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# Intranasal Delivery of pGDNF DNA Nanoparticles Provides Neuroprotection in the Rat 6-Hydroxydopamine Model of Parkinson's Disease

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**Abstract**
Glial cell line-derived neurotrophic factor (GDNF) gene therapy could offer a disease-modifying treatment for Parkinson's disease (PD). Here, we report that plasmid DNA nanoparticles (NPs) encoding human GDNF administered intranasally to rats induce transgene expression in the brain and protect dopamine neurons in a model of PD. To first test whether intranasal administration could transfect cells in the brain, rats were sacrificed 1 week after intranasal pGDNF NPs or the naked plasmid. GDNF ELISA revealed significant increases in GDNF expression throughout the brain for both treatments. To assess whether expression was sufficient to protect dopamine neurons, naked pGDNF and pGDNF DNA NPs were given intranasally 1 week before a unilateral 6-hydroxydopamine lesion in a rat model of PD. Three to four weeks after the lesion, amphetamine-induced rotational behavior was reduced, and dopaminergic fiber density and cell counts in the lesioned substantia nigra and nerve terminal density in the lesioned striatum were significantly preserved in rats given intranasal pGDNF. The NPs afforded a greater level of neuroprotection than the naked plasmid. These results provide proof-of-principle that intranasal administration of pGDNF DNA NPs can offer a non-invasive, non-viral gene therapy approach for early-stage PD.

**Keywords** Intranasal · Parkinson's disease · GDNF · Nanoparticles · Neuroprotection

## Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder of the brain caused by the death of substantia nigra (SN) dopaminergic neurons innervating the corpus striatum. Glial cell line-derived neurotrophic factor (GDNF) has been shown to be a potent neurotrophic factor for these neurons, able to promote their survival and proliferation both in vitro and in vivo [1–4]. The neuroprotective and restorative effects of GDNF in various animal models of PD prompted a series of human clinical trials [5–10] using intracerebral injections or, more recently, convection-enhanced delivery (CED) of GDNF protein or viral vectors encoding GDNF family members [11, 12]. Several of these trials are on-going [13, 14]. However, even if intracerebral infusions of GDNF therapeutics do provide clinical efficacy for PD, the approach requires an invasive surgical procedure that will not be feasible for patients who are unwilling or unable to undergo these procedures. A much larger percentage of early- to mid-stage PD patients could benefit from a GDNF gene therapy given by a non-surgical route.

Here, we investigate a neurotrophic gene therapy for PD using a non-invasive route of administration, intranasal delivery, combined with a novel, non-viral gene vector encoding GDNF. Intranasal administration circumvents the blood–brain barrier (BBB), providing a means of targeting large molecular weight substances to the brain while limiting blood absorption and peripheral exposure to the agent. Macromolecules,

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including proteins, trophic factors, siRNAs, viral vectors, and compacted DNA nanoparticles, have all been shown to pass through spaces in the olfactory epithelium directly into the brain as no BBB exists at this interface [15–25]. Intranasally administered macromolecules have been postulated to move through spaces between olfactory ensheathing cells and olfactory neurons projecting into the nasal cavity [26], ultimately crossing the cribriform plate to reach the cerebrospinal fluid (CSF), which mixes with brain interstitial fluid [27]. Once in the CSF, they are transported through perivascular channels to distant sites in the brain in < 1 h [15, 16, 24, 28]. Transport preferentially follows the olfactory nerve, which projects from the upper nasal cavity to the olfactory bulb and rostral brain, and the trigeminal nerve, which projects from the lower nasal cavity to the midbrain, brainstem, and spinal cord [15–17].

In previous studies, we showed that intranasal administration of a 50 μg dose of GDNF protein to rats 1 h before a unilateral 6-hydroxydopmaine (6-OHDA) lesion provides significant protection of SN dopamine neurons, confirming efficacy in a standard rodent model of PD. Multiple 50 μg doses (daily for 3 days) were even more effective [29]. However, only a tiny fraction of an intranasal dose of protein reaches the brain [16, 18, 30], and concentrations may be too low or too transient to produce clinical efficacy without frequent re-dosing. We reasoned that an intranasal GDNF gene therapy could provide a renewable, scalable source of GDNF within the brain.

To that end, we chose to investigate intranasal delivery of DNA nanoparticles (NPs) compacted with 10-kDa polyethylene-glycol (PEG)-substituted lysine 30-mers (CK30PEG10k). These DNA NPs are comprised of a single molecule of an expression plasmid compacted with CK30PEG10k having an acetate lysine counterion at the time of initial mixing [31]. The NPs have an average diameter of 8–11 nm and exhibit a rod-like shape [32–34]. This DNA NP vector has been shown to be non-immunogenic, non-inflammatory, and small enough to allow entry into the cell's nucleus [32, 34]. These NPs have been shown to successfully deliver expression plasmids and to transfect post-mitotic cells in the brain, lung, and retina for greater than one year [31–38]. We previously reported that intranasal delivery of plasmid DNA NPs encoding enhanced green fluorescent protein (eGFP) under the cytomegalovirus (CMV) promoter yields transfection and expression of eGFP in the rat brain two days after intranasal administration, and the transfected cells are primarily located perivascularly [21]. A clinical gene therapy approach would likely require promoters having a less transient expression pattern than the CMV promoter.

Here, we used a hGDNF transgene expression plasmid, pGDNF_1b [38], transcriptionally controlled by the long-lasting polyubiquitin C (UbC) promoter. Among a series of human GDNF splice variants, the hGDNF_1b variant yielded the highest GDNF protein expression in rat ventral midbrain cultures and produced the highest GDNF expression in rat striatum one week after direct intrastriatal injection [38]. Peak expression occurred 7 to 14 days after brain injection (8- to 16-fold above baseline) and remained 300–400% above baseline for at least 6 months. pGDNF_1b was therefore selected for use in the current studies.

The goals of these studies were to determine if intranasal administration of pGDNF_1b to rats could successfully transfect cells and lead to significant increases in GDNF expression in the brain and to assess whether that level of GDNF expression would be sufficient to provide neuroprotective efficacy in a rodent model of PD.

## Materials and Methods

### GDNF Plasmid Construction

The plasmid used in these studies was derived from the hGDNF plasmid splice variant 1, previously reported by [38].

### Formulation of Compacted DNA Nanoparticles

A total of 20 ml of DNA solution (0.1 mg/ml) was slowly added to a mixing solution of 2.0 ml (3.2 mg/ml) of CK30PEG10k (American Peptide Company, Sunnyvale, CA) having an acetate lysine counterion [21, 32–34]. Compacted DNA was filtered through a sterile 0.2 μm polyethersulfone membrane and then processed with tangential flow filtration in exchange with saline. Further concentration steps were performed using VIVASPIN centrifugal concentrators (MWCO 100 k).

### Animal Treatments

Male Sprague-Dawley (SD) rats weighing 225–250 g (Taconic; Germantown, NY) were used in accordance with approved institutional IACUC protocols. For intranasal delivery, rats were anesthetized with ketamine and xylazine (90/20 mg/kg, i.p.) and placed in the supine position with their noses upright and their heads flat on the surface. Rats were given solutions of either naked plasmid (4.3 mg/ml), compacted plasmid nanoparticles (4.3 mg/ml), or saline via a Hamilton syringe fitted with a short piece of polyethylene tubing. Intranasal doses were given in 2.5 μl increments every minute on both sides, alternating nares, for a total of 20 μl. Rats remained supine for 30 min post-treatment.

### Preparation of Brain Tissue

Brains were fixed for IHC by transcardial perfusion using ice-cold PBS followed by 4% buffered paraformaldehyde (PFA). Brains were removed, post-fixed in 4% PFA for an additional 18 h, and then transferred to 30% sucrose for 48–72 h at 4 °C. The brains were sectioned at 30 μm using a cryostat. The

sections were stored at − 20 °C in a cryoprotectant solution, until further analysis or assay.

Brain tissue used for ELISA was obtained through rapid decapitation under isoflurane anesthesia. Sections were made by razor cuts using a Plexiglas brain matrix. The fresh brain sections were flash frozen in either liquid nitrogen ($N_2$) or on dry ice ($CO_2$) before being stored at − 80 °C. Prior to the ELISA, each section was homogenized in 1 ml of lysis buffer (1% Igepal, 10% glycerol, and 1:100 protease inhibitor (#88666, Pierce Thermo Scientific; Rockford, IL) in PBS) and centrifuged at 4 °C for 30 min at 14,000 RPM. The resulting supernatant was used as the sample for the ELISA.

## Detection of GDNF by ELISA

A 96-well plate was used for all ELISAs, and volumes of added samples were maintained at 100 μl/well. Plates were incubated at room temperature with continuous shaking. Wells were first coated with capture antibody diluted in PBS, 2.0 μg/ml mouse anti-human GDNF, #840189 (R&D Systems; Minneapolis, MN), and then incubated overnight at 4 °C. After this and each subsequent step, the plate was washed three times with room temperature wash buffer (0.05% Tween-20 in PBS). All subsequent incubations were carried out at room temperature. Blocking buffer (5% non-fat dry milk in PBS) was added at room temperature for 1 h prior to addition of standards and samples. GDNF standards (R&D Systems; Minneapolis, MN) made-up in reagent diluent (1% BSA in PBS) were added to rows of wells along one side of the plate. The standards were serially diluted from 1000 to 7.81 pg/ml. Tissue samples required an acid-shock step before adding to the plate. This involved addition of 1 N HCl (10 μl/sample) for 20 min at room temperature, followed by neutralization with 1 N NaOH (10 μl/sample). Reagent diluent was used for blanks. Samples and blanks were then added, and the plate was incubated for 2 h. Next, detection antibody diluted in reagent diluent, 100 ng/ml biotinylated goat anti-human GDNF (#840190; R&D Systems; Minneapolis, MN) was added, and the plates were incubated for another 2 h. For detection, an additional 45 min incubation with secondary antibody (diluted in reagent diluent) was required, 1:200 streptavidin–HRP solution (#890803; R&D Systems, Minneapolis, MN). Color development took place using SureBlue TMB substrate (#52-00-01; KPL, Gaithersburg, MD). The plate was incubated for 10–20 min until full color development took place. The reaction was stopped with 1 N HCl. All ELISAs had optical density read at 450 and 570 nm using a Biotek ELx800 microplate reader (Biotek, Wisnooski, VT) and analyzed using Gen5® software (Biotek, Wisnooski, VT). To express protein in terms of mg protein present in the samples, a BCA protein assay (Thermo Scientific, Rockford, IL) was run concurrent with each ELISA in accordance with the manufacturer's protocol.

## Tyrosine Hydroxylase Immunohistochemistry

Brain sections stored in cryoprotectant were first washed three times with PBS (10 min each) at room temperature and then treated with 0.9% $H_2O_2$ for 15 min to inhibit endogenous peroxidases. Wash steps occurred between each incubation step (3 times for 10 min each). Tissue was blocked with 5% normal goat serum (NGS) in PBS for 30 min at room temperature to decrease non-specific binding. A rabbit anti-TH antibody (#Ab152; Millipore, Billerica, MA) was diluted 1:4000 in 5% NGS in PBS and was used as the primary antibody. Brain sections were incubated overnight at 4 °C on a rotating wheel. On the next day, sections were incubated at room temperature for 1 h with a 1:250 dilution (in PBS) of biotinylated anti-rabbit secondary IgG antibody raised in goat (#BA-1000; Vector Labs, Burlingame, CA). Following this, the sections were incubated for 1 h at room temperature with ABC reagent (#PK-6100; Vector Labs, Burlingame, CA) made according to kit instructions. DAB solution was added for colorimetric detection. Sections were incubated at room temperature for exactly 7 min with DAB to standardize color development.

## 6-OHDA Lesioning

Sterile surgical equipment and aseptic techniques were used for all surgical procedures in compliance with the Northeastern University IACUC protocols. Rats were anesthetized with ketamine and xylazine (90/20 mg/kg, i.p.) and given desipramine (15 mg/kg, i.p.) 30 min prior to 6-OHDA injection to spare noradrenergic neurons. The animals were then placed in the stereotaxic instrument, and their body temperature was maintained at 38 °C. To induce a unilateral lesion of midbrain dopamine neurons, a total of 4 μl of 6-OHDA solution (2 μg/μl in 0.1% ascorbic acid) was injected into the left medial forebrain bundle at a rate of 1 μl/min using the following stereotaxic coordinates: + 1.2 mm lateral to lambda, + 4.4 mm anterior to the lambdoid suture, and − 8.3 mm ventral to the surface of the skull. The needle was left in place for 15 min after the 6-OHDA injection and then slowly withdrawn. Rats were administered a dose of buprenorphine (0.05 mg/kg) after recovery from anesthesia. Rats recovered from the 6-OHDA lesion for 3 weeks, at which time, further behavioral assessment and sacrifice occurred.

## Behavioral Assessment of Unilateral Dopamine Cell Loss

To assess the extent of the 6-OHDA lesion, all lesioned rats were evaluated for rotational behavior following an amphetamine challenge. Just prior to sacrifice 3 weeks after the 6-OHDA lesion, rats were given a dose of *d*-amphetamine sulfate (5 mg/kg, i.p.) and then placed in a large circular pail. A video camera mounted above the pail was used to observe and

record rotations. The number of rotations was counted for a total of 30 min, beginning 15 min after amphetamine injection. Net rotations were expressed as the number of ipsilateral rotations (toward the lesioned side; counterclockwise) minus the number of contralateral rotations (away from the lesion; clockwise).

## Microscopy

Microscopic analyses were performed using an Olympus BX51 with DIC optics for contrast enhancement. Sections were mounted on slides using Fluoromount-G (Southern Biotech, Birmingham, AL). Bright field microscopy was used to detect the chromogenic signal from DAB after TH-IHC.

## Analysis of TH-IHC

The integrated optical density (IOD) of TH staining was assessed in six coronal sections taken at approximately evenly spaced sites along the rostral–caudal axis of the SN using BIOQUANT® image analysis software (Nashville, TN). This software allows reliable and accurate measures of staining density and intensity by integrating both the number of pixels above a selected threshold level, as well as the relative intensity of the staining of the selected pixels. The six sections corresponded to AP levels between − 4.8 and − 6.0 mm from bregma. At the most rostral AP levels, the border between the SN and ventral tegmental area (VTA) was defined by extending a line dorsolaterally from the notch between the cerebral peduncles and the medial mammillary nucleus. At the medial AP levels, the border between SN and VTA was defined by fibers of the medial trigeminal nerve, and at the most caudal AP level, the border was defined by extending a line mediolaterally from the notch between the cerebral peduncles and the interpeduncular nucleus.

Lesion severity in the SN was assessed by comparing the IOD of staining in the lesioned SN versus the intact SN. For each section, the SN on the unlesioned side was first outlined, and the IOD for the encircled region of interest (ROI) was determined. The red/green/blue (RGB) thresholds were set to selectively capture the brown staining from DAB. RGB settings, light settings, and white balance were kept constant for all sections examined. The tracing of the ROI was then flipped and positioned over the SN on the lesioned side, and the IOD was again determined. The IOD of the left (lesioned) SN was divided by that of the right (intact) SN, subtracted from 1, and multiplied by 100 to yield the percent lesion per section. The average percent lesion for each rat was determined by averaging the values for the six sections from that animal.

The integrated optical density (IOD) of TH staining in the striatal sections was assessed in four to six coronal sections taken at approximately evenly spaced sites along the rostral–caudal axis of the striatum. The sections corresponded to AP levels between − 0.12 and + 2.2 mm from bregma. Striatal density measurements were done similarly to those in the SN, except five circular fields (measuring 350 μm in diameter) were analyzed on each side using BIOQUANT® software. These fields were randomly selected to sample the entire dorsal–ventral and medial–lateral striatum of each section. The IOD of staining on the lesioned and intact sides were divided, subtracted from 1 and then multiplied by 100 to obtain the percentage lesion for each section. Values from the striatal sections of each rat were averaged to give the percentage lesion for each animal.

Unbiased dopamine cell counts were also performed by counting all TH-positive cells within the lesioned and unlesioned SN of each section examined. Two-dimensional images of each SN were captured at 4× magnification. Four to six coronal sections corresponding to different rostral–caudal levels of the SN were analyzed from each rat. The perimeter of the SN was first outlined for each section using the anatomical landmarks described previously, and all TH-positive cell bodies within that perimeter were counted using NIH ImageJ software version 1.46 and the CellCounter plug-in. Counting was performed in an unbiased manner by a person blind to intranasal treatments by manually clicking on each cell within the perimeter of the SN and then tabulating the total number of TH positive cells that were marked within that perimeter. Cell counts on the lesioned side were divided by those on the unlesioned side, subtracted from 1 and then multiplied by 100 to obtain the percentage lesion (or % cell loss) for each section.

In intact rats without a lesion, the IOD of TH staining in the SN and striatum and dopamine cell counts in the SN represent the averages for the two sides.

## Statistical Analysis

GraphPad Prism® software, version 4.0, was used to determine significant differences between groups. One-way analysis of variance (ANOVA) with appropriate post-hoc tests was performed to evaluate differences by treatment. Two-way ANOVA with appropriate post-hoc tests was used to determine differences by treatment and by brain region. The $\alpha$-level for significance was set at $P < 0.05$. Data are reported as the mean ± standard error of the mean (SEM).

For analysis of rotational behavior, data from the three treatment groups did not satisfy the ANOVA requirement of homogeneity of variances. Therefore, a square root transformation was carried out prior to analysis. To account for negative values in the cases where contralateral rotations were greater than the ipsilateral rotations, the data was shifted by adding 100. The following formula was used by GraphPad Prism for the square root transformation: Y1 = Square root

of (Y + 100). The transformed data were then analyzed by ANOVA.

## Results

### hGDNF Vector Design

The plasmid map of the hGDNF expression plasmid, pGDNF_1b, is shown in Fig. 1. This plasmid contains the human GDNF_1b splice variant cDNA [38, 39] as well as the UbC promoter, and INF beta and β-globin S/MAR domains.

### Intranasal pGDNF_1b Increases GDNF Levels in the Rat Brain

It was first of interest to determine if intranasal administration of pGDNF_1b could produce measurable increases in GDNF above the endogenous levels present in the brain. Rats were given intranasal saline (control), naked pGDNF_1b, or pGDNF_1b NPs (88 μg DNA). Seven days later, the rats were sacrificed and 2 mm-thick coronal sections were collected and analyzed by GDNF-ELISA. Both naked pGDNF_1b ($n = 6$) and pGDNF_1b NPs ($n = 6$) significantly increased average brain GDNF levels above the baseline levels in saline-treated controls ($n = 4$; Fig. 2). One-way ANOVA indicated significance by treatment, [$F(2,11) = 7.044$, $P = 0.0107$]; Bonferroni's post-test showed significance for both NaCl vs naked pGDNF and NaCl vs pGDNF NPs ($^*P < 0.05$). For both the naked plasmid and the NPs, the whole brain average concentrations of GDNF per mg protein were more than twice the basal levels in saline controls.

### Intranasal pGDNF_1b Is Neuroprotective in the Rat 6-OHDA Lesion Model of Parkinson's Disease

To determine if these increased levels of GDNF production were physiologically relevant, the next studies examined

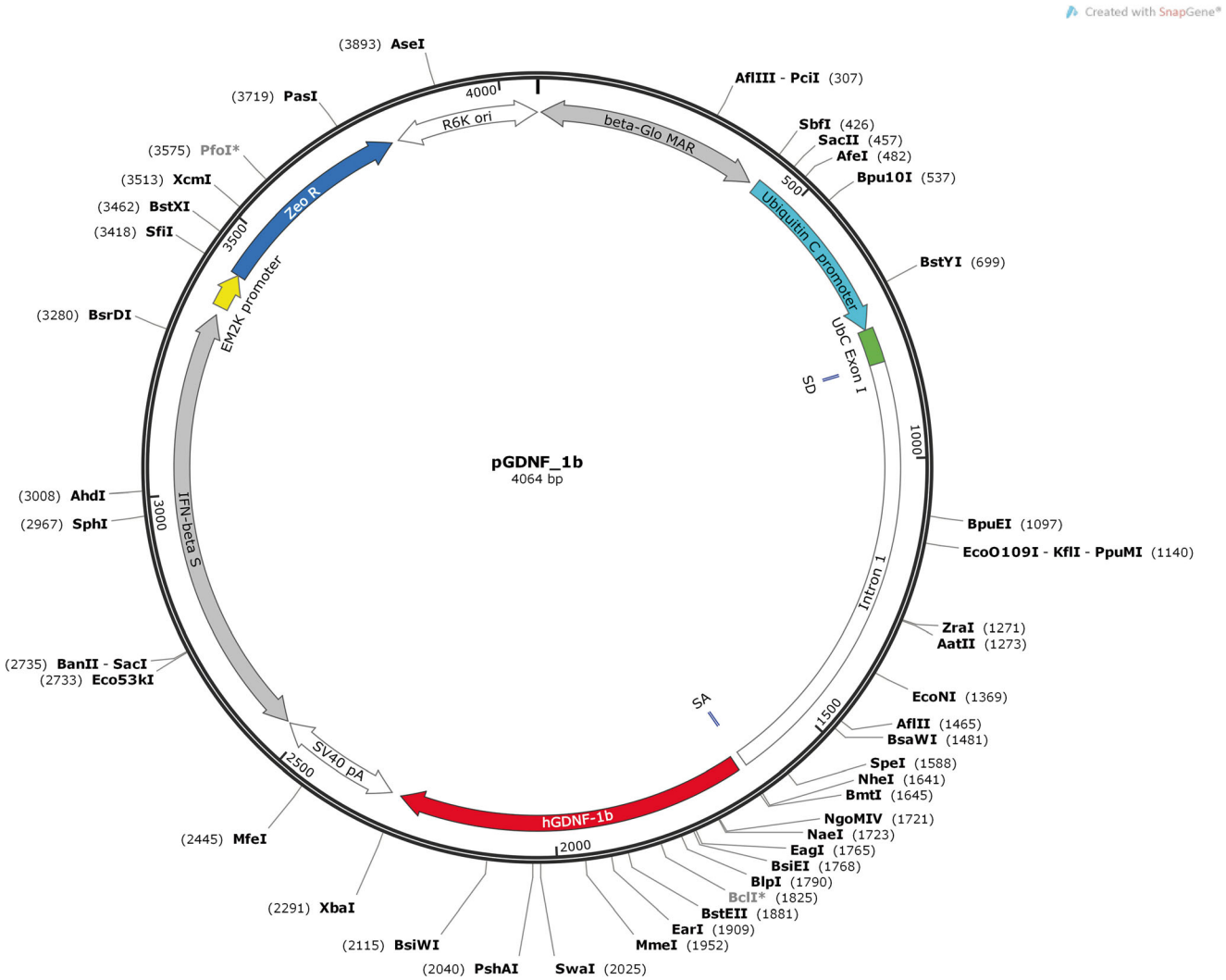


**Fig. 1** pGDNF_1b plasmid map

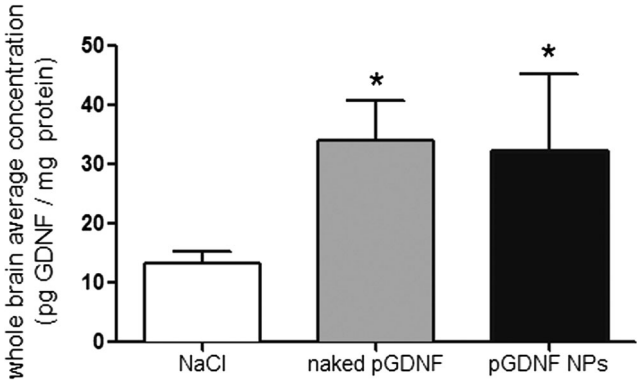


**Fig. 2** Intranasal administration of pGDNF_1b DNA NPs or naked pGDNF_1b increases average GDNF levels in the rat brain. Rats received an intranasal dose of either 20 μl (88 mg DNA) naked pGDNF ($n = 6$), pGDNF NPs ($n = 6$), or saline ($n = 4$) and were sacrificed 7 days later. GDNF levels were measured by ELISA and normalized to mg protein and averaged across brain regions. Bars represent the mean GDNF expression (pg GDNF/mg protein + SEM). One-way ANOVA indicated significance by treatment ($P = 0.017$). Bonferroni's post-test indicated significance for naked pGDNF vs saline and pGDNF NPs vs saline (*$P < 0.05$)

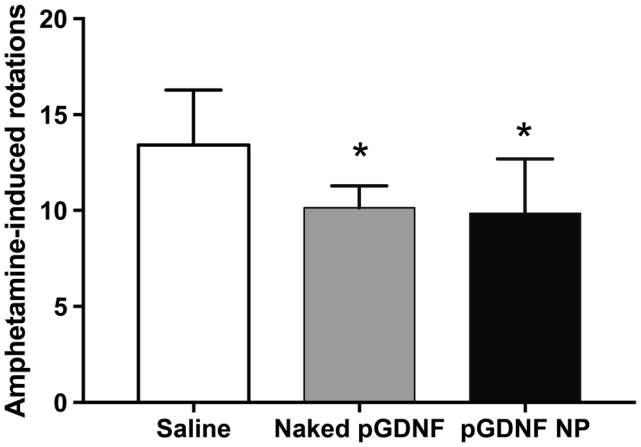


**Fig. 3** Intranasal pGDNF_1b reduces amphetamine-induced rotations in the rat 6-OHDA lesion model of Parkinson's disease. Rats were given an intranasal dose of either naked pGDNF ($n = 7$), pGDNF NPs ($n = 7$), or saline ($n = 8$). One week later, all groups received a unilateral 6-OHDA lesion. Three weeks later, lesioned rats were given *d*-amphetamine (5 mg/kg, i.p.) and net rotations were counted during the following 30 min interval. Bars represent the transformed net rotational frequency + SEM. Saline control rats had the greatest rotational response to the amphetamine challenge. Both naked and pGDNF NP treatments markedly reduced the frequency of ipsilateral rotations, consistent with a neuroprotective effect. The following square root transformation was carried out in order to statistically analyze this data, Y1 = Square root of (Y + 100). One-way ANOVA showed an overall significant effect by treatment ($P < 0.05$), and Tukey's post-test showed significance for pGDNF NPs vs saline (*$P < 0.05$) and naked pGDNF vs saline (*$P < 0.05$)

neuroprotective efficacy in partial 6-hydroxydopamine (6-OHDA)-lesioned rats using the same pGDNF dosing conditions. Seven days after intranasal treatments with saline, naked pGDNF_1b, pGDNF_1b NPs, or 6-OHDA was stereotaxically injected into the left medial forebrain bundle. All rats in this study were lesioned, so the control group given intranasal saline established the severity of the 6-OHDA lesions in the absence of pGDNF treatments. Rats recovered for three weeks and were then assessed using an in vivo test of lesion severity, i.e., rotational behavior following an amphetamine challenge [40–42]. Quantification of net rotations showed that rats given intranasal saline prior to 6-OHDA ($n = 8$) had the greatest ipsilateral rotational response to amphetamine, while rats pre-treated with intranasal naked pGDNF_1b ($n = 7$) or pGDNF_1b NPs ($n = 7$) had significantly reduced rotational behavior (Fig. 3; one-way ANOVA of transformed data indicated significance by treatment, [$F(2,19) = 4.971$, $P = 0.018$]; Tukey's post-test showed significance for pGDNF_1b NPs versus saline, *$P < 0.05$, and for naked pGDNF_1b versus saline, *$P < 0.05$). A square root transformation of the rotation data was performed before analysis by ANOVA to correct for the differing variances between groups. The ANOVA analysis indicated no difference between intranasal naked and pGDNF_1b NP treatments, suggesting that both yielded sufficient transfection and GDNF expression in the brain to attenuate the dopaminergic imbalance caused by a unilateral 6-OHDA lesion, providing behavioral evidence of neuroprotection in this model.

Immediately after the amphetamine challenge, rats were sacrificed and their brains were processed for TH-IHC. BIOQUANT® image analysis software was used to determine lesion severity by comparing the integrated optical density (IOD) of TH-staining in the lesioned versus unlesioned SN for each animal. There was a significant reduction in the % lesion in rats given intranasal pGDNF_1b NPs ($n = 7$) compared to saline controls ($n = 7$), indicating significant protection of SN dopamine neurons and their dendritic fibers (Fig. 4a and Table 1). The more modest reduction in lesion severity in rats given naked pGDNF_1b ($n = 6$) was not significant compared to controls. One-way ANOVA revealed overall significance by treatment [$F(2,17) = 6.48$, $P = 0.008$]; Tukey's post-test showed significance for pGDNF_1b NPs versus saline (*$P < 0.05$). Representative images of the SN of rats from each treatment group are shown in Fig. 4c.

To further assess the neuroprotective effect, dopamine cell counts were performed to assess the % dopamine cell loss in the SN of each of the lesioned rats. Figure 4b and Table 1 show that both intranasal naked pGDNF_1b and pGDNF_1b NPs provided significant protection of dopamine neurons from a 6-OHDA lesion, although the degree of protection was greater with the NPs than the naked plasmid. One-way ANOVA indicated significance by treatment [$F(2,15) = 10.85$, $P = 0.001$]; Tukey's post-test showed significance for the pGDNF_1b NP group versus saline controls (***$P < 0.001$), and for naked pGDNF_1b versus saline controls (*$P < 0.05$).

In addition to the SN, TH-immunostaining densities were also quantified from striatal sections in each treatment group.

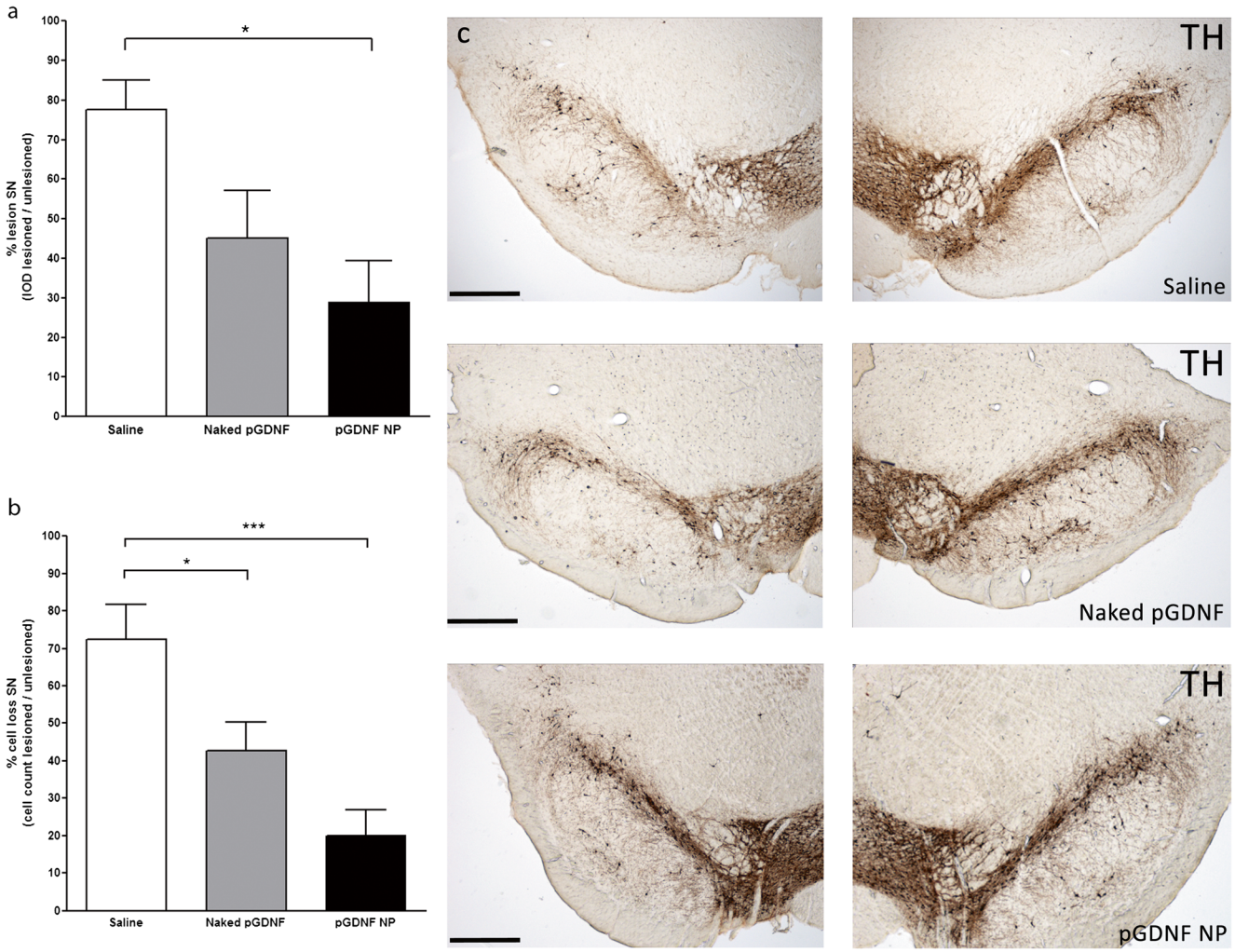


**Fig. 4** Intranasal pGDNF_1b reduces SN dopamine cell loss in the rat 6-OHDA lesion model of Parkinson's disease. TH-IHC was performed on midbrain sections from rats treated with intranasal saline ($n = 7$), naked pGDNF ($n = 6$), and pGDNF NPs ($n = 7$) one week before a unilateral 6-OHDA lesion and sacrificed 3 weeks later. SN sections from each rat were analyzed for the integrated optical density (IOD) of TH immunostaining and dopamine cell counts. **a** Lesion severity assessed by the IOD of TH staining was significantly reduced in the SN of rats given intranasal pGDNF NPs but not in those given the naked plasmid. Bars represent the mean % lesion (+ SEM). One-way ANOVA showed an overall significant effect by treatment ($P < 0.01$), and Tukey's post-test showed significance for pGDNF NPs vs saline ($^*P < 0.05$). **b** Lesion severity assessed by dopamine cell counts was significantly reduced by both intranasal pGDNF NPs and the naked plasmid. Bars represent the mean % cell loss (+ SEM). One-way ANOVA indicated a significant effect of treatment ($P < 0.005$) and Tukey's post-test showed significance for the pGDNF NP group vs saline control ($^{***}P < 0.001$) and for naked pGDNF vs saline control ($^*P < 0.05$). **c** Ventral midbrain images showing the 6-OHDA lesioned side (left) compared to the unlesioned side (right). *Top*, Saline controls exhibited the greatest lesion severity. *Middle*, Rats given intranasal naked pGDNF had partial attenuation of lesion severity based on the IOD of TH staining. *Bottom*, Rats given intranasal pGDNF NPs had the greatest neuroprotection, with a significant reduction in lesion severity based on both IOD of TH staining and dopamine cell loss relative to saline-treated rats. Images were taken at 4× magnification. Scale bars = 250 μm

**Table 1** Average percent lesion assessed by TH-IHC in 6-OHDA-lesioned rats treated with intranasal saline, naked pGDNF_1b, or pGDNF_1b NPs. The % lesion represents the value on the 6-OHDA-lesioned side divided by that on the unlesioned side, subtracted from 1, and then multiplied by 100. TH density in the SN and striatum are the integrated optical density (IOD) of immunostaining in each region

| | % lesion ± S.E.M. | | |
|---|---|---|---|
| | Saline | Naked pGDNF | pGDNF NP |
| SN TH density (IOD) | 77.7% ± 7.4% | 45.0% ± 12.1% | 28.9% ± 10.5% |
| SN dopamine cell count | 72.3% ± 9.3% | 42.6% ± 7.6% | 20.0% ± 6.8% |
| Striatum TH density (IOD) | 83.1% ± 6.3% | 50.7% ± 10.0% | 32.8% ± 4.8% |

Both intranasal naked pGDNF_1b and pGDNF_1b NPs significantly protected striatal dopamine nerve terminals from the 6-OHDA lesion (Fig. 5a and Table 1). One-way ANOVA indicated significance by treatment [$F(2,13) = 12.83$, $P < 0.001$]; Tukey's post-test showed significance for the pGDNF_1b NP group versus saline controls (***$P < 0.001$)

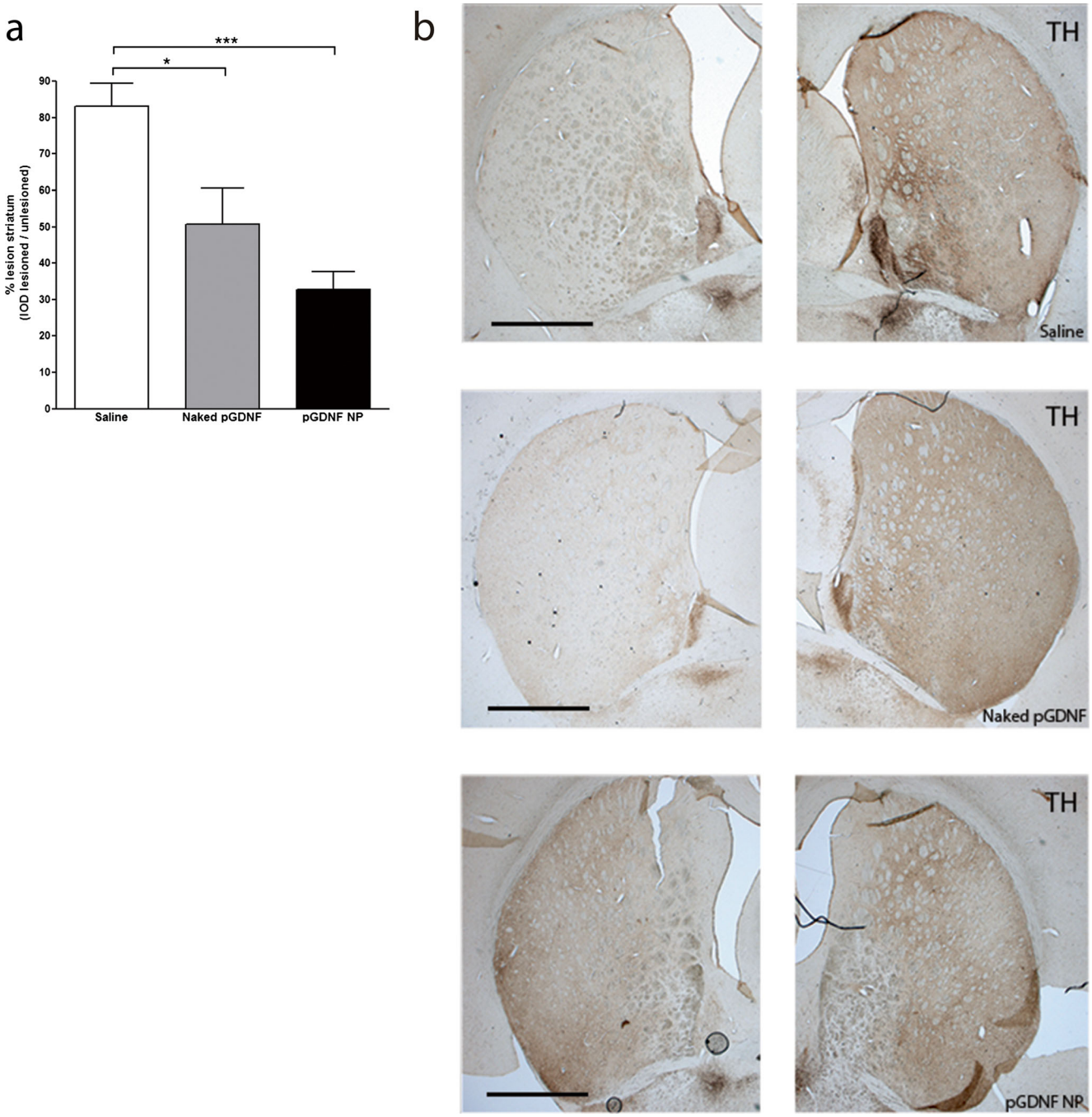


**Fig. 5** Intranasal pGDNF_1b reduces lesion severity in the striatum in the rat 6-OHDA lesion model of Parkinson's disease. **a** TH-IHC was performed on striatal sections from rats treated with intranasal saline ($n = 6$), naked pGDNF ($n = 5$), and pGDNF NPs ($n = 5$) one week before a unilateral 6-OHDA lesion and sacrificed 3 weeks later. Four representative sections spanning the rostral–caudal levels of striatum of each rat were analyzed for the integrated optical density (IOD) of TH immunostaining. Bars represent the mean % lesion (+ SEM). One-way ANOVA indicated a significant effect of treatment ($P < 0.001$) and Tukey's post-test showed significance for the pGDNF NP group vs saline control (***$P < 0.001$) and for naked pGDNF vs saline control (*$P < 0.05$). **b** Striatal images show the 6-OHDA lesioned side (left) compared to the unlesioned side (right). (Top) Saline controls showed the greatest lesion severity. (Middle) Rats given intranasal naked pGDNF had partial but significant protection of staining density. (Bottom) Rats given intranasal pGDNF NPs had the greatest protection of TH staining density. All images are at approximately AP level 0.00 to − 0.12 mm (bregma). All images were taken at 4× magnification. Scale bars = 250 μm

and for naked pGDNF_1b versus saline controls (*$P < 0.05$). There was no significant difference in TH staining density between the naked pGDNF_1b and the pGDNF_1b NP treatment groups. Figure 5b shows representative striatal images from similar rostral–caudal levels illustrating the neuroprotective effect. In summary, pretreatment with intranasal pGDNF_1b one week before a 6-OHDA lesion resulted in significant neuroprotection of dopamine cells in every measure considered. For all immunohistochemical measures, intranasal pGDNF_1b NPs provided a somewhat greater degree of protection than the naked plasmid, although naked pGDNF_1b also yielded significant protection in several of these measures, as well as the behavioral measure.

### Intranasal pGDNF_1b Has no Neurotrophic Effect on SN Dopamine Neurons in the Absence of a Lesion

To next test whether intranasal treatment with pGDNF_1b NPs also exerts a neurotrophic effect on SN dopamine neurons in intact rats, separate groups of rats ($n = 6$/group) were given intranasal saline, naked pGDNF_1b or pGDNF_1b NPs, and sacrificed 4 weeks later. DNA doses, volumes, and intranasal treatments were the same as in the 6-OHDA lesion study. TH IHC was carried out on SN and striatal sections from all rats. Comparing the staining densities between groups in this study required that sections from all rats be assayed together, i.e., sections from the unlesioned animals and all of the 6-OHDA lesioned rats were assayed in a single batch. This insured that any variations in staining intensity between treatment groups reflected differences due to treatments, not assay conditions. No significant differences in TH staining density were observed in either the SN or the striatum of intact, unlesioned rats given intranasal pGDNF_1b NPs, naked pGDNF_1b, or saline (Fig. 6a, c). Similarly, there were no differences in the number of TH-positive neurons in the SN of intact pGDNF_1b-treated rats versus saline controls (Fig. 6b). These results indicate that the intranasal pGDNF_1b treatments did not induce a detectable neurotrophic effect on nigrostriatal dopamine neurons in the absence of a lesion.

We next examined TH immunostaining density and dopamine cell counts in the *unlesioned* SN (control side) of the 6-OHDA lesioned animals in the previous study. In these brains, significant increases in TH IOD were observed on the unlesioned side of pGDNF_1b NP-treated rats relative to intact, unlesioned rats treated with either saline or pGDNF_1b NPs (Fig. 6a; one-way ANOVA [$F(5,30) = 3.33$, $P = 0.016$] with Tukey's post-test, *$P < 0.05$). However, there were no differences in TH-positive cell counts in any of the groups, whether lesioned or unlesioned (Fig. 6b). Thus, intranasal pGDNF_1b NPs increased TH immunostaining density on the intact side of unilateral 6-OHDA-lesioned rats but did not increase TH IOD in the SN of unlesioned rats.

### Intranasal pGDNF_1b Does Not Cause Weight Loss in Lesioned Rats

In previous studies, GDNF has been found to cause significant weight loss in subjects, and this has been postulated to indicate off-target effects at brain sites outside the nigrostriatal system where GDNF might spread following over-expression of a viral vector or intracerebral infusion of GDNF protein [43, 44]. It was therefore of interest to examine whether intranasal pGDNF treatments caused weight loss. Rats were weighed just before their intranasal treatment and again just before sacrifice 3 weeks after the 6-OHDA lesion. No significant weight loss occurred from intranasal treatment with either naked or pGDNF_1b NPs. In fact, rats treated with the NPs gained significantly more weight compared to saline controls and those given intranasal naked pGDNF_1b (one-way ANOVA indicated significance by treatment [$F(2,19) = 7.74$, $P = 0.0035$] with Tukey's post-test, *$P < 0.05$, **$P < 0.01$; Fig. 7).

## Discussion

These studies demonstrate the feasibility of an intranasal GDNF gene therapy using a non-viral vector as a disease-modifying treatment for PD. Current pharmacotherapies provide temporary relief of symptoms but do not prevent or slow the progressive death of SN dopamine neurons. GDNF is a potent neurotrophic factor for dopamine neurons and has demonstrated efficacy in various animal models of PD [2–4, 29, 45] and in some human clinical trials [5–8, 10]. However, the need for surgical infusion of therapeutics into the brain presents formidable obstacles to their widespread clinical application. Intranasal administration offers a largely unexplored, non-surgical means for delivery of large molecular weight therapeutics, including gene constructs, directly to the brain. An intranasal GDNF gene therapy would have the advantages of providing continuous and scalable production of GDNF within the brain via a non-invasive route of administration. Here, we show that intranasal administration of a non-viral vector for hGDNF, unimolecular plasmid DNA NPs compacted with CK30PEG10k, can result in cellular transfection and production of sufficient GDNF in the rat brain to protect SN dopamine neurons in the 6-OHDA model of PD. Our results corroborate and extend those of previous studies [31, 38, 46], which showed that this same formulation of pGDNF NPs is capable of transfecting brain cells in vitro, resulting in increases in GDNF expression and neurotrophic effects on dopamine neurons in rat ventral midbrain cultures. These pGDNF_1b NPs have also been shown to induce transfection and transgene expression in vivo, as indicated by increases in GDNF mRNA expression and partial protection of dopamine neurons in 6-OHDA-lesioned rats when the NPs

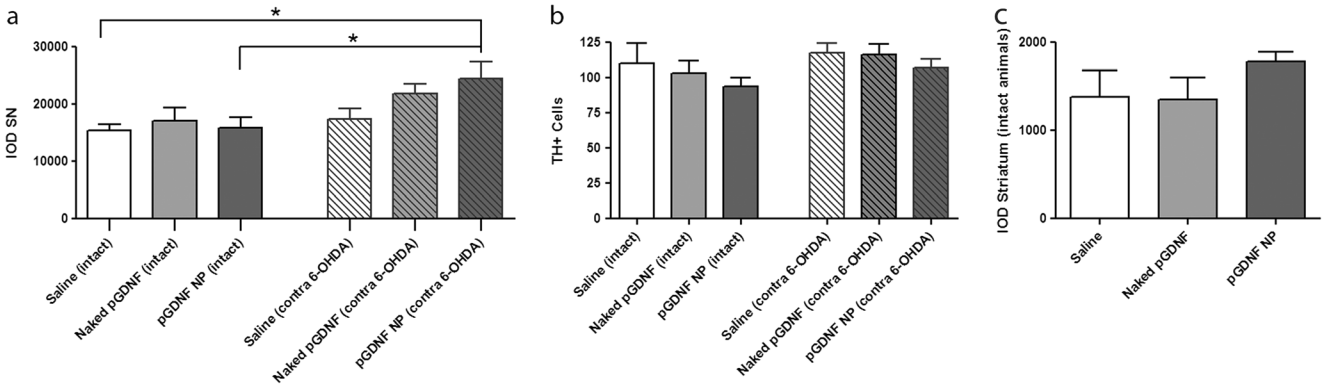


**Fig. 6** Effects of intranasal pGDNF_1b NPs on tyrosine hydroxylase (TH) immunostaining differ between lesioned and unlesioned rats. TH-IHC was performed on midbrain and striatal sections of both intact, unlesioned rats and 6-OHDA-lesioned rats after each group was given intranasal saline, naked pGDNF, or pGDNF NPs ($n = 6$/group). All rats were sacrificed 4 weeks after intranasal administration. **a** IOD of TH staining in the SN of intact rats (solid bars) and in the contralateral SN of 6-OHDA lesioned rats (hatched bars). **b** TH-positive cell counts in the SN of intact rats (solid bars) and on the contralateral SN of 6-OHDA lesioned rats (hatched bars). **c** IOD of TH staining in the striatum of intact rats. One-way ANOVA showed no significant effect of intranasal naked pGDNF or pGDNF DNA NPs on IOD of TH staining in the SN or striatum of intact, unlesioned rats (**a**, solid bars, and **c**). However, the IOD of TH staining was significantly increased in the contralateral (unlesioned) SN of 6-OHDA lesioned rats given intranasal pGDNF NPs compared to unlesioned rats given either intranasal saline or pGDNF NPs (**a**); Tukey's post-test, $^{*}P < 0.05$. There was no significant difference in TH-positive cell counts of lesioned or unlesioned rats given intranasal naked pGDNF or pGDNF NPs (**b**); one-way ANOVA

were injected into the striatum one week earlier. Our current studies now provide evidence of transgene expression and neuroprotective efficacy when these NPs are administered intranasally one week before a 6-OHDA lesion.

In the first case, we showed that intranasal delivery of a single dose of pGDNF_1b NPs increases overall GDNF expression in the rat brain. One week after administration, GDNF levels were significantly higher in the brains of rats given intranasal naked pGDNF_1b or pGDNF_1b NPs relative to saline controls. Since GDNF is endogenously and variably expressed throughout the brain, the amounts derived from pGDNF_1b transfection represent only a fraction of the total GDNF present in each sample. In addition, some proportion of total GDNF could be bound to receptors, interfering with its detection by ELISA [22, 47]. Despite these factors, both intranasal naked pGDNF_1b and pGDNF_1b NPs significantly increased whole-brain GDNF expression. The levels achieved after intranasal administration of naked pGDNF_1b and pGDNF NPs were 2–3-fold above those present endogenously (Fig. 2). Since the concentration of GDNF needed for protection of SN dopamine neurons in vitro is in the picomolar range [1, 48], even modest increases in brain GDNF, especially if sustained over weeks to months, could be sufficient to prevent or delay ongoing neurodegenerative processes. By comparison, the increases we observed are orders of magnitude lower than the 100- to 1000-fold increases reported in the transduced brain regions after direct intracerebral injection of GDNF viral vectors [49–53].

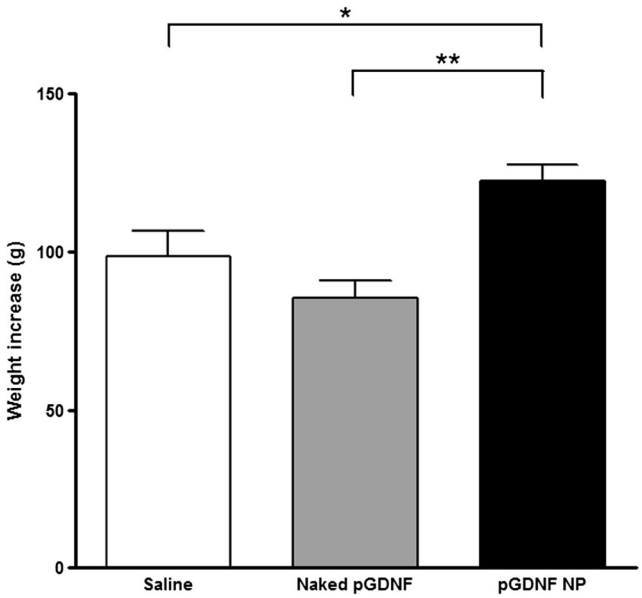


**Fig. 7** Effect of intranasal naked pGDNF_1b and pGDNF_1b NPs on weight gain of 6-OHDA lesioned rats. Rats were weighed before intranasal treatments and four weeks later (three weeks after the unilateral 6-OHDA lesion). Rats that received intranasal pGDNF_1b NPs gained significantly more weight than lesioned rats given intranasal saline or naked pGDNF_1b. One-way ANOVA indicated significance by treatment ($P = 0.0035$) and Tukey's post-test showed a significant effect of pGDNF_1b NP treatment vs saline and naked pGDNF_1b ($^{*}P < 0.05$ and $^{**}P < 0.01$, respectively)

We reasoned that if intranasal pGDNF_1b could generate sufficient transgene expression in one week to be neuroprotective in an acute neurotoxin model, it would merit further development as a PD therapeutic. When tested for efficacy in the rat unilateral 6-OHDA lesion model, both intranasal naked pGDNF_1b and pGDNF_1b DNA NPs provided significant protection against the effects of the lesion. This protection was revealed both behaviorally and neurochemically by TH-IHC of dopamine cells within the SN and their nerve terminals in the striatum. Considering first the behavioral evidence of

neuroprotection, intranasal pGDNF_1b (naked or NPs) significantly reduced amphetamine-induced ipsilateral rotation compared to lesioned rats given intranasal saline. Since rotation is a consequence of an imbalance between dopamine released from intact nerve terminals on the lesioned versus unlesioned side, increasing lesion severity correlates with greater rotational responses and neuroprotection correlates with lower responses. The reduction in rotation therefore indicates that intranasal pGDNF_1b abolishes behavioral evidence of the 6-OHDA lesion.

Next, considering the IHC evidence of neuroprotection, Table 1 shows the average percent lesion in the SN and striatum for rats pretreated 7 days prior to 6-OHDA with intranasal saline, naked pGDNF_1b, or pGDNF_1b NPs. First, comparing the integrated optical density of TH staining in the SN with dopamine cell counts, it is clear that these two measures were consistently very similar within each treatment group. For instance, in the intranasal saline group, the percent lesion was 77.7% using TH staining density and 72.3% using cell counts. This pattern was also observed in the naked pGDNF_1b and pGDNF_1b NP groups, although the treatments attenuated lesion severity to different degrees. This suggests that a close correlation exists between these two measures of dopamine cell integrity and lesion severity. Second, the percent lesion in the SN was generally consistent with that in the striatum within each treatment group, with only slightly higher values for the percent lesion in striatum versus SN. This confirms the internal consistency of the 6-OHDA lesioning protocol, the intranasal treatments, and the immunohistochemical methods used to detect the effects of the intranasal treatments. The similarities seen with the three TH-IHC measures also indicate that the lesion causes parallel changes in dopaminergic functional status at both cell bodies and nerve terminals. Importantly, these results show that a relatively modest increase in GDNF expression generated by intranasal administration of pGDNF NPs is sufficient to provide significant neuroprotection in the 6-OHDA model, a finding in contrast to the general expectation that much higher levels of GDNF would be required in the target brain areas for therapeutic benefit. Indeed, our results corroborate those of Eslamboli et al. [52], who found that similar increases in GDNF (3–4-fold above basal levels) protect nigral dopamine neurons in the 6-OHDA lesion model. However, these and other investigators also observed that long-term over-expression of GDNF by viral vectors can cause unintended consequences, such as aberrant sprouting of dopaminergic fibers and down-regulation of TH [49–51]. Since intranasal administration of our NPs likely does not generate supraphysiological levels of GDNF at target sites or adjacent areas, it is less likely to cause aberrant sprouting of TH-positive fibers or TH down-regulation in either intact or lesioned rats. We found no effect of intranasal pGDNF_1b treatments on TH staining density in the SN or striatum of unlesioned rats (Fig. 6), consistent with the lack of effect of intranasal GDNF protein on TH staining density in the SN of unlesioned rats seen by Migliore et al. [29]. However, in the current study, TH immunostaining density, but not cell counts, was *increased* (as opposed to down-regulated) in the intact SN of 6-OHDA-lesioned rats given intranasal pGDNF_1b NPs. These results suggest a differential sensitivity to increased GDNF levels in lesioned rats, leading to up-regulation of TH by dopamine neurons on the intact side, possibly as a means of compensating for the loss of dopamine on the opposite side. Kozlowski et al. [54] have previously reported that bilateral, compensatory changes in the nigrostriatal system, particularly changes in TH expression, occur in response to a unilateral 6-OHDA lesion in the rat.

Finally, intranasal pGDNF_1b NPs did not cause weight loss in our study, an effect observed with both intracerebral injections of GDNF protein and GDNF viral vectors [43, 44]. In fact, weight gain was significantly greater in 6-OHDA-lesioned rats given intranasal pGDNF_1b NPs than in those given intranasal saline or naked pGDNF_1b during the three weeks post-lesion (Fig. 7). This finding may indicate that the lower levels of GDNF expression generated by our intranasal pGDNF treatments were below the threshold for inducing weight loss in the brain area(s) responsible for this effect, as suggested previously [44].

The most important outcomes of the study are that GDNF generated by intranasal pGDNF_1b significantly reduced 6-OHDA lesion severity by every measure considered without causing effects associated with GDNF over-expression. pGDNF_1b NPs provided roughly twice as much neuroprotection as the naked pGDNF_1b (see Table 1). Although both constructs generated similar levels of GDNF in whole brain one week after intranasal administration (Fig. 2), GDNF expression may have been more sustained over the month after dosing with the NPs than with the naked plasmid, thereby generating more neuroprotection during the course of lesion development. In support of this conclusion, DNA NPs were previously shown to yield longer-lasting expression than the naked plasmid after injection into the rat brain [31, 37].

Unlike current symptomatic treatments for PD, a gene therapy approach aimed at increasing expression of a neurotrophic factor in the nigrostriatal system may provide a direct means of counteracting the underlying processes responsible for damage and the loss of dopamine neurons. While an acute, neurotoxin-induced lesion of SN dopamine neurons is not mechanistically or temporally comparable to the progressive course of human PD, and efficacy in this model cannot be assumed to predict clinical efficacy in PD, the demonstration of neuroprotective efficacy in this model underscores the potential of intranasal pGDNF_1b, and the nanoparticle vector, as means of rescuing injured dopamine neurons in PD.

It must be recognized, however, that the nasal route does not permit targeting to specific brain regions but generates

widespread transfection and protein expression throughout the brain, including unintended brain areas. While this may have advantages in cases where a neurotrophic protein would be desirable in multiple brain areas affected by a neurodegenerative disease, such as the striatum and SN in PD, widespread transgene expression may also present disadvantages, such as off-target effects in unintended brain areas, especially if GDNF concentrations were to greatly exceed normal physiological levels. Under conditions of our study, no changes associated with over-expression of GDNF were observed, but consideration of this possibility must be investigated in future studies. Another issue is whether the relatively low efficiency of intranasal delivery might prevent sufficient, sustained transgene expression in the target brain areas to be of therapeutic benefit in PD. This may be of little concern since dosing could be adjusted or repeated as necessary to insure GDNF production at therapeutic levels in the target brain areas. The non-invasiveness of the intranasal route and the non-immunogenicity of the NPs would permit tailoring doses and dosing intervals to the course of patients' illness. However, balancing these pros and cons and controlling expression levels to achieve therapeutic efficacy without adverse effects will be a necessary step in translating intranasal gene therapy into a clinical application.

## Conclusion

In conclusion, these studies demonstrate the utility and potential of the intranasal route for delivery to the brain of a non-viral gene therapy for PD, and they justify further work to test the approach in primates, where the shape of the nasal cavity, trajectory of nose-to-brain transport, and brain volume all differ substantially from that in the rat, and more closely approximate those of humans. If significant transgene expression can be achieved in the primate brain, it would be compelling to test for clinical neuroprotective efficacy in human PD. Ultimately, intranasal pGDNF_1b NPs could become the first non-invasive gene therapy capable of slowing progression of early stage Parkinson's disease and promoting functional recovery. This non-invasive approach may also be applied to numerous other CNS disorders where GDNF has been shown to be of benefit. Taking this even further, intranasal delivery may provide a means of delivery of other gene therapy vectors to the CNS [25], enabling production of numerous therapeutic proteins with applications well beyond Parkinson's disease.

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## Compliance with Ethical Standards

**Declaration of Interests** AEA, BTH, and BLW declare no conflict of interest. MJC and OSL are current employees of Copernicus Therapeutics, and LP is a past employee. MJC and OSL hold stock/stock options in the company.

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