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Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced parkinsonian monkeys

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Summary

The powerful trophic effects that glial cell line-derived neurotrophic factor (GDNF) exerts on midbrain dopamine neurones suggest its use in treating Parkinson's disease. However, some important questions remain about the possible therapeutic applications of GDNF. Here we demonstrate that the chronic infusion of 5 or 15 μg/day GDNF into the lateral ventricle or the striatum, using programmable pumps, promotes restoration of the nigrostriatal dopaminergic system and significantly improves motor functions in rhesus monkeys with neural deficits modelling the terminal stages of Parkinson's disease. The functional improvements were associated with pronounced upregulation and regeneration of nigral dopamine neurones and their processes innervating the striatum. When compared with vehicle recipients, these functional improvements were associated with (i) >30% bilateral increase in nigral dopamine neurone cell size; (ii) >20% bilateral increase in the number of nigral cells expressing the dopamine marker tyrosine hydroxylase; (iii) >70 and >50% bilateral increase in dopamine metabolite levels in the striatum and the pallidum, respectively; (iv) 233 and 155% increase in dopamine levels in the periventricular striatal region and the globus pallidus, respectively, on the lesioned side; and (v) a five-fold increase in tyrosine hydroxylase-positive fibre density in the periventricular striatal region on the lesioned side. In addition, chronic GDNF treatment did not induce the side-effects generally associated with chronic administration of levodopa, the most widely used treatment for Parkinson's disease. Thus, the results suggest that the prolonged and controlled delivery of GDNF into the brain could be used to intervene in long-term neurodegenerative disease processes like Parkinson's disease. Additional studies are required to determine the potential differences between chronic, intraventricular and intraputamenal (or intranigral) delivery of GDNF to maximize the efficacy of infusion treatments.

Keywords: GDNF; Parkinson's disease; dopamine neurones; regeneration; non-human primates

Abbreviations: DOPAC = 3,4-dihydroxyphenylacetic acid; GDNF = glial cell line-derived neurotrophic factor; HVA = homovanillic acid; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TH = tyrosine hydroxylase

Introduction

Converging evidence from a number of laboratories suggests that glial cell line-derived neurotrophic factor (GDNF) is capable of halting or reversing the progressive degeneration of the nigrostriatal dopamine system in animal models of Parkinson's disease (Tomac *et al*., 1995*a*; Gash *et al*., 1996; Choi-Lundberg *et al*., 1997; Bjorklund *et al*., 2000; Kordower *et al*., 2000). The most likely mechanism is through the trophic actions of GDNF on midbrain dopamine neurones in the substantia nigra. These neurones are the principal target of the pathophysiological processes underlying Parkinson's disease, and the consequent neuronal injury and subsequent degeneration lead to the profound depletion of basal ganglia

dopamine levels that characterizes the disease (Lang and Lozano, 1998*a*, *b*).

While there is good general agreement in results between laboratories using a variety of animal models of Parkinson's disease, some important questions remain about the possible therapeutic applications of GDNF. For instance, GDNF appears to be both neuroprotective and neurorestorative for dopamine neurones (Gash *et al*., 1998; Kordower *et al*., 2000; Costa *et al*., 2001); the protective effects are manifested within hours, whereas the regenerative actions are not evident for days to weeks. In many published animal studies, it is difficult to distinguish between the results that arise from protective (injury prevention) and restorative (recovery after an injury) effects because GDNF treatment is initiated in the hours or days following a lesion while the injury sequelae are still unfolding. However, the distinction is important in assessing treatment strategies for Parkinson's disease using GDNF. If the primary effects were protective, then GDNF treatment would be most beneficial in the early stages of Parkinson's disease, before devastating losses of dopamine neurones have occurred. On the other hand, if the primary actions of GDNF are on restoration, then treatment at all stages of Parkinson's disease could be beneficial.

Another important issue is the titre of biologically available GDNF necessary to produce beneficial effects. Information in this area is especially limited for methods involving the extended release of GDNF into the nigrostriatal pathway in animal Parkinson's disease models using viral vectors or encapsulated cells genetically engineered to produce GDNF (Lindner *et al*., 1995; Choi-Lundberg *et al*., 1997; Bensadoun *et al*., 2000; Kordower *et al*., 2000). While these procedures show promise, techniques for determining and controlling dosing and the timing of delivery are in the developmental stage (Bjorklund and Lindvall, 2000; Olson, 2000). Thus, while significant beneficial effects can be quantified on host dopamine neurones and neuronal processes after viral vector GDNF transfection or implantation of GDNF-producing cells, the levels of biologically available GDNF producing these effects are unclear.

The present study had the following aims. (i) To assess the restorative actions of GDNF under conditions where neuroprotection would have only a minor role, the late stages of human Parkinson's disease were modelled in rhesus monkeys with stable, advanced hemiparkinsonian features induced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Bankiewicz *et al*., 1983; Smith *et al*., 1993; Emborg-Knott and Domino, 1998). In this model, MPTP infusion through the right carotid artery results in ~75% loss of dopamine neurones expressing the phenotypic marker tyrosine hydroxylase (TH) in the right substantia nigra and >99% depletion of dopamine in the right putamen (Gash *et al*., 1996). These reductions are comparable with advanced human parkinsonism, in which cell counts typically show 60–70% loss of nigral dopamine neurones (Jellinger, 1986) and 99% dopamine depletion in the putamen (Kish *et al*., 1988). (ii) To determine the titre of biologically available GDNF necessary to produce beneficial effects, subcutaneously implanted programmable pumps connected to catheters implanted into either the right lateral ventricle adjacent to the striatum or bilaterally into the striatum were used to deliver controlled doses of GDNF or vehicle continuously to the MPTP-injured nigrostriatal system. Behavioural recovery was quantified using standardized videotaped tests; regeneration of the nigrostriatal dopamine system was analysed by quantitative morphology and high-performance liquid chromatography (HPLC) measurements of dopamine levels. The levels of GDNF promoting regeneration of the nigrostriatal system and motor recovery were quantified by enzyme-linked immunosorbent assay (ELISA) and HPLC.

Methods

Animal procedures

All procedures were conducted in the Laboratory Animal Facilities of the University of Kentucky, which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The University of Kentucky's Animal Care and Use Committee approved all protocols. In addition, veterinarians skilled in the health-care and maintenance of non-human primates supervised all animal care. The animals were fed certified primate biscuits supplemented daily with fresh fruits or vegetables, and water was available *ad libitum*. All animals were weighed weekly during the course of the study.

Under sterile field conditions, 13 adult (13 ± 0.6 years old) female rhesus monkeys (*Macaca mulatta*) received right intracarotid artery infusions of 0.4 mg/kg MPTP to induce continuously expressed parkinsonian features (Ovadia *et al*., 1995). The animals were monitored for a minimum of 2 months to ensure that the parkinsonian features expressed were stable. At this point, using stereotaxic procedures guided by MRI, a catheter (outside diameter 1 mm; Medtronic, Minneapolis, MN, USA) was implanted surgically into the right lateral ventricle adjacent to the striatum (n = 8) or bilaterally into the central part of the putamen (n = 5). The catheter(s) were then connected via flexible polyurethane tubing to a programmable pump (SynchroMed™ model 8616–10; Medtronic) implanted subcutaneously in the lateral abdominal region (Grondin *et al*., 2001). The catheters were implanted bilaterally into the putamen in order to parallel the potential bilateral effects of ventricular delivery on nigral neurones (Gash *et al*., 1996). Placement of the catheter(s) was verified by MRI. The animals were anaesthetized with isoflurane (1–3%) during these procedures. Also, an analgesic was given before and after surgery (buprenorphine 0.01 mg/kg intramuscularly); antibiotics were given once daily (gentamicin 2 mg/kg subcutaneously) for 10 days after surgery.

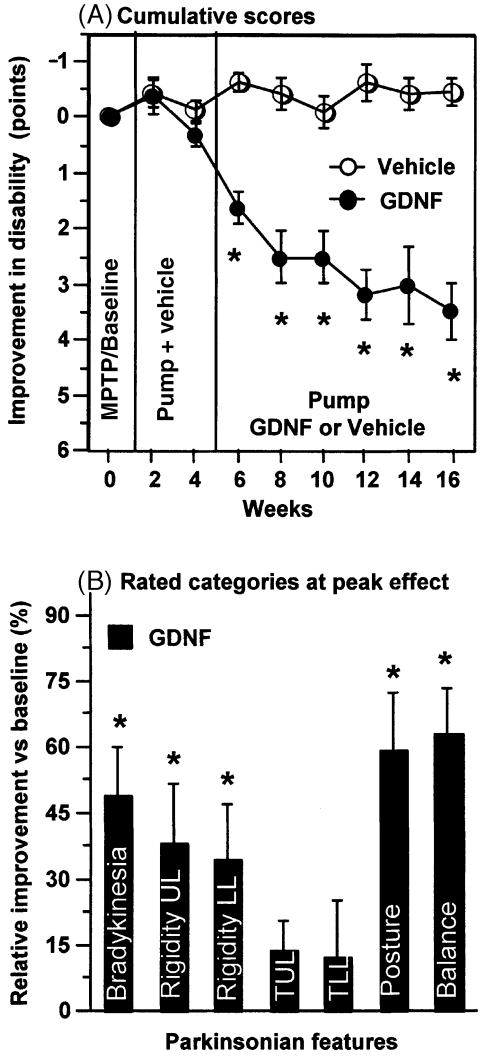

Fig. 1 Behavioural response to daily infusions of GDNF or vehicle. (**A**) Only the GDNF recipients showed a significant and sustained behavioural improvement in their parkinsonian features, of up to 3.5 points, during the treatment period. (**B**) In the GDNF recipients, consistent improvements of up to 60% were evident in bradykinesia, rigidity, balance and posture at peak effect. *$P < 0.05$ versus baseline, same animals. LL = lower limbs; UL = upper limbs; T = tremor.

GDNF treatment and dose levels

The vehicle (10 mM citrate, 150 mM NaCl buffer) was infused daily in all 13 animals during the first month after surgery and continued in five control animals for an additional 3 months (intraventricular, $n = 3$; intraputamenal, $n = 2$). The remaining eight animals received daily infusions of recombinant methionyl human GDNF (Amgen, Thousand Oaks, CA, USA) over the same 3 month period (intraventricular, $n = 5$; intraputamenal, $n = 3$). As a pilot study had shown that the threshold for inducing behavioural improvements in parkinsonian monkeys was >3.75 μg GDNF/day, animals began receiving infusions of nominally 7.5 μg GDNF/day. Four of the animals responded to this dose. In order to achieve a similar level of behavioural improvement among animals (⩾2 points on the rating scale; see 'Behavioural assessments'), the daily dose was increased to nominally 22.5 μg in the other four animals (intraventricular, $n = 2$; intraputamenal, $n = 2$). The pumps were refilled with GDNF or vehicle every 4 weeks by injection through the skin into a fill port (Grondin *et al*., 2001). To estimate the actual doses of GDNF chronically infused into the brain, residual GDNF solutions were removed from the pumps of three animals at the 4, 8 and 12 week time-points of GDNF infusion. Protein loss from adsorption to the pump was estimated by an ELISA essay (Amgen), while the stability of GDNF after 4 weeks in the pumps at 37°C (body temperature) was determined by reverse-phase HPLC.

Behavioural assessments

Motor functions were assessed using our previously published non-human primate parkinsonian scale, patterned after the human Unified Parkinson's Disease Rating Scale (Ovadia *et al*., 1995). Two hours of weekly standardized tests were conducted before and after treatment, and were evaluated from coded videotapes (Ovadia *et al*., 1995). A review of this widely used approach is published elsewhere (Imbert *et al*., 2000). In addition, the tapes were analysed to determine if the animals displayed side-effects from the treatment (Miyoshi *et al*., 1997). Post-MPTP/baseline scores were defined as the averaged scores of two videotaping sessions conducted prior to implanting the pumps. Because the rating scale is non-linear, the cumulative scores obtained weekly in the controls and GDNF recipients were analysed using the non-parametric Friedman test for related samples followed by *post hoc* analysis with the non-parametric Wilcoxon signed rank test on pairs of related samples. For a more detailed analysis of the behavioural response to GDNF, the rating scale was broken into its different components, namely, posture, balance, rigidity, tremor and bradykinesia. For each rated category, the post-MPTP/baseline scores and the scores obtained at peak effect in the GDNF-treated group were compared using the Wilcoxon signed rank test on pairs of related samples. Each animal was used as its own control.

Neurochemistry

After receiving an initial dose of ketamine hydrochloride (20–25 mg/kg), the animals were deeply anaesthetized using pentobarbital sodium (20 mg/kg) and transcardially perfused with heparinized, ice-cold physiological saline. The brains were then recovered and cut into 4 mm thick coronal sections using an ice-cold brain mould. Multiple punch biopsies of brain tissue were taken on both sides of the brain from a single section through the caudate (10–15 biopsies), putamen (10 biopsies) and accumbens (five biopsies) for dopamine, homovanillic acid (HVA) and 3,4-dihydroxy-phenylacetic acid (DOPAC) measurements by HPLC (Cass, 1996). To assess the regional effects of GDNF, the left and right striata

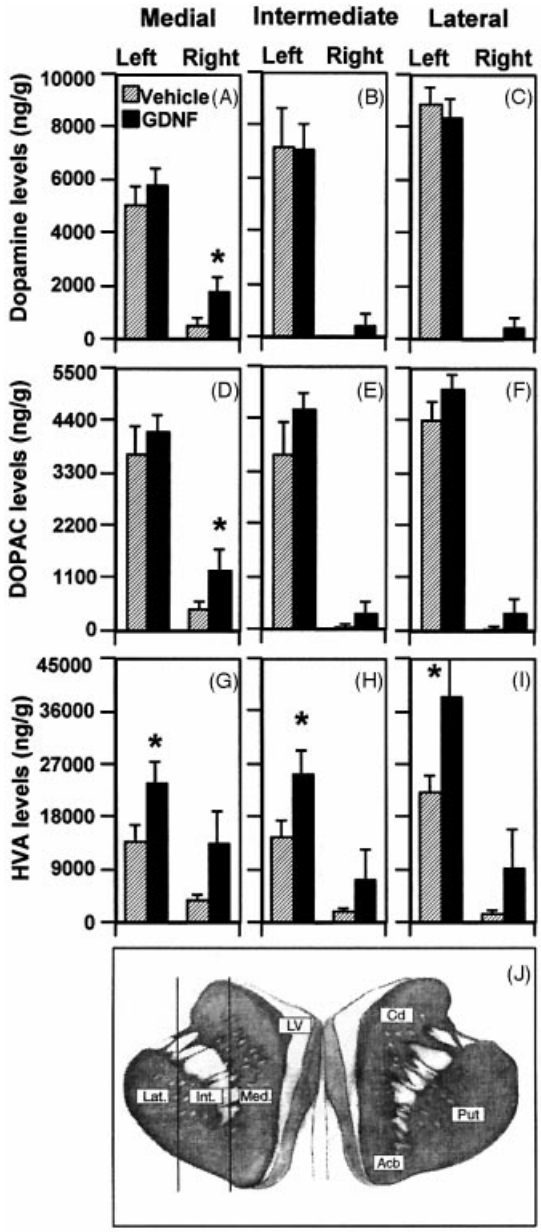

Fig. 2 Striatal levels of dopamine, HVA and DOPAC. As seen in the vehicle recipients, MPTP administration markedly reduced the levels of dopamine, HVA and DOPAC (**A**–**I**) in the medial (Med), intermediate (Int) and lateral (Lat) thirds of the right striatum (see **J**). In contrast, dopamine and DOPAC levels were significantly increased, by 233 and 180%, respectively, in the medial striatum on the lesioned right side of the GDNF recipients (**A** and **D**). HVA levels were elevated by 72, 70 and 73% in the left medial, intermediate and lateral striatum, respectively (**G**–**I**). Values are expressed as ng/g wet weight of tissue. $*P < 0.05$, GDNF versus vehicle, same side. Acb = accumbens; Cd = caudate nucleus; LV = lateral ventricle; Put = putamen.

in the coronal brain section used for tissue punch biopsies were each divided into three regions of ~4 mm each (medial, intermediate, lateral), extending from the lateral ventricle to the lateral border of the putamen (Fig. 2J). All the punch biopsies taken in a given region were averaged, providing a single measure per region per animal for dopamine, DOPAC or HVA. For each hemisphere, independent sample *t* tests were used to estimate differences in dopamine, DOPAC or HVA levels between animals in the control and GDNF treatment groups (assuming unequal variances). Five tissue punch biopsies were also taken from a section of the globus pallidus on both sides of the brain for similar analyses.

Immunohistochemistry and quantitative morphology

Intact 4 mm thick coronal striatal sections, along with the entire midbrain, were immersion-fixed in 4% paraformaldehyde solution at 4°C and processed so that 40 μm thick sections could be cut on a sliding knife microtome through the striatum and the substantia nigra. As described elsewhere (Gash *et al*., 1995), the sections were further processed for immunohistochemical staining for TH (monoclonal antibody, 1 : 1000; Chemicon International, Temecula, CA, USA). The number and perikaryal size of TH^+ midbrain dopaminergic neurones were estimated using an optical fractionator method for unbiased stereological cell counting (Gash *et al*., 1996). The ventral tegmental area was not included in the analysis. For each hemisphere, independent sample *t* tests were used to analyse the effects of GDNF on nigral cell count or cell size between animals in the control and GDNF treatment groups (assuming unequal variances). In addition, a quantitative analysis of TH^+ fibre density was conducted on both sides of the brain on a 40 μm thick coronal section of the striatum. Using the lateral ventricle as the reference point, a 1.2 × 1.2 mm grid was used to quantify (number of pixels; Bioquant Image Analysis System, RadM Biometrics Inc., Nashville, TN, USA) the striatal section dorsoventrally. All the data measured dorsoventrally at a given distance from the lateral ventricle were averaged, providing a single measure per 1.2 mm wide dorsoventral area per animal. The data were analysed using ANOVA (analysis of variance), testing for a within-subject factor of distance from the lateral ventricle and a between-subject factor of treatment group (vehicle versus GDNF). The ANOVA was followed by independent sample *t* tests (assuming unequal variances).

Data analysis

The initial ANOVA testing for the within-subject factors of hemisphere (left versus right) and region (medial, intermediate, lateral) and the between-subject factors of treatment group (vehicle versus GDNF) and route of infusion (intraventricular versus intraputamenal) revealed no main effect for route of infusion for the following measures: TH^+ fibre density; nigral cell size and number; and dopamine and dopamine metabolite levels in the striatum and globus pallidus. Similarly, non-parametric Mann–Whitney *U* tests indicated no effect for route of infusion on the weekly behavioural scores of each treatment group. Thus, in accord-

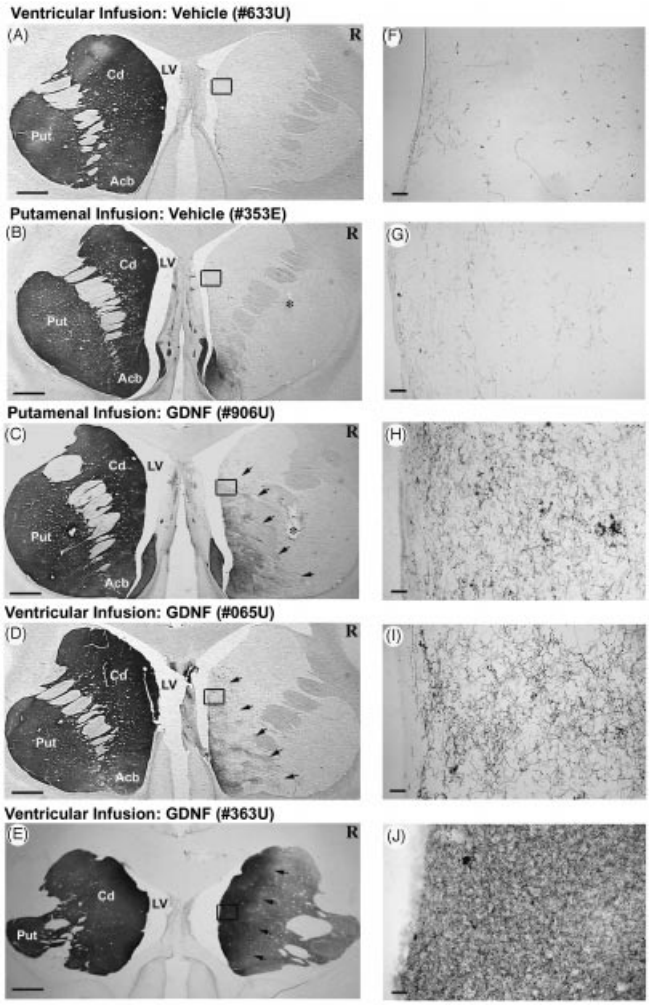

Fig. 3 Qualitative analysis of striatal dopamine fibres expressing TH. As seen in the low-power (*left*) and high-power (*right*) photomicrographs of vehicle recipients, the unilateral carotid artery infusion of MPTP virtually eliminated dopaminergic TH$^+$ fibres in the right striatum (**A**, **B**, **F**, **G**). In comparison, chronic infusion of GDNF increased the number of TH$^+$ fibres in the periventricular region of the right striatum (**H**–**J**). In one animal, the ventricular infusion of GDNF greatly stimulated TH$^+$ fibres in the caudate nucleus (arrows in **E**). The catheter tract is shown by an asterisk (*). The scale bars indicate 2 mm in the left panels and 0.05 mm in the right panels. Acb = accumbens; Cd = caudate; LV = ventricle; Put = putamen; R = right side.

ance with previously published data (Gash *et al*., 1996), all GDNF recipients were treated as one group. $P \leqslant 0.05$ was considered significant in all analyses. In addition to the mixed-group data analysis (Figs 1–4), data from each animal are presented for behaviour, neurochemistry and histology (Tables 1–5).

Results

GDNF stability and dose levels

The nominal concentration of GDNF in the pumps was 25 μg/ml. In residual GDNF solutions removed at the 4, 8 and

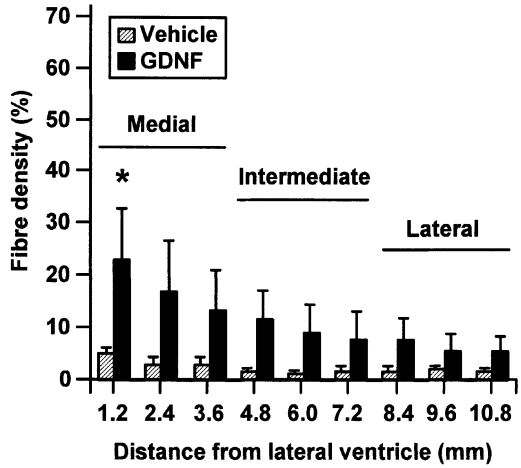

Fig. 4 Quantitative analysis of striatal dopamine fibres expressing TH. While a few residual TH$^+$ fibres could be quantified in the right striatum of vehicle recipients, there was a significant five-fold increase in TH$^+$ fibres in the periventricular striatal region of animals receiving GDNF. TH$^+$ fibres were most evident along the ventricular border of the right striatum, and gradually faded in a gradient from the ventricle to the lateral border of the putamen. One GDNF recipient (no. 224s) was excluded from the analysis due to problems with sectioning of the tissue. *$P < 0.05$, GDNF versus vehicle, right side.

12 week time points of GDNF infusion, GDNF levels of 10.0, 18.3 and 14.5 μg/ml, respectively, were measured by ELISA. Separate measurements showed that the sampling technique (adsorption to the syringe used to recover GDNF from the pump and vials used for storage) accounted for the loss of 3.2 μg/ml GDNF. After adding this back to the ELISA measurements, estimated levels of GDNF after 4 weeks in the pumps at 37°C ranged from 56 to 86% of the nominal value. Reverse-phase HPLC showed that 94% of the residual protein after 4 weeks in a pump eluted in the GDNF native peak, suggesting that the loss was primarily due to adsorption, with little degradation of the remaining protein. On the basis of average ELISA measurements of 71% of nominal levels and 94% levels of native protein, the nominal daily doses of 7.5 and 22.5 μg were estimated to conservatively represent a minimum of 5 and 15 μg/day of GDNF infused into the brain, respectively.

Parkinsonian features and the nigrostriatal system before GDNF treatment

At 45 days after MPTP administration, the animals were assigned to either GDNF ($n = 8$) or vehicle ($n = 5$) test groups so that groups were comparable in their cumulative disability scores prior to catheter implantation (Fig. 1A). To better reflect the improvement in disability, the data were expressed as differences in points between the MPTP/baseline score and post-treatment scores. The parkinsonian features of all 13 monkeys remained stable (no significant changes) from 45 to 60 days after MPTP administration (time 0, Fig. 1A). The

Table 1 *Behavioural response to chronic infusions of GDNF or vehicle (see also Fig. 1)*

Animal	Target	Treatment	Week 4	Treatment	GDNF dose (μg/day)	Week 8	12	16
C224	Putamen	Vehicle	–0.25	Vehicle	0	0.25	0.25	–0.25
353E	Putamen	Vehicle	–0.12	Vehicle	0	–1.12	–1.12	–1.12
J27	Ventricle	Vehicle	0.25	Vehicle	0	–0.50	0.00	–0.25
633U	Ventricle	Vehicle	0.25	Vehicle	0	0.25	–0.75	0.25
6SZ	Ventricle	Vehicle	–0.75	Vehicle	0	–1.00	–1.50	–1.00
			–0.12 ± 0.19			**–0.42 ± 0.29**	**–0.62 ± 0.33**	**–0.47 ± 0.26**
057F	Putamen	Vehicle	–0.25	GDNF	7.5	2.75	2.25	2.00
201W	Putamen	Vehicle	0.50	GDNF	7.5–22.5	0.75	1.25	1.75
906U	Putamen	Vehicle	–0.63	GDNF	7.5–22.5	2.13	4.13	5.13
363U	Ventricle	Vehicle	0.63	GDNF	7.5	4.13	4.13	4.13
065U	Ventricle	Vehicle	0.88	GDNF	7.5	3.13	2.88	2.88
225Z	Ventricle	Vehicle	0.50	GDNF	7.5	4.25	4.75	5.25
224S	Ventricle	Vehicle	0.88	GDNF	7.5–22.5	1.50	2.00	2.50
396U	Ventricle	Vehicle	0.00	GDNF	7.5–22.5	1.38	3.88	4.13
			0.31 ± 0.19			***2.50 ± 0.46**	***3.16 ± 0.44**	***3.47 ± 0.50**

Data are expressed as point differences on the primate rating scale between the MPTP/baseline score and post-treatment scores. Vehicle was infused in all animals for 4 weeks (weeks 1–4). The initial dose of GDNF (7.5 μg/day) was tripled in four animals after 4 weeks of chronic treatment (weeks 5–8) for the rest of the study (weeks 9–16). Negative values indicate a worsening in score and positive values an improvement in score. *$P < 0.05$ versus baseline score for the same animal; non-parametric Friedman test followed by Wilcoxon signed rank test on pairs of related samples; mean ± standard error of the mean. Bold type indicates mean ± SEM.

Table 2 *Levels of dopamine (DA), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in the periventricular striatum (STR) of recipients receiving vehicle and GDNF (see also Fig. 2)*

Animal	Target	Treatment	Left STR			Right STR		
			DA	DOPAC	HVA	DA	DOPAC	HVA
C224	Putamen	Vehicle	2745.5	2213.4	61 22.8	90.4	39.4	1038.9
353E	Putamen	Vehicle	4691.6	2447.6	11 199.9	497.9	286.9	3148.3
J27	Ventricle	Vehicle	5528.6	4463.2	19 141.8	444.7	403.7	4534.4
633U	Ventricle	Vehicle	7204.4	5020.8	19 916.3	324.6	423.0	5247.3
6SZ	Ventricle	Vehicle	4906.2	4341.4	13 594.2	1319.5	1094.1	5717.6
			5015 ± 718	**3697 ± 571**	**13 995 ± 2564**	**535 ± 208**	**449 ± 175**	**3937 ± 844**
201W	Putamen	GDNF	7601.5	3284.2	35 807.8	132.2	80.7	3588.5
057F	Putamen	GDNF	4668.3	3333.0	9025.8	18.7	41.2	1210.9
906U	Putamen	GDNF	8071.5	4165.7	30 853.6	2111.8	1563.6	12 905.2
363U	Ventricle	GDNF	6091.8	4678.3	29 244.6	4137.2	3367.6	47 734.2
065U	Ventricle	GDNF	5154.3	3675.9	35 378.3	1331.1	906.5	17 483.9
225Z	Ventricle	GDNF	3080.1	3636.1	14 607.5	1387.6	621.4	4791.2
224S	Ventricle	GDNF	6765.2	4398.8	19 478.7	2038.9	1086.8	7023.4
396U	Ventricle	GDNF	4801.5	6168.9	17 646.3	3114.0	2405.1	14 356.0
			5779±590	**4168±336**	***24 005±3577**	***1784±494**	***1259±407**	**13 637±5271**

Levels of DA and DA metabolites are expressed as ng/g wet weight of tissue. *$P < 0.05$, GDNF versus vehicle, same side, one-tailed independent sample *t* test; mean ± standard error of the mean. Bold type indicates mean ± SEM.

catheters were implanted at the 60 day time point. All animals recovered without incident following catheter(s) placement and no fatalities were seen during the 4 month study. No significant behavioural changes were seen through the 4 week period of vehicle infusion (Table 1). The parkinsonian features then continued to be expressed at the same level in the five vehicle recipients for the remaining 3 months of the study.

In the vehicle-only recipients at the termination of the study, overall tissue levels of dopamine in the severely

Table 3 *Levels of dopamine (DA), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in the globus pallidus (GP) of recipients receiving vehicle and GDNF*

Animal	Target	Treatment	Left GP			Right GP		
			DA	DOPAC	HVA	DA	DOPAC	HVA
C224	Putamen	Vehicle	186.4	80.1	6066.7	19.6	5.3	1337.5
353E	Putamen	Vehicle	177.3	64.8	8261.6	32.7	11.8	1554.3
J27	Ventricle	Vehicle	204.4	79.0	9569.8	35.9	19.4	1690.1
633U	Ventricle	Vehicle	151.0	126.0	8930.9	64.2	45.0	2052.3
6SZ	Ventricle	Vehicle	249.6	106.4	12 184.2	37.0	19.4	2767.2
			194 ± 16	**91 ± 11**	**9003 ± 990**	**38 ± 7**	**20 ± 7**	**1880 ± 250**
201W	Putamen	GDNF	335.9	87.0	12 395.1	44.9	13.3	1894.6
057F	Putamen	GDNF	199.6	97.9	6806.2	38.9	15.3	1624.6
906U	Putamen	GDNF	924.4	160.5	22 613.4	180.9	160.2	4218.4
363U	Ventricle	GDNF	154.8	103.8	32 199.0	183.8	88.9	3149.0
065U	Ventricle	GDNF	133.6	66.8	10 481.0	48.6	30.5	3518.7
225Z	Ventricle	GDNF	138.1	100.3	15 296.6	32.0	24.3	2333.6
224S	Ventricle	GDNF	236.8	74.8	9201.0	89.9	41.6	1918.5
396U	Ventricle	GDNF	338.5	181.2	11 164.7	167.4	92.4	3488.3
			308 ± 93	**109 ± 14**	***15 019 ± 2978**	***97 ± 24**	***58 ± 18**	***2768 ± 336**

Levels of DA and DA metabolites are expressed as ng/g wet weight of tissue. $*P < 0.05$, GDNF versus vehicle same side, one-tailed independent sample *t* tests; mean ± standard error of the mean. Bold type indicates mean ± SEM.

Table 4 *Quantitative analysis of striatal fibres expressing tyrosine hydroxylase in the dorsoventral region adjacent to the lateral ventricle (0–1.2 mm) of vehicle- and GDNF-treated recipients (Figs 3 and 4)*

Animal	Target	Striatum	
		Left side	Right side
Vehicle			
C224	Putamen	79.0	6.8
353U	Putamen	89.6	7.7
J27	Ventricle	86.1	1.4
633U	Ventricle	91.5	2.8
6SZ	Ventricle	92.4	6.2
		87.7 ± 2.4	**4.9 ± 1.2**
GDNF			
201W	Putamen	91.7	14.6
057F	Putamen	92.2	10.9
906U	Putamen	91.3	30.8
363U	Ventricle	91.4	78.8
065U	Ventricle	89.7	15.4
225Z	Ventricle	91.9	14.1
396U	Ventricle	93.4	6.4
		91.7 ± 0.4	***24.4 ± 9.5**

Data are expressed as percentage of fibre coverage per field of view in 40 μm thick coronal sections. One GDNF recipient (no. 224s) was excluded from the analysis due to problems with sectioning of the tissue. $*P < 0.05$, GDNF versus vehicle same side, one-tailed independent sample *t* tests; mean ± standard error of the mean. Bold type indicates mean ± SEM

lesioned right striatum were 3% of the levels on the left side of the brain (Fig. 2), with the highest residual levels (11%) in the medial striatal region, consisting of the periventricular caudate nucleus and the nucleus accumbens (Table 2). The lowest dopamine levels (<1%) were in the intermediate and lateral regions of the striatum, containing the putamen. Levels of the dopamine metabolites DOPAC and HVA were also highest in the medial striatum (Table 2). MPTP also significantly decreased pallidal levels of dopamine and related metabolites in the vehicle-treated animals. When compared with tissue levels in the left pallidum, an average of 80% depletion of dopamine (194 ± 16 versus 38 ± 7 ng/g wet weight of tissue), HVA (91 ± 11 versus 20 ± 7 ng/g) and DOPAC (9003 ± 990 versus 1880 ± 250 ng/g) was seen in the right pallidum of vehicle recipients (Table 3).

The medial-to-lateral profile of TH^+ fibre density in the right striatum of vehicle recipients mirrored dopamine levels (Figs 3A, B and 4). Overall right striatal TH^+ fibre staining was 3% of the levels in the left striatum. The highest fibre density levels (up to 8%) were in the striatal region directly adjacent to the ventricle (Table 4), with the lowest levels (~2%) found distal to the ventricle. Consistent with the TH^+ fibre reduction in the right striatum, the number of TH^+ neurones in the right substantia nigra of vehicle recipients was reduced to 17.5% of that found on the contralateral side and the neurones were ~100 μm^2 smaller (Table 5).

Antiparkinsonian effects of GDNF infusion

While the chronic infusion of vehicle had no effect on motor functions, a steady improvement was observed in the GDNF-treated animals during the first month after GDNF infusion, reaching an average improvement of 2.5 points on the rating scale (Fig. 1A and Table 1). This level of behavioural improvement was thereafter maintained for an additional

Table 5 *Perikaryal cell size and number of dopamine neurones expressing tyrosine hydroxylase in the substantia nigra (SN) of vehicle and GDNF recipients*

Animal	Target	Treatment	Left SN		Right SN	
			Nigral cell number	Nigral cell size (μm^2)	Nigral cell number	Nigral cell size (μm^2)
C224	Putamen	Vehicle	172 800	309 ± 40	41 175	239 ± 24
353E	Putamen	Vehicle	223 425	351 ± 32	37 125	278 ± 42
J27	Ventricle	Vehicle	191 700	338 ± 23	29 700	212 ± 22
633U	Ventricle	Vehicle	194 400	303 ± 25	32 400	231 ± 9
6SZ	Ventricle	Vehicle	242 325	298 ± 37	36 450	198 ± 19
			204 930 ± 12 367	**320 ± 10**	**35 370 ± 1 986**	**232 ± 14**
057F	Putamen	GDNF	270 675	472 ± 25	67 500	302 ± 22
201W	Putamen	GDNF	228 825	477 ± 44	37 800	195 ± 39
906U	Putamen	GDNF	276 750	634 ± 69	52 650	310 ± 15
363U	Ventricle	GDNF	233 550	396 ± 54	164 025	447 ± 17
065U	Ventricle	GDNF	281 475	340 ± 37	62 775	330 ± 60
225Z	Ventricle	GDNF	194 400	340 ± 24	37 125	218 ± 24
224S	Ventricle	GDNF	242 325	392 ± 47	51 300	309 ± 33
396U	Ventricle	GDNF	214 650	391 ± 34	45 900	315 ± 34
			***242 831 ± 11 053**	***430 ± 34**	***64 884 ±14 662**	***303 ± 27**

*$P < 0.05$, GDNF versus vehicle, same side, one-tailed independent sample t tests; mean ± standard error of the mean. Bold type indicates mean ± SEM.

2 months, averaging 3.5 points by the end of the study (Table 1). This represents an overall 36% improvement in disability. In the GDNF-treated animals, consistent improvements of up to 60% were evident in bradykinesia, rigidity, balance and posture at peak effect (Fig. 1B). Rigidity was defined as decreased limb extension and/or use (Ovadia *et al*., 1995). The chronic infusion of GDNF was well tolerated by all animals, as it did not induce any observable adverse effects, such as dyskinesias, self-mutilation and vomiting. Body weight loss, a side-effect observed with acute injections of GDNF (Gash *et al*., 1996), was not significant in the chronic GDNF recipients during the 4 month study (data not shown).

Upregulation of the nigrostriatal dopaminergic system after GDNF treatment

In comparison with the vehicle recipients, dopamine and its metabolite DOPAC were significantly increased, by 233 and 180%, respectively, in the periventricular striatum on the lesioned right side of the GDNF-infused animals (Fig. 2A and D and Table 2). However, the effects of GDNF treatment varied in the more denervated intermediate and lateral portions of the right striatum. On the left side, HVA levels were significantly increased in the GDNF recipients, by 72, 70 and 73% in the periventricular, intermediate and lateral portions of the striatum, respectively (Fig. 2G–I and Table 2). When compared with tissue levels in the lesioned right pallidum of vehicle recipients, dopamine, DOPAC and HVA levels were significantly increased, by 155, 190 and 47%, respectively, in the right pallidum of the GDNF recipients (Table 3). Only HVA levels were significantly increased, by 67%, in the left globus pallidus of the GDNF recipients (Table 3).

As seen in the vehicle recipients, the direct infusion of MPTP through the right carotid artery almost eliminated TH^+ fibres in the right striatum but largely spared those in the left striatum (Fig. 3A and B). While a few residual TH^+ fibres could be identified at high-power magnification in the right striatum of vehicle recipients (Fig. 3F and G), there was an increase in TH^+ fibres in the periventricular striatal region of animals receiving infusions of GDNF (arrows, Fig. 3H–J). A quantitative analysis of TH^+ fibres present in the striatum revealed a five-fold increase in TH^+ fibre density in the periventricular region of the right striatum compared with vehicle recipients (Fig. 4 and Table 4). TH^+ fibre density was most evident along the ventricular border of the striatum and gradually faded in a gradient from the ventricle to the lateral border of the putamen ($P < 0.025$, linear regression analysis). As evident in both the photomicrographs (Fig. 3) and quantitative measurements (Fig. 4 and Table 4) of striatal dopamine fibres, the periventricular GDNF response ranged from moderate increases in TH^+ fibres in some animals to a dense fibre network in the periventricular striatum of other monkeys. In contrast to the right striatum, there were no significant differences in fibre density between GDNF and vehicle recipients in the left striatum (Table 4).

However, the effects of GDNF on nigral dopamine neurones were bilateral. The number of dopaminergic neurones expressing TH was significantly increased, by >20%, on both the left side and the lesioned right side (Table 5). Similarly, dopaminergic neurone perikaryal size was significantly increased, by >30%, in the left and right substantia nigra (Table 5).

Discussion

The present results demonstrate that the chronic infusion of 5 or 15 μg/day GDNF into the lateral ventricle or the striatum of the advanced parkinsonian brain promotes restoration of the nigrostriatal dopaminergic system and significantly improves motor functions in rhesus monkeys. Although variable, the behavioural improvements seen with either route of delivery were as good as or better than those seen previously in the same model system in response to monthly injections of GDNF or daily administration of levodopa, without inducing side-effects associated with chronic levodopa administration (Gash *et al*., 1996; Miyoshi *et al*., 1997; Zhang *et al*., 1997). In these studies, monthly injections of 150–1000 μg GDNF promoted improvements of between 1.5 and 2.5 points on the non-human primate rating scale in rhesus monkeys, while the present delivery approach resulted in improvements of 1.75–5.25 points (Table 1). Behavioural improvements in the intraputamenal GDNF-treated animals overlapped with those of the intraventricular GDNF recipients, with representatives at both the low (1.75 points) and high (5.1 points) ends of the rating scale. The functional improvements were associated with pronounced upregulation and regeneration of nigral dopamine neurones and their processes innervating the striatum. This was evidenced by (i) >20% bilateral increase in the number of nigral neurones expressing the dopamine marker TH; (ii) >30% bilateral increase in nigral dopamine neuronal cell size; (iii) >70 and >50% bilateral increase in dopamine metabolite levels in the striatum and the pallidum, respectively; (iv) 233 and 155% increase in dopamine levels in the periventricular striatal region and in the globus pallidus, respectively, on the lesioned side; and (v) a five-fold increase in periventricular striatal TH^+ fibre density on the lesioned right side.

The only other chronic delivery study of GDNF in non-human primates has been reported by Kordower *et al*. (2000), using a lentiviral vector to transfect the GDNF gene into the striatum and substantia nigra of aged and parkinsonian rhesus monkeys. They interpreted their results in parkinsonian animals, which were similar to ours, as demonstrating neuroprotection since GDNF was delivered 1 week after MPTP toxicity. However, both restorative and protective actions of GDNF may have been involved as the injury sequelae to MPTP are still unfolding in the weeks immediately after MPTP treatment (Herkenham *et al*., 1991). The GDNF levels produced by transfected cells in their study were not clear, although striatal levels of GDNF were measurable by ELISA 8 months after lentiviral GDNF delivery (Kordower *et al*., 2000). In the present study, the results are primarily attributable to the restorative actions of GDNF, as GDNF was not administered until 3 months after MPTP-induced nigrostriatal injury, a time at which parkinsonian features are expressed stably (Bankiewicz *et al*., 1986; Smith *et al*., 1993).

The effectiveness of the lateral ventricle or striatal region as a site for delivering GDNF in the present study is consistent with rodent studies using either chronic infusion or viral vectors to deliver GDNF to the nigrostriatal dopaminergic system (Connor *et al*., 1999; Bjorklund *et al*., 2000; Kirik *et al*., 2001). Also consistent with rodent studies (Rosenblad *et al*., 1998; Kirik *et al*., 2001), our results emphasize the importance of residual dopamine fibres in the striatum for GDNF-induced recovery. In the vehicle recipients, the highest levels of dopamine fibres and dopamine were present in the periventricular region of the striatum, the area where significant dopamine fibre regeneration and increases in dopamine levels were found in the GDNF recipients. Thus, the effect of GDNF on the lesioned right side of the striatum occurred where surviving elements of the nigrostriatal fibres were concentrated. The actions of GDNF in restoring striatal dopaminergic innervation may be one of the principle components of the recovery seen in the present study. Indeed, measures such as striatal TH^+ fibre density, nigral cell number and cell size were affected similarly by GDNF using either route of administration (Tables 4 and 5). However, as seen in Table 2, the tissue levels of dopamine from individual animals receiving GDNF by intraventricular or intraputamenal routes of administration were variable. Consistent with other studies conducted in MPTP-lesioned monkeys administered GDNF (Gash *et al*., 1996), the data in Table 2 support the hypothesis that behavioural improvements are not always associated with changes in striatal dopamine levels. It may be that changes in dopamine levels in other areas of the basal ganglia and/or changes in the functions of dopamine uptake and release are more important than striatal tissue levels of dopamine for promoting behavioural improvements (Gash *et al*., 1996). Clearly, additional studies are needed to determine the potential differences between chronic intraventricular and intraputamenal delivery of GDNF to maximize the efficacy of infusion treatments.

The substantia nigra is also an important structure in regulating motor functions. Consistent with previous studies with animal models of Parkinson's disease (Tomac *et al*., 1995*a*; Gash *et al*., 1996), we observed pronounced bilateral changes in nigral dopamine neurones after the chronic infusion of GDNF, suggesting an effect on presumably normal nigral neurones on the non-lesioned left side. These effects, along with the bilateral increase in striatal and pallidal dopamine metabolite levels, support the widespread distribution of intracerebrally injected GDNF and are consistent with the diffusion and/or retrograde transport of GDNF from the lateral ventricle or the striatum to the substantia nigra in both rats and monkeys (Tomac *et al*., 1995*b*; Lapchak *et al*., 1998). Evidence suggests that a loss of dopaminergic phenotype precedes the death of nigral neurones in animal models of Parkinson's disease, resulting in quiescent or atrophic dopaminergic neurones (Bowenkamp *et al*., 1996). Thus, a GDNF-responsive cell population that cannot be visualized using markers such as TH might have been present and served as the target for GDNF actions within the brain. In addition, there is now increasing evidence that neurogenesis occurs in several brain regions of adult primates and other

adult mammals, including the neocortex, striatum and substantia nigra (Fallon *et al*., 2000; Zhao *et al*., 2001; Gould and Gross, 2002). Thus, another possibility is that neurogenesis might have occurred in the substantia nigra of the GDNF recipients. The bilateral effects of GDNF on pallidal dopamine and dopamine metabolite levels are also of importance considering that the globus pallidus receives dopaminergic input from the substantia nigra and is involved in regulating motor functions by sending outputs to the motor cortex via the thalamus (Smith and Kieval, 2000). GDNF has been shown to be transported anterogradely from the striatum to the globus pallidus in rats (Kirik *et al*., 2000).

The factors responsible for the variable increases in dopamine and dopamine metabolite levels in the striatum and globus pallidus and the great variability in the regeneration of striatal dopamine fibres are not clear. A similar variable response was seen using a lentiviral vector delivery system for GDNF (Kordower *et al*., 2000). Possible mechanisms include variable rates of degradation of exogenous GDNF by host enzymes, differences in the number of residual dopamine fibres at the onset of treatment and GDNF receptor populations in the host brain. Further research is needed to clarify the primary mechanisms regulating the host response to therapeutic administration of GDNF.

While our study suggests that lateral ventricular or striatal delivery of GDNF could be effective in treating advanced Parkinson's disease, monthly intraventricular injections of up to 300 μg GDNF neither improved clinical parkinsonian features nor induced evident dopaminergic regeneration in one patient with a 23 year history of Parkinson's disease (Kordower *et al*., 1999). Because the human brain is 12–15 times larger than the rhesus brain, the relative GDNF dose administered in this case was significantly lower than the effective dose range seen in this and previous studies in rhesus monkeys (Gash *et al*., 1996; Zhang *et al*., 1997). All available evidence, including this clinical case study, suggests that the dose and timing of delivery (monthly versus daily) are important factors to be considered in order to optimize the benefits and minimize the side-effects of using GDNF. In addition, due to the much larger size of the human brain, GDNF infused into the lateral ventricle may not diffuse far enough from the ventricular wall into the parenchyma of the human striatum to restore key components of the nigrostriatal dopaminergic system. Direct intrastriatal delivery of GDNF may be necessary for therapeutic treatment of Parkinson's disease in humans.

In summary, this study shows that the chronic delivery of 5 or 15 μg/day of exogenous GDNF into the lateral ventricle or striatum of MPTP-lesioned monkeys promotes significant improvements in parkinsonian features and upregulates dopaminergic functions in the striatum, globus pallidus and substantia nigra. The results also provide proof of the concept that indwelling catheters and programmable pumps can be used to chronically deliver controlled doses of GDNF or other drugs into the brain to treat Parkinson's disease and other neurological disorders. Additional studies are needed to determine more precisely the range of therapeutic dose levels of GDNF and to determine whether chronic intraventricular or intraputamenal (or intranigral) delivery produces the best enhancement of motor and dopaminergic functions in MPTP-lesioned non-human primates.

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