

Neurturin gene therapy improves motor function and prevents death of striatal neurons in a 3-nitropropionic acid rat model of Huntington's disease

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Huntington's disease (HD) is a devastating neurodegenerative disease characterized by the selective loss of neurons in the striatum and cerebral cortex. This study tested the hypothesis that an adenoassociated viral (AAV2) vector encoding for the trophic factor neurturin (NTN) could provide neuroprotection in the rat 3-nitropropionic acid (3NP) model of HD. Rats received AAV2-NTN (CERE-120), AAV2-eGFP or Vehicle, followed 4 weeks later by the mitochondrial toxin 3NP. 3NP induced motor impairments were observed on the rotarod test, the platform test, and a clinical rating scale in all groups. However, each of these deficits was attenuated by AAV2-NTN (CERE-120). Stereological counts revealed a significant protection of NeuN-ir striatal neurons from 3NP toxicity by AAV2-NTN. These data support the concept that AAV2-NTN might be a valuable treatment for patients with Huntington's disease.

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Huntington's disease (HD) is an autosomal dominant disorder resulting from an inherited mutation at the IT15 locus of chromosome 4 (The Huntington's Disease Collaborative Research Group, 1993). HD is characterized by a devastating constellation of motor, cognitive and psychiatric symptoms that are currently incurable. These symptoms are attributable to the death of striatal projection neurons and cortical neurons in the brains of HD patients. While the mechanisms of cell death are unclear, mitochondrial deficiency has been implicated in the etiology of striatal and cortical degeneration. In this regard, disruption of mitochondrial function causes deficits in cellular metabolism

resulting in excitotoxicity (Greene and Greenamyre, 1996) and secondary neuronal degeneration.

3-Nitropropionic acid is a mitochondrial toxin that irreversibly inhibits succinate dehydrogenase thereby inhibiting both the Krebs' cycle and complex II of the electron transport chain (Alston et al., 1977). Systemic administration of 3NP causes a disruption in mitochondrial ATP production. When ingested by humans, neuronal death in the caudate and putamen is observed and this neuropathological event is associated with dystonia (Ludolph et al., 1991). Similarly, when administered systemically to rats, 3NP causes selective striatal neuronal death and a progressive decline in gait and motor abilities (Blum et al., 2001).

There are currently no cures or effective treatments for HD. A potent therapy would first involve the protection of striatal neuron function and viability and then prevent the behavioral, cognitive and motor deficits seen in patients. Several neurotrophic factors have been used successfully in preventing the death of specific populations of striatal neurons. Striatal transplantation of fibroblasts, progenitor cells, or stem cells that overexpress the neurotrophins (BDNF, NGF, NT-3, NT-4) prevents the death of medium spiny neurons (Perez-Navarro et al., 2000b). Injections of viral vectors expressing CNTF in a quinolinic acid (QA) model of HD protects DARPP-32 positive striatal neurons (de Almeida et al., 2001). Furthermore, members of the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs) (GDNF and neurturin) also protect select groups of striatal neurons in animal models of HD. In particular, transplantation of cells overexpressing GDNF selectively protects the neurons of the direct circuit in a QA rat model of HD (Perez-Navarro et al., 1999). Our laboratory has previously shown that viral vector delivery of GDNF prevents the motor deficits and striatal degeneration that normally results as a consequence of 3NP toxicity (McBride et al., 2003). In a QA model, neurturin (NTN) has been shown to selectively protect a second population of striatal neurons — those of the indirect circuit (Perez-Navarro et al., 2000a). In HD the striatal neurons of the indirect basal ganglia circuitry are the first to degenerate (Reiner et

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al., 1988). The potential of NTN to prevent the degeneration of these cells may be critical in the treatment of early symptomatology. Although previous studies have shown promising results with NTN, the method of trophic factor administration might be crucial in providing potent neuroprotection. Towards this end, the present study used the adenoassociated virus type 2 (AAV2) vector to administer NTN to the striatum. Gene therapy using viral vectors allows sustained and long-term expression of the gene with robust diffusion of the gene product (Lebherz et al., 2005). The present study examined the effectiveness of *in vivo* adenoassociated viral vector type 2 mediated delivery of neurturin to the striatum in preventing the cellular loss and motor deficits caused by systemic administration of 3NP.

Materials and methods

Animals

Male Lewis rats (150–175 g) were obtained from the Charles River breeding company and housed in the animal care facility at the Rush University Medical Center. They were maintained in a 12-h light–dark cycle and given food and water *ad libitum*. All experiments were performed with the Institutional Animal Care and Use Committee approval and according to both federal and institutional guidelines.

Experimental paradigm

The timeline for all procedures is presented in Fig. 1. All rats underwent baseline behavioral testing at week 1 of the study and at week 2 they received bilateral vector injections into the striatum. Subsequent behavioral tests were conducted weekly until the end of the study. Four weeks after vector surgeries, groups were treated systemically with 3NP (week 6). Animals were sacrificed for histological preparation at the end of week 14.

Treatment groups

Rats were divided into four groups, three of which ultimately received 3NP. Group 1 received bilateral injections of AAV2-NTN into the striatum (AAV2-NTN-3NP; $n=8$). Group 2 received identical injections of AAV-enhanced green fluorescent protein (eGFP) (AAV2-eGFP-3NP; $n=12$). Group 3 received bilateral intrastriatal injections of the formulation buffer (1× PBS with 2 mM magnesium chloride; Vehicle) that was used to prepare and dilute the vector (Vehicle-3NP; $n=11$). Group 4 also received intrastriatal vehicle and saline in lieu of 3NP, 4 weeks later (Vehicle-saline; $n=10$).

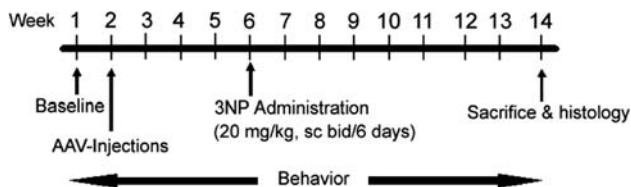


Fig. 1. Timeline of study. All animals underwent baseline testing at week 1. At week 2 they received vector injections. Four weeks following this, at week 6, they were administered 3NP subcutaneously. Behavior testing was conducted weekly until the end of the study at week 14 when the animals were sacrificed.

3NP administration

Four weeks after vector surgeries animals received systemic injections of either 3NP (Groups 1–3) or saline (Group 4). The 3NP was prepared fresh each day and was dissolved in distilled water to obtain a final concentration of 20 mg/ml. Rats received two injections per day, 12 h apart, so that the total concentration of 3NP administered to each rat was 40 mg/kg/day. Injections were given subcutaneously, between the scapulae, for 6 consecutive days. In order to accurately administer each rat with 1 ml/kg of 3NP solution, rats were weighed prior to each injection.

Construction of adenoassociated viral vector

In the present study, we used the CERE-120 vector, an adenoassociated viral vector type 2 (AAV2)-based gene delivery system, to administer NTN. A similar control vector expressing eGFP was also used. The structure of the AAV2 vector used is indicated in Fig. 2A. AAV2 is non-pathogenic and is incapable of replication. All coding sequences were removed, leaving behind only the non-coding inverted terminal repeats of the AAV. The coding sequences were replaced by a NTN expression cassette consisting of a CAG promoter, a region of cDNA encoding the β nerve growth factor pre-pro region (ppNGF) fused to the mature region of NTN, and the polyadenylation sequence from human growth hormone. The CAG promoter consists of a human cytomegalovirus (CMV) enhancer, a chicken β -actin promoter and splice donor and a rabbit β -globin splice acceptor. The ppNGF acts to promote processing and secretion of NTN. The AAV2-eGFP vector is identical to the AAV2-NTN vector except that the ppNGF-NTN cDNA is replaced by an eGFP cDNA. All vectors were produced in human embryonic kidney (HEK) 293 cells using the calcium phosphate triple plasmid transfection method. Three days post transfection, cells were harvested and lysed. The AAV vector was purified from the cell lysates by heparin and ion exchange column chromatography. Purified particles were concentrated by centrifugal filtration and vector titer (vg/ml) was determined by Q-PCR. All vectors were created by Ceregene Inc., San Diego, CA.

Delivery of viral vector

Prior to surgery, rats were anesthetized with Equithesin (3 ml/kg; sodium pentobarbital (3.5 mg/100 g)/chloral hydrate (17 mg/100 g) i.p.). Their heads were shaved, placed in a Kopf stereotaxic frame and sterilized with betadine. A midline incision was made and bilateral burr holes were drilled above the striatum. Using a motorized injector (Stoelting) two injections (2 μ l each, 1×10^{12} vg/ml each) of AAV2-NTN, AAV2-eGFP or Vehicle were made in the striatum bilaterally (coordinates from bregma, site 1: AP +0.7, ML \pm 3.2, DV -5.0 ; site 2: AP +0.2, ML \pm 3.4, DV -5.2). Injections were performed at a rate of 0.2 μ l/min using a 10 μ l Hamilton syringe. The needle was left in place for an additional 5 min to allow diffusion of the vector from the needle tip. The rats were returned to their cage and kept at a steady temperature using a heating pad.

Behavioral tests

Baseline levels of performance were determined 1 week prior to vector surgeries. Subsequently, tests were performed on a weekly basis until the end of the study at week 14.

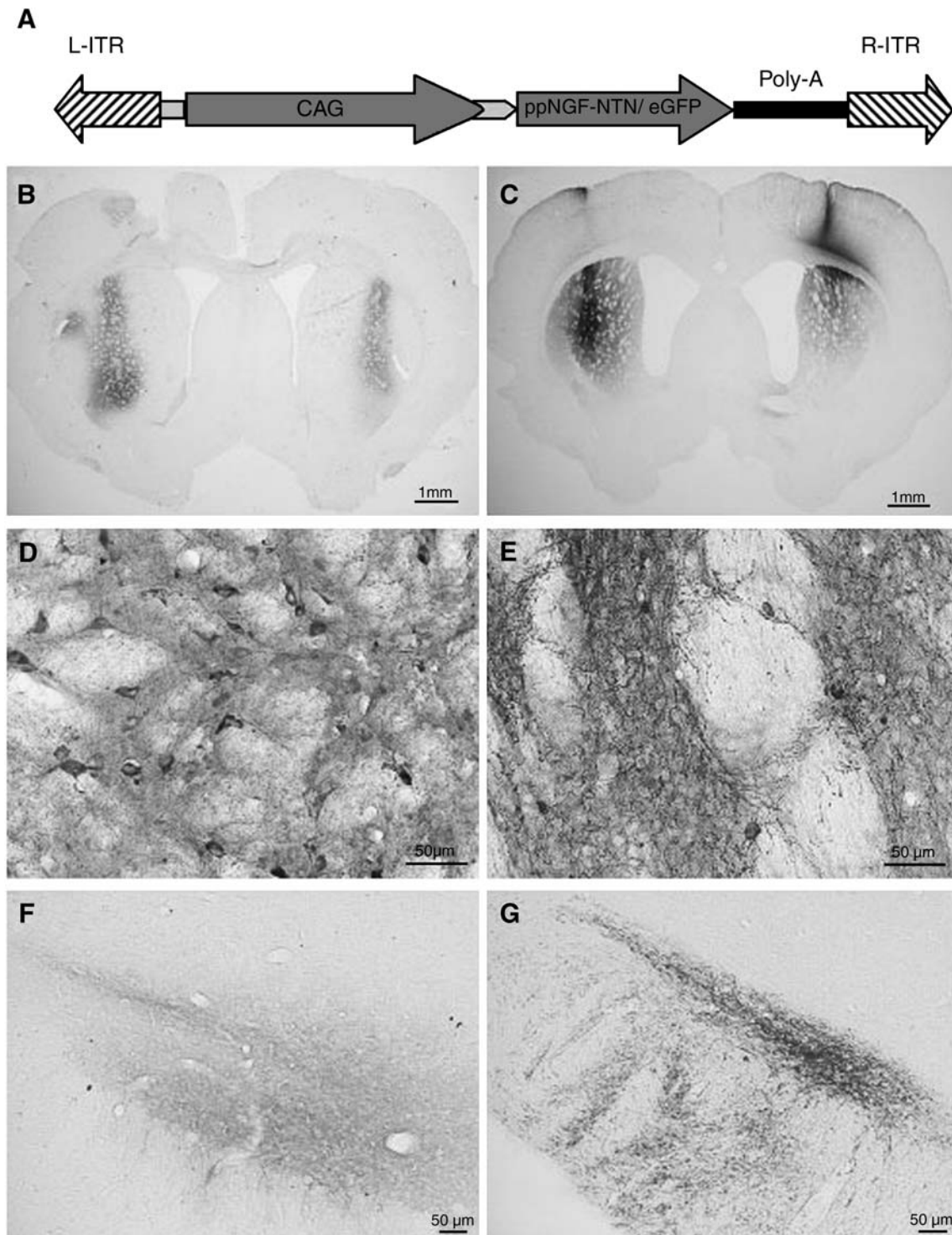


Fig. 2. (A) Construction of adenoassociated viral vector. The CERE-120 vector, an adenoassociated viral vector type 2 (AAV2)-based gene delivery system was used to administer NTN. (B–E) Gene expression. Rats were injected with AAV2-NTN, AAV2-eGFP or Vehicle. Sections were stained immunohistochemically for the expression of NTN or eGFP protein in order to determine the extent of protein expression. (B, C) Both NTN and eGFP staining was seen bilaterally in the striatum as indicated in the low magnification image (1 \times). (D, E) At higher magnification (20 \times), both cell bodies and fibers were seen to stain positive for NTN and eGFP. (F, G) NTN and eGFP staining was also seen in the substantia nigra cell bodies and fibers indicating retrograde and anterograde transport of the secreted protein.

Rotarod

Locomotor coordination was tested using a constant speed rotarod (SDI, San Diego, CA) once a week. The rats were placed on a rod of diameter 6.985 cm and allowed to acclimate for 10 s. The rotarod was then turned on to rotate at a speed of 7 r.p.m. for a maximum time of 120 s. The animal's fall was detected by a photo-beam break and its latency to fall was recorded. A total of three trials were performed per testing day with 1 h between each trial. The best score for each animal on that testing day was used for statistical analysis.

Platform test

The ability of the animal to balance on a raised platform for 20 s was tested twice a week. A small platform was created in wood (9 cm × 5 cm) and raised 10 in. off the base (adapted from Ouary et al., 2000). Scores were recorded as either a 0 (indicating ability to remain on platform for 20 s) or a 1 (indicating inability to perform the task). Percentage of animals in each group that were able to remain on the platform for 20 s was compared across groups. Additionally, the total number of falls over the 14 weeks of study was summed for each animal and averages across the groups were used for statistical comparison.

Quantitative neurological rating scale (QNRS)

Ambulatory capacity was assessed twice a week using the QNRS originally created by Ludolph et al. (1991) and later modified by our group. This is an ordinal scale with a range of 0–6 that evaluates locomotor abilities. The scale is defined as follows: 0—normal ambulatory behavior, 1—slowness and incoordination while ambulating, 2—marked gait abnormality, displacement (slipping) of hindlimbs while ambulating, 3—dystonic movements (twisting of torso), 4—failure to retract hindlimb following manual extension (muscle weakness), 5—inability to use hindlimbs while ambulating, 6—inability to use both hindlimbs and forelimbs while ambulating (Fig. 5A). Total scores were determined for each animal over the 14 weeks and average scores were compared between groups.

Preparation of tissue for analysis

At the end of 14 weeks, rats were deeply anesthetized with Equithesin and sacrificed by cardiac perfusion with 200 ml 0.9% cold saline, followed by 300 ml Zamboni's fixative with 4% paraformaldehyde. Rats were decapitated, the brains removed, and post-fixed in Zamboni's fixative for 2 h. Brains were then transferred to 30% sucrose at 4 °C until completely saturated. Brains were sliced into serial coronal sections (40 μm each) using a microtome and stored in cryoprotectant at –20 °C in 6 sets separated by 180 μm.

Immunohistochemistry

One series of sections each were immunohistochemically labeled for NeuN (1:1000, Chemicon), NTN (1:1000, R&D Systems) or eGFP (1:2000, Clontech) using the biotin-labeled antibody procedure. Following rinsing in a solution of Tris-containing triton-X, endogenous peroxidase activity was removed by incubation in 0.1 M sodium periodate in Tris-buffered saline

(TBS) for 20 min. Non-specific background staining was blocked by incubation in Tris-containing 5% normal serum, and 0.05% triton-X for 1 h. Sections were then incubated with the appropriate primary antibody for 48 h at 4 °C. After thoroughly rinsing off the primary antibody, sections were incubated with the appropriate biotinylated secondary antibody (1:200, Vector Laboratories) for 1 h. After the secondary antibody had been washed off, sections were incubated in Tris-containing triton-X and an ABC reagent (1:500) for 75 min. Sections were visualized using a solution containing 3'3' diaminobenzidine and hydrogen peroxide.

Stereology

Estimates of NeuN-ir cells were performed using a design based unbiased stereological procedure, using a microscope equipped with a camera, motorized stage, and StereoInvestigator software (MicroBrightfield, NJ). NeuN-positive cells were counted in the striatum of five equally spaced serial sections spaced 240 μm apart. Striatal sections chosen were located rostral to the anterior commissure and displayed a prominent 3NP lesion and immunohistochemistry for either NTN or eGFP. Sections at the same levels were chosen from the Vehicle–3NP and Vehicle–saline groups. The striatum was first traced at 4× magnification and counts were performed under oil immersion at 100× magnification using a lens with a 1.4 numerical aperture. At this higher magnification, the section thickness was empirically determined in three separate areas and an average was obtained. The stage was moved until the top of the section came into focus and the stage was zeroed at the z axis. The stage was then moved through the z axis until the bottom of the section was in focus. This yielded an average section thickness of 12 ± 1.1 μm. All cells that fell within an optical disector height of 6 μm were counted allowing for a guard zone of 2 μm from the section top and at least 3 μm from the section bottom. The total number of cells in the five evaluated sections was estimated by the optical fractionator method. To estimate the precision of the cell counts, coefficients of error (CE) were estimated for each group by the program using the Schmitz Hof equation (Chu et al., 2006). The CE values ranged between 0.05 and 0.06 for the AAV2-NTN-3NP group. The CE values ranged between 0.05 and 0.065 for the AAV2-eGFP-3NP group and between 0.06 and 0.07 in the Vehicle-3NP group. The CE for the Vehicle-saline group ranged between 0.05 and 0.06.

Statistical analyses

For the rotarod scores, average weekly scores for each group were compared using a two-way repeated measures analysis of variance (ANOVA). Upon a significant interaction effect, comparisons were made using Student–Newman–Keuls post hoc test. For the QNRS and platform tests, total scores for the 14 weeks of testing were generated per animal and averages were compared between groups using a Kruskal–Wallace test for non-parametric data. A Dunn's test was used to obtain specific group wise differences. For neuronal cell counts, a two-way ANOVA was used to determine differences between cell counts in the right and left striata. When a lack of such a difference was established ($p > 0.05$), data from both hemispheres were averaged and a one-way ANOVA was used to compare group differences using a Student–Newman–Keuls post hoc test.

Results

Gene delivery of NTN or eGFP: gene expression

To explore transgene expression following AAV2-NTN and AAV2-eGFP injections, sections were stained immunohistochemically for the NTN or eGFP protein. Robust staining of both NTN and eGFP was seen bilaterally in the striatum of all animals receiving AAV2-NTN or AAV2-eGFP respectively (Figs. 2B, C). Both cell bodies and fibers stained positive for NTN and eGFP (Figs. 2D, E). In Vehicle- and AAV2-eGFP-3NP-treated rats, there was no detectable NTN-ir, indicating that there was no endogenous upregulation of NTN following 3NP treatment or following the control intrastriatal injections. In addition to the detection of NTN and eGFP within the striatum, both proteins were observed within the substantia nigra pars compacta indicating retrograde transport while fiber staining was seen in the globus pallidus, entopeduncular nucleus and substantia nigra pars reticulata indicating anterograde transport of the secreted protein or virus (Figs. 2F, G). Since it is known that eGFP is not a secreted protein, its presence in the pars compacta of the substantia nigra suggests that this staining represents retrograde transport of the AAV vector to these non-injected loci, while staining in fibers of the pars reticulata represents anterograde transport of the eGFP protein within transduced striatonigral neurons.

Rotarod performance

To compare recovery of each treatment group from 3NP induced motor deficits, rats were tested on a constant speed rotarod. Average weekly rotarod scores were compared between groups for each of the for 14 weeks (Fig. 3). A repeated measures ANOVA revealed significant effect of group ($F(3,37)=6.465$; $p<0.001$), of time ($F(13,37)=39.627$; $p<0.001$) and a group by time interaction ($F(3,13)=3.246$; $p<0.001$). 3NP induced significant deficits on the rotarod in all groups beginning week 6. Transient deficits were seen in the Vehicle-saline-treated group at week 6 which was probably due to stress from being injected or context-dependent learning (Amstage and Schmidt, 2003). How-

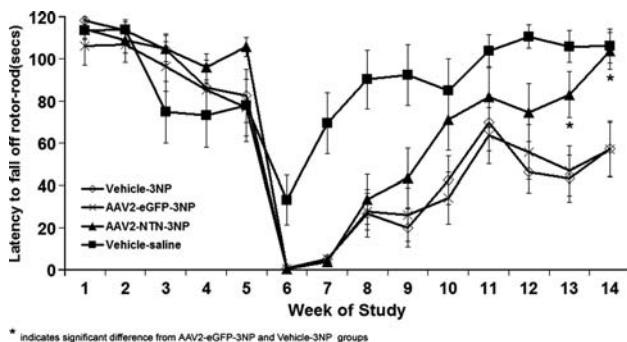


Fig. 3. Rotarod scores. 3NP administration at week 6 induced deficits on the Rotarod in all treated groups ($p<0.05$). Starting week 11 the AAV2-NTN-3NP rats began to recover from their deficits and their performance did not differ from that of the saline-treated controls ($p>0.05$). At weeks 13 and 14, the performance of AAV2-NTN-3NP-treated animals was also significantly better than that of the AAV2-eGFP-3NP-treated and Vehicle-3NP-treated animals ($p<0.05$). AAV2-eGFP-3NP and Vehicle-3NP rats never recovered from their toxin induced Rotarod deficits ($p<0.05$).

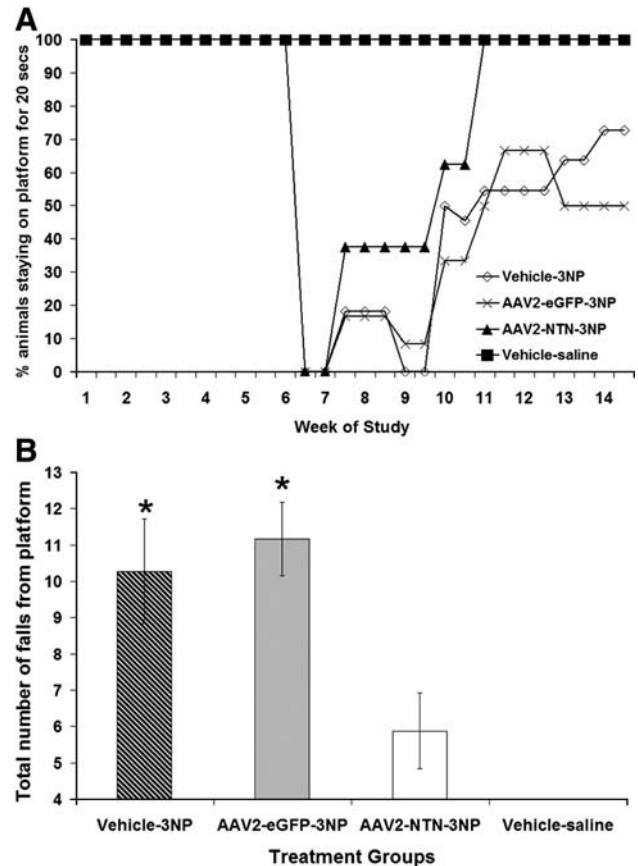


Fig. 4. Platform test. (A) 3NP caused dystonic movements of the limbs and tail hindering the performance of rats on this test. Immediately following 3NP treatment all the rats fell off the platform prematurely. All the AAV2-NTN-3NP animals completely regained their ability to stay on the platform at week 11. By the end of the study at week 14 only 50% of AAV2-eGFP-3NP treated and 72% of the Vehicle-3NP animals regained their ability to perform on this test. (B) AAV2-eGFP-3NP and Vehicle-3NP groups committed a significantly higher total number of falls from the platform for the entire study compared to Vehicle-saline-treated controls ($p<0.05$). AAV2-NTN-3NP animals did not perform significantly differently than Vehicle-saline animals.

ever, this group recovered quickly and returned to baseline levels of performance. AAV2-eGFP-3NP and Vehicle-3NP rats were significantly impaired on this task beginning week 6 and never recovered, always differing from Vehicle-saline controls ($p<0.05$). In contrast, AAV2-NTN-3NP rats began to recover from their deficits on the Rotarod task beginning week 10 and performed significantly better than the AAV2-eGFP-3NP and Vehicle-3NP animals at weeks 13 and 14 ($p<0.05$). From week 10 onward, the performance of AAV2-NTN-3NP rats was statistically similar to Vehicle-saline-treated controls ($p>0.05$).

Platform scores

3NP administration causes dystonic movements of the limbs and tail and these alterations in motor function can be captured on the platform test. Prior to 3NP administration, all rats were able to remain on the platform for 20 s. However, immediately after 3NP treatment, all the rats fell off the platform prematurely. All the AAV2-NTN-3NP animals completely regained their ability to stay

on the platform by week 11 and continued to do so for the remainder of the experiment (Fig. 4A). At week 11, only 54.5% of Vehicle 3NP and 50% of AAV2-eGFP-3NP rats could stay on the platform. By the end of the study at week 14, still only 50% of AAV2-eGFP-3NP and 72% of the Vehicle-3NP animals regained their ability to perform on this test (Fig. 4A). The total number of falls from the platform for the entire time-course was calculated for each animal and group averages were compared. The AAV2-eGFP-3NP group committed an average of 11.17 falls, the Vehicle-3NP group 10.27 falls but the AAV2-NTN-3NP group only committed 5.88 falls. A one-way ANOVA for ranks revealed a significant difference in the performance between groups ($H(3,37)=27.407$; $p<0.001$). Both AAV2-eGFP-3NP and Vehicle-3NP groups showed similar performance to each other and performed significantly worse than Vehicle-saline-treated controls ($p<0.05$; Fig. 4B). Following 3NP treatment, in contrast, AAV2-NTN-3NP injected rats performed significantly better than AAV2-eGFP-3NP and Vehicle-3NP animals ($p<0.05$). Furthermore, AAV2-NTN-3NP-treated rats were similar in performance to Vehicle-saline rats that did not receive 3NP ($p>0.05$).

QNRs scores

3NP administration causes severe acute deficits in locomotor activity. Immediately after 3NP treatment, rats demonstrated a high rating on the blinded QNRs from which they progressively recovered. Rats were rated twice a week for ambulation using the QNRs. A one-way ANOVA on ranks revealed a significant difference in performance between groups ($H(3,37)=25.929$; $p<0.001$). Both AAV2-eGFP-3NP and Vehicle-3NP groups had significantly higher total scores (indicating worse function) than Vehicle-saline animals ($p<0.05$; Fig. 5B). However, the scores of AAV2-NTN-3NP animals did not differ significantly from the Vehicle-saline-treated control animals; a group that had a total score of 0. Additionally, individual Kruskal-Wallis tests were conducted to determine group differences at each of the 9 weeks of post 3NP treatment. Significant group differences existed between weeks 6 and 14 (all $p<0.001$; Fig. 5C). The AAV2-eGFP-3NP and Vehicle-3NP groups performed significantly worse than the Vehicle-saline animals for all of the 9 post-treatment weeks. The AAV2-NTN group improved significantly beginning week 11 and their performance did not differ significantly from that of the Vehicle-saline animals between weeks 11 and 14 ($p<0.05$ for each of these weeks).

Neuronal counts in the striatum

3NP treatment caused death of neurons within the striatum. Estimates of the number of NeuN-positive cells were performed for both the right and left striata. Statistical analyses determined that there was no significant difference between the number of NeuN-positive cells between hemispheres ($p>0.05$) and these numbers were then averaged for each animal in all four groups. The average

number of striatal neurons was then compared between groups. A one-way ANOVA revealed a significant difference in the number of cells between groups ($F(3,37)=132.71$; $p<0.001$; Fig. 6A). The Vehicle-saline injected rats that did not receive 3NP had on average $1.66 \times 10^6 \pm 16,053$ (mean \pm SEM) cells. Estimates of NeuN-ir neuronal number in the AAV2-eGFP-3NP and Vehicle-3NP groups averaged of $8.32 \times 10^5 \pm 4060$ and $8.54 \times 10^5 \pm 6152$ NeuN-ir neurons in the striatum respectively. These numbers represent a 49.7% neuronal loss in the AAV2-eGFP-3NP group and a 48.4% loss in the Vehicle-3NP group, both of which reflect significant reductions in cell number compared to the Vehicle-saline group. In contrast, AAV2-NTN-3NP-treated rats averaged $1.20 \times 10^6 \pm 16,102$ neurons. This represents a 27.4% loss of NeuN-ir neurons relative to non-lesioned animals. The AAV2-NTN-3NP group had significantly higher numbers of surviving cells compared to the AAV2-eGFP-3NP and Vehicle-3NP groups ($p<0.001$ for comparisons with both groups). Consistent with the finding of robust cell death in the AAV2-eGFP-3NP- and Vehicle-3NP-treated animals, animals in these groups were found to have an apparent enlargement of the lateral ventricles compared to AAV2-NTN-3NP injected animals (Figs. 6B–E).

Discussion

In the present study, gene delivery of NTN using an AAV2 vector delivery system (CERE-120) provided structural and functional neuroprotection in a rat model of Huntington's disease. 3NP, a mitochondrial neurotoxin that is a well-established model of HD (Lee and Chang, 2004), induced performance deficits on the Rotarod test, the platform test, and the QNRs. Furthermore, 3NP lesions induced significant cell death within the striatum. On all of these measures, AAV2-NTN (CERE-120) provided partial protection.

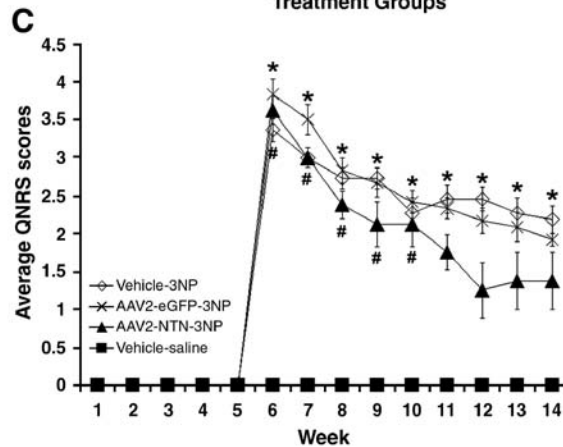
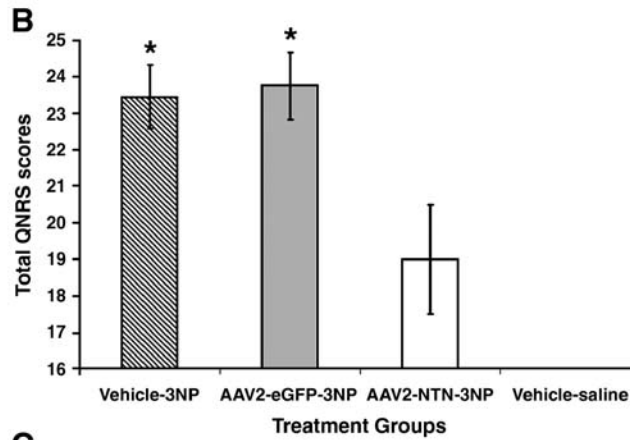
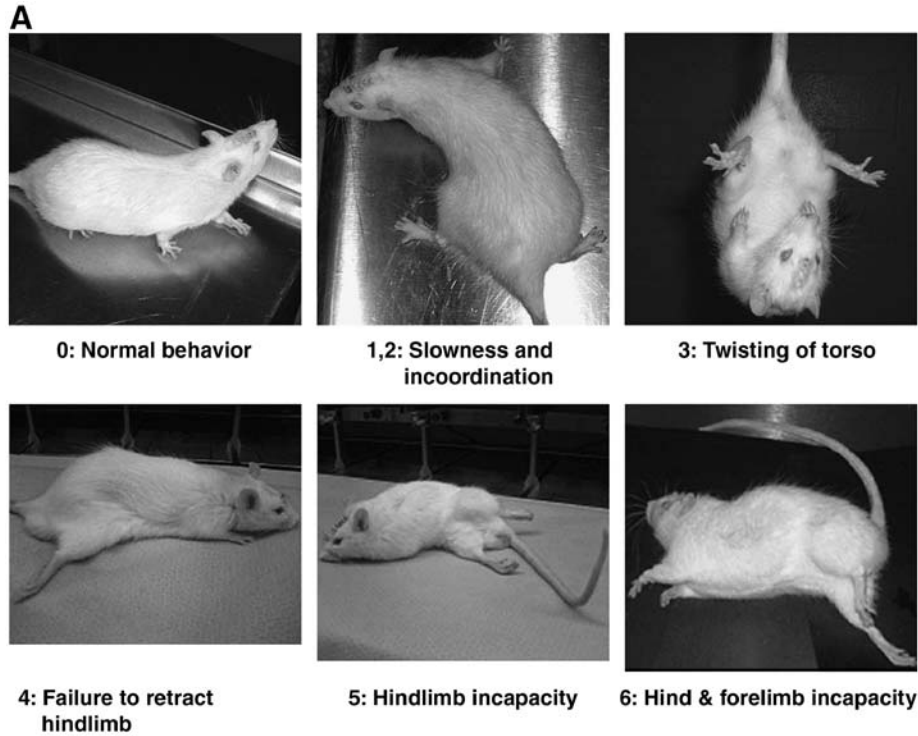
Previous studies have shown that systemic 3NP administration to Lewis rats results in reproducible motor deficits such as incoordination, brief hyperactivity followed by hypoactivity, dystonia and gait abnormalities (Blum et al., 2001). In our study, reduction in striatal cell death from AAV2-NTN treatment was associated with a reversal of behavioral deficits. AAV2-NTN-treated animals recovered quickly from their deficits on the rotarod starting at week 10 (Fig. 3). Control (both AAV2-eGFP and Vehicle) animals that received 3NP, on the other hand, did not return to baseline levels of performance for the duration of the study. Rotarod performance requires intact balance and coordination of both forelimbs and hindlimbs. These functions require a relatively intact striatum. Control animals receiving 3NP suffered from severe dystonia of the limbs and tail which resulted in deficits in performance on this task. AAV2-NTN improved locomotor activity on this task, consistent with the preservation of a significant number of striatal neurons destined to die following 3NP.

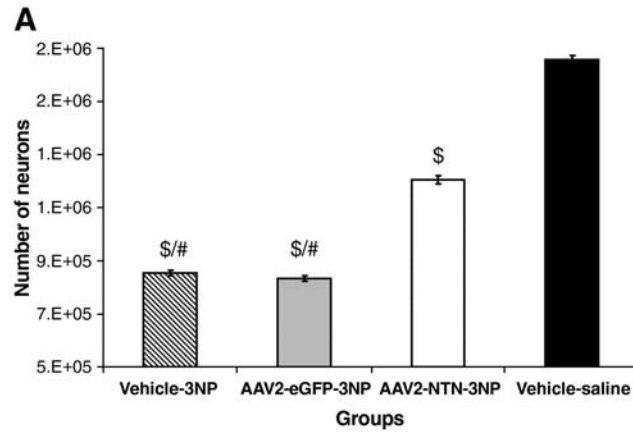
AAV2-NTN treatment also improved the performance of 3NP-treated animals on the platform test and by week 11, all rats

Fig. 5. QNRs scores. (A) 3NP caused acute deficits in ambulatory behavior as assessed by the QNRs. Animals were rated on their locomotor abilities on a scale of 0–6. The scale is defined as follows: 0—normal ambulatory behavior, 1—slowness and incoordination while ambulating, 2—marked gait abnormalities, slipping of hindlimbs while ambulating, 3—dystonic movements (twisting of torso or tail), 4—failure to retract hindlimb following manual extension (muscle weakness), 5—inability to use hindlimbs while ambulating, 6—inability to use both hindlimbs and forelimbs while ambulating. (B) Animals were tested twice a week for 14 weeks. Total scores were determined for each animal for the duration of the study and average scores were compared between groups. All saline-treated control animals showed normal locomotion and had a total score of 0. AAV2-eGFP-3NP and Vehicle-3NP groups had significantly higher scores than the saline-treated controls ($p<0.05$). AAV2-NTN-3NP animals did not perform significantly different than the saline-treated control animals.

receiving the active gene therapy treatment could remain on the platform. Similar to the rotarod, the platform test requires balance and coordination of limbs and tail. Control (both eGFP- and

Vehicle-treated) 3NP animals have severely dystonic hindlimbs, tail and torso that prevent them from maintaining their balance on the elevated platform. In contrast, substitution of the *NTN* gene





\$# = indicates significant reduction from AAV2-NTN-3NP and Vehicle-saline groups
 \$ = indicates significant reduction from Vehicle-saline group and significant increase from Vehicle-3NP and AAV2-eGFP-3NP groups

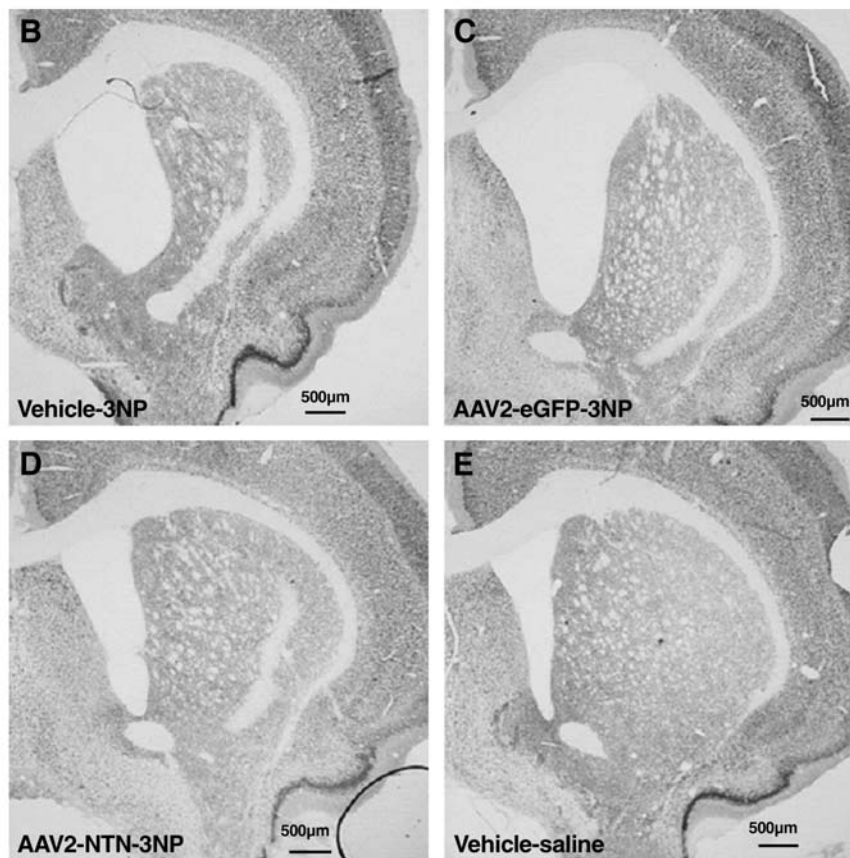


Fig. 6. Neuronal counts. (A) NeuN-positive cells were counted in the striatum to quantify 3NP induced cell death. The average number of striatal neurons was determined for each animal and compared between groups. The AAV2-eGFP-3NP group had an average of $57,505 \pm 1448$ cells (mean \pm SEM) (42.2% cell loss) in the striatum and the Vehicle-3NP group contained $56,406 \pm 1420$ cells (43.3% cell loss). The number of NeuN positive cells in these control groups was a significant reduction from the $99,513 \pm 2502$ cells in the saline-treated control group ($p < 0.001$). There was a partial neuroprotection of striatal neurons in the AAV2-NTN-3NP group where there was only a 23.7% loss of cells ($75,928 \pm 1886$ neurons). There was however a significant difference in the number of cells between the AAV2-NTN-3NP group and the Vehicle-saline-treated control animals. (B–E) Pronounced cell death was seen in the lateral portions of the striatum accompanied by enlargement of the lateral ventricles. The AAV2-eGFP-3NP and Vehicle-3NP animals had qualitatively larger ventricles than the AAV2-NTN-3NP animals.

for the eGFP gene or Vehicle, led to a significant decline in the performance of 3NP-treated rats on the platform test. The AAV2-eGFP-3NP and Vehicle-3NP groups show progressive but slower improvements on this test indicating that the peripheral effects of

3NP may have subsided and the deficits in the last few weeks may be due to lesions in the brain. More pronounced differences in this test may have been revealed if the test had been prolonged for more than 20 s. The AAV2-NTN-3NP group on the other

hand, showed a much quicker and complete recovery on this test at week 11.

Administration of 3NP caused a progressive decline in motor function that was captured using the QNRS scale. Saline-treated animals show normal ambulatory behavior and rate a 0 on this scale. In the first few days of 3NP administration animals show slowness and incoordination while ambulating (score 1). As this progresses the hindlimbs of the rats becomes displaced (slips) to the side when walking (score 2). More severe dystonic movements are seen when the rat is suspended by its tail. The rat begins to twist its torso dramatically (score 3). A score of 4 is assigned to an animal when it shows a failure to retract its hindlimb following manual extension. The rat rates a 5 when it loses its ability to use its hindlimbs while ambulating and finally a 6 when it loses its ability to use both hindlimbs and forelimbs and lays recumbent (Fig. 5A). AAV2-NTN (CERE-120)-treated rats displayed significantly lower scores (i.e., improvement) on the QNRS rating scale compared to both AAV2-eGFP and Vehicle 3NP animals. Since AAV2-NTN treatment provided striatal neuroprotection, symptoms associated with striatal degeneration were reduced. In these animals there was a reduction in dystonia, muscle weakness, hypokinesia and other gait abnormalities. Similar to the platform test, AAV2-eGFP–3NP and Vehicle–3NP groups exhibit progressive improvements on their ambulatory scores. However, this improvement does not occur as swiftly for these groups as it does for the AAV2-NTN–3NP group. The results on the platform test and the QNRS may translate into a delaying of behavioral deficits in HD patients. AAV2-NTN has a potential to significantly delay the onset of motor symptoms in HD patients when administered prior to the onset of deficits.

NTN binds to the GDNF receptor GFR α -2 which normally is not expressed in the adult striatum. However, at the levels of NTN expressed following gene delivery, NTN is promiscuous and binds to GFR α -1 receptors which are abundant in the striatum (Burazin and Gundlach, 1999). Our interest in NTN as a neurotrophic factor for HD is based upon its important role in the growth, development and trophic support of striatal neurons. In this regard, treatment of GABAergic neurons in ventral mesencephalic cultures with NTN increases their cell density and neurite outgrowth (Ducray et al., 2006). In response to intrastriatal injections of quinolinic acid (QA) in a rat model of HD, endogenous NTN mRNA levels are upregulated in the striatum, likely in an attempt to stave off inevitable excitotoxic cell death (Marco et al., 2002a). At high enough doses, exogenous administration of NTN will protect striatal neurons from cell death in both a quinolinic acid and kainic acid model of HD (Gratacos et al., 2001). In the QA model, NTN selectively protects striatal projection neurons of the indirect circuit, the striatal cell population that is the first to die in HD patients (Reiner et al., 1988; Albin et al., 1992). In the present study, bilateral AAV2-NTN (CERE-120) treatment prior to 3NP administration partially protected striatal neurons from cell death and prevented ventricular enlargement as a result of reduction in striatal volume.

In previous studies employing rat models of HD, cells have been genetically engineered to overexpress NTN (Perez-Navarro et al., 2000a; Marco et al., 2002b; Alberch et al., 2002). Studies in other neurodegenerative models have administered bolus injections of NTN directly to the striatum (Hamilton et al., 2001; Oiwa et al., 2002) or into the lateral ventricle (Rosenblad et al., 1999). These methods of NTN delivery, while effective in the short run, are likely not ideal for long-term sustained expression of the protein.

Since Huntington's disease is a progressive disorder, sustained expression of NTN will likely be required over several years. Direct infusion of NTN protein to the brain requires repeated injections of the factor to maintain high levels of expression within the striatum for a long term (Rosenblad et al., 1999) and this may be difficult to achieve clinically. Infusions using a pump may be limited due to poor spread of the trophic factor delivered from a single point source. Under these circumstances, the infused trophic factor may be suboptimally distributed and thus may provide minimum clinical benefit. Patients with HD die 15 to 20 years after the first signs appear, necessitating trophic factor expression that lasts for this period of time and beyond. Studies using adenoassociated viral (AAV) vectors have shown sustained gene expression for many years and theoretically could last the lifetime of the patient (Lebherz et al., 2005; Kaplitt et al., 1994; Xiao et al., 1996). Thus vector delivery of trophic factors may be superior to bolus or chronic NTN protein delivery. Indeed we have performed other studies that demonstrate the safety and tolerability of high doses of AAV2-NTN in rats and nonhuman primates with long-term high levels of transgene expression.

This study examined the neuroprotective potential of neurturin in a model of Huntington's disease. AAV2 vector surgeries were administered prior to 3NP administration and therefore prior to the onset of cell death and motor symptoms. Since HD is a genetic disease with an autosomal dominant inheritance pattern, familial history of the disease can be used as a guide for predicting disease risk. Genetic testing for HD has been around since the 1980s (Harper et al., 1990; Koller and Davenport, 1984) and is readily available to detect the presence or absence of the HD mutation in at risk patients (Silber et al., 1998; Creighton et al., 2003; Ramos-Arroyo et al., 2005). By early prediction of disease status in HD patients, neurturin gene therapy can potentially be administered prior to onset of symptoms so as to prevent striatal cell death early on. Since AAV vectors can sustain long-term gene expression (Lebherz et al., 2005), NTN induced neuroprotection can persist for several years without repeated surgeries. In contrast to HD, Parkinson's disease patients generally do not exhibit symptoms until they have lost over 80% of striatal dopamine. Thus HD may contain an optimal patient population to ultimately demonstrate functional neuroprotection using trophic factors delivered via gene therapy.

Our study supports the concept that NTN is neuroprotective in rodent models of HD. We demonstrate in the present study, that delivery of NTN using an AAV2 vector provides widespread gene delivery that prevents striatal neuronal cell death and the functional disability associated with striatal lesions. Further efficacy studies employing transgenic mice, coupled with ongoing safety studies in patients with Parkinson's disease (PD), will determine the feasibility of this approach for patients with HD. It is important to consider that given the lack of any meaningful therapy for this catastrophic disease, efficacy in transgenic mice and safety in primate models and patients with PD might be sufficient for the initiation of clinical trials.

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