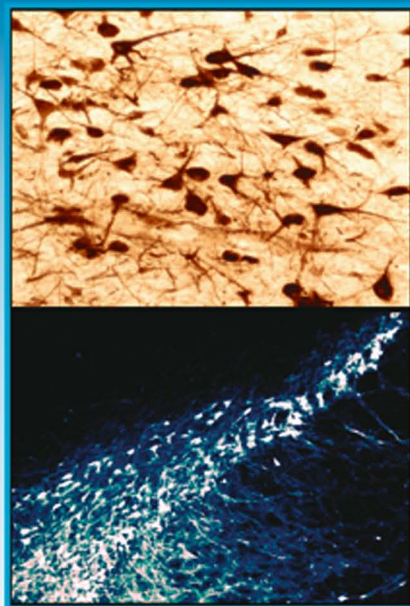
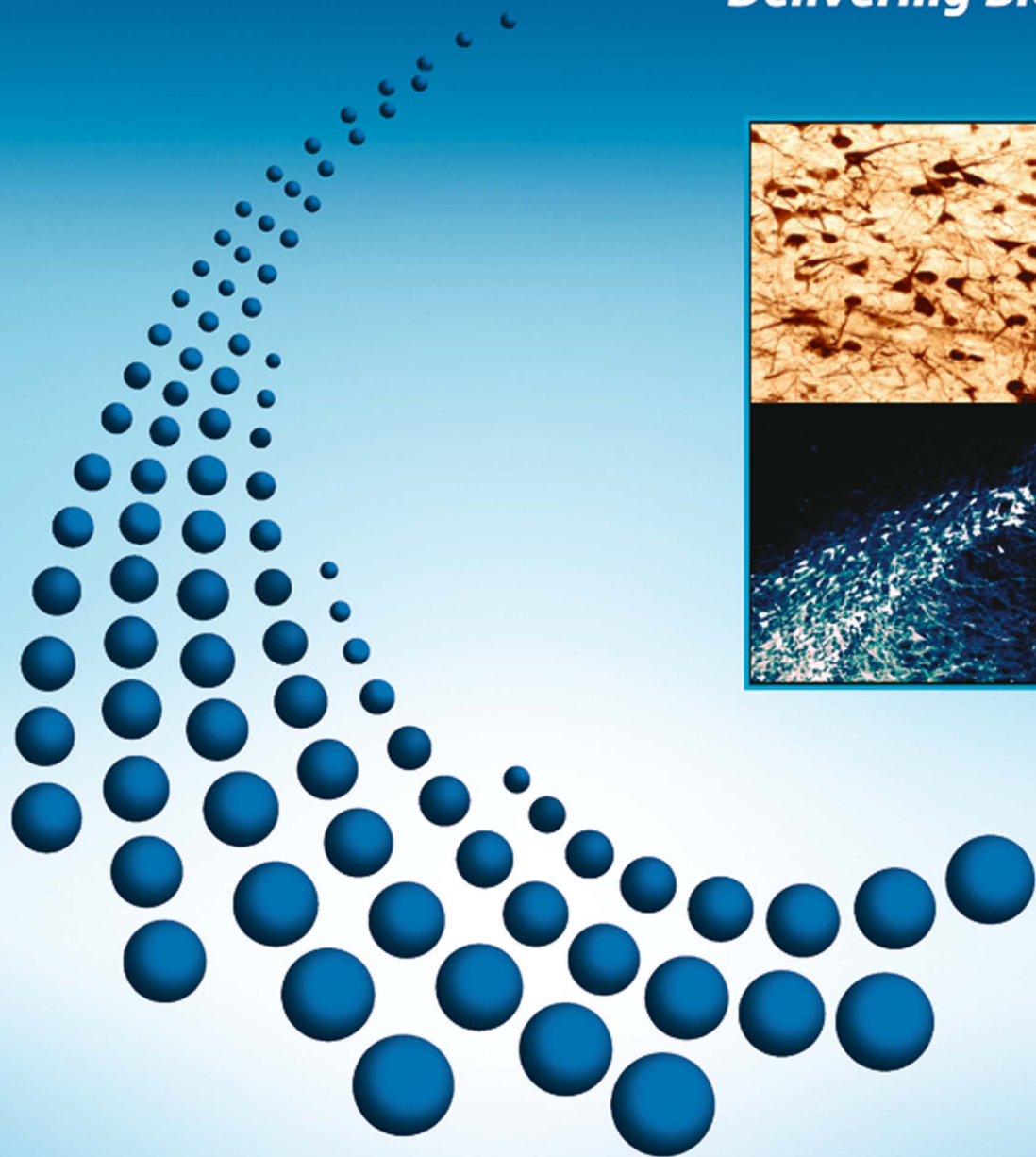


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Gene transfer provides a practical means for safe, long-term, targeted delivery of biologically active neurotrophic factor proteins for neurodegenerative diseases

Christopher D. Herzog · Kathie M. Bishop ·
Lamar Brown · Alistair Wilson · Jeffrey H. Kordower ·
Raymond T. Bartus

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Abstract Efforts to develop neurotrophic factors to restore function and protect dying neurons in chronic neurodegenerative diseases like Alzheimer's (AD) and Parkinson's (PD) have been attempted for decades. Despite abundant data establishing nonclinical proof-of-concept, significant delivery issues have precluded the successful translation of this concept to the clinic. The development of AAV2 viral vectors to deliver therapeutic genes has emerged as a safe and effective means to achieve sustained, long-term, targeted, bioactive protein expression. Thus, it potentially offers a practical means to solve those long-standing delivery/translational issues associated with neurotrophic factors. Data are presented for two AAV2 viral vector constructs expressing one of two different neurotrophic factors: nerve growth factor (NGF) and neurturin (NRTN). One (AAV2-NGF; aka CERE-110) is being developed as a treatment to improve the function and delay further degeneration of cholinergic neurons in the nucleus basalis of Meynert, the degeneration of which has been linked to cognitive deficits in AD. The other (AAV2-NRTN; aka CERE-120) is similarly being developed to treat the degenerating nigrostriatal dopamine neurons and major motor deficits in PD. The data presented here demonstrate: (1) 2-year, targeted, bioactive-protein in monkeys, (2)

persistent, bioactive-protein throughout the life-span of the rat, and (3) accurately targeted bioactive-protein in aged rats, with (4) no safety issues or antibodies to the protein detected. They also provide empirical guidance to establish parameters for human dosing and collectively support the idea that gene transfer may overcome key delivery obstacles that have precluded successful translation of neurotrophic factors to the clinic. More specifically, they also enabled the AAV-NGF and AAV-NRTN programs to advance into ongoing multi-center, double-blind clinical trials in AD and PD patients.

Keywords Protein delivery · Gene transfer · Neurotrophic factors · Neurodegenerative disease · Translational research

Introduction

Neurotrophic factors are endogenous proteins that are responsible for neuronal differentiation, survival and establishing appropriate efferent connectivity of the nervous system during ontogeny and early maturation [1–3]. Each of the many different neurotrophic factors thus far identified serves these roles for only a relatively small number of neuronal types or populations. Once the nervous system has developed, the levels of these proteins decline and they assume a more maintenance-like function during adulthood and throughout the aging process [1, 4]. However, research over the past several decades has demonstrated that under conditions of neurodegeneration, supra-physiological (i.e., biopharmaceutical) levels of a neurotrophic factor can induce neuronal repair genes in degenerating neurons. Induction of these repair genes routinely leads morphological and functional restoration of the

C. D. Herzog · K. M. Bishop · L. Brown · A. Wilson ·
R. T. Bartus (✉)
Ceregene, Inc.,
9381 Judicial Drive, Suite 130,
San Diego, CA 92121, USA
e-mail: rtbartus@ceregene.com

J. H. Kordower
Department of Neurological Sciences,
Rush University Medical Center,
Chicago, IL 60612, USA

neurons, significant slowing of further neurodegeneration and even protection against death [4]. It is therefore not surprising that several investigators hypothesized decades ago that neurotrophic factors might be used to treat human neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (i.e., ALS or Lou Gehrig's disease) [4–6]. In fact, numerous clinical trials involving several different neurotrophic factors and neurodegenerative diseases have now been conducted, spanning over two decades [7–17], but no clear evidence of clinically robust efficacy has yet been achieved in a controlled trial.

Most authorities agree that the lack of success in translating the concept and therapeutic potential of neurotrophic factors to the clinic is directly related to a long-standing inability to overcome significant obstacles specifically related to delivering proteins to select sites in the brain [14, 15, 18–22]. As proteins, neurotrophic factors cannot be taken orally, do not cross the blood–brain barrier (BBB) and do not often have a sufficiently long half-life. These characteristics preclude the majority of traditional pharmaceutical formulations and delivery approaches as viable options for administering neurotrophic factors to patient's brains. Thus, a number of innovative methods have been attempted to deliver these proteins to the central nervous system (CNS), but all have suffered serious limitations. For example, various efforts to transport neurotrophic factors across the BBB following systemic administration have been attempted. Those demonstrating the greatest promise exploited endogenous transport receptor-mediated systems located on the abluminal surface of cerebral capillaries (e.g., transferrin transport receptors). While early nonclinical, conceptual success was achieved [23–26], the approach ultimately proved impractical due to serious peripheral side effects induced via exposing nontargeted tissue outside the brain to the neurotrophic factor following intravenous injections (e.g., see [27, 28]). Efforts to develop biodegradable polymeric microsphere formulations to provide sustained release of biologically active proteins also met with some success (e.g., see [29, 30]), but an inability to solve the need for frequent injections into the brain to maintain therapeutic levels of the neurotrophic factor rendered this approach impractical for chronic neurodegenerative diseases. Infusions of neurotrophic factor proteins directly into the ventricles of the brain showed early promise in animal studies of neurodegeneration, but induced significant side effects in humans because of reactions of the periventricular tissue following exposure to high concentrations of the neurotrophic factor [10, 14, 18, 22, 31]. While subsequent infusions of the proteins directly into the degenerating parenchyma using chronically indwelling pumps and cannula reduced some, but not all safety issues [15, 32], poor diffusion of the protein from a single point source severely limited exposure of the protein to the area of degenerating tissue, thus likely restricting clinical benefit [15, 20, 21].

More recently, gene transfer (sometimes also called gene therapy) has emerged as a practical means of potentially overcoming all the obstacles associated with delivering neurotrophic factors to the brain, thus possibly providing the “enabling” technology required for translating the use of these proteins into viable biotherapeutics for human neurodegenerative diseases. As currently practiced, gene transfer involves the use of a harmless viral vector to deliver the gene for the therapeutic protein directly to a targeted site so that the cells in close proximity to that site will be transduced and programmed to express and secrete the protein in a continuous, long-term fashion. Thus, rather than attempting to exogenously deliver the large, three-dimensionally complex protein directly to the targeted site, the gene for the protein is delivered to the targeted site, thereby inducing local cells to manufacture and secrete the protein through their endogenous systems.

Adeno-associated virus (AAV) has emerged as a favorite vector for translational purposes for at least two key reasons. First, it is unusually safe, with over 1,000 patients administered a variety of AAV gene transfer constructs and no serious safety issues with its general use arising (*Journal of Gene Therapy*: <http://www.wiley.com/legacy/wileychi/genmed/clinical>). Secondly, most authorities agree it generally provides potentially life-long expression of the protein in the targeted area of the body following a single administration of the viral vector [33, 34]. Two instances exist where the gene transfer of neurotrophic products have recently advanced into multi-center, double-blinded, controlled Phase 2 efficacy trials, both of which use AAV as the viral vector. One is AAV2-nerve growth factor (NGF; aka CERE-110) for Alzheimer's disease (<http://clinicaltrials.gov>; identifier NCT00985517) and the other AAV2-neurturin (NRTN; aka CERE-120) for Parkinson's disease (<http://www.clinicaltrials.gov>; identifier NCT00876863). NGF has been shown to exert robust neurotrophic effects on basal forebrain cholinergic neurons, whose loss of function has been linked to the cognitive deficits in early stage disease [35–37]. NRTN has been shown to exert robust neurotrophic effects on nigrostriatal dopamine neurons, the loss of which has been linked to the major motor deficits characteristic of Parkinson's disease [38]. In both cases, an adeno-associated viral vector, serotype-2 (i.e., AAV2) is genetically engineered to express and secrete only the human neurotrophic factor (i.e., either NGF or NRTN). Prior studies in multiple animal models of Alzheimer's disease (AD) and Parkinson's disease (PD)-like neurodegeneration established that AAV2-NGF can prevent the degeneration and enhance the function of degenerating basal forebrain cholinergic neurons, while AAV2-NRTN can provide similar benefits to degenerating nigrostriatal dopamine neurons, with each protecting its respective neuronal popula-

tion from further degeneration and death. For both AD and PD, the most widely accepted models of neurodegeneration were included, providing initial preclinical proof of concept for using gene transfer to deliver neurotrophic factors in a safe and effective manner [39–45].

While these prior studies in animal models also established that dose-related protein expression in the normal intact brain can reliably predict expression in appropriate AD and PD models of neurodegeneration [40, 42, 46], a number of other translational objectives must be established in order to more fully support and validate the concept of using gene transfer to deliver neurotrophic factors to treat chronic, human neurodegenerative diseases. For example, it is important to establish that the volume of protein expression and its location is predictable and controlled, and is limited to the targeted brain site, with no significant changes in volume or location of expression over long periods of time. Additionally, it is essential that persistent, long-term expression be achieved and that the protein remains biologically active in order to justify the neurosurgical procedure required for administering the vector. Moreover, neither the viral vector, nor the expression of the transgene protein, can be associated with serious side effects. Finally, because this approach involves “site-specific” delivery within the brain, it is important that the dosing parameters required for humans be successfully tested in relatively large-brained primates, so that when the parameters are scaled for human application, their safety and effectiveness are likely to be preserved.

We report here the results of four separate studies that were specifically conducted to address these major translational, delivery issues. In the first experiment, we demonstrate that following AAV2-NGF (CERE-110) delivery to the cholinergic neurons located in the nucleus basalis of Meynert of nonhuman primates, steady, long-term (2-year), expression of biologically active NGF is achieved, with expression restricted to the targeted tissue, thus supporting both the safety and effectiveness of this approach. In the next experiment, we further extend and generalize those results by establishing similarly long-term expression of a second neurotrophic factor (neurturin or NRTN) in another brain site (striatum), using a different species (rats). Importantly, the long-term (18 to 20 months) expression of NRTN achieved in rats represents successful protein delivery for ~80% of the mean life-span of the rat following a single gene transfer procedure, thus establishing that age-related changes in the brain that are known to occur during the lifetime of that species have no impact on the expression, safety, or bioactivity of the protein delivered via gene transfer. In the third experiment, we confirm that AAV2-NRTN is equally effective in transducing neurons and expressing human NRTN when administered to aged rats (20 months old at the time of dosing), further

supporting the viability of this approach for treating many neurodegenerative diseases, since for many such as AD and PD, age is a major risk factor. Finally, in the last experiment, we evaluated a number of dosing parameters in nonhuman primates (i.e., injection volumes, number of delivery sites, and infusion flow rates) to better understand those that will most likely provide safe and effective delivery of AAV2-NRTN and AAV2-NGF to human patients, while also establishing the means to scale the application of gene transfer to the larger human brain so that reliable therapeutic protein expression in the targeted human brain regions might be reasonably predicted. Collectively, the four independent experiments presented in this paper address key translational issues associated with the use of gene transfer for chronic human, age-related neurodegenerative diseases and thus more firmly establish the empirical foundation supporting that concept. Moreover, these data provided instrumental support to enable both CERE-110 (AAV2-NGF) and CERE-120 (AAV2-NRTN) to proceed into testing in human patients and eventually to advance safely to the ongoing double-blinded controlled Phase 2 studies that are currently in progress.

Materials and methods

Viral vectors

CERE-110 (AAV2-NGF) and CERE-120 (AAV2-NRTN) are adeno-associated serotype 2 viral vectors genetically engineered to express human NGF and human NRTN, respectively. The design and production of both CERE-110 and CERE-120 have been described in detail previously [39, 42]. Briefly, the genome for both vectors consists of AAV2 inverted terminal repeats flanking either an NGF or NRTN expression cassette containing a CAG promoter, the human NGF cDNA (CERE-110) or human mature NRTN cDNA fused to the human β -nerve growth factor pre/pro-sequence (CERE-120), and a human growth hormone polyadenylation signal. Viral vectors are formulated in 2 mM magnesium chloride, in 1× phosphate buffered saline (formulation buffer, FB).

Experiment 1: can AAV2 gene transfer provide safe, long-term, bioactive expression of NGF, accurately targeted to the nucleus basalis in nonhuman primate brain?

The purpose of this GLP-compliant experiment was to characterize the effects of AAV2-NGF delivery on long-term human NGF expression over a range of doses and to further assess the bioactivity and safety of AAV2-NGF when delivered to the nonhuman primate nucleus basalis of Meynert (NBM).

Subjects

Twenty-six cynomolgus monkeys (*Macaca fascicularis*; 11 males, 15 females; ≥ 2 years of age and 2–3 kg at the time of dosing) served as subjects. Animals were housed in individual stainless steel cages, were provided food and water ad libitum, and were maintained on a 12:12-h light/dark cycle. Environmental enrichment was provided according to study facility guidelines, and all procedures were conducted in accordance with Institutional Animal Care and Use Committee protocols.

Surgical procedures and AAV2-NGF administration

Experimental groups are summarized in Table 1. Each animal received a total of four 10 μ L injections (two injections per hemisphere) of FB or AAV2-NGF targeting the NBM at a rate of 1 μ L/min. Following each injection, the needle remained at the target site for 5 min and was then retracted 2 mm and left in place for an additional 5 min to minimize the potential backflow of fluid up the needle track.

Stereotaxic targeting of the NBM was accomplished using magnetic resonance imaging (MRI; Picker, 1.0 T) scans taken prior to the day of surgery. Animals were anesthetized with medetomidine hydrochloride and ketamine HCl, placed in an MRI-compatible head frame, and three volume scans (1 mm slice, no gap) were performed on each animal: T1-weighted coronal, T1-weighted sagittal, and a TE 7 volume scans (1 mm slice, no gap) were performed. On the day of surgery, animals were pretreated with atropine sulfate followed with ketamine HCl to induce sedation, intubated, and maintained at a surgical plane of anesthesia with halothane or isoflurane (2% in O₂). A midline incision was made over the calvarium, and the injection needle was advanced to the appropriate stereotaxic coordinates through bilateral craniotomies. Injections were performed using a 25- μ L Hamilton 700 syringe attached to a blunt 22-gauge S-type needle. Prior to use, each needle/syringe was cleaned, sterilized, and rinsed with viral vector to prevent adsorption of the vector to the stainless steel needle during dosing. Following the completion of injections, craniotomies were closed with dental acrylic and incisions were closed with sutures and tissue adhesive.

Table 1 Experiment 1: study design and endpoints

Design (i.e., treatment groups and parameters)					
AAV2-NGF dose (vg/animal)	AAV2-NGF concentration (vg/ml)	Total animals (<i>n</i>)	3-month group (<i>n</i>)	12-month group (<i>n</i>)	24-month group (<i>n</i>)
FB	n/a	7	2	3	2
2.0 $\times 10^9$	5.0 $\times 10^{10}$	2	2	0	0
4.0 $\times 10^9$	1.0 $\times 10^{10}$	3	0	3	0
2.0 $\times 10^{10}$	5.0 $\times 10^{11}$	7	2	3	2
8.0 $\times 10^{10}$	2.0 $\times 10^{12}$	7	2	3	2

Study Endpoints	Frequency/timing
Clinical Observations	Daily
Body weight	BL, weekly
Morbidity and Mortality Observations	Twice daily
Food consumption	Daily
Neurological and physical exams	BL, day 3, months 1, 3, 6, 9, 12, 15, 18, 24
Hematology and serum chemistry	BL, months 1, 3, 6, 9, 12, 15, 18, 24
Serum antibodies to AAV and human NGF	BL, months 1, 3, 6, 9, 12, 15, 18, 24
Serum levels of NGF	BL, months 1, 3, 6, 9, 12, 15, 18, 24
CSF levels of NGF	BL, necropsy
CSF AAV2 biodistribution by QPCR	BL, necropsy
CSF chemistry and hematology	BL, necropsy
Brain and organ system histopathology	Necropsy
Brain NGF expression	Necropsy
Bioactivity of NGF by cell size	Necropsy
Brain immune/inflammatory markers	Necropsy
Schwann cell hyperplasia and axon sprouting analyses in medulla and spinal cord	Necropsy

FB formulation buffer control, vg vector genomes, BL baseline

In-life clinical observations and examinations

The schedule of in-life assessments is summarized in Table 1. Clinical observations (i.e., signs of clinical effects, illness, and/or death), food consumption, and body weights were recorded. Neurological examinations included: level of consciousness, eye tracking, motor function, orbicularis oculi reflex, pupillary reflex, corneal reflex, sensory foot reflex, knee jerk, cutaneous, proprioceptive, and tail reflexes. Physical examinations included: measurement of heart rate, body temperature, and respiration as well as examination of eyes, ears, skin and nails, oral cavity, abdomen (via palpation), lymph nodes, thoracic auscultation, gait, and disposition. All evaluations were conducted according to standard operating procedures of the testing facility.

Necropsy and histopathological examination

Animals were subjected to full necropsy at the time of sacrifice (3, 12, or 24 months following dose administration). Animals were sedated with ketamine HCl, provided a bolus of heparin sodium (200 IU/kg, i.v.), and maintained on a halothane/oxygen mixture. A comprehensive necropsy was performed including examination of a total of 46 tissues and organs. Selected organs were weighed before fixation and organ-to-body weight and organ-to-brain weight ratios were calculated. Animals were then perfused via the left cardiac ventricle with saline followed by 0.2% parabenzoquinone (PBQ)/2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Brains and spinal cords were harvested, post-fixed at 2–8°C in 0.2% PBQ/2% PFA in 0.1 M phosphate buffer for 2 h, and then cryoprotected in a 30% sucrose solution.

A panel of tissues was collected, preserved in 10% neutral buffered formalin, and transported to Pathology Associates, Inc. (Frederick, MD, USA) for histopathological analyses. Samples of the following tissues were embedded in paraffin, processed to slides, and stained with hematoxylin and eosin (H&E): larynx, tongue, skin with mammary gland, eyes with optic nerve, lymph nodes, gross lesions, gastrointestinal organs (esophagus, stomach, gall bladder, duodenum, jejunum, ileum, cecum, colon, rectum), respiratory organs (trachea, lung), reproductive organs (ovaries, testes with epididymides, uterus), endocrine organs (adrenals, pancreas, pituitary, prostate, salivary gland, thyroids with parathyroids, thymus), circulatory organs (heart, aorta, liver, spleen), musculoskeletal organs (femur with marrow, joint with bone, skeletal muscle, sternum [bone and marrow]), and urogenital organs (kidney, ureter, urinary bladder, urethra, vagina). Tissues from the sciatic nerve, tibial nerve, sural nerve, and trigeminal ganglion were embedded in plastic and stained with H&E.

Coronal sections (40 µm) through the brain, cervical spinal cord, and medulla were generated using a sliding–freezing microtome. All tissues were evaluated histologically by a board-certified veterinary pathologist.

Evaluation of brain and spinal cord

Evaluation of NGF expression in the brain In order to assess the degree and spread of NGF protein expression following AAV2-NGF delivery, NGF immunohistochemistry was performed using a polyclonal anti-NGF antibody (generously provided by J. Connor, UCSD; 1:1,000 dilution) according to standard techniques. For control, sections were treated identically, except substituting an irrelevant IgG for the primary antibody. Sections were mounted onto glass slides, dehydrated, cover slipped, and the extent and degree of NGF staining throughout the brain was examined qualitatively. In addition, the volume of NGF expression in the NBM brain parenchyma was quantified with the aid of a computerized microscope with SPOT Advanced software (v3.4, Diagnostic Instruments, Sterling Heights, MI) according to 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.

Quantification of choline acetyltransferase antibody cell size in the NBM To examine the size of cholinergic neurons within the NBM following AAV2-NGF delivery, sections throughout the basal forebrain were immunolabeled using a choline acetyltransferase antibody (ChAT; Chemicon; 1:1,000). A series of 40-µm-thick sections through the NBM were identified in the anterior–posterior plane by the appearance and completion of the ansa peduncularis passage through the NBM at its ventrolateral extent and subsequent grouping of cholinergic neurons into a single cluster at the posterior NBM. The volume of ChAT positive cells in the NBM was quantified with the aid of a computerized microscope, high magnification (100×) oil immersion objective, and the Stereo Investigator software (MicroBrightfield Inc.). All analyses were performed by an investigator blinded to treatment conditions.

Evaluation of immune/inflammatory response in brain To examine potential local immune or inflammatory responses in the brain following AAV2-NGF delivery, a series of brain sections were immunolabeled for glial fibrillary acidic protein (GFAP, a marker of an astrocytic response; Chemicon; 1:2,000 dilution), CD45 (a pan-leukocyte marker; BD Pharmingen; 1:500 dilution), and CD68 (a marker of a microglial response; Oncogene; 1:400 dilution) using standard techniques. Immunolabeled sections were evaluated using microscop-

py and a 5-point intensity rating scale by an individual blinded to treatment assignment.

Evaluation of Schwann cell hyperplasia and axonal sprouting Schwann cell hyperplasia and axonal sprouting on the dorsal surface of the medulla oblongata and/or spinal cord have been observed following high-dose intraventricular infusions of NGF. A series of sections were therefore stained with cresyl violet and immunolabeled for tyrosine hydroxylase (TH, Chemicon; 1:3,000 dilution) and calcitonin gene-related peptide (CGRP; Chemicon; 1:8,000 dilution) which would reveal potential sympathetic or sensory axonal sprouting, respectively. Quantitative assessment of pia mater thickness and qualitative assessment of aberrant axonal sprouting or potential neurite outgrowth into the pial layer were performed by an individual blinded to treatment assignment.

Analysis of serum and cerebrospinal fluid

Chemistry and hematology assessments Standard serum chemistry and hematology assessments were conducted using blood samples collected from the femoral vein of fasted animals (see Table 1 for time points). Cerebrospinal fluid (CSF) samples were collected via cisterna magna taps from fasted, anesthetized animals at baseline and at necropsy, and analyzed for total cell count and chemistry parameters (albumin, sodium, total protein, glucose, phosphate, chloride, calcium, potassium).

Distribution of NGF and AAV2 in serum and cerebrospinal fluid Serum concentrations of NGF were measured by enzyme-linked immunosorbent assay (ELISA; see Table 1 for time points). NGF in the CSF was measured at baseline and at necropsy by ELISA. The NGF ELISA employed flat-bottom 96-well plates coated with a polyclonal (NGF Emax ImmunoAssay System, Promega) or monoclonal (Human beta-NGF DuoSet ELISA, R&D Systems) anti-NGF antibody. After application of diluted test samples and a recombinant NGF standard curve to each plate, bound soluble NGF was detected by the addition of either a rat monoclonal anti-NGF antibody, followed by horseradish peroxidase (HRP)-conjugated anti-rat IgG or by the addition of a biotinylated goat anti-NGF polyclonal antibody and streptavidin–HRP. For detection, the chromogenic substrate tetramethylbenzidine (3,3',5,5'-tetramethylbenzidine) was then applied. After stopping the color development reaction by the addition of acid, the optical density was quantitated using a 96-well plate reader set at 450 nm. The lower limit of quantitation of the ELISA was determined to be 63 pg/mL for CSF samples and 156 pg/mL for serum samples.

CSF samples collected at baseline and at necropsy were analyzed for the presence of the CERE-110 vector genome by real-time Quantitative TaqMan® PCR, where the primers and probe amplify a 110-bp region of the genome spanning the 3' end of the CAG promoter and the 5' end of the NGF pre/pro-coding region. The CSF samples were assayed based on input of initial sample volume and included 40 µL equivalent of the original sample per reaction, in triplicate reactions. The limit of detection (LOD) was ten copies per reaction and the limit of quantitation was 46 copies per reaction.

Serum antibody analysis To detect potential immune responses to either the AAV2 viral vector or the subsequently produced NGF, sera were evaluated by ELISA for antibody titers to AAV2 and human NGF (see Table 1 for time points). Serum samples were initially diluted at 1:500 (AAV2 assay) or 1:100 (human NGF assay), and then as a threefold dilution series. Serum dilutions were assayed for reactivity against intact AAV2 capsids or purified recombinant human NGF protein and assigned an antibody titer value based on comparison to known negative and positive monkey sera (for AAV2) or rat sera (for human NGF).

Experiment 2: can AAV2 gene transfer provide safe, bioactive expression of NRTN, accurately targeted to the striatum in the brain, throughout the life-span of the rat?

Experiment 2 examined the long-term expression, bioactivity, and safety of NRTN protein delivery in rats, when delivered to the striatum. Animals were injected at approximately 8 weeks of age, with subsequent analyses over the course of 18–20 months of life. Thus, the animals were euthanized at 20 to 22 months of age and as the mean life expectancy of this rat strain is ~24 months [47], this experiment examined the stability of NRTN expression, localization, bioactivity, and general tolerability of AAV2-mediated expression of NRTN for a duration of ~80% of the expected life-span of these rats, during which time significant age-related changes are known to occur.

Subjects

A total of 36 male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing between 274 and 380 g at the time of dosing were housed on a 12-h light/dark cycle in cages with access to food and water ad libitum. All animal care and experimental procedures were performed with approval of the Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical procedures and AAV2-NRTN administration

Animals received bilateral intrastriatal injections (two injections per hemisphere) of one of two doses of AAV2-NRTN (8×10^9 total vg or 4×10^{10} total vg) or FB control injections using standard rodent stereotaxic surgical techniques. Briefly, animals were anesthetized with an anesthetic cocktail (ketamine/xylazine/acepromazine) and placed into a stereotaxic frame (Stoelting, Wood Dale, IL). Stereotaxic coordinates used for targeting the anterior injection sites relative to bregma were: anterior/posterior (AP) +1.0, medial/lateral (ML) ± 3.0 , and dorsal/ventral (DV) -5.0 (from dura); and posterior injection sites were: AP -0.6 , ML ± 3.5 , and DV -5.0 (from dura). All infusions were made using a Hamilton syringe with a 26s gauge needle at a volume of 2 μL per injection at a flow rate of 0.5 $\mu\text{L}/\text{min}$. Prior to use, each needle/syringe was cleaned, sterilized, and rinsed with viral vector to prevent adsorption of the vector to the stainless steel needle during dosing. The needle was left in place for 1 min after each injection, retracted +1.0 mm, then held in place for one additional minute prior to withdrawal.

In-life observations and functional observation battery

All animals were observed daily for changes in general appearance or behavior. Starting at 6 months following AAV2-NRTN delivery and continuing at ~ 3 month intervals throughout the duration of the study, animals underwent formal neurobehavioral assessments using a functional observational battery (FOB). The 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. Variations of this formal test battery have been used extensively in rodent safety/toxicology studies (e.g., [48, 49]). 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.

Euthanasia and histological analyses

Animals were euthanized at age 20 to 22 months of age (i.e., 18–20 months following AAV2-NRTN delivery). 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

microtome. Potential neurotoxicity was assessed on H&E stained sections throughout the brain by a board-certified (DACVP) veterinary pathologist, who was blind to the treatment group. Transgene expression throughout the brain was examined using goat polyclonal anti-human NRTN (R&D Systems; 1:250) using standard immunohistochemical techniques [39, 40]. Volumetric analyses of NRTN distribution were performed on a series of sections using a microscope interfaced with a camera and SPOT Advanced software (Diagnostic Instruments) 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. Potential bioactivity of NRTN on neurons in the substantia nigra was evaluated following immunolabeling for phosphorylated extracellular signal-regulated kinase 1/2 (pERK 1/2; Cell Signaling; 1:1,000), a cellular marker of activation of a neurotrophic factor-mediated intracellular signaling cascade. For control, sections were treated identically, except substituting an irrelevant IgG for the primary antibody.

Experiment 3: can AAV2 provide safe, bioactive expression of NRTN predictably targeted to the aged rat brain?

This experiment was conducted to confirm that AAV2-NRTN is effective in transducing neurons and expressing biologically active NRTN in aged rats, further extending the expression data previously obtained with AAV2-NGF and AAV2-NRTN following gene transfer. Advancing age is one of the more significant risk factors of neurodegenerative diseases. Moreover, because functional and neurochemical deficiencies in the nigrostriatal system have been reported in the aged rat without frank dopaminergic cell loss [50–52], the aged rat may be useful in evaluating the cellular and functional effects of potential therapeutics within the neurological milieu of age-related functional deficits. This experiment therefore provided important information for evaluating (and potentially supporting) the viability of employing viral vector-mediated delivery of a neurotrophic factor for treating age-related neurodegenerative diseases.

Subjects

A total of 15 aged male Fischer 344 rats (21 months old at time of surgery, 436–501 g; Harlan, Inc.) were used. All animal care and experimental procedures were performed with approval of the Institutional Animal Care and Use Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Rats were pair housed in a ventilated cage system under a 12-h light/dark

cycle with food and water available ad libitum. In addition to standard rat chow, their diet was supplemented with a mixture of chocolate chip and peanut butter cookies, coconut flakes, and breakfast cereals. Half to one cup of this mix was provided in the home cage once per day.

Surgical procedures and AAV2-NRTN administration

Rats were anesthetized using isoflurane (induction 2–5%; maintenance 1–2%) and secured in a stereotaxic frame (Stoelting). AAV2-NRTN (4.0×10^9 total vg/rat; $n=11$) or FB (PBS with 2 mM $MgCl_2$; $n=4$) was injected unilaterally into the right striatum in two sites (2 μ L/site) at the following coordinates relative to bregma: (1) +1.0 mm AP, –3.0 mm ML, and –5.0 mm DV (from dura) and (2) –0.6 mm AP, –3.5 mm ML, and –5.0 mm DV (from dura). Injections were made as previously described in Experiment 2.

In-life observations/functional observational battery

Rats were monitored daily by cage-side observations, and body weights were recorded every 1–2 weeks. A FOB was performed at baseline and monthly thereafter until sacrifice, as described in Experiment 2.

Sacrifice and histological analyses

Rats were sacrificed at 3 months (13 weeks) following AAV2-NRTN administration by perfusion with ice-cold saline followed by Zamboni's fixative as described in Experiment 2. Brains were removed and cryoprotected in 30% sucrose/0.1 M phosphate buffer. Serial coronal brain sections (40 μ m) were collected using a freezing microtome. Immunohistochemistry was performed according to standardized methods [41, 42]. Goat anti-NRTN (R&D Systems; 1:250), rabbit anti-pERK 1/2 (Cell Signaling; 1:250), and rabbit anti-TH (Pel-Freez; 1:500) primary antibodies were used. Quantification of TH and pERK 1/2 positive cell number was performed on nonoverlapping fields throughout the substantia nigra using unbiased stereology as described previously [40]. In addition, cell size was determined by measuring the cross-sectional area of each TH-positive cell using computer software during the cell counting process.

Immunofluorescent staining for immune and inflammatory markers was performed on a series of sections for each marker using rabbit anti-GFAP (GFAP; Dako; 1:400), mouse anti-CD45 (Chemicon; 1:1,000), and mouse anti-CD68 (Serotec; 1:250), then visualized with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch; 1:250). For control, sections were treated identically, except substituting an irrelevant IgG for the primary antibody. In addition, a series

of H&E-stained brain sections were analyzed by a board-certified (DACVP) veterinary pathologist.

Experiment 4: testing variations in gene transfer dosing parameters in nonhuman primates as a prelude to human testing

The present experiment evaluated a number of dosing parameters in nonhuman primates to better understand which parameters will most likely provide safe and effective AAV2-mediated delivery of either NGF or NRTN to human patients, while also establishing the means to scale the dosing parameters to the larger, targeted, human brain structures.

Subjects

Eighteen cynomolgus monkeys ≥ 5 years of age and weighing between 4 and 10 kg at time of dosing served as subjects. Animals were housed in individual stainless steel cages, were provided food and water ad libitum, and were maintained on a 12:12-h light/dark cycle. Environmental enrichment was provided according to study facility guidelines and all procedures were conducted in accordance with Institutional Animal Care and Use Committee protocols.

Experimental design

Animals were randomly assigned to receive one of several injection parameters either into the left or right putamen in order to determine the effect of each variable on the volume of NRTN expression. To maximize use of nonhuman primates, both hemispheres of all monkeys were used to obtain independent observations for the following specific variables, and as summarized in Table 2: (1) volume of infusion, (2) delivery method, and (3) rate of infusion. With respect to AAV2-NRTN volume, the effects of delivering two 5- μ L deposits separated by 4 mm along a single needle tract on NRTN expression was compared to a volume of 10 and 50 μ L in a single deposit. These parameters were

Table 2 Experiment 4: summary of study design

Volume	Delivery	2 μ L/min	5 μ L/min
5 μ L \times 2	Pump (continuous)	$n=3$	–
	Manual (pulsatile)	$n=3$	–
10 μ L	Pump	$n=5$	$n=6$
	Manual	$n=3$	–
50 μ L	Pump	$n=6$	$n=7$
	Manual	–	–

n indicates the number of primate hemispheres devoted to that parameter in the experimental design; the hyphen indicates parameter not evaluated

chosen since the two 5- μ L injection scheme was used in our initial Phase 1 trial [53]. With respect to delivery method, we compared continuous advancement of the syringe plunger using an automated infusion pump to manual delivery, accomplished by manually depressing the syringe plunger approximately 1 μ L every 30 s, thus producing pulsatile waves of infusion. Infusion rates of 2 or 5 μ L per minute were used.

Surgical procedures and AAV2-NRTN administration

Stereotactic targeting and general surgical procedures employed in this study were similar to those described previously in nonhuman primate studies [43, 46]. Stereotaxic coordinates for surgery were obtained using magnetic resonance (MR) images collected using a 3.0 Tesla General Electric MR scanner.

For intracranial injections, animals were tranquilized with ketamine HCl, intubated and anesthetized with isoflurane, and placed in the same stereotaxic head frame in an identical head position as the one used for the MR scans. Vital signs were monitored continuously throughout the procedure. The automated, continuous delivery of AAV2-NRTN was performed using a Stoelting infusion pump, programmed to infuse at a rate of either 2 or 5 μ L per minute, and interfaced to a Hamilton syringe. Manual delivery of AAV2-NRTN was performed by advancing the plunger on the syringe approximately 1 μ L every 30 s (as was done in prior AAV2-NRTN clinical protocols), thus producing a pulsatile infusion. Following each injection, the needle was allowed to remain in place for 3 min to decrease the potential for efflux of viral vector up the needle track. Following this 3-min hold, the injection hardware was slowly removed and the next injection was performed, or the animal was sutured and allowed to recover following the last injection.

In-life observations/measures

Animals were observed daily for general appearance, signs of toxicity, distress, and changes in behavior or general mobility. Body weights were recorded at baseline prior to dosing, throughout the study, and at the time of sacrifice.

Serum collection and analyses Blood samples were collected from the femoral vein of a subset of six monkeys by venous puncture prior to surgery and at sacrifice. Three aliquots of each serum sample were subsequently prepared and stored at approximately $\leq 60^\circ\text{C}$ for analyses of antibodies to AAV2 and NRTN. Titers for antibodies to AAV2 and human NRTN were determined by ELISA as described in detail previously [46]. Briefly, for each ELISA, serial sample dilutions were prepared and assayed for reactivity

against intact AAV2 capsids or purified recombinant human NRTN protein. Antibody titer values were then assigned by comparison to known negative and positive monkey sera (for AAV2) or rat sera (for human NRTN).

Cerebrospinal fluid collection and analyses CSF was collected (as much as possible) from all monkeys at the time of sacrifice via intracisternal puncture and was stored at $\leq 60^\circ\text{C}$ until shipped to Ceregene, Inc. on dry ice for analyses of NRTN and CERE-120 concentrations. The NRTN concentration in the CSF of each monkey was determined by NRTN ELISA as described previously [46].

The concentration of AAV2-NRTN vector in the CSF of each monkey was determined by real-time Quantitative PCR (QPCR). Sample DNA was isolated using the Qiagen BioRobot EZ1 and the Qiagen EZ1 DNA Tissue Kit. The isolated DNA was then analyzed for the presence of the CERE-120 genome using QPCR. The QPCR method LOD had been determined to be ten copies per reaction and the lower limit of quantitation to be 46 copies per reaction.

Sacrifice and histological analyses All animals were sacrificed 8 weeks following AAV2-NRTN administration. Animals were anesthetized with ketamine and pentobarbital and were sacrificed by exsanguination via transcardial perfusion of 0.9% saline followed by a modified Zamboni's fixative (see Experiment 2). Brains were extracted and post-fixed in modified Zamboni's fixative at $2\text{--}8^\circ\text{C}$ overnight, after which they were transferred to increasing gradients of sucrose/0.1 M PB at $2\text{--}8^\circ\text{C}$ over the course of several days and stored in 30% sucrose until sectioned. Brains were sectioned in the coronal plane using a freezing sliding microtome at a thickness of 40 μm . Immunohistochemistry for NRTN (anti-NRTN goat IgG, 1:200, R&D Systems) was performed on a series of sections through the brain according to standard laboratory protocols as described previously [43]. Sections were processed to glass slides and using light microscopy, the volume of NRTN protein detected by immunohistochemistry was determined in each hemisphere as described in Experiments 1 and 2 above.

Results

Experiment 1: single gene transfer administration provides safe, bioactive-protein, accurately targeted in nonhuman primate brains for at least 2 years

In-life observations and examinations

Animals from all groups appeared normal and healthy throughout the duration of the experiment. Daily clinical

observations did not reveal any adverse effects related to AAV2-NGF over the course of the 2 years following delivery to the brain.

Dose-related, long-term, targeted expression of NGF in basal forebrain

Qualitative analysis of NGF immunohistochemical staining revealed a dose-related increase in area and staining intensity of NGF expression (Fig. 1b). Stereological quantification and statistical analyses confirmed an overall effect of AAV2-NGF dose on the volume of NGF expression ($F(2,13)=5.99$; $p<0.014$; Fig. 1a). Subsequent pair-wise comparisons revealed a significant difference between the low and high dose ($p=0.012$) but no significant

difference between the mid-dose and either the low or high dose. In addition, qualitative analysis of NGF immunolabeling revealed no increase in area or intensity of NGF staining between 3 and 24 months following AAV2-NGF administration. The small number of animals in the 24-month cohort ($n=2$ /group receiving either FB control or the two higher doses of AAV2-NGF) prevents a quantitative analysis of NGF expression at this time point. However, volumetric analyses did reveal that the volume of NGF expression, irrespective of viral vector dose, did not increase between 3 and 24 months suggesting that NGF expression was accurately restricted to the target and stable over time (mean volume \pm SEM) of NGF immunolabeling was 16.44 ± 5.37 (mid-dose, $n=4$ hemispheres) and 15.94 ± 5.40 mm³ (high dose, $n=4$ hemispheres; see Fig. 1a).

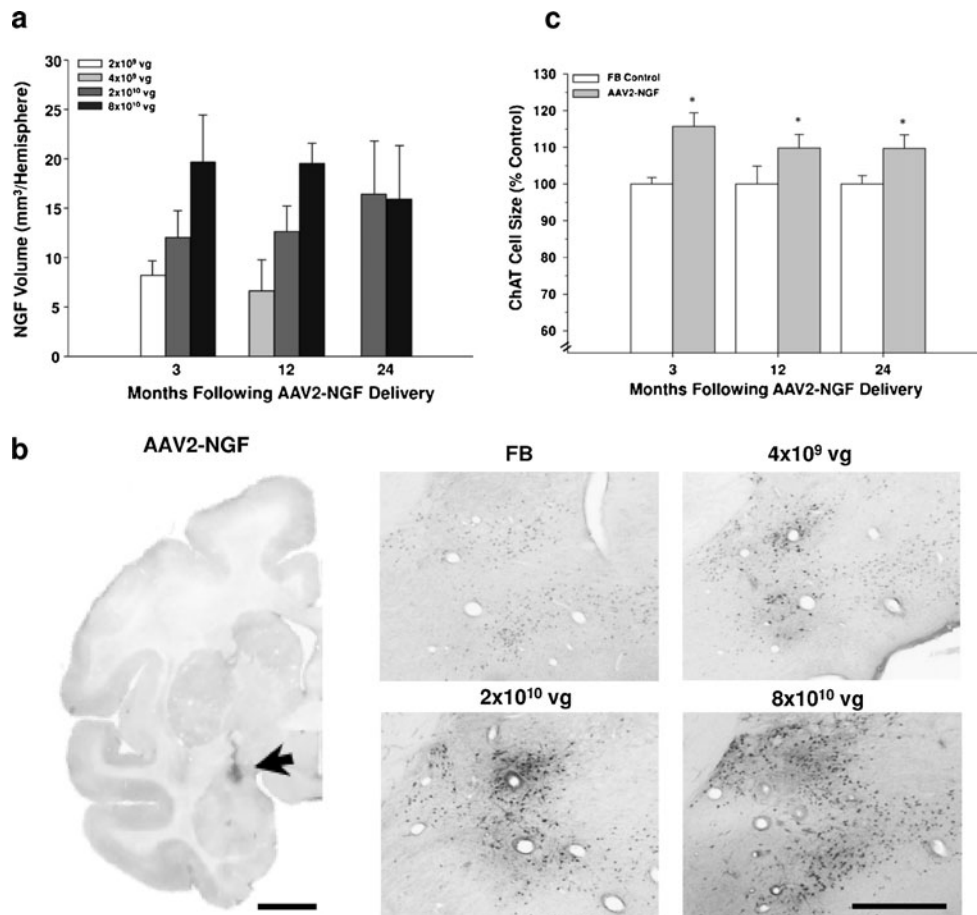


Fig. 1 Persistent, stable, dose-related expression and bioactivity of NGF in nonhuman primates following gene transfer of NGF to the cholinergic neurons of the NBM (nucleus basalis of Meynert). **a** Quantitative analyses showing a statistically significant, dose-related increase in the volume of NGF ($p<0.014$) following AAV2-NGF delivery. Data are presented as mean NGF volume (mm³) \pm SEM. **b** Low power photomicrograph (*left panel*) illustrating NGF immunolabeling in the targeted NBM 12 months following AAV2-NGF injection (*scale bar*=5 mm). Higher power photomicrographs (*right*

panels) illustrating a dose-related increase in NGF immunolabeling in the NBM 12 months following delivery of AAV2-NGF (*scale bar*=1 mm). **c** Quantitative analyses revealed a significant and sustained increase (10–15%) in the size of ChAT positive cells within the NBM at 3, 12, and 24 months following AAV2-NGF delivery, demonstrating neuronal hypertrophy qualitatively and quantitatively typical for elevated NGF, therefore confirming persistent bioactivity. Data are expressed as mean (\pm SEM) percent FB control cell size for all AAV2-NGF groups at each respective time point ($*p<0.05$)

Long-term bioactive effects of AAV2-NGF on basal forebrain cholinergic neurons following gene transfer

In this study, we tested the basic hypothesis that AAV2-NGF delivery resulted in cholinergic cell hypertrophy throughout the 2-year term of this study. This would provide important confirmation for persistent, long-term bioactivity of NGF delivered via gene transfer. Due to the relatively small number of animals at some of the doses and time points, we pooled all doses to provide a single AAV2-NGF group per time point, and ChAT soma size for the treated animals was compared to the FB control groups at each time point. Quantitative analyses of ChAT positive cells in the NBM of AAV2-NGF and FB control treated animals revealed a significant and sustained increase (10–15%) in soma size across all time points examined (see Fig. 1c). A two-way ANOVA on soma size relative to FB controls at each respective time point revealed a significant effect of AAV2-NGF treatment [$F(1,41)=8.96$, $p=0.005$], with no effect of time and no interaction between these two variables [both values of $F<1.0$, $p>0.05$] (see Fig. 1c).

No evidence of systemic or neurological toxicity or any side-effects up to 2 years following AAV2-NGF delivery

Body weight, food consumption, and neurological and physical examinations Daily observations of food consumption and weekly measurements of body weight revealed no adverse effects of AAV2-NGF for up to 2 years following administration. Animals gained weight over the course of the study. No adverse effects of AAV2-NGF delivery were revealed by neurological or physical exams conducted at baseline and every 3 months throughout the 2-year study.

Clinical chemistry and hematology, necropsy, and organ histopathology AAV2-NGF caused no alterations in serum chemistry or hematology parameters during the course of the study. Furthermore, AAV2-NGF caused no gross lesions, changes in absolute or relative (to body and brain weight) organ weights, nor any histopathological changes in organs or tissues.

Brain and spinal cord histopathology Examination of brain and spinal cord sections revealed no abnormal pathology associated with AAV2-NGF. Injection sites for all animals (including FB controls) were characterized by minimal hemosiderin pigment and gliosis believed to be an increase of astrocytes adjacent to the injection tracts. The microscopic changes at the injection tracts were similar between the FB control and AAV2-NGF treated animals. Microscopic changes away from the injection sites in all tissues,

including those outside the nervous system, were considered to be spontaneous, seen in all animals including FB animals, and not related to AAV2-NGF.

Schwann cell hyperplasia and axonal sprouting Microscopic evaluation of TH- and CGRP-immunolabeled as well as cresyl violet-stained sections showed no evidence of Schwann cell hyperplasia or aberrant axonal sprouting in the medulla or cervical spinal cord in any animal from 3 to 24 months following AAV2-NGF delivery, confirming lack of NGF bioactivity in or near the untargeted ventricles.

CSF chemistry and hematology No alterations attributed to AAV2-NGF were observed in total cell count or CSF chemistry (albumin, sodium, total protein, glucose, phosphate, chloride, calcium, potassium) at any time point throughout the study.

No evidence of immune/inflammatory reaction in brain Immunohistochemistry for GFAP (a marker of astrocytic responses), CD45 (a pan-leukocyte marker), and CD68 (a marker of activated microglia) revealed that there was no AAV2-NGF-related immune or inflammatory reactions in the brain at any time point following AAV2-NGF delivery to the NBM. However, positive immunolabeling was observed for each marker in the vicinity of the needle tracks in both AAV2-NGF and FB control treated animals. Additionally, sporadic weak to moderate CD68 immunolabeling was observed outside the vicinity of the needle tracks in both AAV2-NGF and FB control animals; this was primarily restricted to the corpus collosum, the anterior commissure, or cortical white matter, and the intensity and pattern of CD68 labeling were similar between the treatment groups, including FB control. The local immune/inflammatory response observed only in the needle track, including equally robust with FB control, is a subclinical response to minor tissue damage associated with insertion of the injection needle. This is consistently observed in animals across species following any needle entry into brain and has never created a safety problem, nor comprised the expression or bioactivity of protein following gene transfer.

Serum antibody analysis No biologically meaningful or consequential increases in serum antibody titers to human NGF were observed over 24 months. The majority of animals exhibited anti-AAV2 antibody titers at baseline ranging from 1:500 to 1:13,500, suggesting exposure to naturally occurring AAV2 prior to study initiation. No consistent AAV2-NGF-related antibody responses to AAV developed over 24 months, although a few animals in both the control and AAV2-NGF groups exhibited mild transient increases in titer.

No NGF protein in serum and CSF or AAV2-NGF vector in CNS No detectable increase in NGF concentration in the serum or CSF was observed across the 24 months following AAV2-NGF delivery to the NBM. No detectable levels of AAV2-NGF vector DNA were observed in the CSF at baseline or at the 3-, 12-, or 24-month sacrifice time points.

Experiment 2: single gene transfer administration provides safe, brain-targeted, bioactive protein throughout life-span of the rat

In-life observations and functional observation battery

Animals were observed daily throughout the duration of the in-life portion of the study, and a FOB was performed on all animals every 3 months beginning at 6 months following AAV2-NRTN delivery (see Table 3). The FOB, conducted by experienced individuals blinded to treatment group, revealed that all animals appeared normal for their appropriate age at testing (e.g., no salivation, lacrimation, piloerection, involuntary motor behavior, or abnormalities in posture). Additionally, no changes over time or differences between groups were observed any of the measurements, including reactivity and sensorimotor responses to handling, and tests of neurological reflexes (e.g., palpebral, pinna, and flexor). In addition, no changes, differences, or abnormalities in gait, posture, exploratory activity, or any other behavior were observed in open-field behavior. Consistent with normal aging of this rat strain, several animals from each group were either found dead or were euthanized due to their moribund condition, starting at

approximately 15 months post-injection (i.e., approximately 17 months of age). There were no statistically significant, between-group differences (including FB controls) in morbidity/mortality during the course of this long-term study (chi-square test; $p=0.41$).

Robust NRTN expression in nigrostriatal system 18–20 months after AAV2-NRTN

Immunohistochemical analyses of rats allowed to grow old (i.e., 20 to 22 months of age), following AAV2-NRTN treatment at 2 months of age, revealed robust NRTN expression in all AAV2-NRTN-injected animals. In the low dose group (4×10^9 vg/hemisphere), NRTN expression was largely confined to the striatum and anatomically connected regions of the striatum (i.e., globus pallidus, entopeduncular nucleus, nigrostriatal pathway, and substantia nigra both pars compacta and pars reticulata) and cortical regions containing the needle track. Volumetric analysis of NRTN labeling was performed within the targeted striatum for all low dose injected animals surviving to the end of the 18–20-month study. The volume of NRTN was within the range we had predicted (12 to 14 mm³), based on our prior experience administering similar doses of AAV2-NRTN to young rats (see Fig. 2a, b). Similarly, NRTN expression in the high dose group (2×10^{10} vg/hemisphere) was mostly confined to the striatum and anatomically connected regions of the striatum, including the SN. Unlike what was observed with the lower dose of AAV2-NRTN, where NRTN protein was largely restricted to the targeted nigrostriatal system, this intentionally very high dose (intended to identify possible toxicities) resulted in

Table 3 Functional observation battery (FOB) used to assess neurologic and behavioral side effects in Experiment 2 every 3 months during lifetime of rats

In-life safety measurements	6 months	9 months	12 months	15 months	18–20 months
Cage-side observations					
Salivation, lacrimation, piloerection, involuntary motor behavior, postural abnormalities	No differences observed in any behavior	No differences observed in any behavior	No differences observed in any behavior	No differences observed in any behavior	No differences observed in any behavior
Hand-held examination					
Reactivity to handling; sensorimotor response to handling, physical exam	No changes observed	No changes observed	No changes observed	No changes observed	No changes observed
Cranial/spinal reflexes					
Palpebral, pinna, flexor	All reflexes intact	All reflexes intact	All reflexes intact	All reflexes intact	All reflexes intact
Body temperature	No changes observed	No changes observed	No changes observed	No changes observed	No changes observed
Body weight	No changes observed	No changes observed	No changes observed	No changes observed	No changes observed
Open-field behavior					
Exploratory behavior, activity level, ambulatory and non-ambulatory movements	All behaviors appeared normal	All behaviors appeared normal	All behaviors appeared normal	All behaviors appeared normal	All behaviors appeared normal

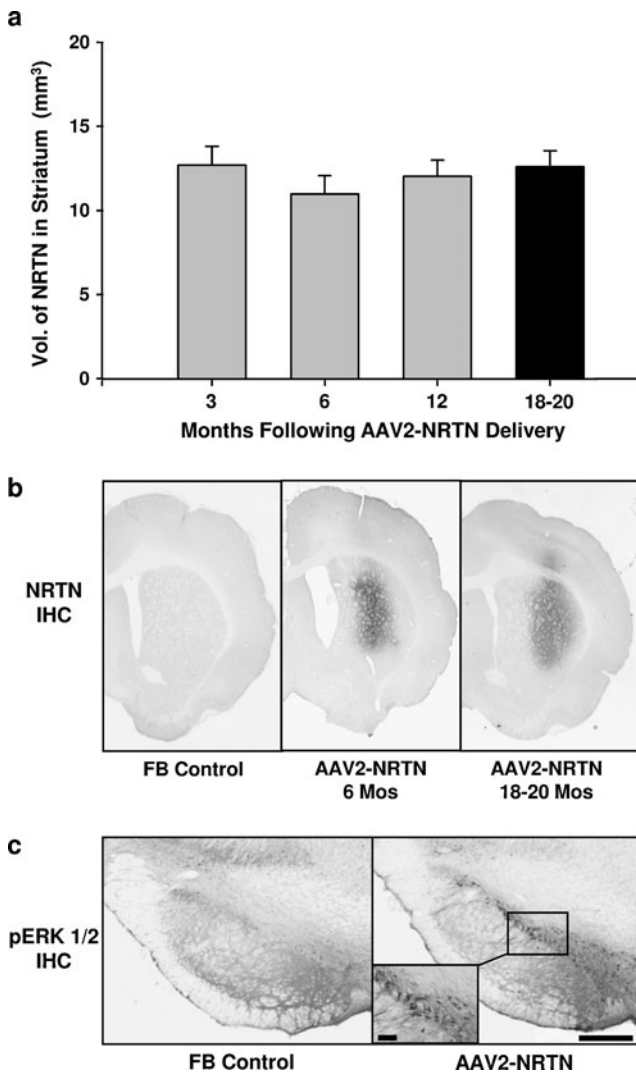


Fig. 2 Delivery of AAV2-NRTN to rat striatum results in stable, persistent, targeted and bioactive NRTN expression 18–20 months in rats (longest time point examined). **a** Quantitative analyses at 18–20 months after AAV2-NRTN delivery (4×10^9 vg/hemisphere) revealed a volume of NRTN immunolabeling in the rat striatum (*black histogram*) equivalent to that previously observed at shorter time intervals (data plotted in *gray* reflect data previously published in ([39] and presented here, again, to facilitate comparison). **b** Representative photomicrographs of coronal sections through the rat striatum illustrate persistent NRTN expression consistently restricted to the targeted striatum (terminal field of nigrostriatal dopamine neurons), along with expected NRTN in cell bodies of targeted neurons located in substantia nigra (data not shown), as well as with minor expression along needle track at 6 and 18–20 months following gene transfer via AAV2-NRTN delivery. **c** Representative photomicrographs of coronal sections through the rat midbrain illustrating pERK 1/2 immunolabeling (a marker for neurotrophic signaling by NRTN) in the substantia nigra pars compacta (i.e., brain area or origin for the dopamine cells that project to the targeted striatum). Note less intense endogenous signal in formulation buffer control section (FB con), relative to that representative of AAV2-NRTN treated rats 18–20 months following delivery to the striatum, demonstrating persistent bioactivity of NRTN on nigral dopaminergic neurons

NRTN expression in additional regions of the brain, including the cortex (in regions including the needle track, as well as cortical regions not including the needle track) and the medial and reticular thalamus.

Bioactivity of NRTN: activation of pERK 1/2 in the substantia nigra 18 to 20 months following AAV2-NRTN

Representative photomicrographs of coronal sections through the rat midbrain illustrating pERK 1/2 immunolabeling in the SN pars compacta in FB control and AAV2-NRTN-injected rats 18–20 months following delivery to the striatum are presented in Fig. 2c. Consistent with persistent bioactive effects of NRTN on nigral dopaminergic neurons, blinded raters were able to consistently identify sections from the AAV2-NRTN-injected animals, which showed enhanced number of pERK + cells and processes and enhanced pERK-staining intensity in the SN (relative to all control sections), but with no apparent differences between the two AAV2-NRTN doses.

No evidence of neurotoxicity in 20 to 22-month-old rats, 18 to 20 months following AAV2-NRTN treatment

Histopathological analyses on H&E-stained sections throughout the brains of animals surviving to the 18–20-month time point was performed by a board-certified veterinary pathologist blinded with respect to treatment condition. There were no pathological findings related to AAV2-NRTN administration. Additional brain and organ histopathology was performed on tissues from animals that died prior to the end of the 18–20-month study. An independent histopathologist concluded cause of death to be normal age-related pathology (and not due to AAV2-NRTN delivery), with progressive nephropathy being the most common necropsy observation in animals, including the formulation buffer-treated control animals.

Experiment 3: gene transfer in aged rat brain provides safe and accurate expression of bioactive protein equivalent to that obtained in young rodents

In-life observations and functional observation battery

During the course of the study, three animals died or were euthanized ($n=1$ FB, $n=2$ AAV2-NRTN). In all three cases, post-mortem analyses revealed death was attributed to spontaneous age-related disease (either lymphocytic leukemia or chronic progressive nephropathy), and no neurotoxicity was observed. Those animals surviving to the scheduled sacrifice ($n=12$) appeared healthy and maintained stable body weight throughout the study. No neurological or behavioral abnormalities were identified in any animals during monthly FOB testing.

Robust NRTN expression in the nigrostriatal system of aged rat

Immunohistochemical staining for NRTN revealed robust transgene expression predominantly in the striatum (Fig. 3a) and SN (Fig. 3b) in the right (AAV2-NRTN-injected) hemisphere of all rats. The average volume of NRTN distribution within the striatum was $12.96 \pm 0.90 \text{ mm}^3$ (mean \pm SEM). This volume of distribution was virtually identical to that seen in young adult Sprague-Dawley rats following administration of the same dose of AAV2-NRTN reported previously from 3 to 12 months [40] (see Fig. 2a) and at 18–20 months following delivery (Experiment 2 of the present manuscript). Also, similar to what is observed in young rats, the SN pars reticulata ipsilateral to the injection exhibited diffuse labeling, indicative of anterograde transport of NRTN protein and subsequent secretion from the axon terminals. NRTN positive cell bodies in the SN pars compacta were observed and likely resulted from retrograde transport of the protein and/or vector in aged rats (Fig. 3b), as occurs in young animals. Additional NRTN expression was observed in some rats around the needle track in the cerebral cortex and in the reticular thalamic nucleus. As in young animals, the cortical NRTN expression, for the most part, appears to be the result of a small amount of back flow of viral vector during the dosing procedure. However, some NRTN signal in the cortex may also be due to retrograde transport of protein and/or vector from the striatum to the cortex by way of the corticostriatal pathway. The NRTN in the reticular thalamus is most readily explained by transport of protein from the SN, globus pallidus, and/or cortex. No NRTN immunohistochemical signal was present in the contralateral, uninjected, or FB-injected control hemispheres.

Enhanced tyrosine hydroxylase positive cell size and pERK 1/2 labeling in the substantia nigra

No significant difference between the number of TH-positive cells in the SN pars compacta in AAV2-NRTN-injected hemispheres ($9,595 \pm 456$; mean \pm SEM) compared to the contralateral uninjected sides ($9,333 \pm 530$) was observed [$t_8 = -0.410$, $p = 0.693$]. Significant hypertrophy of TH-positive SN neurons as a result of AAV2-NRTN was observed [paired $t_8 = -6.959$, $p < 0.001$] (Fig. 3c; mean percent increase of 13%). In uninjected and FB control-injected hemispheres, weak endogenous levels of pERK 1/2 immunoreactivity were apparent in the SN compared to negative controls. AAV2-NRTN-injected hemispheres, however, displayed a dramatic increase in pERK 1/2 labeling throughout the cell bodies and processes of SN compacta neurons, similar to what was observed in young animals in Experiment 2 (see Fig. 2c). Stereological quantitation revealed

a significant increase ($\sim 155\%$) in the number of pERK 1/2-positive cells in AAV2-NRTN-injected hemispheres ($4,852 \pm 272$) compared to the contralateral uninjected sides ($1,909 \pm 203$) [paired $t_8 = -13.01$, $p < 0.001$] (Fig. 3c).

No evidence of neurotoxicity or immune/inflammatory response in brain

Detailed histopathological evaluation of H&E-stained sections throughout the brain conducted by a veterinary pathologist blind with respect to treatment condition revealed no microscopic abnormalities related to AAV2-NRTN. Brain sections examined for evidence of inflammation/immune response by semi-quantitative assessment of immunoreactivity for GFAP, CD45, or CD68 (astrocytes, pan-leukocyte and activated microglia markers, respectively) presented similar staining patterns for all groups. Thus, the immune and inflammatory reactions observed are most likely due to focal damage from the injection needle alone (and unrelated to injection of AAV or expression of NRTN). Also consistent with this hypothesis, no evidence of gliosis, leukocyte infiltration, or activated microglia accumulation was seen in any other brain region. The local immune/inflammatory response observed only in area of the needle tracks a subclinical response to minor tissue damage associated with insertion of the injection needle. It occurred equally robust with FB (formulation buffer) controls animals. This response is consistently observed in animals across species following any needle entry into brain. Importantly, the subclinical reaction does not interact with aging or with the local neuropathology in the disease models we have used, e.g., [41, 43, 44] and, as has been true for all nonclinical studies, has not posed any safety problems in any of the human patients similarly dosed.

Experiment 4: gene transfer dosing parameters can be scaled in monkeys to inform predictions for targeted brain expression in humans

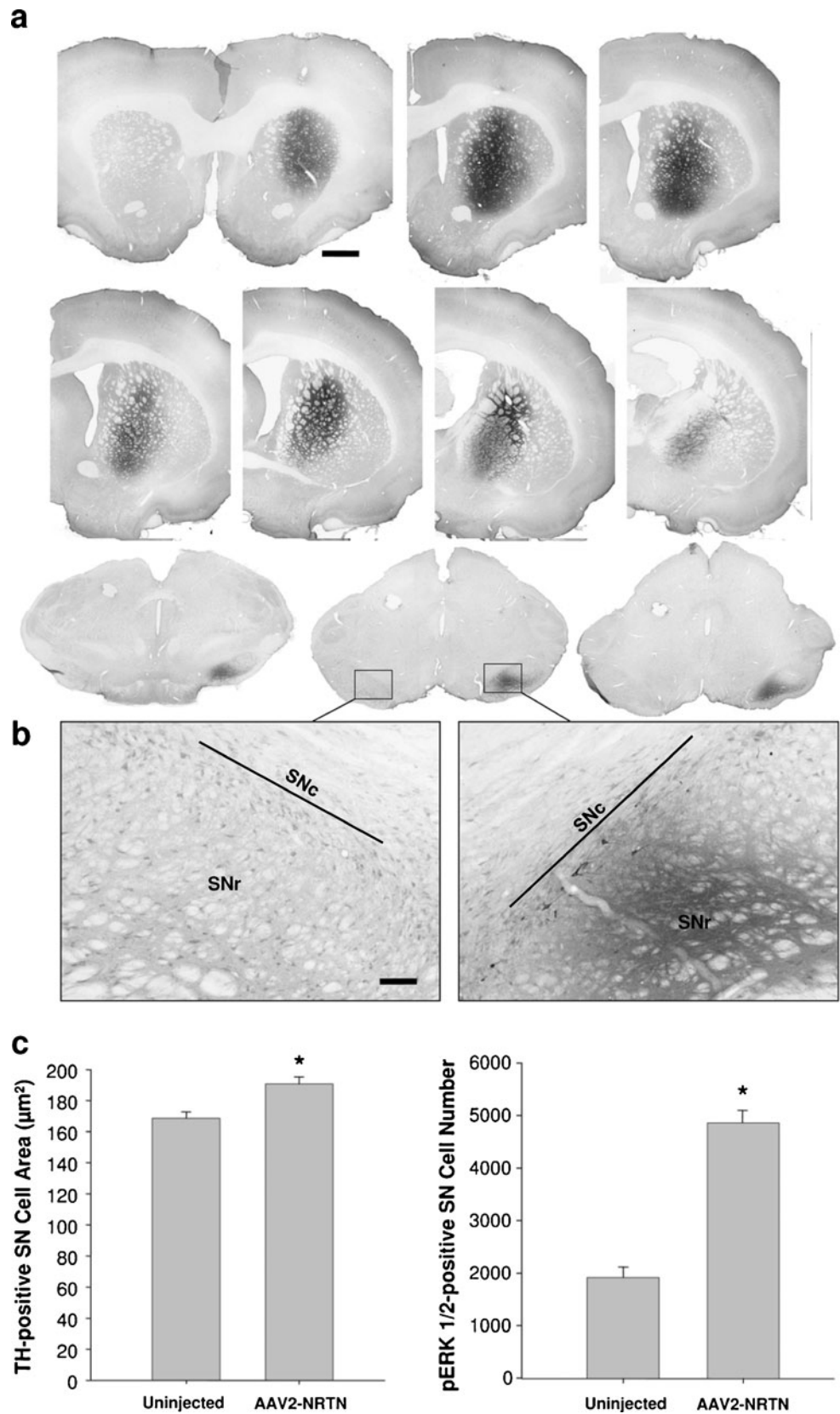
In-life observations and body weight

Animals appeared normal and healthy throughout the duration of the study. There were no alterations in body weight throughout the study attributed to AAV2-NRTN using any of the various dosing conditions.

Effect of delivery method and dosing parameters

The volume of NRTN expression achieved via an automated infusion pump (delivering a constant, continuous flow of AAV2-NRTN) versus manual delivery (which produces a pulsatile flow) was compared using two dosing parameters: (1) two 5- μL volume deposits separated by 4 mm along a

Fig. 3 Robust expression and bioactivity of NRTN in the nigrostriatal system of aged rats following AAV2-NRTN delivery to the striatum. **a** Coronal sections through the striatum and midbrain illustrating representative NRTN labeling 3 months following injection of AAV2-NRTN (4×10^9 vg) into the right striatum of aged rats (20 months old at time of dosing; scale bar=1.0 mm). **b** Higher magnification photomicrographs illustrating NRTN immunolabeling in the substantia nigra (SN) of the control, uninjected (left panel) and AAV2-NRTN-injected (right panel) hemispheres from the same animal. Note the NRTN labeling in both the SN pars compacta and SN pars reticulata, demonstrated expected retrograde and anterograde transport following delivery of AAV2-NRTN to the terminal field in the striatum; see text for the discussion (scale bar=100 μ m). **c** Quantitative analyses revealed significant hypertrophy of TH-positive cells (left panel) in the SN 3 months following AAV2-NRTN delivery to the aged rat striatum, as well as marked increase in the number of pERK 1/2-positive cells in the SN (right panel) following AAV2-NRTN delivery in aged rats, confirming persistent bioactivity in aged rats. Data are expressed as mean (\pm SEM) cell area (μ m²); * p <0.001



single needle tract at a flow rate of 2 μ L/min and (2) one 10 μ L volume at delivered at 2 μ L/min. These parameters

were chosen since our initial Phase I and Phase II trials utilized the first dosing parameter and we wanted to test whether the

second parameter would be superior. The volume of NRTN expression across groups is presented in Fig. 4a. Statistical analyses (two-way ANOVA) revealed that the volume of NRTN expression was found to be comparable when delivering AAV2-NRTN using either an automated infusion pump or manual delivery [$F(1,13)=0.73$, $p=0.41$]. In addition, no difference in NRTN expression was observed

when delivering AAV2-NRTN in two 5 μL volumes injected into a single needle tract or in one 10 μL volume (both delivered at a flow rate of 2 $\mu\text{L}/\text{min}$) [$F(1,13)=1.25$, $p=0.29$], and there was no interaction [$F(1,13)=0.06$, $p=0.82$] between these two variables [i.e., infusion method (pump vs manual) and delivery approach (5 $\mu\text{L}\times 2$ or 10 $\mu\text{L}\times 1$).

Effects of increasing the dose of AAV2-NRTN and the rate of infusion

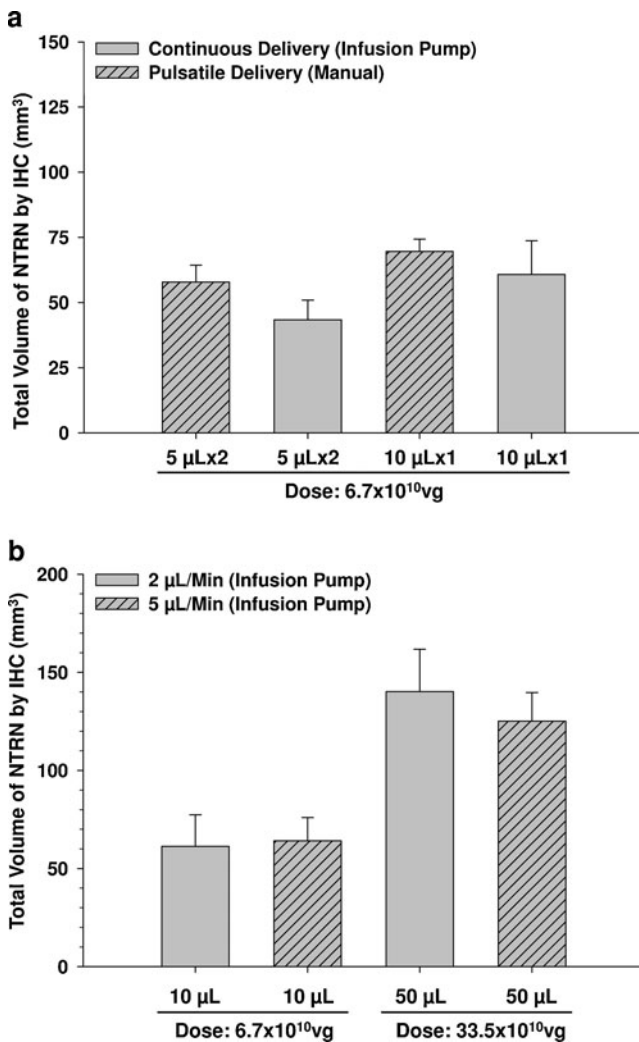


Fig. 4 Effects of varying the volume, flow rate, and method of infusion on the volume of NRTN immunolabeling in striatum of nonhuman primates. **a** The volume of NRTN expression was found to be comparable when delivering AAV2-NRTN using either an automated infusion pump (with constant delivery) or manual infusion (with pulsatile delivery). Additionally, no difference in NRTN expression was observed when the same dose of AAV2-NRTN was delivered via two 5- μL volume injections separated by 4 mm or a single 10- μL volume injection, both at 2 $\mu\text{L}/\text{min}$. **b** Increasing the volume (and therefore, the dose) of AAV2-NRTN by fivefold produced a significant increase in the volume of NRTN expression. However, modifying the rate of infusion (2 versus 5 $\mu\text{L}/\text{min}$) did not impact volume of NRTN expression, independent of whether 10 or 50 μL of AAV2-NRTN was infused. These data and the parameters manipulated provided important information to help establish and support the dosing parameters used in subsequent human trials

Figure 4b illustrates the difference in volume of NRTN expression obtained with two dose levels, fivefold different from each other (produced by manipulating volume of AAV2-NRTN), as well as the lack of an effect on volume of NRTN staining using two different infusion rates (fivefold different). Statistical analyses (two-way ANOVA) confirmed a highly significant effect of dose (50 μL volume produced larger volume of NRTN expression; [$F(1,18)=19.58$, $p<0.001$], no effect of rate of infusion [$F(1,18)=0.13$, $p=0.72$], and no interaction between dose of AAV2-NRTN and infusion rate [$F(1,18)=0.34$, $p=0.57$]. Consistently with the results of earlier-dose-response studies in nonhuman primates and rodents [40, 42, 46], the increase in volume of NRTN expression volume at the higher dose was curvilinear (i.e., a “law of diminishing returns” was observed) [39, 42, 46, 54]. Nonetheless, we were able to more than double the volume of NRTN expression in the putamen with the larger dose (produced by administering a larger volume). Equivalent volumes of NRTN expression were observed with 2 and 5 $\mu\text{L}/\text{min}$ infusion rates, independent of dose (i.e., whether 10 or 50 μL volumes of AAV2-NRTN were infused). As described in more detail below, we observed no evidence of toxicity or trauma to the brain with the higher infusion rate or volume, nor any evidence of greater backflow of viral vector up the needle tract based on the pattern of NRTN immunolabeling. In summary, these data supported the use of larger vector volumes as well as higher flow rates for the ongoing clinical trials.

No evidence of toxicity in brain

Coronal sections through the brain were examined microscopically to determine whether any of the dosing conditions induced any evidence of neurotoxicity. All brains appeared normal, with no evidence of physical duress to the brain parenchyma, including no evidence of hemorrhage, edema, or profound inflammation.

Serum antibody analyses and CSF analyses

No serum human NRTN antibody titers increased in any of the six animals tested. Serum AAV2 antibody titers

increased modestly in four of the six animals tested, but was unrelated to any of the infusion conditions or to AAV2-NRTN dose level, since we balanced the total dose received per monkey by selecting appropriate combinations of doses for each of the two hemispheres (resulting in nearly equivalent total doses received per monkey). Moreover, and in keeping with prior findings [46, 54], the induction of AAV2 antibodies had no impact on NRTN expression, NRTN bioactivity, or any safety measure.

No evidence of NRTN or AAV2-NRTN viral vector in cerebrospinal fluid

No quantifiable NRTN or AAV2-NRTN was found in the CSF of any animal.

Discussion

Neurotrophic factors have long been known to have both neurorestorative and neuroprotective properties for damaged and dying neurons. While many investigators have recognized their unique therapeutic potential to treat neurodegenerative diseases in humans, no trial has yet provided clear, unambiguous evidence of clinically meaningful efficacy. In the nearly two decades since the first clinical trials were initiated to test their safety and efficacy in neurodegenerative patients, much has been learned about the biology of neurotrophic factors. This cumulative insight provides important details about the translational obstacles that must be confronted and solved before neurotrophic factors might be developed as successful human therapeutics. For example, to treat chronic neurodegenerative diseases, adequate levels of neurotrophic factors must be maintained for very long periods of time (i.e., for years), for once the proteins return to basal levels, their benefit typically reverses and is therefore lost [1, 3–5]. Similarly, it is important that an appreciable volume of the degenerating cell population be exposed to the neurotrophic factor in order to produce sufficient restoration of neuronal function and thus achieve measureable clinical improvement (though the exact proportion required for therapeutic benefit is currently not clear). Because serious side effects have been observed when delivery of neurotrophic factors have inadvertently exposed nontargeted brain sites (e.g., periventricular tissue), the importance of accurately predicting, controlling, and restricting protein delivery specifically to the intended target has become apparent [10, 14, 18, 22, 31]. Further complicating the translation of neurotrophic factors, because they are proteins, they cannot be taken orally, do not cross BBB naturally, and thus cannot be administered systemically, even if linked to a BBB carrier, due to side

effects induced by exposing systemic organs and tissue [27, 28]. These issues, individually and collectively, render safe and effective delivery of neurotrophic factors extremely challenging. Thus, it is for this reason that a consensus opinion has emerged among investigators that the successful translation of neurotrophic factors to the human clinic will first require that these crucial delivery issues be resolved [15, 20, 21, 55].

In the past decade, the development and use of AAV2 as a safe and effective viral vector, as a means of delivering the genes for therapeutic proteins and thus providing long-lasting, targeted expression of the protein offers a possible solution to these long-standing delivery obstacles. Importantly, many studies indicate that AAV, when combined with the common CAG promoter, likely provides life-long expression of the therapeutic protein [33, 34]. AAV2 is easily engineered so that it contains none of the viral-coding sequences and thus expresses only the therapeutic human protein of interest. Moreover, AAV2 has established itself as a very safe delivery vehicle, for no significant safety issues have been noted following its administration to over a thousand patients in scores of trials involving many different diseases, with several years of follow-up (*Journal of Gene Therapy*: <http://www.wiley.com/legacy/wileychi/genmed/clinical>). Thus, AAV2, in conjunction with gene transfer, may provide a practical means to overcome the long-standing complications of delivering neurotrophic factor proteins to treat neurodegenerative diseases. Indeed, research conducted in various animal models of AD and PD, including the most widely accepted models of neurodegeneration for each disease, have consistently shown an ability to restore and protect vulnerable neurons via NGF and NRTN, respectively, when delivered via an AAV vector in a safe and effective manner [39, 40, 42–45]. Moreover, the data from these studies confirms that the expression characteristics and bioactivity of the protein achieved in normal brain generally predicts that obtained in the disease model [40, 42, 46].

The data presented in this manuscript, using young and aging animals, provide important, new information that expand the existing database for AAV2 as a solution to the long-standing obstacles of delivering neurotrophic factors to the central nervous system. Thus, it helps translate the concept of gene transfer of neurotrophic factors to potential use in the human clinic. The first experiment, a GLP study in nonhuman primates, replicated and extended the long-term expression database for AAV2 by providing the first evidence that gene transfer can result in safe, bioactive expression of a NGF for at least 2 years. Importantly, no side effects, antibodies to NGF or toxicity were observed during this entire period, while stable neurotrophic activity was maintained, as evidenced by a classic neurotrophic response (i.e., cell hypertrophy or enhancement in the size

of the targeted neurons) in the targeted NBM throughout the duration of the experiment. Moreover, these data provide further evidence that the amount and location of protein expressed remains relatively stable and confined to the targeted brain region for at least two years in nonhuman primates. Finally, the data provide further support that the volume of protein expression can be manipulated by the dose of AAV2 vector administered.

In the second experiment, we extend those long-term safety and expression data, using another neurotrophic factor, NRTN, while evaluating its expression, bioactivity and safety over a major portion of the life-span of the host. AAV2-NRTN was administered to the striatum of rats when 2 months old and the animals euthanized 18 to 20 months later (i.e., age 20 to 22 months old). We again observed, predictably targeted, long-term protein expression following gene transfer, with NRTN accurately targeted to the striatum and the substantia nigra (due to transport of the protein from the terminals and neurons in the injected striatum, along their afferent and efferent pathways). Once more, no side effects, toxicity, or safety issues were seen. Finally, long-term bioactivity of the NRTN was confirmed, by induction of pERK in the cell bodies of the targeted neuron in the substantia nigra; induction of pERK is part of the MAP kinase pathway known to mediate neurotrophic activity and has been linked to induction of cellular repair genes by neurotrophic factors, such as NRTN [2]. The data in this experiment, therefore, provide novel evidence for stable, long-term expression and bioactivity of a neurotrophic factor over a time-frame comprising over 80% of the rats' life-span, following a single gene transfer treatment—the longest relative time-frame thus far ever reported. No apparent change in the expression, safety, or bioactivity of AAV2-NRTN was seen, despite significant age-related changes known to occur over this time-frame.

In the third experiment, these data were further extended by administering AAV2-NRTN to rats already senescent (i.e., 20 months old—the age at which the long-term expression rats in Experiment 2 were euthanized). The data demonstrated successful prediction and control of both the location and volume of NRTN expression over the next 3 months in aged rats, while confirming safe and bioactive protein in the aged rat brain. Additionally, we demonstrate that NRTN can produce the desired and predicted neurotrophic response (both cellular hypertrophy and induction of pERK in the targeted neurons), substantially expanding the age-related data initially published with AAV2-NRTN in 3 aged monkeys [44] and AAV2-NGF with aged rats [42]. Thus, these data further support the use of AAV-mediated gene transfer for age-related neurodegenerative diseases, for it has been argued long ago that aged animals can provide important information as models for age-related neurodegenerative

diseases [56–58] and indeed aged animals have since been used as models to address certain translational questions for both AD [35, 37, 59–61] and PD [62].

The final experiment yielded valuable information regarding dosing parameters required to translate the animal data with AAV2-NRTN to the larger-brained human. The data offered important guidance for our most recent, ongoing clinical trial, providing the means to help predict the spread of protein following higher volumes and doses of vector, thus supporting the selection of more safe and potentially effective doses for human testing. Additionally, the studies supported the safety data of employing higher volumes of vector (therefore enabling larger doses per injection site), fewer needle passes (therefore reducing risk to patients), and higher infusion rates (therefore reducing procedure time in the operating room). Finally, the experiment also provided data that supported a more efficient and simplified dosing scheme for the clinic, supporting the use of an automated infusion pump, which reduced the potential for human error and eliminated significant human tedium. Not only did these studies directly support the ongoing AAV2-NGF (CERE-110) and AAV2-NRTN (CERE-120) gene transfer programs for AD and PD, respectively, but the information achieved and the dosing variables studied should also be of more general use to others who may wish to similarly target the human CNS with viral vectors or possibly even proteins.

The emergence of gene transfer, in conjunction with the development of AAV2 as a safe and dependable viral vector construct, represents a potentially “paradigm-shifting” event in the quest for long-term expression of bioactive proteins and other biologics, particularly when organ or tissue-specific (as opposed to systemic) targeting is desired or required. Moreover, most investigators believe that AAV viral vectors uniquely provide life-long expression of the therapeutic protein following a single administration. To date, scores of gene transfer clinical trials have been completed, safely administering AAV vectors to well over 1,000 subject, including to the brains of well over 100 with various neurological diseases, including delivering an aspartoacylase-encoding gene to Canavan's disease patients [63], delivering the neuronal 2 gene to late infantile neuronal ceroid lipofuscinosis patients [64] and delivering the glutamic acid decarboxylase (GAD65/67) genes to the subthalamic nucleus [65–67] and the aromatic acid decarboxylase gene to the putamen [68] in two different Parkinson's disease programs. Several years of long-term follow-up data have accumulated with no serious issues identified particularly if extremely high titers of vector are not exposed to systemic circulation. While AAV is relatively non-immunogenic, most adults have been exposed to the natural form of the virus with many people exhibiting neutralizing antibodies (though no clinical

symptoms or disease has even been linked to this exposure). While the existence of circulating antibodies has not affected the ability of AAV to transduce neurons in the brain nor express bioactive protein over long periods of time [54] (Experiment 1 in this manuscript), it remains to be determined whether subsequent re-administration of vector might be as effective.

To date, only two neurotrophic programs applying gene transfer delivery methods have advanced into clinical trials: AAV2-NGF for AD (called CERE-110) and AAV2-NRTN for PD (called CERE-120), both of which are the focus of the present manuscript. Over 20 AD patients have already been safely treated with AAV2-NGF and 65 PD patients with AAV2-NRTN. Both programs have advanced to multi-center, double-blinded, controlled efficacy trials (CERE-110: <http://www.clinicaltrials.gov>; identifier: NCT00985517 and CERE-120: <http://www.clinicaltrials.gov>; identifier: NCT00876863) making these among the most advanced clinical programs using either neurotrophic factors for any indication or gene transfer for any CNS disorder [53, 69, 70].

While traditional small molecule treatments have been developed, approved and used with varied success for both diseases, neither AD nor PD is adequately treated. For AD, cholinesterase inhibitors remain the mainstay of treatment for the cognitive deficits, but their effects are modest and occur only in an unpredictable portion of the AD population, particularly in the earlier stages of the disease [35, 37]. Significant, dose-limiting side effects dampen what might otherwise be possible from enhancing cholinergic neuronal function, but given the ubiquitous status of neurons using acetylcholine throughout the brain and body, that scenario is unlikely to improve (and has not in the decades following the initial approval of this class of drugs). Moreover, even in the best case, they only offer symptomatic treatment, by no means completely reverse the cognitive symptoms and do nothing to prevent the inevitable progression of the disease toward full-blown dementia and eventual “loss of self” [35, 37]. During the initial stages of PD, the dopamine agonists and the dopamine precursor L-dopa (combined with carbidopa, a peripheral inhibitor of DOPA decarboxylase or DDC, to assure maximum conversion of dopamine in the brain) provides satisfactory relief of symptoms. However, as time passes with chronic use and further disease progression, the benefits of these drugs wane [71, 72]. In time, the therapeutic index becomes intolerably narrow, leading to “wearing off” phenomenon (i.e., lack of efficacy despite optimized medication) periodically throughout the day and peak-dose dyskinesias (i.e., uncontrolled movements) related to high plasma levels of L-dopa. While adjunctive therapies (e.g., dopamine receptor agonists, enzyme inhibitors) exist which help modulate dopaminergic function and thus help relieve these problems, in time, nothing is effective

and the patient’s condition continuously worsens [71, 72]. Thus, for both AD and PD, the possibility that neurotrophic factors might enhance the function of degenerating neurons, possibly restore their health and delay or even prevent their eventual death offers significant hope to an otherwise dire medical prognosis. It is for these reasons that neurotrophic factors are seen as the best available means to significantly improve the status of degenerating diseases like AD and PD. Whether gene transfer will indeed provide the enabling delivery technology that permits them to be successfully translated to the clinic remains to be seen. Nonetheless, the data presented in this manuscript provide further support toward that goal, while also providing key information that allowed both the AAV2-NGF and AAV2-NRTN programs to progress forward. This information presented should also be helpful to others as they continue efforts to develop these and other potentially powerful proteins and biotherapeutics as treatments for human diseases.

Concluding remarks

The data presented in this manuscript demonstrate that gene transfer may indeed provide the means to overcome several of the long-standing delivery obstacles that have precluded neurotrophic factors from being successfully translated to the clinic. We show that one can achieve predictably stable, safe, targeted, bioactive protein over the course of 2 years in monkeys (the longest time point ever established for a neurotrophic factor). We also show similarly stable, safe, targeted, bioactive protein throughout most of the life-span of the rat (a concept never before tested), during a time when many known age-related changes are taking place in the targeted brain. These data show that these changes have no impact on the long-term and stable expression, safety or bioactivity of the neurotrophic factor. We also show that similarly safe, predictable and effective protein can be expressed even after these age-related changes have occurred, thus further supporting the translation of this approach for age-related neurodegenerative diseases. Finally, we demonstrate that the dosing parameters initially established and tested in much smaller-brained rats and monkeys can be modified and scaled to accommodate the much larger human brain. The modified doses, volumes, and infusion rates provided guidance to define parameters in humans that should provide similarly safe and predictably effective protein expression in the targeted human brain regions.

These studies collectively provided important information that supported and guided the translation of AAV2-NGF and AAV2-NRTN into ongoing Phase 2 clinical trials in AD and PD, respectively, while also offering insight to others similarly interested in achieving sustained levels of bioactive protein in targeted tissue for therapeutic purposes.

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