

GDNF fails to exert neuroprotection in a rat α -synuclein model of Parkinson's disease

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The neuroprotective effect of the glial cell line-derived neurotrophic factor has been extensively studied in various toxic models of Parkinson's disease. However, it remains unclear whether this neurotrophic factor can protect against the toxicity induced by the aggregation-prone protein α -synuclein. Targeted overexpression of human wild-type α -synuclein in the nigrostriatal system, using adeno-associated viral vectors, causes a progressive degeneration of the nigral dopamine neurons and the development of axonal pathology in the striatum. In the present study, we investigated, using different paradigms of delivery, whether glial cell line-derived neurotrophic factor can protect against the neurodegenerative changes and the cellular stress induced by α -synuclein. We found that viral vector-mediated delivery of glial cell line-derived neurotrophic factor into substantia nigra and/or striatum, administered 2–3 weeks before α -synuclein, was inefficient in preventing the wild-type α -synuclein-induced loss of dopamine neurons and terminals. In addition, glial cell line-derived neurotrophic factor overexpression did not ameliorate the behavioural deficit in this rat model of Parkinson's disease. Quantification of striatal α -synuclein-positive aggregates revealed that glial cell line-derived neurotrophic factor had no effect on α -synuclein aggregation. These data provide the evidence for the lack of neuroprotective effect of glial cell line-derived neurotrophic factor against the toxicity of human wild-type α -synuclein in an *in vivo* model of Parkinson's disease. The difference in neuroprotective efficacy of glial cell line-derived neurotrophic factor seen in our model and the commonly used neurotoxin models of Parkinson's disease, raises important issues pertinent to the interpretation of the results obtained in preclinical models of Parkinson's disease, and their relevance for the therapeutic use glial cell line-derived neurotrophic factor in patients with Parkinson's disease.

Keywords: Parkinson's disease; alpha-synuclein; GDNF; lentiviral vector; adeno-associated viral vector

Abbreviations: AAV = adeno-associated viral; GDNF = glial cell line-derived neurotrophic factor; GFP = green fluorescent protein; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; VMAT2 = vesicular monoamine transporter 2

Introduction

Parkinson's disease is characterized by the progressive loss of dopamine neurons in the substantia nigra. The resulting decrease in the striatal level of dopamine leads to the development of characteristic motor symptoms that are central to the diagnosis of the disease in patients. Beyond the symptomatic relief provided by dopamine substitution therapy and deep-brain stimulation, there is an unmet need for the identification of neuroprotective/neurorestorative agents that can modify the progression of the underlying disease processes. Among all the candidate molecules tested to date for neuroprotection in Parkinson's disease, glial cell line-derived neurotrophic factor (GDNF) has gained most attention due to its robust effects in preventing degeneration of the nigrostriatal system in the commonly used neurotoxin-based preclinical models of the disease. Infusion and viral-mediated delivery of GDNF, as well as transplantation of GDNF-producing cells, afford substantial neuroprotection in rodents and primate models of Parkinson's disease induced by either 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Bjorklund *et al.*, 2000; Kirik *et al.*, 2004; Ramaswamy *et al.*, 2009; Aron and Klein, 2010).

These promising preclinical results have led to clinical trials designed to evaluate the efficacy of GDNF and neurturin in patients with Parkinson's disease. However, the results obtained in the clinical trials conducted so far remain inconclusive (Gill *et al.*, 2003; Nutt *et al.*, 2003; Lang *et al.*, 2006; Bartus *et al.*, 2010). This raises the important issue of the relevance or predictability of the toxin-based animal models for the human disease. Several key aspects of the disease are not faithfully replicated in the neurotoxin models. One of the characteristic histological features of Parkinson's disease is the presence of cytoplasmic inclusions containing aggregates of α -synuclein in the surviving nigral dopamine neurons reflecting the involvement of α -synuclein in the neurodegenerative process (Spillantini *et al.*, 1997). This aspect of the disease has been possible to replicate by viral vector delivery of α -synuclein to the dopamine neurons of the nigrostriatal pathway. Targeted overexpression of human wild-type α -synuclein using recombinant adeno-associated viral (AAV) or lentiviral vectors mimics many of the typical neuroanatomical, behavioural, biochemical and molecular features of the human disease (Kirik *et al.*, 2002; Klein *et al.*, 2002; Lo Bianco *et al.*, 2002; Yamada *et al.*, 2004).

In a previous study, Lo Bianco *et al.* (2004) reported the lack of neuroprotection of GDNF delivered using a lentiviral vector in rats where the A30P mutant form of human α -synuclein was overexpressed using the same type of vector. It remains unclear, however, whether this negative result was due to the use of the mutant form of α -synuclein (A30P), which is suggested to be more susceptible to aggregation and more toxic *in vivo* (Lee *et al.*, 2002). Moreover, this study explored only one site of delivery, i.e. into the substantia nigra. Therefore, it is still to be determined if striatal delivery of GDNF, which is the target site for GDNF delivery in the clinical trials, can protect from toxicity induced by overexpression of wild-type human α -synuclein.

In the present study, we investigated whether GDNF delivery into the substantia nigra or striatum, or in both sites combined, can provide neuroprotection against the toxicity induced by overexpression of human wild-type α -synuclein. Our data reveal that none of these modes of delivery prevent the loss of nigral dopamine neurons or their terminals in the striatum. In addition, we did not observe any changes in the number of α -synuclein-positive aggregates in the GDNF-treated rats.

Materials and methods

Vector preparation

Production of recombinant adeno-associated viral vectors

Transfer plasmids carrying AAV2 inverted terminal repeat coding for either a human wild-type α -synuclein or enhanced green fluorescent protein (GFP) downstream of a cytomegalovirus enhancer hybrid synthetic chicken β -actin (CBA) promoter were generated. Two hundred and ninety-three cells were grown in cell factories to ~70–80% confluency. The transfection was carried out using the calcium phosphate method and included the appropriate transfer plasmid encoding-enhanced GFP, human GDNF or human wild-type α -synuclein and the packaging plasmids pDG or pDP6 encoding for the AAV2 or AAV6 capsid proteins, respectively (Zolotukhin *et al.*, 1999; Grimm, 2002). The 293 cells were transfected with 2.5 mg of DNA with equimolar amounts of helper and transfer DNA. Transfected cells were incubated for 3 days before being harvested in phosphate buffered saline–EDTA. The cell pellet was treated with a lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.4) and lysed by performing freeze–thaw cycles in a dry ice/ethanol bath. The lysate was then treated with 21 U/ml benzonase (Sigma) for nuclear digestion. The crude lysates were purified first by ultracentrifugation (1.5 h at 350 000 g at 18°C) in a discontinuous iodixanol gradient and the virus-containing fractions were purified with ion-exchange chromatography using fast protein liquid chromatography. The virus suspension was then concentrated using a concentrator (Millipore Amicon Ultra, 100 kDa molecular weight cut-off) at 1500 g and 18°C in two consecutive steps by adding phosphate buffered saline. The physical titres of recombinant AAV2 vectors were determined using dot blot quantification as described previously (Zolotukhin *et al.*, 1999). Genome copy titres were determined using real-time quantitative polymerase chain reaction. Briefly, the viral particles were digested using 20% sodium dodecyl sulphate and a standard 40 cycle TaqMan polymerase chain reaction amplification was employed and the quantities were calculated using 10-fold serial dilutions of CBA standards. The genome copy titres were 2.5×10^{12} , 1×10^{12} and 3.7×10^{13} genome copies/ml for AAV-GFP, AAV-GDNF and AAV- α -synuclein, respectively.

Production of recombinant lentiviral vectors

The lentiviral vectors used in Experiment 1 were generated by co-transfecting 293T cells with the transfer constructs encoding enhanced GFP or human GDNF under the control of the cytomegalovirus promoter, the packaging construct pCMVR8.91, and the envelope plasmid encoding the vesicular stomatitis virus G-protein. The cell culture media containing the viral particles were collected at 48 and 72 h after transfection and concentrated by ultracentrifugation for two rounds at 75 000 g (1.5 h/round). The final pellet was dissolved in Dulbecco's modified Eagle's medium, 10% foetal bovine serum and

1% glutamine. The number of transduction units of the concentrated lentiviral-GFP vector stock was determined on 293T cells. The cells were plated at a density of 1×10^5 cells per well in six-well tissue culture dishes. Serial dilutions of the vector stock were added, and the number of GFP-expressing cells was analysed 48 h later. An RNA slot blot technique (von Schwedler *et al.*, 1993) was subsequently used to determine viral particle titre for both the GFP and GDNF vector stocks. The number of transduction units of the GDNF-expressing vector was estimated on the basis of the ratio between viral particle titre and transduction units as determined for the lentiviral-GFP vector. The final titre of the lentiviral-GFP vector stocks was $2.9\text{--}4.6 \times 10^8$ transduction units/ml, and the titre of the lentiviral-GDNF vector stocks was estimated at $1.8\text{--}2.3 \times 10^8$ transduction units/ml. The stock solution was diluted to 1×10^7 transduction units/ml before injection.

Animals

Adult female Sprague Dawley rats ($n = 40$), 225–250 g at the time of surgery, were purchased from Charles River and housed two to three per cage with *ad libitum* access to food and water during a 12 h light/dark cycle. All procedures were conducted in accordance with guidelines set by the Ethical Committee for the use of laboratory animals in the Lund–Malmö region and the European Ethical Committee (86/609 EEC).

Surgical procedure

All surgical procedures were performed under general anaesthesia using either isoflurane or a 20:1 mixture of fentanyl citrate (Fentanyl) and medetomidin hydrochloride (Dormitor) (Apoteksbolaget injected i.p.). Rats were placed in a stereotaxic frame (Kopf) and vector solutions were injected using a 5 μ l Hamilton syringe fitted with a glass capillary (outer diameter of 60–80 μ m). Vector solutions were infused at a rate of 0.2 μ l/min, and the needle was left in place for an additional 3–5 min before it was slowly retracted.

In a first set of experiments (Experiment 1), lentiviral-GDNF or lentiviral-GFP was injected in the striatum or above the substantia nigra. Two weeks later, animals received an intranigral injection of AAV- α -synuclein.

In a second set of experiments (Experiment 2), AAV-GDNF or AAV-GFP was delivered in the striatum and above the substantia nigra. Three weeks later, rats received an intranigral injection of AAV- α -synuclein.

A total of 3 or 4.5 μ l of the vector solutions were injected above the substantia nigra and in the striatum, respectively. The injections were made with the head placed in a flat-skull position at the following coordinates for substantia nigra: anterior–posterior: –5.3 mm, medial–lateral: –1.7 mm, dorsal–ventral: –7.2 mm below dural surface; and for striatum (distributed over three deposits): anterior–posterior: 1.0 mm, medial–lateral: –2.6 mm, dorsal–ventral: –5.0 mm and –4.0 mm and anterior–posterior: –0.5 mm, medial–lateral: –4.0 mm, dorsal–ventral: –4.0 mm as calculated relative to bregma according to the stereotaxic atlas (Paxinos and Watson, 1986).

Behavioural testing

Eight weeks after nigral delivery of AAV- α -synuclein, the rats in Experiment 2 were assessed for motor function-based amphetamine-induced rotational behaviour. D-Amphetamine sulphate (2.5 mg/kg) (Apoteksbolaget) was delivered intraperitoneally. Analysis of rotations

was performed in automated rotational bowls (AccuScan Instruments) coupled to the rotameter software as described previously (Ungerstedt and Arbuthnott, 1970). Right and left full body turns were recorded over a period of 90 min and data are expressed as net full turns per minute and turns ipsilateral to the injection side were assigned a positive value.

Tissue processing and immunohistochemistry

Eight weeks after AAV- α -synuclein injection, the rats were deeply anaesthetized with 1.2 ml sodium pentobarbital intraperitoneal (Apoteksbolaget) and then perfused through the ascending aorta with 50 ml saline (0.9% w/v) at room temperature, followed by 250 ml ice-cold paraformaldehyde (4% w/v in 0.1 M phosphate buffered saline). The brains were removed, post-fixed for 2 h in 4% paraformaldehyde and cryoprotected overnight in sucrose (25% w/v in 0.1 M phosphate buffered saline) before being sectioned on a freezing microtome (Leica). Coronal sections were collected in six series at a thickness of 35 μ m.

Immunohistochemical stainings were performed on free-floating sections using antibodies raised against tyrosine hydroxylase (rabbit IgG, 1:1500; Chemicon), GFP (chicken IgG, 1:5000; R&D systems), vesicular monoamine transporter 2 (VMAT2) (rabbit IgG, 1:5000; Abcam), GDNF (Goat IgG, 1:1000, R&D systems) and human α -synuclein (Ab 211, mouse IgG, 1:10000; courtesy of Dr Virginia M. Lee, University of Pennsylvania). Sections were rinsed three times in potassium-phosphate buffer between each incubation period. All incubation solutions contained 0.25% Triton X-100 in potassium-phosphate buffer. The sections were quenched for 10 min in 3% H₂O₂/10% methanol. One hour of pre-incubation with 5% normal goat serum or normal horse serum was followed by incubation overnight with the primary antibody in 2% serum at room temperature and incubation with 1:200 dilution of biotinylated goat anti-rabbit antibody or horse anti-mouse (Vector Laboratories), followed with avidin–biotin–peroxidase complex (ABC Elite; Vector Laboratories), and visualized using 3,3-diaminobenzidine as a chromogen, mounted and coverslipped using DePex mounting medium.

Cell counting and optical densitometry analysis

Assessment of the total number of tyrosine hydroxylase-positive neurons in the substantia nigra was made according to the optical fractionator principle, using a Nikon 80i microscope and the NewCast software, as described previously (Kirik *et al.*, 1998). Every sixth section covering the entire extent of the substantia nigra was included in the counting procedure. A coefficient of error was calculated according to Gundersen and Jensen (1987) and an error <0.10 was accepted.

Photographs of forebrain sections were captured using a high-resolution scanner (Scanscope CS, Aperio Technologies). Striatal tyrosine hydroxylase- and VMAT2-positive fibre density was measured by densitometry at four coronal levels (+1.2, 0.8, 0.0 and –0.4 mm relative to bregma) using the ImageJ software (Version 1.32j, National Institutes of Health). The measured values were corrected for non-specific background staining by subtracting values obtained from the cortex. The data are expressed as a percentage of the corresponding area from the intact side.

Analysis of α -synuclein-positive aggregates

The number of α -synuclein-positive aggregates was analysed in a medial, lateral and central region of the striatum on one coronal section (0.0 mm relative to bregma). The z-stack pictures 1 μ m apart were taken throughout the thickness of the section using the $\times 40$ objective on a light microscope (Olympus AX70) and images were compiled using the Velocity software (Perkin Elmer). This 3D reconstruction of the structure allowed the detection and counting of the α -synuclein-positive swellings and dystrophic terminals (Fig. 4A). α -Synuclein-positive structures with a volume $>0.8 \mu\text{m}^3$ were considered as aggregates and quantified. Data are represented as the total number of aggregates in the three areas measured.

GDNF determination by enzyme-linked immunosorbent assay

Three weeks after AAV-GFP ($n = 4$) or AAV-GDNF injection ($n = 4$), animals were sacrificed and nigral and striatal concentration of human GDNF were determined using a method previously described (Georgievska *et al.*, 2002a, b, 2004). Briefly, brains were removed; ventral mid-brains and striata were dissected and quickly frozen on dry ice. Tissue samples were sonicated in buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100, 1.7 $\mu\text{g}/\text{ml}$ phenylmethylsulphonyl fluoride, 1 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin and 1 $\mu\text{g}/\text{ml}$ pepstatin) at a concentration of 30 mg/ml. Tissue levels of human GDNF were determined on these homogenates by enzyme-linked immunosorbent assay according to the supplier's recommendations (G7620, Promega).

Statistical analysis

All statistics were conducted using the GraphPad Prism software (version 5.0). Group comparisons were performed using a *t*-test for Experiment 1 and one-way ANOVA followed by the Tukey *post hoc* test for Experiment 2. Statistical significance was set at $P < 0.05$. All values are presented as mean \pm SEM.

Results

Transgene expression

We used immunohistochemistry to assess the expression patterns of the three different transgenes (GDNF, GFP and α -synuclein), delivered using lentiviral or AAV vectors injected in mid-brain and/or striatum. Lentiviral- and AAV-mediated delivery of GDNF in the substantia nigra resulted in a good transduction of the nigral dopamine neurons as well as expression of the proteins in the striatum subsequent to anterograde axonal transport (Fig. 1A, C, F and G). Injection of the vectors in the striatum resulted in a high expression of the transgenes in the striatal region. In addition, we detected positive cells in the substantia nigra pars compacta and fibres in the substantia nigra pars reticulata, resulting from the retrograde and anterograde transport of the protein from the striatum to the substantia nigra pars reticulata, respectively (Fig. 1B and D).

As assessed by the intensity of the immunostaining, the level of GDNF expression in the substantia nigra and striatum observed in the present study is similar to that previously obtained in neuroprotection experiments in 6-hydroxydopamine lesioned rats, using the same lentiviral and AAV vector constructs, where substantial dopamine neuron protection and GDNF-induced axonal sprouting was achieved (Mandel *et al.*, 1997; Kirik *et al.*, 2000; Georgievska *et al.*, 2002a, b), suggesting that the GDNF was expressed at sufficient levels to exert functional effects.

AAV-mediated delivery of human wild-type α -synuclein in substantia nigra resulted in a good transduction of neurons distributed throughout the substantia nigra pars compacta. The protein was also transported towards the striatum, as suggested by the high α -synuclein immunoreactivity observed in the axonal terminals distributed throughout the striatum (Fig. 1E).

We previously showed by enzyme-linked immunosorbent assay measurement that lentiviral-mediated over-expression of GDNF, with a number of $\mu\text{g}/\text{ml}$ comparable with what we used here in Experiment 1, induced high and sustained levels of GDNF in the striatum (2–4 ng/mg tissue) and substantia nigra (1–4 ng/mg tissue) (Georgievska *et al.*, 2002, 2004). Likewise, 3 weeks after viral vector delivery we determined the levels of nigral and striatal GDNF in Experiment 2. No detectable levels of GDNF could be measured in the substantia nigra and the striatum of AAV-GFP-injected animals. The levels of GDNF in the transduced substantia nigra (4.12 ± 0.61 ng/mg tissue) and striatum (5.48 ± 0.38 ng/mg tissue) were well above that needed to afford near-complete neuroprotection in the 6-hydroxydopamine lesion model (~ 2 ng/mg tissue in striatum) (Georgievska *et al.*, 2002a, b).

GDNF overexpression does not protect nigral neurons against α -synuclein-induced toxicity

In these experiments the lentiviral- or AAV-GDNF injections were made 2–3 weeks before AAV- α -synuclein, and the rats were perfused for immunohistochemical analysis at 8 weeks after the AAV- α -synuclein injection. To evaluate the neurodegeneration induced by overexpression of human wild-type α -synuclein and the potential neuroprotective effect of GDNF, we estimated the number of tyrosine hydroxylase-positive neurons in the substantia nigra by stereological quantification.

Tyrosine hydroxylase immunostaining of mid-brain sections showed a substantial loss of nigral dopamine neurons, regardless of treatment, in both Experiments 1 and 2 (Fig. 2A–D, F–G). Eight weeks after AAV- α -synuclein vector injection, stereological counts revealed a significant loss of nigral dopamine neurons both in Experiment 1 ($53 \pm 10\%$ in lentiviral-GFP substantia nigra group; $57 \pm 3\%$ in lentiviral-GFP striatum group compared with intact side, $P < 0.01$ for both groups) and in Experiment 2 ($57 \pm 9\%$ in the AAV-GFP group compared with intact side, $P < 0.01$) (Fig. 2E and H). Lentiviral-mediated delivery of GDNF in substantia nigra or striatum did not prevent the loss of dopamine neurons induced by α -synuclein ($54 \pm 10\%$ in lentiviral-GFP substantia nigra group; $59 \pm 16\%$ in lentiviral striatum group)

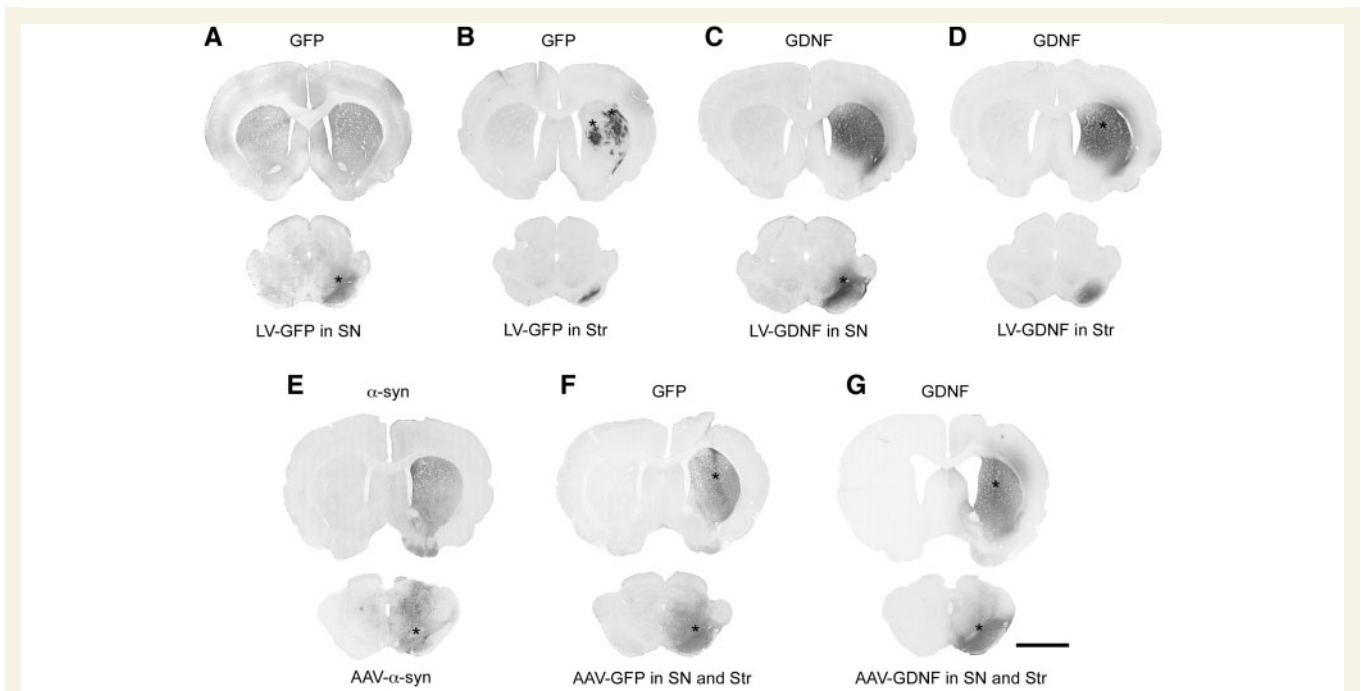


Figure 1 Pattern of expression of the different transgenes in the nigrostriatal system. Expression of GFP (A, B, F), GDNF (C, D, G) and α -synuclein (E) in the mid-brain and forebrain after lentiviral (A–D) or AAV (E–G) vector injection in the substantia nigra (A, C, E), striatum (B, D) or both sites (F, G). Nigral injection of vectors results in the expression of the transgenes in both the mid-brain and the striatum. Following striatal delivery of the vectors, immunoreactivity is observed in the striatum and at the level of the substantia nigra pars compacta and reticulata. Asterisk shows the site of delivery of the viral vector. The vectors injected and the stainings used are stated below and above the images, respectively. Scale bar = 300 μ m for forebrain sections; 400 μ m for mid-brain sections. LV = lentiviral; SN = substantia nigra; str = striatum.

compared with the respective lentiviral-GFP-treated groups ($P > 0.05$) (Fig. 2E).

Similarly, AAV-mediated overexpression of GDNF in both substantia nigra and striatum did not affect the loss of nigral dopamine neurons ($59 \pm 5\%$) compared with the GFP control group ($P > 0.05$) (Fig. 2H).

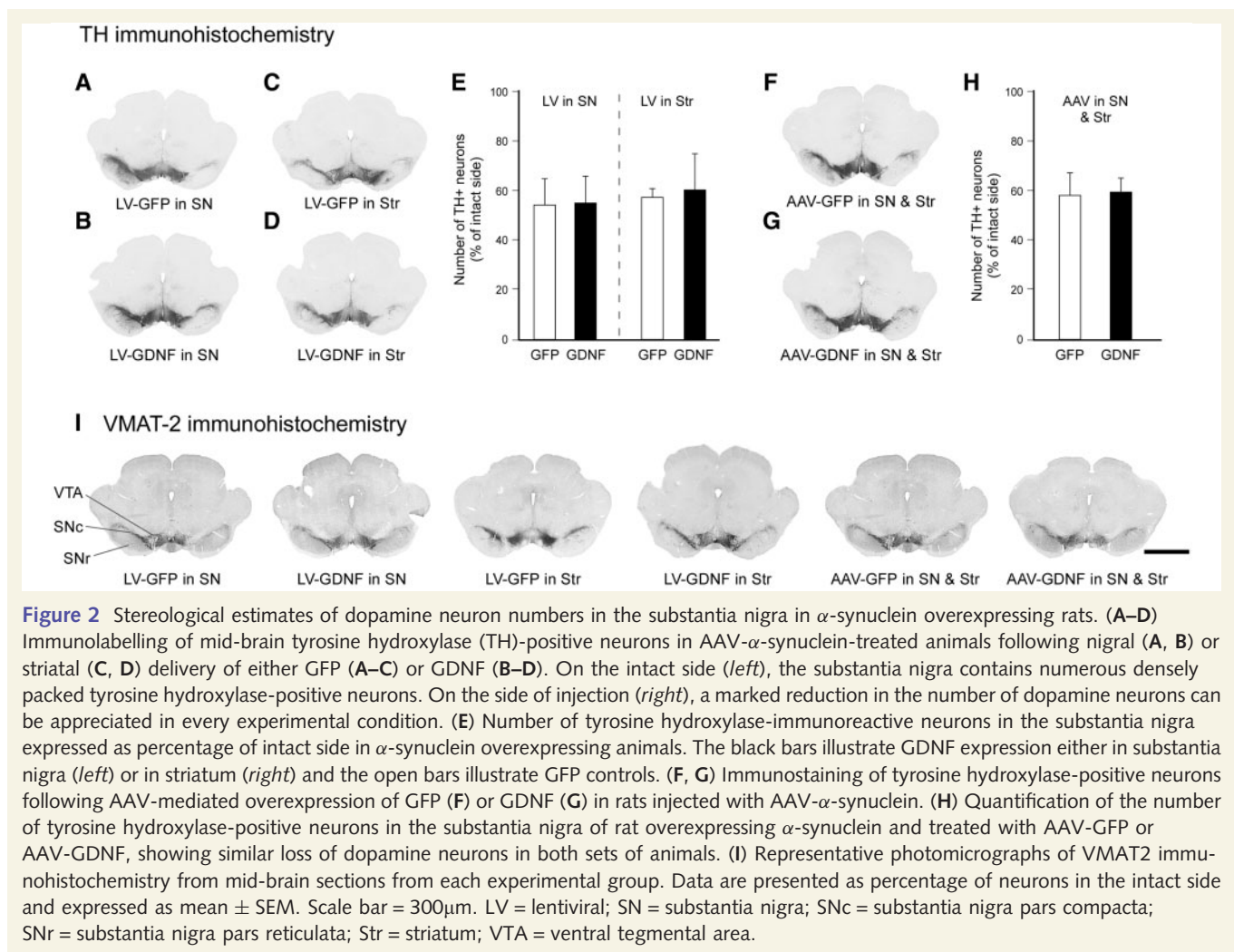
In order to ascertain that the failure to detect any neuroprotective effect of GDNF on dopamine neurons was not due to any effect of the treatment on tyrosine hydroxylase expression, we stained a set of brain sections for VMAT2. Similar to what we observed from the tyrosine hydroxylase-stained sections, GFP-treated animals showed a marked loss of nigral VMAT2-positive neurons compared with the intact side and GDNF overexpression and did not protect the VMAT2-positive mid-brain dopamine neurons against α -synuclein-induced degeneration in either Experiment 1 or 2 (Fig. 2I).

Failure of GDNF to protect the striatal dopamine innervation against α -synuclein damage

A large number of studies have demonstrated that GDNF can exert a potent neuroprotective or regenerative effect on dopaminergic terminals in toxin models of Parkinson's disease (Bjorklund *et al.*, 2000; Kirik *et al.*, 2004; Ramaswamy *et al.*, 2009; Aron and Klein, 2010). In line with these data, we investigated whether

despite the lack of protective effect observed on dopamine nigral neurons, GDNF overexpression could prevent the loss or stimulate sprouting of striatal dopaminergic terminals. Striatal tyrosine hydroxylase-positive fibre innervation was determined by optical densitometry in the different experimental conditions. Compared with the contralateral intact side, we observed a substantial reduction in the density of tyrosine hydroxylase-positive fibres in the striatum in all groups of animals (Fig. 4A–D, F–G). Quantitative analysis confirmed the significant reduction of striatal tyrosine hydroxylase immunoreactivity (Experiment 1: $54 \pm 8\%$ in the lentiviral-GFP substantia nigra group; $54 \pm 10\%$ in the lentiviral-GFP striatum group; Experiment 2: $53 \pm 8\%$ in the AAV-GFP group compared with intact side; $P < 0.01$ for all groups) induced by α -synuclein overexpression (Fig. 3E, H). Lentiviral-mediated delivery of GDNF in substantia nigra or striatum led to an even more pronounced loss of tyrosine hydroxylase density (29 ± 6 and $27 \pm 9\%$, respectively, as % of intact side; $P < 0.05$ compared with GFP control groups) (Fig. 3C–E). Likewise, overexpression of GDNF in both substantia nigra and striatum using the AAV-GDNF vector had no protective effect on striatal dopaminergic terminal density. Indeed, a significant reduction in the intensity of tyrosine hydroxylase innervation was observed in rats treated with GDNF ($26 \pm 11\%$) compared with control group ($P < 0.05$) (Fig. 3G–H).

Since sustained overexpression of GDNF can impact the expression of tyrosine hydroxylase by reducing the levels of the protein



(Georgievska *et al.*, 2004) we performed similar analysis of striatal innervation density, as described above, using VMAT2 as marker, which is known not to be affected by long-term expression of GDNF. As observed for tyrosine hydroxylase immunoreactivity, the density of striatal VMAT2-positive fibres was reduced after α -synuclein expression in the GFP-treated controls ($66 \pm 5\%$ in the lentiviral-GFP substantia nigra group; $67 \pm 9\%$ in the lentiviral-GFP striatum group; and $60 \pm 3\%$ in the AAV-GFP group compared with intact side) (Fig. 3I, J, M, N and P). In both sets of experiments, overexpression of GDNF did not protect dopaminergic terminals, as suggested by the non-significant difference in VMAT2 immunoreactivity between GDNF-treated groups and their respective control groups ($70 \pm 6\%$ in the lentiviral-GFP substantia nigra group; $64 \pm 5\%$ in the lentiviral-GFP striatum group; and $71 \pm 3\%$ in the AAV-GFP group compared with intact side) ($P > 0.05$) (Fig. 3K–M, O, P).

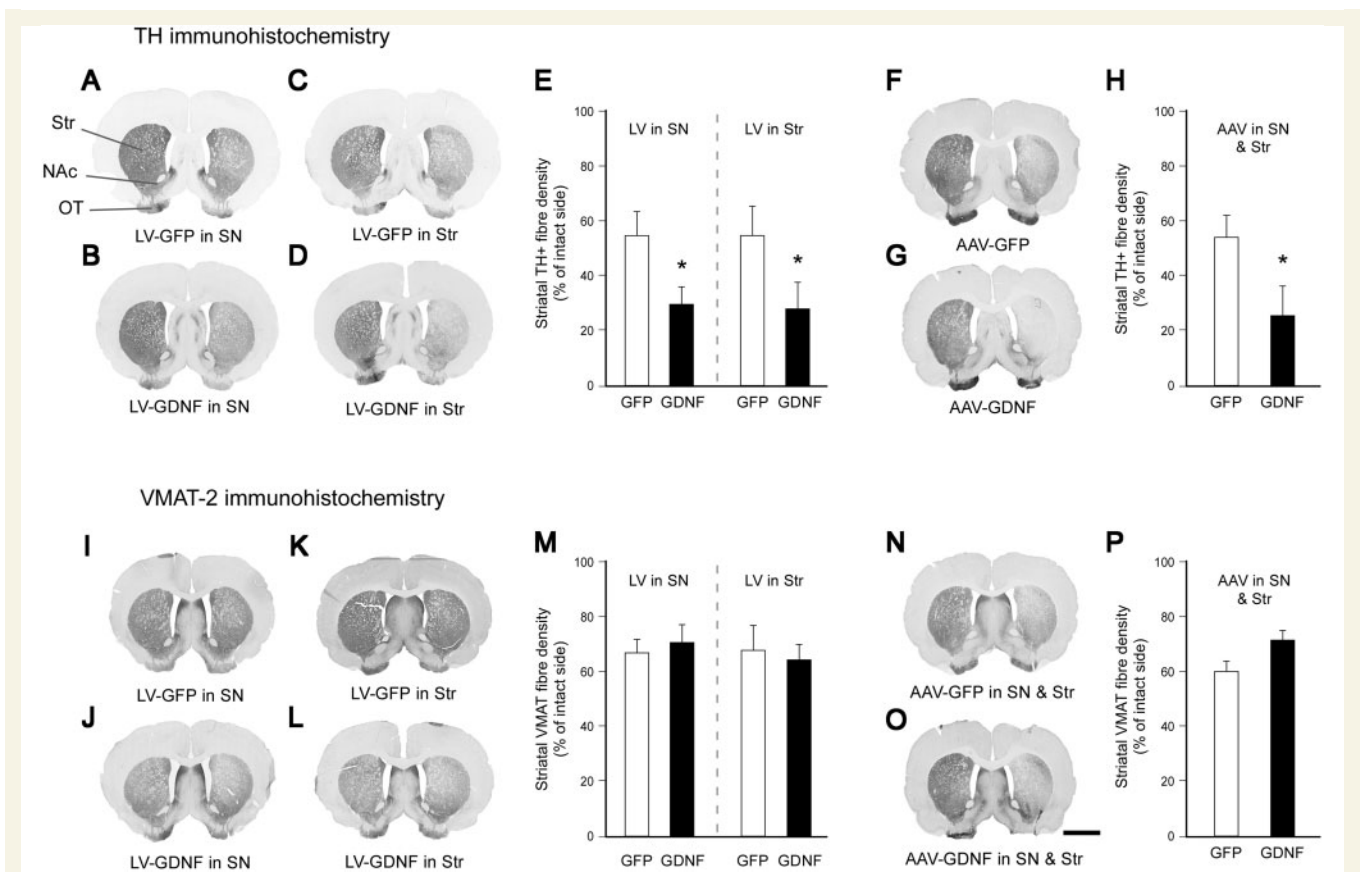
Failure to restore motor function in the drug-induced rotation test

In Experiment 2, we used amphetamine-induced rotation to assess changes in motor behaviour in the AAV- α -synuclein-transduced

animals. Consistent with the morphometric data, we observed a significant motor impairment in the α -synuclein-treated control rats that received AAV-GFP in mid-brain and in striatum (3.9 ± 1.5 turns/min). In addition, we showed that AAV-mediated delivery of GDNF in both regions did not ameliorate the behavioural impairment induced by α -synuclein overexpression compared with GFP-treated group (6.5 ± 1.6 turns/min; $P > 0.05$).

The process of α -synuclein aggregation is unaffected by GDNF

Recent studies suggest that oligomeric fibrils of α -synuclein represent the toxic form for dopamine neurons (Karpinar *et al.*, 2009). The progression of the aggregation process eventually leads to the formation aggregates reminiscent of the α -synuclein aggregates, Lewy bodies and Lewy neurites, characteristic of the human disease. In the present study, we investigated the impact of GDNF overexpression on the number of striatal α -synuclein-positive aggregates. To this end, we collected z-stacked pictures from three different areas of the striatum (dorso-medial, dorso-lateral and central) and quantified the number of α -synuclein-positive axonal swellings, defined as aggregates, using a computer-based



analysis (Fig. 4A). As previously described (Kirik *et al.*, 2002), long-term overexpression of α -synuclein in the nigrostriatal system leads to the development of dystrophic dopaminergic terminals containing α -synuclein-positive aggregates, as observed in the GFP-treated control animals (Fig. 4B and C) (4335 ± 485 in the lentiviral-GFP substantia nigra group, 5415 ± 1019 in the lentiviral-GFP striatum group in Experiment 1; 2806 ± 166 in Experiment 2). In Experiment 1, neither nigral nor striatal delivery of GDNF exerted any influence on the number of α -synuclein-positive aggregates in the striatum (5569 ± 556 and 5736 ± 256 , respectively) compared with the GFP-treated control animals ($P > 0.05$) (Fig. 4B). Similarly, AAV-mediated overexpression of GDNF in Experiment 2 did not influence the number of striatal α -synuclein-positive aggregates (2259 ± 206) ($P > 0.05$) (Fig. 4C).

Discussion

Our study demonstrates that viral vector-mediated delivery of GDNF, at levels known to be efficient in the toxin models, is unable to prevent the degeneration of the nigrostriatal dopamine neurons induced by overexpression of human wild-type α -synuclein. Targeted delivery of lentiviral-GDNF into the striatum or the substantia nigra, or AAV-mediated overexpression of GDNF in both regions, did not protect dopamine neurons or their terminals against α -synuclein-induced toxicity. Furthermore, the process of α -synuclein accumulation in dopaminergic terminals in the striatum was unaffected by GDNF overexpression. Consistent with the histological observations, we showed in one set of experiments that GDNF overexpression failed to improve the

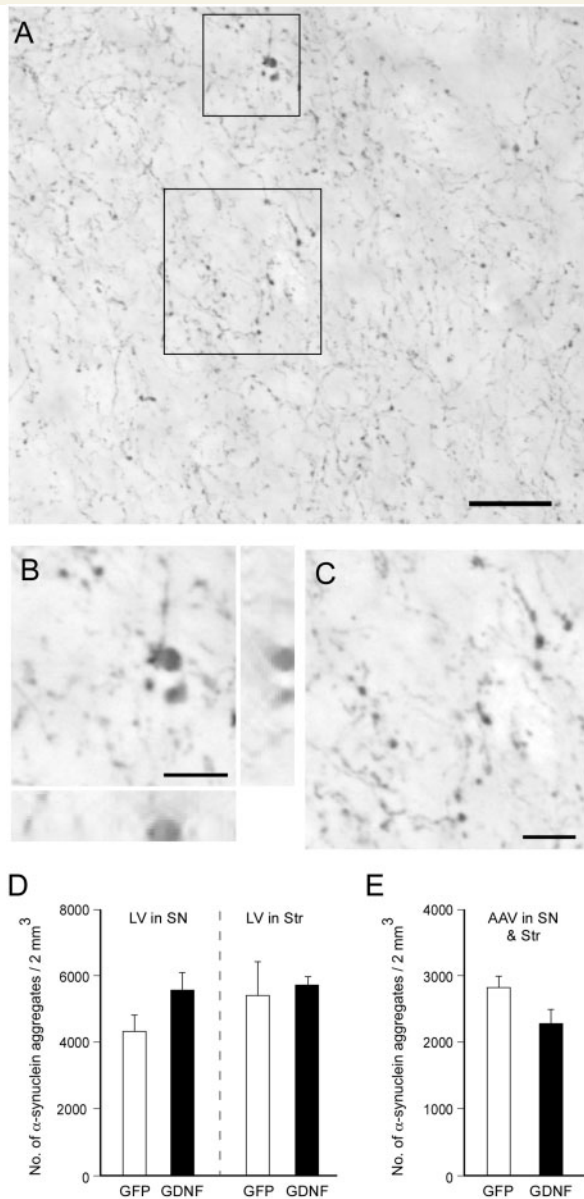


Figure 4 Analysis of α -synuclein-immunoreactive axonal swellings (aggregates) in the striatum. (A–C) Low (A) and high-power magnification (B, C) photographs showing the abundance and shape of α -synuclein-immunoreactive dystrophic fibres and axonal swelling, defined as aggregates. Quantification was performed in three regions of the striatum, medial, lateral and central, using the Velocity software (Perkin). A 3D reconstruction of the section generated from pictures were taken 1 μ m apart throughout the thickness of the section and the total number of aggregates with a size $>0.8 \mu\text{m}^3$ was assessed for each measuring field. (D, E) Quantification of the number of α -synuclein-positive aggregates in the striatum of α -synuclein-overexpressing rats treated with GFP or GDNF using lentiviral (B) or AAV (C) vectors. Viral vector-mediated delivery of GDNF in the substantia nigra or the striatum has no effect on the number of aggregates as compared with GFP-treated group. Scale bar = 100 μ m for the low-magnification image (A) and 30 μ m for the high-magnification images (B–C). Data are expressed as mean \pm SEM. LV = lentiviral; SN = substantia nigra; Str = striatum.

α -synuclein-induced motor impairment as seen in the amphetamine-induced rotation test.

These results are in line with those reported previously by Lo Bianco *et al.* (2004), where lentiviral-mediated delivery of GDNF into the substantia nigra failed to protect against the toxicity induced by nigral overexpression of mutant (A30P) α -synuclein. However, it was unclear whether the lack of protection observed in this experiment could be due to the site of delivery of GDNF (into substantia nigra) or to more pronounced toxicity of the mutant form of α -synuclein. Therefore, we felt that it would be important to confirm and extend the findings of Lo Bianco *et al.* (2004) and investigate whether delivery of GDNF into the striatum or into substantia nigra and striatum can protect dopaminergic neurons against the toxicity induced by the wild-type form of α -synuclein.

Previous neuroprotection studies using GDNF in toxin models of Parkinson's disease have revealed three modes of action of GDNF: protection of the cell bodies in the substantia nigra; induction of axonal sprouting both inside the striatum and in extrastriatal areas (such as globus pallidus and substantia nigra); and upregulation of dopamine function in the spared nigrostriatal projections, as revealed in the amphetamine rotation test (Kirik *et al.*, 2004; Ramaswamy *et al.*, 2009). None of these effects of GDNF were detectable in the AAV- α -synuclein overexpression model. Notably, the design of the two sets of experiments (lentiviral-GDNF in Experiment 1 and AAV-GDNF in Experiment 2) mimics closely the protocols used in previous studies in 6-hydroxydopamine lesioned rats (Mandel *et al.*, 1997; Kirik *et al.*, 2000; Georgievska *et al.*, 2002a, b). In these studies, GDNF overexpression resulted in almost complete protection of dopamine neurons against 6-hydroxydopamine-induced toxicity.

The discrepancy in the results obtained between the toxic models (6-hydroxydopamine and MPTP) and the α -synuclein model of Parkinson's disease raises important issues relevant to the use of GDNF in neuroprotective therapies. The deleterious mechanisms underlying 6-hydroxydopamine/MPTP-induced degeneration and those induced by α -synuclein overexpression are fundamentally different. The damage caused by injection of exogenous toxins is acute, and not protracted in time, and result from the induction of oxidative stress and mitochondrial dysfunction. In contrast, α -synuclein is an endogenous protein that normally plays a role at the level the dopaminergic synapse by regulating synthesis, release and reuptake of the neurotransmitter, as well as in cellular trafficking (Ulusoy *et al.*, 2010). Thus, in the context of α -synuclein overexpression, the α -synuclein overexpressing dopamine neurons are likely to be dysfunctional. In addition, α -synuclein is prone to forming toxic intermediates, oligomers and protofibrils and generates intracellular aggregates and inclusions. The formation of α -synuclein-positive aggregates in turn, may disrupt both anterograde and retrograde transport within the affected neurons further adding to cellular dysfunction (Chung *et al.*, 2009). Any attempt of GDNF to promote axonal sprouting, therefore, may fail as axonal sprouting and regrowth will depend on efficient anterograde transport of material to the axonal terminal. Similarly, impaired axonal transport may interfere with retrograde transport of GDNF to the substantia nigra, which may be essential for the neuroprotective effect obtained from

GDNF delivered to the striatum (Tsui and Pierchala, 2010). Indeed, Bartus *et al.* (2010) have suggested that the failure to obtain significant neuroprotective effect in patients with Parkinson's disease receiving AAV-mediated delivery of neurturin, in contrast to what have been observed in MPTP-treated primates, may be due to impaired axonal transport that will limit the access of the neurotrophic factor to its site of action.

Our data indicate that GDNF overexpression in this model of Parkinson's disease has no impact on the number of α -synuclein-positive dystrophic axonal swellings, here defined as aggregates, in the striatum, suggesting that GDNF is unable to counteract this process. Regardless of which form of α -synuclein is most toxic, and whether α -synuclein-positive inclusions, such as those observed in human Parkinson's disease, are toxic or protective, one would expect that an efficient disease-blocking therapeutic molecule would interfere in the process of α -synuclein aggregation, or enhance the degradation of these aggregates.

An interesting issue, which remains to be addressed, is whether the lack of protective effect in the AAV- α -synuclein model is specific for GDNF. Indeed, other neurotrophic factors, such as neurturin, MANF or CDNF, have been shown to afford significant protection in toxin models of Parkinson's disease (Gasmi *et al.*, 2007; Lindholm *et al.*, 2007; Voutilainen *et al.*, 2009). To what extent the mechanisms underlying the neuroprotective action of these compounds are limited to conditions related to oxidative stress or mitochondrial damage (as seen in the 6-hydroxydopamine- and MPTP-induced models), or whether any of these compounds can act more broadly to counteract the toxic impact of α -synuclein overexpression, is an interesting issue that needs to be explored in future studies. Testing of molecules that afford neuroprotection against 6-hydroxydopamine or MPTP in the α -synuclein overexpression model may help to evaluate their therapeutic potential in human Parkinson's disease.

In conclusion, our study highlights the importance of performing preclinical testing of potential therapeutic compounds in mechanistically different models of Parkinson's disease. The neuroprotective properties of candidate compounds have previously been evaluated almost exclusively in the standard 6-hydroxydopamine- and MPTP-induced models of Parkinson's disease. Our insights into the pathogenic mechanisms of Parkinson's disease have undergone considerable development over the last decade, and it is now clear that mechanisms related to free radical stress and mitochondrial damage (as modelled in the 6-hydroxydopamine and the MPTP-treated animals) is only part of the intricate neuropathological cascade leading to neuronal dysfunction and cell death. Future development of neuroprotective and/or disease-modifying therapies, therefore, have to be carried out more broadly, using a combination of preclinical models that address different aspects, or different pathogenic mechanisms, relevant to the human disease.

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