

Striatal Delivery of Neurturin by CERE-120, an AAV2 Vector for the Treatment of Dopaminergic Neuron Degeneration in Parkinson's Disease

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Glial cell line-derived neurotrophic factor (GDNF) or its naturally occurring analog, neurturin (NTN), can potentially improve the function and delay the rate of degeneration of dopaminergic neurons in Parkinson's disease (PD). However, their delivery to the central nervous system has proven to be a significant challenge. Viral vector-mediated gene transfer offers a practical means to continuously supply neurotrophic factors in targeted areas of the brain. CERE-120 is an adeno-associated viral vector encoding NTN, developed for the treatment of PD. We found that the kinetics and pattern of NTN expression in the rat striatum following injection of CERE-120 is rapid, increases significantly up to 4 weeks, and exhibits a stable volume of distribution thereafter for at least 1 year, the longest time-point evaluated. Quantitative enzyme-linked immunosorbent assay confirmed that steady-state levels are maintained from 4 weeks onward. We demonstrated that NTN volume of distribution can be controlled by varying the dose of vector injected and that NTN delivered via CERE-120 was bioactive, as evidenced by the neuroprotection of DA neurons in the rat 6-hydroxydopamine lesion model. These data provided the foundation for further non-clinical development of CERE-120, leading to an ongoing clinical trial in PD patients.

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INTRODUCTION

Parkinson's disease (PD) motor symptoms result from the progressive loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* of the basal ganglia.^{1,2} Current PD therapies provide temporary relief of motor symptoms but do not prevent disease progression. Therefore, a significant unmet need exists for a therapy that might restore DA function and also slow, halt, or even reverse the ongoing neurodegeneration.

Because of their neuroprotective and neuronal function enhancing properties, neurotrophic factors are attractive candi-

dates for the treatment of neurodegenerative disorders. For PD in particular, glial cell line-derived neurotrophic factor (GDNF) and its naturally occurring analog neurturin (NTN),³ have been shown to enhance survival and function of DA neurons both *in vitro* and in animal models of PD.⁴⁻⁹ To obtain the desired clinical effect, treatment with neurotrophic factors must achieve sustained delivery of an effective dose of the protein in a targeted fashion. It is equally important that unintended spread of the factor beyond the regions of interest be prevented to reduce the risk of adverse events. To date, attempts to treat patients by intraventricular¹⁰ or intraputamenal¹¹⁻¹⁵ infusions of GDNF did not provide anticipated or consistent clinical benefits, respectively, most likely in large part due to the limited diffusion of the protein throughout the region of DA neurodegeneration. Viral vector gene delivery offers the means to achieve a continuous and selectively distributed supply of neurotrophic factors to degenerating neurons in specifically targeted sites within the CNS. Indeed, several groups have established the proof of principle of this method using GDNF gene delivery directly to the nigrostriatal system in animal models of PD (reviewed in McBride and Kordower¹⁶).

In this study we tested the hypothesis that CERE-120, an adeno-associated virus type 2 (AAV2)-derived vector encoding a modified form of the human NTN cDNA could provide localized and sustained delivery of biologically active NTN to the nigrostriatal system. NTN and GDNF signal through the Ret transmembrane protein kinase, upon binding to their cognate receptor, GFR α 2 and GFR α 1, respectively.¹⁷⁻¹⁹ Given their structural homology, NTN and GDNF are each capable of inducing a neurotrophic response through either receptor when exogenous, pharmacological levels are delivered.^{18,20,21} This phenomenon explains why NTN is as potent as GDNF on the adult nigrostriatal system that expresses low levels of GFR α 2 but expresses high levels of GFR α 1.²²⁻²⁵ In the studies presented herein we sought to determine the onset, kinetics, duration, and localization of NTN expression upon delivery of CERE-120 into the rat striatum. This information was necessary to establish the basic pattern of transgene expression and the factors that affect expression in order to design a preclinical development program

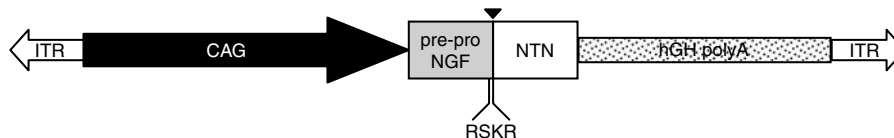


Figure 1 Structure of the CERE-120 vector genome. AAV2 ITRs flank the NTN expression cassette, which consists of the CAG promoter, the pre-pro-NGF-NTN hybrid cDNA and the human growth hormone gene polyadenylation signal. The location of the canonical RXXR sequence derived from the NGF pro-domain, and the cleavage site are shown.

for CERE-120. Our findings indicate that CERE-120 promotes the rapid and long-lasting expression of biologically active human NTN. We also present preliminary evidence that NTN volume of distribution can be controlled, by manipulating the dose of CERE-120 injected, thus achieving targeted delivery of the protein. Finally we report preliminary data on CERE-120 bioactivity in the 6-hydroxydopamine (6-OHDA) rat model of DA neurodegeneration. These studies provided the empirical foundation that justified and guided more extensive efficacy, safety, and tolerability studies in rats and monkeys, which in turn led to the initiation of the ongoing clinical trial in PD patients.²⁶

RESULTS

In vitro CERE-120-derived NTN secretion and bioactivity

The structure of the CERE-120 genome is shown in **Figure 1**. Protein sequence analysis performed on purified, secreted mature NTN from the pre-pro nerve growth factor (NGF)/NTN hybrid cDNA expressed in human cells revealed complete identity of its first five amino terminus residues (Ala-Arg-Leu-Gly-Ala) with those of the naturally secreted NTN (data not shown). The bioactivity of NTN expressed from CERE-120 genome was evaluated *in vitro* using Neuro 2A-GFR α 1 cells. As shown in **Figure 2**, Neuro 2A-GFR α 1 cells treated with NTN from the conditioned medium of CERE-120 plasmid transfected cells underwent differentiation, as evidenced by cell soma hypertrophy and the development of neurites, similar to cells treated with either recombinant human (rh) NTN or rhGDNF. In contrast, cells treated with conditioned medium from cells transfected with an AAV2-GFP plasmid did not differentiate.

Onset and kinetics of NTN expression

Following intrastriatal administration of CERE-120, immunostaining for NTN through serial coronal brain sections revealed that the protein was detectable, mostly in the cytoplasm of striatal cells, as early as 2 days postvector injection, the earliest time point evaluated (**Figure 3**). By day 5 postinjection, NTN was clearly present in the striatal extracellular matrix and a further increase in the volume of staining was observed between day 5 and day 10 (1.06 ± 0.2 to 2.3 ± 0.17 mm³). One-way analysis of variance confirmed a significant effect of time on volume of NTN distribution in the striatum ($F(2,15) = 51.16$, $P < 0.001$) from 2 to 10 days.

Analysis of the longer-term kinetics of NTN expression up to 12 weeks revealed a significant increase in volume of NTN distribution as a function of time ($F(6,34) = 24.57$, $P < 0.001$) (**Figure 4a**). *Post hoc* analysis (Tukey test) confirmed the

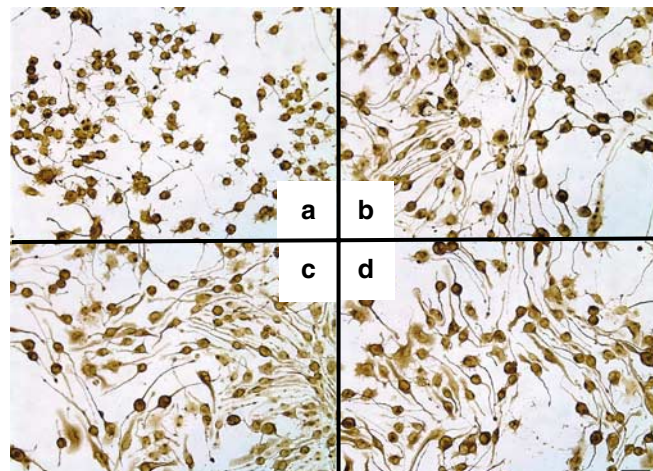


Figure 2 Bioactivity of CERE-120-derived NTN in Neuro2A-GFR α 1 cells. Representative photomicrographs of β -tubulin immunolabeled Neuro2A-GFR α 1 cells following treatment with conditioned medium from cells transfected with AAV2-GFP (**a**) or conditioned media from cells transfected with the CERE-120 genome diluted to contain 50 ng/mL NTN (**b**). Positive controls of differentiation were obtained by treating cells with 50 ng/mL of either rhNTN (**c**) or rhGDNF (**d**). Note the somatic hypertrophy and neurite outgrowth in differentiated cells **b-d** compared to undifferentiated cells **a**. Bar = 50 μ m.

significant and pronounced increase in NTN volume over the first 4 weeks following CERE-120 injection ($P < 0.001$) with no significant additional increase from 6 to 12 weeks postdosing ($P > 0.05$; **Figure 4a**).

In other non-clinical studies conducted to establish the safety and efficacy of CERE-120 in naive or 6-OHDA-lesioned rats (EP Brandon *et al.*, MA Printz *et al.*, manuscripts submitted), animals received a dose of 4×10^9 vector genomes (vg)/striatum of vector divided in two equal injections. In a total of five studies spanning 4 to 55 weeks, measures of NTN volume of distribution further confirmed that protein spread did not increase past week 4 following vector administration (**Figure 4b**). Additionally, no appreciable expansion beyond the border of the striatal target occurred over 12 months post-CERE-120 injection, with NTN mainly confined to the striatum and its neuroanatomically related regions (*i.e.*, globus pallidus, nigrostriatal pathway, and substantia nigra (SN)), though a faint signal was seen in the cortex in the vicinity of the needle track (**Figure 4c**). In another study where animals were injected with 4×10^9 vg/striatum, ELISA was used to quantify the amount of NTN protein in dissected striatal homogenates over several time points up to 14 weeks post-CERE-120 administration. In addition, a subset of animals from this study were used to

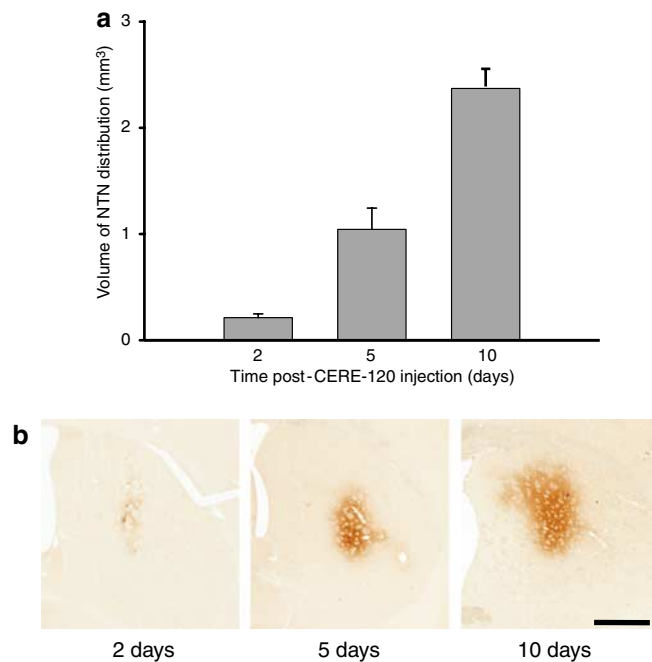


Figure 3 Onset of NTN expression following CERE-120 injection. Animals received single bilateral injections of 2×10^9 vector genomes (vg) of CERE-120 per striatum and were killed at 2, 5, and 10 days following injection. **(a)** Histogram presenting the mean volume of NTN protein detected by IHC throughout the striatum. Error bars represent SEM, $n=6$ per time point. **(b)** Representative coronal sections illustrating the NTN IHC signal in the striatum for each time point. Bar = 1.0 mm.

Table 1 NTN protein in striata via ELISA

Weeks post-CERE-120 injection	NTN (ng/striata) ^a
2	5.8 ± 1.9
4	14.7 ± 2.8
6	15.0 ± 1.9
8	14.7 ± 0.6
10	18.2 ± 2.1
12	9.1 ± 0.9
14	12.1 ± 1.6

^aValues represent the sum of the amount of NTN measured in the left and the right striata of rats injected with 4×10^9 vg of CERE-120 per striatum (mean \pm SEM, $n=4$). ELISA, enzyme-linked immunosorbent assay; NTN, neurturin; vg, vector genomes.

determine volume of distribution of NTN by immunohistochemistry (IHC) at 2, 4, 8, and 12 weeks postinjection. Data show that similar to what was previously observed, significant increase of NTN protein in the striata occurs over the first 4 weeks following injection, with no further significant increase up to 14 weeks, the longest time point evaluated ($F(6,21) = 5.26$, $P = 0.002$; Tukey *post hoc* $P > 0.05$). IHC analyses from this study also confirmed that NTN volume of distribution does not increase significantly after 4 weeks post-CERE-120 injection (Table 1).

Dose response and volume of distribution

NTN volume of distribution produced by different doses of CERE-120 was measured 4 weeks postinjection (*i.e.*, after steady-state expression had been reached). The volume of distribution of NTN increases sharply with the dose of CERE-120 from 7×10^7 to 6×10^8 vg (1.15 ± 0.26 to 3.15 ± 0.50 mm³), but begins to approach a plateau at a three-fold higher dose; *i.e.*, 4.01 ± 0.68 mm³ for 2×10^9 vg (Figure 5). In a separate study, we found that at steady state of expression, the volume of transgene distribution is independent of the volume of injection, as a single dose of 2×10^9 vg delivered in 1, 3, or 10 μ L resulted in a volume of distribution of NTN averaging at 7.6 ± 0.3 mm³ (data not shown). Thus, the total dose of CERE-120 (in vg/striatum) appears to be far more important than either the concentration or the total volume delivered.

In vivo bioactivity of CERE-120 in 6-OHDA rat model of nigral DA neuron degeneration

In this study, rats received CERE-120, AAV2-GDNF, or formulation buffer (FB) 2 weeks prior to a 6-OHDA injection. NTN and GDNF IHC analyses confirmed the robust expression of both neurotrophic factors 4 weeks post-vector administration (*i.e.*, two weeks post-lesion; data not shown). TH immunolabeling in coronal sections through the SN from animals of each treatment group showed clear neuroprotective effects of both CERE-120 and AAV2-GDNF on TH-positive cells (Figure 6a). Consistent with the well-characterized effects of striatal 6-OHDA administration, animals that received FB exhibited a profound loss of TH signal in the hemisphere receiving the toxin, compared to the intact contralateral side. Unbiased stereological estimates revealed a 70% decrease in the number of SN TH-positive DA neurons in the FB treatment group (Figure 6b). In contrast, animals treated with either CERE-120 or AAV2-GDNF showed significant protection of SN TH-positive neurons from the 6-OHDA lesion ($F(2,23) = 41.97$, $P < 0.001$). *Post hoc* Tukey test analysis revealed equivalent TH-positive cell number in both vector-injected groups ($P > 0.05$).

DISCUSSION

The neurotrophic factors GDNF and its naturally occurring structural and biological analog, NTN, have the potential to restore function and prevent degeneration of DA neurons in PD. However, their use as therapeutic agents has been hampered by the lack of an effective method to deliver them properly to the target brain regions, namely the striatum and SN. The importance of adequate distribution of neurotrophic factors in achieving robust therapeutic effects was recently underlined in a study performed in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine primate model of PD. Animals' motor symptom improvement following midbrain infusion of GDNF correlated significantly with the volume of distribution of the neurotrophic factor through the targeted parenchyma.²⁷ This study reinforced the need for a delivery method that can achieve widespread coverage of protein throughout the targeted region. It has also been recognized for years that sustained levels of the protein must also be achieved. Viral vector gene transfer constitutes a

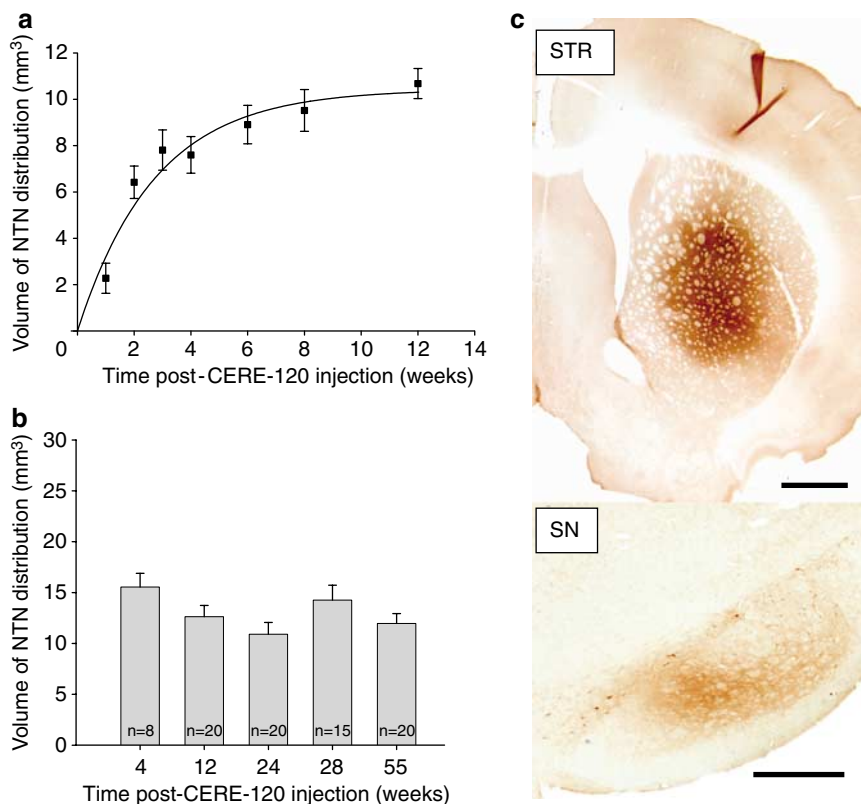


Figure 4 Long-term kinetics of NTN expression in the rat striatum following CERE-120 injection. **(a)** Animals received a dose of 2×10^9 vg of CERE-120 in each striatum and were killed at various time points over a 12-week period. For each time point, mean volumes were obtained from both hemispheres of 3 animals ($n=6$, except for the 8 week time point $n=5$). Error bars represent \pm SEM. **(b)** Analysis of NTN volume of distribution across five different studies spanning different time points between 4 and 55 weeks. In each experiment, animals received two $2 \mu\text{L}$ injections of CERE-120 for a total dose of 4×10^9 vg/striatum either unilaterally or bilaterally, depending on the study. NTN expression persisted up to 55 weeks post-vector administration and did not increase significantly once asymptote is reached, *i.e.*, at week 4. Error bars represent the SEM. **(c)** Sustained and localized expression of NTN 12 months following striatal CERE-120 injection. Representative photomicrograph of coronal sections illustrating robust detection of NTN in the striatum (STR) and SN of rats injected with CERE-120 at a dose of 4×10^9 vg/hemisphere. Bar = 1.0 mm (STR) and 0.5 mm (SN).

potential means to fulfill these two essential delivery requirements. In this report, we present evidence demonstrating that CERE-120, an AAV2 vector encoding human NTN can deliver biologically active protein to a large portion the nigrostriatal system in a sustained, targeted, and controlled manner. The results from these studies provided the foundation for further development of CERE-120 for the treatment of DA neurodegeneration in PD.

AAV2 vectors offer many advantages over other vector systems. They generally do not induce inflammatory reaction upon injection to the brain, and the transferred vector genome remains almost exclusively episomal,²⁸ minimizing the possibility of insertional mutagenesis such, as described with the use of retroviruses.²⁹ In addition, AAV2 based vectors provide long-term expression in the brain (over 4.5 years in monkeys³⁰), and have recently been used safely in clinical trials for several neurological disorders, including PD,^{31,32} Alzheimer's disease,³³ and inborn errors of metabolism.^{34,35}

In the CERE-120 vector genome, the NTN pre-pro region was replaced with that of human β -NGF in order to promote efficient secretion from transduced cells. The poor secretion of NTN with its natural pre-pro domain had previously been

identified and similar hybrid constructs were employed in other NTN gene transfer studies.^{6,36} The CERE-120 NGF/NTN hybrid cDNA was designed so that upon cleavage, the secreted mature NTN peptide would be released from the NGF pro-domain, therefore generating two products naturally encountered in the human brain. Protein sequencing of the secreted NTN resulting from CERE-120 genome expression revealed complete conservation of the protein N terminus, confirming that, as anticipated, the protein precursor cleavage occurred at the NGF pro-domain RSKR sequence. The modification of the NTN cDNA did not affect the bioactivity of the secreted molecule either *in vitro* or *in vivo*.

The kinetics and pattern of expression of NTN following CERE-120 injection in the rat striatum were analyzed in various studies using two different doses of vector (2×10^9 and 4×10^9 vg/striatum), delivered as $2 \mu\text{L}$ injections in one or two sites per striatum, respectively. Using IHC and ELISA methods, we found that NTN is rapidly expressed upon CERE-120 administration, reaches steady state after 4 weeks, and remains stable with no further increase for up to 12 months, the longest time point examined. In the course of our rat studies, we had determined that the volume of distribution of NTN began to

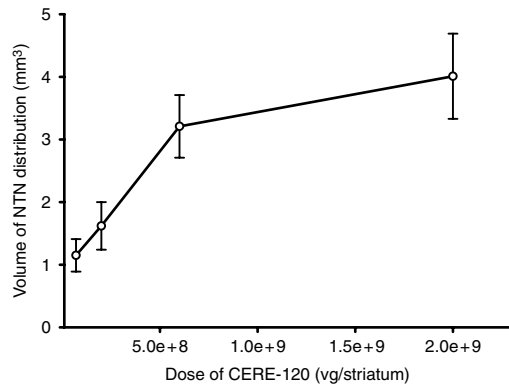


Figure 5 CERE-120 dose response in the rat striatum. A range of CERE-120 doses (three-fold increments: 7×10^7 , 2×10^8 , 6×10^8 , and 2×10^9 vg) were injected in single sites in each striatum of 12 rats ($n = 6$ /dose). Rats were killed 4 weeks post-vector administration and mean NTN volume of distribution for each dose was plotted against CERE-120 dose administered. At steady state of expression, NTN striatal distribution approaches asymptote at a dose of approximately 6×10^8 vg/striatum. Errors bars represent the SEM.

reach a plateau at approximately 2×10^9 vg/striatum (**Figure 5**), and that in order to cover the widest striatal area possible two injections of vector would be needed in efficacy studies in the 6-OHDA rat model of nigrostriatal neurodegeneration. The apparent plateau achieved with NTN volume of distribution following a single site infusion of CERE-120 is consistent with the saturation of transduceable cells in the vicinity of vector injection and the limited spread of vector to a wider area that would otherwise allow for transduction of a larger number of cells. Accordingly, multiple injection paradigms were adopted for the subsequent monkey studies as well as for the ongoing clinical trial.

IHC analysis over 12 months postinjection established that the NTN signal was mostly restricted to the striatum and its anatomically related structures. The presence of NTN signal in the SN was expected because of anterograde and retrograde transport of the protein as well as retrograde transport of the vector from the striatum.³⁷ Using AAV2-GFP, we confirmed that some of the vector indeed is retrogradely transported from the striatum, transducing DA neurons in the SN (data not shown). This aspect of AAV2 gene transfer should allow NTN to exert a protective effect in a paracrine fashion to neighboring cells whose striatal terminals have degenerated and therefore could not otherwise benefit from exogenous NTN administered in the striatum.

In the absence of a practical and safe system for regulating transgene expression in humans, it was particularly important to establish that the levels and location of NTN expression can be predicted and controlled by targeted delivery of CERE-120 dose. The data from these rat studies demonstrate that the volume of NTN distribution can be controlled by the dose of vector injected and thus with proper targeting of the vector injection, the expression and spread of protein could be predicted and limited to the targeted brain structures. These findings in the rat were corroborated in monkey studies (the details of which are presented elsewhere:³⁸⁻⁴⁰ and CD Herzog, manuscript

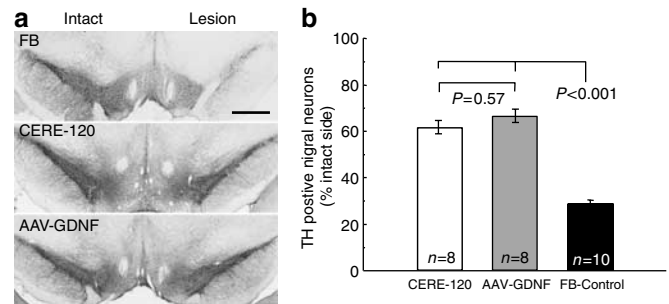


Figure 6 (a) Protection of TH positive cells in the SN following CERE-120 or AAV2-GDNF delivered to the striatum. Representative photomicrographs of TH IHC staining in the SN from 6-OHDA lesioned and intact hemispheres of animals injected with FB, CERE-120, or AAV2-GDNF. A profound reduction in the number of TH positive cells on the lesioned side in FB-treated animals is observed (upper panel). In comparison, the TH signal on the lesioned side of animals treated with either CERE-120 or AAV2-GDNF is virtually indistinguishable from that of the intact side, confirming CERE-120 bioactivity *in vivo*. Bar = 0.5 mm. (b) Nigral TH-positive cells survival 2 weeks after striatal 6-OHDA lesion. Values shown are the mean percentage of the number of TH positive neurons relative to the unlesioned side. There is a significantly greater number of TH positive cells in the vector treated animals compared to FB treated ones. The effect size of CERE-120 is not different from that of AAV2-GDNF. Error bars represent \pm SEM.

submitted), which confirmed that upon CERE-120 infusion in the striatum, the spread of NTN is stable over time once steady state is reached, and expression is confined to the targeted nigrostriatal system.

Lastly, we confirmed that NTN expressed via CERE-120 is biologically active *in vivo*, by demonstrating neuroprotection of rat DA neurons exposed to the neurotoxin 6-OHDA. Clear efficacy with CERE-120 was demonstrated, comparable to that of AAV2-GDNF tested in parallel, and of similar magnitude to that demonstrated in other reports of nigral cell protection following delivery of NTN or GDNF via various methods.^{5,9,16,36,41-44}

In conclusion, the data presented herein demonstrate that: (1) delivery of CERE-120 to the rat striatum results in rapid, robust, and persistent expression of NTN; (2) NTN volume of distribution in the rat brain can be predicted and controlled by manipulating the dose of CERE-120 administered; (3) a large portion of the rat striatum can be covered with CERE-120-derived NTN without substantial protein distribution to non-targeted sites in the brain; and (4) NTN expressed via CERE-120 is biologically active and is as efficient as GDNF in preserving DA neurons from 6-OHDA induced degeneration. Together with results obtained from longer term studies aimed at determining the safety/toxicology and efficacy of CERE-120 in both rats and monkeys, these data established the rationale for the initiation of a clinical program to evaluate the safety and efficacy of CERE-120 in advanced PD patients.²⁶

MATERIALS AND METHODS

Vector plasmid constructs. The CERE-120 vector genome contains the AAV2 inverted terminal repeats (ITRs) flanking a transgene expression cassette containing the CAG promoter⁴⁵ and the human growth hormone gene polyadenylation signal (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 NGF by recombinant PCR method.⁴⁶ The details of cloning procedures and primer sequences are available upon request. Plasmid clones identity was confirmed by restriction digestions and nucleotide sequence determination.

Cell culture and vector production. Vectors were produced by standard triple plasmid transfection technique in 293 cells⁴⁷ and purified by filtration, affinity (heparin), and ion exchange chromatography followed by centrifugal filtration/concentration and resuspended in FB (PBS, 2 mM MgCl₂). Vector titer was determined by quantitative PCR using vector specific primers.

NTN ELISA. The NTN ELISA developed at Ceregene uses a recombinant form of the NTN GFR α 2 receptor to capture bioactive NTN dimers. Details are presented in the **Supplementary material**.

For *in vivo* expression analysis, the left and right striata of injected animals were freshly dissected and separately homogenized in HB (20 mg wet tissue/mL in 250 mM NaCl, 0.5% BSA, 5 mM EDTA, 2% CHAPS in PBS) containing 0.125% protease inhibitor cocktail using the FastPrep lysing matrix system (Qbiogene, Carlsbad, CA). Upon centrifugation, supernatants of each containing 20 mg equivalent of wet tissue were submitted to NTN ELISA analysis. The data are reported as the mean of the sum of the amount of NTN (ng) measured in the left and right striata ($n = 4$).

Bioactivity of CERE-120-derived NTN in vitro. CERE-120-derived NTN bioactivity *in vitro* was determined on Neuro2A-GFR α 1 cells (gift from E. Johnson, Washington University, St Louis, MO). Neuro2A-GFR α 1 is a murine neuroblastoma cell line stably transfected with GFR α 1 conferring the capability to differentiate in the presence of either GDNF or NTN. Upon differentiation, cells increase in size and develop neurite-like processes that can be clearly visualized at low power magnification after immunostaining for β III-tubulin, a marker of cellular processes.

Neuro2A-GFR α 1 cells plated onto chamber slides (Nalge Nunc International, Naperville, IL) were incubated for 72 h with 50 ng/mL rhNTN (Peprotech, Rocky Hill, NJ), 50 ng/mL rhGDNF (Peprotech), or conditioned media from CERE-120 genome (normalized to 50 ng/mL NTN) or AAV2-GFP genome transfected cells. Treated Neuro2A-GFR α 1 cells were then fixed and processed for anti- β -tubulin (β III) IHC using a rabbit polyclonal antibody (Covance Research Products, Berkeley, CA). Growth factor-induced differentiation was compared between treatment groups by examination of the relative size of cell bodies and the presence of neurite extensions.

Surgical procedures. All animal care and procedures were in accordance with IACUC and NIH guidelines. Sprague-Dawley rats (Harlan, Indianapolis, IN) were used in all *in vivo* experiments. Animals received injections of CERE-120, AAV2-GDNF, or FB at a continuous flow rate of 0.5 μ L/min using standard stereotaxic surgical procedures. Vectors were diluted to the appropriate concentration with FB as needed.

For the kinetics of NTN expression analyses, rats received 2 μ L injections bilaterally in single sites at coordinates AP +0.4, ML \pm 3.0, DV -5.0, of 2 \times 10⁹ CERE-120 vg per striatum. For the analysis of NTN volume of distribution across studies (**Figure 4b**), animals received two 2 μ L injections of CERE-120 for a total dose of 4 \times 10⁹ vg/striatum either unilaterally or bilaterally depending on the study. Coordinates were AP +1.0, ML \pm 3.0, DV -5.0 and AP -0.6, ML \pm 3.5, DV -5.0. For the neuroprotection study, animals received two 2 μ L injections in the right striatum only for a total dose of 4 \times 10⁹ vg of either CERE-120 or AAV2-GDNF (positive control) as described above. Negative control animals

received the same injection volumes of FB. Two weeks postvector or FB administration, animals received, in the same side as treatment, a single 5 μ L injection of 20 μ g 6-OHDA (Acros Organics, Geel, Belgium) dissolved in a solution of 0.05% ascorbic acid in 0.9% saline. The coordinates for the injection were: AP +0.2, ML -3.0, DV -5.0. Following surgeries, the incisions were closed and animals were allowed to recover in a clean cage placed on a heating pad and with free access to food and water.

Histology and immunohistochemistry analyses. At the appropriate time point, animals were deeply anesthetized with an anesthetic cocktail consisting of ketamine, xylazine, acepromazine, and intra-cardially perfused with 150–200 mL of saline followed by 300–400 mL of a modified Zamboni's solution (4% paraformaldehyde, 0.18% picric acid). Brains were removed and further processed for sectioning using a freezing-stage microtome (details available in **Supplementary material**).

For all IHC staining procedures, brain sections were pretreated with hydrogen peroxide in TBS and processed for NTN IHC, GDNF IHC, and TH IHC using a goat anti-human NTN polyclonal antibody (R&D Systems), a goat anti-GDNF polyclonal antibody (R&D Systems) and a rabbit anti-TH polyclonal antibody (Pel-Freez, Milwaukee, WI), respectively. IHC procedure details are available in the **Supplementary material** section. Stained sections were placed onto glass slides, dried overnight, mounted with DPX Mounting Medium (Electron Microscopy Sciences, Ft, Washington, PA), and coverslipped.

Volumetric analysis of striatal NTN distribution. For volumetric analysis, images were captured from 1-in-12 series of brain sections spanning the striatum using a microscope interfaced with a camera and SPOT Advanced software (v3.4, Diagnostic Instruments, Sterling Heights, MI). The area of NTN distribution was subsequently determined by measuring the area of positive staining in mm². The total volume was then calculated using Cavalieri's formula: $V = A \times F \times T$ where A is the total sum of areas calculated across all measured sections; F is the sampling frequency (*i.e.*, 12 for a 1-in-12 series); and T is the sections thickness.

TH positive nigral cell counts. The optical fractionator method⁴⁸ was used to estimate the total neuronal cell population in the *substantia nigra pars compacta* using a computerized microscope with the StereoInvestigator software v5.0 (MicroBrightField Inc., Colchester, VT). The sections used for counting the TH-positive neuronal cell population spanned the rostral tip of the *substantia nigra pars compacta* to the caudal end of the *substantia nigra pars reticulata*. Results are presented as the percentage of TH-positive cells in the lesioned hemisphere relative to the number of neurons present in the intact hemisphere.

Striatal fiber optical densitometry. Optical densitometry was performed on TH-positive fibers in the striatum using the NIH Image J software v1.3 (National Institute of Health). For each animal, the average optical density of four separate sections through the striatum was determined. According to the atlas of Paxinos and Watson,⁴⁹ the approximate sections were as follows relative to bregma: +0.22 mm, -0.26 mm, -0.74 mm, and -1.22 mm. Optical densitometry from a region of cortex immediately adjacent to the corpus callosum was used to correct striatal optical densitometry measurements for non-specific background.

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SUPPLEMENTARY MATERIAL

Materials and Methods.

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