

AAV2-mediated delivery of human neurturin to the rat nigrostriatal system: Long-term efficacy and tolerability of CERE-120 for Parkinson's disease

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Neurturin (NTN) is a neurotrophic factor with known potential to protect and restore the function of dopaminergic substantia nigra neurons whose degeneration has been most closely linked to the major motor deficits in Parkinson's disease (PD). CERE-120, an adeno-associated virus serotype 2 (AAV2)-based gene delivery vector encoding human NTN, is being developed as a potential therapeutic for PD. In a series of preclinical studies reported herein, CERE-120 delivery to the striatum produced a dose-related neuroprotection of nigrostriatal neurons in the rat 6-hydroxydopamine (6-OHDA) lesion model. Long-lasting efficacy of CERE-120 was evidenced by substantia nigra cell protection, preserved fiber innervation of the striatum, and behavioral recovery for at least 6 months. In addition, striatal infusion of CERE-120 was found to have a safety and tolerability profile devoid of side effects or toxicological responses, for at least 12 months post-treatment, even at dose multiples 125 times that of the lowest efficacious dose tested. These results support the ongoing CERE-120 clinical program in PD patients.

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Introduction

Because neurotrophic factors can enhance neuronal function and protect neurons from further degeneration, they are attractive candidates for the treatment of neurodegenerative disorders. For PD in particular, glial cell line-derived neurotrophic factor (GDNF) and its naturally occurring structural and functional analog,

neurturin (NTN) (Kotzbauer et al., 1996), have been shown to enhance survival and function of dopaminergic (DA) neurons both *in vitro* and in animal models of PD (Hoane et al., 1999; Horger et al., 1998; Oiwa et al., 2002; Rosenblad et al., 1999; Tseng et al., 1998). A major hurdle in the development of neurotrophic factor therapy is achieving sustained delivery of an effective dose of the protein throughout the targeted region. Among other possibilities, it has been hypothesized that the inconsistent clinical benefits of striatal GDNF infusion in PD patients (Gill et al., 2003; Lang et al., 2006; Love et al., 2005; Slevin et al., 2005) could in part be explained by poor diffusion of the protein throughout the targeted striatum from the single point source (Salvatore et al., 2006; Sherer et al., 2006). 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. Indeed, several groups have established proof of principle for GDNF gene delivery directly to the nigrostriatal system in animal models of PD (McBride and Kordower, 2002). Our group has recently demonstrated that striatal administration of an adeno-associated virus type 2 (AAV2)-derived vector encoding a modified form of the human NTN cDNA (also called CERE-120) resulted in localized and sustained delivery of biologically active NTN to the nigrostriatal system in rats (Gasmi et al., 2007) and provided symptomatic and anatomical benefit in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkey model of PD (Kordower et al., 2006). AAV serotype 2 (AAV2) offers a number of advantages for purposes of near-term vector product development and approval. AAV2-derived vectors are the most well-characterized AAV vectors and have been shown to be safe and well tolerated in several clinical CNS trials (Crystal et al., 2004; During et al., 2001; McPhee et al., 2006). In addition, AAV2 has a very low potential for insertional mutagenesis, exhibits a virtually exclusive neurotropism in the brain and promotes sustained transgene expression in cerebral tissue (reviewed in McCown,

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2005). These characteristics have elevated AAV2 to the preferred vector for the majority of new gene transfer programs for CNS applications.

As an important prerequisite to clinical testing of CERE-120 in PD patients, an extensive series of preclinical studies testing the efficacy and safety of CERE-120 in rats and monkeys has been conducted. Here we report the results of several independent studies which collectively demonstrate that expression of neurturin following CERE-120 administration: (1) occurs in an orderly, dose-related fashion; (2) is stable up to 12 months, the longest time point examined; (3) produces long-term, dose-related anatomical and functional efficacy in the rat 6-hydroxydopamine (6-OHDA) lesion model of nigrostriatal degeneration; and (4) is safe and well tolerated, producing no adverse effects on any of the numerous safety and toxicology endpoints examined, even at doses up to 125 times higher than those required for efficacy. These results add to the growing wealth of data establishing the safety and efficacy of CERE-120 in animals, thus supporting an ongoing clinical program in PD.

Materials and methods

Viral vector construction and production

CERE-120 vector design, construction, production and purification methods were as recently described (Gasmı et al., 2007). Upon purification, vectors were resuspended in formulation buffer (FB) (PBS, supplemented with 2 mM MgCl₂) and vector titers were determined by QPCR and expressed in vector genomes/mL (vg/mL). CERE-120 lots ranged from 2.1×10^{12} vg/mL to 8.3×10^{12} vg/mL. Because of the large volume of data previously published with AAV2–GDNF, it was included as a positive control for select outcomes in the 6-OHDA efficacy study. It was produced using methods identical to those for CERE-120 and its titer was 9.6×10^{11} vg/mL.

Animals

All experiments were conducted in accordance with the guidelines of the Office of Laboratory Animal Welfare and the Ceregene, Inc. Institutional Animal Care and Use Committee. A total of 283 rats Sprague Dawley rats (Harlan, Indianapolis, IN) were used for these experiments. For the dose–response and long-term 6-OHDA lesion model experiments, a total of 73 male rats were used. 60 male and 60 female young adult Sprague-Dawley rats (approximately 10 weeks old and 230–360 g (males) or 205–290 g (females) were used in the long-term safety experiment. Rats were provided with food and water *ad libitum* and maintained on a 12-h light/dark cycle. In addition, 90 animals (males and females of similar age and weight as above) were used in a separate study where cerebellar toxicity was assessed.

Surgical procedures

Vector administration

Animals were anesthetized with ketamine/xylazine/acepromazine cocktail and injections were made using a stereotaxic frame (Stoelting, Wood Dale, IL). Either two 2- μ L or two 5- μ L injections of vector were made per injected hemisphere in each experiment. Injection coordinates to target the striatum were: (Injection 1) +1.0 mm anterior to bregma, ± 3.0 mm from midline,

and 5.0 mm below the dura and (Injection 2) –0.6 mm posterior to bregma, ± 3.5 mm from the midline, and 5.0 mm below the dura. Injections were performed with a 26 G stainless steel needle attached to a glass syringe (Hamilton), pre-coated with concentrated vector to prevent vector adsorption and assure accurate dosing. Injections were made at a rate of 0.5 μ L/min using an automated pump (Stoelting).

Vectors were diluted with formulation buffer (FB) to achieve the desired doses normalized by vector genome for each experiment. In the dose–response efficacy study employing the 6-OHDA lesion model, three groups of rats received unilateral injections of CERE-120 in the right striatum at 3 different doses 4.0×10^9 vg/striatum ($n=8$), a 5-fold lower dose of 8.0×10^8 vg/striatum ($n=8$), or a 25-fold lower dose of 1.6×10^8 vg/striatum ($n=8$). A fourth group received FB injections for control purposes ($n=10$). In the long-term efficacy study employing the 6-OHDA lesion model, rats received unilateral injections of CERE-120 injections at (3.7×10^9 vg/striatum; $n=15$), or AAV2–GDNF (3.7×10^9 vg/striatum; $n=13$, as a positive control), or FB control ($n=11$).

In the long-term safety/tolerability experiment, normal unlesioned rats were injected bilaterally with a “higher dose” of CERE-120 (2.0×10^{10} vg/striatum; 4.0×10^{10} vg/rat), a “lower dose” of CERE-120 (4.0×10^9 vg/striatum; 8.0×10^9 vg/rat), or FB control. Thirty rats ($n=10$ /group) were sacrificed at 3 months, 59 rats ($n=19$ – 20 /group) at 6 months, and 31 rats ($n=10$ – 11 /group) at 12 months (55 weeks), with approximately equal numbers of each sex at each time point. Cerebellar toxicity was assessed in a separate study in 90 animals at 3, 30 and 90 days post higher dose injection (8.0×10^{10} vg/rat, $n=10$ rats), lower dose injection (4.0×10^{10} vg/rat, $n=10$) or FB injection ($n=10$).

Note that we elected to use FB (formulation buffer) as the negative control for all of these studies, rather than a vector expressing a reporter gene such as AAV-GFP. AAV-GFP has been used by many laboratories (including ours) as a control in gene transfer studies (e.g., see Kirik et al., 2000; Kordower et al., 2006; Mandel et al., 1999; Mandel et al., 1997; Wang et al., 2002) because it provides the means to expose the brain to both the AAV viral vector as well as expressed exogenous protein. Collectively, these studies have consistently and convincingly demonstrated that AAV-GFP lacks neurotrophic-related bioactivity, including using many of the identical endpoints used in this study. Therefore, not only has the use of AAV-GFP as a control gradually become less pertinent, but for purposes of ascertaining the safety, toxicity and tolerability of CERE-120, the most appropriate control is clearly the biologically inert formulation buffer (FB) (otherwise, possible toxicity of CERE-120 might not be noted because of similar effects induced by either AAV and/or GFP expression in the control group).

6-OHDA lesions

For the dose–response and long-term efficacy experiments, 6-OHDA injections were performed 2 weeks post vector or FB administration. Rats were anesthetized and injected at a single site in the right striatum with 20 μ g of 6-OHDA (Acros Organics, Geel, Belgium) dissolved in 5 μ L of 0.02% ascorbic acid/0.9% saline at 2 weeks after vector or FB administration. The 6-OHDA injection site was located between the 2 sites used for vector or FB delivery at +0.2 anterior to bregma, –3.0 mm from the midline, and –5.0 mm below the dura. For the dose–response efficacy study, animals were sacrificed 4 weeks post vector or FB administration (2 weeks post-lesion). For the long-term efficacy study, animals

were sacrificed 28 weeks post vector (or FB) administration (26 weeks post-lesion).

Amphetamine-induced rotational behavior

Amphetamine-induced rotation testing was performed at 8, 12 and 24 weeks post 6-OHDA lesion as described previously (Ungerstedt and Arbuthnott, 1970). Briefly, rats received 5 mg/kg of D-amphetamine intraperitoneally and were attached to a rotational counting device in a test bowl and rotations were automatically counted for 60 min using computerized software (Med Associates, St. Albans, VT). The number of net ipsilateral (ipsilateral minus contralateral) rotations per minute was calculated.

Cylinder task of spontaneous forelimb use

The cylinder test was performed 8, 12 and 24 weeks post 6-OHDA lesion. In a dark testing room, rats were placed individually in an acrylic cylinder (20 cm in diameter and 45 cm in height) in front of a mirror and their activity was recorded with a video camera. Left and right forepaw contacts with the wall of the cylinder were quantified by a trained observer blinded to treatment. A minimum of 20 total forepaw contacts was required to complete each test session. Data are presented as percent of forepaw contacts contralateral (left paw) to the side of lesion.

Functional observational battery (FOB) testing

The FOB consists of gross physical, behavioral, and neurological assessments that provide general information about potential effects of a test article on the nervous system. FOB assessments included explicit observations made while the rat was in its homecage (general health and presence or absence of involuntary movements), moving freely in an open field (involuntary movements or abnormal gait), and while being handled (palpebral reflex, pinna reflex, and flexor reflex). Body weight was also recorded. All FOB assessments were made by an investigator blinded with respect to treatment group.

Histological analyses

At the scheduled time point, animals were sacrificed and brain tissue was processed as described elsewhere (Gasmi et al., 2007), except for cerebellar tissue (see Supplementary data). For animals in the long-term safety studies, organs and tissues were collected and placed in 10% formalin solution and shipped to Comparative Biosciences (Sunnyvale, CA) for histopathological analyses. 40- μ m-thick cryostat sections from the lower medulla through the hypoglossal nuclei were also collected on slides for potential Schwann cell hyperplasia and axon sprouting analyses. Neurotoxicity was assessed by a board-certified (DACVP) veterinary pathologist (details are available in the Supplementary data).

Immunohistochemistry (IHC)

Transgene expression was analyzed using goat polyclonal anti-human NTN and anti-human GDNF (R&D Systems) immunohistochemistry (IHC) as described previously (Gasmi et al., 2007). The presence of dopaminergic cells and fibers in brain tissue was visualized by tyrosine hydroxylase (TH) and vesicular monoamine

transporter type 2 (VMAT-2) IHC using rabbit anti-TH (Pel-Freez) and guinea pig anti-VMAT2 (Sigma) antibodies diluted 1:500 and 1:10,000, respectively. Schwann cell hyperplasia was evaluated by calcitonin gene-related peptide (CGRP) IHC using a rabbit antibody (Chemicon) diluted 1:12,000.

Potential immune and inflammatory reactions in injected brain tissue were evaluated by CD45 (panleukocyte marker), CD68 (monocyte marker), and glial fibrillary acidic protein (GFAP, astrocyte marker) IHC. Details are provided in Supplementary data.

Quantification

The Optical Fractionator method (West et al., 1991) was used to estimate the total number of TH-positive cells in the substantia nigra using Stereo Investigator software v5.0 (MicroBrightField Inc., Williston, VT). An investigator blinded with respect to treatment group counted cells in a 1:6 series of 40- μ m sections spanning the rostral tip of the substantia nigra pars compacta to the caudal end of the substantia nigra pars reticulata. Optical densitometry (OD) of TH- or VMAT-2-positive fibers in the striatum was performed using NIH Image J software (v1.3; National Institute of Health) by an investigator blinded to treatment group. Details are available in the Supplementary data. Volumetric quantification of NTN or GDNF distribution was performed as described in Gasmi et al. (2007). Detailed methods used to assess potential Schwann cell hyperplasia are described in the Supplementary data.

Serum measurements

Hematology and serum chemistry

Blood was collected at baseline (prior to vector or FB injections) and at sacrifice for analyses of serum chemistry and hematology (LabCorp Preclinical Services). Details on the panel of measurements are provided in the Supplementary data.

Serum antibodies to AAV2 and human NTN

The development of potential anti-AAV2 or anti-human NTN humoral immune responses to AAV2 and human NTN was analyzed by ELISA techniques developed at Ceregene Inc. Details are provided in the Supplementary data.

Statistical analyses

Statistical analyses were performed using SigmaStat v2.03 (SPSS). Preliminary analyses were performed to verify that the data were normally distributed. Differences between groups were assessed using Student's *t*-test or parametric analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc testing, as appropriate. For data that were not normally distributed, a Mann–Whitney rank sum test or a Kruskal–Wallis one-way ANOVA on ranks was performed followed by Dunn's multiple comparison tests, as appropriate. A significant difference between groups was established if $p < 0.05$.

Results

CERE-120 produces dose-related neuroprotection in the 6-OHDA lesion rat model

Immunohistochemical (IHC) staining confirmed NTN expression in the right striatum at all CERE-120 dose levels at 4 weeks

post vector injection (data not shown). 6-OHDA injections in control animals treated with formulation buffer (FB) resulted in a 76% reduction in TH-positive nigral neurons, as determined by tyrosine hydroxylase (TH) immunohistochemical staining and unbiased stereological quantification (Fig. 1A and Table 1). In contrast, animals injected with CERE-120 showed a significant dose-related neuroprotection of SN dopaminergic neurons over a 25-fold range of doses of CERE-120 [$F_{(3,30)}=24.52$, $p<0.001$] (Fig. 1B and Table 1).

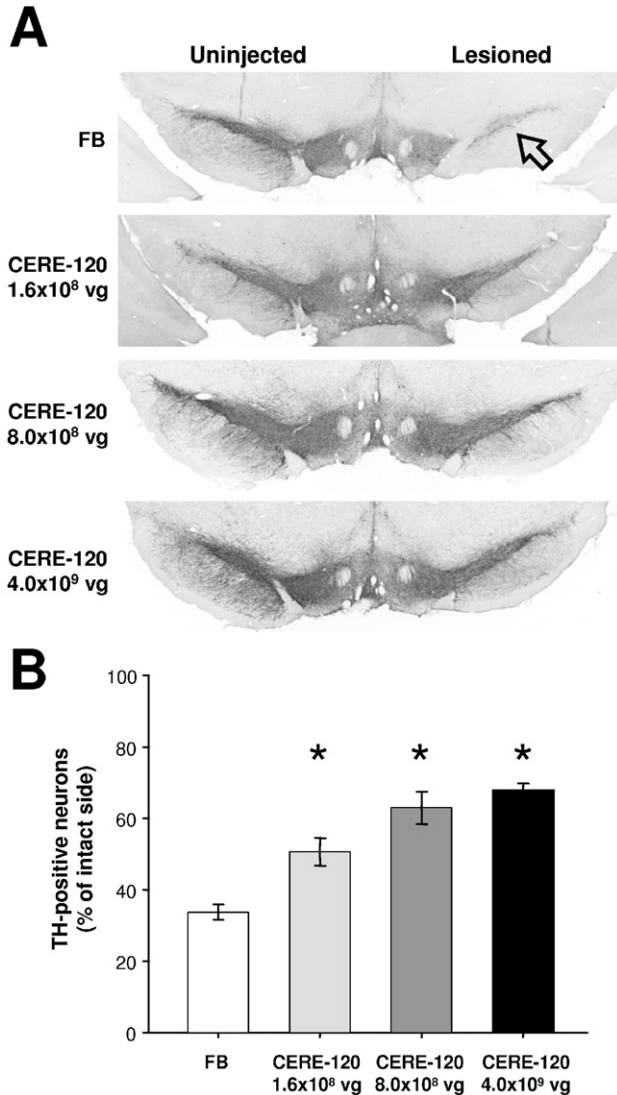


Fig. 1. CERE-120 delivery to the striatum provides dose-related neuroprotection of dopaminergic substantia nigra neurons. (A) Representative sections illustrate that the 6-OHDA lesion resulted in a dramatic depletion of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra of formulation buffer (FB)-injected animals (upper panel). Note the increasing neuroprotective effect with increasing dose of CERE-120 delivered (3 lower panels). (B) Stereological quantification confirmed a significantly greater number of TH-positive neurons (mean±SEM) in the substantia nigra for all of the CERE-120 dose groups (shaded bars) compared to formulation buffer controls (open bar). *Significantly different from control at $p<0.05$.

Table 1

Number of TH-immunoreactive cells in the substantia nigra in the dose-response and long-term rat 6-OHDA lesion studies (mean±SEM)

	Unlesioned side	Lesioned side	% surviving neurons
Dose-response study			
FB control	9754±283	3316±273	33.71±2.13
1.6×10 ⁸ vg CERE-120	9613±506	4780±296	50.61±3.88 ^a
8.0×10 ⁸ vg CERE-120	10,332±442	6488±539	62.94±4.54 ^a
4.0×10 ⁹ vg CERE-120	10,295±761	6970±515	67.94±1.78 ^{a,b}
Long-term study			
FB control	12,756±674	2290±298	18.48±2.57
3.7×10 ⁹ vg CERE-120	11,745±366	6781±580	58.08±4.41 ^c
3.7×10 ⁹ vg AAV2-GDNF	12,169±359	8801±457	72.56±3.28 ^c

FB=formulation buffer; vg=vector genomes.

^a $p\leq 0.003$ relative to FB control.

^b $p=0.004$ relative to lowest dose of CERE-120.

^c $p<0.001$ relative to FB control.

CERE-120 produces long-term expression and neuroprotection in the 6-OHDA lesion rat model

NTN expression

IHC staining revealed robust expression of NTN or GDNF in the nigrostriatal system of all animals injected with CERE-120 or AAV2-GDNF, respectively, at 28 weeks post-vector injection (Fig. 2). Intense growth factor immunoreactivity was evident in the striatum (Fig. 2A), globus pallidus, and SN (Fig. 2B), and weak immunoreactivity was observed in cortical areas adjacent to the needle tracks. Within the SN, cells in the *pars compacta* and fibers in the *pars reticulata* stained positive for each neurotrophic factor, consistent with the retrograde transport of vector and/or protein and anterograde transport of protein (Fig. 2B). The volume of striatal NTN or GDNF immunohistochemical distribution was 14.43±1.47 mm³ for NTN (mean±SEM) and 20.82±1.64 mm³ for GDNF [$t(26)=2.91$, $p<0.01$], which represent approximately 58% and 83% of the rat striatal volume, respectively. The apparent difference in volume of expression observed following administration of a single dose each of AAV-NTN (CERE-120) and AAV-GDNF, may reflect possible differences in sensitivity of antibodies, diffusion of protein and/or more efficient transgene expression.

Behavioral function

The effects of AAV2-GDNF and CERE-120 injection on rotational behavior showed a trend towards behavioral improvement at 8 weeks, which became significant in the case of CERE-120-treated animals at 12 and 24 weeks post-lesion (Fig. 3A). Rotational behavior of AAV2-GDNF-injected animals continued to show a trend toward improvement at time points longer than 8 weeks, but this improvement was not statistically different from FB control animals or CERE-120-injected animals at either 12 weeks (2.27±0.88 versus 4.80±1.40 for FB controls; $p>0.05$) or 24 weeks post-lesion (0.48±0.16 versus 1.94±0.74 for FB controls; $p>0.05$).

Results of the spontaneous forelimb use in the cylinder test showed that at baseline, prior to striatal delivery of FB, AAV2-GDNF or CERE-120, all groups displayed normal forelimb contacts (Fig. 4B). From 8 weeks through 24 weeks post-6-OHDA, a consistent and dramatic decrease in forepaw use in the

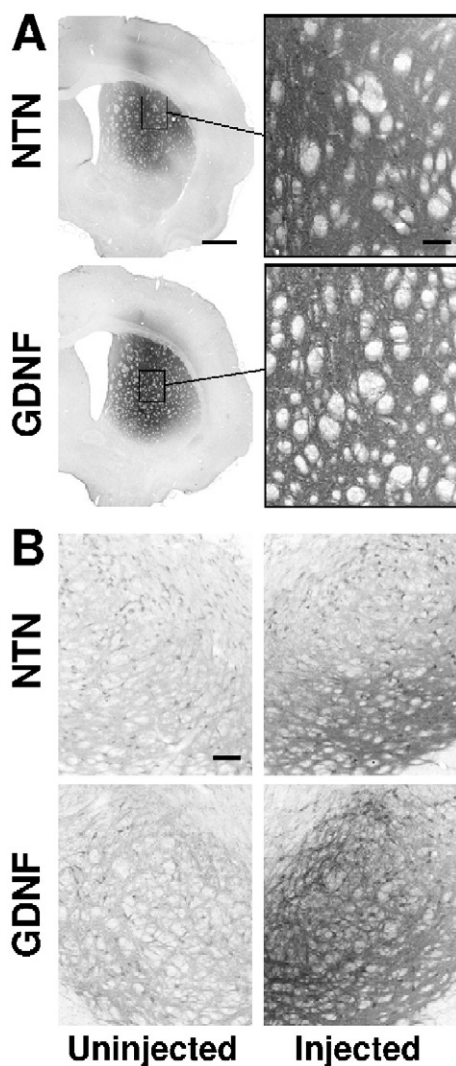


Fig. 2. NTN and GDNF expression in the nigrostriatal system of 6-OHDA-lesioned rats at 28 weeks after vector administration. (A) Characteristic detection of NTN and GDNF in the striatum, as detected by immunohistochemistry. (B) Characteristic expression of NTN and GDNF in the injected and un.injected control substantia nigra, as detected by immunohistochemistry. Scale bars: 1.0 mm (A, left panels), 0.1 mm (A, right panels; B).

limb contralateral to the injected hemisphere was observed across all groups. A two-way ANOVA with time and treatment group as factors revealed no effect of treatment [$F_{(2,30)}=0.651$, $p>0.05$], a significant effect of time [$F_{(3,90)}=167.86$, $p<0.001$], and no interaction between the two [$F_{(6,90)}=0.177$, $p>0.05$]. A significant effect of time, and the lack of an interaction between treatment and time, reflect the persistent decrease in forelimb use that was equivalent between groups across time as a consequence of the unilateral 6-OHDA lesion.

Neuroprotection

Unbiased stereological quantification showed that FB-injected animals had an extensive loss of TH-positive neurons in the lesioned side of the SN at 26 weeks post-lesion (Table 1 and Fig. 3). In contrast, animals injected with CERE-120 or AAV2-GDNF exhibited an obvious sparing of TH-positive SN neurons (Table 1 and Fig. 3) [$F_{(2,36)}=51.07$, $p<0.001$]. Although CERE-120 and

AAV2-GDNF showed qualitatively similar neuroprotective effects (see Fig. 3A), there were more TH-positive SN neurons remaining at 26 weeks post-lesion in AAV2-GDNF-treated than in CERE-120-treated animals ($p=0.019$) in spite of superior behavioral recovery of function seen on amphetamine-induced rotation by CERE-120-treated rats.

Dopamine fiber preservation

To assess the effect of NTN on fiber preservation, striatal sections of CERE-120- and FB-injected animals were stained for vesicular monoamine transporter (VMAT-2) by IHC. VMAT-2 is normally expressed in dopaminergic neurons and their processes in the striatum, and since expression of VMAT-2, unlike TH, does not appear to be undergo a phenotypic downregulation as a consequence of striatal GDNF over-expression in the rat (Rosenblad et al., 2003), it serves as a more reliable indicator of the anatomical integrity of nigrostriatal projections. VMAT-2 immunolabeling in

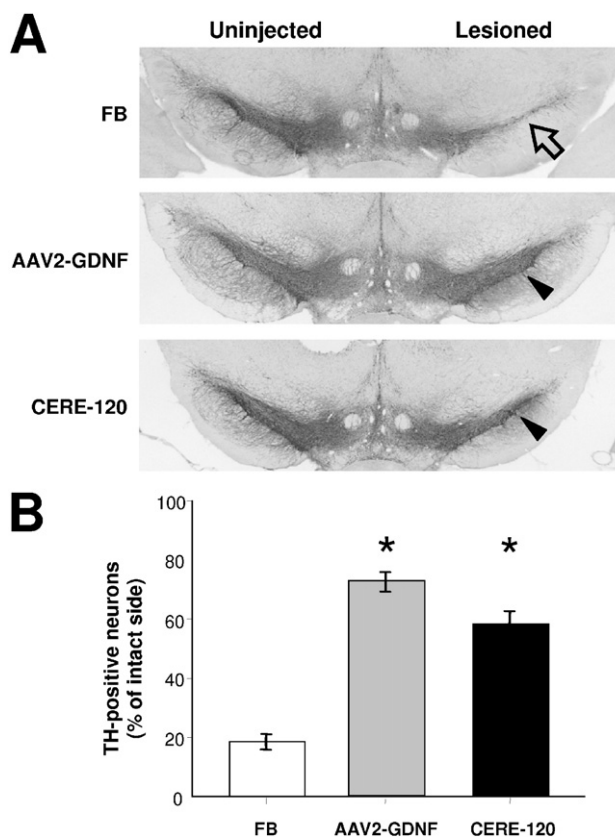


Fig. 3. CERE-120 and AAV2-GDNF delivery to the striatum provide long-term neuroprotection of dopaminergic substantia nigra neurons. (A) Representative sections illustrate that the 6-OHDA lesion resulted in a dramatic depletion of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra of formulation buffer (FB)-injected animals at 26 weeks post-lesion (upper panel). In contrast, animals injected with CERE-120 and AAV2-GDNF showed significant neuroprotection at 26 weeks post-lesion (middle and bottom panels, respectively). (B) Stereological quantification confirmed significant neuroprotection with AAV2-GDNF (gray bar) and CERE-120 (black bar) compared to formulation buffer controls (open bar) at 26 weeks post-lesion. Plotted data are mean±SEM; *significantly different from FB control at $p<0.001$; CERE-120 and AAV2-GDNF were also significantly different from each other at $p<0.05$, see text for statistical details.

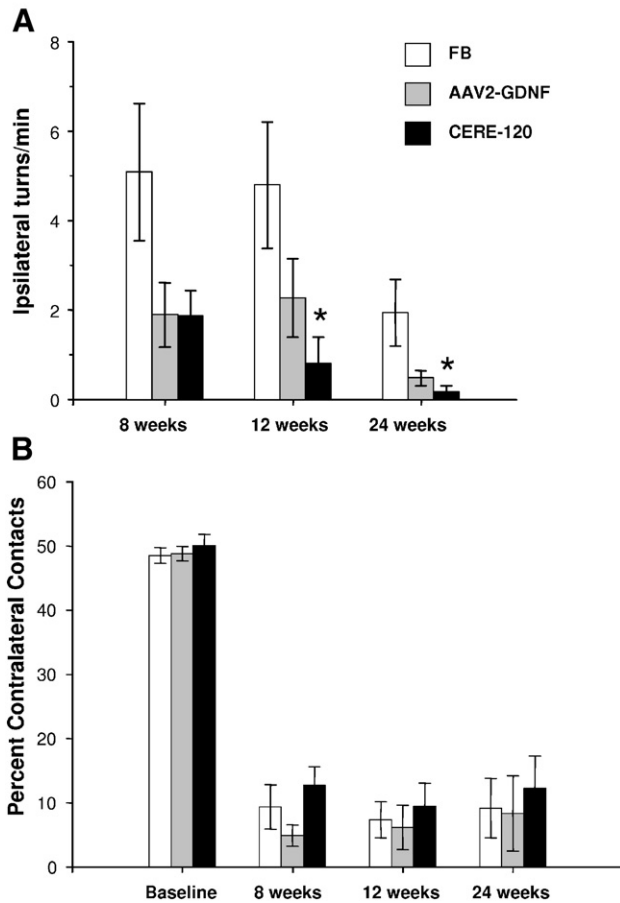


Fig. 4. CERE-120 has significant functional effects on the nigrostriatal system in 6-OHDA-lesioned rats, revealed by a decrease in amphetamine-induced rotation, but does not alter spontaneous forepaw use in the cylinder test. (A) At 8 weeks post-lesion, both AAV2-GDNF and CERE-120 groups demonstrated a trend toward a reduction in amphetamine-induced turning, compared to formulation (FB) controls. At both 12 and 24 weeks post-lesion, CERE-120 animals exhibited a significant reduction in amphetamine-induced turning compared to formulation buffer controls ($*p < 0.05$). Plotted data are mean \pm SEM net ipsilateral turns per minute. (B) All groups showed an equivalent number of left and right forepaw contacts on the wall of the cylinder during exploratory activity prior to 6-OHDA lesion. At 8, 12, and 24 weeks post-lesion, all groups were found to have a dramatic decrease in the number of wall contacts made with the forepaw contralateral to the lesioned hemisphere, consistent with a persistent motor deficit as a consequence of lesion. Data are presented as mean (\pm SEM) percent contralateral contacts; see text for statistical details.

the uninjected hemisphere of FB- and CERE-120-injected animals revealed a normal pattern of staining throughout the striatum that was comparable between the two groups (Fig. 5A). In contrast, qualitative and quantitative analysis revealed a reduction of VMAT-2 optical density in the 6-OHDA-injected hemisphere (~73% relative to the intact side) of FB-injected animals, while only an approximate 16% reduction in VMAT-2 optical density was observed in the 6-OHDA-lesioned hemisphere of CERE-120-injected animals. Statistical analysis performed on OD values revealed a significant difference between groups, with CERE-120-injected animals displaying more intense VMAT-2 striatal immunoreactivity in the lesioned hemisphere compared to FB-injected control animals (Mann-Whitney rank sum, $T = 50.00$,

$n_1 = 8$, $n_2 = 11$, $p = 0.015$). Mean OD values for each group in the lesioned striatum relative to the control, intact striatum were as follows: FB = $26.60 \pm 8.07\%$; CERE-120 = $83.58 \pm 15.51\%$ (\pm SEM). Because these VMAT data merely replicate, with AAV-NTN, several prior reports that used GDNF (Georgievska et al., 2002; Georgievska et al., 2004; Rosenblad et al., 2003), we did not include GDNF as a positive control for this endpoint.

TH staining in the striatum of CERE-120-, AAV2-GDNF- and FB-injected animals revealed normal pattern of intense immunolabeling in all groups on the uninjected side (data not shown). In contrast, the 6-OHDA-lesioned hemisphere displayed approximately 60% depletion in fiber staining across all groups. One-way ANOVA performed on relative OD values (i.e. percent intact hemisphere) confirmed no differences between groups in striatal TH staining intensity [$F_{(2,34)} = 0.23$, $p > 0.05$]. Mean OD values for each group in the lesioned striatum relative to the control, intact striatum were as follows: FB control = $41.10 \pm 7.63\%$; AAV2-GDNF = $45.54 \pm 4.29\%$; CERE-120 = $45.76 \pm 4.11\%$ (\pm SEM).

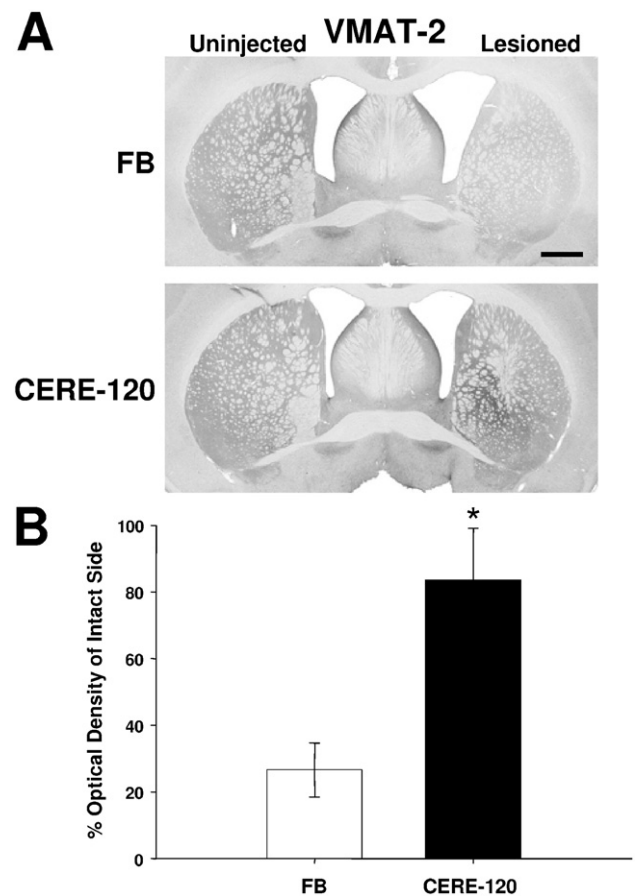


Fig. 5. Vesicular monoamine transporter 2 (VMAT-2)-immunoreactive fibers in the striatum are protected/restored by CERE-120 in the long-term 6-OHDA lesion model study. (A) Representative sections illustrate a considerable loss of VMAT-2 immunolabeling in the lesioned striatum of formulation buffer (FB)-injected animals relative to the unlesioned side at 26 weeks post-lesion. In contrast, animals injected with CERE-120 displayed substantially more intense striatal VMAT-2 immunoreactivity compared to FB controls. (B) Optical densitometry confirmed a significant higher density of VMAT-2-immunoreactive fibers relative to the unlesioned striatum in CERE-120-injected animals compared to FB controls ($*p < 0.05$). Plotted data are mean \pm SEM. Scale bar = 1.0 mm.

CERE-120 is safe and well tolerated at long time points and high doses following delivery to the rat striatum

Long-term, stable NTN expression

NTN IHC in the striatum of all CERE-120-injected rats revealed a stable, dose-dependent expression of the protein up to 12 months post vector injection (longest time point examined). Administration of 8.0×10^9 vg per animal (4.0×10^9 vg/striatum) of CERE-120 resulted in NTN immunoreactivity largely confined to the striatum and anatomically connected structures such as the SN, globus pallidus, and the reticular thalamus at all time points. No statistically significant differences in the striatal volume of NTN protein distribution was observed at 3 months (12.79 ± 1.12 mm³), 6 months (11.08 ± 1.09 mm³), or 12 months (12.13 ± 0.97 mm³) [$F_{(2,27)} = 0.66$, $p > 0.05$]. At a 5-fold higher dose (4.0×10^{10} vg per animal, 2.0×10^{10} vg/striatum) NTN immunoreactivity was observed in additional brain regions including the cerebral cortex and medial thalamus (Fig. 6). At this higher dose, strong NTN staining was also visible along of the needle track and particularly in the cortex, likely due to vector reflux during the injection procedure (Fig. 6).

No evidence of neurotoxicity or neuroinflammatory responses

To broadly assess neurological function over the 12-month in-life portion of this study, all rats were tested using a “functional observation battery” (FOB) pre-surgery and at 3, 6, 9, and 12 months post-surgery. The FOB is composed of observations of gross behavioral and neurological function and provides general information about the potential functional consequences of long-term AAV2-mediated NTN expression in the brain. No behavioral abnormalities were observed in any rat at either dose at any time point using this assay.

To investigate the potential toxicity of CERE-120 both at the delivery site as well as more extensively throughout the brain, histopathological analysis of H&E stained sections was performed by a board-certified pathologist at 3, 6, or 12 months post-administration. No histological abnormalities were observed in the numerous brain structures examined, including but not limited to striatum, forebrain, cerebral cortex, septum, hippocampus, thalamus, hypothalamus, amygdala, midbrain (including the substantia

nigra) brainstem, and upper spinal cord. The brains of CERE-120- and FB-injected rats equally exhibited evidence of the intraparenchymal injections in the striatum, consisting of a needle track characterized by a line of hemosiderin deposit. No cerebellar toxicity or inflammation was detected up to 90 days post vector injection at any of the two doses.

Potential activation of inflammatory or immune responses by CD45, CD68 and GFAP IHC at 3, 6 and 12 months post-treatment revealed a characteristic response along the needle track in the striatum of all animals, consisting of mild leukocyte infiltration and activation of microglia and astrocytes. As with H&E staining, this response was similar in brains injected with either dose of CERE-120 or FB control. Beyond the needle track, no consistent CERE-120-related changes in immune or inflammatory markers were observed in any part of the brain, including regions where NTN was expressed at high levels.

No evidence for adverse effects previously associated with non-targeted neurotrophic factors

No abnormal behavior nor histological evidence of non-targeted neurotrophic factor activity were found in CERE-120-injected animals. All animals gained weight normally over the course of the study. No treatment-related differences in body weight were observed from 3 through 12 months following CERE-120 delivery. The mean percent change from baseline for CERE-120-injected animals was comparable to FB-injected controls [12-month high dose males: $t(10) = -0.487$, $p > 0.05$; 12-month high dose females: $t(9) = -0.484$, $p > 0.05$]. The pia-arachnoid thickness across the medulla, an indicator of Schwann cell hyperplasia, was not significantly different in FB control (37.51 ± 0.57 μ m, mean \pm SEM), lower dose CERE-120 (39.25 ± 0.52 μ m), or higher dose CERE-120 (39.49 ± 0.97 μ m) animals at 3 months [$F_{(2,27)} = 2.31$, $p > 0.05$], 6 months [FB control = 38.49 ± 0.83 μ m; lower dose CERE-120 = 39.92 ± 0.59 μ m; higher dose CERE-120 = 40.33 ± 0.70 μ m, $F_{(2,27)} = 1.837$, $p > 0.05$], or 12 months [FB control = 40.83 ± 0.25 μ m; lower dose CERE-120 = 40.73 ± 0.15 μ m; higher dose CERE-120 = 40.56 ± 0.50 μ m, $F_{(2,28)} = 0.130$, $p > 0.05$]. In addition, microscopic examination of sections did not reveal aberrant sprouting in the medulla or upper spinal cord, as assayed by staining for TH, a marker of sympathetic fibers, and calcitonin

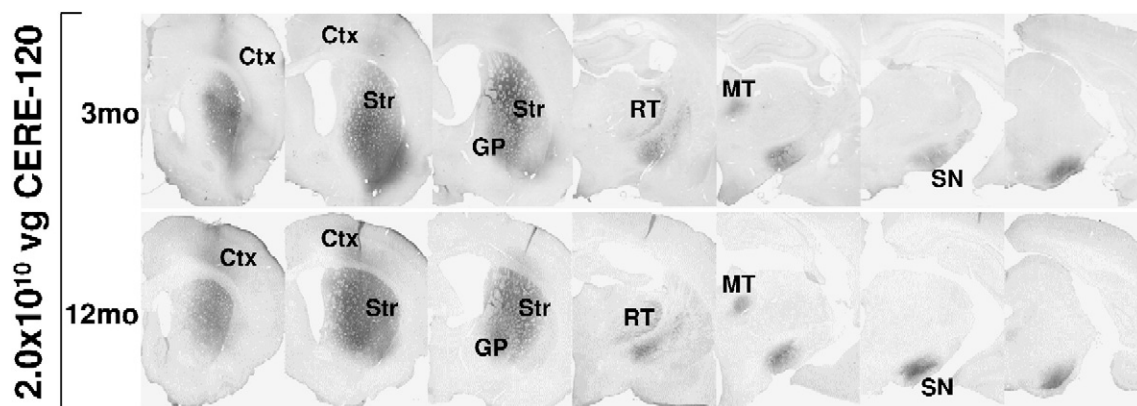


Fig. 6. NTN protein is expressed in a restricted, stable manner at 3 and 12 months after CERE-120 delivery to the rat striatum. NTN immunohistochemistry in representative coronal brain sections at 3 and 12 months following intrastriatal delivery of 2.0×10^{10} vg of CERE-120. NTN-positive brain structures include nigrostriatal system: striatum (Str) and substantia nigra (SN) as well as its anatomically related regions: reticular thalamus (RT), medial thalamus (MT), globus pallidus (GP) and cerebral cortex (Ctx). A strong signal is also seen in the cerebral cortex (Ctx) in the vicinity of the needle track. Sections shown are spaced approximately 1.5 mm apart and are arranged in order from rostral (left) to caudal (right).

gene-related peptide (CGRP), a marker for sensory fibers at 3, 6, or 12 months.

No systemic or peripheral adverse effects

To assess the systemic safety and tolerability of CERE-120, standard clinical chemistry and hematology and histopathological evaluation of peripheral organs were performed at 3, 6, and 12 months post-treatment. No CERE-120-related alterations in clinical chemistry or hematological parameters were observed. Histopathological analyses performed by a board-certified pathologist revealed no CERE-120-related abnormalities in any of the peripheral tissues examined.

No biologically meaningful antibody responses to human NTN were observed in CERE-120-injected rats, with all changes in titer $<5\times$ compared to baseline, with the exception of a single lower dose (8.0×10^9 vg) rat at 3 months ($25\times$ change in titer from baseline). Antibody responses to the AAV2 capsid were observed in only a few lower dose (8.0×10^9 vg) CERE-120-injected animals [0/10 at 3 months, 2/10 at 6 months ($25\times$ and $625\times$ changes in titer from baseline), and 1/9 at 12 months ($>25\times$ change in titer from baseline)]. In higher dose (4.0×10^{10} vg) CERE-120-injected rats, the majority of animals at all time points exhibited an antibody response to the AAV2 capsid, with responses ranging from $5\times$ to $3125\times$ increases in titer from baseline. No clinical or histopathological changes were noted as a consequence of these antibody responses, nor were any differences in NTN expression or bioactivity observed.

Discussion

Neurotrophic factors, such as NTN, have the potential to improve upon the treatment of neurodegenerative diseases such as PD. However, a major hurdle in the therapeutic development of neurotrophic factors has been to find the means to safely and effectively deliver them to targeted brain regions. We have previously shown that the *in vivo* gene delivery of NTN using an AAV2-based vector (CERE-120) resulted in the distribution of NTN protein throughout the targeted brain region in a predictable, controlled and persistent fashion (Gasmı et al., 2007; Herzog et al., *in press*; Kordower et al., 2006). Here we describe the long-term efficacy and safety/tolerability of this treatment in rats and provide supportive evidence that it may represent a viable therapeutic approach for PD.

Results from efficacy experiments in the rat 6-OHDA lesion model, reported here, indicate substantial neuroprotection of CERE-120 on nigrostriatal neurons. CERE-120 delivery to the striatum resulted in significant, dose-related dopaminergic SN cell survival over a 25-fold range of doses. CERE-120 also produced significant long-term SN cell protection, increased striatal innervation and enhanced nigrostriatal function over approximately 6 months post vector administration. These results augment and extend the growing body of literature on the bioactive effects of NTN on the dopaminergic nigrostriatal system (Fjord-Larsen et al., 2005; Gasmı et al., 2007; Horger et al., 1998; Kordower et al., 2006; Oiwa et al., 2002; Rosenblad et al., 1999), confirming its place with its close naturally occurring structural and functional analog, GDNF, as promising trophic factors for PD.

The anatomical and functional effects of NTN expression observed in the long-term 6-OHDA experiment are consistent with what has been observed in prior studies designed to examine the potential neuroprotective effects of long-term GDNF expres-

sion in 6-OHDA rats. Significant preservation of dopaminergic cells in the nigra and evidence of preservation and/or restoration of dopaminergic processes within the striatum have been observed previously following long-term AAV2 or lentiviral mediated delivery of GDNF to the striatum (Georgievska et al., 2002; Kirik et al., 2000). Although a significant reduction in amphetamine-induced rotation was observed in the present study following CERE-120 delivery (but not following AAV2–GDNF treatment), the cylinder task failed to demonstrate any benefit of either GDNF or NTN. These data are consistent with the literature related to functional improvement on behavioral tasks following 6-OHDA lesion and GDNF treatment. While some studies reported positive effects (Kirik et al., 2000; Rosenblad et al., 1998), others have not (Georgievska et al., 2002; Winkler et al., 1996), and it seems likely that differences in the lesion parameters, relative timing of the lesion and growth factor treatment and the kinetics of expression and/or diffusion of the growth factor might all collectively work to produce variable data from different groups using this very aggressive model of nigrostriatal degeneration. Nevertheless, our observations of consistent and robust reduction in amphetamine-induced rotational behavior following long-term viral-vector mediated NTN delivery, together with significant sparing of nigrostriatal neurons, are consistent with a trophic-factor mediated potentiation of dopaminergic activity and subsequent restoration of motor behavior following amphetamine administration.

Our direct comparison of AAV2–GDNF and CERE-120, using only a single dose for each, provides general support to the fact that GDNF and NTN offer similar therapeutic potential. While the rats treated with AAV2–GDNF showed modestly more nigral cell sparing than those injected with CERE-120, the same rats did not reveal as robust and consistent behavioral improvement as those injected with CERE-120. Thus, we do not believe that these subtle and inconsistent outcomes reflect genuine biological differences between GDNF and NTN. This position is in agreement with several previous reports that had shown the similar biological effects of NTN and GDNF (Akerud et al., 1999; Gasmı et al., 2007; Horger et al., 1998; Kotzbauer et al., 1996). However, for any definitive conclusions to be reached regarding the relative bioactivity of GDNF vs. NTN, full dose–response comparison of the two molecules would be required and this has yet to be performed by anyone and falls well outside the scope of this study.

Long-term (12 months) studies also establish that CERE-120 produces no evidence of neurotoxicity in any of multiple cerebral regions examined, no systemic toxicity, and no adverse effects associated with non-targeted growth factor exposure such as weight loss, or aberrant Schwann cell hyperplasia or sympathetic or sensory nerve sprouting in the spinal cord (Boyd and Hovland, 2004; Eriksson-Jonhagen et al., 1998; Kordower et al., 1999; Nutt et al., 2003). In addition, no histopathological changes were observed in the cerebellum of CERE-120-injected rats, similar to what was observed in CERE-120-injected monkeys (Kordower et al., 2006). This is in contrast to the apparent cerebellar toxicity observed in a monkey study where animals received intraputamenal infusion of GDNF via an indwelling catheter (discussed in Sherer et al., 2006). Peripherally, no histopathological abnormalities or changes in either clinical chemistry or hematology parameters were observed and no consistent antibody response to human NTN was detected. Again, this is in contrast to anti-growth factor antibody responses previously reported following

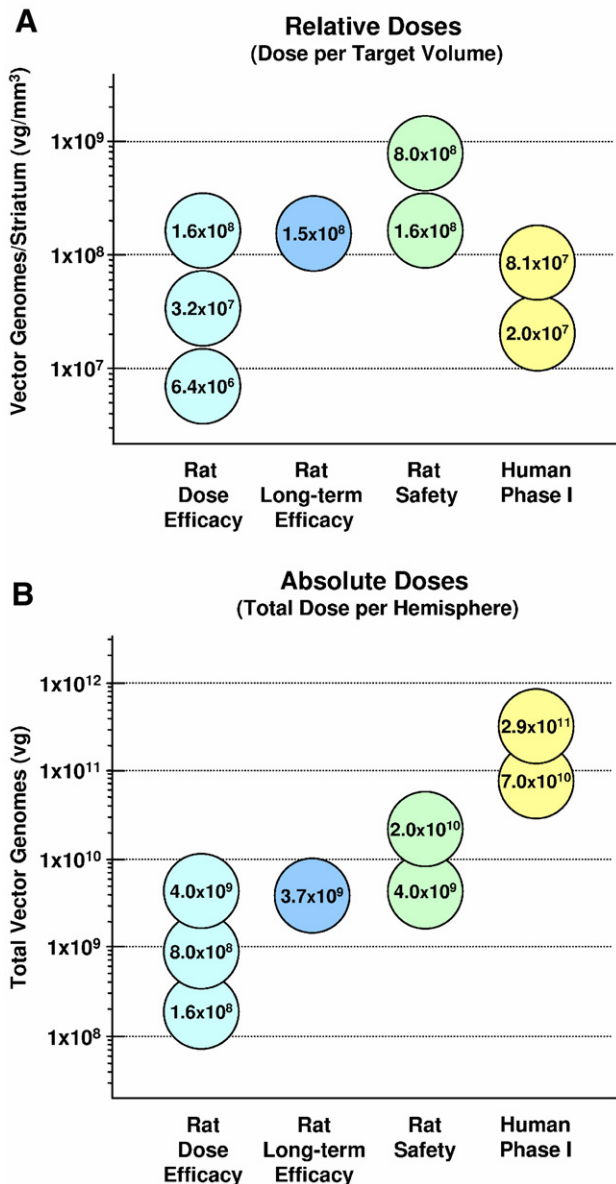


Fig. 7. Comparison of CERe-120 doses across three rat studies (dose–response efficacy, long-term efficacy, safety) versus those used in the human (Phase I) study. (A) Relative equivalent CERe-120 doses in vector genomes per target volume (vg/mm³). The total CERe-120 vector genomes administered per hemisphere was normalized for species-specific target volume, where volumes for rat striatum and human putamen are 25 and 3500 mm³/hemisphere, respectively. The relative equivalent doses for the Phase I clinical trial are well below those demonstrated to be safe and well tolerated in rats, but at the same time are within the range of doses shown to be efficacious. Adjusting the doses by brain or body weight rather than target size yields an even greater safety margin (>100×) between the rat and human studies. (B) Absolute CERe-120 doses per hemisphere (total vg/hemisphere).

intraparenchymal infusion of GDNF (discussed in Sherer et al., 2006). Antibody responses to the AAV2 capsid did develop following CERe-120 delivery to the striatum, particularly in higher dose-injected rats, but no clinical manifestations of this response were observed, and no effect on NTN expression was evident.

The efficacy and safety/tolerability studies described herein tested a range of vector doses and indicate a broad therapeutic index for CERe-120. Doses of CERe-120 used in the rat efficacy studies relative to those of the rat safety study are illustrated in Fig. 7, expressed in relation to striatal target volume (panel A), and as absolute CERe-120 dose (panel B). The lowest CERe-120 dose demonstrated to be efficacious in the rat 6-OHDA lesion model experiments is 125 times lower than the highest dose tested, which was shown to be safe and well tolerated. The dose levels of CERe-120 tested in a Phase I clinical study in subjects with PD (Marks et al., 2006b) are also illustrated in Fig. 7, for comparison purposes. CERe-120 human dose equivalents adjusted for target volume (Fig. 7A) fall in the middle of the range of doses found to be efficacious in the rat 6-OHDA lesion model experiments described herein. Human clinical dose equivalents are also well below the CERe-120 doses shown to be safe and well tolerated in these rat studies. Thus, comparison of the CERe-120 Phase I clinical doses and CERe-120 doses shown to be efficacious and safe in rat preclinical studies suggest that the human doses selected should fall well within the established effective dose range for CERe-120 and well below any dose that might produce undesirable side effects.

The studies described herein represent a portion of an extensive program examining the efficacy and safety of CERe-120 in rats and monkeys to support the development of CERe-120 as a therapeutic for PD (Dass et al., submitted for publication; Gasmi et al., 2007; Herzog et al., in press; Kordower et al., 2006). Results from these studies have consistently revealed bioactive effects of CERe-120 on the dopaminergic nigrostriatal system at dose levels far below those tested and shown to be safe. Based on these data, this program has advanced into testing in PD patients in an initial open-label study that was recently completed successfully (Marks et al., 2006a, b,c; Starr et al., 2007) and a multi-center, double-blind, sham surgery controlled study is underway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2007.04.003.

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