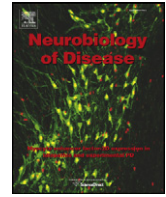




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Properly scaled and targeted AAV2-NRTN (neurturin) to the substantia nigra is safe, effective and causes no weight loss: Support for nigral targeting in Parkinson's disease[☆]

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ABSTRACT

Recent analyses of autopsied brains from subjects previously administered AAV2-neurturin (NRTN) gene transfer argues that optimizing the effects of neurotrophic factors in Parkinson's disease (PD) likely requires delivery to both the degenerating cell bodies (in substantia nigra) and their terminals (in striatum). Prior to implementing this novel dosing paradigm in humans, we conducted eight nonclinical experiments with three general objectives: (1) evaluate the feasibility, safety and effectiveness of targeting the substantia nigra (SN) with AAV2-NRTN, (2) better understand and appraise recent warnings of serious weight loss that might occur with targeting the SN with neurotrophic factors, and (3) define an appropriate dose of AAV2-NRTN that should safely and effectively cover the SN in PD patients. Toward these ends, we first determined SN volume for rats, monkeys and humans, and employed these values to calculate comparable dose equivalents for each species by scaling each dose, based on relative SN volume. Using this information, we next injected AAV2-GFP to monkey SN to quantify AAV2-vector distribution and confirm reasonable SN coverage. We then selected and administered a ~200-fold range of AAV2-NRTN doses (and a single AAV2-GDNF dose) to rat SN, producing a wide range of protein expression. In contrast to recent warnings regarding nigra targeting, no dose produced any serious side effects or toxicity, though we replicated the modest reduction in weight gain reported by others with the highest AAV2-NRTN and the AAV2-GDNF dose. A dose-related increase in NRTN expression was seen, with the lower doses limiting NRTN to the peri-SN and the highest dose producing mistargeted NRTN well outside the SN. We then demonstrated that the reduction in weight gain following excessive-doses can be dissociated from NRTN in the targeted SN, and is linked to mistargeted NRTN in the diencephalon. We also showed that prior destruction of the dopaminergic SN neurons via 6-OHDA had no impact on the weight loss phenomenon, further dissociating neurotrophic exposure to the SN as the culprit for weight changes. Finally, low AAV2-NRTN doses provided significant neuroprotection against 6-OHDA toxicity, establishing a wide therapeutic index for nigral targeting. These data support targeting the SN with AAV2-NRTN in PD patients, demonstrating that properly targeted and scaled AAV2-NRTN provides safe and effective NRTN expression. They also provided the means to define an appropriate human-equivalent dose for proceeding into an ongoing clinical trial, using empirically-based scaling to account for marked differences in SN volume between species.

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Introduction

Neurotrophic factors have long been recognized to provide the potential to repair damaged and degenerating neurons, restore their function and protect them from further degeneration and death. Interest in using neurotrophic factors to treat serious neurodegenerative diseases has thus persisted for decades (Apfel et al., 1998, 2000; Eriksdotter Jonhagen et al., 1998; Miller et al., 1996; Seiger et al., 1993), even though no clear clinical success has yet been achieved. Indeed, efforts to treat Parkinson's disease (PD) with GFLs (i.e., Glial

cell line-derived Family of Ligands, like GDNF and NRTN) have actually intensified over the past decade, despite no controlled-study yet showing a significant benefit on the primary endpoint (Lang et al., 2006; Marks et al., 2010; Nutt et al., 2003). Many have concluded that the key to eventually translating the scientific promise of neurotrophic factors to successful clinical treatment rests with solving the significant challenges associated with delivering sustained concentrations of protein throughout the targeted cell populations deep within the brain (e.g., Salvatore et al., 2006; Sherer et al., 2006). Gene therapy offers a practical means to overcome these challenges by expressing proteins directly to the desired brain targets in a controlled and sustained fashion following a single treatment (Bartus et al., 2007). However, recent insight gained from a clinical trial with AAV2-NRTN in advanced PD patients revealed still other delivery issues using neurotrophic factors for chronic neurodegenerative diseases, with gene therapy no more immune to these new hurdles than other delivery modalities (Bartus et al., 2011).

Specifically, traditional research with neurotrophic factors established that their effectiveness is predicated on their ability to induce repair genes by signaling pathways in the neuronal cell body (Hefti, 1997; Snider and Johnson, 1989), emphasizing the importance of achieving adequate concentrations of protein in or near the soma. At the same time, research using animal models of PD emphasized that it was also important to expose the degenerating terminals to high concentrations of neurotrophic factor (Kirik et al., 2004). Studies in animal models of PD demonstrated that substantial protection of degenerating substantia nigra (SN) neurons could be achieved whether the neurotrophic factor was delivered to the terminal area (e.g., striatum) or to the cell bodies (in the SN) (e.g. Bilang-Bleuel et al., 1997; Connor et al., 1999; Kirik et al., 2000a; 2000b), presumably because the factor and critical signaling components could be efficiently retrogradely transported from terminals to the cell body. Because the literature also suggested that functional recovery occurs more reliably when dopamine fibers are preserved (Kirik et al., 2000b, 2004), along with simplicity of targeting, the terminal area became the preferred site of delivery for the majority of animal studies, as well as subsequent clinical efforts. However, recent data from autopsied PD brain suggest a fundamental difference likely exists between how effectively neurotrophic factors are transported along the axons in traditional animal models of PD versus in the advanced human PD nigrostriatal system (Bartus et al., 2011). These data support an emerging hypothesis that axonal transport mechanisms in advanced PD are deficient and thus unable to efficiently transport protein (or vector) from the terminal area in the putamen to the cell body in the substantia nigra pars compacta (Braak et al., 1999; Cheng et al., 2010; De Vos et al., 2008; Morfini et al., 2009; Roy et al., 2005). These data, therefore, provide a potential explanation for the lack of reliable improvement using neurotrophic factors in past clinical trials in PD (Lang et al., 2006; Nutt et al., 2003) and argue that if maximal benefit of either GDNF or NRTN is to be achieved in moderately advanced PD, it is likely necessary to deliver the protein directly to both the cell bodies as well as the terminal field, which involves two distinct areas of the brain (i.e. the SN and striatum, respectively).

Because the SN is a relatively small structure, lying deep within the midbrain, it presents more difficult challenges for stereotactic targeting. Additionally, because the SN lies in close proximity to many other densely distributed anatomical structures that support a wide variety of functions, targeting the SN requires special consideration and caution. Indeed, recent papers from two laboratories warn that dire weight loss side effects are likely to occur if one targets the SN with GFL's (Manfredsson et al., 2009a; 2009b; Su et al., 2009). Therefore, to help establish the feasibility and safety of targeting the SN with AAV2-NRTN, before attempting to test this novel dosing paradigm in PD patients, a series of eight nonclinical translational experiments was conducted.

First, we empirically determined the volumes of rat and monkey SN, given the paucity of published information, and compared these to

the values published for human SN (Ahsan et al., 2007; Foster et al., 2008; Krabbe et al., 2005). By scaling the doses based on relative SN volumes, we were able to make direct dose-comparisons between species, allowing the results from one to be extrapolated to the other and eventually providing the information required to define what is predicted to be a safe and effective dose for human testing in PD.

Using these volumetric comparisons, along with prior data targeting other brain sites in rats and monkeys, we then calculated a dose of AAV2-GFP (green fluorescent protein, a commonly used reporter protein) that should transduce a significant number of SN neurons, thus providing reasonable SN coverage without exceeding its borders. After we confirmed that this approach could reasonably predict SN coverage, we further built on these data by conducting a large AAV2-NRTN (CERE-120) study in rats to test the safety, feasibility and potential risk:benefit of surgically targeting and then expressing NRTN in and around the SN. Four dose levels were tested, selected to provide a range that would include one intended to appropriately and accurately cover the majority of the SN, one that would be significantly lower (to help define a therapeutic index) and others intended to be much higher than required for appropriate SN coverage to help test for possible toxicity and side effects. Volume and location of NRTN expression was quantified as a function of dose, providing important information regarding spread of vector and protein around SN. Several other studies were then conducted to more fully understand the relationship between dose of AAV2-NRTN and protein expression, as well as the potential for toxicity and efficacy. Finally, additional studies were directed toward understanding and better elucidating the weight loss problem recently reported by others following GDNF expression in the SN. Collectively, these data provided strong support for the safety, feasibility and potential usefulness of targeting the SN with AAV2-NRTN in advanced PD patients while also offering a rational, empirically-supported means of defining a dose likely to be safe and effective for translation to testing in humans.

Materials and methods

Animals

Male Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 275–325 g at the time of surgery served as subjects as described in individual experiments below. Three adult monkeys (*Macaca fascicularis*; 6.4–10 kg at time of surgery) housed at the AAALAC-accredited, Biological Resources Laboratory at the University of Illinois (UIC), served as subjects. All rodent and nonhuman primate experiments were performed with approval of the appropriate Institutional Animal Care and Use Committee and in accordance with NIH Guide for the Care and Use of Laboratory Animals. For quantification of the volume of the SN, tissue from the present experiments as well as tissue from prior studies was utilized (Bartus et al., 2011; Herzog et al., 2009).

Viral vectors

Viral vector design, construction, production and purification methods were as described previously (Gasmı et al., 2007b). The AAV2-NRTN stock was 1.2×10^{13} vg/mL, while AAV2-GDNF and AAV2-GFP were 1.6×10^{13} and 5.8×10^{12} vg/mL, respectively. Vectors were diluted with formulation buffer (FB, PBS with 2 mM magnesium chloride) to the desired concentrations prior to intracranial injections on the day of surgery for each experiment. All doses have been expressed as vg/hemisphere, and the exact doses are detailed in individual experiments below.

Rodent surgical procedures

Animals were anesthetized with an intraperitoneal injection of an anesthetic cocktail (ketamine, xylazine, acepromazine) and

intracranial injections were made using standard stereotaxic techniques. All injections were made using a Hamilton syringe with a 26 s gauge stainless steel needle at a flow rate of 0.5 $\mu\text{L}/\text{min}$. Prior to use, the needle/syringe was cleaned, sterilized, and primed with viral vector to prevent adsorption of the vector to the stainless steel needle during dosing. Studies employing 6-OHDA lesions (as described below) involved unilateral delivery of AAV or FB control to the SN (1.6, 2, or 5 μL in 1 site per hemisphere at AP-5.0, ML \pm 2.0 mm from bregma, DV-7.2 mm from dura). All other studies employed bilateral injections into the SN. When appropriate, 6-OHDA (Acros Organics) was delivered to the striatum in a single deposit of 20 μg in 5 μL (0.02% ascorbic acid/0.9% saline) at the following coordinates relative to bregma: AP +0.2, ML -3.0 mm from bregma, DV -5.0 from dura. For the Supplement to Experiment 2 (see below), which was designed to characterize the effect of AAV2-NRTN injection volume (1.0, 3.2 and 10 μL per site) on the subsequent volume of NRTN expression, rats received single injections (2×10^9 vg/hemisphere) into the left and right striata (AP +0.4, ML \pm 3.5 mm from bregma, DV -5.0 from dura) and were sacrificed 4 weeks later for histological analyses.

Neurobehavioral assessments: functional observational battery

The Functional Observational Battery (FOB) consists of standardized physical, behavioral, and neurological assessments that are performed by experienced individuals, blinded to treatment group. It provides formal information about adverse neurological changes and thus is useful for assessing potential problems related to expression of NRTN or GDNF. Variations of this formal test battery have been used extensively in rodent safety/toxicology studies (e.g., Moser, 2000; Ross, 2002). Assessments included explicit observations made while the rat was in its home cage, evaluation of behavior in an open field, hand-held observations and assessments, assessment of specific cranial/spinal reflexes and routine recording of body weight and temperature.

Histology and immunohistochemistry

At sacrifice, animals were deeply anesthetized and intracardially perfused with saline followed by a modified Zamboni's solution (4% paraformaldehyde, 0.18% picric acid). Brains were removed and placed in a 30% sucrose solution prior to sectioning (40 μm) frozen on a sliding knife microtome. Potential neurotoxicity was assessed on H&E stained sections throughout the entire brain in the initial dose range study (i.e., Experiment 2, below) by a board-certified (DACVP) veterinary pathologist, who was blind to the treatment group. Immunohistochemistry was utilized to assess transgene expression throughout the brain, using goat polyclonal anti-human NRTN (1:250; R&D Systems) and anti-human GDNF (1:1000; R&D Systems). Tyrosine hydroxylase labeling of dopaminergic cells and fibers was performed using a rabbit anti-TH antibody (1:500; PelFreez).

Quantification of tyrosine hydroxylase positive nigral neurons and volume of transgene expression

The optical fractionator method (West et al., 1991) was used to estimate the total number of TH-positive cells in the substantia nigra (SN) as described previously (Gasmi et al., 2007a). Results are presented as the percent of TH-positive cells in the lesioned hemisphere relative to the intact hemisphere. Volumetric analyses of NRTN and GDNF distribution were performed on a series of sections using a microscope interfaced with a camera and SPOT Advanced software (Diagnostic Instruments). The area of transgene expression was determined on a series of sections spanning from approximately -2.3 mm to -7.6 mm relative to bregma according to the atlas of Paxinos and Watson (Paxinos and Watson, 2004) and total volume was subsequently calculated using Cavalieri's principle and the

following formula: $V = A \times F \times T$ where A is the sum of the area across all measured sections, F is the sampling frequency (i.e. 6 for a 1-in-6 series), and T is the section thickness. For the Supplement to Experiment 2, NRTN volumetric analyses were performed on a series of sections spanning the entire rat striatum using Cavalieri's principle as described above.

Statistical analyses

Between-group differences were assessed using parametric analyses of variance (ANOVA) followed by Tukey's multiple comparison post-hoc testing, when appropriate, with significant difference between groups established if $p < 0.05$. Correlational analyses were performed using Pearson's product moment correlation coefficient.

Prologue experiment: quantifying inter-species nigra volumes in order to scale and calculate between-species dose equivalents

We assessed 84 rat SN hemispheres and 16 *Macaca mulatta* (rhesus) monkey SN hemispheres, providing a more comprehensive assessment of the absolute and relative SN volumes in both species than previously available in the literature. Contours around the SN were made on images captured throughout the rostrocaudal plane of the structure on a per hemisphere basis using TH- and/or NRTN-labeled sections to permit calculation of volume of SN in each hemisphere (see calculation of volume above). Estimates of human SN volume were derived from a more-extensive published literature (Ahsan et al., 2007; Foster et al., 2008; Krabbe et al., 2005). (See Table 1).

Experiment 1: targeting the primate SN with AAV-GFP to confirm initial dose predictions

AAV2-GFP (5.8×10^{12} vg/mL) was administered to the SN in the right hemisphere of 3 monkeys (*M. fascicularis*) in a volume of 10 μL at 2 $\mu\text{L}/\text{min}$, using MRI-guided stereotaxic delivery. Eight weeks later the monkeys were euthanized and histological analyses were performed. Surgical and histological analyses were as described in detail previously (Kordower et al., 2000; 2006). Microscopic analyses of the volume of GFP expression was performed using Cavalieri's principle as described above.

Experiment 2: targeting the SN with a range of AAV2-NRTN doses in rats to test the safety and feasibility, as well as spread of protein

In this experiment, 6 groups of naïve Sprague Dawley rats ($n = 7-8$ per group) were employed (see Table 2). Surgical procedures were as described above. Animals underwent formal neurological assessment via FOB testing at 13 weeks post nigral dosing (see above). Histological analyses following sacrifice at 6 or 13 weeks included assessment of transgene expression and potential neurotoxicity on H&E stained sections evaluated by an independent histopathologist, blind with respect to treatment group.

Supplement to Experiment 2: comparison of different vector volumes on NRTN expression in rat brain

Because it was necessary to give a larger, 5 μL volume of vector to achieve the highest AAV2-NRTN dose (Dose D; Table 2), we conducted a supplemental experiment to help assure that the difference in volume did not affect volume of protein expression and that any differences observed as a consequence of this highest dose were therefore only related to that dose. AAV2-NRTN (2×10^9 vg/hemisphere) was administered to the striatum (see additional detail in the surgical procedure section above) bilaterally in 9 animals using a within subject design such that the volume of transgene expression was determined in

Table 1

Nigral volumes and comparable dose equivalents, scaled across species.

Study or paradigm	SN volume (mm ³)	Injection vol. per hem. (μL)	Normalized injection vol. per hem. (μL/mm ³)	SN dose per hem. (vg)	Scaled SN dose (vg x10 ⁹ /mm ³)	SN dose multiples (compared to rat targeting dose)
AAV2-NRTN Rat targeting dose (Current studies)	2.5 – 3.0	2.0 *	0.74	1.6 x 10 ⁹	0.6	-----
AAV2-NRTN Monkey targeting dose (Projected)	50 – 60	10	0.18	32 x 10 ⁹	0.6	~ 1X
AAV2-NRTN Human clinical dose (Implemented: Ph1/2b)	300 – 500	30	0.075	200 x 10 ⁹	0.5	~ 1X
AAV5-GDNF Rat dose (Manfredsson et al., 2009a, 2009b)	2.5 – 3.0	2.0	0.74	8 x 10 ⁹	3	~ 5X
AAV2-GDNF Monkey dose (Su et al., 2009)	50 – 60	50	0.9	550 x 10 ⁹	10	~ 16X

Note: for all rat dosing calculations, a SN volume of 2.7 mm³ was used; for monkey calculations, a SN volume of 55 mm³ was used; for human calculations, a SN volume of 400 mm³ was used.

* Volume is higher than required for dose but was selected to replicate Manfredsson et al. (2009a, 2009b).

a total of 6 hemispheres per condition (1.0, 3.2 and 10 μL per site). Animals were euthanized 4 weeks following injections and the volume of NRTN protein expression in the striatum was determined as described above.

Experiment 3: linking the emergence of reductions in weight gain to location of NRTN expression

Doses of AAV2-NRTN employed were 1.6 x 10⁹ vg/hemisphere—Dose B (with which no weight changes occurred in Experiment 2) and an approximately 40-fold higher dose (60 x 10⁹ vg/hemisphere; Dose D) which produced reductions in weight gain in the initial study, as early as 3 weeks following nigral delivery. All rats (n = 6 per group) were euthanized at 3 weeks post-dosing and the volume of NRTN protein expression was determined as detailed above. These data were used to perform correlational analyses on the change in weight and the location of NRTN expression at the time the changes in weight emerged.

Experiment 4: Determination of whether SN neurons are required to produce the reduction in weight gain

Three groups of rats (n = 7 to 10 per group) were tested, with change in weight and daily cage-side observations being the primary endpoints examined over a period of 6 weeks following AAV2-NRTN

delivery to the SN. These in-life measures were followed by histologic examination of brain for NRTN expression and survival of TH-positive dopamine neurons in SN. The three groups of rats were as follows: 1) intrastriatal injection of 6-OHDA followed by intranigral injection of AAV2-NRTN, 2) intrastriatal injection of 6-OHDA followed by intranigral injection of FB control or 3) intranigral injection of AAV2-NRTN only.

Experiment 5: protection against 6-OHDA following nigral targeting of AAV2-NRTN

Two lower doses of AAV2-NRTN (i.e. 0.32 x 10⁹—Dose A and 1.6 x 10⁹ vg/hemisphere—Dose B) were tested for their ability to protect SN neurons against 6-OHDA toxicity (n = 8 per group). Two weeks prior to unilateral 6-OHDA administration to the striatum, rats received either one of two doses of AAV-NRTN or FB to the SN, as described in the Materials and methods. All animals were sacrificed 2 weeks later (i.e. 4 weeks after AAV2-NRTN delivery), and the ability of each treatment to protect TH-positive nigral neurons against toxin-induced cell death was assessed stereologically.

Experiment 6: mitigating the weight reduction by dietary supplementation

Two groups of rats (n = 8 per group) received injections of the high dose of AAV2-NRTN (60 x 10⁹ vg/hemisphere; Dose D) and a

Table 2

Experimental design for Experiment 2; 'targeting the SN with a range of AAV2-NRTN doses in rats to test the safety and feasibility, as well as spread of protein'.

Group	# Rats	Injection vol. (μL)	Dose/Hem (vg)	Designated dose	Dose description
Formulation buffer	8	2 or 5	NA	FB	Control
AAV2-NRTN	8	2	0.32 x 10 ⁹	Dose A	1/5th dose projected to cover majority of SN; volume chosen to replicate Manfredsson et al. (2009a, 2009b)
AAV2-NRTN	8	2	1.6 x 10 ⁹	Dose B	Dose successfully projected to cover majority of rat SN
AAV2-NRTN	8	2	8 x 10 ⁹	Dose C	5X higher dose than required to cover SN, but equivalent to Manfredsson et al. (2009a, 2009b)
AAV2-NRTN	7	5	60 x 10 ⁹	Dose D	~40X higher than required to cover SN
AAV2-GDNF	8	2	8 x 10 ⁹	GDNF	'GDNF reference dose' reported by Manfredsson et al. (2009a, 2009b) to produce body weight changes

FB = formulation buffer; vg = vector genomes; SN = substantia nigra; NA = not applicable.

third group (n=8) received FB into the SN. All animals were maintained on a standard rodent chow diet (Pro Lab RMH 2500) with food available *ad libitum* throughout the study. Half of the AAV2-NRTN-injected animals were provided additional food on a daily basis for 8 weeks following SN injections, consisting of a mixture of various cereals, cookies, coconut flakes and peanut butter. Body weights over time and daily cage-side observations were the primary endpoints in this study.

Results

Prologue experiment: quantifying inter-species nigra volumes in order to scale and calculate between-species dose equivalents

In order to compare data between rats and monkeys and establish relative dose equivalents between rats, monkeys and humans, we first determined the relative volumes of the SN (which included the pars compacta and reticulata) for each species. This was considered an essential translational step for implementing a strategy to target the SN with AAV2-NRTN, for it provided a rational means to generalize the protein expression observed in either animal species to the other, and eventually from both rats and monkeys to humans, for potential use in a PD clinical trial. Because of the paucity of published data regarding SN volume in rats and monkeys, we determined volumes for each prior to initiating studies to test the safety and feasibility of targeting the SN, as described in **Materials and methods**.

Using 84 SN hemispheres from rats, we calculated a mean (\pm SEM) volume for the SN to be 2.68 (\pm 0.03) mm³; using 16 different SN hemispheres from monkeys (*M. mulatta*), we calculated a mean volume for SN to be 55.89 (\pm 2.90) mm³. Our assessment of human SN volume was based on data from existing, published literature, and was determined to be between 300 and 500 mm³ (Ahsan et al., 2007; Foster et al., 2008; Krabbe et al., 2005). Using relative between-species SN volumes, relative dose equivalents based for each species could be established. For example, when calculated on a vg/mm³ basis, 1.6 \times 10⁹ vg into the rat SN (2.7 mm³ in volume) is roughly equivalent to 32 \times 10⁹ vg into the monkey SN (55 mm³ in volume), which in turn is roughly equivalent to 200 \times 10⁹ vg into the human SN (400 mm³ in

volume). These dose equivalents allowed for direct comparisons of the effects achieved with AAV-GDNF and AAV-NRTN between species and laboratories, as well as provided the means to project an appropriate SN-targeted human dose (see **Table 1**).

Experiment 1: targeting the primate SN with AAV-GFP to confirm initial dose predictions

As an initial experiment to target the SN, we assessed spread of AAV2-vector within the SN, providing information helpful for determining the range of doses of AAV2-NRTN to test in rodents (and eventually humans). Using the calculations of monkey SN volumes obtained in the Prologue Experiment, and data collected from several previous studies where volume of protein expression was quantified following AAV2-GFP, AAV2-NRTN and AAV2-NGF injections into other brain sites (Bishop et al., 2008; Herzog et al., 2007; 2008; 2009; Kordower et al., 2006), we estimated that 58 \times 10⁹ vg AAV2-GFP would likely cover a significant portion of the monkey SN with vector (taking into account that GFP is not secreted and thus AAV2-GFP will only reflect volume of neurons transduced), while importantly, not exceeding its boundaries. Quantification of the volume of GFP positive immunolabeling in the region of the SN revealed a mean of 31.3 mm³ (SEM \pm 3.2) for the 3 AAV injected monkeys (*M. fascicularis*). This pattern of expression revealed that the AAV2-GFP injections into the SN reasonably covered the targeted tissue (~50–60%). These data, therefore, helped confirm that AAV2 vector spread (and protein expression) in and around SN was not substantially different from other brain regions we had previously targeted (e.g., caudate, putamen, nucleus basalis of Meynert) with AAV2 (Bishop et al., 2008; Gasmi et al., 2007a; 2007b; Herzog et al., 2007; 2008; 2009; Kordower et al., 2006).

This information was then used to define the dose range of AAV2-NRTN tested in rats in Experiment 2, taking into account the fact that, contrary to GFP, NRTN is secreted from the cell bodies and thus produces somewhat greater coverage with a comparable vg dose. Together with that data obtained from the rat dose-response study, these data were essential for calculating the ‘human-equivalent’ doses eventually used in an ongoing trial administering AAV2-NRTN to the

Table 3
A ~200-fold range of AAV2-NRTN (CERE-120) doses to substantia nigra caused no serious toxicity or side effects over a range of blinded measures and methods.

In-life safety/tolerability	Outcome
“In-life” observations/measurements	
<ul style="list-style-type: none"> Daily cage-side observations <ul style="list-style-type: none"> e.g., salivation, lacrimation, piloerection, involuntary motor behavior, postural abnormalities Three times per week body weight Weekly food intake 	<ul style="list-style-type: none"> No differences in general health/appearance <ul style="list-style-type: none"> No differences noted in any behavior Modest reduction; highest dose only Modest reduction; 1st 6 weeks
Neurobehavioral examination	
<ul style="list-style-type: none"> Hand-held examination <ul style="list-style-type: none"> Reactivity to handling; sensorimotor response to handling, physical exam Cranial/spinal reflexes <ul style="list-style-type: none"> Palpebral, pinna, flexor Body temperature Open field behavior <ul style="list-style-type: none"> Exploratory behavior, activity level, ambulatory and non-ambulatory movements 	<ul style="list-style-type: none"> No changes noted All reflexes intact No change or difference All behaviors appeared normal
Transgene expression	
<ul style="list-style-type: none"> NRTN labeling throughout brain Quantification of NRTN distribution 	<ul style="list-style-type: none"> Dose-related increase in volume of NRTN expression from targeted SN
Neurotoxicity	
<ul style="list-style-type: none"> H&E staining throughout brain Blinded histopathological analysis 	<ul style="list-style-type: none"> No neurotoxicity, cell loss, inflammation, gliosis, cellular proliferation, hemorrhage or other effect of vector or transgene

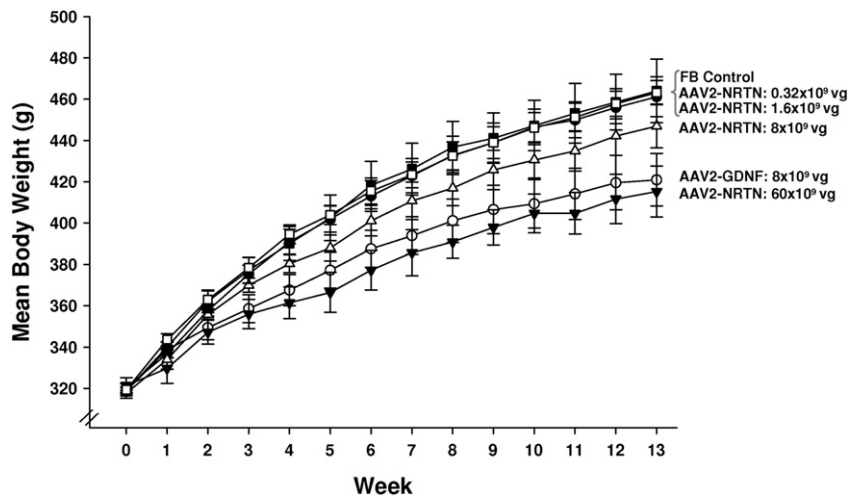


Fig. 1. Dose-related effect of bilateral nigral-targeted AAV2-NRTN and AAV2-GDNF on body weight in rats over time (Experiment 2). Note that only the highest dose of AAV2-NRTN (i.e., Dose D: 60×10^9 vg/hemisphere) produced a reliable, statistically significant reduction in weight gain (approximately 12% less weight gain over 13 weeks; (p 's < 0.02–0.05; see Results for additional statistical detail). This dose is roughly 40 times the rat dose successfully projected to provide appropriate NRTN coverage (i.e., 1.6×10^9 vg/hemisphere: Dose B) and thus 40 times higher than the projected human dose, by relative volume of SN in rats versus humans). No other AAV2-NRTN dose had an effect. The single dose of AAV2-GDNF tested (i.e., 8×10^9 vg/hemisphere, equivalent to the AAV5-GDNF dose reported in the literature to cause weight loss) produced an equivalent reduction in weight as the highest AAV2-NRTN dose. All doses are defined on the basis of vg/hemisphere.

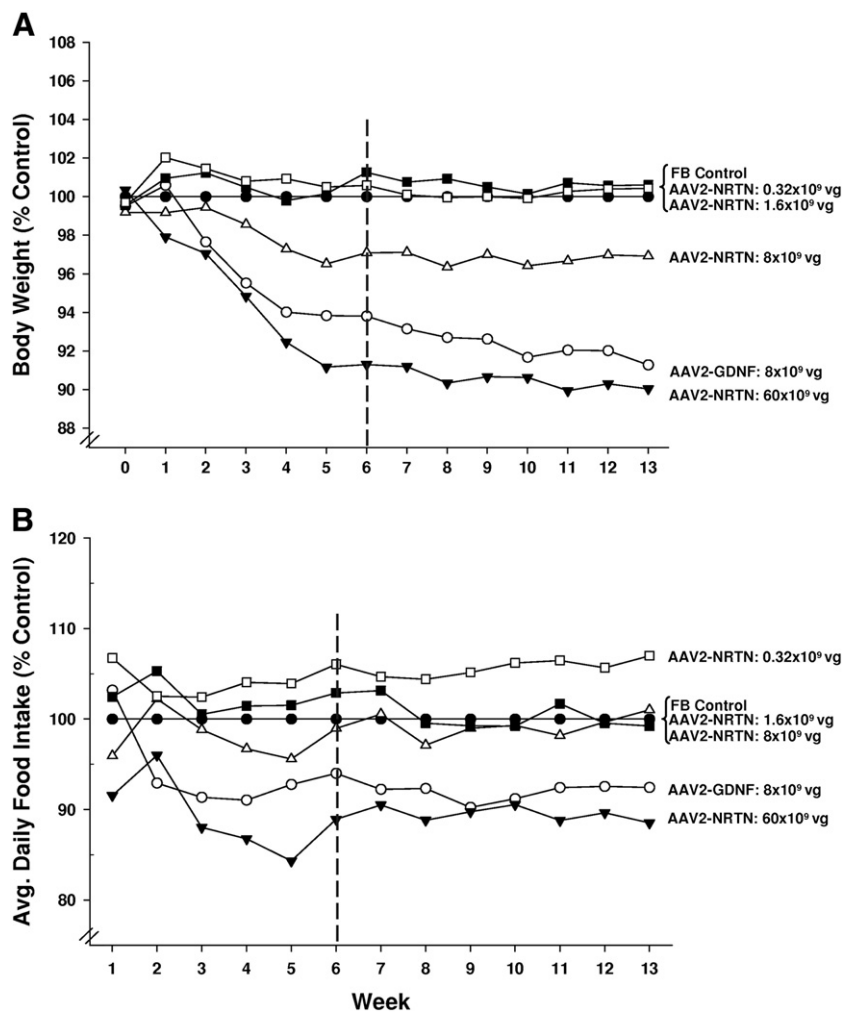


Fig. 2. Effect of bilateral nigral GFLs (i.e., GDNF and NRTN) on weight changes and food intake as a function of dose and time from Experiment 2. (A) Differences in body weight as a function of dose (plotted as % of controls); note that by approximately week 6, dose-related differences in weight have stabilized and no further differences are seen (i.e., weight curves are parallel over remaining time points); (B) decrease in food per week as function of dose (plotted as % of controls); note also that by week 6, differences in food intake between dose groups stabilized, mirroring stabilization of weight changes in panel A.

SN and putamen of PD patients (i.e., CERE-120; ClinicalTrials.gov identifier NCT00985517; see Table 1).

Experiment 2: targeting the SN with a range of AAV2-NRTN doses in rats to test its safety and feasibility, as well as spread of protein

Based on the results of Experiment 1, complemented with data from past studies, we selected a range of AAV2-NRTN doses to test the safety and volume of NRTN expression following delivery to the SN in rats. These doses included one that we projected to adequately cover the SN with NRTN, as well as doses both lower and higher, in order to adequately characterize the targeting, potential benefits, as well as the potential risks and side effects of injecting AAV2-NRTN into the SN (see Table 2). We also included a dose of AAV2-GDNF that was equivalent to the dose of AAV5-GDNF reported by Manfredsson et al. (2009a; 2009b) to cause weight loss when injected into the SN (e.g., 8×10^9 vg/hemisphere), thus providing an opportunity to independently replicate and extend those data. We employed the same volume, infusion rate and vector concentration that they used for our AAV2-GDNF as well as most of our CERE-120 groups, but because they employed AAV5, which is known to bind with lower affinity to heparin sites in the brain and therefore spreads more widely throughout the parenchyma, our use of AAV2 necessarily provided a conservative estimate of the GDNF expression previously obtained with the same vg/hemisphere dose of AAV5-GDNF (see Tables 1 and 2).

Despite testing a wide (~200-fold) range of AAV2-NRTN doses, no apparent side effects or other serious deleterious consequences were seen during the course of this study. The formal 'Functional Observational Battery' (FOB), conducted by experienced individuals blinded to treatment group, revealed that all animals appeared normal during cage-side observations (e.g. no salivation, lacrimation, piloerection, involuntary motor behavior or abnormalities in posture). Additionally, all animals from all groups were found to display normal reactivity and sensorimotor responses to handling, and were found to have intact neurological reflexes (e.g. palpebral, pinna and flexor). In addition, no abnormalities in gait, posture, exploratory activity, or any

other behavior were observed while animals were individually placed in an open field for formal observation (Table 3). Finally, no between-group differences were seen in body temperature, though a modest, decrease in weight gain was observed with the highest AAV2-NRTN, as well as the single AAV2-GDNF dose (Fig. 1).

Statistical analyses on weekly body weight data for all animals through week-13 (two-way repeated measures ANOVA) revealed a significant effect of treatment group [$F(5,23) = 3.4$, $p < 0.025$, a significant effect of time [$F(13,299) = 661.5$, $p < 0.001$], and a significant interaction between the two [$F(65,299) = 3.5$, $p < 0.001$]. However, a post-hoc Tukey test confirmed that only the highest AAV2-NRTN dose (60×10^9 vg/hemisphere) and the single AAV2-GDNF dose tested (8×10^9 vg/hemisphere—Dose D) produced a significant, but relatively modest reduction in weight (10 to 12% decrease relative to FB controls; $p < 0.02$ – 0.05 ; see Fig. 2A). No other AAV2-NRTN groups differed from FB controls at any time point.

Several aspects of the reduction in weight gain deserve special attention. First, the dose-related decrease on weight gain was linked to a similar dose-related decrease on food intake (see Figs. 2A and B). Secondly, the time course of the dose-related effect on weight and food intake co-varied, with both showing no further between-dose changes after week-6 (see Figs. 2A and B). These data argue that the reduced weight gain is most likely a direct result of a self-limiting reduction in food intake.

Formal, independent histopathology examination of the brains from all animals revealed no evidence of toxicity or histopathology as a result of AAV2-NRTN. A mild trauma-induced inflammatory reaction was observed along the needle tract across all treatment groups and was not seen elsewhere (see Fig. 3). The independent neuropathologist (ToxPath Specialists, Hagerstown, MD) who examined the coded sections (blinded to treatment groups) stated that the major reaction at the injection site was gliosis (astrocytosis), which is consistent with what we have previously confirmed in several previous studies using immunohistochemistry (e.g., GFAP antibody) where a similar needle tract was produced during delivery (Gasmi et al., 2007a; Herzog et al., 2007,2008, 2009). While, in this study, we did not perform a panel of immunohistochemical markers to more fully characterize the local-site

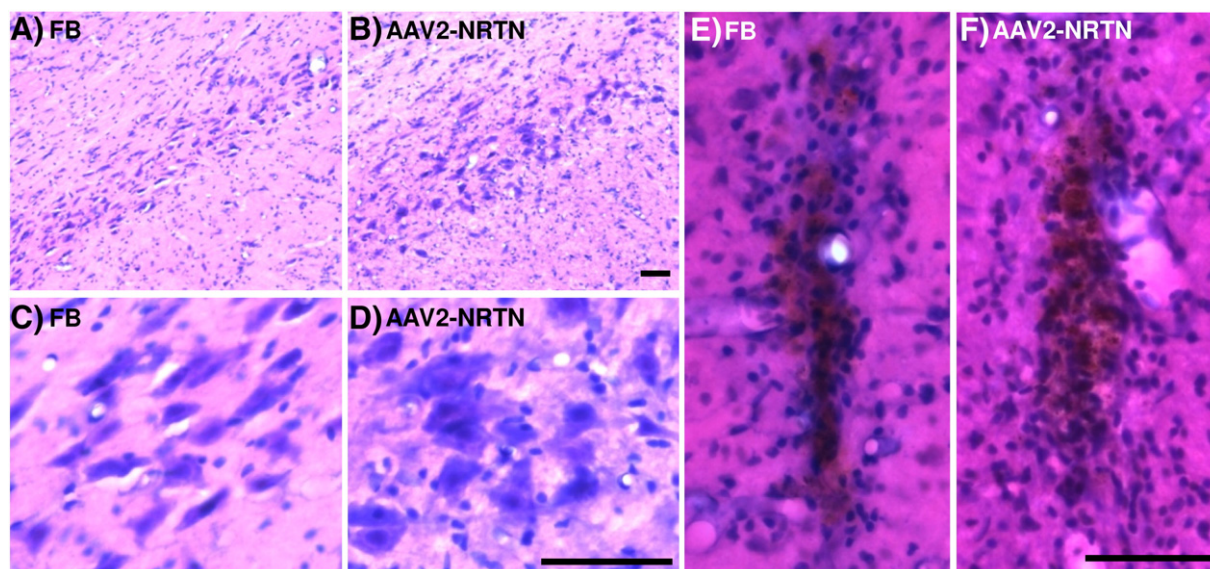


Fig. 3. Hematoxylin and eosin (H&E) stained coronal sections through the rat midbrain, collected in Experiment 2. Panels A (FB: formulation buffer control) and B (highest dose AAV2-NRTN) present low magnification photomicrographs from regions in the substantia nigra where NRTN immunolabeling was most intense (see Fig. 4), illustrating the normal pattern of staining expected in the SN (including pars compacta and reticulata). Panels C (FB) and D (highest dose AAV2-NRTN) are higher magnification photomicrographs illustrating the normal appearance of individual neurons within the pars compacta in Panels A and B. No evidence of neurotoxicity or any other pathology was found in any section from any group. Note that hypertrophy (increase in neuronal size) was observed following AAV2-NRTN, which reflects a normal and common, positive neurotrophic response to NRTN. Panels E (FB) and F (high dose AAV2-NRTN) illustrate the typical pattern of staining we observed in the region proximal to the needle tract (only), showing clear evidence of a mild inflammatory reaction, gliosis, and hemosiderin deposits. These changes reflect the only histopathological abnormality seen in these studies and were qualitatively similar and equally robust in AAV2-NRTN and FB control animals, reflecting an expected reaction to trauma induced by the injection needle. Scale bar in Panels B, D and F represents 50 μ m.

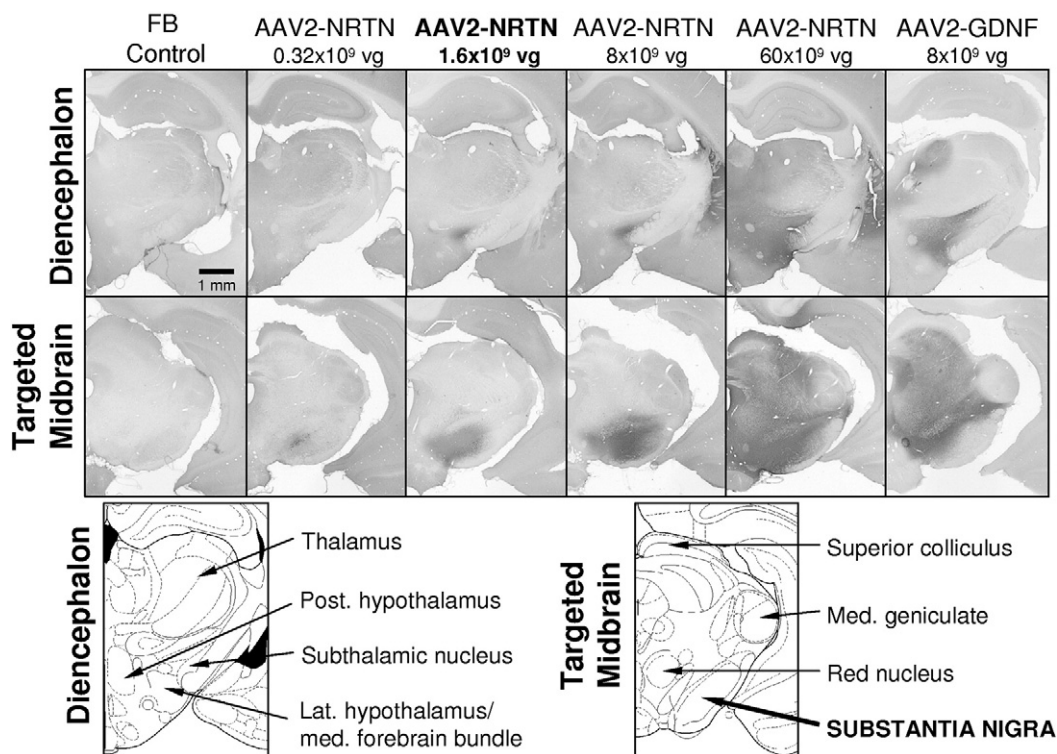


Fig. 4. Dose-related NRTN and GDNF expression following bilateral injections of vector into substantia nigra (SN) from Experiment 2 (see Tables 1 and 2 for details regarding doses). Top row shows representative photomicrographs of coronal sections through the diencephalon area of the brain (see left panel atlas drawing below photomicrographs for specific structures). The second row shows similar sections from the same rats, cut through the midbrain, containing SN (see right panel atlas drawing for specific structures). Note that the lower doses of AAV2-NRTN produce NRTN expression throughout, and to varying degrees in reasonable proximity to the SN, while the highest dose of AAV2-NRTN (Dose D) and AAV2-GDNF produce protein far outside the targeted SN and midbrain, and well into the diencephalon. All doses are defined on the basis of vg/hemisphere. Coronal sections illustrating anatomical boundaries are -3.6 and -5.8 mm in the AP plane relative to bregma from the atlas of Paxinos and Watson (Paxinos and Watson, 2004).

reaction we can state with a high level of certainty, that in addition to increased astrocytes, a more modest increase in microglia/monocytes and pan-leucocytes (detected in the past via CD68 and CD45, respectively) most likely also occurred in all groups, including formulation buffer control (Gasmı et al., 2007a; Herzog et al., 2007, 2008, 2009). The pathologist concluded, “All tissue changes were similar (across all groups) and were thus due to the mechanical trauma associated with the injection procedure. There was no evidence of neuronal damage in any animal.” Note that robust hypertrophy

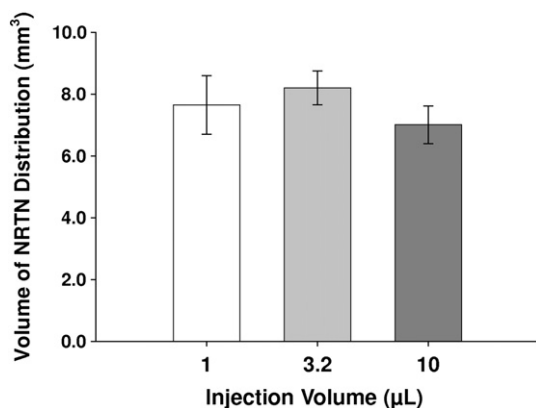


Fig. 5. Volume of neurturin (NRTN) expression in rat striatum following AAV2-NRTN, delivered via three different volumes (1, 3.2 and 10 μL) with the concentration adjusted to provide the same vector genome dose (2×10^9 vg/hemisphere). Note that equivalent volume of NRTN distribution is achieved with the same AAV-NRTN dose, despite a 10-fold difference in volume of vector. These data corroborate earlier evidence that volume of transgene distribution is influenced strongly by total vector genome dose (e.g., see, Bishop et al., 2008; Herzog et al., 2008; 2009) and that within the range of volumes tested, total volume delivered is relatively unimportant.

(increase in neuronal size) was observed following AAV2-NRTN (see Figs. 3C and D). This is a normal and common phenomenon indicative of a positive neurotrophic response, and has been reported previously in the rat (Bartus et al., 2007) and nonhuman primate (Herzog et al., 2009) following NRTN expression in the nigrostriatal system.

An evaluation of NRTN staining in the brains revealed significant, dose-related increases [Mean (± SEM) total volume of NRTN detected for Dose A: $2.0 (\pm 0.4)$ mm³; Dose B: $4.6 (\pm 0.6)$ mm³; Dose C: $7.5 (\pm 1.1)$ mm³; Dose D: $41.1 (\pm 9.7)$ mm³; AAV2-GDNF: $27.2 (\pm 3.4)$ mm³]. Importantly, the rat dose closest to the primate equivalent (i.e., 1.6×10^9 vg/hemisphere; Dose B) provided appropriate NRTN staining throughout the nigra, with relatively little NRTN in surrounding brain sites, demonstrating reasonable between-species predictability using relative SN volumes to calculate between-species dose equivalents. In contrast, the two higher AAV2-NRTN doses (Doses C and D; see Table 2), as well as the ‘AAV2-GDNF reference dose’, produced extensive protein well outside the targeted nigra, including far into the diencephalon (i.e., a significant distance from the SN; see Fig. 4). It is also noteworthy that far greater spread of GDNF to untargeted tissue was seen, compared to that for NRTN, when equivalent doses were compared; a similar difference was also seen with the effects on body weight (see Fig. 1).

Supplement to Experiment 2: comparison of different vector volumes on NRTN expression in rat brain

No differences were noted in the distribution and pattern of NRTN expression in the striatum between three groups given a 10-fold range of vector volumes (but the same vg dose, accomplished by varying vector concentration). Blinded, quantitative, volumetric analyses confirmed no differences in volume of NRTN expression

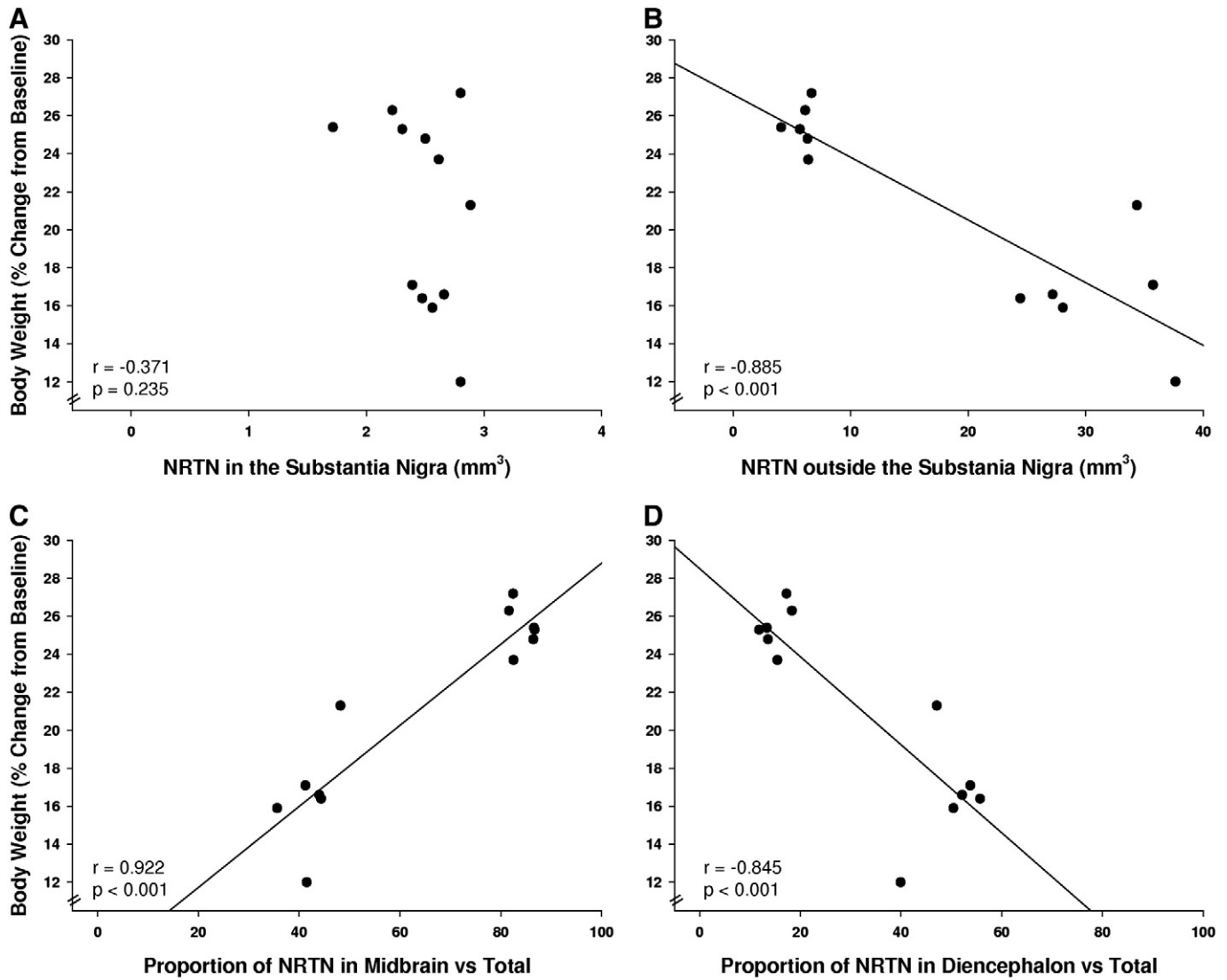


Fig. 6. Graphs depicting correlational analyses from individual rat data, performed on weight gained in rats euthanized at week 3, post AAV-NRTN versus location/amount of NRTN protein at week 3 (Experiment 3). Rats used for this analysis were given a dose that adequately exposes the SN with NRTN (Dose B: 1.6×10^9 vg/hemisphere) and one 40 times higher (Dose D: 60×10^9 vg/hemisphere) that caused reliable reductions in weight gain in Experiment 2 as early as 3 weeks following dosing. These analyses were intended to identify where NRTN protein exists when the reduction in weight gain first begins to emerge. (A) Lack of correlation between weight gained and volume of NRTN expressed in SN, further corroborating conclusion that changes in weight are not related to NRTN expression in SN. Note also that no further increase in NRTN volume of expression in SN is seen with 40-fold increase in dose because much lower dose was sufficient to cover volume of SN and thus additional vector only caused protein to be expressed far outside targeted SN; (B) strong *negative* correlation between weight gained and volume of NRTN expression outside nigra, further suggesting weight effect is mediated by NRTN expression somewhere outside SN; (C) strong *positive* correlation between weight gained and proportion of NRTN expressed within midbrain (i.e., SN and surrounding structures), relative to non-midbrain sites. These data illustrate that when NRTN is mainly restricted to midbrain, animals gain weight normally; indeed, the more NRTN expression that occurs in the midbrain (i.e., SN and surrounding structures) relative to non-midbrain structures, the more weight gained; (D) Strong *negative* correlation between weight gained and proportion of NRTN expressed in diencephalon. These data illustrate that the more the diencephalon is exposed to NRTN, the less weight that is gained.

(Fig. 5; $F(2,15) = 0.68$, $p = 0.52$) as a consequence of delivering the same AAV2-NRTN dose over a range of volumes from 1 to 10 μ L.

Experiment 3: linking the emergence of reductions in weight gain to location of NRTN expression

In order to better elucidate the location of NRTN expression responsible for the weight changes observed in Experiment 2, an additional study was performed where two doses of AAV2-NRTN or FB control were administered bilaterally to the SN. One dose of AAV2-NRTN was 1.6×10^9 vg/hemisphere (Dose B), which caused no weight changes in Experiment 2, and the other was approximately 40-fold higher (60×10^9 vg/hemisphere; Dose D) which produced the reductions in weight gain in the initial study, as early as week-3, post-dosing. By comparing NRTN expression at 3 weeks in rats given a dose known to cause diminished weight gain at this time point, with

another dose shown not to affect weight at any time point, we were able to correlate the changes in weight induced by administration of AAV2-NRTN and the location of NRTN expression at the time the weight changes emerged.

Reliable reductions in weight gain were once again observed with the high dose of AAV2-NRTN (60×10^9 vg/hemisphere) as early as week 2, which continued to the week-3 termination-point. Once again, no change in weight was seen with the lower dose of AAV-NRTN (1.6×10^9 vg/hemisphere), which properly targeted the SN. Statistical analyses on percent-change in body weight from baseline confirmed a significant effect of group [$F(2,15) = 7.5$, $p < 0.01$], significant effect of time [$F(2, 30) = 579.6$, $p < 0.001$], and a significant interaction between the two [$F(4,30) = 16.4$, $p < 0.001$], with post-hoc analyses revealing significant reduction in weight gain following the higher 60×10^9 vg/hemisphere AAV2-NRTN dose, compared to both the lower 1.6×10^9 vg/hemisphere dose and FB injected animals at

week 2 and 3 (p 's < 0.02–0.05). The absolute and relative differences in weight gain closely mirrored those previously obtained for the same groups on weeks 1 through 3, as reported in Experiment 2 (Fig. 1).

Immunohistochemical analysis of NRTN staining throughout the brain from these rats was performed at week 3 and the pattern of staining did not differ substantially from that seen previously at week 13 (i.e., Fig. 4). Detailed analyses, based on quantification of NRTN expression within the rat brain following the two AAV2-NRTN doses are summarized in Fig. 6. Fig. 6A shows that no correlation exists between the amount of weight gained during the course of this study versus volume of NRTN protein expressed in the SN (Pearson $r = -0.37$, $p > 0.05$). Note that both doses of AAV2-NRTN exposed the majority of SN with NRTN (estimated to be 2.5 to 3 mm³) and thus while the 40-fold difference in dose produced a marked difference with regard to effect on weight, there were no measurable differences with regard to volume of NRTN within SN. Fig. 4B, however, shows a strong *negative* correlation (Pearson $r = -0.89$, $p < 0.001$) between the volume of NRTN expression *outside* the SN versus the amount of weight gained, showing that as more protein is expressed *outside* the nigra, the less weight that is gained.

Figs. 6C and D follows the insight derived from Figs. 6A and B by addressing the question, 'where outside the nigra must the protein be expressed to produce the change in weight?' Fig. 6C, shows a *positive* correlation between the weight gained versus the proportion of NRTN protein expressed in the midbrain, relative to the total volume (Pearson $r = 0.92$, $p < 0.001$). Thus, as more NRTN is expressed in the midbrain (i.e., the SN and the several surrounding brain structures), relative to other non-midbrain regions (i.e., primarily diencephalon), the more normal was the weight gained. In contrast, Fig. 4D shows that a strongly *negative* correlation was obtained between weight gained versus the proportion of NRTN expressed in the diencephalon, relative to the total volume of NRTN (Pearson $r = -0.85$, $p < 0.001$). In other words, the greater the NRTN expression in the diencephalon, the less weight the rats gained.

Experiment 4: determination of whether SN neurons are required to produce the reduction in weight gain

If nigral neurons are responsible for GFL-mediated reduction in weight gain, then destroying these neurons prior to administering GFLs should prevent GFL-mediated reductions in weight gain. To further test this, we first destroyed the majority of dopaminergic SN neurons with a unilateral striatal injection of 6-OHDA (6-hydroxydopamine) and then administered the highest dose of AAV2-NRTN 3 weeks later ipsilaterally to the SN (i.e., after the majority of the dopamine neurons would be destroyed). This dose of AAV-NRTN had previously been shown to produce reliable reductions in weight gain in Experiments 2 and 3, above.

Quantification of TH-immunolabeled neurons in the SN confirmed that pre-treatment with 6-OHDA to the striatum successfully destroyed the majority of TH-positive neurons in the SN. On the intact side, the mean number of TH-positive cells was 11,593 (± 154) for FB controls and 11,047 (± 381) for AAV2-NRTN animals. On the lesioned side 2216 (± 256) remained in controls and 2696 (± 154) in AAV2-NRTN-injected animals. Immunohistochemical analysis of NRTN staining revealed that the pattern and extent of NRTN staining for both doses was indistinguishable from that produced by the same two doses in Experiments 2 and 3, except that NRTN expression was restricted to the ipsilateral hemisphere following the unilateral dosing employed in this experiment.

Quantification of body weight over time produced data similar to that seen in Experiment 2. That is, a significant decrease in weight gain was seen following administration of the high AAV2-NRTN dose (60×10^9 vg/ single hemisphere) during the 6 week duration of this study (Fig. 7A). Importantly, despite extensive (i.e. 80%) destruction of dopamine nigral neurons, the high AAV2-NRTN dose, when injected

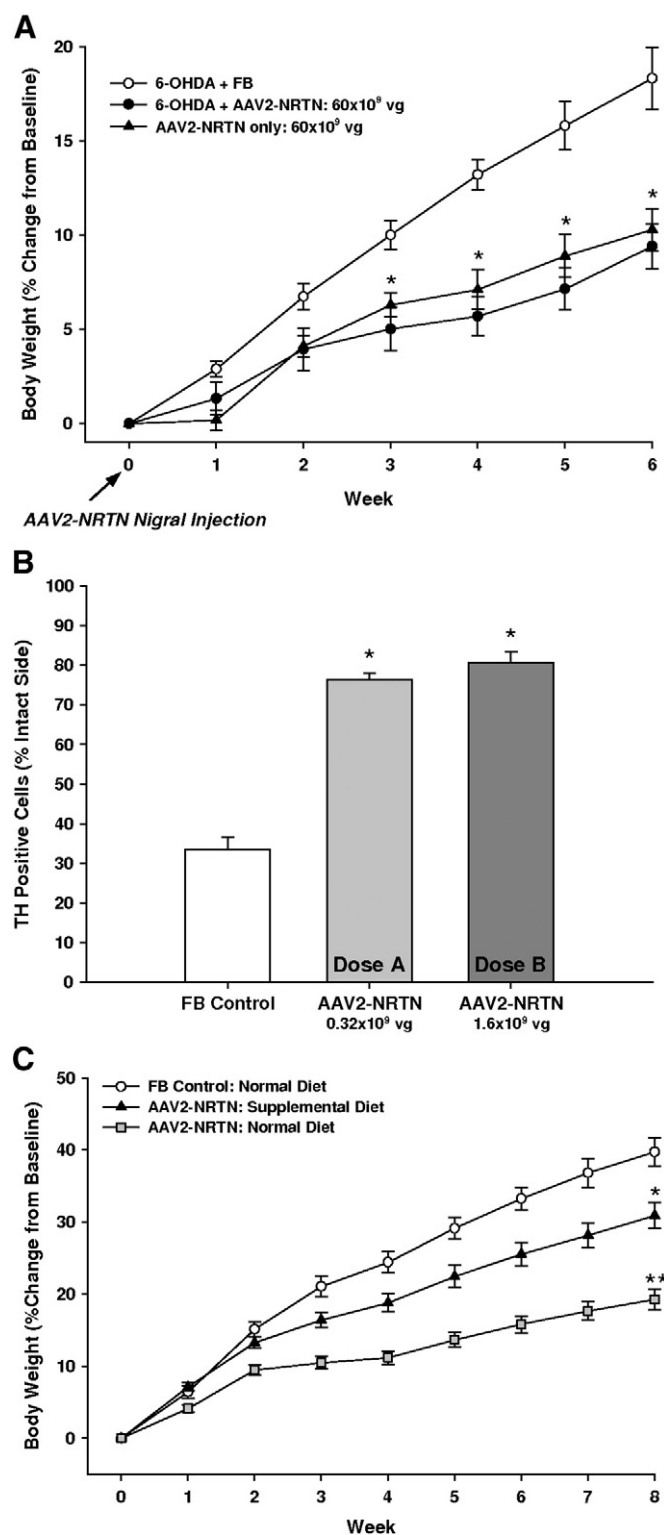


Fig. 7. (A) Prior destruction of nigral DA neurons (via unilateral 6-OHDA) has no impact on weight changes induced by a high dose AAV2-NRTN (Dose D) administered unilaterally to the SN as in Experiment 4 (* represents p 's < 0.02–0.001 compared to controls). (B) Preservation of nigrostriatal neurons following two doses of AAV2-NRTN (Doses A and B) in 6-OHDA lesioned rats (Experiment 5). Note that both these doses covered major portions of the SN without substantial protein expression far outside its boundaries. The data demonstrate that both doses were able to protect against unilaterally administered 6-OHDA toxicity (* represents $p < 0.001$ compared to FB controls). (C) Simple dietary supplementation of normal rat chow with preferred, high caloric food mitigates ~50% of weight changes in rats produced by bilateral, high dose of AAV2-NRTN (Dose D) injected into the SN (Experiment 6).

into the area of the SN, had an equivalent effect on body weight, compared to rats with a completely intact dopaminergic SN cell population.

Statistical analyses revealed significant between-group effects on body weight, [$F(2, 20) = 12.1, p < 0.001$], a significant effect of time [$F(6, 120) = 189.2, p < 0.001$], and a significant interaction between these two variables [$F(12, 120) = 11.0, p < 0.001$]. Post-hoc analyses (Tukey test) confirmed that both AAV2-NRTN groups were found to differ significantly from FB controls beginning at week 3 and persisting through the end of the study (all p 's < 0.02 – 0.001). There were, however, no differences in body weight between 6-OHDA lesioned animals that received AAV2-NRTN injections, compared to intact AAV2-NRTN-injected animals (Tukey test, p 's > 0.05). These data therefore corroborate the prior conclusions of Experiments 2 and 3, that the weight changes seen with the high dose AAV2-NRTN and AAV2-GDNF are not related to SN-exposure, but likely due to mistargeted protein expression far outside the targeted SN.

Experiment 5: protection against 6-OHDA following nigral targeting of AAV2-NRTN

This experiment tested whether the two lowest doses of AAV2-NRTN (which involved the dose we had projected to cover the SN, based on prior scaling exercises described in the Prologue Experiment and Experiments 1 and 2 (1.6×10^9 vg/hemisphere), and one five times lower (0.32×10^9), which still covered a significant proportion of the SN; see Fig. 4) were able to provide significant neuroprotection of dopaminergic nigral neurons against 6-OHDA toxicity. As shown in Fig. 7B, both doses provided robust neuroprotection when administered to the SN (one way ANOVA: $F(2, 15) = 106.1, p < 0.001$; post-hoc analyses: both AAV2-NRTN doses produced comparable, robust neuroprotection relative to FB controls; $p < 0.001$).

Experiment 6: mitigating the weight reduction by dietary supplementation

As shown in Fig. 7C, supplementing the normal rat diet with preferred, high caloric foods mitigated at least 50% of the weight changes induced by the high dose of AAV2-NRTN. Statistical analyses (two-way ANOVA) confirmed there was a significant difference between treatment groups [$F(2,33) = 31.1, p < 0.001$], time [$F(8, 16) = 610.8, p < 0.001$], and an interaction between group and time [$F(16,264) = 28.7, p < 0.001$]. Beginning at week 3 and persisting throughout the remainder of the 8 week study, AAV2-NRTN-injected animals that received the supplemental diet consistently gained more weight than those that received the 'normal' diet (all p 's < 0.003 – 0.001), though the supplemental diet did not completely reverse the effects of mistargeted NRTN and thus both groups gained less weight compared to FB plus normal diet controls ($p < 0.005$ – 0.001).

Discussion

Background and relevance of general findings

Recent analyses of NRTN expression and nigrostriatal TH-induction following administration of AAV2-NRTN to PD subjects challenges traditional approaches of targeting the nigrostriatal neurons in PD. These analyses suggest that if delivery of neurotrophic factors to the PD brain is to be optimally effective, it will likely require targeting the cell bodies of degenerating neurons (in the SN), along with the more traditional approach of targeting the terminal fields (in the putamen) (Bartus et al., 2011; Marks et al., 2010). We report here, a series of nonclinical translational experiments testing the safety, feasibility and effectiveness of targeting the SN with AAV2-NRTN to support clinical studies attempting to enhance the trophic response in PD dopaminergic neurons via this dosing paradigm. A wide, ~200-fold range of AAV2-NRTN doses was shown to have no observable toxicity or serious side effects when injected into the SN of rats. While we were able to replicate

the relatively modest reduction in weight gain recently reported by others in rats (Manfredsson et al., 2009a; 2009b) and monkeys (Su et al., 2009) following AAV-GDNF injections to the SN, we show that this effect is clearly not due to appropriately scaled and targeted NRTN or GDNF in the SN, but rather to mistargeted protein expressed in the distant diencephalon due to use of a single, very high, excessive dose level. Thus, the recent warning of serious weight loss from targeting the SN with GFLs (Glial cell line-derived Family of Ligands, like NRTN and GDNF) seems inconsistent with the vast weight of evidence that exists (discussed in detail below). Moreover, we show that significantly enhanced neuronal survival against 6-OHDA can be achieved with AAV2-NRTN doses appropriately scaled to accurately target the SN, which are only 1/40th to ~1/200th those required to reduce weight gain, thereby establishing a wide therapeutic index and safety margin for SN-targeted AAV2-NRTN.

It is important to note that the neuroprotection study (Experiment 5) was not intended to demonstrate that nigral targeting, alone, might be sufficient for effective neurotrophic therapy in PD. We recognize the published literature suggests that a better functional outcome is correlated with preservation of fibers projecting to the striatum and that this occurs more reliably when neurotrophic factors are delivered to the terminal field (see Kirik et al., 2004 for detailed discussion). Rather, our conclusion from recent autopsy analyses (Bartus et al., 2011), is that, in contrast to accounts in animal models of PD, the degenerating nigrostriatal system of PD patients does not permit optimal exposure of the neurotrophic factor to the degenerating cell bodies when it is only delivered to the degenerating nerve terminals, thus compromising the ability of the neurotrophic factor to induce repair genes. For this reason, we have argued that, contrary to what is effective in typical animal models of PD, when treating advanced PD patients, it is necessary to also target the cell bodies directly. From this perspective, the results of Experiment 5 demonstrate that very low doses of AAV2-NRTN (CERE-120) are adequate to achieve robust preservation of degenerating neurons (effectively demonstrating that appropriate repair genes were indeed activated) and that this occurs at doses far lower than those required to cause the changes in weight or any other side effect (though no other was observed). Thus, when combined with appropriate targeting in the striatal terminal field, human-equivalent doses of AAV2-NRTN should provide optimal neurotrophic effects from NRTN expression and maximal functional improvement and protection.

Establishing the safety of AAV2-NRTN targeted to the SN and the importance of proper scaling

The studies reported here, and the interpretation of the data generated, were greatly aided by a prologue study performed to first calculate the SN volume of rats and monkeys (e.g. see Table 1). This information provided the means to scale doses between-species and calculate dose equivalents, which then allowed direct comparisons to be made regarding the effects of AAV-GDNF and AAV-NRTN across species and laboratories. This, and the wide range of doses tested here, allowed us to establish that when AAV-NRTN is appropriately targeted to the SN, no toxicity or side effects are seen at doses far in excess of those that elicit a robust neurotrophic response in rats and should be required for effective therapy in PD subjects.

The safety endpoints included formal, blinded histopathologic examination by a Board-certified pathologist, as well as formal assessment of neurological symptoms by trained personnel blinded to treatment group. They also included monitoring changes in body weight three times per week for several months. No evidence of any side effect (except the modest reduction in weight gain, discussed in detail below) was noted at any of the AAV2-NRTN or single AAV2-GDNF dose (see Table 3). Specific tests of nigrostriatal motor function were not employed in this study because ample evidence in the literature already existed that doses of GDNF orders of magnitude greater than expressed here via AAV2-NRTN, when delivered to the SN, produce no deficits, and indeed even improve nigrostriatal

dopamine function when administered to neurons subjected to 6-OHDA-induced degeneration (e.g. Lapchak et al., 1997; Hoffer et al., 1994; Bowenkamp et al., 1995; Hoffman et al., 1997). Similar results have been reported with lenti- and AAV-vectors expressing comparable amounts of GFLs as we estimate are expressed with AAV2-NRTN (e.g. McGrath et al., 2002; Dowd et al., 2005; Kozlowski et al., 2000; Rosenblad et al., 2000a, 2000b).

What had not yet been adequately addressed in the literature is whether other, more general behavior or neurological function might be impaired with GFLs administered in high concentrations to this area of the brain. For this reason, we employed the 'Functional Observation Battery' (FOB) which has been accepted by the FDA and many laboratories as providing a reasonable survey of general neurological status and health (e.g. see Moser, 2000; Ross, 2002).

Examination of H&E stained tissue near the injection site and throughout the brain revealed nothing remarkable, except for modest evidence of infiltration of inflammatory cells near the area of the needle tract which was generally equivalent in the formulation buffer controls and all AAV2-NRTN doses. While further characterization using immunohistochemical methods was not performed to identify the type of inflammatory cells, the H&E response in the direct vicinity of the needle tract is common to every other AAV-NRTN and AAV-NGF study we performed and we can thus conclude with confidence based on those studies that the primary cell type is astrocytes, with more modest and less consistent participation of microglia/monocytes and pan leukocytes (Gasmi et al., 2007a; Herzog et al., 2007; 2008; 2009).

Establishing rational 'dose equivalents' to account for between-species differences in SN volumes

Translating data from nonclinical studies to define clinical parameters is a notoriously difficult and often daunting challenge. However, several principles based on extensive empirical observations greatly simplified this task with AAV2-NRTN (which likely extends to similar efforts by others using other neurotrophic factors and/or delivery approaches). For example, we have established in multiple studies extending many years that if sufficient NRTN is expressed in the targeted cell bodies to be easily detected via immunohistochemistry, a reliable neurotrophic response is generally achieved (Bartus et al., 2007; Gasmi et al., 2007a,b; Herzog et al., 2008, 2009). Furthermore, we have also consistently observed that doses of AAV2-NRTN hundreds (and at the injection site, even thousands) of times higher than required for efficacy, when accurately administered to the targeted region of the brain, are without any notable toxicity, pathology or side effect (Gasmi et al., 2007a; Herzog et al., 2007, 2008, 2009). These observations, therefore, provided the guiding principle to define doses to translate to the human clinic, with the objective of covering the targeted brain region as effectively as possible, without causing significant protein expression in non-targeted regions.

The studies reported here establish that AAV-NRTN doses targeted to the SN that are ~200 times greater than those required to produce the desired trophic response appear safe and without serious side effects in rats. This provides a rational means and the empirical support to scale and project a dose for PD patients that should be similarly safe and effective. A review of several papers in the published literature (Ahsan et al., 2007; Foster et al., 2008; Krabbe et al., 2005), establishes the volume of the human SN to be approximately 400 mm³, or roughly 7.3 times the volume of the monkey SN and about 145 times larger than the rat SN (see Table 1). Thus, we conservatively projected a 'human-equivalent dose' for targeting the SN in Parkinson's patients to be 2 × 10¹¹ vg per hemisphere (i.e., a dose equivalent to 1.6 × 10⁹ vg/hemisphere used here for rats when relative inter-species volumes of SN are considered, but 125 times greater in absolute terms). This dose should adequately cover the SN in PD patients, provide the desired trophic response to the degenerating SN neurons, but limit spread of NRTN outside the SN border and thus pose relatively little risk of serious

side effects. Thus a dose of 2 × 10¹¹ vg per SN was therefore selected for SN targeting in our ongoing Phase 1/2b AAV2-NRTN trial in PD (i.e., CERE-120; ClinicalTrials.gov identifier NCT00985517).

Clarifying the issue of possible weight loss following SN targeting of GFLs

Aside from the wide safety margin and therapeutic index of AAV-NRTN established by these studies, several other aspects of the data deserve discussion. For example, while replicating the reduction in weight gain reported by others for AAV-GDNF (Manfredsson et al., 2009a; 2009b; Su et al., 2009) with our highest dose of AAV2-NRTN (only) and a 'replication dose' of AAV2-GDNF, we also found that the effect on weight was linked to a reduction in food intake and that both the effect on weight and food intake appeared to be self-limiting. That is, animals in all groups gained the same amount of weight after week 6, while differences in food intake stabilized at the same time point, though the reduced weight gain that occurred earlier was not recovered by week 13. Importantly, quantitative analysis of immunohistochemical staining throughout the brain demonstrated that, at the dose of AAV-NRTN and AAV-GDNF required to induce the weight changes, NRTN and GDNF expression extended well outside the targeted nigra, into distant midbrain and diencephalon structures. At lower doses, where NRTN staining was more restricted to the targeted SN, normal weight gain occurred. However, even at a dose 5-times higher than required to adequately cover the substantia nigra (i.e., 8 × 10⁹ vg, or Dose C), which caused mistargeted protein expression in many non-SN midbrain structures, no significant effect on weight was seen. These data emphasize the high dose required to produce, and wide-spread, mistargeted protein associated with, the changes in weight reported by others recently and replicated by us here.

Previous investigators have targeted the SN with GDNF protein or viral vectors expressing GDNF and we noted that while many reported no reductions in weight or any weight-related issue (Connor et al., 1999; Gash et al., 2005; Hebert and Gerhardt, 1997; Kozlowski et al., 2000; Rosenblad et al., 2000a, 2000b), a small number had reported a reduction in weight (or weight gain) when higher doses were used (Gash et al., 1995; Lapchak et al., 1997; Martin et al., 1996). While it was therefore somewhat surprising when three more recent papers by two groups described significant weight loss following AAV-GDNF targeting to the SN, warning of substantial safety concerns if GDNF or similar trophic factor (e.g., NRTN) was to be injected into SN in any clinical trial (Manfredsson et al., 2009a; 2009b; Su et al., 2009), we also noted that none of the prior publications had apparently attempted to scale the dose administered to the SN and few had tested sufficient dose levels to truly understand what dose might be required for efficacy and how that dose compared to any observed effect on weight. We reviewed the methods and data presented in those papers warning of serious weight loss with particular care and believe the data that each generated are merely the result of employing a single, unscaled dose of AAV-GDNF that was far in excess of what was needed to appropriately target the SN, thus producing mistargeted protein far outside the targeted SN.

Specifically, calculating dose equivalents using the relative volumes of SN in rats, monkeys and humans described earlier, it becomes clear that the single AAV-GDNF dose tested by Su et al. (2009), as well as that tested by Manfredsson et al. (2009a; 2009b) was excessively high for appropriate "targeting" of the SN (see Table 1). For example, Su et al. (2009) used 'convection-enhanced delivery' (CED) to inject 5.5 × 10¹¹ vg unilaterally into each monkey nigra. Based on relative inter-species differences in SN volume, that dose corresponds to about 20 times greater than used here to successfully cover the rat nigra, and also 20 times greater than we calculate to be an appropriate human-equivalent dose. Indeed, the photomicrograph published by Su et al. (2009) clearly shows wide-spread GDNF expression far outside the targeted SN, expanding beyond the midbrain—a point not addressed in that paper. Similarly,

Manfredsson et al. (2009b) injected 8×10^9 vg per hemisphere to produce their reductions in weight, which is 5 to 25 times greater than the two doses we demonstrated to be effective against 6-OHDA in rats, and at least 5 times greater than the human-equivalent dose we project to be appropriate for dosing the SN in PD patients. Although Manfredsson et al. (2009b) did not publish sufficient photomicrographs or quantitative data to provide clear information regarding protein spread outside the SN, our research revealed that the same vector genome dose used in their study (i.e., 8×10^9 vg) produced wide-spread, mistargeted protein far outside the targeted SN (see Fig. 3). The replication and extension of their data clearly point to mistargeted, non-nigral protein as responsible for the weight changes they report.

Several other factors likely caused even greater spread of mistargeted protein in those studies, relative to what we report here, including: (1) use of convection-enhanced delivery (CED) by Su et al. (2009), which intentionally forces greater spread by inducing convection currents. As an aside, while the use of CED to enhance spread of vectors and proteins is justifiably popular today (Fiandaca et al., 2009; Sanftner et al., 2005; Szerlip et al., 2007), the approach nonetheless is notorious for increasing uncontrolled spread of vector and protein along paths of least resistance (e.g., white matter tracts and around large blood vessels), often exacerbating mistargeted protein. This likely contributed further to the weight loss reported by Su et al. (2009); (2) use of AAV5 by Manfredsson et al. (2009a; 2009b), which has a lower affinity for heparin-binding sites, compared to AAV2 we used (Davidson and Bohn, 1997; Dodiya et al., 2009), thus producing greater spread from the injection site, and (3) the use of GDNF by both laboratories, which is known to distribute more widely than NRTN, also due to lower affinity for heparin-binding sites decorating the intracellular matrix within brain parenchyma, thus also causing greater spread of protein from the injection site (Alfano et al., 2007; Hamilton et al., 2001). This characteristic of GDNF likely explains why the equivalent dose of AAV2-NRTN (8×10^9 vg) did not produce the same weight loss as AAV2-GDNF (Experiment 1) and yet prior studies comparing the identical two vector constructs against 6-OHDA demonstrated equivalent potency and efficacy (Gasmi et al., 2007a). In the weight loss comparison, the broader distribution of GDNF led to even greater mistargeted protein than NRTN, producing the mistargeted-dependent side effect (and only at higher doses of AAV2-NRTN; e.g., 60×10^9 vg was mistargeted protein sufficient to produce this side effect). In the prior 6-OHDA experiments appropriately targeted doses of NRTN and GDNF were employed (i.e., protein restricted to the striatum) and thus no differences in the two proteins were observed. Thus, the difference between GDNF and NRTN in the present weight loss comparison (but not the 6-OHDA comparison) reflects mere bioavailability differences due to broader distribution of GDNF protein and not greater potency or efficacy.

All in all, the data presented in this paper strongly argue that the protein expression produced by the doses and methods used in the papers warning of dire weight changes following “SN targeting” (Manfredsson et al., 2009b; Su et al., 2009), in reality, greatly exceeded the targeted SN, and like the high dose of AAV2-NRTN and AAV2-GDNF we report here, exposed significant portions of diencephalon. Thus, we believe their data and ours are entirely consistent and mutually corroborating, with both providing strong evidence that the changes in weight that can accompany viral vector-mediated delivery of GFLs into the ‘nigra’ are due to mistargeted GFLs, far removed from the nigra. In this regard, all these data are entirely consistent with the well-known potential risks of anorexia and weight loss in humans and nonhuman primates subjected to inappropriately, mistargeted GDNF, whether delivered to the ventricles (Kordower et al., 1999; Nutt et al., 2003), putamen (Hovland et al., 2007), or nigra (present discussion).

Additional studies with AAV2-NRTN reported here further support the conclusion that mistargeted GFLs (and not protein in the SN) is responsible for the weight reductions. For example, we noted in

Experiment 2 that the weight effects reliably begin to emerge by 2 to 3 weeks after administration of AAV2-NRTN, which we reasoned provided an important opportunity to gain a biological snapshot for where in the brain NRTN must be expressed for the weight reductions to first be induced. This study clearly implicated structures in the diencephalon as mediating the onset of weight reduction and equally excluded the SN and surrounding midbrain structures as being involved (Experiment 3). Manfredsson et al. (2009b) had previously suggested an important role of hypothalamus in this phenomenon and our data implicating diencephalon structure(s) are generally consistent with that aspect of their hypothesis, though our data and the pattern of NRTN and GDNF expression we observe seem more consistent with simple protein spread from the site of injection than transport along axons, as they suggest.

Moreover a transport explanation for the weight changes becomes even more implausible in light of the results from the experiment where we lesioned the SN prior to high dose AAV-NRTN and achieved the same reduction in weight gain (Experiment 4). By first destroying the dopamine neurons in the SNc, we eliminated the only ventral midbrain neurons capable of such transport, for TH+ neurons make up 98+% of all neurons in the SNc, with virtually all projecting to striatum and no clearly established projections innervating hypothalamus (A. Bjorklund, personal communication, April, 2011). These data further demonstrate that neither the SN neurons, their axons, or transport along the axons, plays a major role in mediating the weight changes.

The observation that the extent of NRTN expression throughout the midbrain did not correlate with the weight changes offers further confidence in the safety of targeting the SN in addition to the putamen, as was recently implemented for AAV2-NRTN treatment for PD patients (i.e., CERE-120; ClinicalTrials.gov identifier NCT00985517). That is, even in the face of serious stereotactic mistargeting that might expose adjacent midbrain structures to NRTN, there exists no evidence that detectable side effects will be induced. Our data also argue that the central trigeminal structures, whose destruction had long-ago been shown to produce aphagia and weight loss (Zeigler and Karten, 1974), are not involved in the reduced weight gain phenomenon, since many of these structures are located in the tissue comprising our midbrain assessment. Further comfort in targeting the SN with GFLs can be gleaned from our ability to mitigate a substantial portion of the weight effect following mistargeted expression with simple dietary supplementation of high caloric, preferred foods, especially since other data presented here also shows that the weight changes are likely mediated by decreases in food intake. In sum, these data support the safety and feasibility of targeting the SN to enhance neurotrophic therapy for Parkinson's disease.

Conclusions

The novel data reported in this paper demonstrate the safety and feasibility of targeting the SN with AAV2-NRTN. Over a range of doses, no neurohistopathology was observed. Moreover, a formal ‘Functional Observation Battery’ of tests revealed no functional or behavioral changes, except relatively modest reductions in food intake and weight gain, which only occurred following the highest dose and were dissociated from NRTN exposure to SN neurons and clearly linked to NRTN far outside the targeted SN and into the diencephalon. Finally, the neuroprotection against 6-OHDA achieved with the two lowest doses supports a wide therapeutic index and thus wide safety margin for AAV2-NRTN targeted to the SN. Together, these data argue that the novel paradigm of targeting the SN with AAV2-NRTN appears safe and feasible, and is also likely to be effective in assuring adequate protein reaches the degenerating cell bodies in PD patients to stimulate the desired trophic response. Finally, the data provide an empirically-supported approach to properly scale the dose to account for the volume of the human SN, thus providing a rationale for selecting a

dose for human testing in PD that might be expected to be equally safe and effective.

Conflict of interest

The authors declare the following potential conflicts of interest. All of the authors, except for Drs. Johnson and Kordower are employees of Ceregene (the company developing AAV2-NRTN for Parkinson's disease) and receive salary and stock options. Drs. Johnson and Kordower are members of Ceregene's SAB and receive consulting remuneration and stock options.

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