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Transgene expression in the striatum following intracerebral injections of DNA nanoparticles encoding for human glial cell line-derived neurotrophic factor (hGDNF)

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Abstract

A goal of our studies is to develop a potential therapeutic for Parkinson's disease (PD) by a human GDNF (hGDNF) expression plasmid administered to the rat striatum as a compacted DNA nanoparticle (DNP) and which will generate long-term hGDNF expression at biologically active levels. In the present study we used a DNA plasmid encoding for hGDNF and a polyubiquitin C (UbC) promoter that was previously shown to have activity in both neurons and glia, but primarily in glia. A two-fold improvement was observed at the highest plasmid dose when using hGDNF DNA incorporating sequences found in RNA splice variant 1 compared to splice variant 2; of note, the splice variant 2 sequence is used in most preclinical studies. This optimized expression cassette design includes flanking scaffold matrix attachment elements (S/MARs) as well as a CpG-depleted prokaryotic domain and, where possible, eukaryotic elements. Stable long-term GDNF activity at levels 300–400% higher than baseline was observed following a single intracerebral injection. In a previous study DNPs plasmids encoding for reporter genes had been successful in generating long-term reporter transgene activity in the striatum (>365 days) and in this study produced sustained GDNF activity at the longest assessed time point (6 months).

Keywords

plasmid optimization; Parkinson's disease; gene therapy; splice variant

INTRODUCTION

Recent gene therapy studies have shown promising results using viral vector gene delivery systems in the central nervous system (Manfredsson and Mandel, 2010). Although striatal delivery of various neurotrophic factors have been shown to provide modest improvement in a rat model of PD, members of the GDNF family have provided the most beneficial effects and it is these factors that have moved on to clinical trials. Neuroprotective and

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neurorestorative effects for GDNF have been demonstrated using a variety of viral vectors: adeno-associated (AAV) in rodents (Mandel et al., 1997, Kirik et al., 2000) and non-human primates (Eslamboli et al., 2005, Elsworth et al., 2008, Eberling et al., 2009), lentivirus (LV) in rodents (Bensadoun et al., 2000, Georgievska et al., 2002, Azzouz et al., 2004) and non-human primates (Kordower et al., 2000, Palfi et al., 2002), adenovirus (Ad) in rodents (Choi-Lundberg et al., 1998, Connor et al., 2001) and herpes simplex virus (HSV) in rodents (Natsume et al., 2001, Sun et al., 2005).

Previously, we used a plasmid (pUL; see Figure 1) compacted into DNA nanoparticles (DNPs) to achieve long-term and stable transgene activity of a reporter gene, luciferase, in the brain; transgene activity was sustained for >365 days in the mouse (Yurek et al., 2011). To develop a non-viral therapy for PD preclinical models, we developed DNPs containing a pUL derivative encoding rat GDNF cDNA. GDNF was chosen for its potent neurotrophic effect on dopaminergic neurons; firstly, to ameliorate the neurodegenerative effects of Parkinson's disease and secondly, for morphological differentiation of dopaminergic neurons (Lin et al., 1993). We observed sustained GDNF over-expression after single injections of rat GDNF DNPs into the striatum (Yurek et al., 2009b); these same GDNF DNPs imparted a neurotrophic effect on grafted fetal ventral mesencephalic cells when injected into the brain 1 week prior to the grafting procedure (Yurek et al., 2009a). Given these encouraging results, we now develop optimized human GDNF (hGDNF) DNPs to produce high level and prolonged duration of hGDNF in the rat striatum. A panel of DNPs was formulated to evaluate expression plasmids encoding hGDNF splice variants 1 and 2: 1) splice variants of human GDNF cDNA; 2) splice variants of a custom-designed codon-optimized and CpG-depleted hGDNF; 3) splice variants of a custom-designed hGDNF additionally modified to minimize heterochromatin formation. The prokaryotic backbone and, where possible, eukaryotic elements were CpG depleted. An optimized Kozak sequence was included in every construct to ensure efficient translation initiation. Splice variants 1 and 2 of GDNF produce the same mature, secreted hGDNF protein, although the shorter splice variant 2 has a 78 bp deletion in the pro-region of the GDNF coding sequence (Figure 1) (Schaar et al., 1993, Trupp et al., 1995, Grimm et al., 1998). The motivation for testing these splice variants is a report suggesting that the shorter splice variant results in less secreted hGDNF (Wang et al., 2008). Additionally, since heterochromatin formation can reduce levels and duration of transgene expression, strategies to reduce heterochromatin formation have been proposed (Luykx et al., 2006, Radwan et al., 2008). Based on prolonged production of biologically active levels of hGDNF, an optimized hGDNF DNP has been selected for the next phase of preclinical testing.

EXPERIMENTAL PROCEDURES

Plasmid Construction

DNA vectors were constructed using standard molecular biology techniques, followed by restriction analysis and sequencing of subcloned regions (Sambrook et al., 1989). Detailed methodology for the pUL plasmid construction is reported in a previous publication (Yurek et al., 2009b). Figure 1 shows the pUL plasmid map from which the GDNF plasmids were derived.

Preparation of Condensing Peptide

L-Cysteinyl-poly-L-lysine 30-mers (UCB Bioproducts, Inc.) were conjugated with 10-kDa polyethylene glycol (PEG) (Nektar Therapeutics) as described in (Liu et al., 2003) except that trifluoroacetate counterion was replaced with acetate by size-exclusion chromatography on Sephadex G-25 before lyophilization of the PEGylated peptide.

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Formulation of DNA Nanoparticles

Compacted DNA was manufactured by adding 20.0 mL of DNA solution (0.1 mg/mL in water) to 2.0 mL of PEGylated condensing peptide (3.2 mg/mL in water) at a rate of 4.0 mL/min by a syringe pump and through sterile tubing ended with a blunt cannula. During this addition, the tube with peptide was vortexed at a controlled rate so that the two materials mixed instantaneously. Peptide and DNA were formulated at a final amine-to-phosphate ratio of 2:1. The DNP was then filtered through a vacuum-driven sterile filter with 0.2-μm polyethersulfone membrane. The filtered DNP was then processed with tangential flow filtration to remove excess peptide and exchange solvent for saline, and then was concentrated 20–30 fold using VIVASPIN centrifugal concentrators (MWCO 100k). The final concentrations of DNA were 4.1 μg/μl, 4.4 μg/μl, 4.1 μg/μl, and 4.3 μg/μl for *pGDNF_1a*, *pGDNF_1b*, *pGDNF_2b*, and *pGDNF_3b*. After formulation, the DNPs underwent several quality control tests, including sedimentation, turbidity, gel electrophoresis, and transmission electron microscopy, as described in (Liu et al., 2003, Ziady et al., 2003). Also, endotoxin levels were checked using an ENDOSAFE® PTS (Portable Test System) manufactured by Charles River Laboratories. Estimated number of transfecting nanoparticles: *pGDNF_1a* = 9.39×10^{11} particles/μl; *pGDNF_1b* = 9.88×10^{11} particles/μl; *pGDNF_2b* = 9.21×10^{11} particles/μl; *pGDNF_3b* = 9.66×10^{11} particles/μl.

Transfection of Ventral Midbrain Culture

The ventral mesencephalon from E14 fetuses were dissected on ice and placed into oxygenated calcium/magnesium-free buffer (CMF; 0.15 M NaCl, 8.0 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM $KHPO_4$, 26.0 mM $NaHCO_3$, 0.1% glucose, 100 mg/ml streptomycin, 2.5 mg/ml fungizone, pH 7.2). Embryonic tissue was pooled and rinsed with CMF; a cell suspension was prepared using a 0.125% trypsin solution (Invitrogen; Carlsbad, CA) followed by inactivation with 1.0 ml newborn calf serum (Invitrogen; Carlsbad, CA) and 500 μl of DNase [0.5 mg/ml] in 3.5 ml Hank's Balanced Salt Solution (Invitrogen; Carlsbad, CA). Cells were triturated with a 22g needle 15 times, placed drop-wise on top of sterile fetal bovine serum (Invitrogen; Carlsbad, CA), and spun at 1000 RPM for 10 minutes. Cells were plated on 24 well poly-D-lysine coated plates at a density of 3.75×10^5 cells per well in DMEM/F12/N2 medium. At 72 hours post-plating, cultured ventral midbrain cells were treated with 1 μg of naked GDNF plasmid in the presence of Lipofectamine™ and incubated for 4 hours. After 4 hours of incubation, medium was removed and DMEM/F12/N2 was added. Cells were fed every other day until day 7. The medium was removed and stored at −80°C for analysis of secreted GDNF protein. 200 μl of 1X cell culture lysis reagent (Promega; Madison, WI) was added to each well. Plates were placed on shaker in cold room for 30 minutes and samples were transferred to microcentrifuge vials. Samples were spun at 10,000 × g for 10 minutes at 4°C. Supernatant was stored at −80°C for intracellular GDNF protein analysis.

Animals

Male Sprague-Dawley rats at ages 4–5 months were obtained from Harlan Farms and used exclusively in this study. All rat procedures were conducted in strict compliance with approved institutional protocols, and in accordance with the provisions for animal care and use described in the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, NIH, 1985).

Stereotactic Microinjection of DNPs into Brain

DNA nanoparticles or plasmids alone were suspended in sterile saline and loaded into a sterile 30 gauge Hamilton syringe needle. Injections were made stereotactically into the brain of isoflurane-anesthetized rats at a rate of 0.5 μL/min for 4 minutes per site. DNPs

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were delivered to the brain using two needle tracts for injections made into the striatum. For each tract the injector was lowered to the deepest DV coordinate (DV_1), a 2.0 μL deposit of DNPs was made at this site, the injector was then retracted to the second site (DV_2) and a second 2.0 μL deposit was made at this site. Stereotactic coordinates: *Tract 1* AP +0.5, ML +3.6, DV_1 −6.5, DV_2 −5.0; *Tract 2* AP +0.5, ML +2.4, DV_1 −6.5, DV_2 −5.0. All stereotaxic adjustments were made with the skull in the flat position: AP and ML adjustments were made from the intersection of the sagittal and coronal sutures (bregma), and DV adjustments were made from the top of the skull; all stereotactic coordinates used in these studies were determined using a stereotaxic atlas (Paxinos and Watson, 2007).

6-hydroxydopamine Lesion

Some rats were given unilateral 6-hydroxydopamine (6-OHDA) lesions of the left nigrostriatal pathway; 6-OHDA (Sigma) was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 3.0 μg/μl and stereotaxically injected into the nigrostriatal pathway of anesthetized rats at a rate of 1.0 μl/min for 2 min. Each rat received two injections of 6-OHDA: one injection in the medial forebrain bundle (AP −4.4, ML 1.2, DV −7.5) area and the other in the rostral pars compacta of the substantia nigra (AP −5.3, ML 2.0, DV −7.5); all coordinates reported in this study represent millimeter adjustments from bregma (AP, ML) and below the dural surface (DV) with the top of the skull in a flat position. This technique routinely produces complete lesions of dopamine neurons in the A9 and A10 midbrain regions, and near complete denervation of dopaminergic fibers innervating the ipsilateral striatum (Thomas et al., 1994).

Tissue Dissection for ELISA Assays

For striatal dissections, brains were removed from euthanized animals and then placed upside down (ventral surface facing upwards) into a stainless steel brain matrix (RMB-4000C, ASI Instruments). For the following descriptions, the approximate stereotaxic coordinates are listed in parentheses. The initial coronal cut was targeted at a level just caudal to the optic chiasm (~AP −0.5) and a second cut was made 3.0 mm anterior to the initial cut. The 3.0 mm thick coronal slab formed by these two cuts was removed from the brain matrix and then divided into two pieces along the midline. Both the left and right striatum were dissected from the two pieces. A rectangular piece of tissue was dissected by making the following cuts: 1) a cut parallel to the midline was made just lateral to the lateral ventricle, 2) a cut parallel to the midline was made just medial to the lateral aspect of striatum at the striatum/corpus callosum border, 3) a cut perpendicular to the midline was made just ventral to the dorsal aspect of the striatum at the striatum/corpus callosum border, and 4) a cut perpendicular to the midline was made just dorsal to the anterior commissure. Dissected tissues were weighed, immediately frozen on dry ice and stored at −80°C until analyzed for protein content.

Quantification of GDNF by Enzyme-Linked immunosorbent Assay (ELISA)

Protein levels of GDNF were measured in culture media, VM culture cell lysates or tissue samples. Subsequently, each tissue sample was homogenized in 300 μl volumes of homogenate buffer [400 mM NaCl, 0.1% Triton-X, 2.0 mM EDTA, 0.1 mM benzethonium chloride, 2.0 mM benzamidine, 0.1 mM PMSF, Aprotinin (9.7 TIU/ml), 0.5% BSA, 0.1 M phosphate buffer, pH=7.4]. The homogenate was centrifuged for 10 minutes at 10,000 × g at 4°C. Tissue homogenates, VM culture cell lysates and culture media samples were assayed for GDNF content using a GDNF Emax™ ImmunoAssay System (Promega; Madison, WI).

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Statistical Analyses

The α level of significance was set at $p < 0.05$. Analysis of variance (ANOVA) was used for statistical analyses; choice of test was dependent on the experimental design. Descriptive statistics: means are reported with their corresponding standard error of the mean (s.e.m.). For the *in vivo* plasmid dose-response study, we used a 3-way ANOVA; the 3 factors were *plasmid*, *dose*, and *side* (left or right hemisphere). For the longitudinal analysis of GDNF protein levels, groups of treated animals were sacrificed at each time point and the amount of GDNF in the injected striatum was normalized to the amount in the non-injected (contralateral) striatum; a $\log_{10}$ transformation of the normalized data was performed in order to carry out a 1-way ANOVA to analyze for the factor of time after DNP injection.

RESULTS

Plasmid Optimization for human pGDNF

Primary ventral midbrain cultures were established and then transfected with the six different plasmids described in Table 1; all plasmids were derived from the pUL plasmid (Figure 1) and encoded for hGDNF splice variants 1 or 2. Four days after transfection, media was removed and analyzed for secreted and intracellular GDNF using ELISA (Figure 2). Statistical analyses of GDNF protein values revealed a significant treatment effect for secreted GDNF [$F(5,6)=19.03$, p<0.001] as well as intracellular GDNF protein [$F(5,6)=5.16$, p=0.035]. From the protein analyses generated from the VM culture studies, we chose the 4 best plasmids (*pGDNF_1b, pGDNF_2b, pGDNF_1a, and pGDNF_3b*) and then we compacted each plasmid individually into DNPs for an *in vivo* dose-response study.

DNA nanoparticles containing *pGDNF_1b, pGDNF_2b, pGDNF_1a,* or *pGDNF_3b* were unilaterally injected into the striatum of naïve rats at the following doses: 0, 2.0, 4.0, 8.0, 16.0, or 32.0 μg; all injections were made in an equivalent volume (8.0 μl) of sterile 0.9% saline as described in the Methods section. One week later, the injected and non-injected striata were dissected from each animal and GDNF content in each sample was determined using ELISA. Figure 3 summarizes the results from the *in vivo* dose-response study. Three-way ANOVA of dose-response data revealed a significant *Plasmid × Dose × Side* interaction [$F(12,154)$= 11.39, p<0.001]. At the highest dose (32.0 μg), *pGDNF_1b* and *pGDNF_2b* DNPs produced the highest levels of striatal GDNF protein that were not statistically different from one another but both were significantly greater than GDNF levels produced by the two other DNPs. However, GDNF levels for *pGDNF_2b* DNPs dropped precipitously for the next two lower doses while GDNF levels remained fairly stable for the next two lower doses of *pGDNF_1b* DNPs. At the 8.0 μg dose, *pGDNF_1b* DNPs produced GDNF levels that were significantly greater and >10 fold higher than GDNF levels for all other DNPs; at this dose (8.0 μg), GDNF levels were not significantly greater than baseline levels for all compacted plasmids except for *pGDNF_1b*. The two control injections, sterile saline (vehicle; 8.0 μl) or pUL3 DNPs (16.0 μg) produced no significant change in striatal GDNF levels when compared to baseline GDNF levels. Based upon the results of this dose-response study, we chose to examine the long-term transgene expression of GDNF following intrastriatal injection of *pGDNF_1b* DNPs at the 16 μg dose level.

DNA nanoparticles containing *pGDNF_1b* were injected in the left striatum of naïve young adult rats and animals were euthanatized at 7, 14, 28, 56, 98, 130, 150, or 180 days post-injection, at which time tissue from the right (non-injected) and left (injected) striata was dissected and subsequently analyzed for GDNF protein content. At initial time points (7 and 14 days), GDNF protein values in the injected striatum were 8–16 fold higher than in the non-injected striatum (Figure 4). One month following the injection, GDNF protein levels in

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the injected striatum stabilized at values that were 3–6 fold higher than in the non-injected striatum for the remaining 5 months of the study.

A second group of animals received 6-OHDA lesions 4 weeks before the injection of either DNPs or saline and were euthanatized at 28 and 98 days post-injection. Statistical analysis of striatal GDNF protein values revealed a significant treatment effect [$F(3,26) = 136.0$, $p<0.001$]. DNA nanoparticles injected into the intact or lesioned striatum produced significantly greater GDNF levels than in the intact or lesioned striatum of saline treated animals (Figure 5). Injections of DNPs into the lesioned striatum produced significantly higher GDNF levels than those measured in the intact striatum of DNP treated animals.

DISCUSSION

Our initial studies examined *in vitro* GDNF expression in cells that were treated with plasmids encoding for splice variants 1 or 2 of human GDNF cDNA and further modified to encode for either a codon-optimized and CpG-depleted synthetic 'gene' and a variant with nucleosome non-friendly elements. In theory, insertion of nucleosome non-friendly elements into the plasmid should reduce the number of DNA-histone complexes and thus provide a more favorable state for DNA transcription. Likewise, a reduction in the number of CpG dinucleotides in coding and non-coding regions of the plasmid can improve the rate of gene transcription because methylation of CpGs located close to the promoter can increase the likelihood of gene silencing (Kass et al., 1997). All vector types were screened in our *in vitro* studies and at least one natural cDNA, codon-optimized, or nucleosome non-friendly hGDNF plasmid was compacted into DNPs and tested *in vivo*. In terms of GDNF transgene expression in both the *in vitro* and *in vivo* studies, plasmids encoding for natural cDNA or custom designed synthetic hGDNF 'genes' as longer splice variant 1 performed better than the analogous plasmids encoding for the shorter splice variant 2. These findings are consistent with an earlier report suggesting the longer splice variant generates improved levels of secreted GDNF protein (Wang et al., 2008). At the 8 and 16 μg *in vivo* doses, DNPs containing plasmids encoding for natural hGDNF cDNA for splice variant 1 produced statistically significant higher levels of GDNF than DNPs containing plasmids encoding for natural hGDNF cDNA for splice variant 2. Even though the highest *in vivo* dose (32.0 μg) of *pGDNF_2b* DNPs produced comparable levels of GDNF to *pGDNF_1b* DNPs, GDNF levels dropped precipitously for the next two lower doses of *pGDNF_2b* DNPs while GDNF levels remained stable for *pGDNF_1b* DNPs at these same doses. It is noteworthy that the hGDNF cDNA produced higher GDNF levels than the two synthetic GDNF DNA 'genes' tested. The lack of effectiveness of a codon-optimized GDNF compared to the natural cDNA is perhaps not too surprising, since the ability of codon optimization to improve gene expression is variable (Welch et al., 2009). The *pGDNF_1b* DNPs appeared to have the best *in vivo* GDNF expression profile and for this reason we moved forward with *pGDNF_1b* DNPs in our longitudinal studies.

While we observed significantly higher GDNF transgene expression in the striatum transfected with *pGDNF_1b* DNPs when compared to the striatum transfected with *pGDNF_1b* DNA alone or basal levels in the non-transfected striatum, the tissue level of transgene activity is lower than that achieved with viral vectors [18]. We observed sustained GDNF transgene activity at levels 300–400% above basal levels for a 6 month period after a single dose of DNPs while AAV-GDNF can increase GDNF tissue levels to 100–1,000 fold higher than baseline over the same period. However, it is not known exactly what tissue levels of GDNF need to be achieved for therapeutic purposes, and this level may be close to that achieved by DNPs. Furthermore, continuous high level expression of GDNF is undesired and may lead to unwanted side effects, such as down-regulation of tyrosine hydroxylase (Georgievska et al., 2004). Levels of GDNF transgene expression at several

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fold above baseline may be sufficient for phenotypic correction. For example, AAV-GDNF treatments that produced a 300% increase of GDNF protein levels in striatal tissue over basal levels were shown to be effective in protecting the nigrostriatal pathway against 6-OHDA neurotoxicity (Eslamboli et al., 2005). This is the same level of GDNF over-expression that we observed at the end point of our experiment using DNPs; of note, we observed fairly stable luciferase transgene activity in the striatum of mice for a period greater than 1 year following a single dose of DNPs (Yurek et al., 2011). In a previous study we used rat GDNF DNPs to transfect the striatum prior to engrafting fetal dopaminergic neurons and observed enhanced survival of grafted TH+ cells when compared to grafted cell survival in the non-transfected striatum (Yurek et al., 2009a); whether or not the increased neurotrophic activity was limited to the injection site of the DNPs remains to be determined. Secondly, we also observe that intrastriatal injections of DNPs do not result in transport of the vector and/or transgene protein to the SN (Yurek et al., 2009b). On the other hand, intrastriatal injections of AAV can increase transgene protein levels in the SN as well as in the striatum. This may be simply due to the fact that AAV produces high-level transgene expression, and excess protein is transported via striatonigral neurons to the SN while low-level production of GDNF using DNPs is not transported to the SN to any significant extent. Alternatively; it may also be the case that AAV itself is transported to the ventral midbrain and transduce cells in this region. For some clinical indications, such as PD, only focal therapeutic gene expression may be desired, whereas multi-region spread of expression may lead to toxicities.

We observed that the same dose of *pGDNF_1b* DNPs injected into the lesioned striatum of young adult rats produces significantly higher levels of GDNF transgene expression than when injected into the intact striatum of young adult rats. While we have shown in previous studies that the lesion itself may induce a compensatory increase in endogenous GDNF levels, the lesion-induced increase is transient and typically returns to baseline levels several weeks following the lesion (Yurek and Fletcher-Turner, 2001); therefore, it is unlikely that endogenous GDNF contributed to the increased GDNF levels observed in the lesioned/transfected striatum 4–14 weeks post-lesion. It is not exactly clear why the lesioned striatum would show a higher rate of transfection than the intact striatum; however, one distinct possibility may be related to how the target cells of DNPs might be altered by the degeneration process itself. In our previous studies using DNPs to deliver reporter genes to the brain, we observed long-term transgene activity occurred primarily in astrocytes (Yurek et al., 2009b). Several studies have shown degeneration of the nigrostriatal pathway can lead to profound increases in GFAP [astrocyte] expression in the striatum and ventral midbrain. For instance, Strömberg *et al.* (Stromberg et al., 1986) was first to demonstrate a significant up-regulation of astrocytes in the striatum and substantia nigra following lesions of the nigrostriatal pathway using 6-OHDA or MPTP in rats or mice, respectively, that persisted for at least one month post-lesion. Subsequently, Sheng *et al.* (Sheng et al., 1993) reported 6-OHDA lesions produced a persistent up-regulation of GFAP+ cells in the lesioned striatum that was 170% greater than in the control striatum at 28 days post-lesion while Dervan *et al.* (Dervan et al., 2004) reported a >200% increase in the number of astrocytes in the striatum of mice 6–8 weeks following MPTP administration. Similarly, Rodriques *et al.* (Rodrigues et al., 2004) reported 166% increase in GFAP IHC in the ipsilateral ventral midbrain of 6-OHDA treated rats at a 22 days post-lesion time point. In a rat model of PD, Gordon *et al.* (Gordon et al., 1997) demonstrated that there is an exaggerated astrocyte reactivity in striatum of aged animals treated with 6-OHDA when compared to younger lesioned animals. Clearly, these results provide intriguing evidence that the cells targeted by DNPs, astrocytes, increase significantly as a result of nigrostriatal pathway degeneration, and it may be the case that the observed increase in GDNF levels in the lesioned striatum treated with DNPs is related to this increase in GFAP activity. This is significant because the pathological state of neurodegeneration may actually lay a foundation that is actually

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beneficial for this particular type of gene therapy while other gene therapy techniques, *e.g*, AAV, that target neurons are actually transducing cells that are decreasing as the disease progresses. From this standpoint, viral and non-viral gene therapies may actually complement one another in that they can approach the same disease state by affecting different cell types.

Highlights

- ➢ We tested a non-viral gene transfer technique in brain.
- ➢ Plasmids were optimized to achieve long-term expression in brain tissue.
- ➢ Optimized plasmids were compacted into DNA nanoparticles and injected into brain.
- ➢ One plasmid/DNP complex achieved long-term transgene activity in brain.
- ➢ DNA nanoparticles may be a potential therapeutic for Parkinson's disease.

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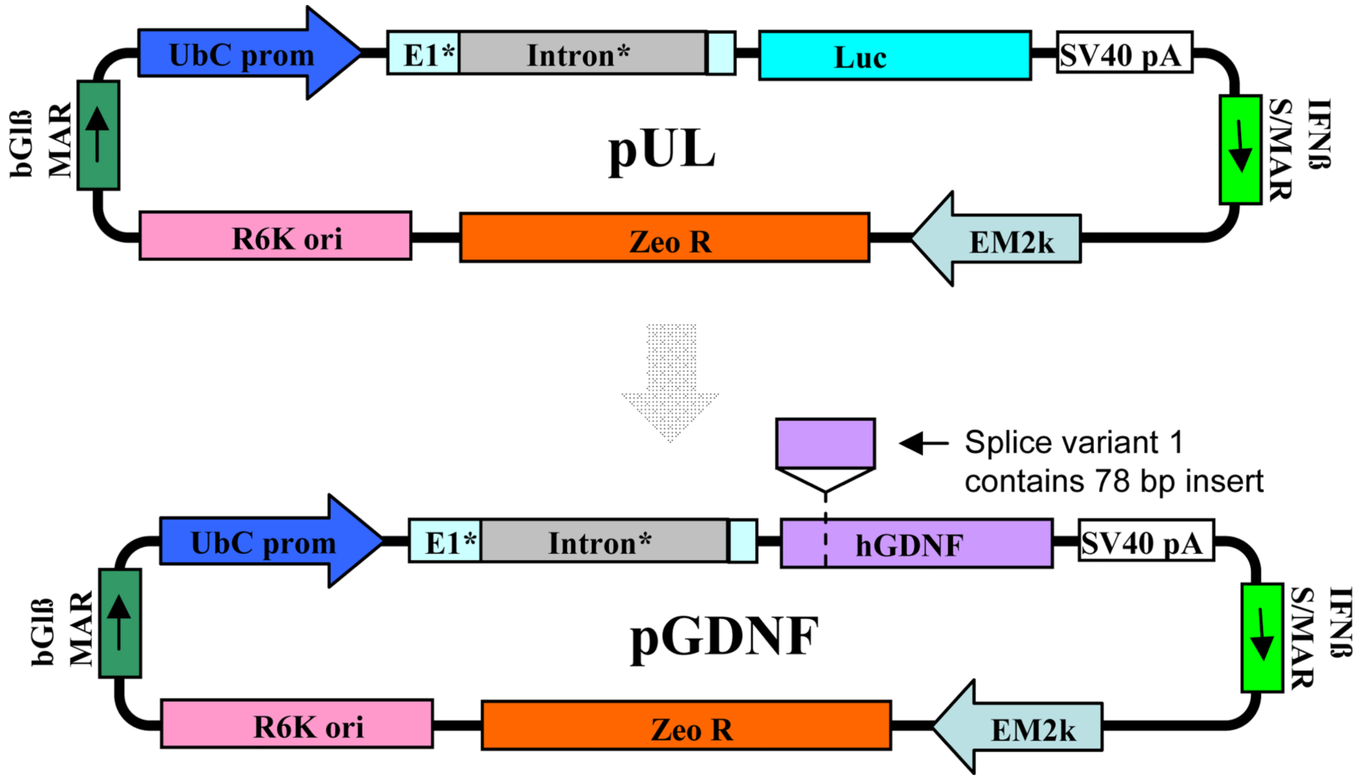

Figure 1.
Plasmid maps for pUL and pGDNF. The pGDNF plasmid used in this study was derived from the pUL plasmid, which had been successfully tested in previous studies (Yurek et al., 2011). Six different derivatives of pGDNF were tested in this study, as described in the text.

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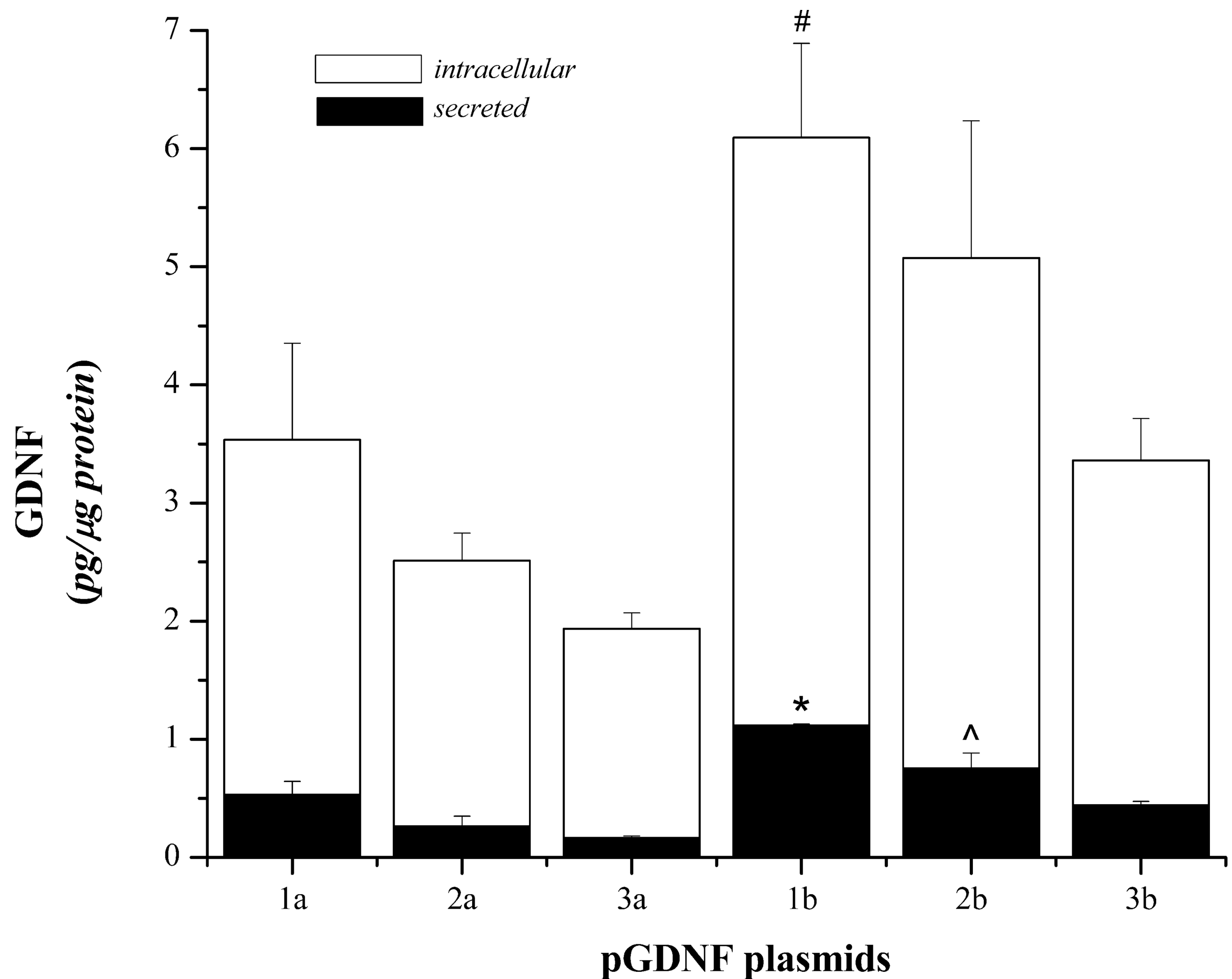

Figure 2.

Secreted and intracellular GDNF in primary ventral midbrain cultures transfected with pGDNF plasmids. Ventral midbrain cultures were established as described in the Methods section and transfected with one of the following plasmids: *pGDNF_1a* (1a), *pGDNF_1b* (1b), *pGDNF_2a* (2a), *pGDNF_2b* (2b), *pGDNF_3a* (3a), or *pGDNF_3b* (3b). Each bar represents the average (± s.e.m.; n=5) amount of GDNF protein detected in cells or secreted in the culture media. *p<0.05, 1b vs. 1a, 2a, 3a, 2b, 3b; ^p<0.05 2b vs. 3a, 2a; #p<0.05 1b vs 2a, 3a, 3b.

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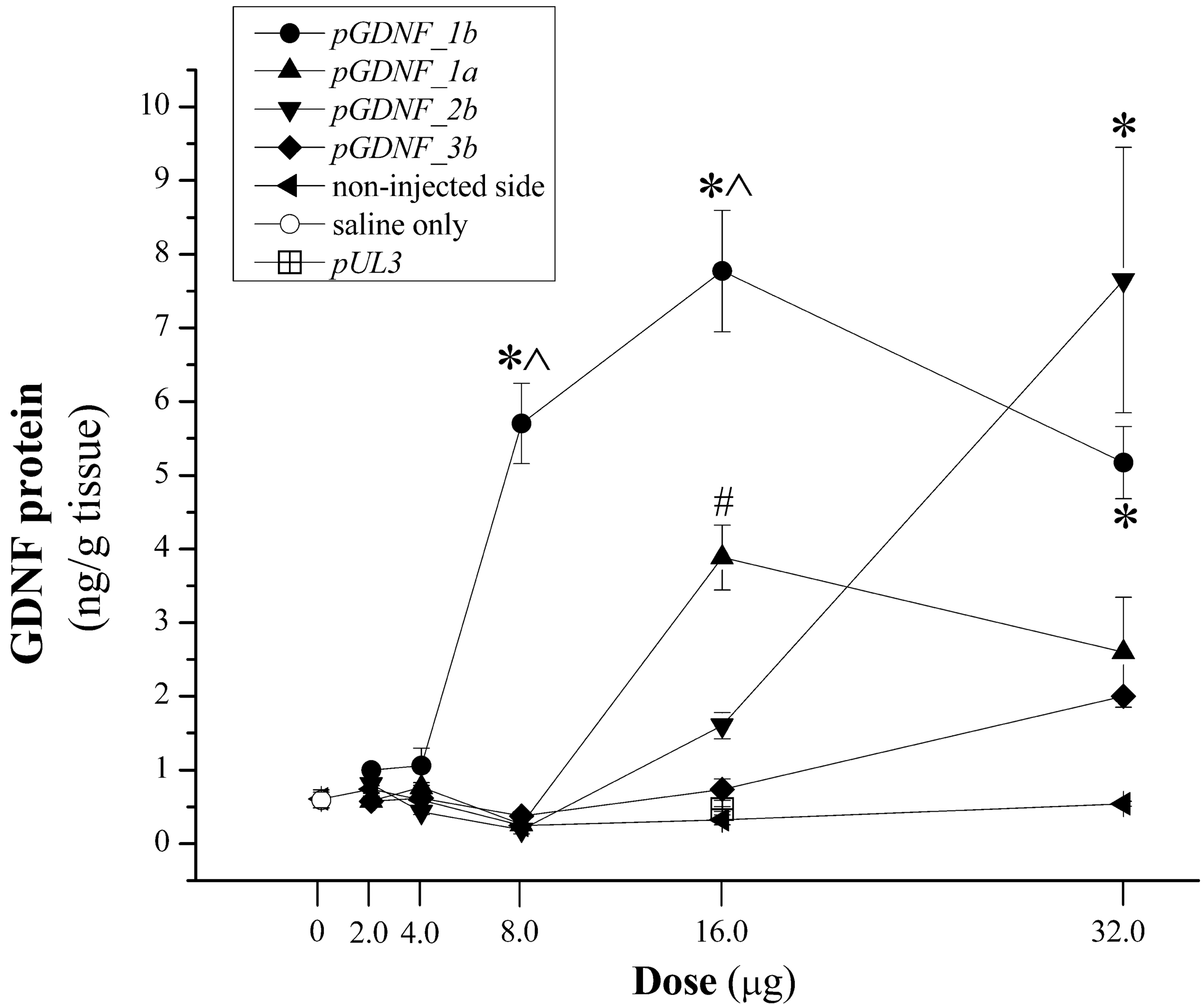

Figure 3.

Dose-response study for intracerebral injections of DNPs containing *pGDNF_1a*, *pGDNF_1b*, *pGDNF_2b*, or *pGDNF_3b*. Various doses of DNPs containing 1 of the 4 plasmids were injected in equivalent volumes (8.0 μl) of sterile saline into the left striatum. One week after DNP injection GDNF protein was measured in the injected or non-injected striatum using ELISA, and each point on the graph represents the mean (± s.e.m.; n=5) of GDNF protein for each plasmid at each dose. There is only one point for two control conditions: saline (8.0 μl; n=5) and pUL3 (16.0 μg; n=5); pUL3 is a plasmid encoding for luciferase and does not encode for GDNF. *p<0.05 vs. *pGDNF_1a, pGDNF_3b, non-injected side*; ^p<0.05 vs. *pGDNF_2b*; #p<0.05 vs. *pGDNF_2b*, *pGDNF_3b*, *non-injected side*.

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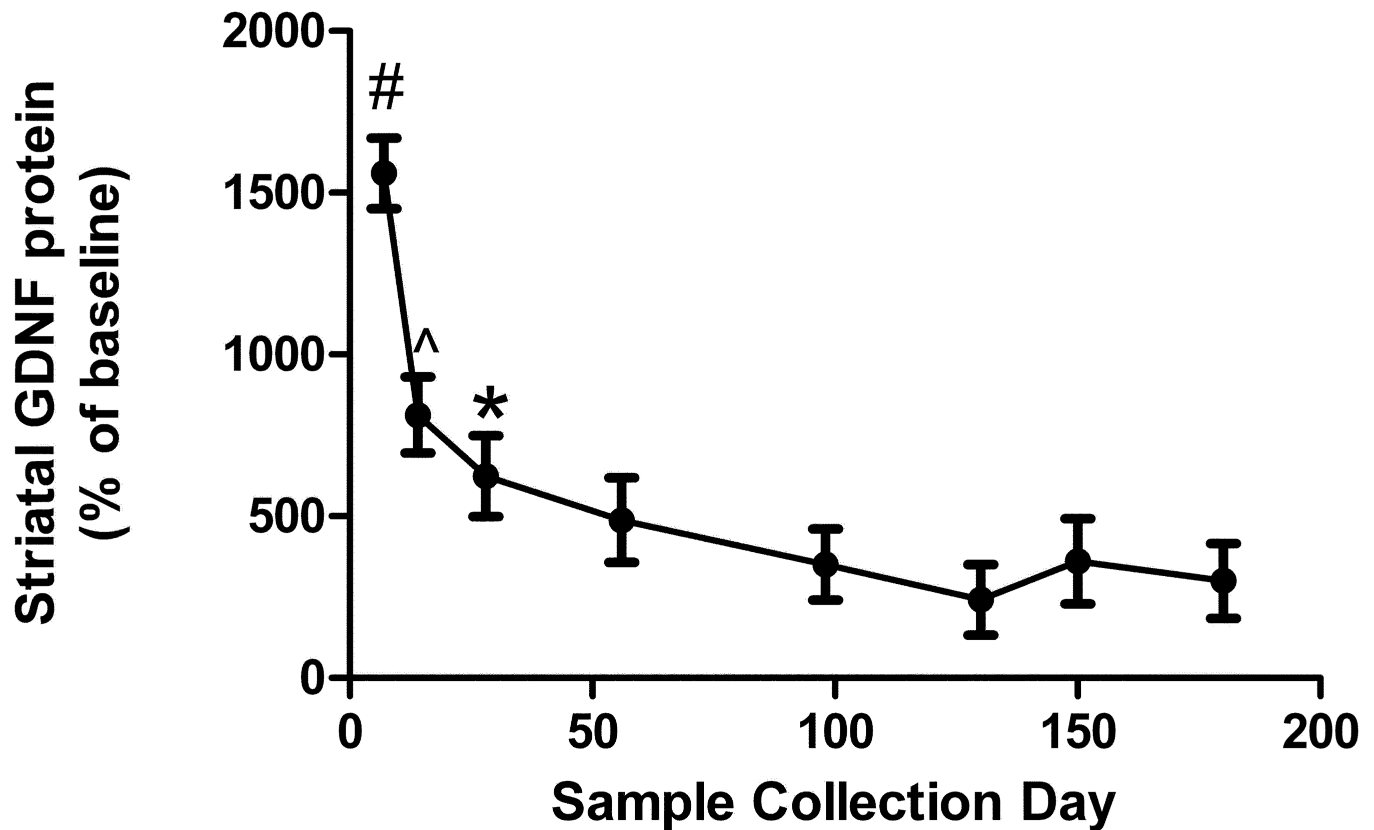

Figure 4.

Time course of GDNF protein expression in the striatum during a six month period following a single administration of DNPs containing *pGDNF_1b* (16.0 μg). Animals were euthanized in groups of 5 at each time point and the injected and non-injected striatum were dissected from the brain. In each dissected sample GDNF protein content was determined by ELISA and data are graphed as a percent of baseline; that is, GDNF protein values for the injected side were divided by the values for the non-injected side (baseline). ^$p<0.05$ vs. Days 98, 130, or 180; #$p<0.05$ vs. all other days; *$p<0.05$ vs. Day 130.

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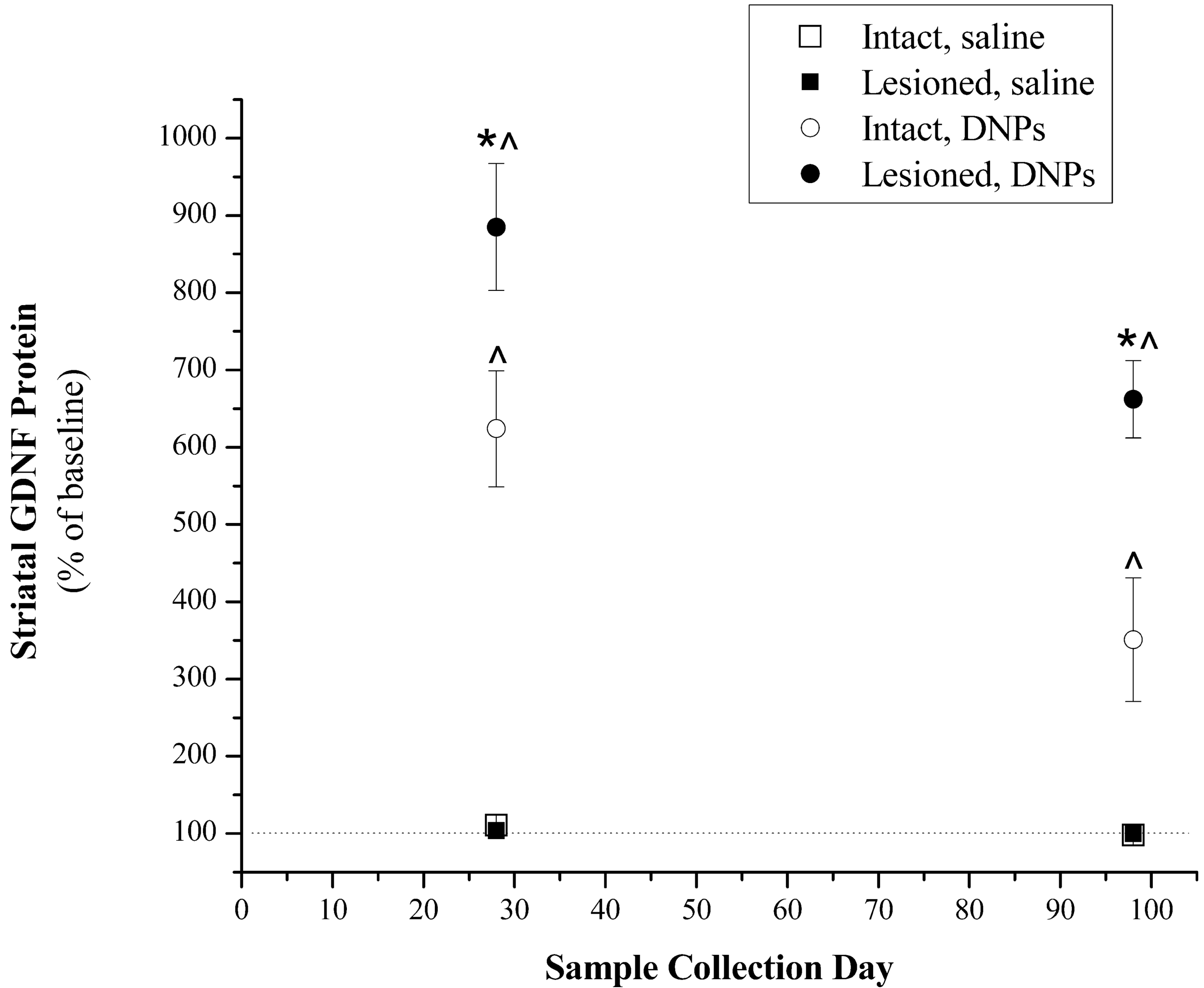

Figure 5.

Striatal GDNF protein levels at 28 or 98 days following a single injection of *pGDNF_1b* DNPs or saline into the intact or lesioned striatum. This study used naïve rats or rats with unilateral 6-OHDA lesions. Saline (8.0 µl) was injected into the left striatum of naïve rats (Intact, saline; n=5) or into the denervated striatum 4 weeks after 6-OHDA treatment (Lesioned, saline; n=5). *pGDNF_1b* DNPs (16.0 µg) was injected into the left striatum of naïve rats (Intact, DNPs; n=5) or into the denervated striatum 4 weeks after 6-OHDA treatment (Lesioned, DNPs; n=5). Animals were euthanized either 28 or 98 days following the saline or DNP injection and the injected and non-injected striatum were dissected from the brain. In each dissected sample GDNF protein content was determined by ELISA and data are graphed as a percent of baseline; that is, GDNF protein values for the injected side were divided by the values for the non-injected side (baseline). * $p<0.05$ Lesioned, DNPs vs. Intact, DNPs at 28 and 98 days; ^$p<0.05$ Lesioned, DN Ps or Intact, DNPs vs. Intact, saline or Lesioned, saline at 28 or 98 days.

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Table 1

Vector	Splice Variant	Vector Description
pGDNF_1a	2 (short)	pUL derivative with natural hGDNF cDNA
pGDNF_1b	1 (long)	
pGDNF_2a	2	pUL derivative with codon optimized hGDNF
pGDNF_2b	1	
pGDNF_3a	2	pUL derivative with nucleosome non-friendly hGDNF
pGDNF_3b	1	