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RESEARCH ARTICLE

Comparative Studies of Continuous Versus Pulsatile Delivery of a Novel Mammalian Cell-Derived Variant of GDNF (GDNFv) into the Rhesus Macaque Striatum

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) remains a promising disease modifying therapeutic agent for the dopamine-containing neurons that are affected in Parkinson's disease and recent clinical findings show renewed promise for its use in patients with Parkinson's disease. However, translating this approach from research laboratories to the clinic has been met with obstacles, including insufficient brain biodistribution, immunogenicity, and poor stability of unglycosylated wildtype GDNF produced from bacteria. We have previously reported that continuous infusion of a novel glycosylated mammalian variant of GDNF (GDNFv) has increased biodistribution compared to wildtype GDNF along with increased dopamine turnover in the non-human primate brain. Here, we extend these findings by comparing continuous versus pulsatile intrastriatal infusion of GDNFv in intact rhesus macaques. Intermittent, pulsatile delivery paradigms were explored to possibly enhance drug distribution in the brain while decreasing the total amount of drug and infusion volume needed to achieve target activation. Vehicle or GDNFv was directly administered into the putamen via a pump and catheter system using a constant flow rate or using pulsatile profiles of two patterns: pulsatile infusion of 24-hour duration or 48-hour duration. Study endpoints involved comparisons of brain biodistribution, retrograde transport to nigral neurons and dopamine turnover. Each catheter was placed in or near the center of the putamen as confirmed by post-operative magnetic resonance imaging. Our results support that continuous and pulsatile administration of GDNFv was well tolerated in all animals. In addition, pulsatile delivery of GDNFv demonstrated favorable physiological activity of potential therapeutic value with biodistribution, retrograde transport to nigral cells and significant dopamine turnover modulation comparable or better than that achieved with continuous flow delivery. More importantly, the animals administered GDNFv via pulsatile protocols only received half the total drug amount and half the infused volume used in the continuously-infused animals, while still attaining a similar efficacy in increasing dopamine turnover. These data suggest that pulsatile delivery of trophic factors, such as GDNFv, may be a viable disease altering strategy for patients with Parkinson's disease by offering a means to reduce the drug amount needed to improve dopamine function while limiting potential therapeutic barriers.

Introduction

More than 30 years after its original discovery, glial cell line-derived neurotrophic factor (GDNF)[1] continues to be a promising molecule for improving the function of dopamine (DA) neurons in patients with Parkinson's disease (PD). Bacteria-derived, full-length, wildtype human GDNF (GDNFwt) has demonstrated both neuroprotective and neurorestorative properties *in vitro*[1-2] and in multiple species *in vivo* including rodent and non-human primate PD models[3-7]. Due to inherent blood-brain barrier limitations involved in delivering drugs to the brain coupled with the heparin-binding characteristics of GDNF, paradigms investigating how to best deliver GDNFwt intracranially to the brain have been extensively investigated[8-10]. Studies have focused on determining the best dosing protocol that provides therapeutic benefit and correlations with distribution, dose and other drug delivery variables such as pulsatile delivery. Using convection-enhanced delivery to distribute GDNFwt over a large brain volume is a promising delivery approach particularly when considering achieving adequate distribution in the human brain. However, increasing GDNFwt dose did not confer further behavioral improvement in hemiparkinsonian non-human primates, suggesting that once a critical threshold is attained, GDNFwt dose reaches a plateau for trophic stimulation of DA neurons[11].

As an added challenge, translating GDNFwt delivery approaches from animal studies to the clinic has proven to be difficult. Eight clinical trials testing intraparenchymal delivery of GDNF or neurturin, a member of the same neurotrophic factor family, have been conducted in >200 participants with PD and reported in peer-reviewed scientific journals[12-19]. At least four different methods for drug delivery into the brain have been used: 1) bolus injections into the cerebral ventricles, 2) continuous putamenal infusion, 3) intermittent convection-enhanced delivery to the putamen and 4) gene delivery via direct infusion to various brain targets including the putamen or both the putamen and substantia nigra. The open-label Phase 1 studies have repeatedly shown the safety of the drug and delivery strategies. Although these Phase 1 safety trials did not have a control arm, significant improvements in parkinsonian symptoms seen in drug recipients suggested efficacy. However, randomized, controlled, and double-blinded Phase 2 trials did not demonstrate efficacy over the placebo control group. Placebo-related improvements in clinical trials require consideration since they can be pronounced and long lasting in patients with PD[20]. Nevertheless, the absence of clinical efficacy in the Phase 2 trial participants has been attributed to several other factors[10], including: 1) insufficient dose[21], 2) insufficient tissue distribution of GDNFwt in the brain parenchyma[22], 3) immunogenicity[23] and/or 4) poor stability of the unglycosylated, *E. coli*-produced GDNFwt[24]. Moreover, a recent extended treatment study supports that a minimum of 18 months of neurotrophic factor treatment may be necessary to help control for placebo effects and to see potential clinical efficacy more clearly in disease progression[25].

Strategies to achieve optimal delivery and distribution of GDNF are of continued interest[18] with recent Phase 1 clinical trials utilizing real-time intraoperative MRI to investigate distribution of GDNF in patients with PD using intracranial infusion of a surrogate tracer along with adeno-associated virus serotype 2 vectors containing the human GDNF transgene[26]. In addition, systemic delivery using viral vector variants has also been investigated as an alternative approach to direct brain infusion methods but requires higher doses and lacks target specificity[19]. To address some of these challenges, a novel form of GDNF expressed and purified from mammalian cells was developed by Smith and collegues[27]. This glycosylated GDNF variant (GDNFv) was modified to increase tissue distribution by reducing binding to the extracellular matrix through removal of the N-terminal heparin-binding domain. In addition, key amino acids were substituted to improve chemical stability. GDNFv was demonstrated to be biologically comparable to GDNFwt in a series of *in vitro* studies based on several key outcome measures including: 1) binding to the human and rat GFRα-1 receptors, 2) c-Ret phosphorylation, and 3) neurite outgrowth in PC-12 cells[27]. GDNFv was also shown to have increased chemical stability and lower predicted clinical immunogenicity compared to GDNFwt[27]. Importantly, and consistent with reduced heparin binding to the extracellular matrix, GDNFv showed increased biodistribution versus GDNFwt in intact rats administered a single drug injection into the striatum[27]. Furthermore, GDNFv was shown to increase DA turnover up to 10 days post infusion of a single intrastriatal injection in intact rats and to protect midbrain DA neuron function for at least 21 days in 6-hydroxydopamine-lesioned rats treated with a single intrastriatal injection of GDNFv[27]. We have previously reported that continuous, intrastriatal administration of GDNFv has increased biodistribution compared to GDNFwt along with increased DA turnover in the larger non-human primate brain[10]. Together, these

data support that the functional effects observed with GDNFv compare favorably to GDNFwt when sufficient biodistribution is attained in the brain regions of interest.

The current study was designed to extend these findings in non-human primates administered GDNFv or vehicle (PBS) into the putamen via a pump and catheter delivery system using a constant flow rate or using pulsatile profiles of two patterns for comparisons of brain biodistribution and DA turnover. Due to the long-lasting pharmacodynamic effects of GDNF, the pulsatile delivery approach is of interest and may be preferred clinically by offering important advantages to patients with PD, including reduced drug exposure and reduced infusate volume. These factors are clinically relevant because a reduction in drug exposure to the brain and reduction in the amount of drug delivered would avoid continuous activation of GFRα1 signaling pathways and reduce the amount of drug needed to achieve therapeutic levels for the treatment of patients with PD. Moreover, the most recent clinical trial of GDNFwt used intermittent, convection-enhanced delivery of GDNFwt[17,27]. Thus, comparative studies of continuous versus pulsatile infusion of GDNF in the non-human primate are needed to further investigate the capabilities of intermittent, pulsatile delivery of GDNF.

Materials and Methods

ANIMALS

Twenty female rhesus macaques (*Macaca mulatta*) ranging in age from 4 to 16 years old and weighing 4 to 10 kg were obtained from commercial vendors and housed in the AAALAC-accredited vivarium at the University of Kentucky Division of Laboratory Animal Resources. All procedures were approved by the University of Kentucky Institutional Animal Care and Uses Committee. Each animal was identified by a permanent skin tattoo. While on study, the animals were housed individually to help preserve the integrity of the delivery system and maintained on a 12-hour light/12-hour dark cycle in temperature- and humidity-controlled rooms. Animals were provided toys and other sterilized items for enrichment. Except prior to procedures requiring sedation, each animal was fed a standard, commercial diet of non-human primate chow (Harlan 2050 Teklad Global 20% Protein Primate Diet). Feed was given once in the morning and supplemented in the afternoon with fresh fruit or vegetables. Municipal tap water, purified by reverse osmosis, was available *ad libitum* via an automated watering system.

STUDY DESIGN AND CATHETER SPECIFICATIONS

The studies were designed to determine brain biodistribution and functional effects on dopaminergic neuronal systems following MRI-guided intraputamenal delivery of GDNFv using pulsatile versus continuous infusion patterns in the normal adult non-human primate brain. GDNFv was delivered via a surgically implanted programmable pump coupled to an experimental catheter (1.0 mm O.D.) made of 55D urethane (model 8683NHP, Medtronic Inc., Minneapolis, MN) and terminating in a closed-off, platinum iridium needle tip (0.20 mm O.D.) designed to minimize tissue damage. Two lateral side ports (0.10 mm in diameter) were positioned at 0.5 mm from the most distal end of the closed-off tip.

Vehicle (PBS) was used as control. All group assignments were randomly selected. First, all animals were ranked in descending weight. Next, the randomization involved sequentially assigning the rank-ordered animals to one of four treatment groups, A through D (N=5 animals per group). Then, a commercial statistics software (Minitab 15) was used to randomize the groups across all surgery days. All animals were infused with PBS/vehicle at neutral pH for 7 days post-operatively. Following the surgical recovery period, animals were administered either PBS or GDNFv provided in sterile, coded vials (A through D). Group membership was unblinded at the end of the study, upon collection of all raw data. As outlined in **Table 1**, animals were dosed for 17 days with either a constant flow rate of 216 µL/day (GDNFv) or with one of two pulsatile profiles: i.e., an infusion rate of 216 µL/day for 24 hours then dropping to a lower basal infusion rate of 48 µL/day for 6 days (GDNFv) or an infusion rate of 216 µL/day for 48 hours then dropping to a lower basal infusion rate of 48 µL/day for 5 days (GDNFv or PBS). In this way, all animals in the pulsatile treatment groups received three bolus infusions of either 24- or 48-hour duration over the 17-day infusion period (**Figure 1**) for a total dose of 0.40 mg and 0.55 mg, respectively. In contrast, the animals continuously infused with GDNFv received a total dose of 1.10 mg. These are estimates because pump programming (performed between 10am and 12pm) and brain collection (performed 17 days later between 10am and 3pm) did not take place at the exact same time of day for some animals, accounting for a maximum difference of 1.2% in total dose.

Table 1: Test Groups (N = 5 animals / group) and Dosing Parameters

Infusate	Concentration (mg/mL)	Infusion pattern (bolus duration)	Infusion rate (µL/day)	Infusion period (days)	Total volume (mL)	Total dose (mg)
GDNFv	0.3	Continuous	216	17	3.67	1.10
GDNFv	0.3	Pulsatile (24hr-long bolus)	216 (bolus rate) 48 (basal rate)	3 (bolus infusion) 14 (basal infusion)	1.32	0.40
GDNFv	0.3	Pulsatile (48hr-long bolus)	216 (bolus rate) 48 (basal rate)	6 (bolus infusion) 11 (basal infusion)	1.82	0.55
PBS (vehicle)	0	Pulsatile (48hr-long bolus)	216 (bolus rate) 48 (basal rate)	6 (bolus infusion) 11 (basal infusion)	1.82	0.00

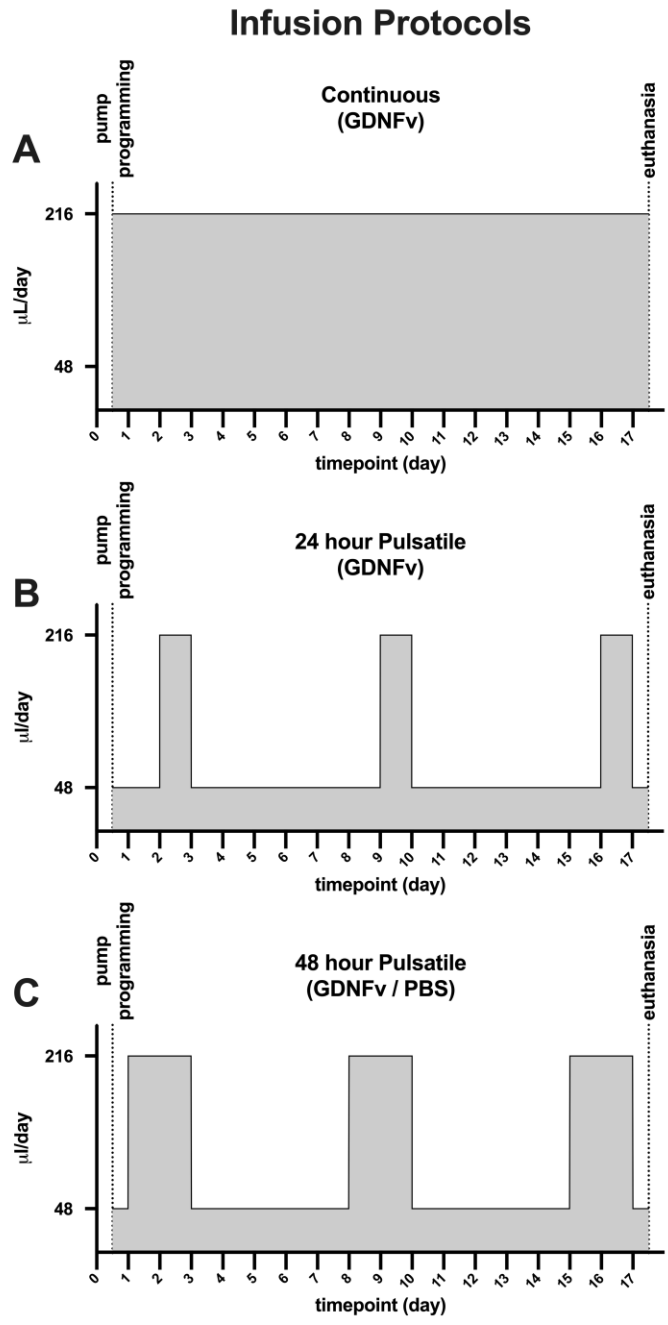

Figure 1:
The infusion pump was programmed to deliver the dosing solution via one of three patterns: A) Continuous, B) Pulsatile with a 24-hour infusion bolus duration or C) Pulsatile with a 48-hour infusion bolus duration. GDNFv was used in all infusion protocols (panels A-C). PBS was used in the 48-hour pulsatile pattern only (panel C).

GDNFv

GDNFv was expressed in Chinese Hamster Ovary (CHO) cells. The gene encoding human GDNFv was prepared using standard molecular biology and sub-cloned into the Glutamine Synthetase (GS)-containing expression plasmid backbones (pEE12.4-based plasmids, Lonza Biologics, Slough, UK) in frame with the murine kappa signal sequence[27]. GDNFv was purified to homogeneity through 4-step column chromatography as per methods described by Smith and colleagues[27]. GDNFv was modified from GDNFwt in two important ways: 1) N-terminal truncation of 31 amino acids and 2) amino acid substitutions at positions 38 (N38Q) and 95 (D95E) of the original GDNFwt sequence[27].

MRI-GUIDED STEREOTACTIC SURGICAL PROCEDURES

Animals were sedated with ketamine (10-20 mg/kg, IM) admixed with atropine (0.02-0.04 mg/kg, IM) and intubated for administration of isoflurane gas anesthesia (1-4%) in 100% oxygen to effect. The anesthetized animals were placed in a stereotaxic headframe in a ventral-lateral position for subsequent magnetic resonance imaging (MRI) acquisition taken on a Siemens 3T Tim Trio scanner to guide unilateral catheter placement into the right putamen. Heat support was provided during the imaging sessions via a water-circulated pad and vital signs were monitored by a trained technician.

Following acquisition of brain coordinates by MRI, the animals were given a slow-release analgesic (buprenorphine SR, 0.06−0.09 mg/kg, IM) and moved to a sterile surgical suite. Next, the abdominal and cranial surgical sites for pump and catheter implantation, respectively, were prepped using antiseptic techniques. Then, an incision was made through the scalp under sterile field conditions and the skin and muscles overlying the skull were reflected. Antero-posterior, lateral and vertical coordinates for stereotaxic catheter placement were determined immediately prior to the surgery and derived from T1-weighted coronal brain images for each animal in the following ranges: 16-25 mm at the antero-posterior coordinate (i.e., distance from the interaural line to the target), 9-12 mm at the lateral coordinate (i.e., distance from the sagittal sinus/third ventricle to the target) and 20-24 mm at the vertical coordinate (i.e., distance from the surface of the brain to the target). A small burr hole was drilled in the skull directly over the right putamen using the antero-posterior as well as lateral coordinates and the overlying meninges were punctured to expose the surface of the brain. The experimental catheter (model 8683NHP, Medtronic Inc., Minneapolis, MN) was lowered for insertion into the center of the putamen using the vertical coordinate and subsequently connected to an anchor (model 8684NHP, Medtronic Inc., Minneapolis, MN) attached to the skull with titanium screws. Next, a 20-mL capacity SynchroMed® II pump (model 8637-20, Medtronic Inc., Minneapolis, MN) was surgically implanted into the lateral abdominal region and connected to the skull anchor by tubing tunneled under the skin. After completion of the procedures, the incisions were sutured over the exposed areas. Heat support and fluid replacement were provided during the surgical procedures and vital signs were monitored by a trained technician. Following the surgical procedures, a contrast agent (5mM Magnevist®) was briefly infused (100 µL over 10 minutes) to help assess catheter placement and patency using a post-surgical MRI acquired within 60 minutes after completion of contrast infusion[28,29].

CLINICAL AND BEHAVIORAL ASSESSMENT

Cage-side observations for morbidity and mortality were performed daily for each animal through the day of necropsy. Cage-side observations included evaluations at the incision sites (e.g., redness, swelling, discharge), abnormal behaviors (e.g., immobile, uncoordinated, loss of limb use), and notations for any other abnormal clinical signs (e.g., vomiting, seizures). Body weights were measured periodically throughout the study.

NECROPSY AND WHOLE BRAIN SECTIONING

Animals were euthanized via pentobarbital overdose (>50 mg/kg) and transcardially perfused with 4-6 L of 0.9% ice-cold saline. Next, the brains were removed, placed ventral side up in a chilled adult rhesus brain mold (Ted Pella Inc. Redding, CA), and sectioned into 2 mm-thick coronal tissue slabs. The slabs were removed from the mold in the rostral-to-caudal direction and placed on pre-labeled foam pads with slab number and animal ID number. Brain slabs were notched on the edge of the left hemisphere to maintain orientation and were photographed. The brain slab with the infusion site, targeting the putamen, was determined by comparing the coronal tissue slabs with the anatomical MRI images containing the cranial needle catheter and/or gross examination of the tissue slabs for the needle tract. The slab that contained the infusion site was used for GDNFv immunocytochemistry (ICC). From this origin, adjacent slabs were punched for pharmacodynamic DA turnover measures and then

slabs alternated between analysis for ICC and DA turnover in both the rostral and caudal direction. Slabs selected for neurochemical analysis were frozen using dry ice. Slabs selected for GDNF ICC were post-fixed in 4% paraformaldehyde.

GDNFv IMMUNOCYTOCHEMISTRY

Post-fixed slabs were cryoprotected and sectioned to produce a uniform, level surface. Forty (40) micron-thick sections were collected from brain slabs containing the putamen for GDNFv biodistribution. In addition, sections from the slab containing the substantia nigra were also analyzed for evidence of GDNFv retrograde transport from the putamen. Briefly, and as previously reported[10,30], endogenous peroxidase was blocked with 0.2% hydrogen peroxidase for 10 minutes. Free-floating sections were incubated with Goat anti-GDNF primary antibody (1:200, R&D Systems, Minneapolis, MN) for overnight at 4°C, and then exposed to biotinylated Horse anti-Goat IgG (1:500, Vector Labs, Burlingame, CA) for 1 hour. Sections were incubated in the Avidin-biotin-Peroxidase complex using the Elite ABC Vectastain Kit (Vector Labs) and visualized using 3,3'diaminobenzidine as the chromogen with nickel enhancement.

NEUROCHEMICAL ANALYSES OF BRAIN TISSUE FOR DOPAMINE AND DOPAMINE METABOLITES

Multiple, grey matter tissue punches were taken from selected coronal brain slabs adjacent to the infusion site using sterile, disposable biopsy needles (3 mm O.D.). Tissue punches were obtained from rostral and caudal aspects of the putamen. In total, ten (10) punches were taken from the right putamen per animal along the brain rostral-to-caudal axis. Additional punches were taken in the right caudate nucleus (3 punches/animal) and right nucleus accumbens (1 punch/animal) from the slab immediately rostral to the infusion site. Each tissue punch was quickly weighed, snap frozen on dry ice and transferred to storage (<70°C) until processed for neurochemical analyses using HPLC coupled to electrochemical detection in accordance with previously published procedures[31,32]. Tissue levels of DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were expressed as ng/g wet weight tissue. Dopamine turnover measures were calculated as (DOPAC+HVA)/DA.

STATISTICAL ANALYSES

Quantitative data are presented as mean ± standard error of the mean (SEM) values. Parametric data sets were analyzed using a one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparisons test, as applicable. A p value ≤ 0.05 was considered significant in all analyses.

Results

CATHETER PLACEMENT AND CLINICAL OBSERVATIONS

Post-surgical MRI scans taken within 60 minutes of completing the surgical procedures indicated that all catheters were placed in the center region of the putamen and were patent as indicated by the presence of MRI contrast in the putamen in all animals. In addition, administration of up to 1.10 mg GDNFv over the 17-day infusion period was well tolerated in all animals as there were no clinical signs of systemic toxicity observed in any of the GDNFv-treated animals, whether vomiting, loss of consciousness, or seizures. In addition, body weights remained stable over the course of GDNFv infusion at/near baseline levels (data not shown).

GDNFv BIODISTRIBUTION

Postmortem analyses of coronal brain sections containing the infusion catheter were performed to further evaluate catheter patency and GDNFv distribution. Immunocytochemistry for GDNFv supports that catheter patency was maintained throughout the study in all 15 GDNFv-treated animals as shown in **Figure 2**. Additionally, GDNFv distribution in the putamen detected by immunostaining was comparable between GDNFv-treated recipients, whether GDNFv was delivered using continuous or pulsatile infusion protocols, and extended beyond the infused target to adjacent brain regions including the caudate nucleus and nucleus accumbens. GDNFv was also detected in brain sections that were both rostral and caudal to the infusion site in every GDNFv-infused animal (data not shown).

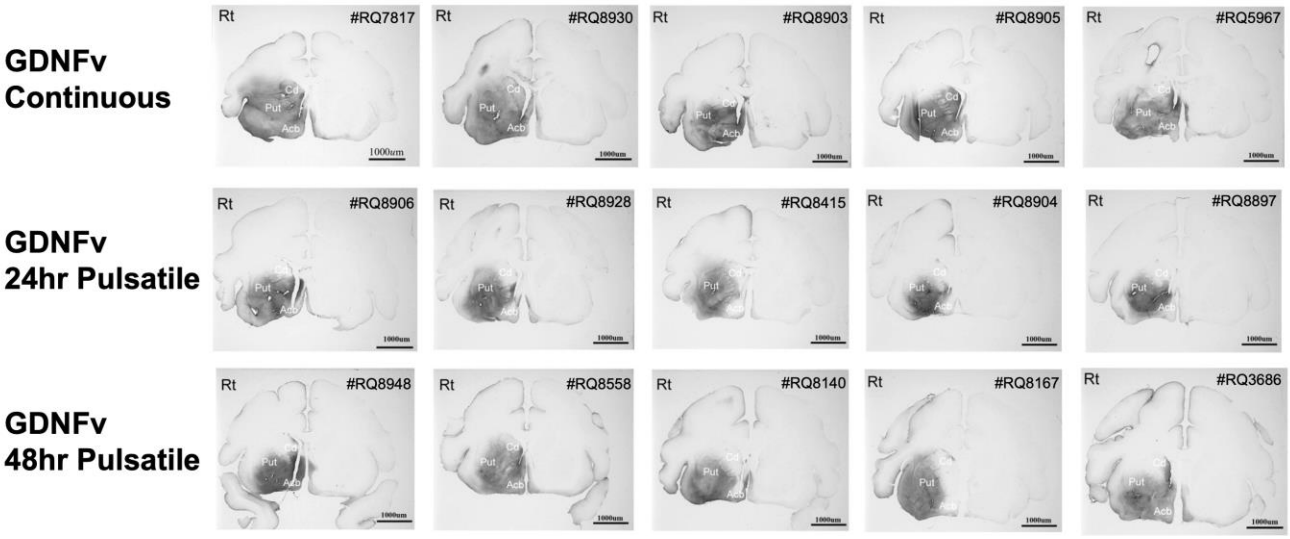

Figure 2. GDNFv-immunostained coronal sections at/near the infusion site are shown for all animals from each GDNFv treatment group (N = 5 animals / group). GDNFv (dark) was detected in the putamen and surrounding regions in all GDNFv-treated animals. Nucleus accumbens (Acb); Caudate nucleus (Cd); Putamen (Put); Right (Rt). Scale bar = 1,000 µm.

RETROGRADE TRANSPORT OF INFUSED GDNFv FROM THE PUTAMEN TO THE SUBSTANTIA NIGRA

Retrograde transport of GDNFv from the putamen to the substantia nigra was evaluated using immunocytochemistry. Substantia nigra neurons retrogradely labeled for GDNFv were found in all GDNFv-treated animals (**Figure 3**). Unbiased stereological cell counting was used to estimate the number of GDNFv-positive nigral neurons ipsilateral to the infusion site[6]. That number averaged 533±133 with a median of 385 in the animals continuously infused with GDNFv. By comparison, the number of GDNFv-positive nigral neurons ipsilateral to the infusion site in the animals receiving GDNFv by pulsatile infusion of 24-hour duration averaged 351±64 with a median of 352 and 457±20 with a median of 448 for those receiving GDNFv by pulsatile infusion of 48-hour duration. While the pattern of retrograde transport labeling of substantia nigra neurons varied between the three infusion protocols, there was no statistically significant difference between the number of GDNFv-positive nigral neurons in the animals continuously infused with GDNFv versus those receiving GDNFv by pulsatile infusion.

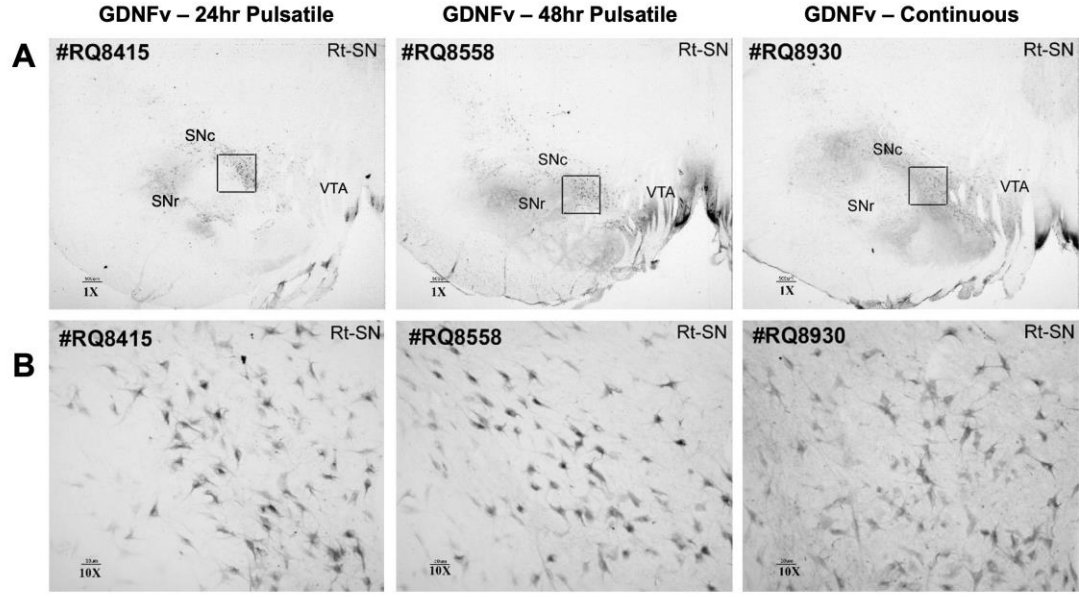

Figure 3. GDNFv-immunostained sections of the right substantia nigra (Rt-SN) are shown for three representative animals of each treatment group. Row A is a low power (1X) view. Row B shows GDNFv-positive nigral cells at higher magnification (10X). Substantia nigra pars compacta (SNc); Substantia nigra pars reticulata (SNr); Ventral tegmental area (VTA). 1X scale bar = 500 µm; 10X scale bar = 50 µm.

DOPAMINE TURNOVER CHANGES IN GDNFv-TREATED ANIMALS

Neurochemical analyses were performed to compare and evaluate functional effects of continuous versus pulsatile GDNFv on dopaminergic systems. Multiple tissue punches taken in the right putamen were obtained from three coronal slabs along the brain rostral-to-caudal axis. For each animal, three punches were taken in the slab immediately anterior to the infusion site and four punches were taken in the slab immediately posterior to the infusion site. An additional three punches were taken in the slab containing the caudal putamen (i.e., posterior to the anterior commissure) for a total of 10 putamenal punches in the right putamen per animal. Brain tissue punches were also taken in the right caudate nucleus (3 punches/animal) and right nucleus accumbens (1 punch/animal) from the slab immediately rostral to the infusion site.

Dopamine turnover [(HVA+DOPAC)/DA] was used as a functional marker for the effects of GDNFv on nigro-striatal neurons as has been previously reported[10]. Data were analyzed using a one-way ANOVA followed by Dunnett's *post hoc* comparisons. In these studies, analysis of DA turnover ratios showed a significant effect of GDNFv treatment ipsilateral to the infusion for all three GDNFv-treated groups across all three putamenal levels versus PBS-infused, control animals (**Figure 4**). All three GDNFv treatment groups showed comparable increases in putamenal DA turnover, whether GDNFv was delivered via continuous or pulsatile infusion protocols (**Figure 4**). The greatest increases in DA turnover ratios were seen in the animals receiving GDNFv by pulsatile infusion of 24-hour duration as follows: 73.5% in the putamen immediately anterior to the infusion site; 69% in the putamen immediately posterior to the infusion site and 41% in the caudal putamen.

Additional comparisons with control animals in the right caudate nucleus and right nucleus accumbens immediately rostral to the infusion site showed significant increases in DA turnover following GDNFv administration using either continuous or pulsatile infusion protocols versus PBS (data not shown). As reported in the putamen, the greatest increases in DA turnover ratios were seen in the animals receiving GDNFv by pulsatile infusion of 24-hour duration as follows: 73% in the caudate nucleus and 71% in the nucleus accumbens (data not shown).

Discussion

GDNFv is a mammalian cell-derived form of wildtype human GDNF previously shown to have improved tissue distribution in the rat brain along with increased stability and expected reduced clinical immunogenicity[27]. We extended these findings in the rat by also showing the enhanced biodistribution potential of GDNFv versus wildtype GDNF using continuous infusion methods in the non-human primate brain[10]. Here, we report the first comparative study in non-human primates examining the effects of intraputamenal GDNFv on brain distribution, retrograde transport to midbrain substantia nigra neurons and dopaminergic function using pulsatile and continuous infusion protocols.

No clinical signs of systemic toxicity were observed in any of the GDNFv-treated animals indicating that intraputamenal GDNFv delivered via a pump and catheter system was well tolerated using these protocols. Also, GDNFv distribution in the putamen detected by immunostaining was comparable between GDNFv-treated recipients, whether GDNFv was delivered using continuous or pulsatile infusion protocols. Similarly, there was no statistically significant difference between the number of GDNFv-positive nigral neurons in the animals continuously infused with GDNFv versus those receiving GDNFv by pulsatile infusion. The presence of GDNFv in midbrain substantia nigra neurons supports that GDNFv retrograde transport is possible using either continuous or pulsatile delivery protocols. This is important because other studies using neurturin, a neurotrophic factor structurally related to GDNF, reported the absence of retrograde transport in severely compromised disease states (e.g., in advanced stages of PD), which may contribute to a lack of neurorestorative effect[16]. Thus, comparable studies in DA depletion models, such as the 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP)-lesioned hemiparkinsonian monkey model, are needed using GDNFv to further examine the full potential of GDNFv in the treatment of neurodegenerative disease, particularly in disorders like PD where disease progression is inevitable, current therapies prove less efficacious with time and do not slow the disease progression.

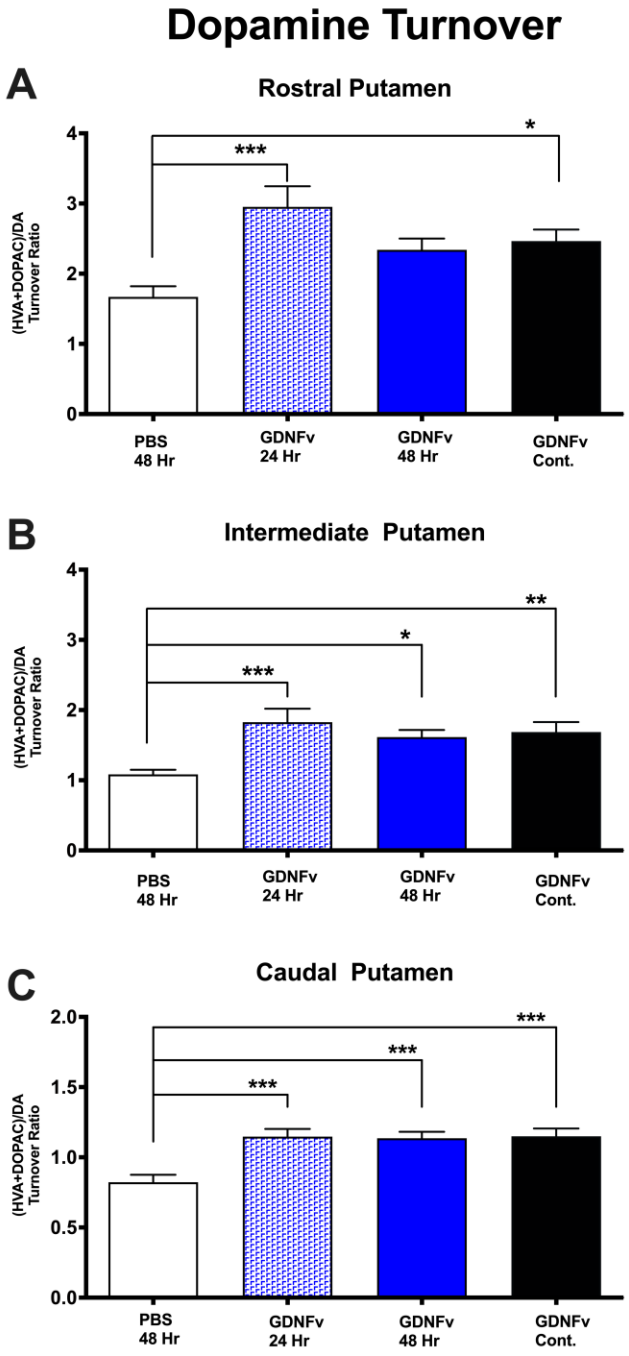

Figure 4. Tissue punches from the putamen were analyzed for neurochemical content and used to calculate dopamine turnover ((HVA+DOPAC)/DA) in the slab immediately anterior (rostral putamen) and posterior (intermediate putamen) to the infusion site. Punches were also taken in the slab containing the caudal putamen. Data are shown as mean ± SEM: *p < 0.05, **p < 0.01, ***p < 0.001.

Dopamine turnover [(HVA+DOPAC)/DA] was used in our prior work as a functional marker of target activation *in vivo* to assess the effects of trophic factors both in intact[10] and hemiparkinsonian non-human primates with decreased DA function[6,7]. Our prior results in rhesus macaques administered GDNFv using continuous infusion protocols showed increases in DA turnover in putamenal tissue compared to control[10]. In fact, the turnover ratios of GDNFv compared to wildtype GDNF in this earlier non-human primate study showed similar DA turnover across the range of doses tested suggesting at least the same relative potency for GDNFv compared to wildtype GDNF[10]. Here, we used DA turnover to provide a more definitive understanding of GDNFv target activation *in vivo* when administered using continuous and pulsatile infusion protocols. The current neurochemical data show that GDNFv administered directly into the intact rhesus macaque putamen using pulsatile infusion protocols significantly increased striatal DA turnover to a level comparable to that achieved with continuously infused GDNFv in multiple basal ganglia regions relevant to PD, including the putamen, caudate nucleus and nucleus accumbens. Of note, and as shown in Table 1, the animals administered GDNFv via pulsatile protocols only received half the total drug amount and half the infused volume used in the continuously-infused animals to achieve

comparable changes in dopaminergic function. These results not only lend further support to the favorable physiological activity of GDNFv as demonstrated previously using continuous infusion studies in non-human primates[10], but significance across the pulsatile and continuous GDNFv treatment groups support that lower doses of GDNFv could be used to activate DA systems while still attaining a similar efficacy in increasing DA turnover. As such, pulsatile delivery may be a viable approach to achieve *in vivo* effects while minimizing the total amount of GDNF needed for clinical benefits. This possibility is relevant clinically as it may offer unique advantages and solutions to established challenges in clinical care. Reducing exposure to GDNF, both within the CNS and systemically, is of clinical relevance to avoid continuous activation of GFRα1 signaling pathways and reduce the amount of drug needed while still reaching therapeutic levels. Additionally, reducing the amount of drug required to reach therapeutic benefit provides another possibility of decreasing the incidence of toxic side-effects. Reported results from clinical trials have described that >50% of the subjects treated with wildtype GDNF resulted in 18 cases of anti-GDNF antibodies with 4 subjects developing neutralizing antibodies[23], making antigenicity of GDNF one of the important clinical criteria to consider. Therefore, determining an optimum delivery mode for improved distribution and functional recovery at the minimum dose possible to mitigate any potential side-effects is key in drug development.

The present study has focused on direct delivery of a large protein, GDNFv, to the brain of non-human primates using an intracranial catheter and implanted pump system to achieve targeted distribution of this potent neurotrophic factor. This drug delivery approach has also been used in clinical trials on the use of GDNF in participants with PD[18]. The open-label Phase 1 trials of GDNF showed great potential for improving motor function in PD. This was not replicated in the blinded Phase 2 trials. The Phase 1 trials employed catheter delivery of GDNF using either pulsatile delivery or convection enhanced delivery while the Phase 2 trials used low constant flow to deliver GDNF to the putamen[12,14,15]. We previously proposed that the promising open-label Phase 1 trials were possibly related to greater volume of distribution of the GDNF protein as compared to that achieved using a low constant flow[22]. More recent studies in non-human primates also support that catheter patency is best achieved via pulsatile methods for drug delivery to the brain[29]. Thus, the present study strengthens the concept that pulsatile drug delivery can help achieve improved volume of distribution, lower total drug dose, and possibly improved catheter patency during clinical trials.

Conclusions

In summary, the current studies highlight the potential of intrastriatal GDNFv to reach target neuron populations that are depleted in PD and to deliver physiological effects at lower doses when combined with pulsatile infusion protocols. The possibility of using pulsatile delivery approaches with lower total doses of GDNFv, combined with previous reports that GDNFv demonstrated a lower risk of clinical immunogenicity compared to GDNFwt[27], offers a means to limit potential side effects and/or barriers to therapeutic usefulness that have been reported in the clinic[23]. Together, these data suggest that new forms of GDNF, such as GDNFv, still hold promise as a disease altering therapy for PD. Additional studies are warranted to investigate GDNFv in DA depletion animal models to further assess its clinical potential, particularly in neurodegenerative diseases like PD for which palliative treatments prove less efficacious over time and no therapeutic options currently exist to slow disease progression.

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Conflicts of Interest

GAG and DMG are founders of Avast Therapeutics Corp. There are no other conflicts of interest to declare.

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