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Response of Aged Parkinsonian Monkeys to *In Vivo* Gene Transfer of GDNF

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Abstract

This study assessed the potential for functional and anatomical recovery of the diseased aged primate nigrostriatal system, in response to trophic factor gene transfer. Aged rhesus monkeys received a single intracarotid infusion of MPTP, followed one week later by MRI-guided stereotaxic intrastriatal and intranigral injections of lentiviral vectors encoding for glial derived neurotrophic factor (lenti-GDNF) or beta-galactosidase (lenti-LacZ). Functional analysis revealed that the lenti-GDNF, but not lenti-LacZ treated monkeys displayed behavioral improvements that were associated with increased fluorodopa uptake in the striatum ipsilateral to lenti-GDNF treatment. GDNF ELISA of striatal brain samples confirmed increased GDNF expression in lenti-GDNF treated aged animals that correlated with functional improvements and preserved nigrostriatal dopaminergic markers. Our results indicate that the aged primate brain challenged by MPTP administration has the potential to respond to trophic factor delivery and that the degree of neuroprotection depends on GDNF levels.

Keywords

Macaque; MPTP; GDNF; gene transfer; Parkinson's disease; aging

INTRODUCTION

Modeling Parkinson's disease (PD) in non-human primates, albeit challenging has proven essential to understand PD, develop and test new therapies (Capitanio and Emborg, 2008; Emborg, 2007). As the cause of sporadic PD is unknown and monkeys do not present PD, risk factors, like aging and exposure to environmental neurotoxins (Granholt et al., 2008; Korell et al., 2005; Tanner et al., 1999) have been applied for model development. Aged monkeys,

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like humans, show motor impairments (Bachevalier et al., 1991; Irwin et al., 1994; Emborg et al., 1998; Zhang et al., 2000) and nigrostriatal dysfunction (Wenk et al., 1989, Goldman-Rakic and Brown 1981; Irwin et al., 1994; Collier et al., 2007; Gerhardt et al., 1995; Emborg et al., 1998; Sidiqi and Peters, 1999; De Jesus et al., 2001) resembling early PD. Yet, aged monkeys as models have limited value, as their symptoms are subtle and varied between individuals, positive response to DA replacement therapy can only be recorded with sensitive methods (Grondin et al., 2000), and neurodegeneration is slow and minimal compared to PD (Bohn and Choi-Lundberg, 1988). The most used PD monkey model is based on the neurotoxin 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP). The characteristics of the induced syndrome vary depending on the method of MPTP administration (Capitanio and Emborg, 2008; Emborg, 2007). Intracarotid artery infusion can quickly induce a replicable hemiparkinsonian syndrome that is stable over time (Emborg-Knott and Domino, 1998). Its reliability and stability are key to assess neuroprotective strategies, as the first weeks after neurotoxin challenge provide a window of opportunity to start neuroprotective strategies (Eberling et al., 1997; Emborg, 2004). But the MPTP model also has limitations (Emborg et al., 2007). Moreover, MPTP is usually administered to young adult monkeys, which limits the capacity to forecast if a therapy will benefit old PD patients. Combining the age factor to neurotoxin administration maybe a solution for more accurate prediction of clinical outcomes.

Glial derived neurotrophic factor (GDNF) is a member of the transforming growth factor- β family and a potent neurotrophic factor for dopaminergic neurons (Lin et al., 1993). GDNF can not cross the blood brain barrier and, thus, requires direct intracerebral delivery by direct protein delivery by cannulas and pumps or viral gene transfer. Evidence of GDNF neuroprotective effects by either of these methods has been collected in numerous studies using PD animal models (Ai et al., 2003; Choi-Lundberg et al., 1997; Eslamboli et al., 2003; Grondin et al., 2003; Kishima et al., 2004; Kojima et al., 1997; Kozlowski et al., 2000; Kordower et al., 2000; Mandel et al., 1999; Sautter et al., 1998; Tseng et al., 1997). Based on these data, clinical trials for direct intracerebral protein infusion were started yet their results have been controversial. Problems in the methods of delivery and dosing have been suggested (Sherer et al., 2006). Although several factors may have affected the outcome of GDNF clinical trials, a question that lingers is the reliability of the models used to test the strategy. Most PD patients are old, yet GDNF preclinical evaluations focused on one type of degeneration model at a time: age- or toxin- induced. Would the combination of both factors affect the response to GDNF?

In the present study we attempted to answer this question by testing in a combined aged-neurotoxin model the effects of lentiviral delivery of GDNF. The experimental design of this project was chosen to closely resemble the design of our previous published gene transfer study in young MPTP-treated monkeys (Kordower et al., 2000) in order to facilitate comparisons between data sets.

MATERIALS AND METHODS

Subjects

Aged male rhesus monkeys (*Macaca mulatta*; 24–30 years old, 7–14 kg.) were used in this study. This age range was selected because the majority of PD patients are over 55 yrs old and postmenopausal. An equivalent age condition for rhesus monkeys corresponds to over 24–25 yrs. (Walker and Herndon, 2008)

Animals were housed individually on a 12-hour light/dark cycle and received food and water *ad libitum*. The experiment was performed according to the federal guidelines of animal use and care and with approval of the local IACUC.

Experimental design

Fourteen aged monkeys started this study. All the animals were trained to perform a fine motor skills task and evaluated with a clinical rating scale, as well as general health measures (CBC, blood chemistry, weights). After baseline magnetic resonance imaging (MRI) and [^{18}F] fluorodopa (FD) positron emission tomography (PET) scan data were obtained, all animals received a single intracarotid infusion of MPTP under sterile surgical conditions. MPTP dosages were calculated according to weight and corresponded to two-thirds of the amount normally administered to young adult rhesus monkeys, to safely induce an equivalent PD syndrome. One week later animals were evaluated with a clinical rating scale. The animals with a score of more than 9 points were matched into two groups that were then randomly assigned a treatment. The selected animals received MRI-guided stereotaxic injections of lentiviral vectors encoding for GDNF (n=5) or lacZ (n=4) into the striatum and the substantia nigra. Five of the 14 original animals were excluded from this study due to: clinical score lower than nine points after MPTP (one animal), death after MPTP administration (one animal), death after lentiviral vector surgery (one lenti-LacZ treated animal), and stroke-like lesions in the basal ganglia (one lenti-LacZ treated animal and one lenti-GDNF treated animal). An extensive analysis of these lesions has been published (Emborg et al., 2006). Behavioral evaluations continued until the end of the study. Three months post lentiviral surgeries the animals received a second FD PET scan. Forty-eight hours later the animals were euthanized by transcardiac perfusion of saline, their brains quickly retrieved and prepared for chemical and anatomical analysis. Full body necropsies were also performed. Investigators blind to the treatment groups performed all data collection and analysis.

Behavioral evaluations

Throughout the study animals were evaluated by a trained observer blind to the treatment groups using a previously validated clinical rating (CR) scale (Kurlan et al., 1991; Emborg et al., 1998, 2006). Observation began two weeks before any experimental intervention to establish a baseline rating score. The score was obtained as the sum of the features, out of a total of 32 points, with 0 corresponding to normal scoring and 32 to extreme severe disability. Occurrence of dyskinesias, psychological disturbances, and vomiting was also recorded.

Upper limb fine motor ability was measured using a monkey movement analysis panel (mMAP) in a food retrieval task (Gash et al., 1999). Animals were transferred into a testing cage in a quiet room separate from their home cage and without distraction of other animals. The mMAP was placed on the testing cage and animals were trained to retrieve a small piece of fruit by maneuvering their arm through the panel. Only one limb at a time could be used, as the researcher could limit access to the mMAP by closing access ports. The testing was performed for each animal three times weekly, and each test consisted of twelve total trials alternating between arms, six per side. Delaying feeding time until after the task was completed ensured animals' compliance with the test. Data collected included the time taken for the animal to move its hand into the chamber where the fruit was located (reaction time), the time taken to pick up the fruit while the hand was in the chamber (reception time) and the total time taken to move the hand into the chamber, retrieve the fruit and bring the hand back out of the panel and into the cage (total time). The animals were trained to a consistent level of performance before any surgical procedures took place.

In vivo imaging

All animals were scanned with a GE Signa 3T Magnetic Resonance Imaging (MRI) scanner prior to surgical intervention for baseline purposes and to obtain stereotaxic target coordinates as previously described (Emborg et al., 2006; Kordower et al., 1999, 2000).

All animals received a baseline [^{18}F] Fluorodopa Positron Emission Tomography (FD PET) scan one month before MPTP treatment, and another scan 3 months post lentiviral injection as described elsewhere (Kordower et al., 2000, Emborg et al., 2006). The image data were co-registered with T1-weighted morphological MR data from each subject using locally developed methods. Regions corresponding to left and right caudate nucleus and putamen, and bilateral occipital cortex, were identified and used to calculate striatal/cortical uptake ratios based on the average image values during the last 30 min of the study (60 to 90 min post- injection).

MPTP administration

All monkeys received a single intracarotid artery infusion of 2–3 mg of MPTP-HCl (depending on their weight) in 20 ml of saline at a rate of 1.33 ml/min under sterile surgical conditions as previously described (Emborg-Knott and Domino, 1998; Emborg et al., 2006). This dose represents two-thirds of the dose needed to create a stable PD model in young adult macaque monkeys.

Preparation of Lentiviral Vectors

The cDNA coding for a nuclear-localized β -galactosidase (lacZ) and the human GDNF containing a Kozak consensus sequence (a 636-bp fragment: position 1 to 151 and 1 to 485; GenBank accession numbers L19062 and L19063) were cloned in the SIN-W-PGK transfer vector (Zufferey et al., 1999). The packaging construct and vesicular stomatitis virus G protein (VSV-G) envelope used in this study were the PCMVDR8.92, PRSV-Rev, and the PMD.6 plasmids described previously (Zufferey et al., 1997, Hottinger et al., 2000). The viral particles were produced in 293T cells as previously described (Kordower et al., 1999). The titer (3 to 5×10^8 TU/ml) of the concentrated lacZ-expressing viruses (440,000 ng p24/ml) were determined on 293T cells. The GDNF-expressing viral stocks were normalized for viral particle content using p24 antigen measurement (150,000 ng p24/ml).

Intracerebral viral vector injection

One week after the MPTP procedure, the animals received MRI-guided intracerebral injections of lentiviral vectors under sterile surgical conditions as previously described (Kordower et al., 2000; Emborg et al., 2006). Six needle tracks ipsilateral to MPTP infusion hemisphere were done into: the head of the caudate nucleus (10 μ l), body of caudate nucleus (5 μ l), anterior putamen (10 μ l), commissural putamen (10 μ l), postcommissural putamen (5 μ l), and substantia nigra (5 μ l). The needle was left in place for 3 minutes after each injection to allow complete diffusion of lentivirus from the needle before withdrawal.

Necropsy, preparation of tissue

Three months post-lentivirus infusions the monkeys were anesthetized with pentobarbital (25 mg/kg, iv.) and perfused transcardially with warm (100 ml) followed by ice-cold (400 ml) normal saline. The brain was rapidly removed from the calvaria and immersed in ice-cold saline for 10 minutes. The brain was then slabbed (4 mm thickness) in the coronal plane using a calibrated Lucite brain slice apparatus. Slabs through the striatum were placed on a frozen Petri dish and punches of the caudate nucleus, putamen, nucleus accumbens, frontal cortex, and cerebellum were taken bilaterally, placed in centrifuge tubes containing antioxidant, and frozen for ELISA analysis of GDNF levels. The slabs were then immersed in a 4% paraformaldehyde fixative for 48 hours of fixation and cryoprotected by immersion in a graded (10–40%) sucrose/0.1 M phosphate buffered saline (PBS, pH 7.2) solution. The tissue slabs were cut frozen (40 μ m thickness) on a sliding knife microtome. All the sections were stored in a cryoprotectant solution before processing. Full body pathology was also performed.

GDNF ELISA

An ELISA measured GDNF concentration in brain samples. Total protein was isolated by homogenization in 300 μ l lysate buffer (0.1% Tween-20/0.5% BSA/2mM EDTA in PBS containing protease inhibitors). Total protein concentration was determined by the Lowry assay, and GDNF concentration was determined by GDNF ELISA (R&D Systems). GDNF ELISA analysis was performed as suggested by the manufacturer and all procedures were performed at room temperature. Briefly, a 96 well plate was coated with the human GDNF capture antibody, washed, and then blocked to reduce non-specific binding. Isolated protein lysate samples from various regions of the monkey brain were then added along with a known concentration of GDNF protein to produce a standard curve (diluted to a range of 2 ng – 0 ng). After 2 hrs of incubation, the plate was washed and incubated with the GDNF detection antibody, washed again, incubated with HRP, and then a substrate solution. Finally, the reaction was stopped using 2N H₂SO₄ and measured on a spectrophotometer at 405nm. The data were expressed as total GDNF (ng)/total protein (mg).

Immunohistochemistry

Coronal brain sections were used for immunohistochemical staining according to our previously published protocols (e.g.: Emborg et al., 2006; Kordower et al., 2000; Stephenson et al., 2005). Antibodies used include TH (1:20,000; Chemicon Inc., CA), GDNF (1:250; R&D Systems, Minneapolis, MN), lacZ (1:3,000; Novus Biologicals, Littleton, CO), GFAP (1:2,000; DakoCytomation, Glostrup, Denmark) and CD68 (1:3,000; DakoCytomation, Glostrup, Denmark).

Quantification of nigrostriatal TH immunoreactivity

The density of TH positive fibers was quantified within ventral, medial and dorsal sections of both the caudate and putamen using NIH ImageJ software. Images of six coronal sections per monkey approximately 2 mm apart were captured using an Epson 1640XL-GA high-resolution digital scanner. ImageJ was calibrated using a step tablet, grey scale values were converted to optical density (OD) units using the Rodbard function, and the mean OD for each area of interest was recorded.

The number of TH-ir neurons in the SN was estimated using unbiased stereologic cell-counting methods previously described (Emborg et al., 1998, 2006).

RESULTS

Behavioral evaluations

Clinical evaluation of the aged animals prior to MPTP administration showed mild slowness of movement, gait disturbances, and slight tremors, which varied between individuals. The monkeys scored between one and five on the CR scale (mean $\pm$ S.E.; lenti-GDNF, 2.6 $\pm$ 0.89; lenti-LacZ, 2.75 $\pm$ 0.48; Fig. 1), typical scores of naïve, aged rhesus monkeys. After intracarotid infusion of MPTP and before lentiviral injections, animals in both groups developed a distinctive hemiparkinsonian syndrome in the side contralateral to MPTP administration, which consisted of tremors, slowness and decreased amount of movement, as well as balance and gait impairments. Animals with a CR score over 9 points were selected for this study and randomly assigned to one of the two treatment groups (lenti-GDNF, 11.16 $\pm$ 1.4; lenti-LacZ, 12.0 $\pm$ 1.5). It is our experience that this degree of disability ensures a stable parkinsonian lesion (Emborg-Knott and Domino, 1998). After lentiviral treatment the lacZ group did not show changes in their hemiparkinsonian syndrome, continuing to score over 10 points in the CR scale. The lenti-GDNF group began to show a steady improvement in CR score during the second month after gene therapy treatment that continue until necropsy. The group differences

achieved significance at the post-treatment in the 3rd month post lentiviral surgery (Mann-Whitney U test $p < .004$; Fig. 1). Analysis of specific features revealed significant improvements in bradykinesia ($p < .005$), gross motor skills ($p < 0.044$) and gait ($p < .018$).

Evaluation of the fine motor skills of the aged monkeys prior to MPTP administration showed a symmetrical performance in the mMAP task for the right and left hand (mean $\pm$ S.E.; lenti-LacZ, 0.86 ± 0.11 ; lenti-GDNF, 0.96 ± 0.15). After MPTP and lenti-LacZ surgeries these animals showed difficulties to complete the mMAP task with the hand contralateral to the treatment (3 of the 4 lacZ animals). The lenti-GDNF group as a whole performed the task with the left hand (contralateral to surgical procedures) more quickly than the lenti-LacZ group. Three of the five lenti-GDNF aged animals returned to their baseline left hand times by the end of the study, while one of the remaining two was slower, and one was unable to complete the task. However, statistical significance was not achieved at any time point on the mMAP test with the left hand (last FMS data for left hand: lenti-LacZ, 23.05 ± 8.09 ; lenti-GDNF, 7.66 ± 6.35). The right hand did not show any significant group differences throughout the study (last FMS data for right hand: lenti-LacZ, 1.0 ± 0.24 ; lenti-GDNF, 1.27 ± 0.36).

PET FD Uptake

Baseline FD uptake levels showed symmetrical function in the striatum of all the aged monkeys. Three months post MPTP and lentiviral treatment the FD uptake rate constant, K_{occ} , revealed that the lenti-LacZ group lost more dopaminergic storage capacity than the lenti-GDNF group. The lenti-LacZ group decreased K_{occ} 81.0 ± 0.1 % in the caudate, and 77.5 ± 0.1 % in the putamen, representing a significant loss (ANOVA $p < 0.05$). In comparison, the lenti-GDNF group displayed K_{occ} values statistically similar to normals, although there was some absolute loss in FD uptake capacity in the caudate (28.8 ± 0.3 %) and putamen (31.6 ± 0.3 %; Fig. 2). Comparison between groups showed that the mean FD uptake of GDNF monkeys was 300% higher than that in the LacZ animals, yet the group difference did not reach statistical significance due to individual differences and the small sample size. While 3 GDNF monkeys had symmetrical FD uptake (equivalent to baseline levels), 2 animals had similar levels to controls.

Lentiviral gene expression and host immune reaction

GDNF immunostaining showed unilateral expression of GDNF in the caudate, putamen and SN of the lenti-GDNF treated monkeys. The intense pattern of GDNF expression followed the target areas of transfection and expanded beyond the needle tracks as a halo in the surrounding areas, including globus pallidus, corpus callosum, nucleus accumbens, subthalamic nucleus and red nucleus. Lenti-LacZ aged monkeys did not show expression of GDNF detectable with immunohistochemistry. As previously described (Kordower et al., 1999, 2000; Emborg et al., 2006) these animals exhibited lacZ immunoreactive cells in the targeted areas of the striatum (not shown) and SN (Fig. 3A, C). GDNF ELISA confirmed GDNF expression in the target areas of the lenti-GDNF group (Fig. 3B, D). GDNF immunoreactivity was generally undetectable in either hemisphere of the lenti-LacZ group. Areas of highest concentration of GDNF in the lenti-GDNF group were the right ventral putamen (6.37 ± 6.03 ng/mg) right medial putamen (2.96 ± 1.45 ng/mg) and the right ventral caudate (1.78 ± 1.42 ng/mg). Comparatively, the highest concentration of GDNF in the lenti-LacZ aged animals was in the left nucleus accumbens (0.008 ± 0.008 ng/mg). Interestingly, there was a slight elevation in the GDNF level of the left nucleus accumbens in the lenti-GDNF treated animals as well (0.132 ± 0.053 ng/mg).

Evaluation of immune markers at the injection sites revealed mild GFAP positive-astrogliosis as well as the presence of CD68 positive microglia/macrophages. CD68 was also found in the white-matter areas adjacent to the needle tracts, in particular the corpus callosum of both treatment groups (Supplemental material – Figure 1). As expected, GFAP-ir and CD68-ir cells

were found dispersed in the SN ipsilateral to MPTP administration of all monkeys (McGeer et al., 2003; O'Callaghan et al., 1990). One animal (R06462) in the GDNF group showed isolated strong CD68 response in both lateral geniculate nuclei and bilaterally throughout the optic tract (Figure 6).

Quantification of nigrostriatal TH immunoreactivity

Neuropathological observation of TH immunostaining in the striatum revealed unilateral loss of TH positive fibers in the caudate and putamen ipsilateral to MPTP administration of the lenti-LacZ treated animals (Fig. 4A). The lenti-GDNF monkeys showed differing degrees of TH expression in the striatum, but in general exceeded the levels seen in lenti-LacZ animals (Fig. 4B). Quantification of TH positive immunostaining confirmed significant more TH expression in the putamen ipsilateral to MPTP administration of lenti-GDNF treated aged animals compared to lenti-LacZ (67% preservation compared to contralateral side; ANOVA $p < .0001$, post Hoc Scheffe $p < .045$; Fig. 5A) and a trend for the ipsilateral caudate nucleus (84% preservation compared to contralateral side but with great variability inter-individuals; ANOVA $p < .002$, post Hoc Scheffe $p < .068$; Fig. 5A).

Mesencephalic TH immunostaining revealed unilateral loss of neurons in the SNpc ipsilateral to the MPTP administration with preservation of cells in the ventral tegmental area of all lenti-LacZ treated monkeys (Fig. 4C, E). In comparison, lenti-GDNF monkeys showed different degrees of TH-positive cell preservation as well as increases in the neuropile and sprouting of fibers from the SNpc (Fig. 4D, F). Unbiased stereological counting of SNpc TH positive neurons showed that lenti-LacZ had a significant loss of cells in the side ipsilateral to MPTP administration, compared to the contralateral side of the same animals (80.57%; ANOVA $p < .0001$, post Hoc Scheffe $p < .0001$). Lenti-GDNF animals showed preservation in the number of TH positive neurons. Although comparison of the right and left SN in the same animals showed a significant loss (34%; ANOVA $p < .0001$, post Hoc Scheffe $p < .02$; Fig. 5B), comparison between treatment groups revealed a significantly higher number of TH positive cells in the GDNF treated group in the treated side compared to lenti-LacZ (ANOVA $p < .0001$, post Hoc Scheffe $p < .003$; Fig. 5B).

DISCUSSION

The present study demonstrated that the aged non human primate brain with ongoing toxin-triggered neurodegeneration of the nigrostriatal system has the capacity to respond to GDNF gene transfer. The experimental design of this project has high translational value for the assessment of a neuroprotective strategy. As the majority of PD patients are over 55 yrs. old, the approach of using aged rhesus monkeys provides a more accurate representation of the disease condition in humans. As previously described, the aged rhesus monkeys used in this study presented a mild spontaneous decline in their motor performance and preserved nigral cells in the side contralateral to MPTP administration indicating that all of them had mild, age-related nigrostriatal dysfunction. The unilateral administration of MPTP induced an accelerated unilateral PD syndrome. One week after neurotoxin and when the PD syndrome was evident, we administered gene therapy. Our goal was to replicate in the model the patients' ongoing neurodegeneration (Bezard et al., 2006). We have previously validated this paradigm in young adult rhesus monkeys (4–5 yrs old; Kordower et al., 2000), showing that lenti-GDNF treatment was neuroprotective. In the current study we purposefully replicated the experimental design using aged animals to facilitate comparisons between results.

We confirmed gene expression of GDNF 3 months post-surgery by ELISA and immunohistochemical methods. Moreover, GDNF levels correlated with behavioral, imaging and anatomical measures of nigrostriatal DA neuroprotection (see Supplemental material - Figure 2). This type of analysis is not usually reported, as in most experiments biochemical

assays are performed in different animals than the ones used to assess behavioral and morphological changes. Our data showed variations in GDNF intracerebral amounts between subjects. This could be due to individual differences in transfection efficiency and/or gene expression. Gene transfer variability is known to occur and has been documented for adeno-associated viral vectors (Burger et al., 2004). Further investigations to create predictable transfection methods is needed to ensure successful clinical application of gene therapies.

The area of highest concentration of GDNF was in the right ventral and medial putamen, with slightly lower levels in the dorsal putamen. Correlation analysis showed that expression in the medial putamen was most closely related to striatal FD uptake and putaminal TH OD, which also correlated with behavioral measures (see Supplemental material – Figure 2). These findings suggest that GDNF expression in the putamen protected dopaminergic terminals that critically affect motor function. It should be noted that in the early stages of human PD, DA storage capacity is first reduced in the dorsal putamen (Morrish et al., 1995; Kish et al., 1988). Our analysis also indicates that the aged animals with GDNF putaminal levels over 2 ng/mg presented the best nigrostriatal preservation, which correlated with better behavioral performance. Although striatal levels of GDNF in the young MPTP treatment paradigm were not obtained, we have reported the data from 2 intact monkeys that received identical vector injections and were necropsied 8 months later (Kordower et al., 2000). Striatal GDNF levels in those animals ranged between 2.5–3.5 ng/mg protein in the striatum and supports the concept that these levels are appropriate to observe efficacy of the treatment in rhesus monkeys that receive GDNF after the MPTP lesion. This differs from findings in common marmosets monkeys lesioned with 6-OHDA that responded to as little as 0.04 ng/mg tissue of GDNF delivered by adeno associated viral vectors (Eslamboli et al., 2004) that were transfected 8 weeks before lesioning. This is also different from the results in MPTP-treated rhesus monkeys receiving between chronic 7.5 to 22.5 µg/ml/day of GDNF protein infusions 2 months post MPTP intoxication (Grondin et al., 2002). Comparison between reports suggests that the variation on efficacious dosing seem to be dependant on the specie, method of delivery and lesion paradigm.

In this and our previous young rhesus study (Kordower et al., 2000) the animals showed a positive behavioral response to lenti-GDNF, although with some differences between age groups. Compared to the young monkeys, the old rhesus presented spontaneous PD-like signs and their baseline CR scores were higher (old, 2.7; young, 0). After intracarotid MPTP the aged animals were slightly more affected (12) than the young ones (10.6). Interestingly, the last average score after lenti-GDNF treatment was very similar (young, 6.5; old, 7.5) and the percentage of improvement in the CR score was equivalent for both age groups (approximately 40%). In both studies improvements started approximately 6 weeks post lentiviral transfection and the difference between treatment groups reached statistical significance at the beginning of the 3rd month post treatment and persisted until necropsy.

Some of the old monkeys that we were able to obtain for this study had missing fingers, which impaired their ability to scoop treats from wells to test their FMS (pick up test, Kordower et al., 2000). As an alternative, we used the level 1 of the mMAP test, in which the animals took food rewards from a flat surface (Zhang et al., 2000). Although the FMS tests were different between young and old animal studies, some observations can be made. In the young MPTP paradigm, the lenti-GDNF monkeys performed significantly better than the control animals, while in the aged monkey study, although the lenti-GDNF animals performed better (3 of 5 monkeys returned to baseline levels), as a group they did not reach statistical significance. It should be noted that in both studies some lenti-GDNF animals presented serious difficulties in the FMS task (young, 1 of 4; old, 2 of 5). Interestingly, the two aged animals showed the lower levels of putaminal GDNF and lower TH OD and TH nigral cells counts, suggesting a relationship between GDNF levels, functional and anatomical improvements. One of the 4

aged lenti-LacZ improved its performance in the FMS task but none of the 5 young lenti-LacZ. This combination of factors (and the limited number of animals used in the study) may have impeded to obtain statistically significant differences between treatment groups for the FMS task in the old monkey study. It is interesting that one of the lenti-GDNF aged monkey (R06266) that was unable to complete the test with the left hand, also showed difficulties on the ipsilateral (right hand) side, which was not observed in the young monkeys, or other aged animals. R06266 was much slower after both surgeries (from 0.21s pre-surgery to 3.51s post-surgery) and progressively improved but did not return back to baseline (0.89s in final week of testing). The finding that R06266's TH nigral cell count and striatal TH on the "intact" side was average suggests that the behavioral impairments were maybe a functional consequence of multiple surgical interventions or, that nigrostriatal plasticity was taking place but not yet fully developed.

Preservation of the nigrostriatal DA system was measured *in vivo* and *post mortem* in the young and aged monkey studies. All LacZ animals, regardless of age, presented unilateral loss of DA markers ipsilateral to the side of MPTP administration, while GDNF treatment improved DA markers, but in different degrees. The average striatal FD uptake in young lenti-GDNF animals was >300% higher than in young controls, while aged animals was approximately up to 300% higher in the putamen. Statistical significant differences between groups were only observed in the putamenal measures of the young animals. In both studies, some lenti-GDNF animals (young, 2 of 4; old, 3 of 5) had symmetrical (no differences between MPTP and intact side) FD uptake. Interestingly, while the young GDNF animals that showed a unilateral loss had more FD uptake than the control animals, the GDNF aged animals' loss was equivalent to controls. Postmortem analysis of DA markers revealed that the young monkeys had 70% preservation of fibers in the caudate nucleus and 75% in the putamen, both reaching statistically significance when compared to LacZ animals. The aged monkeys as a group had 84% preservation of fibers in the caudate but due to individual variability was non significant, yet the putamen had a significant 67% preservation of TH fibers. Stereological TH nigral cell counts in young or aged rhesus revealed statistically significant more neurons in lenti-GDNF monkeys compared to LacZ animals. Most importantly, while the young animals presented 32% more neurons than controls, the aged monkeys had 34% less neurons, suggesting that GDNF in the young MPTP monkeys compared to the aged ones was able to protect more DA neurons, induce neurogenesis and/or upregulate TH production in "dormant" cells. Collectively, these data support the concept that lenti-GDNF treatment to young and aged monkeys can protect against MPTP functional and anatomical consequences. The magnitude of the effect seems to depend on GDNF levels and the age of the subjects.

The presence of GFAP and CD68 immunoreactive cells across the different injection sites of both lenti-LacZ and lenti-GDNF treated monkeys suggests that intracerebral injections of lentivirus, regardless of treatment, may induce a persistent reaction in the host brain, similar to our previous observations with lenti- or adeno-associated viral vectors (Kordower et al., 2000; Emborg et al., 2007). Although quantifications of GFAP-ir or CD68-ir were not performed in the young monkey study, qualitative observations suggest that they presented less astro- and microglial reaction to the lentiviral treatment than their aged counterparts. Old age is associated with increase spontaneous glial activation (Conde and Treit, 2006; Sheffield and Berman, 1998) which may affect the long term effect of gene transfer treatments.

One lenti-GDNF monkey (R06462) of the original 14 aged animals in the study, showed numerous CD68-ir cells bilaterally throughout the optic tracts and lateral geniculate nuclei. A possible explanation is that the myelin surrounding the optic tract degenerated with age and attracted activated macrophages or microglia, which in turn began to phagocytize the detritus, following the axonal path into the lateral geniculate nucleus, as described in aged monkeys by Sandell and colleagues (2001).

A critical issue for the performance of this experiment was the overall morbidity (Emborg et al., 2006). Aged monkeys require more intense postoperative care compared to young animals. As aged animals are more sensitive to MPTP (Ovadia et al., 1995), we administered 2/3 of the original dose, in order to induce in the aged animals a PD syndrome similar to the one induced in young rhesus. It could be argued that the dose was still high. Yet, it should be recalled that one animal was excluded due to the failure to develop the pre-defined PD state after an equivalent dose and that one animal of the lenti-LacZ group spontaneously recovered in the fine motor task. With regards to the death after lentiviral surgery, it should be mentioned that in our young monkey study two rhesus died after lentiviral surgery (one lenti-LacZ and one lenti-GDNF). In both studies, the deaths after intracerebral injections could be related to the stress of receiving two consecutive surgeries. We should reiterate that the animals were carefully evaluated throughout the study by veterinarian staff and that the surgical procedures occurred separated by one week. Overall, our results suggests that with older age there is an increase in individual differences in the response to MPTP and that experiments and budgets need to be powered accordingly. These findings also reflect that old rhesus, like humans (e.g.: Turrentine et al., 2006) may have higher surgical and post surgical morbidity than younger counterparts.

In conclusion, this study shows that aged parkinsonian non human primates can respond to gene transfer delivery of GDNF. Our results also suggest that trophic factor levels and age affect the magnitude of the response and that the surgical invasive procedure needed to deliver the therapy presents risks to old monkeys. These data strongly support the use of aged rhesus for preclinical analysis but also cautions about its difficulties. Availability and costs limit the use of old monkeys in preclinical research, yet the information that can be gathered from them can have high clinical impact and may affect the way that new clinical trials are designed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Clinical rating score of aged rhesus monkeys treated with a unilateral injection of MPTP into the carotid artery, followed one week later by MRI-guided stereotaxic intracerebral injections of lentiviral vectors encoding for GDNF (lenti-GDNF) or β -galactosidase (lenti-LacZ). A significant improvement in the lenti-GDNF treated monkeys at the beginning of the third month post lentiviral surgery (*Mann-Whitney U test $p < .004$).

Figure 2.

A-Coronal images of FD PET in the striatum of aged rhesus monkeys treated unilaterally with MPTP and lentiviral vectors encoding for GDNF or LacZ. Note the preservation of FD uptake in the lenti-GDNF treated animal (lenti-GDNF image corresponds to the animal with highest FD uptake). B-Quantification of FD uptake in the caudate and putamen nucleus revealed significant loss of uptake in the side ipsilateral to MPTP administration for the Lac-Z group but not for the GDNF animals (*ANOVA, $p < .05$, post Hoc Scheffe $p < .05$).

Figure 3.

Low (A, B) and high (C, D) magnification microphotographs of Lac-Z (A, C) and GDNF (B, D) immunostained coronal brain sections at the level of the substantia nigra. Arrowhead in A and D indicates the presence of neuromelanin. Lac-Z expression was observed filling up numerous neuronal- and astrocyte-like cells (C). Strong GDNF expression was observed in the target areas of the lenti-GDNF monkeys, filling up cell bodies and covering the surrounding neuropile. Scale bar: A, B=2.0mm; C, D=100µm

Figure 4. Microphotographies of TH immunostained coronal sections at the level of the striatum (low magnification: A, B.) and SN (low magnification: C, D; high magnification: E, F) of lenti-LacZ (A, C, E) and lenti-GDNF (B, D, F) treated monkeys. Note the symmetrical TH staining in the striatum of lenti-GDNF treated monkey, lenti-LacZ treated animal (A, B). Preservation of fibers was associated with preservation of TH positive neurons in the SN (D, F). Scale bar: A, B=7.5mm; C,D=1.0mm; E, F=100 μ m

Figure 5.

A-Optical density of putaminal TH immunohistochemistry. Preservation of dopaminergic fibers was observed as increased TH optical density (*ANOVA $p < .0001$, post Hoc Scheffe $p < .0001$). B-Stereological cell counts of TH positive nigral neurons revealed statistically significant higher numbers for lenti-GDNF treated monkeys compared to lenti-LacZ (*ANOVA $p < .0001$, post Hoc Scheffe $p < .003$).

Figure 6.

A-Low magnification microphotography of a CD68 immunostained coronal section at the level of the lateral geniculate nucleus (LGN) of lenti-GDNF monkey R06462. Note the bilateral CD68 immunoreactivity in the LGN. B corresponds to higher magnification of image of black square area in A; C corresponds to higher magnification of image of black square area in B. Scale bar: A=4mm; B=1.5mm; C=150 μ m