–C) and a PC12 neurite regeneration assay (Fig. 5D–F). Withdrawal of serum from the culture medium of PC12 cells caused >90% cell death after 3 days (Fig. 5A) that was rescued by addition of conditioned media from 293T cells transfected with the NGF transfer plasmid (Fig. 5B), or by recombinant NGF (Fig. 5C). Recombinant NGF also promoted neurite outgrowth in primed PC12 cells (Fig. 5D), as previously described (33). The same effect was obtained with conditioned medium from 293T cells transfected with the NGF transfer plasmid (Fig. 5E) but not with control media from untransfected cultures (Fig. 5F), as expected.

The NGF-encoding lentiviral vector was used to transduce cultures of NT2 cells. To determine the percentage of cells that had been transduced, NGF-transduced and control untransduced cultures were screened for *hNGF* mRNA by in situ hybridization. Similar to the transduction efficiency with the eGFP lentiviral vector, the NGF lentiviral vector transduced approximately 80% of the NT2 cells, as shown by their hybridization with the *NGF* antisense riboprobe (Fig. 5G). Control untransduced NT2 cells did not react with the *NGF* antisense riboprobe (Fig. 5H). Both transduced and untransduced NT2 cells did not hybridize with the control *NGF* sense riboprobe (Fig. 5I, J), indicating that the reaction was specific for *NGF* mRNA expressed from the vector genome in the transduced cells.

The amount of NGF secreted by transduced and untransduced NT2 cells was measured using an NGF-specific ELISA (Fig. 5K). NGF-transduced cells secreted 7669 ± 913.9 pg NGF/day/10^5 cells. Untransduced NT2 cells secreted 14.2 ± 0.72 ng/day/10^5 cells, or 540-fold less NGF than the transduced NT2 cells.

Adult mice (n = 32) were subjected to brain injury using a CCI device (35), which produces immediate damage to the cortex and delayed cell death in the underlying hippocampus. This results in the loss of cholinergic neurons of the medial septum, which are dependent on the hippocampus for NGF trophic support. Because the septo-hippocampal pathway is important for spatial learning, we assessed performance of the CCI-injured mice using the Morris Water Maze test of visuo-spatial learning at 1 month post-injury.

Brain-injured mice or sham-injured (surgery without injury) mice were subjected to cell transplantation or injection of medium at 24 h after the injury. In brain-injured mice, NGF-transduced or untransduced NT2N neurons were transplanted into the medial septum ipsilateral to the injury. As controls, sham-injured and brain-injured animals were injected with an equal volume of the same medium (serum-free DMEM) that was used to resuspend the neurons. At 1 month post-transplant, groups of 8 animals were evaluated for their ability to learn the position of a hidden platform in the Morris Water Maze (Fig. 5L). The test consisted of 24 trials (8 trials/day × 3 consecutive days), and the time for each mouse to reach the platform (latency) was measured. It has been demonstrated that swim speeds and patterns do not differ between injured and uninjured mice in the CCI model (35). Brain-injured animals had a marked cognitive deficit compared to the uninjured animals, indicating a significant injury effect at 1 month post-injury. Brain-injured animals that had received transplants of NGF-expressing NT2N neurons into the medial septum performed significantly better than brain-injured animals that had received either a

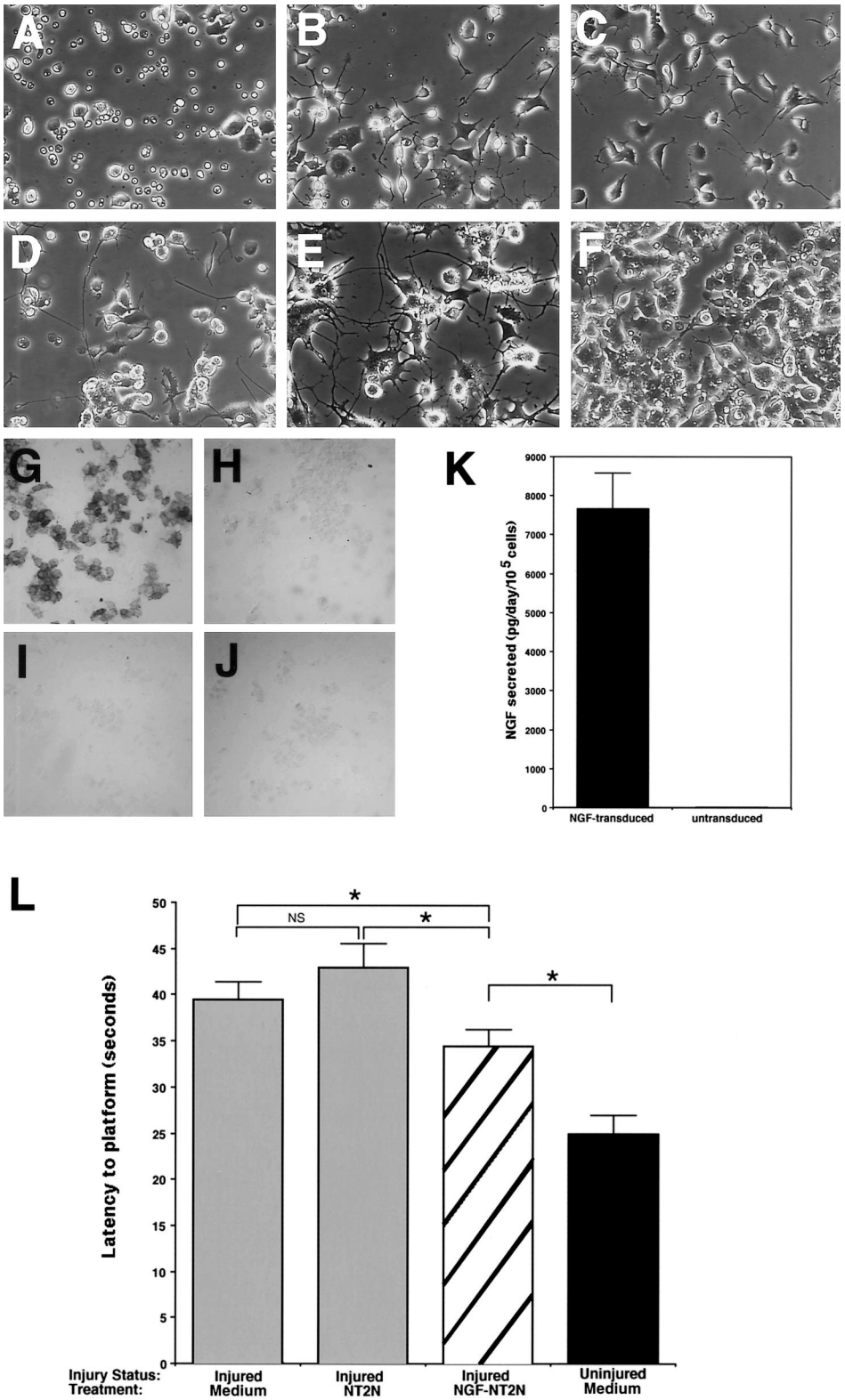

Fig. 5. Characterization of NGF-expressing NT2 cells and neurobehavioral improvement after NGF-NT2N transplantation into brain-injured mice. **A–C:** PC12 survival assay for NGF bioactivity. Serum withdrawal results in the death of PC12 cells after 72 hours (**A**). Cells survived in serum-free medium supplemented with either 50 ng/ml recombinant human NGF in the positive control (**B**), or 50% conditioned medium from 293T cells transfected with the lentiviral NGF transfer plasmid (**C**). **D–F:** PC12 neurite regeneration assay for NGF bioactivity. PC12 cells extended processes after plating in serum-free medium plus either 50 ng/ml recombinant human NGF (**D**) or 50% conditioned medium from NGF-transfected cells (**E**), but not 50% conditioned medium from control untransfected cells (**F**). **G–J:** In situ hybridization histochemistry to detect *NGF* mRNA in transduced

transplant of untransduced NT2N neurons ($p < 0.05$) or an injection of media ($p < 0.05$), indicating that the NGF-expressing cells improved learning and that a neuronal transplant by itself did not have a therapeutic effect.

DISCUSSION

We show that human neuronal cells that were engineered to express a transferred gene can engraft into the host CNS architecture, extend processes in a regionally appropriate manner, and survive and express the transferred gene for at least 6 months in vivo. Furthermore, NT2N neurons genetically engineered to express NGF were able to improve cognitive function when transplanted into brain-injured mice. These data indicate that human NT2N neurons represent a practical and effective platform for ex vivo gene therapy in the CNS.

NT2 cells have particular experimental advantages due to their origin as a clonal cell line that can be easily propagated in culture. Transduction with a pseudotyped lentiviral vector was stable, efficient (obviating the need for a selectable marker), and nontoxic. The ability to achieve stable gene expression in NT2 cells and NT2N neurons in this study is primarily due to 3 factors: 1) The eukaryotic promoter we chose (EF1α) is constitutively active, in contrast to strong viral promoters which can be down-regulated. In fact, the commonly used cytomegalovirus immediate-early promoter is actively repressed in NT2 cells (data not shown and [36]); 2) We used an integrating lentiviral vector that results in the transmission of the vector genome to daughter cells; and 3) Lentiviral vectors are very efficient and nontoxic, in contrast to the toxicity of adenovirus and SFV (V.M-Y.L, unpublished data), poor infection of NT2 cells and NT2N neurons by wild-type herpes simplex virus type I (HSV-1) (37), lysis of NT2 cells by a neuroattenuated strain of HSV-1 (38), and the inefficiency of AAV [25]). Although recombinant vaccinia virus (VV) has been successfully used with differentiated NT2N neurons without cytotoxicity, VV infection of the NT2 precursor cells was cytotoxic (24). Furthermore, VV is a nonintegrating virus.

We also found that multiple coding sequences can be introduced by using multiple vectors (data not shown). Another alternative is the use of bicistronic or tricistronic vectors that include regulatable or constitutively active promoters. Individual cell clones can be isolated, expanded, and characterized for the levels of expression of the introduced gene, the number of neurons generated upon exposure to retinoic acid, and the sequence of the genomic integration site.

Earlier studies showed that cultured NT2N neurons express markers of immature CNS neurons, including neurofilament proteins and MAP2 (4), APP695 (39), N- and L-type calcium channels (40), GABA receptors (41), and acetylcholinesterase (42, 43). Cultured NT2N neurons form functional synapses in vitro and use both glutamatergic and GABAergic neurotransmission (44, 45). When differentiated NT2N neurons are transplanted and allowed to survive for more than 4 months in vivo in the CNS of immunodeficient mice, maturation occurs with the expression of highly phosphorylated NF-H and the adult form of the microtubule-associated protein, tau (7). Transplantation of NT2N neurons in or near white matter tracts provides a permissive environment for axonal outgrowth (7, 16). Genetically modified NT2N neurons should allow molecular characterization of proteins that facilitate guidance, axonal outgrowth, and fasciculation in vitro and in vivo.

Long-term survival of marked neural grafts in vivo allows the opportunity to investigate several other important questions in CNS transplant biology. The ability to identify grafted neurons in live slice cultures will permit electrophysiological characterization of synaptic activity (if any) in both immature and mature (>4 month) transplanted neurons. Synaptic connections between the transplanted neurons and between the host and the graft can be investigated. Also, the possibility for the graft to become an abnormally active epileptogenic focus can be studied. Marked neurons can also be used to determine whether guidance cues in the injured brain recruit transplanted neurons to the damaged tissue. Finally, down-regulation of expression of genes transferred by viral vectors has been a long-standing concern in the field of gene therapy (46). Genetically marked NT2N neurons, which

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NT2 cells. The antisense *NGF* riboprobe hybridized with ~80% of the transduced NT2 cells (**G**) but not control untransduced NT2 cells (**H**). A control sense riboprobe did not hybridize to either *NGF*-transduced (**I**) or untransduced (**J**) NT2 cells. **K:** Quantitation of the amount of NGF secreted by transduced and untransduced NT2 cells. Bar represents standard error of the mean ($n = 3$). **L:** Latencies to reach the submerged platform in the Morris Water Maze over 24 trials of testing at 1 month post-injury for brain-injured animals injected with media or untransduced NT2N neurons (grey bars), brain-injured animals injected with NGF-NT2N neurons (striped bar), or sham-injured animals injected with medium (black bar). Error bars are standard error of the mean. Sham-injured animals injected with medium had significantly shorter latencies to reach the platform than brain-injured animals injected with medium, indicating that there was still a cognitive deficit due to the injury after 4 weeks. In the brain-injured animals treated with NGF-NT2N neurons (striped bar), there was a significant improvement compared to the brain-injured animals injected with untransduced NT2N neurons ($p = 0.024$) or with control medium ($p = 0.036$). Multivariate ANOVA ($p < 0.01$) followed by a Tukey-Kramer multiple comparison test. *$p < 0.05$.

are identifiable in vivo with human-specific antibodies, can be used to examine this issue.

Experimental rodent models of TBI have been shown to consistently produce both short (days to weeks) and prolonged (months, up to 1 year) deficits in learning and memory that can be evaluated using the visuospatial learning task in the Morris Water Maze (35, 47–52). These models accurately reproduce many of the most common cognitive deficits associated with TBI in humans, including problem solving, learning, and memory (53–55). Intraparenchymal (directly into the injured cortex) or intracerebroventricular (ICV) administration of NGF has been shown to have beneficial effects on cognitive outcome in experimental studies using rodent models of TBI (56–58). However, clinical application of neurotrophin infusions has been problematic due to the global side effects associated with ICV administration, so this approach may therefore not be suitable for long-term therapeutic intervention (59). Our approach using NT2N neurons as a platform for ex-vivo gene therapy may provide an advantage for long-term neurotrophin delivery into selectively vulnerable regions of the injured brain.

It is of interest that the untransduced grafts had no effect on cognition in this setting. Previous studies in the traumatically injured CNS (18, 60) also did not find cognitive or motor recovery at 2 or 4 weeks after transplantation of untransduced NT2N neurons in the injured cortex. Borlongan et al (22) described a therapeutic effect of NT2N grafts in ischemic rats at 1 month post-injury, but in other models of neurodegenerative disease, therapeutic effects of NT2N grafts on motor function were observed only at more chronic (>10 weeks) post-transplant time points (17, 20). Experimental animals in our study were analyzed at 4 weeks post-transplant. One potential mechanism to account for the improved Morris Water Maze performance of the NGF-NT2N grafted animals involves the trophic actions of NGF on medial septal neurons and preservation of the septo-hippocampal pathway (57, 61, 62). The behavioral recovery might therefore be further improved by transplantation of a larger NGF-expressing graft, improved graft survival, or increased NGF secretion from the graft. These results demonstrate that the ability to genetically engineer NT2N neurons can greatly expand their potential utility as therapeutic tools for the CNS.

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