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EXPRESSION, BIOACTIVITY, AND SAFETY 1 YEAR AFTER ADENO-ASSOCIATED VIRAL VECTOR TYPE 2-MEDIATED DELIVERY OF NEURTURIN TO THE MONKEY NIGROSTRIATAL SYSTEM SUPPORT CERE-120 FOR PARKINSON'S DISEASE

OBJECTIVE: Parkinson's disease is characterized by profound motor deficits that result mainly as a consequence of degeneration of midbrain dopaminergic neurons. No current therapy slows or halts disease progression. Neurturin (NTN) and glial cell line-derived neurotrophic factor have potent neuroprotective and neurorestorative effects on dopaminergic neurons, but their use in treating Parkinson's disease has been limited by significant delivery obstacles. In this study, we examined the long-term expression, bioactivity, and safety/tolerability of CERE-120, an adeno-associated virus type 2 vector encoding human NTN, after bilateral stereotactic delivery to the striatum of nonhuman primates.

METHODS: Twelve naïve rhesus macaques received bilateral stereotactic injections of 1 of 2 CERE-120 doses or vehicle to the caudate and putamen. Neurological and clinical parameters were monitored for up to 1 year postadministration, after which animals were sacrificed for histological analyses.

RESULTS: Dose-related NTN expression was observed at 1 year and was associated with enhanced tyrosine hydroxylase immunolabeling in the striatum, hypertrophy of tyrosine hydroxylase-positive cells in the substantia nigra, and induction of extracellular signal-regulated kinase signaling in the substantia nigra. Extensive, formal analyses, conducted in accordance with Good Laboratory Practice Regulations, across multiple time points revealed no evidence of clinical, neurological, or systemic toxicity.

CONCLUSION: The present study provides evidence of long-term expression and bioactivity of NTN on the dopaminergic nigrostriatal system after bilateral stereotactic delivery of CERE-120 to the striatum. Furthermore, no evidence of any adverse effects for up to 1 year postadministration was observed. These findings reveal a wide safety margin for CERE-120 and collectively support the ongoing clinical testing of the efficacy and safety of CERE-120 in patients with Parkinson's disease.

KEY WORDS: Adeno-associated virus, CERE-120, Gene therapy, Neurotrophic factor, Neurturin, Parkinson's disease

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Over the past several decades, it has been well established that neurotrophic factors have the capacity to enhance the function of degenerating neurons, as well as protect them from further cellular damage

and death. However, thus far it has proven impossible to safely deliver adequate amounts of any of these proteins throughout the targeted region in patients to produce the desired biological effect, while also limiting the spread of non-

ABBREVIATIONS: AAV2, adeno-associated virus type 2; CGRP, calcitonin gene-related peptide; CSF, cerebrospinal fluid; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; FB, formulation buffer; GDNF, glial cell line-derived neurotrophic factor; Ig, immunoglobulin; MRI, magnetic resonance imaging; NTN, neurturin; PD, Parkinson's disease; pERK, phosphorylated extracellular signal-regulated kinase; TH, tyrosine hydroxylase; vg, vector genomes

targeted protein, the latter of which is known to induce problematic side effects. For example, although glial cell line–derived neurotrophic factor (GDNF) has robust neuroprotective and neurorestorative effects on dopamine neurons—the cell population that degenerates as a hallmark of Parkinson’s disease (PD)—a neurosurgical procedure is required to deliver it to the central nervous system. Even using neurosurgically supported methods of delivery, initial attempts to treat patients with PD using intracerebroventricular infusion of GDNF failed, presumably because of inadequate diffusion of the protein from the ventricular system to the nigrostriatal targets (19, 27). Subsequent intraparenchymal infusion studies produced mixed results. Although 2 open-label trials showed evidence of efficacy with this approach (9, 33), a randomized placebo-controlled, double-blinded trial did not (20). These findings led to the assumption that the GDNF protein was inadequately delivered to the large human striatum via a chronically indwelling cannula that provided a single point source for protein diffusion (26, 31, 32).

Gene transfer offers a genuine opportunity to overcome the problem of inadequate coverage of the targeted brain regions with a neurotrophic factor. Viral vector–mediated delivery of a neurotrophic factor can confer a more widespread and continuous exposure throughout the targeted brain area. This approach also eliminates the complications associated with chronically indwelling hardware in the brain and the periodic refilling of implanted infusion pumps, which can lead to antibody formation resulting from leakage of the protein into the body cavity. For these reasons, we have developed CERE-120, an adeno-associated viral vector type 2 (AAV2)–based system for delivery of neurotrophic factor neurturin (NTN) to the striatum for the potential treatment of PD. NTN, and its structural and functional analog GDNF, have potent neuroprotective and neurorestorative effects on the nigrostriatal system in animal models of PD (6–8, 10, 13, 16–18, 23, 28, 30). AAV2 is well characterized and has been shown to be safe and well tolerated in several clinical trials after delivery to the central nervous system (3, 5, 15, 22, 25). AAV2 has a very low potential for insertional mutagenesis, exhibits virtually exclusive neurotropism in the brain, and promotes sustained long-term transgene expression (1, 24).

Herein, we present findings in nonhuman primates that establish long-term, dose-related expression and bioactivity of CERE-120 at 1 year after bilateral stereotactic delivery to the striatum, as well as the safety/tolerability of CERE-120 by assessments of in-life behavioral and clinical parameters, organ histopathology (including analysis of brain and spinal cord), serum and cerebrospinal fluid (CSF) levels of NTN protein, potential systemic immune responses to AAV2 or NTN, and potential local immune/inflammatory reaction to AAV2 and/or NTN protein.

MATERIALS AND METHODS

Viral Vector

CERE-120 (AAV2-NTN) construction and production has been described in detail previously (7). Vector genomes consist of AAV2

inverted terminal repeats flanking a transgene expression cassette containing a CAG (hybrid chicken β -actin/human cytomegalovirus) promoter and a human growth hormone gene polyadenylation signal (Stratagene, La Jolla, CA). Human NTN was expressed from a hybrid complementary deoxyribonucleic acid (DNA), where the NTN pre-pro domain was replaced by that of the human nerve growth factor to enhance NTN secretion.

Animals

Twelve naïve rhesus monkeys (*Macaca mulatta*; n = 6 per sex), 3 to 5 years of age at time of dosing, were used in this experiment. All animals were individually housed at Northern Biomedical Research, Inc., in Muskegon, MI, in standard monkey cages and maintained on a 12-hour light/dark cycle with food (20 biscuits/day of PMI Certified Primate Diet; PMI Nutrition International, Richmond, IL) and water (ad libitum) provided. Animals were fasted overnight before conducting a subset of experimental procedures (e.g., blood collection, stereotactic surgery). Animals received environmental enrichment per the Northern Biomedical Research Program of Animal Care, and all experimental procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of Northern Biomedical Research, Inc.

Doses of CERE-120 and Infusion Parameters

For the high-dose group, the maximum feasible dose of CERE-120 was delivered to the monkey striatum based on the following parameters: the number of injection sites per animal, the injection volume per site, the surgical and infusion time required for each animal, and the highest manufactured concentration of CERE-120 (7.1×10^{12} vector genomes [vg] per milliliter). It was determined that the highest feasible dose that could be practically delivered was 3.6×10^{12} total vg/animal, delivered in 5 sites per hemisphere (2 sites in the caudate and 3 sites in the putamen), in a volume of 50 μ L of CERE-120 per site, resulting in delivery of a total of 0.5 mL of CERE-120 per animal during a single 7- to 8-hour surgical procedure. Low-dose animals received 5.8×10^{11} total vg/animal delivered in a volume of 30 μ L per site. Control animals received injections of 50 μ L of formulation buffer (FB), phosphate-buffered saline with addition of 2 mmol/L magnesium chloride, per site. All striatal injections were performed at a flow rate of 2 μ L per minute (i.e., infusion durations of 15 or 25 minutes per injection for 30 and 50 μ L volumes, respectively). At the completion of each injection, the needle/syringe was allowed to remain in the brain for an additional 5 minutes to minimize potential efflux of vector up the needle track.

Stereotactic Delivery of CERE-120 or Formulation Buffer Control

Stereotactic targeting of the caudate and putamen was performed based on presurgery magnetic resonance imaging (MRI). Animals were anesthetized with medetomidine HCl (0.08 mg/kg intramuscularly) and ketamine HCl (2.5 mg/kg intramuscularly). A catheter was placed in the saphenous vein for delivery of additional anesthesia as needed. Animals were placed in a custom-made MRI-compatible nonhuman primate stereotactic instrument (Northern Biomedical Research, Inc.). Care was taken to reposition the animal in the frame in the same position as that for the MRI by using recorded measurements from Vernier scales on the ear bars placed into the auditory meatus and with adjustable infraorbital clamps. In addition, measurement of the height of the left canine facilitates replacing the animal in the frame during surgery in exactly the same dorsal-ventral position. This is the only plane that is critical to measure because

direct visualization of the midsagittal sinus is used during surgery to set the medial-lateral plane.

MRI was conducted (1.0 T; Picker T55D-1006B; Picker International, Inc., Cleveland, OH [T1-weighted coronal, T1-weighted sagittal, and a TE 7 volume scan with 1-mm slices, no gap]) while each animal was positioned in the stereotactic frame. On the day of surgery, animals were given atropine sulfate (0.04 mg/kg subcutaneously) and, approximately 15 minutes later, an intramuscular injection of ketamine HCl (8.0 mg/kg). They were then intubated and maintained at a surgical plane of anesthesia with isoflurane (1%–2% in O₂), and were placed in the same stereotactic frame used to collect presurgery MRI scans. The targets for the 10 injections per animal were selected based on their potential to maximize the distribution of CERE-120 or FB throughout all 3 dimensions of each brain region. Injections were performed using a Hamilton syringe attached to a blunt 22-gauge S-type needle. Needle/syringe sets used for injections were pretreated by performing 20 volume exchanges of either FB or CERE-120 as appropriate to prevent vector adsorption. After completion of all injections, the burr hole was filled with gel foam and dental acrylic, and the incisions were sutured in anatomic layers.

Food Consumption, Body Weight, and Neurobehavioral Function

Clinical observations and food consumption monitoring (number of biscuits consumed) were performed daily, body weight was recorded weekly, and physical examinations and neurobehavioral assessments were conducted at baseline (presurgery), day 3, and monthly thereafter during the study. Physical examinations included formal, documented assessment of heart rate, body temperature, and respiration; abdominal palpation; and examination of eyes, ears, skin, nails, oral cavity, lymph nodes, thoracic auscultation, gait, and disposition according to approved standard operating procedures. Neurobehavioral examinations included formal, documented assessment of tracking, general motor function, pupillary reflex, orbicularis oculi reflex, corneal reflex, sensory foot reflex, knee jerk, cutaneous response, proprioceptive response, and tail reflex according to approved standard operating procedures.

Necropsy and Histopathology

Full necropsy was performed on all animals after they were sacrificed. Monkeys were anesthetized with ketamine (8 mg/kg intramuscularly) and maintained on an isoflurane/oxygen or halothane/oxygen mixture, given an intravenous bolus of heparin sodium (200 IU/kg), and transcardially perfused with 0.9% saline followed by fixation with a modified Zamboni's solution (4% paraformaldehyde and 0.18% picric acid in 0.1 mol/L phosphate buffer). The brains and spinal cords were then removed, immersed in a modified Zamboni's solution overnight at 2°C–8°C, and then cryoprotected in a sucrose solution (30%) and maintained at 2°C–8°C until equilibrated. Coronal sections through the brain and upper spinal cord were cut frozen on a sliding microtome at 40- μ m thickness and stored in a cryoprotectant solution. A series of sections throughout the brain (including cerebellum) and upper spinal cord were mounted onto glass slides and stained with hematoxylin and eosin and evaluated by a board-certified veterinary pathologist blinded to the treatment condition. A standard panel of organs/tissues was also collected from all animals and placed in 10% neutral, buffered formalin. Tissues from the heart, lung, liver, testes/ovaries, prostate, intestine, spleen, thymus, adrenals, kidneys, and cervical lymph nodes were processed into paraffin blocks, sectioned, stained with hematoxylin and eosin, and evaluated by a board-certified veterinary pathologist.

Immunohistochemistry and Immunofluorescence on Brain, Spinal Cord, and Cerebellum

All procedures were performed according to well-established, standardized methods. Briefly, primary antibodies were anti-NTN goat immunoglobulin (Ig)G (1:200; R & D Systems, Minneapolis, MN), anti-tyrosine hydroxylase (TH) mouse IgG (1:20000; ImmunoStar, Inc., Hudson, WI), anti-phosphorylated extracellular signal-regulated kinase 1/2 (pERK 1/2) rabbit IgG (1:200; Cell Signaling Technology, Danvers, MA), anti-calcitonin gene-related peptide (CGRP) rabbit IgG (1:8000; Chemicon International, Temecula, CA), anti-CD68 mouse IgG (1:250; VWR International, West Chester, PA), anti-CD45 mouse IgG (1:500; BD Biosciences Pharmingen, San Diego, CA), and anti-glial fibrillary acidic protein mouse IgG (1:2000; Chemicon). Excluding the primary antibody or substituting the primary antibody with an irrelevant IgG served as controls. For immunohistochemistry (NTN, TH, pERK 1/2, CGRP), visualization of antibody binding was performed using an avidin-biotin procedure (Vector Laboratories, Burlingame, CA) with nickel sulfate (omitted for TH and CGRP) and diaminobenzidine as the chromogen. For immunofluorescence (CD45, CD68, and glial fibrillary acidic protein), sections were incubated with Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

Optical Densitometry and Stereology

Optical densitometry of TH-positive fibers in the striatum was performed using NIH ImageJ software (v1.3; National Institutes of Health, Bethesda, MD) by an investigator blinded to the treatment group. For each animal, the mean staining intensity of both the left and right caudate and putamen was determined from 9 sections matched for anatomic level. For each of the left and right caudate and putamen, on each of the 9 sections, an optical density value was determined. Additionally, for each section analyzed, an optical density value from the corpus callosum was determined, and that background intensity was subtracted from that obtained within the structure. A mean TH optical density value was subsequently obtained for each structure for each animal. All sections were stained at the same time under identical conditions to eliminate potential variability in staining intensity caused by immunohistochemical processing. All immunohistochemical methods and densitometry analyses were based on well-established, previously reported techniques (18, 19, 29).

Unbiased stereological estimates of the number of TH- and pERK 1/2-positive cells in the substantia nigra were generated from 12 sections through the right hemisphere using a design-based counting method (optical fractionator, Stereo Investigator; MicroBrightField, Inc., Williston, VT) by an investigator blinded to the treatment groups. While performing the analysis of cell number using this software, the cell area of the sampled TH-positive cells was also determined. The intensity of pERK 1/2 immunostaining in the substantia nigra was also quantified by optical densitometry by an investigator blinded to the treatment condition. Sections from each animal were stained at the same time under identical conditions to eliminate potential variability in staining intensity caused by immunohistochemical processing. Additionally, for each section analyzed, the optical density of the corpus callosum was ascertained and this background level of staining intensity was subtracted from the measured pERK 1/2 optical density displayed by nigral neurons.

Schwann Cell Hyperplasia and Axonal Sprouting

Intraventricular delivery of trophic factors has previously been found to cause Schwann cell hyperplasia and aberrant axonal sprouting (4). To determine whether such treatment-related side effects

occurred in the present study, the lower medulla through the hypoglossal nuclei and the upper spinal cord were sectioned on a cryostat at a thickness of 40 μm . A 1-in-6 series of sections was mounted onto slides and stained with cresyl violet. To assess potential Schwann cell hyperplasia, the pia-arachnoid thickness surrounding the medulla and upper spinal cord was quantified. Photomicrographs were captured using digital software (Diagnostic Instruments), and the mean pia thickness for each animal was calculated from a total of 6 measurements taken from the left and right dorsolateral regions of the upper spinal cord. A 1-in-6 series of sections through the medulla and spinal cord were immunolabeled for CGRP, a marker of sensory neurites, and TH, a marker of sympathetic axons, to assay for potential aberrant axonal sprouting and a qualitative assessment of potential neurite outgrowth into the pia layer was performed.

CSF and Serum Analyses

Clinical Chemistry and Hematology

Blood samples were collected from the femoral vein of fasted animals at baseline (presurgery), 1 month after surgery, and every 3 months throughout the remainder of the study, and after the animals were killed. A standard hematology and serum chemistry panel was performed. CSF samples were collected via cisterna magna taps at baseline and after the animals were sacrificed, and the following analyses were performed: total cell count, total protein, and levels of glucose, phosphate, chloride, calcium, and potassium.

Biodistribution of NTN to Serum and CSF

The serum concentration of NTN from each monkey receiving the highest dose of CERE-120 (3.6×10^{12} vg) was determined using NTN enzyme-linked immunosorbent assay (ELISA) at baseline, Weeks 5, 14, 27, 40, and at the time of death. CSF NTN concentrations for all monkeys were determined using NTN ELISA at baseline and at time of death. The NTN ELISA consisted of flat-bottom 96-well ELISA plates coated with a recombinant form of the NTN cognate receptor, GFR α -2 (R & D Systems). After application of the diluted CSF or serum sample, bound soluble NTN was detected by the addition of a biotinylated goat anti-NTN polyclonal antibody, followed by a streptavidin-horseradish peroxidase conjugate and the chromogenic substrate, TMB One (3,3',5,5'-tetramethylbenzidine). A dilution series of purified recombinant human NTN was used to generate a standard curve. After adding acid to stop the color development reaction, optical density was quantitated using a 96-well plate reader set at 450 nm. CSF and serum samples were run in triplicate wells and the mean of the triplicates were reported when samples fell within the quantitative range. The limits of quantitation of the NTN ELISA for CSF and serum samples were 196 pg/mL (lower limit) and 25 ng/mL (upper limit).

Biodistribution of CERE-120 to CSF

At baseline and at death, the biodistribution of CERE-120 vector to the CSF was assayed by real-time quantitative TaqMan polymerase chain reaction in all animals. The quantitative polymerase chain reaction assay amplifies a 110 base pair region of the CERE-120 genome spanning the 3' end of the CAG promoter and the 5' end of the nerve growth factor pre/pro coding region. The CSF samples were assayed by quantitative polymerase chain reaction based on input of the initial sample volume, and included a 20- μL equivalent of the original sample per reaction, in triplicate reactions. For each sample, 2 of 3 reactions contained sample DNA alone, and the 3rd reaction contained sample

DNA plus a spike of 100 copies of CERE-120 vector DNA to test for the presence of polymerase chain reaction inhibition. The limit of detection of the method was 7 copies/reaction and the limit of quantitation was 32 copies/reaction.

Serum Anti-AAV and Anti-NTN Antibody Analyses

To assess potential humoral responses against the vector capsid or transgene product of CERE-120, titers for antibodies to AAV2 and NTN were assayed by ELISA at baseline, weeks 5, 14, 27, 40, and at death. Serum test samples were initially diluted 1/500 for the AAV2 assay or 1/100 for the human NTN assay and then as a 3- or 4-fold dilution series (as necessary). Serum dilutions were then assayed for reactivity against intact AAV2 capsids or purified recombinant human NTN protein and assigned an antibody titer value based on comparison to known negative and positive monkey sera (for AAV2) or rat sera (for human NTN). Neutralizing antibodies to AAV2 were assessed in baseline samples and the time point showing peak anti-AAV2 response as determined by ELISA. To determine AAV2 neutralizing antibody titers, serum test samples were initially diluted 1:20 and then as a 2-fold dilution series. Sample dilutions were combined with AAV2-*LacZ* vector and tested for transduction activity by application to Huh 7 cells and subsequent chemiluminescent assay for β -galactosidase activity. Neutralizing antibody titers were assigned to sample dilutions imparting at least a 50% decrease in transduction activity relative to controls. Changes in serum antibody titer between baseline and necropsy were calculated for each individual animal and are reported as the ratio difference in titer (necropsy titer/baseline titer). The AAV2 antibody ELISA and AAV2 neutralizing antibody assays are capable of detecting both antibodies generated specifically against AAV serotype 2, as well as antibodies generated against other closely related serotypes of AAV that can cross-react to AAV2. Therefore, these assays cannot distinguish between responses generated specifically against CERE-120 and naturally occurring AAV infections.

RESULTS

Long-term, Dose-Related Expression of Human NTN in the Nigrostriatal System

One year after striatal CERE-120 administration, NTN expression was confirmed in all animals. In contrast, no NTN expression was observed in any brain region in FB-injected control monkeys. At the injection sites of CERE-120-injected animals, NTN immunolabeling was localized to the targeted caudate and putamen. In addition, NTN immunoreactivity was observed in striatal target sites such as the globus pallidus (external and internal divisions) and the pars reticulata (Fig. 1, A–C). In the substantia nigra pars compacta, neurons were stained positive for NTN in CERE-120-injected animals (Fig. 1, D–I). The NTN detected outside of the targeted or above-mentioned brain regions was predominantly limited to cortical regions and/or underlying white matter adjacent to the needle tracks. Diffuse, light staining for NTN was also observed in a small region of the medial thalamus in 2 of 8 hemispheres in the low-dose group (5.8×10^{11} vg), and 4 of 8 hemispheres in the high-dose group (3.6×10^{12} vg). Quantitative analyses revealed a dose-related increase in the volume of NTN distribution in the caudate and putamen. Mean volume (\pm standard

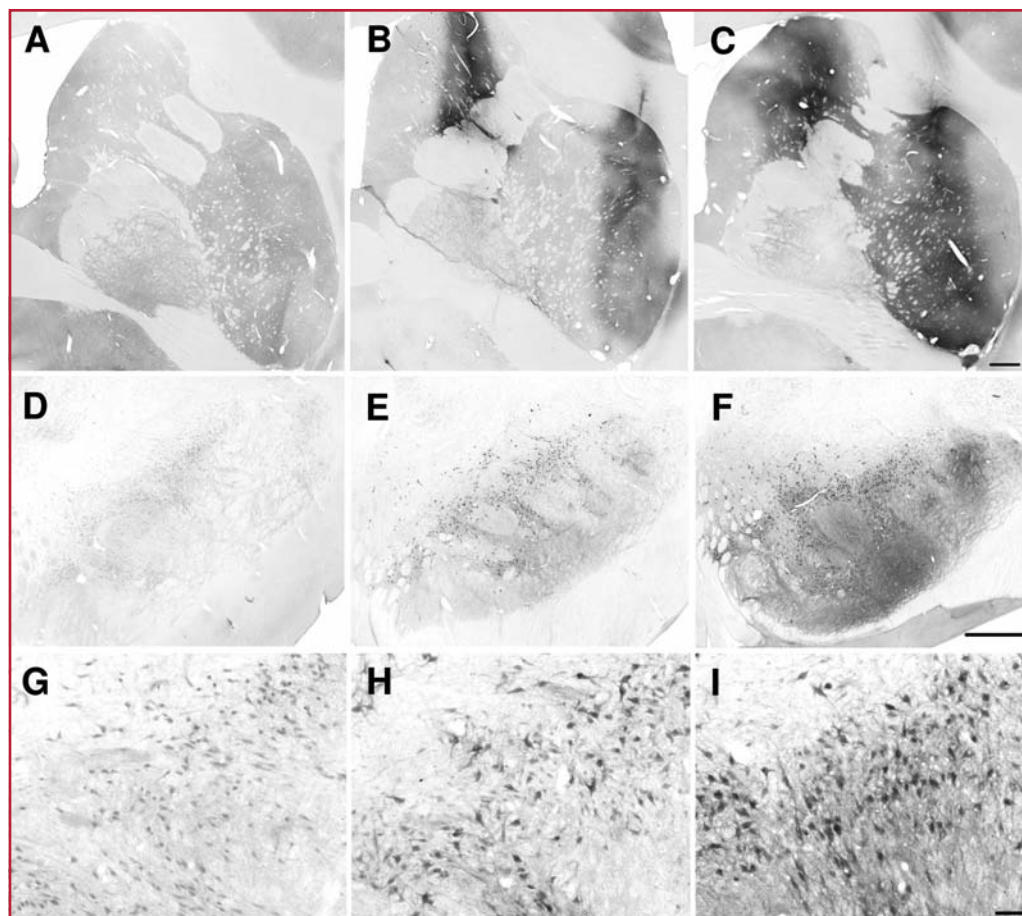


FIGURE 1. Long-term, dose-related expression of human neurturin (NTN) in the nigrostriatal system. Representative low-power photomicrographs of NTN immunolabeling in coronal sections through the caudate and putamen (A–C), and high-power photomicrographs through the substantia nigra (D–I) of the formulation buffer (FB) control (A, D, G) and CER-120–injected monkeys at 1 year after striatal administration (5.8×10^{11} total vector genomes [vg] of CER-120/animal presented in panels B, E, and H; 3.6×10^{12} total vg of CER-120/animal presented in panels C, F, and I). NTN expression was confirmed in all animals and was localized mainly to the targeted caudate and putamen in a dose-related manner. In addition, cells in the pars compacta and fibers in the pars reticulata of the substantia nigra were immunolabeled for NTN in a dose-related manner. Scale bar, 1.0 mm (C and F) and 0.1 mm (I).

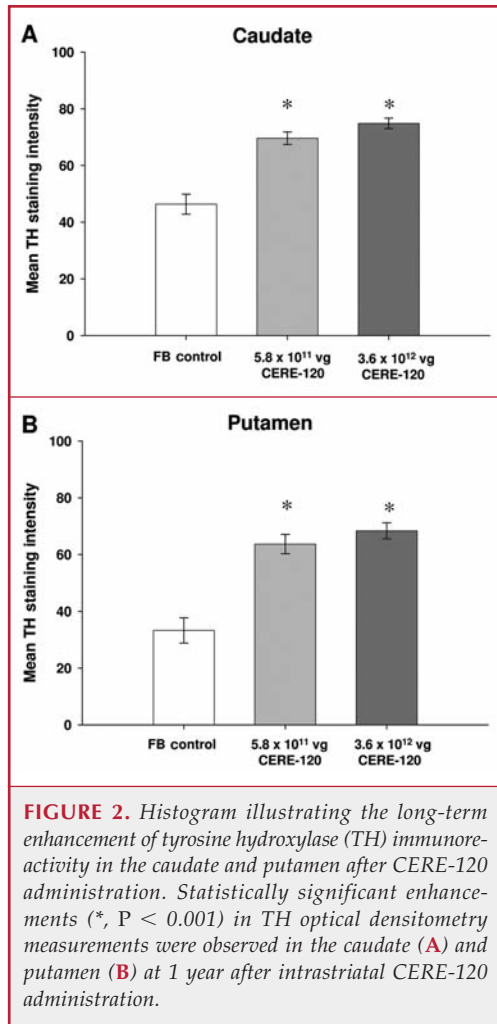
error of the mean) of striatal NTN immunolabeling was $258.08 \text{ mm}^3 (\pm 33.54 \text{ mm}^3)$ for low-dose (5.8×10^{11} vg) and $473.35 \text{ mm}^3 (\pm 40.94 \text{ mm}^3)$ for high-dose (3.6×10^{12} vg) CER-120–injected animals ($t_{14} = -4.07$, $P = 0.001$).

Long-term Bioactivity of CER-120 on the Nigrostriatal Dopaminergic System

A robust increase in TH immunoreactivity in both the caudate and putamen was observed in CER-120–injected monkeys 1 year after delivery (Fig. 2). Quantitative analyses (one-way analysis of variance) revealed a highly significant increase in TH-positive fiber-staining intensity in both of these brain regions (caudate, $F_{2,9} = 33.13$, $P < 0.001$; putamen, $F_{2,9} = 27.73$, $P < 0.001$). Post hoc analyses revealed that both CER-120 dose groups differed significantly from FB-injected control animals in

both brain regions ($P < 0.001$), but did not differ from each other ($P \geq 0.40$) (Fig. 2). Quantitative analyses also revealed a significant enhancement of TH-positive cell size in the substantia nigra 1 year after CER-120 ($F_{2,9} = 8.25$, $P = 0.009$) (Table 1). As was observed in the striatum, post hoc analyses revealed that both CER-120 dose groups differed significantly from FB-injected control animals ($P < 0.03$), but did not differ from each other ($P = 0.79$) (Table 1). Importantly, the number of TH-positive cells in the substantia nigra did not change as a consequence of CER-120 injection, as no between-group differences were observed ($F_{2,9} = 0.18$, $P = 0.84$) (Table 1). As anticipated, pERK 1/2–positive nuclei in the substantia nigra were observed in both FB control and CER-120–injected animals. Cells in the substantia nigra of FB-injected control monkeys showed essentially no pERK 1/2 staining in the cytoplasm (Fig. 3, A and B). In contrast, a robust and reliable increase in the frequency of cells showing cytoplasmic pERK 1/2 immunoreactivity, as well as enhanced staining intensity within those cells showing cytoplasmic staining, were observed in

CER-120–injected animals (Fig. 3, C and D). Quantitative analyses revealed significantly greater numbers of cells with pERK 1/2 labeling in the cytoplasm after CER-120 injection compared with FB controls ($F_{2,9} = 11.45$, $P = 0.003$), with no differences between the 2 doses of CER-120 ($P > 0.05$) (Fig. 3E). Optical densitometry also revealed a significant increase in cytoplasmic staining intensity after CER-120 injection ($F_{2,9} = 20.96$, $P < 0.001$) and both CER-120 groups were significantly different from FB controls ($P < 0.002$), but not each other ($P = 0.55$) (Fig. 3F). Additional immunofluorescence analyses using double labeling for TH and pERK 1/2 within the substantia nigra revealed robust and consistent colabeling of these markers (Fig. 4), confirming the dopaminergic phenotype in cells displaying enhanced cytoplasmic pERK 1/2 as a consequence of CER-120 injections.



No Evidence of Neurological Toxicity or of Side Effects Potentially Related to Nontargeted NTN Distribution

Body Weight, Food Consumption, and Neurobehavioral Assessments

Daily observations of food consumption and weekly measures of body weight revealed no adverse effects of CERE-120 delivery for up to 1 year postadministration. Animals in all groups exhibited normal fluctuations in food consumption and maintained or gained weight throughout the duration of the study. No adverse effects of CERE-120 administration were revealed by neurological or physical examinations conducted monthly up to 3 months, and every 3 months thereafter to 1 year.

Brain Histopathology, Including Cerebellum

No abnormal pathology related to CERE-120 administration was observed in histopathological evaluation of sections throughout the brain and upper spinal cord. Focal accumulation of hemosiderin within macrophages, focal fibrosis, and sporadically focal gliosis were consistently observed at the needle tracks

TABLE 1. Quantitation of tyrosine hydroxylase-positive cells in the substantia nigra^a

Group	TH + cell area (μm^2)	TH + cell no.
FB control	384.91 \pm 17.55	140 495.42 \pm 4937.14
CERE-120		
5.8 \times 10 ¹¹ vg	455.55 \pm 13.10 ^b	136 011.56 \pm 5584.55
3.6 \times 10 ¹² vg	470.38 \pm 16.68 ^b	135 129.95 \pm 9206.65

^a TH, tyrosine hydroxylase; FB, formulation buffer; vg, vector genomes. Data are mean \pm standard error of the mean.

^b $P < 0.03$ versus FB control.

within the striatum in animals across all treatment groups. In addition, occasional, sporadic mononuclear cell infiltration at the striatal injection sites, not associated with treatment group, was observed. No CERE-120-related alterations were observed in any other brain region, including the cerebellum.

Immune/Inflammatory Markers in Brain, Including Cerebellum

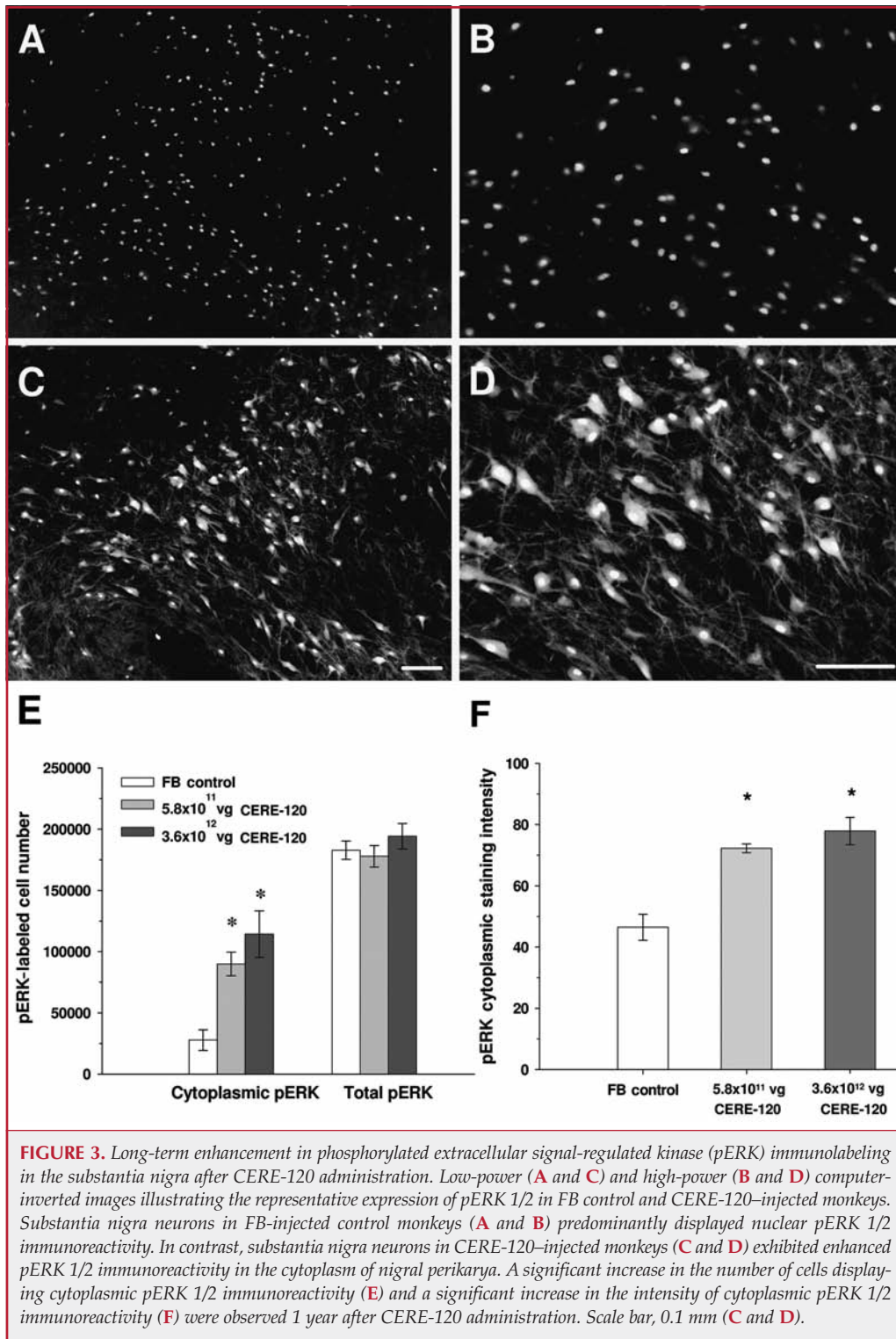
Overall, immunolabeling for CD45 (a pan-leukocyte marker), CD68 (a marker of activated microglia), and glial fibrillary acidic protein (an astrocytic marker) on sections throughout the rostrocaudal extent of the cerebrum revealed no differences between FB control and CERE-120-injected animals. Positive immunolabeling for each of these markers was consistently observed in restricted brain regions immediately adjacent to the needle tracks such as cortical white matter, corpus callosum, caudate, and putamen in animals from all groups, including FB controls. Instances of positive staining outside of, but often in close proximity to, the needle track, occurred in multiple brain regions sporadically in animals in all groups, including FB controls. Blinded analyses of the pattern of CD45 and CD68 immunolabeling in the cerebellum also revealed comparable staining patterns between FB control and CERE-120-injected animals.

Potential Schwann Cell Hyperplasia and Axonal Sprouting

Microscopic analyses of medulla and spinal cord sections revealed a normal pattern of TH and CGRP staining in all animals at 1 year postadministration indicating no aberrant sprouting as a result of striatal delivery of CERE-120. In addition, no evidence of pia thickening associated with Schwann cell hyperplasia at the level of the medulla or upper spinal cord was observed, as quantification of pial thickness at the level of the upper spinal cord revealed no effects of CERE-120 ($F_{2,9} = 0.31$, $P > 0.05$).

CSF Chemistry Analysis

No CERE-120-related alterations were observed on CSF total cell counts or chemistry (total protein, and levels of glucose, phosphate, chloride, calcium, and potassium) at 1 year postadministration.



Serum and CSF Biodistribution

CSF NTN concentrations were below the limit of quantitation of the ELISA assay (196 pg/mL) for all baseline and

necropsy samples tested, except for 1 baseline sample in 1 monkey (276 pg/mL). Serum NTN concentrations for animals that received the highest dose of CERE-120 (3.6×10^{12} vg) were all below the limit of quantitation of the ELISA assay (<196 pg/mL) at all time points tested. No CERE-120 vector DNA was detected in any of the CSF samples collected at baseline. At necropsy, CSF samples from 2 of 4 monkeys in the CERE-120 high-dose group (3.6×10^{12} vg) yielded detectable quantities of CERE-120 vector DNA; however, the levels were below the limit of quantitation (<32 copies/20 μ L of CSF).

Systemic Toxicity

Clinical Chemistry and Hematology

No adverse effects of CERE-120 administration were observed on any of the hematology or serum chemistry parameters examined at any time point up to and including 1 year (baseline, monthly up to 3 months, and every 3 months thereafter).

Organ/Tissue Histopathology

No CERE-120-related effects were observed in histopathological assessment of heart, lung, liver, testes/ovaries, prostate, intestine, spleen, thymus, adrenals, kidneys, and cervical lymph nodes at 1-year postadministration.

Humoral Immune Responses

Serum Antibody Analyses

No changes in serum antibody titers to human NTN were observed in any monkey more than 1 year after CERE-120 delivery. Five of 12 animals had moderately increased AAV2-reactive titers at baseline ranging

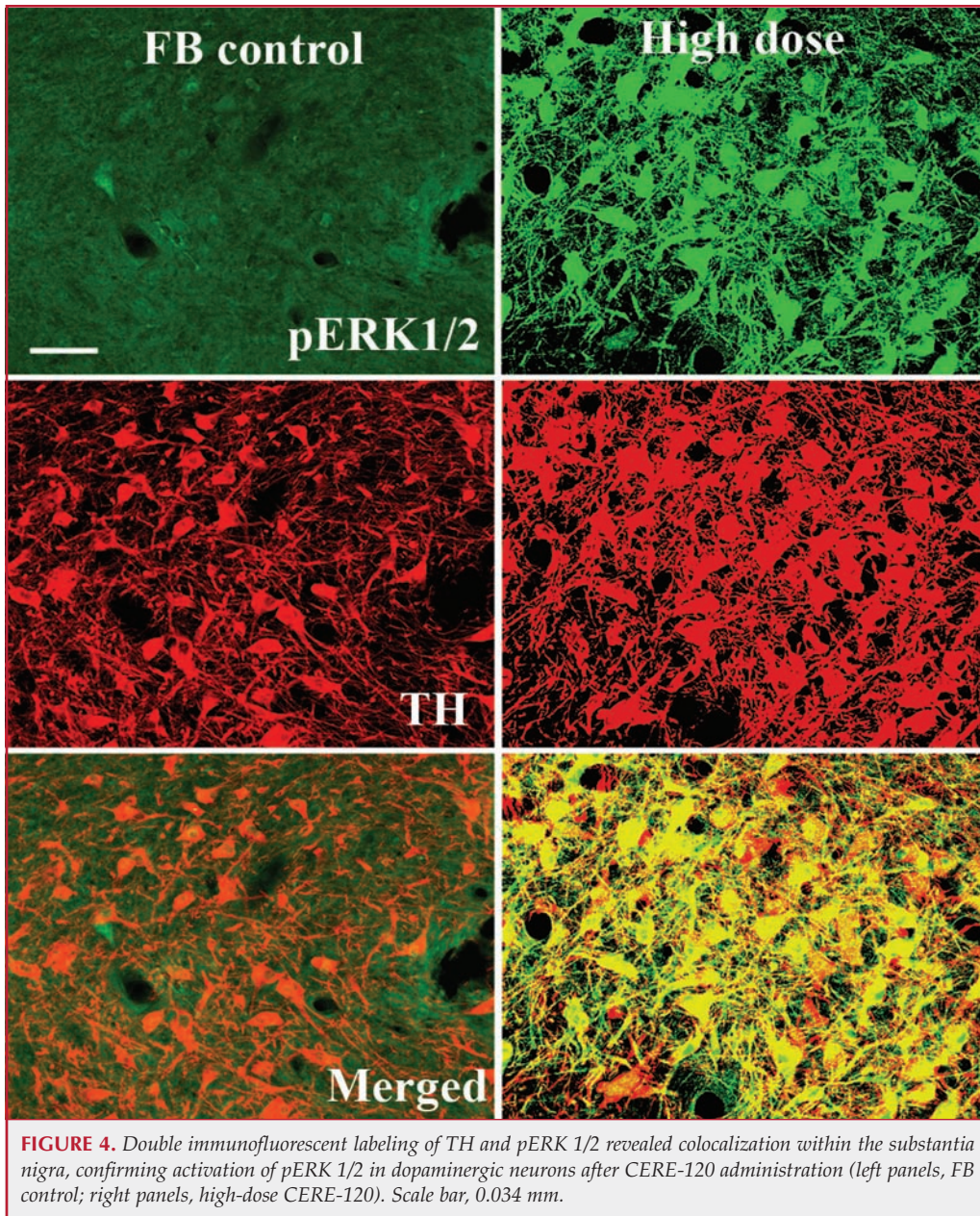


FIGURE 4. Double immunofluorescent labeling of TH and pERK 1/2 revealed colocalization within the substantia nigra, confirming activation of pERK 1/2 in dopaminergic neurons after CERE-120 administration (left panels, FB control; right panels, high-dose CERE-120). Scale bar, 0.034 mm.

from 1:1500 to 1:8000 as measured by ELISA. This might represent a preexisting immunity to AAV in these monkeys. Postdosing serum antibody analyses revealed dose-dependent antibody responses to AAV2 within 5 to 14 weeks after CERE-120 delivery to the striatum. In all cases, the antibodies were capable of neutralizing AAV2 transduction activity in vitro, and antibody titers decreased in all animals over subsequent time points. Despite the presence of preexisting neutralizing antibodies in 2 low-dose (5.8×10^{11} vg) and 2 high-dose (3.6×10^{12} vg) monkeys, as well as the formation of antibody responses to AAV2 in initially naïve CERE-120-injected monkeys, robust NTN protein expression was evident in brains of all monkeys

injected with CERE-120 (see results above). In addition, no clinical signs or pathological abnormalities were associated with the responses to AAV2, including assessments of immune and inflammatory markers in the brain.

DISCUSSION

The therapeutic potential of neurotrophic factors has been recognized for some time, given their potent capacity to restore the function of degenerating neurons and protect them from further cellular damage and death (2, 11). Adequate delivery of these proteins, however, has continued to be the single greatest obstacle clinically and has precluded their successful development and use in patients for the treatment of neurodegenerative diseases. Although the neuromedical community has largely concluded that a neurosurgical procedure will be required to appropriately deliver the neurotrophic factors to the targeted regions of the central nervous system to exploit their therapeutic power, previous attempts at infusing the proteins via a single point source (e.g., via indwelling cannulae) have been collectively disappointing for a variety of safety and efficacy reasons (9, 20, 33). Gene transfer is an alternative approach

to exploit the therapeutic power of neurotrophic factors, because it potentially offers widespread, permanent coverage of the targeted region by the protein after a single neurosurgical procedure. It was with this approach in mind that CERE-120 was constructed and developed for the treatment of PD, given the well-known neurotrophic effects of NTN and its structural analog, GDNF, in animal models of nigrostriatal degeneration (6, 8, 10, 13, 16–18, 23, 28, 30). The findings from the current study demonstrate that stereotactic delivery of an AAV2 viral vector encoding human NTN results in orderly, dose-related, long-term expression of NTN throughout the nonhuman primate striatum. Although we have previously published data

demonstrating that viral vector-mediated expression of growth factors (e.g., GDNF and NTN) confers anatomic and functional benefit to the nigrostriatal system in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) lesioned (17, 18) and aged nonhuman primates (13), the present report is the first to describe the persistent, longer-term, dose-related expression of NTN (for at least 1 year) after CERE-120 administration as well as the persistence of NTN bioactivity for that duration. Moreover, this is the first formal evidence for the longer-term (12 months) safety of AAV2-mediated expression of NTN in nonhuman primates. Collectively, the data demonstrate the following: 1) CERE-120-mediated delivery of NTN maintains relatively constant volume of expression up to 1 year after administration to the striatum compared with findings at a shorter interval postadministration (12); 2) the protein is biologically active, evidenced by induction of 2 biomarkers of dopaminergic activity, TH and pERK 1/2; and 3) administration of CERE-120 and subsequent expression of NTN seem to cause no safety or tolerability issues in nonhuman primates up to 1 year, even at the highest dose feasible (see Materials and Methods section for details). Thus, these data add to the weight of evidence supporting the use of CERE-120 as a treatment to restore function and possibly prevent further degeneration of nigrostriatal dopamine neurons, the integrity of which has a major role in the pathogenesis and symptomatology of PD.

Long-term, dose-related expression of human NTN in the monkey nigrostriatal system was confirmed in all animals that received CERE-120 at 1 year after striatal delivery. NTN protein was mainly confined to the caudate, putamen, substantia nigra, and the globus pallidus. The observation of robust NTN immunoreactive fibers in the substantia nigra pars reticulata is consistent with anterograde transport of NTN protein via striatonigral projections, and has been observed previously (12, 13). NTN-positive perikarya and processes within the substantia nigra pars compacta were also consistently observed, and reflect either retrograde transport of NTN from the striatum, retrograde transport of the vector and subsequent transduction of dopaminergic neurons, or both of these phenomena, as they are not mutually exclusive and might collectively account for this pattern of NTN labeling. The pattern and distribution of NTN immunolabeling throughout the targeted nigrostriatal system are similar to those observed at 3 and 8 months after CERE-120 administration to the monkey striatum (12, 13). In addition, these data are consistent with published reports from other laboratories revealing long-term, stable AAV2-mediated transgene expression after intraparenchymal delivery to the brain (1). It has been suggested that one of the potentially limiting factors in using chronic delivery of exogenous GDNF protein to the striatum as a therapy for PD involves inefficient striatal protein distribution and therefore limited bioavailability of GDNF (9, 26, 31, 32). Delivery of multiple deposits of CERE-120 throughout the striatum, however, results in widespread distribution of human NTN (as detected by immunohistochemistry) not only throughout the terminal fields of the nigrostriatal dopaminergic cells, but also to the location of the cell bodies within substantia nigra. This enhanced bioavail-

ability of AAV2-mediated NTN compared with traditional protein infusion approaches could in theory result in increased efficacy in PD patients.

A robust increase in the labeling intensity of TH immunoreactive fibers was observed in the caudate and putamen at 1 year after administration of CERE-120. Additionally, within the substantia nigra, cellular hypertrophy of dopaminergic cells and robust activation of pERK 1/2, a signal transduction marker for activation of the downstream mitogen-activated protein kinase intracellular signaling pathway, were observed. Collectively, this augmentation of markers of dopaminergic activity is consistent with NTN-mediated bioactivity within midbrain dopaminergic neurons and their processes, and is consistent with previous observations in young intact (12), young parkinsonian (17, 18), and aged nonhuman primates (13, 18) at shorter intervals of time (i.e., 3–10 months) after either CERE-120 or lentiviral vector-mediated GDNF delivery, and further extends these observations to 1 year. Importantly, the present study replicates and extends prior findings observed at 3 months after CERE-120 delivery (12) to include a 6-fold higher dose level, and further establishes that long-term expression of NTN produces no evidence of toxicity in the brain or periphery. That is, no evidence of neurological toxicity or side effects potentially related to nontargeted NTN distribution were observed, as there were no adverse effects on body weight, food consumption, or neurobehavioral and physical examinations over the course of this 1-year study. There was no evidence of adverse effects on serum chemistry, hematology, humoral immune responses, or on peripheral tissues/organ pathology. Pathological analyses of the brain (including cerebellum) and spinal cord, using several histological markers of potential neurotoxicity, axonal sprouting, and immune/inflammatory responses, revealed no evidence of adverse effects of long-term CERE-120-mediated expression of NTN. These findings are in contrast to the dose-limiting side effects that have been reported after intraventricular and/or intraparenchymal infusion of growth factors via indwelling cannulae in rodents, monkeys, and humans (4, 14, 27). Although chronic GDNF protein delivery in nonhuman primates has been found to result in pathological changes that are the likely cause of some of the adverse effects observed in humans (e.g., Schwann cell hyperplasia or axon sprouting in the medulla or upper spinal cord [14], no evidence of these phenomena was observed in the present study at 1 year after delivery of CERE-120. It has also been reported that 3 months after cessation of chronic delivery of GDNF protein to the putamen, focal cerebellar lesions were observed in monkeys (14, 32). Nevertheless, extensive analyses conducted throughout the brain, including the cerebellum, have consistently revealed no histological evidence of neurotoxicity and no evidence of an immune/inflammatory response as a consequence of long-term CERE-120-mediated NTN expression, up to and including 1 year postadministration (12, 13, 18). The potential for development of neutralizing antibodies to the therapeutic protein has also been a concern in clinical trials involving chronic exogenous protein delivery

(20, 32, 33). In the present study, consistent with the observations that no human NTN was detected in the serum or CSF, no antibodies to human NTN were detected up to 1 year after CERE-120 delivery to the striatum. Although antibody responses to the AAV2 capsid did develop in a dose-related manner, there were no clinical manifestations of this response or effects on NTN expression, as widespread visualization of NTN was observed in all animals.

For subsequent clinical trials testing CERE-120 in PD patients (21, 22), a different dosing strategy was used based on the information from these and other nonhuman primate data (12, 13, 18). The clinical dosing strategy was not intended to deliver as much CERE-120 as possible in order to search for possible toxicity (the primary objective of the present study); rather, it was intended to maximize coverage of the putamen with NTN protein as much as possible, while avoiding untargeted protein spread to brain areas outside of the putamen. With a much larger target brain structure in the human compared with the monkey, the location of each needle pass and subsequent injections is planned with the intent to maximize potential coverage of the putamen with NTN in all dimensions, and minimize the potential "overlap" of NTN protein from the individual injections. This dosing strategy varies greatly from that required to deliver the very high doses used in this monkey study (i.e., injection of the highest possible volume and concentration of CERE-120 and injections less widely distributed because of the size of the monkey putamen compared with human, resulting in a high degree of "overlap" of NTN protein).

These findings, as well of those from additional nonclinical studies conducted in rodents (7, 8) and nonhuman primates (12, 13, 18), reveal a broad therapeutic index for CERE-120 and collectively support the ongoing clinical testing of CERE-120 delivery to the putamen in PD patients. The relative equivalent doses for a recently completed open-label phase I clinical study (22) are well below those demonstrated to be safe and well tolerated in monkeys, yet are within the range of doses shown to be effective in monkeys (12, 18). Adjusting the doses by brain weight rather than target size yields an even greater safety margin, with a greater than 400-fold difference between the highest dose tested in nonhuman primates (3.6×10^{12} total vg) and the lowest dose tested in humans (1.4×10^{11} total vg), and greater than 100-fold difference between the highest dose tested in nonhuman primates and the highest dose tested in humans (5.8×10^{11} total vg). Data collected to date from this open-label phase I trial have revealed no serious adverse events, with a significant improvement in the Unified PD Rating Scale III off-medication scores at 1 year compared with each subject's pretreatment baseline (22). These intriguing but preliminary open-label results have led to the initiation of a larger, multicenter, double-blind, sham-surgery controlled phase II study.

CONCLUSION

The present study provides evidence of long-term transgene expression and bioactivity of NTN on the dopaminergic nigrostriatal system after bilateral stereotactic delivery of CERE-120 to

the striatum. Furthermore, no evidence of any adverse effects was observed for up to 1 year postadministration. These findings reveal a broad therapeutic index for CERE-120 and collectively support the ongoing clinical testing of the efficacy and safety of CERE-120 in PD patients.

Disclosure

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COMMENTS

Targeted gene transfer of neurotrophic factors is one of the most promising approaches to halt the progression of Parkinson's disease (PD) as well as other conditions involving neurodegeneration. PD is a particularly good candidate for gene therapy because it involves the loss of a discrete cell type (i.e., dopaminergic neurons) in a highly localized brain region (substantia nigra pars compacta). Previous studies from this laboratory have shown that adeno-associated virus type 2 (AAV2) delivery of neurturin (NTN), designated CERE-120, was neuroprotective in rat and nonhuman primate models of PD. The work of this group has culminated in clinical trials of CERE-120 stereotactic injection for PD, including a recently completed open-label phase I study that supported the safety and potential efficacy of CERE-120.

In this context, this study is an essential contribution because it provides clear evidence that CERE-120 is capable of eliciting long-lasting and safe NTN expression in the striatum of nonhuman primates. Moreover, this study also presents evidence of NTN bioactivity, including elevated tyrosine hydroxylase labeling in the striatum and substantia nigra. This indicates that by acting as a neuroprotective agent for dopaminergic neurons in PD, CERE-120 enhances the function of the dopaminergic circuitry. Overall, this study represents a substantial advance in the development of a promising new PD therapy.

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This article describes the preclinical laboratory studies that in part provided the basis for the recently completed phase I trial of intrastriatal injection of AAV-NTN (brand name, CERE-120) for PD (5). The report documents safety of the biological at doses higher than those used in the human trials and shows vector expression at 1 year.

As a participant in both the phase I and phase II clinical trials of this technique, I remain concerned about issues surrounding the scaling of the therapy from the macaque brain to the human brain. There have now been many promising trials in PD using delivery of growth factors or cells in attempts to restore dopaminergic function. Despite excellent basic science work, none have lived up to their promise. This may have been related in part to problems with cell or drug delivery. Will CERE-120 share that fate?

In their results, Herzog et al. state, "Mean volume (\pm standard error of the mean) of striatal NTN immunolabeling was 258.08 mm³ (\pm 33.54 mm³) for low-dose (5.8×10^{11} vg) and 473.35 mm³ (\pm 40.94 mm³) for high-dose (3.6×10^{12} vg) CERE-120-injected animals . . ." The volume affected in the macaque in the low-dose group was 0.25 mL. This was a similar dose to the highest dose used in human studies (5.8×10^{11} total vg). In the human studies, 40 μ L per hemisphere was delivered

along 4 injection tracts (5), compared with 150 μL per hemisphere (low dose) or 250 μL per hemisphere (high dose) using 5 injection tracts in this macaque study. Thus, in the human, I would expect, on the basis of the present preclinical study, at most 0.25 mL of striatum to be transduced (same total dose as low dose in the macaque, but in a smaller injection volume). Yet the volume of the human putamen is approximately 3.5 cm^3 , 3 times the size of the macaque striatum (putamen and caudate). Is coverage of less than 10% of the striatum adequate to expect a therapeutic effect in humans? In a related preclinical study by Kordower et al. (4), which showed efficacy of CERE-120 for alleviating parkinsonian motor signs in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated macaque model of PD, a larger proportion of the striatum was likely transduced, compared with the human trials.

The volume of human putamen that needs to have transgene expression, for clinical efficacy in growth factor therapy, is not known. In tract tracing studies in intact rodents, single substantia nigra pars compacta axons branch fairly widely in the striatum (2). Thus, focal injections in the striatum could ultimately affect a larger region than the original injection volume, by retrograde transport to surviving substantia nigra pars compacta axons or nuclei followed by an anterograde affect on substantia nigra pars compacta arbors innervating striatal regions that were not originally injected.

Finally, the authors state that their article is the “first to describe the persistent, longer-term, dose-related expression of NTN (for at least 1 year) after CERE-120 administration as well as the persistence of NTN bioactivity for that duration.” Although technically true, this statement is somewhat misleading. Kordower et al. (3), using lentivirus-mediated gene delivery of glial cell-derived neurotrophic factor, showed expression of a neurotrophic factor gene at 8 months postinjection. In their article studying AAV-NTN (CERE-120) in the 1-methyl-4-phenyl-

1,2,3,6-tetrahydropyridine-treated macaque, Kordower et al. (4) showed behavioral changes induced by AAV-NTN up to 10 months postinjection. Expression of transgenes from AAV vectors in macaque brain has been shown at up to 6 years by other groups, although the gene expressed was not one encoding a neurotrophic factor (1). Nevertheless, the data in this report represent an important stepping-stone to the human trials of AAV-NTN.

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