

Delivery of Neurturin by AAV2 (CERE-120)-Mediated Gene Transfer Provides Structural and Functional Neuroprotection and Neurorestoration in MPTP-Treated Monkeys

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Objective: We tested the hypothesis that gene delivery of the trophic factor neurturin could preserve motor function and protect nigrostriatal circuitry in hemiparkinsonian monkeys.

Methods: An adeno-associated virus–based vector encoding human neurturin (AAV2-NTN; also called CERE-120) was injected into the striatum and substantia nigra of monkeys 4 days after a unilateral intracarotid injection of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) rendered them hemiparkinsonian. Control hemiparkinsonian monkeys received either AAV2 encoding green fluorescent protein or formulation buffer.

Results: Although stable deficits were seen in all control monkeys, AAV2-NTN significantly improved MPTP-induced motor impairments by 80 to 90% starting at approximately month 4 and lasting until the end of the experiment (month 10). AAV2-NTN significantly preserved nigral neurons, significantly preserved striatal dopaminergic innervation, and activated phospho-extracellular signal–regulated kinase, consistent with a mechanism involving a trophic factor–initiated molecular cascade. Histological analyses of numerous brain regions, including the cerebellum, showed normal cytoarchitecture and no aberrant pathology.

Interpretation: These data demonstrate that AAV2-NTN (CERE-120) can preserve function and anatomy in degenerating nigrostriatal neurons and are supportive of ongoing clinical tests in Parkinson's disease patients.

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The cardinal signs of Parkinson's disease (PD), bradykinesia, resting tremor, rigidity, and postural instability result from striatal dopamine insufficiency secondary to degeneration of dopaminergic neurons within the substantia nigra pars compacta.¹ Numerous dopaminergic therapies provide substantial symptomatic benefit to PD patients (e.g., *L*-dopa). However, over time, most PD patients suffer debilitating treatment-induced side effects such as dyskinesias and motor fluctuations, and for many, the therapeutic window in which they receive benefit without side effects eventually diminishes to near zero.^{2,3} Deep-brain stimulation has recently become a valuable tool in the armament against PD.⁴ However, substantial benefit occurs in only approximately 30% of patients and issues related to the patency of the implanted hardware and the duration of battery life complicate this procedure.⁴ More critically,

neither existing drug therapies nor approved surgical procedures confer neuroprotection or alter the natural course of disease progression.

Toward this end, neurotrophic factors hold great promise in preventing dopaminergic neuron degeneration and enhancing nigrostriatal function in PD. By slowing or stopping the degenerative process, trophic factors have the potential to alter the natural course of disease progression and could potentially be a powerful approach for long-term therapy. Although nigral neurons are responsive to a variety of trophic factors,⁵ they are exquisitely sensitive to glial cell line–derived neurotrophic factor (GDNF) and its naturally occurring structural and functional analog, neurturin (NTN). Both GDNF and NTN enhance dopaminergic neuron survival and nigrostriatal function in animal models of PD. In side-by-side comparisons, both factors provide

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equal protection from 6-hydroxydopamine-induced degeneration in rats.⁶ Intracerebral delivery of the GDNF protein or the GDNF complementary DNA (cDNA) via gene delivery provides neuroprotection and neuroregeneration in parkinsonian monkeys.^{7–9} Although evidence of efficacy has been obtained using a chronic, point source of GDNF in PD patients,¹⁰ a controlled study failed to observe comparable results.¹¹ Thus, the utility of GDNF infused into the relatively large putamen target with a single point source of protein remains controversial,^{12–15} and further product development using this approach appears to have been abandoned.¹⁶ Gene delivery of NTN potentially provides a more effective means of delivering neurotrophic support for degenerating nigrostriatal neurons, in that bilateral long-term expression in the majority of the putamen can be achieved in a single surgical setting, with no need for chronically implanted hardware, or problems with controlling pump flow rates, protein diffusion, or convection from a relatively small point source. To investigate this possibility, we have performed a large series of experiments with AAV2-NTN (CERE-120) in young and aged rodents,⁶ as well as young¹⁷ and aged¹⁸ nonhuman primates. These studies have demonstrated that AAV2-NTN is safe at dose multiples more than 100 times greater than those required for efficacy and preserves and/or enhances dopaminergic function in a persistent, dose-dependent manner. As part of our nonclinical program before initiating clinical trials in PD, we performed this study in parkinsonian monkeys to demonstrate efficacy and provide additional support for the safety of AAV2-NTN (CERE-120). We report that delivery of AAV2-NTN to the nigrostriatal system of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys reverses motor deficits for up to 10 months, prevents the loss of nigral neurons, and partially preserves dopaminergic striatal innervation. These data, taken together, support the concept that gene delivery of NTN via CERE-120 may be safe and efficacious for the treatment of PD and merits testing in human subjects.

Materials and Methods

All experimentation was performed with the approval of the Institutional Animal Care and Use Committee and Institutional Biosafety Committee at Rush University, the University of Illinois in Chicago Medical Centers, and Ceregene (San Diego, CA).

Experimental Animals

Twenty male rhesus monkeys (4–10kg) were administered MPTP as described below. Based on an assessment of their hemiparkinsonian symptoms, 10 monkeys were selected for stereotaxic injection of AAV2-NTN (CERE-120) or control articles. All monkeys were housed individually and provided food and water ad libitum.

MPTP Treatment

All monkeys received a right intracarotid injection of MPTP as described in detail previously.⁹ In brief, under isoflurane anesthesia, the right carotid bifurcation was exposed surgically, the right external carotid artery was permanently ligated, and 3mg MPTP-HCl was injected into the right common carotid artery in the direction opposite to blood flow in a 20ml volume at a rate of 1.33ml/min.

Vector Construction

Gasmi and colleagues (manuscript provisionally accepted) describe the construction of CERE-120 (AAV2-NTN) and AAV2 encoding green fluorescent protein (AAV2-GFP) vectors used in this study. In brief, vector genomes consisted of the AAV2 inverted terminal repeats flanking a transgene expression cassette containing the CAG promoter and the human growth hormone gene polyadenylation signal (polyA) (Stratagene, La Jolla, CA). In the CERE-120 vector genome, human NTN is expressed from a hybrid cDNA, where the NTN pre-pro domain was replaced by that of the human nerve growth factor (NGF) to enhance NTN secretion. The AAV2-GFP vector is identical to the AAV2-NTN vector except that the ppNGF-NTN cDNA is replaced by an enhanced GFP cDNA. All vectors were produced in human embryonic kidney 293 cells using the calcium phosphate triple plasmid transfection method. Three days after transfection, cells were harvested and lysed. AAV2 vector was purified from the cell lysates by heparin and ion exchange chromatography. Purified particles were concentrated by centrifugal filtration, and vector titer (vector genome per milliliter (vg/ml)) was determined by quantitative polymerase chain reaction. All vectors were created by Ceregene.

Stereotaxic Surgery

Delivery of AAV2-NTN, AAV2-GFP, and formulation buffer (FB; phosphate-buffered saline with 2mM magnesium chloride) was performed according to previously published protocols.⁹ In brief, based on clinical rating scale assessments taken 3 to 4 days after MPTP treatment, 10 monkeys displaying the classic crooked arm posture and general slowness on the side opposite of the MPTP infusion were selected from the larger group and continued in the study. It is our experience that animals displaying this phenotype do not spontaneously recover over time. Based on clinical rating scale scores, they were matched into two groups ($n = 5$ each) and received either AAV2-NTN (CERE-120) or control treatments (AAV2-GFP [$n = 2$] or FB [$n = 3$]). For all dependent measures, the data for the two control groups were similar, and thus were combined for statistical analyses. Four days after MPTP treatment, monkeys received stereotaxic injections of AAV2 or FB into the brain hemisphere ipsilateral to MPTP treatment. Two injections were made into the caudate nucleus (15 μ l each), three into the putamen (15 μ l each), and one into the substantia nigra (10 μ l). The total dose for each monkey injected with AAV2-NTN or AAV2-GFP was 1.7×10^{11} vg. Injection coordinates were based on magnetic resonance imaging guidance.

Clinical Rating Scale Analysis

All behavioral assessments were performed by a single investigator blinded to the experimental conditions. Beginning 3 to 4

days after MPTP (baseline), then at 1 week after surgery, and ongoing thereafter for 10 months, monkeys were assessed 1 to 3 times per week for parkinsonian features using a previously described clinical rating scale (9). All scores over each week of testing were averaged and analyzed via both parametric and nonparametric statistical models (see description in Results).

Histological Analysis

At 10 months after surgery, monkeys were anesthetized with pentobarbital (25mg/kg intravenously) and killed via perfusion with 0.9% saline followed by fixation with a modified (4%) Zamboni's fixative. Brains were removed from the calvaria, immersed in 30% sucrose in phosphate-buffered saline, and sectioned frozen (40 μ m) on a sliding knife microtome. Tissue sections were stored in a cryoprotectant solution at 4°C. Sections were stained immunocytochemically for NTN (1:1,000; R&D Systems, Minneapolis, MN), tyrosine hydroxylase (TH; 1:20,000; Chemicon, Temecula, CA), GFP (1:1,000; Clontech, Palo Alto, CA), or phospho-extracellular signal-regulated kinase (phospho-ERK; 1:200; Cell Signaling Technology, Beverly, MA) using the avidin-biotin procedure and 3'3'-diaminobenzidine as the chromogen. Immunohistochemical visualization of NTN used an antigen retrieval procedure followed by standard immunohistochemistry with nickel intensification. For each antibody, all animals in the study were stained at the same time to control for potential variability in staining intensities across immunohistochemical runs. In addition, sections throughout the brain, including the substantia nigra, striatum, and cerebellum were stained with hematoxylin and eosin followed by histopathological analysis by a board-certified veterinary pathologist who was blinded to treatment condition.

Stereological Counts of Tyrosine Hydroxylase Immunoreactive Nigral Neurons

Estimates of dopaminergic nigral cell number were performed bilaterally using an unbiased design-based counting method (optical fractionator, StereoInvestigator; MicroBrightfield, Williston, VT). All counts were performed by a single investigator blinded to the experimental conditions. Using a random start, we outlined the substantia nigra under low magnification (1.25 \times objective) and sampled 20% of the treated nigra or 5% of the intact nigra in a random but systematic manner. Statistical comparisons between AAV-NTN and control-treated monkeys were made using analysis of variance followed by Tukey post hoc tests.

Optical Density of Striatal Tyrosine Hydroxylase

All optical densitometry was performed by a single investigator blinded to the experimental conditions. Five coronal sections matched for anatomic level were quantified for the optical density of TH immunoreactivity (TH-ir) using the National Institutes of Health Image system.⁹ Two sections were rostral to the anterior commissure, one was at the level of the anterior commissure, and two were posterior to the commissure. The light levels from the microscope, as well as the ambient light in the room, were kept constant for the entire analysis. Using 20 \times magnification, we randomly sampled the caudate nucleus and putamen at approximately 100 sites each, and the mean optical density of TH staining was

quantified by the National Institutes of Health image program. From this sampling scheme, a mean optical densitometry measurement was obtained for each animal. Statistical comparisons between groups were made using analysis of variance followed by Tukey post hoc tests.

Results

Neuroprotective Effects of Adeno-associated Virus-Based Vector Encoding Human Neurturin on Motor Function

Four days after MPTP treatment, monkeys were evaluated on an established clinical rating scale analogous to the human Unified Parkinson's Disease Rating Scale.^{9,19,20} Ten of the 20 MPTP-injected monkeys displayed clear signs of hemiparkinsonism after a single intracarotid MPTP injection, and only these monkeys were enrolled in the study. Monkeys were matched into groups, based on clinical rating scale scores, and received either AAV2-NTN ($n = 5$), AAV2-GFP control ($n = 3$), or FB control ($n = 2$) 4 days after MPTP. AAV2-GFP- and FB-treated animals were statistically similar on all outcome measures, and thus were combined into a single control group. After gene transfer, AAV2-NTN and control groups diverged over time on their clinical rating scale responses (Fig 1). The five control monkeys remained stable in their parkinsonian disability throughout the 10 months of the study and none spontaneously recovered. In contrast, the five AAV2-NTN-treated monkeys progressively recovered functionally. Due to the zero clinical rating scores in some animals after AAV2-NTN and the apparent stabilization of group means after month 4, a repeated-measures analysis was performed only for data between months 0 and 4. Al-

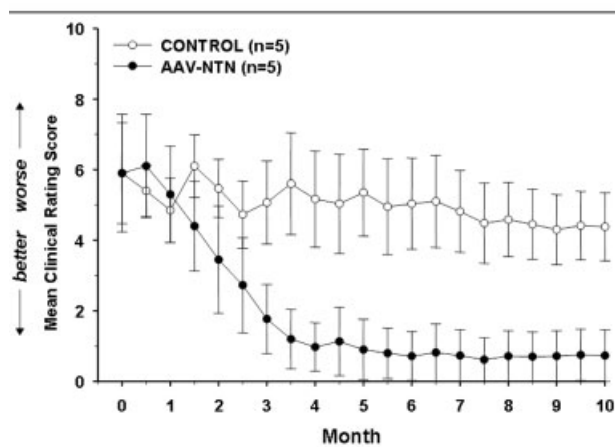


Fig 1. Clinical rating scale scores illustrating stable parkinsonian features in control-treated monkeys (open circles; $n = 5$) and a progressive and sustained functional benefit in adeno-associated virus-based vector encoding human neurturin (AAV2-NTN)-treated monkeys (solid circles; $n = 5$). Significant differences between groups were observed beginning month 4 and continue for the duration of the experiment (month 10).

though the original clinical rating scores were ordinal, their monthly means showed a continuous and normal distribution. A mixed model for repeated-measures analysis was therefore used instead of a generalized estimated equation method for ordinal data. The model included three terms: Month, Group, and a Month \times Group interaction. We also used an unstructured covariance structure, random intercept, fixed covariates, and Kenward–Roger denominator degrees of freedom. There was a statistically significant interaction between Month and Group ($p = 0.0041$), indicating that the temporal pattern of mean clinical rating scores differed between the two groups. Wilcoxon rank sum test showed that significant differences between groups first emerged at month 4 (exact $p = 0.032$), and this effect was sustained for the duration of the experiment. Indeed, four of the five treated monkeys showed complete recovery on this motor task, whereas the fifth monkey showed significant, albeit partial, recovery (see Case Study: Monkey 7177 section later in this article). In contrast with control monkeys, AAV2-NTN–treated monkeys displayed a mean 88% reduction in their parkinsonian score at the last time point measured, 10 months after treatment.

Transgene Expression in MPTP-Treated Monkeys

Detection of NTN immunoreactivity (NTN-ir) in the caudate, putamen, and substantia nigra confirmed that the stereotaxic injections were well targeted. The distribution of NTN-ir was primarily limited to these targeted sites and their anatomically related regions, as well as cortical regions and white matter around the needle track (Fig 2). Anterograde transport of NTN was observed in the globus pallidus, entopeduncular nucleus, and substantia nigra pars reticulata. Although the intranigral injection of AAV2-NTN precludes a definitive interpretation of retrograde transport of NTN and/or AAV2-NTN to the substantia nigra, the granular nature of NTN-ir in nigral neurons at a distance from the AAV2-NTN injection site suggests that NTN and/or AAV2-NTN were retrogradely transported from the striatum to the substantia nigra, consistent with observations in rats, intact young monkeys, and aged monkeys (manuscripts in preparation) who received injections of AAV2-NTN into the stratum only. To varying degrees in four of the five AAV2-NTN–injected animals, we observed NTN-ir in the mediodorsal nucleus of the thalamus. Mediodorsal thalamic staining was robust in a few fibers, diffuse in the neuropil, and moderate in cell bodies.

Adeno-associated Virus–Based Vector Encoding Human Neurturin-Induced Enhancements in Striatal Tyrosine Hydroxylase Immunoreactivity Optical Densitometry

In control monkeys, qualitative analyses showed a comprehensive loss of TH-ir in the caudate and putamen

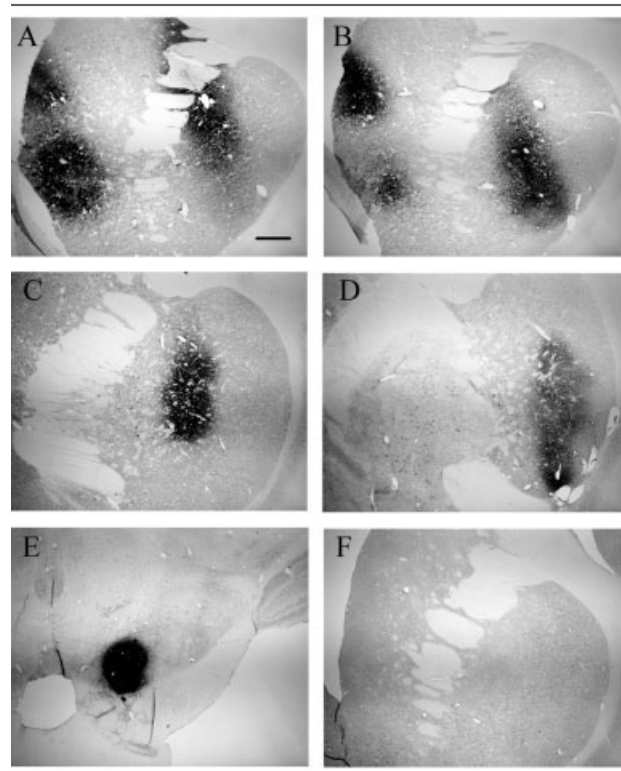


Fig 2. Neurturin (NTN) immunohistochemistry illustrating the appropriate targeting and spread of NTN protein for each of the five injection sites. (A) Head of caudate and rostral putamen. (B) Commissural putamen. (C, D) Postcommissural putamen. (E) Substantia nigra. (F) Control-treated monkey stained for NTN illustrating the lack of endogenous upregulation of NTN after control injection. Scale bar = 1mm.

(Fig 3). In contrast, all AAV2-NTN–treated monkeys displayed greater striatal TH-ir than control animals. However, in none of these animals was striatal TH-ir at normal (ie, unlesioned) levels. Quantitative optical densitometry measurements were performed bilaterally on sections through the caudate and putamen for all monkeys (see Fig 3E). For the caudate, a two-way analysis of variance demonstrated no significant effect of treatment ($F_{(1,16)} = 0.36$; $p > 0.05$), a significant effect of hemisphere ($F_{(1,16)} = 123.57$; $p < 0.001$), and a significant treatment by hemisphere interaction ($F_{(1,16)} = 7.84$; $p < 0.05$). For the caudate nucleus on the intact side, control and AAV2-NTN–treated monkeys displayed similar TH-ir optical densitometry values (mean \pm standard error of the mean: 69.63 ± 5.7 and 59.97 ± 5.5 arbitrary units, respectively; $p > 0.10$). On the MPTP lesioned side, control monkeys had a mean optical densitometry value of 8.58 ± 1.2 , representing 12.3% of the value from the intact side ($p < 0.001$). A partial preservation of TH-ir in the caudate nucleus was seen in AAV2-NTN–treated monkeys, who had a mean optical densitometry value of 23.48 ± 3.6 , representing 39.2% of the intact side,

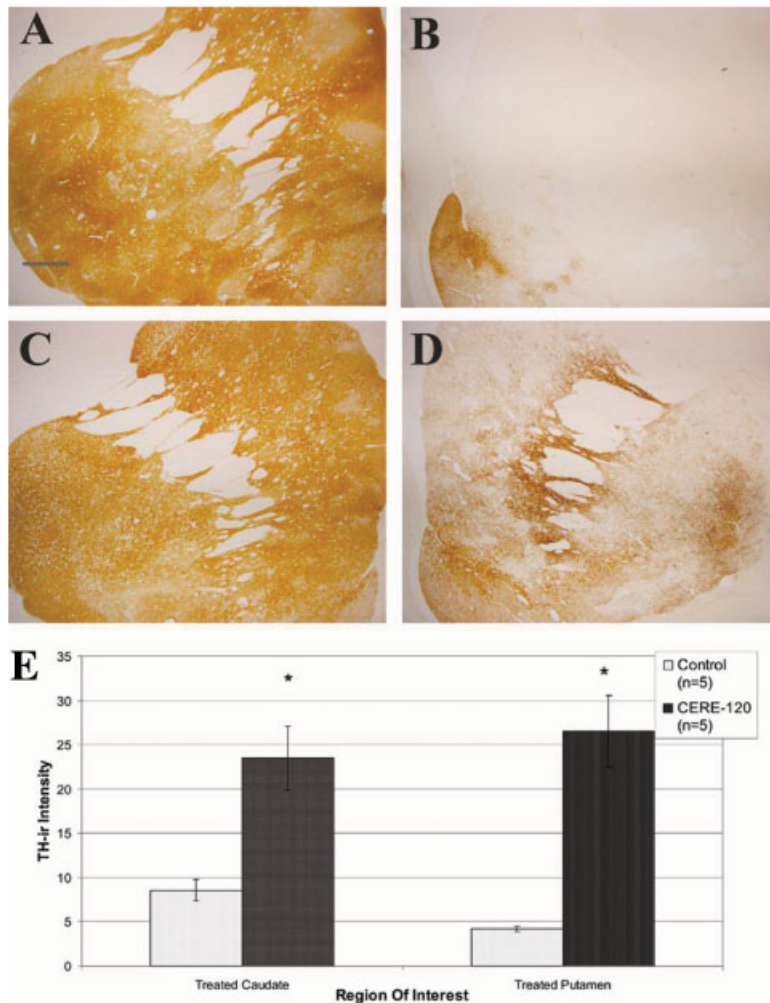


Fig 3. Preservation of striatal tyrosine hydroxylase (TH) after AAV2-NTN delivery. (A, C) Intact sides. (B) The comprehensive loss of striatal TH immunoreactivity (TH-ir) on the N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) side with control treatment. (D) In contrast, preservation of striatal TH occurred on the MPTP side with adeno-associated virus-based vector encoding human neurturin (AAV2-NTN) treatment. (E) Histogram quantifying the preservation of TH-ir optical densitometry in AAV2-NTN-treated monkeys (CERE-120; dark bars; $n = 5$). Light bars designate controls ($n = 5$). Scale bar = 1mm (A–D). * $p < 0.01$.

which is significantly greater than in control animals ($p < 0.05$). A two-way analysis of variance performed on TH-ir optical densitometry values in the putamen demonstrated no significant effect of treatment ($F_{(1,16)} = 0.81$; $p > 0.05$), a significant effect of hemisphere ($F_{(1,16)} = 92.23$; $p < 0.001$), and a significant treatment by hemisphere interaction ($F_{(1,16)} = 12.58$; $p < 0.05$). In the intact putamen, control (71.27 ± 6.7) and AAV2-NTN (57.97 ± 6.3)–treated monkeys had similar TH optical densitometry values ($p > 0.05$). On the lesioned side, control monkeys had optical densitometry values in the putamen of 4.20 ± 0.33 , representing 5.9% of the value of the intact side ($p < 0.001$). A partial preservation of TH-ir was seen in the lesioned putamen of AAV2-NTN-injected monkeys, where putamenal optical densitometry was 26.51 ± 4.0 . This level of TH-ir represents 45.7% of

the intact side and was significantly greater than in control animals ($p < 0.01$).

Adeno-associated Virus–Based Vector Encoding Human Neurturin–Induced Neuroprotection of Nigral Cell Number

In control monkeys, a comprehensive loss of TH-ir in the substantia nigra was observed on the hemisphere of MPTP administration (Fig 4). In contrast, on the treated side of AAV2-NTN-injected animals, the breadth and intensity of nigral TH staining was notably greater than what was seen in control monkeys (see Fig 4). Stereological estimates of TH-positive nigral neurons were performed bilaterally for all monkeys (see Fig 4). A two-way analysis of variance showed a significant effect of treatment ($F_{(1,16)} = 12.56$; $p < 0.005$),

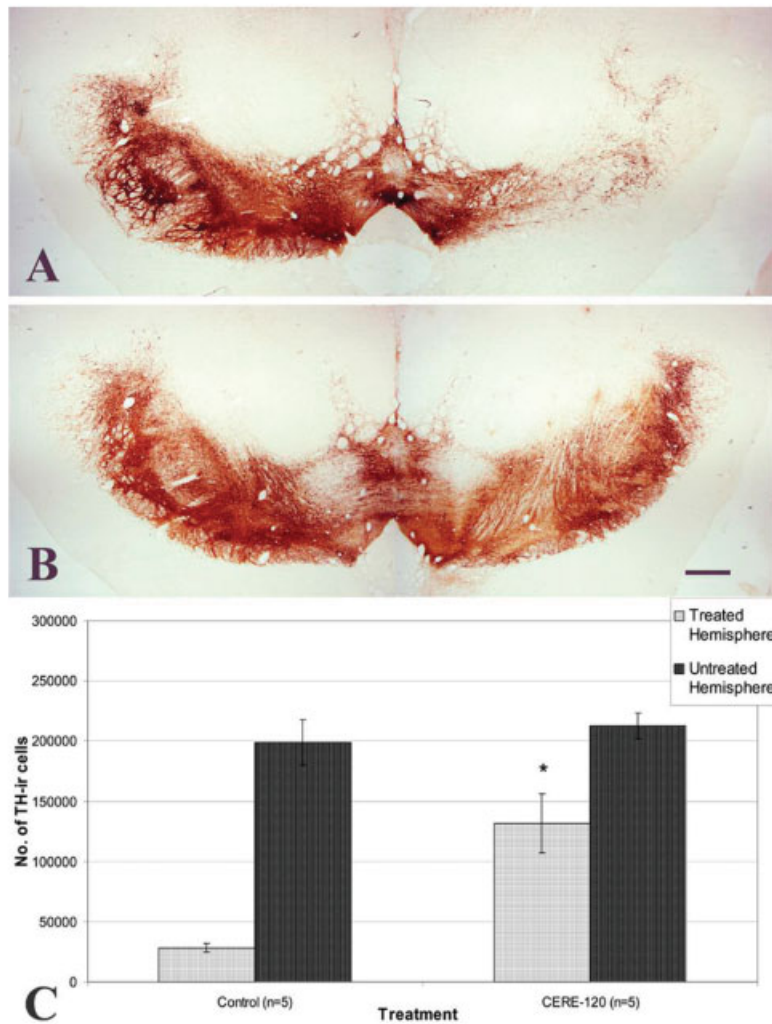


Fig 4. Preservation of nigral tyrosine hydroxylase (TH) after adenoassociated virus-based vector encoding human neurturin (AAV2-NTN). (A) In control treated monkeys, there is a comprehensive loss of TH-ir neurons on the lesioned (right) side relative to the intact (left) side. (B) In contrast, AAV2-NTN prevented the loss of TH-ir nigral neurons on the side of MPTP treatment. (C) Histogram illustrating the preservation of TH-ir nigral neurons in AAV2-NTN treated monkeys. Gray bars indicate treated hemisphere; dark bars indicate untreated hemisphere. Scale bar in B represents 1 mm A and B. * $p < 0.01$.

a significant effect of hemisphere ($F_{(1,16)} = 58.30$; $p < 0.001$), and a significant treatment by hemisphere interaction ($F_{(1,16)} = 7.48$; $p < 0.05$). On the intact side, control and AAV2-NTN-injected monkeys had similar numbers of TH-positive nigral neurons (average \pm standard error of the mean: $198,959 \pm 18,745$ and $212,287 \pm 10,795$, respectively; $p > 0.05$). On the MPTP lesioned side, control monkeys had $28,090 \pm 3,529$ TH-ir nigral neurons, representing 14% of the number on the intact side ($p < 0.001$). In contrast, AAV2-NTN-treated monkeys had $131,558 \pm 24,604$ TH-ir neurons on the lesioned side. This represents 62% of the number of TH-ir neurons on the intact side, a value significantly greater than in control monkeys (Tukey test, $p < 0.001$; see Fig 4).

Adeno-associated Virus-Based Vector Encoding Human Neurturin Activates Phospho-extracellular Signal-Related Kinase in the Substantia Nigra

As a part of the intracellular response to neurotrophic factor stimulation in nigral neurons, ERK is phosphorylated and consequently activated. In an unstimulated cell, ERK expression is limited to the nucleus, but on phosphorylation, ERK translocates to the cytoplasm where it further activates downstream signaling molecules. Thus, both the amount and subcellular localization of phosphorylated ERK immunoreactivity (pERK-ir) reflect the biological response to a neurotrophic factor such as NTN. Robust specific pERK-ir was consistently observed in numerous brain regions including the cerebral cortex, hippocampus, red nucleus, and

substantia nigra. In the substantia nigra, on the intact side in all monkeys, pERK-ir was observed almost exclusively within the nucleus and rarely in the cytoplasm (Fig 5). In control monkeys, far less nigral pERK-ir cells were observed on the MPTP-treated side than on the intact side (due to the lesion, as described earlier for TH-positive cells). However, the subcellular localization of the existing pERK-ir was the same on both sides, being almost exclusively nuclear. In contrast, AAV2-NTN-treated monkeys displayed many more pERK-ir nigral cells and robust pERK-ir was observed in the cytoplasm of these protected cells, indicative of the activation of ERK in response to NTN delivered via AAV2.

General Histopathology

After staining of coronal sections throughout the cerebrum, midbrain, brainstem, and cerebellum with hematoxylin and eosin, detailed histopathological analyses were performed. No evidence of any abnormal pathology was observed in any control or AAV2-NTN injected animal in coronal section or brain region.

Case Study: Monkey 7177

Of the five AAV2-NTN-treated monkeys, all but one showed complete recovery of motor performance (see results of motor tests earlier in this article). Several anatomic findings appeared to correlate with the incom-

plete functional recovery observed in this one monkey. First, it had the least NTN expression within the targeted nigrostriatal system, suggesting less accurate targeting of the striatum compared with the other treated monkeys. Not surprisingly, this monkey also had the least number of TH-positive cells in the nigra (approximately 30% of the intact hemisphere compared with a mean of 70% of the intact hemisphere for all other AAV2-NTN-injected monkeys) and the least TH-positive staining intensity in the both the caudate and putamen (approximately 20% of the intact hemisphere compared with a mean of 50% of the intact hemisphere in all other AAV2-NTN-injected monkeys). Finally, this monkey also displayed the lowest level of pERK activation, compared with the other AAV2-NTN-treated monkeys. Thus, for each treatment-related histological end point evaluated, this monkey displayed results that were between the other treated monkeys and all the control monkeys (ie, more intense signal than all the control monkeys, yet less intense than all the other treated monkeys).

Discussion

Although antiparkinsonian treatments can be effective for a number of years, no current treatment is able to halt, retard, or reverse the progressive loss of motor control or the underlying degenerative process. GDNF, and its naturally occurring structural and functional

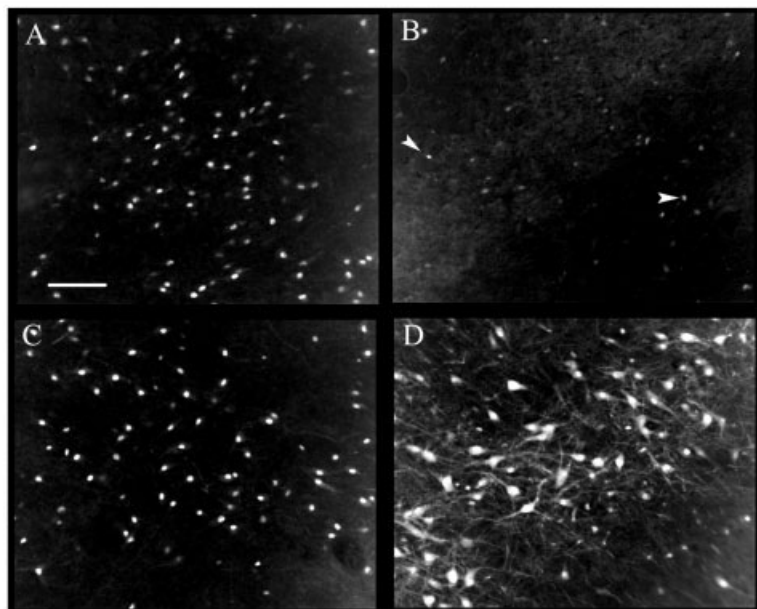


Fig 5. Computer-inverted images illustrating the expression of phosphorylated extracellular signal-regulated kinase (pERK). (A, C) On the control, nonlesioned side, like in N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned control-treated animals, pERK was almost exclusively localized to the nucleus. (B) On the MPTP side of adeno-associated virus-based vector (AAV2) encoding green fluorescent protein-treated monkeys, less pERK-immunoreactive neurons were seen and virtually all display nuclear immunoreactivity (arrowheads). (D) In contrast, robust cytoplasmic pERK was seen in nigral neurons on the MPTP-lesioned, AAV2 encoding human neurturin-treated side. Scale bar = 500 μ m (A–D).

analog NTN, possess the capacity to restore function of nigrostriatal dopamine neurons, retard their degeneration, and protect them from death. Thus, these molecules hold great promise for their potential ability to alter the degenerative process and change the natural course of PD. Although efforts to treat PD patients with brain infusions of GDNF have produced inconsistent and at times disappointing results, the efforts have nonetheless been highly informative. For example, intraventricular delivery of the GDNF protein did not provide expected clinical benefits²¹; the postmortem examination of the brain of patient who had died from causes unrelated to the trial demonstrated that little or no protein reached the degenerating nigral cells from the cerebrospinal fluid.²² Subsequent studies attempting to deliver GDNF protein directly to the postcommissural putamen have reported mixed success,^{10,11,23} whereas the approach continues to be fraught with controversy.^{12–15} Nonetheless, from these efforts a consensus has emerged that accurate and effective targeting and distribution of the protein to the site of neural degeneration is as essential as the trophic molecule used.¹³

Our groups have been collaborating in performing a series of experiments that test the overarching hypothesis that delivery of the NTN cDNA represents a safe and effective means of treating PD. This study provides key evidence in support of this concept. Monkeys rendered parkinsonian with MPTP that received AAV2-NTN (ie, CERE-120) became virtually asymptomatic within a few months after gene delivery and remained so for 10 months after treatment, the final time point examined. In contrast, control animals that received either FB or AAV2-GFP instead of AAV-NTN presented persistent parkinsonian symptoms for the duration of the study. The restoration of motor function achieved with AAV2-NTN was associated with robust preservation of dopaminergic nigral perikarya and a partial preservation of striatal innervation. The link between NTN expression in the targeted striatum, these neuroanatomic measures, and the functional recovery we observed is particularly well-supported by the data of a single AAV2-NTN-treated monkey (Monkey 7177). This monkey displayed the least degree of functional recovery, as well as the least amount of NTN expression, within the targeted striatum. Importantly, this monkey also displayed the greatest TH-ir nigral cell loss and lowest TH-ir striatal optical densities within the treated group, with values roughly midway between the treated and control monkeys. In addition, this monkey displayed the least activation of p-ERK, relative to all the other AAV2-NTN-treated cohorts. Thus, in a monkey where the targeting of AAV2-NTN appeared, by chance, to be suboptimal (based on volume of NTN expression), the behavioral recovery, the restoration of DA-related histological markers, and induction of pERK were all less than all

other treated monkeys (though clearly still greater than that seen in any of the control monkeys). The effects of NTN on dopamine neurons in brain likely occurs through multiple mechanisms. The robust activation of p-ERK, a molecule central to the cascade of events underlying a trophic response in nigral neurons, supports our interpretation that the functional improvement generated by AAV2-NTN on the clinical rating scale in parkinsonian monkeys was due, in part, to atrophic factor mechanism and suggests that this treatment in PD patients could alter the natural progression of the disease. Indeed, that nigral neurons do not degenerate after intracarotid MPTP until well after maximal NTN expression supports the concept that at the level of the substantia nigra, AAV2-NTN is providing neuroprotection. NTN has also been shown to be neurorestorative by promoting the phenotypic upregulation of dopamine neurochemical pathways.¹⁷ Thus, we cannot currently differentiate whether the functional benefit observed in this study was primarily due to a symptomatic benefit of treatment via the neurorestorative process of enhanced dopamine function or via the neuroprotective process of enhanced preservation of dopamine neurons. However, it is this dual mechanism of action of neurotrophic factors such as NTN, providing neuroprotection and symptomatic benefit, that suggests this approach may offer the optimal means to provide potent benefit for PD patients that is quick to achieve and is long-lasting.

Although GDNF has received the most attention as a trophic factor transgene for PD, to date it is notable that the magnitude of *functional recovery*, as determined by the change in clinical rating scale scores presented in this study with AAV2-NTN, is virtually identical to that seen in a similar study that we previously performed with lenti-GDNF.⁹ This is true even though the parkinsonian disability was less severe in this study due to batch-to-batch differences in MPTP potency. Indeed, this reduced level of parkinsonian disability may be fortuitous because it better models early-stage patients, a population that might benefit best from a trophic factor therapy. Indeed, if one considers the percentage change in antiparkinsonian benefit after treatment, AAV2-NTN compares favorably with lenti-GDNF. Although the degree of *neuroanatomical protection* afforded by these two gene therapy approaches appears to favor lenti-GDNF, this apparent difference is entirely consistent with known differences in the kinetics of peak protein expression established for AAV2 (4 weeks) versus lentivirus (2 days) vectors.²⁴ A rapidly progressing nonhuman primate model of PD, such as the MPTP model used here, places AAV2 at a distinct, but artificial (and clinically meaningless), disadvantage; that is, the aggressive, day-by-day nature of the dopamine neuron degeneration in this model does not mimic the gradual year-by-year pace of degen-

eration seen in sporadic human PD. Thus, the delay to achieve peak expression with AAV2 to salvage dopamine fibers in the MPTP model should not hamper prevention of neurodegeneration, which occurs over the course of decades. The robust functional recovery seen after AAV2-NTN, coupled by the substantial neuroanatomic preservation of the degenerating nigrostriatal system under experimental conditions that do not favor such protection, support the concept that AAV2-NTN could provide potent clinical benefit for patients with PD. Interestingly, significant functional recovery was observed 4 months after AAV2-NTN, which is 3 months after maximal gene expression. Indeed, we have previously seen a similar discord between maximal gene expression and functional recovery after lenti-GDNF treatment in parkinsonian monkeys.⁹ It is likely that the molecular and structural events required for functional recovery are complex, and time-dependent events such as synaptic reorganization may be requisites for functional recovery to occur.

Both NTN and GDNF signal through the (REarranged during Transfection) RET receptor, which is synthesized within nigral perikarya.^{25–27} Although at normal physiological levels GDNF preferentially binds to GFR- α_1 and NTN binds to (GFR)- α_2 ,^{27–29} at the supraphysiological levels achieved with exogenous protein delivery or in vivo gene transfer, the two analog proteins each stimulate both receptors. Once either protein is bound to the α receptor, the trophic factor/receptor complex is transported retrogradely to nigral perikarya to bind with RET and initiate cell signaling. Interestingly, in the adult striatum, GFR- α_1 is abundant whereas little GFR- α_2 is expressed.²⁹ The comparable effects of NTN and GDNF in multiple rodent models, as well as young and aged primates,^{6,17} confirms that at the levels delivered by exogenous means, NTN can, indeed, exert robust trophic responses to nigrostriatal neurons through the GFR- α_1 receptor.

Although NTN clearly has the potential to improve function of dopamine neurons and protect them from degeneration and death, harnessing that capacity in the form of an effective and practical treatment for PD (or any other human disease) has remained a formidable challenge. Information gradually accumulated through past efforts suggests that sustained expression of the neurotrophic factor over a substantial proportion of the targeted area (eg, putamen) is likely to be required to achieve consistent and robust therapeutic benefit. Attempts to achieve this with artificial hardware implanted into the brain, whereby a relatively small point source of protein is gradually infused, have not been consistently successful. In addition, attempts to deliver the protein via implanted hardware has raised significant safety concerns in both human (where significant hardware-related adverse events have been noted¹¹) and primate studies (where evidence of protein leakage

from the targeted site is apparent^{30,31}), possibly producing neurotoxicity within the cerebellum far removed from the targeted site.¹³

In contrast with the use of implanted hardware, use of gene transfer offers a practical means of solving these problems, whereas providing continuous and selective expression of the protein throughout the targeted site. Current gene transfer promoters and vectors (such as CAG and AAV2, respectively) can provide long-term (ie, perhaps permanent), targeted expression of the protein throughout the putamen. Among the vectors currently available for gene transfer in humans, AAV2 provides a number of important advantages. It is non-pathogenic in humans, in that it is not associated with any disease or clinical symptoms, despite the fact that the majority of humans have been exposed to it during their normal lives. Numerous studies in the central nervous system have demonstrated that AAV2 induces no inflammatory reactions. Moreover, it is defective for replication in its wild state, and the vector currently used for gene transfer has been completely stripped of its genes and has no capacity for replication. Importantly, as a vector, AAV2 does not integrate into the host chromosome, but rather forms a stable episome in the nucleus. Thus, the therapeutic protein is continuously expressed with little or no risk for insertional mutagenesis. Finally, the kinetics of transgene expression are well established, with stable expression confirmed to persist for years.³² Thus, CERE-120 (AAV2-NTN) appears to possess the essential characteristics required for a safe and effective means of providing long-term trophic support of degenerating dopamine neurons in PD. The data presented in this article offer valuable evidence that it can, indeed, restore and salvage dopamine neurons destined to die in a widely accepted primate model of PD, thus preserving motor function analogous to that lost in PD. These data, therefore, add to the nonclinical results suggesting that CERE-120 (AAV2-NTN) may represent a novel and potentially powerful treatment for PD, and thus support ongoing clinical trials in PD patients.³³

Conclusion

In summary, this study demonstrates that delivery of AAV2-NTN (CERE-120) to the nigrostriatal system of MPTP-treated monkeys virtually eliminates parkinsonian symptoms for up to 10 months, protects dopaminergic nigral neurons, and partially preserves striatal dopaminergic innervation. These functional benefits were associated with increased activation of nigral p-ERK, in support of a trophic mechanism of action. No functional or structural adverse events were observed in this study. Results from this study, coupled with results from numerous other safety and efficacy studies, support the concept that AAV2-NTN (CERE-

120) may potentially be a potent therapy for patients with PD.

Disclosure

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