

Striatal Delivery of CERE-120, an AAV2 Vector Encoding Human Neurturin, Enhances Activity of the Dopaminergic Nigrostriatal System in Aged Monkeys

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Abstract: Neurturin (NTN) is a potent survival factor for midbrain dopaminergic neurons. CERE-120, an adeno-associated virus type 2 (AAV2) vector encoding human NTN (AAV2-NTN), is currently being developed as a potential therapy for Parkinson's disease. This study examined the bioactivity and safety/tolerability of AAV2-NTN in the aged monkey model of nigrostriatal dopamine insufficiency. Aged rhesus monkeys received unilateral injections of AAV2-NTN into the caudate and putamen, with each animal therefore serving as its own control. Robust expression of NTN within the nigrostriatal system was observed 8 months postadministration. ¹⁸F-fluorodopa imaging using positron emission tomography revealed statistically significant increases in ¹⁸F-fluorodopa uptake in the injected striatum compared with the uninjected side at 4 and 8 months. In addition, at 8 months postadministration, a sig-

nificant enhancement in tyrosine hydroxylase immunoreactive fibers and an increase in the number of tyrosine hydroxylase immunoreactive cells was observed in the AAV2-NTN injected striatum compared with the uninjected side. Robust activation of phosphorylated extracellular signal-regulated kinase immunoreactivity in the substantia nigra was also observed. Histopathological analyses revealed no adverse effects of AAV2-NTN in the brain. Collectively, these results are consistent with the neurotrophic effects of NTN on the dopaminergic nigrostriatal system and extend the growing body of evidence supporting the concept that AAV2-NTN may have therapeutic benefit for Parkinson's disease. © 2007 Movement Disorder Society

Key words: AAV2; gene therapy; neurturin; CERE-120; Parkinson's disease; GDNF

Parkinson's disease (PD) is a progressive, debilitating motor disorder that affects over 1 million people in North America and is associated with the degeneration of dopamine-producing neurons in the substantia nigra. Loss of the normal dopaminergic innervation of the striatum as a consequence of nigral degeneration results in the cardinal symptoms of PD, including tremor, rigidity, and

akinesia.¹ Although current therapies can provide symptomatic benefit for a period of time, no approved therapy slows or halts disease progression. One particularly attractive approach for improving function of nigrostriatal neurons, while also slowing or halting further degeneration, is the delivery of neurotrophic factors to the affected neuronal population. Data collected over recent years have demonstrated that several growth factors have neurorestorative and neuroprotective effects on dopaminergic neurons within the substantia nigra. Most notably, glial cell line-derived neurotrophic factor (GDNF) and its naturally occurring structural and functional analog neurturin (NTN) have potent neurotrophic effects on this cell population both in vitro and in vivo.²⁻⁵ For example, NTN or GDNF recombinant protein delivered to either

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the substantia nigra or dopaminergic terminal field in the striatum promotes robust anatomical, neurochemical, and behavioral recovery in several animal models of nigrostriatal degeneration.⁵⁻¹⁶

A challenge in harnessing the potential utility of growth factors for the treatment of neurodegenerative disease lies in achieving sustained, localized delivery to the targeted neuronal population. Initial attempts delivering GDNF protein to the ventricular system failed to demonstrate efficacy and resulted in side effects related to broad distribution of protein affecting nontargeted brain systems.^{17,18} More recent studies utilizing chronic delivery of GDNF protein directly to the putamen have yielded some promising, but mixed results,¹⁹⁻²² and sub-optimal distribution of GDNF throughout the putamen has been hypothesized as one likely reason for lack of reliably robust results.²³ One attractive alternative delivery method is the use of viral vectors. This approach affords the opportunity for much broader exposure of these neurotrophic factors to the targeted nigrostriatal system, while at the same time providing long-term, stable levels of transgene expression.²⁴ We are therefore developing CERE-120, an adeno-associated type 2 viral vector encoding human NTN (AAV2-NTN) as a novel, potentially potent treatment for PD.

In the nonhuman primate, anatomical and neurochemical changes have been observed in the brain with advanced aging. Specific changes such as loss of dopaminergic phenotype and accumulation of α -synuclein in the substantia nigra are similar to what is seen in aged humans and in PD.^{11,25-28} Using the aged monkey as a model system for nigrostriatal degeneration, prior studies demonstrated that delivery of GDNF, either by intraparenchymal infusion of the recombinant protein or by lentiviral-mediated delivery, results in anatomical, neurochemical, and functional restoration of the nigrostriatal dopaminergic system of aged monkeys.^{11,12,15} The purpose of the present study was to assess the potential long-term effects of AAV2-mediated NTN expression on anatomical and functional markers of dopaminergic activity in the nigrostriatal system of aged monkeys.

SUBJECTS AND METHODS

Animals

Three female rhesus monkeys (*Macaca mulatta*) that were of mean age (based on recorded dates of birth) 23.03 ± 0.88 (\pm SEM) years at the time of surgery served as subjects. Food and water were available ad libitum in a temperature-controlled room on a 12-hour light:dark cycle. All experimental procedures were performed according to Rush University Institutional Ani-

mal Care and Use Committee and Institutional Biosafety Committee approved protocols.

CERE-120 (AAV2-NTN) Vector Construction

CERE-120 (AAV2-NTN) was created as detailed previously.⁵ 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 construct, human NTN was expressed from a hybrid cDNA, where the NTN pre-pro domain was replaced by that of human nerve growth factor, to enhance NTN secretion.

Surgical Procedures

Stereotaxic injection sites were selected to evenly distribute AAV2-NTN vector through the majority of the caudate and putamen, based on sites reported previously.¹² AAV2-NTN (3×10^{11} vector genomes) was delivered to the left caudate ($2 \times 30 \mu\text{L}$ deposits) and left putamen ($3 \times 30 \mu\text{L}$ deposits) using a Hamilton syringe and a microinfusion pump at $2.0 \mu\text{L}/\text{min}$. The right brain hemisphere of each animal served as an uninjected control side.

Daily Observations and Body Weight

Animals were observed daily by veterinary staff experienced with the normal care, handling, and behavior of aged nonhuman primates. Body weight was recorded on the day of surgery, periodically throughout the study, and at the time of sacrifice.

¹⁸F-fluorodopa Positron Emission Tomography

All animals underwent ¹⁸F-fluorodopa positron emission tomography (PET) imaging at 4 and 8 months following AAV2-NTN delivery. Animals were placed in a stereotaxic head holder and positioned in a Concorde P4 microPET scanner. ¹⁸F-fluorodopa (185 MBq) was administered over 30 seconds and a 90-min three-dimensional dynamic emission scan was performed. A method modified from that described previously²⁹ was used to quantitate ¹⁸F-fluorodopa uptake rate constants K_{occ} (min^{-1}) in every image pixel in the caudate and putamen and mean K_{occ} values were determined for the left and right caudate and putamen for all animals.

Necropsy

Animals were sacrificed 8 months following AAV2-NTN delivery. Monkeys were deeply anesthetized and perfused transcardially with 0.9% saline followed by fixation with a modified Zamboni's fixative (4% paraformaldehyde/0.18% picric acid in 0.1 M phosphate buff-

ered saline). The brains were removed, immersed in fixative overnight at 4°C, and then cryoprotected. Brains were cut (40 µm thickness) on a microtome and sections were stored in cryoprotectant.

Immunohistochemical Procedures

All immunohistochemical procedures were performed according to well-established, standardized methods. Briefly, primary antibodies were anti-NTN goat IgG (1:200; R&D Systems), anti-TH (tyrosine hydroxylase) sheep IgG (1:20,000; Chemicon), anti-phospho-ERK 1/2 rabbit IgG (1:200; Cell Signaling), anti-CD68 mouse IgG (1:250; VWR), anti-CD45 mouse IgG (1:500; BD Pharmingen), and anti-GFAP mouse IgG (1:2,000; Chemicon). Following labeling, sections were mounted onto glass slides and cover-slipped. Controls consisted of substituting the primary antibody solvent or an irrelevant IgG for the primary antibody.

Quantification of Volume of NTN Protein Labeling in the Striatum

Volumetric analysis of NTN immunolabeling in the striatum was performed with the aid of a computerized microscope with SPOT Advanced software (v3.4, Diagnostic Instruments, Sterling Heights, MI). The total volume was then determined according to the method of Cavalieri as discussed in Ref. 30. Images were digitized from a 1-in-12 series of brain sections spanning the striatum and the area of NTN distribution was subsequently determined by measuring the area of positive staining in mm².

Quantification of TH Immunoreactive Fibers in the Striatum

For optical densitometry measurements, the intensity of TH immunoreactive staining was quantified using the Scion Image system on five sections through the striatum of each animal. Two sections were rostral to the anterior commissure, one at the level of the anterior commissure, and two posterior to the commissure. Briefly, sections were visualized through an Olympus BH2 microscope at 20× magnification and images were digitized by a Javelin Chromachip II camera. The caudate nucleus and putamen were randomly sampled at approximately 100 sites each and the mean optical density of TH staining was quantified. The ambient and microscope light levels were maintained constant across all measurements. Random fields were captured throughout the caudate and putamen, such that approximately 100 measurements were performed per section. Three measurements were also taken from the unstained corpus callosum of each section to estimate background staining intensity, and the

mean of these was subtracted from each recorded striatal intensity measurement to obtain a final optical densitometry value.

Quantification of TH and Phosphorylated Extracellular Signal-regulated Kinase Immunoreactive Cells

Unbiased stereological estimates of the total number of TH and phosphorylated extracellular signal-regulated kinase (pERK 1/2) immunoreactive cells in the SN were performed with the optical fractionator method³¹ using an Olympus BX51 microscope hard-coupled to a Ludl computer-controlled x-y-z motorized stage, a high-sensitivity Hitachi 3CCD video camera system (Hitachi, Japan), and the aid of computer software (StereoInvestigator 2000 software; Micro-BrightField, Colchester, VT). An investigator blinded to the treatment groups performed all analyses. Twelve equispaced sections along the entire SN were evaluated from the caudal level of the mammillary bodies to the caudal level of the inferior colliculus. Quantification of the number and size of TH positive cells was also performed on five sections through the striatum of each animal. Two sections were rostral to the anterior commissure, one at the level of the anterior commissure, and two posterior to the commissure.

Histopathological Analyses

Hematoxylin and eosin staining was performed on a 1-in-24 series of sections through the entire brain, including the cerebellum (approximately 40 total sections per monkey) and evaluated by a board certified veterinary pathologist blind with respect to injection hemisphere. In addition, a 1-in-24 series of sections throughout the brain was stained for activated microglia (CD68), activated leukocytes (CD45), or astrocytosis (GFAP) and analyzed using a qualitative 5-point scale.

RESULTS

General Safety/Tolerability: In-life Measures

No abnormalities in general appearance or in normal behavior were observed during the 8-month duration of this study. No effects were observed on measures of body weight, as all animals either maintained, or slightly increased their body weight. Mean (\pm SEM) body weight was 8.51 (\pm 0.20) kg at surgery and 9.60 (\pm 0.35) kg at sacrifice.

Evaluation and Quantification of NTN Expression

NTN expression by immunolabeling was confirmed in the AAV2-NTN injected hemisphere 8 months following gene delivery and was localized mainly to the targeted

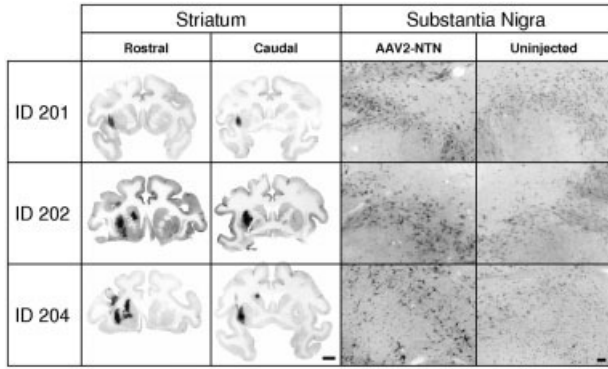


FIG. 1. Coronal sections through the striatum and substantia nigra illustrating positive labeling for NTN within the caudate, putamen (left panels), and substantia nigra (right panels) in the AAV2-NTN injected left hemisphere. Scale bar = 5.0 mm for striatal sections, and 0.1 mm nigral sections ($\times 10$ magnification).

caudate, putamen, and anatomically related regions in the injected hemisphere (Fig. 1). NTN positive fibers in the globus pallidus and substantia nigra, pars reticulata were observed, consistent with anterograde transport of NTN protein by striatal neurons to their terminals. NTN positive perikarya were observed in the pars compacta division of the substantia nigra, a finding consistent with retrograde transport of the vector and/or NTN protein from the striatum. Some NTN immunoreactive staining was also observed in cortical gray and white matter, but was clearly limited to the sites within or adjacent to the injection tracks. Volumetric analysis of striatal NTN labeling revealed between-subject variation that was related to incomplete stereotaxic targeting of all five injections in all three animals (i.e., some injections were placed dorsal to the target into the corpus colosum, or laterally into white matter tracts). The volume of NTN immunolabeling in the caudate and putamen was lowest in the animal with the least accurate number of injections (one of five injections were entirely in the target region; Monkey 201; volume 42.4 mm³), and highest in the animal with the most accurate targeting (all five injections were entirely within the target region; Monkey 204; 251.7 mm³; See Table 1).

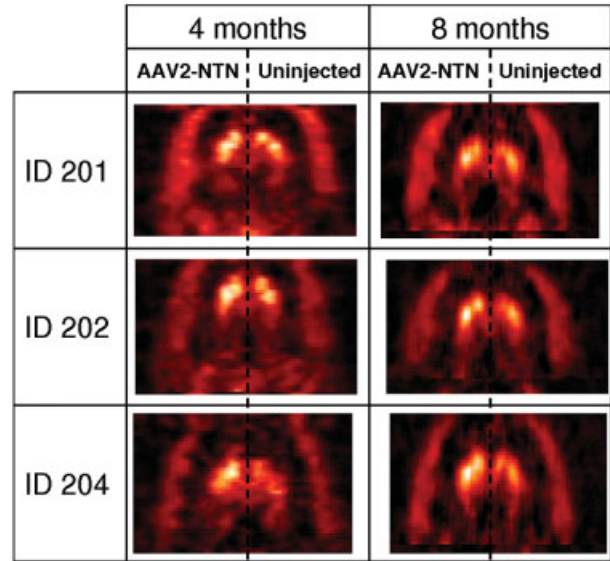


FIG. 2. PET imaging scans illustrating ¹⁸F-fluorodopa uptake in each monkey 4 and 8 months following injection of AAV2-NTN. ¹⁸F-fluorodopa uptake signal was enhanced in the left, AAV2-NTN injected hemisphere at both time-points compared with the uninjected control side.

tions were entirely within the target region; Monkey 204; 251.7 mm³; See Table 1).

¹⁸F-fluorodopa PET

Visual examination of the intensity of ¹⁸F-fluorodopa uptake in PET radiograms of all 3 animals revealed an apparent increase in ¹⁸F-fluorodopa uptake in the caudate and putamen in the AAV2-NTN injected hemisphere at 4 and 8 months following delivery (see Fig. 2 for representative images). The qualitative increase in uptake was confirmed by quantitative analysis of rate uptake constants K_{OCC} (min⁻¹) in the striatum. Rate constants were determined for each monkey by combining the uptake value for each caudate and putamen. Statistical analyses (paired *t* tests) revealed a relatively consistent (~20%) and significant increase in ¹⁸F-fluorodopa uptake in the

TABLE 1. Analyses between individual animals

Monkey ID	Striatum									
	Caudate				Putamen				Substantia Nigra	
	NTN volume (mm ³)	Fluorodopa uptake (% control)	TH OD (% control)	Number of TH+ cells (% control)	NTN volume (mm ³)	Fluorodopa uptake (% control)	TH OD (% control)	Number of TH+ cells (% control)	Number of TH+ cells (% control)	cytoplasmic pERK (% control)
201	23.5	125.61	191.37	502.26	18.9	107.78	171.08	139.10	138.58	1137.48
202	51.3	121.11	205.37	530.81	139.0	121.11	240.75	933.40	147.96	1997.42
204	92.3	124.44	247.82	1524.1	159.5	120.22	261.24	1062.25	113.80	606.07

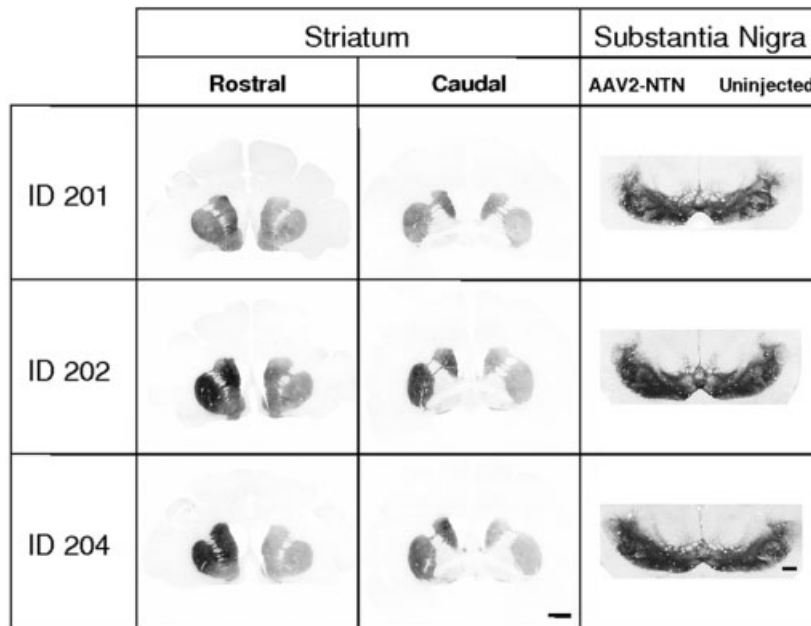
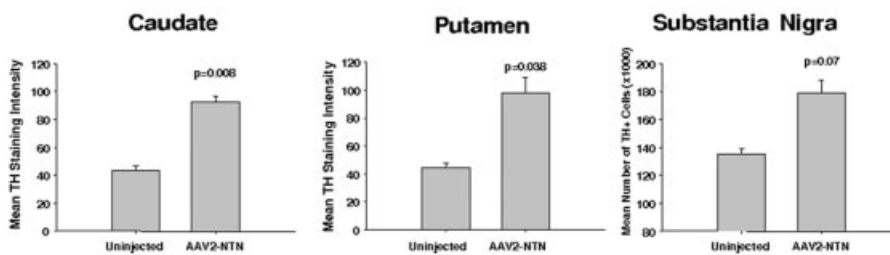


FIG. 3. Coronal sections through the striatum (left) and substantia nigra (right) of each animal illustrating enhanced labeling for TH in the AAV2-NTN injected hemisphere. Scale bar = 5.0 mm for striatal sections, and 0.1 mm for nigral sections ($\times 10$). Quantitative analyses of mean TH staining intensity and mean number of TH positive cells in the substantia nigra are also presented.



AAV2-NTN injected hemisphere at 4 months [$t_2 = 45.03$, $P < 0.001$] which persisted at 8 months [$t_2 = 9.52$, $P = 0.01$] following AAV2-NTN delivery (see Table 1 for the relative difference in ^{18}F -fluorodopa uptake between the 2 hemispheres of each animal at the 8 month time point).

TH Positive Fibers and Cells in the Nigrostriatal System

A robust increase in TH staining intensity in both the caudate and putamen of the AAV2-NTN injected hemisphere was observed in all three monkeys (Fig. 3; Table 1). Quantitative analysis confirmed a significant increase in the staining intensity of TH positive fibers in both the caudate [$t_2 = 11.21$, $P = 0.008$] and putamen [$t_2 = 4.98$, $P = 0.038$] of the AAV2-NTN injected hemispheres, compared with the control hemispheres. Unbiased stereological estimates of the number of TH positive cells in the substantia nigra also revealed an apparent increase in the AAV2-NTN hemisphere (Fig. 4; Table 1), although this effect failed to meet conventional levels of statistical significance [$t_2 = 3.51$, $P = 0.07$].

Given the small sample size and the high degree of between-subject variability, quantification of TH positive striatal cells failed to reveal a significant difference in the AAV2-NTN injected hemisphere compared with the uninjected hemisphere [$t_2 = -2.19$, $P = 0.16$]. However, on average, there were 750% and 545% more TH positive cells observed in the caudate and putamen, respectively, in the AAV2-NTN injected hemisphere (caudate: 70399.67 ± 28677.67 ; putamen: 76101.67 ± 33898.41 ; mean \pm SEM) compared with the uninjected control hemisphere (caudate: 8204.67 ± 1057.24 ; putamen: 11803.67 ± 2239.03) (Fig. 4; Table 1). There was a significant increase in the volume of TH positive striatal cells in the AAV2-NTN injected caudate of approximately 235% [$t_2 = 4.59$, $P = 0.04$] and a trend for an increase (approximately 135%) in the putamen [$t_2 = 3.32$, $P = 0.08$], even given this small sample size. Qualitatively, TH positive cells in the striatum of the AAV2-NTN injected hemisphere were also more intensely stained than those in the uninjected control side.

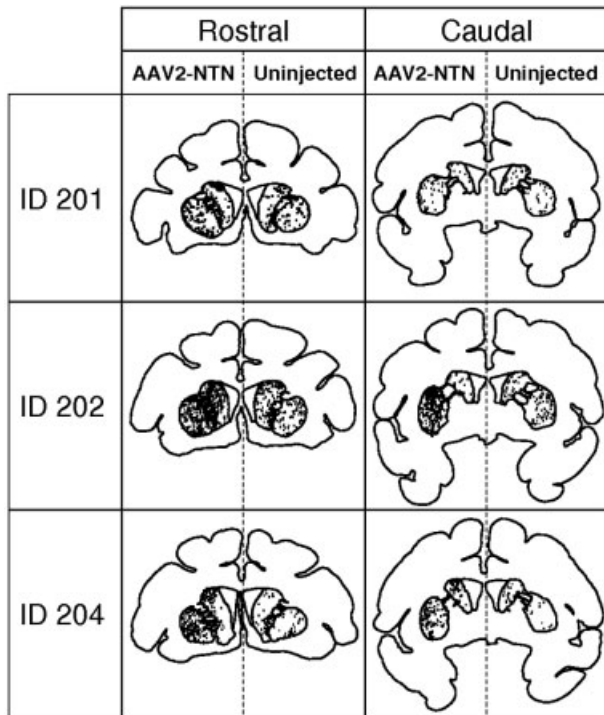


FIG. 4. Coronal reconstructions of the number and location of TH positive cells intrinsic to the caudate and putamen are illustrated for each animal.

pERK 1/2 in the Substantia Nigra

The total number of cells expressing pERK 1/2 was similar in the injected hemispheres and uninjected control hemispheres. However, clear differences in the sub-cellular localization of pERK 1/2 were observed (Fig. 5). In the control hemisphere, pERK 1/2 staining was principally localized to the nuclei of nigral perikarya. In contrast, nigral neurons displayed robust pERK 1/2 immunoreactivity throughout the cytoplasm and nucleus in the AAV2-NTN injected hemisphere. Quantification revealed a significant increase in cytoplasmic pERK 1/2 in the substantia nigra of the AAV2-NTN injected hemisphere [$t_2 = -5.41$, $P = 0.03$], consistent with an activated cellular response as a consequence of the bioactive effects of NTN (also see Table 1).

Histopathology and Immune/Inflammatory Assessments in the Brain

Detailed histopathological analyses performed on sections throughout the brain, including the cerebellum, revealed no microscopic abnormalities related to AAV2-NTN. Staining for immune/inflammatory markers revealed CD45, CD68, and GFAP labeling in regions immediately adjacent to the needle tracks. No other abnormalities were present in any other brain region in

either the AAV2-NTN injected or uninjected hemispheres.

DISCUSSION

This study assessed the long-term anatomical and functional effects of AAV2-mediated NTN expression within the dopaminergic nigrostriatal system of aged monkeys using CERE-120, a vector that is currently being tested in the clinic as a potential therapy for PD. It also provided data on the safety and tolerability of CERE-120 delivery and subsequent NTN expression in aged monkeys.

Expression of NTN protein was observed in all aged animals 8 months following striatal delivery of AAV2-NTN. NTN protein was principally confined to the caudate and putamen, while NTN immunoreactivity was also observed within processes in the globus pallidus and substantia nigra pars reticulata, indicative of anterograde transport of NTN protein. We also observed intense NTN positive immunoreactive cell bodies in the pars compacta region of the substantia nigra, which may be either due to retrograde transport of the vector and subsequent transduction of nigral neurons or retrograde transport of NTN protein. Since these phenomena are not mutually exclusive, and without the ability to effectively distinguish between them, both have to be considered as potential mechanisms underlying the potent bioactive effects of AAV2-NTN in the substantia nigra following delivery to the striatum. Although intersubject variability in the volume of NTN expression in the striatum was observed in this study, all animals displayed enhancements in each measure of bioactivity, as further discussed later.

PET imaging revealed a significant increase in ^{18}F -fluorodopa uptake in the AAV2-NTN injected striatum compared with the uninjected control hemisphere. This effect ($\sim 20\%$ increase) was observed both at 4 months and 8 months post AAV2-NTN delivery. A similar up-regulation of ^{18}F -fluorodopa uptake has been seen previously following vector mediated expression of GDNF within the nigrostriatal system of both young MPTP treated and aged monkeys.¹² It is interesting to note that the magnitude of enhanced ^{18}F -fluorodopa uptake was very similar (20%) between all 3 monkeys at both time points, even though there was variation in the volume of NTN in the striatum as revealed by immunohistochemistry. While preliminary, these data may suggest a ceiling effect on ^{18}F -fluorodopa uptake in aged monkeys, and if true, may be interpreted to suggest it may not be necessary to target 100% of the striatum in order to achieve maximized restoration of nigrostriatal dopamine activity. This concept might be a focus of future studies.

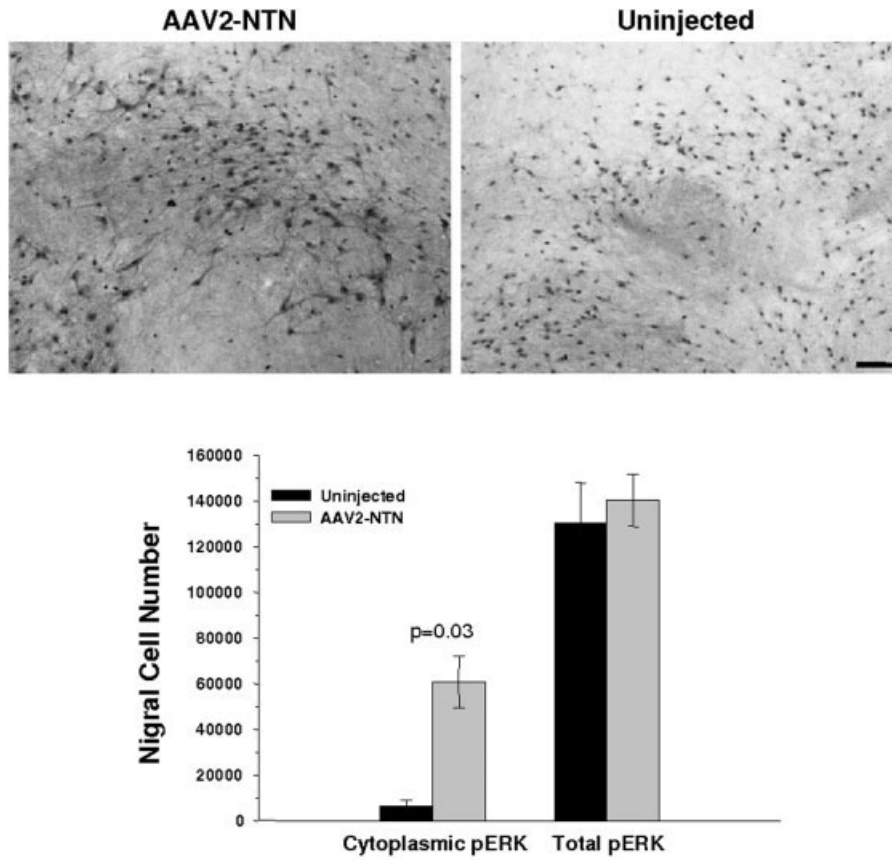


FIG. 5. Photomicrographs illustrating enhanced cytoplasmic pERK 1/2 labeling in the substantia nigra of the AAV2-NTN injected hemisphere compared with the uninjected control side (Monkey 202; median response) and results from quantitative analysis of pERK 1/2 labeling in all animals (Mean \pm SEM).

A robust increase in the labeling intensity of TH immunoreactive fibers was observed in the AAV2-NTN injected striatum of all 3 monkeys, consistent with the predicted bioactive effects of NTN on dopaminergic neurons. Prior studies in young and aged animals have demonstrated that delivery of GDNF and NTN to the monkey nigrostriatal system results in a phenotypic up-regulation of TH.^{12,32} Consistent with a previous report in aged monkeys examining vector-mediated overexpression of GDNF in the nigrostriatal system,³² an increase in the, size, and staining intensity of TH positive cells intrinsic to the striatum was also observed in this study. This population of cells was initially described in the nonhuman primate by Dubach and colleagues.³³ Subsequent studies indicate that these cells coexpress the dopamine transporter,^{32,34} suggesting that they have the capacity to produce dopamine. Thus, the induction of TH in this striatal cell population as a consequence of NTN expression could theoretically provide an additional, local source of dopamine within the striatum, independent from the nigrostriatal projection.

Enhanced pERK 1/2 serves as a marker for the activation of the downstream intracellular signaling mecha-

nisms of both NTN and GDNF on dopaminergic neurons.³⁵ Increased immunoreactivity for pERK 1/2 in the cytosolic compartment of neurons in the substantia nigra was observed in the AAV2-NTN injected hemisphere of all monkeys. Although this finding is consistent with the predicted neurotrophic effects of NTN on this dopaminergic cell population, there have been some reports of potentially deleterious effects following dopamine-mediated activation of pERK 1/2 in the striatum.³⁶ However, no adverse effects of long-term nigral pERK 1/2 activation were observed in the present study (i.e., no abnormal findings when examining the striatum or substantia nigra histopathologically, and there was no loss of TH positive cells in these brain regions). Thus, not only does NTN have neurotrophic effects that are evident in the targeted terminal field of DA neurons (i.e., enhanced ¹⁸F-fluorodopa uptake), but also at the level of the dopaminergic cell bodies, far from the site of injection. A trophic response at the level of the substantia nigra likely reflects retrograde transport of the vector and/or NTN protein, as well as possibly a retrograde signal mediated by exposure of DA terminals to the NTN protein.

It is important to note that enhanced functional and anatomical markers of dopaminergic activity have been previously reported in MPTP treated young monkeys, and in aged monkeys following vector-mediated delivery of GDNF,¹² as well as in MPTP treated young monkeys following delivery of AAV2-NTN³⁷ using an injection paradigm that included both the striatum and the substantia nigra. The present study employed delivery of AAV2-NTN exclusively to the striatum in aged monkeys, resulting in effective distribution of NTN protein throughout the caudate, putamen, and substantia nigra with subsequent robust enhancements in several markers of nigrostriatal function at both the level of the striatum and the substantia nigra.

Studies in rats, monkeys, and humans have previously indicated that nontargeted delivery of growth factors consistently results in adverse side-effects.^{18,38-42} Observational and histopathological analyses were therefore included in this study to characterize the general safety/tolerability of AAV2-NTN delivery to the striatum in aged monkeys. Striatal delivery of AAV2-NTN in aged monkeys was safe and well tolerated, as there were no adverse effects on daily observations or measures of body weight throughout the 8-month study. Furthermore, histopathological analyses performed on sections throughout the brain failed to reveal any adverse effects of AAV2-NTN delivery. In addition, analyses of sections throughout the brain did not reveal any immune or inflammatory reaction on a panel of markers.

The present study therefore provides clear evidence of potent enhancement of functional and anatomical markers of activity of the dopaminergic system in the aged nonhuman primate as a consequence of striatal delivery of AAV2-NTN. Furthermore, this study provides evidence that striatal delivery of AAV2-NTN and subsequent long-term expression of NTN in the aged nigrostriatal system in nonhuman primates is safe and well tolerated. Collectively, these data support the testing of CERE-120 in ongoing clinical trials as a potential therapy for PD, particularly given that age is one recognized risk factor for PD.

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