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A model of GDNF gene therapy in mice with 6-hydroxydopamine lesions: time course of neurorestorative effects and ERK1/2 activation

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Abstract

BACKGROUND: Glial cell line-derived neurotrophic factor (GDNF) is the most promising neurotrophin for restorative treatments in Parkinson's disease, but its biological effects are not completely understood.

OBJECTIVE: To define a model of GDNF gene therapy in the mouse, we studied the long-term effects of lentiviral GDNF delivery in mice with striatal 6-hydroxydopamine (6-OHDA) lesions.

METHODS: Lentiviral vectors coding for GDNF or green fluorescent protein (GFP) were injected unilaterally in the striatum two weeks prior to the 6-OHDA lesion. Mice were monitored on tests of spontaneous activity and amphetamine-induced rotation at 1, 4, 10 and 35 weeks post-lesion. Brains were processed immunohistochemically for tyrosine hydroxylase (TH) and markers of extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation at the same time points.

RESULTS: Lentiviral GDNF significantly inhibited both spontaneous and amphetamine-induced rotation. Compared to the control vector, lentiviral GDNF resulted in a partial protection of TH-positive cells in the substantia nigra, and in a nearly total restoration of striatal TH immunostaining by 35 weeks. A progressive sprouting of TH-positive neurites occurred in both the globus pallidus and the substantia nigra, reaching a 4-5 fold increase above controls by 35 weeks. This effect was paralleled by a long-term supranormal activation of ERK1/2 and its downstream target, phospho-Ser31 TH.

CONCLUSIONS: Lentiviral GDNF delivery produced robust long-term signaling responses and neurorestoration. This experimental model of GDNF gene therapy will be particularly suitable to study the molecular mechanisms of dopaminergic fiber sprouting, a long-term response to GDNF delivery that also occurs in Parkinson's disease patients.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is regarded as the most potent neurotrophic factor for nigrostriatal dopamine (DA) neurons, and the one closest to clinical application. The strengths of GDNF consist both in its potency and in the range of effects that it can achieve. This neurotrophin has the ability to protect nigral DA neurons from neurotoxic insults, as seen *in vitro* [1], *in vivo* in the rat [2,3], in the mouse [4], and in the non-human primate [5]. Moreover, GDNF can induce sprouting of DA axon fibers, as seen *in vitro* [1], *in vivo* in the rat [6], and also in human patients [7]. Last but not least, GDNF can increase the activity of the rate-limiting enzyme in DA biosynthesis, tyrosine hydroxylase (TH). This effect is mediated by phosphorylation of TH on specific seryl sites [8]. The latter action of GDNF can boost the function of residual DA neurons in Parkinson's disease (PD) [9], potentially yielding a symptomatic benefit independent of structural neurorestoration.

The molecular mechanisms mediating the various effects of GDNF *in vivo* are poorly understood, and signalling pathways downstream of GDNF have thus far been characterized almost exclusively in cell models. These studies have shown that the effects of GDNF are mediated by the GFR α 1 receptor [10], which forms a complex with the transmembrane receptor tyrosine kinase Ret triggering its autophosphorylation [10-12]. Ret then translocates to the lipid raft, and initiates further downstream signalling events [13] including the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) through a mitogen-activated protein kinase (MAPK) cascade [14-16]. In nigrostriatal DA neurons, ERK1/2 activation may confer protection against toxic damage. Indeed, pharmacological inhibition of ERK1/2 signalling has been shown to prevent the neuroprotective action of GDNF against 6-hydroxydopamine (6-OHDA)-induced toxicity in a dopaminergic cell line [17]. It remains to be shown whether ERK1/2 signaling also mediates GDNF-induced DA axon sprouting, an effect of GDNF documented to also occur in PD patients [7], reviewed in [18].

This work was undertaken in order to generate a mouse model of GDNF gene therapy that would pave the way for using genetically engineered mice in molecular investigations of GDNF action mechanisms. To this end, we carried out an extensive time course study of the effects of GDNF in mice with unilateral 6-hydroxydopamine (6-OHDA) lesions. A lentiviral vector construct previously tested in the rat [6] was used to deliver GDNF to the mouse striatum. Mice were studied for up to 37 weeks following the lentiviral injections. Behavioural observations were combined with morphological analyses of the nigrostriatal DA pathway, and with immunohistochemical detection of phosphorylated (p) ERK1/2 and p-TH.

MATERIALS and METHODS

Subjects

The study was performed in male C57BL/6 mice (Charles River Laboratories, Germany) weighting 25 g when purchased (corresponding to approx. 2 months of age). Mice were housed under a 12-h light/dark cycle with free access to food and water. All experimental procedures had been approved by the Malmö-Lund Ethical Committee on Animal Research.

Experimental Design

The timeline of the experiment is shown in Fig. 1. Animals were injected with lentiviral vectors encoding either GFP or GDNF (hereafter termed LvGFP and LvGDNF, respectively) two weeks prior to 6-OHDA lesion. They were then injected with 6-OHDA or saline (sham lesion) into the striatum (time point 0). Animals were sacrificed at time points, 1, 4, 10 and 35 weeks after lesion (n = 7-9 per group).

Lentiviral vectors

The rLv-CMV-GFP-W and rLv-CMV-GDNF-W vectors were generated as described

previously [19]. Briefly, the transfer constructs and the helper plasmids pCMVDR8.91 and pMD.G were cotransfected into 293T cells. Virions released into the media were collected, the media centrifuged 1.5 h at 26 000 r.p.m, and the pellet dissolved in DNMM. The titer of infectious particles was determined by serial dilution of the GFP vector on 293T cells. An RNA slot blot technique [20] was subsequently used to determine viral particle titer for both the GFP and GDNF vector. The transducing units (TU) of the GDNF expressing vector were estimated based on the ratio between viral particle titer and TU as determined for the rLv-GFP vector [6]. The viral vector dose used in this study was determined to be 4.1×10^5 TU/injection. In a separate group of intact mice it was determined that this LvGDNF vector dose led to striatal GDNF protein concentrations always above 0.1 ng/mg of tissue from 4 to 20 weeks post-transduction (which was the longest time point examined), representing an increase > 25-fold relative to time-matched LvGFP control mice. It has been previously established that the majority of cells transduced by this LvGDNF construct in the striatum are medium spiny neurons [6,21,22].

Delivery of lentiviral vectors

Mice were anesthetized with a mixture of 4% Isoflurane in air (Isoba®vet Apoteksbolaget, Sweden) for 5 min, placed in a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with a Cunningham mouse adaptor (Stoelting, Wood Dale, IL, USA), and kept anaesthetized using 2% Isoflurane. One μ l of suspension with vectors encoding for either GDNF or the control protein GFP were injected at a rate of 0.5 μ l/min into the right striatum using a glass capillary attached to a 10 μ l Hamilton syringe, at the following coordinates (in mm from bregma and the skull): AP +0.8, ML -2.0, DV -3.5. The syringe was left in place 2 min before and 3 min after the infusion was terminated to allow for diffusion into tissue and minimize leakage up the needle track.

6-OHDA lesions

Lesion surgery was performed two weeks after Lv injection, a time interval sufficient to afford expression of the transgenes using lentiviral vectors [23]. Animals were anesthetized with isofluorane and placed in a Kopf stereotaxic frame as described above. 6-OHDA hydrochloride (Sigma Aldrich AB, Sweden) was dissolved at a fixed concentration of 3.0 µg/µl free-base in 0.02% ice-cold ascorbate-saline and used within 2 h. The mice received two injections of 6-OHDA (2 µl each) into the right striatum [24,25] at the following coordinates (in mm from bregma and the skull): (i) AP +1.0, ML -2.1, DV -3.3; (ii) AP +0.3, ML -2.3, DV -3.3. Sham-lesioned mice were injected with 0.02% ascorbate-saline devoid of 6-OHDA.

Behavioral testing

All the mice in this study underwent two types of behavioral tests, i.e. an open field test of spontaneous activity, and an amphetamine-induced rotation test (see Fig 1).

Open field test

This test was used to monitor overall horizontal activity and spontaneous rotational behavior during weeks 1, 2, 4 and 10 post-lesion (thereafter the test was not repeated further because spontaneous rotation had completely subsided also in the LvGFP group). The open field apparatus consisted of a Plexiglas box (36 cm x 36 cm) with non-transparent walls with a grid (9 x 9 cm) painted on its floor. The animals were put in the center of the box and video recorded for 10 min immediately thereafter. The video-recordings were used to measure the following parameters: (1) number of lines crossed during the monitoring period (a line crossing was counted when the mouse moved the whole body from one square to another), (2) number of 180° turns in the direction ipsilateral (right) and contralateral (left) to the lesion.

Only turns where the mouse body remained confined within 1-3 squares in the grid were counted. In the following, only the counts of spontaneous ipsilateral turns will be presented, while the counts of line crossings (horizontal activity) will not be shown due to the lack of any significant difference between groups or time points.

Amphetamine-induced rotation

Amphetamine-induced rotation was recorded during weeks 2, 4, 10 and 35 post-lesion. Mice were placed individually in glass bowls with an upper diameter of 20 cm and attached via a specially adapted harness to an automated rotometer (Rotamex, Columbus Instruments, Columbus, OH). They were allowed to habituate to this environment for 10 min before being injected with d-amphetamine (Apoteksbolaget AB, Sweden; 5 mg/kg, i.p.). Turns of 90 degrees turns in the directions contralateral and ipsilateral to the lesion were recorded for 40 min. Results were expressed as net number of 90° ipsilateral turns/10min.

Histological tissue preparation

Mice were deeply anesthetized with sodium pentobarbital (240 mg/kg, 10ml/kg body weight, i.p.; Apoteksbolaget AB, Lund, Sweden) and perfusion-fixed by 10 ml 0.1 M phosphate buffer (pH 7.4) containing 1.0 mM sodium fluoride (a phosphatase inhibitor designed to stabilize phosphoepitopes) and 1.0% sodium nitrate (vasodilator) followed by 20 ml of 4% formaldehyde containing 1.0 mM sodium fluoride. Brains were rapidly extracted and post-fixed in the same solution for 12 hr at 4°C, and then cryoprotected in 25% sucrose in 0.1 M phosphate buffer (pH 7.4) for 48 hr at 4° C. Sections were cut at 40 µm in the coronal plane on a freezing microtome from the front of the striatum to the end of the retrorubral field ^[26]. Unless otherwise noted, every sixth section was incubated with any given primary antibody.

Bright-field quantitative immunohistochemistry

All reagents for immunohistochemistry were diluted in 0.1 M phosphate buffered saline (PBS; pH 7.6), which was also used for washes between incubations unless specified otherwise. Sections to be incubated with antibodies against phosphorylated ERK1/2 (see below) were pretreated with 0.5% sodium borohydride and then washed. All sections were pretreated with 0.1% hydrogen peroxide for 15 min, followed by several changes of buffer. As a blocking step, sections were then incubated in 10% normal horse serum or normal goat serum and 0.3% Triton-X 100 for 1-2 hrs at room temperature. This was followed by incubation in primary antibody diluted in 1% normal (horse or goat) serum and 0.3% Triton-X 100 for 48 hrs on a rotator at 4° C. The following primary antibodies were used: rabbit polyclonal for phospho-Thr202/Tyr204 ERK1/2 (pERK1/2) (1:200; Cell Signaling Technologies, Danvers, MA), goat polyclonal for GDNF (1:1000; R & D Systems, Minneapolis, MN), rabbit polyclonal for TH (1:1,000; Pel-Freeze, Rogers), and rabbit polyclonal for phospho-Ser31-TH (THSer31p; 1:200; Chemicon). After incubation with the primary antibody, sections were rinsed and then incubated for 90-120 min at room temperature with appropriate biotinylated secondary antibodies (goat anti-rabbit, or horse anti-goat; 1:200; Vector Laboratories, Burlingame, CA) in the same buffer solution. This was followed by Vectastain Elite avidin-biotin reagents (Vector Laboratories) with 0.3% Triton-X (90-120 min) on a rotator at room temperature. Sections were finally incubated in 3-3'-diaminobenzidine (DAB, Sigma-Aldrich, 0.02% in Tris Buffer, pH 7.2), and hydrogen peroxide was added to a final dilution of 0.01%. The reaction was terminated within 10 min, after which the sections were rinsed, mounted and allowed to dry overnight at room temperature. The sections were then dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped with DPX mounting medium (BDH; UK).

Dual-antigen immunofluorescence

To detect pERK1/2 expression in DA neurons, we used double immunofluorescent labeling with rabbit anti-pERK1/2 (1:150) and monoclonal mouse anti-TH antibodies (1:1000; Chemicon, Temecula, CA), diluted in PBS containing 1% normal (donkey or goat) serum, and 0.3% Triton-X 100. Sections were subsequently incubated with Alexa Fluor 488-conjugated goat anti-mouse antibodies (1:200; Molecular Probes, Invitrogen, CA) and Cy3-conjugated donkey anti-rabbit antibodies (1:100, Jackson ImmunoResearch, West Grove, PA), diluted in 0.3% Triton X-100-PBS (2 hrs incubation at room temperature). Sections were then washed, mounted, coverslipped, and viewed under a Zeiss LSM 710 NLO confocal laser scanning fluorescence microscope (Carl Zeiss Microimaging Inc; Thornwood, NY).

Image analysis and expression of the data

Densitometric analysis of TH, p-TH, and p-ERK immunostaining on regions of interest were performed using the freeware *NIH Image J 1.43* (National Institute of Health, Bethesda, MD, 2007; downloadable from <http://rsbweb.nih.gov/>). Images were digitized through a Nikon 80i microscope connected to a digital camera (Nikon DM1200F) and analyzed in a blind fashion. Staining intensities were calibrated on optical density (O.D.) standards provided by the software. Measurements were carried out in 8 rostro-caudal sections per animal throughout striatum and globus pallidus (GP), and 4 sections throughout the substantia nigra (SN). The average O.D. in the structure of interest was calculated after background subtraction. To compensate for small differences in absolute levels of stainings between sections, measurements from the lesion side were expressed as a percentage of the values from the contralateral side.

Stereological cell counts

Following TH immunostaining, a set of sections through the SN were counterstained with cresyl violet (Nissl staining), dehydrated, and coverslipped. The total number of TH positive cells in the SN pars compacta was estimated with unbiased stereology using an optical fractionator method [27] by an investigator blind to the group allocations of the mice. Seven coronal 40 μm sections throughout the SN were sampled from each animal. Analysis was performed employing the CAST system (Cast version 2.3.2.0, Visiopharm, Denmark) consisting on a Nikon 80i microscope with an x-y motor stage controlled by the CAST software. Images from the microscope were acquired with a digital camera (Nikon DM1200F) and displayed live on a monitor screen. The SN pars compacta (A9 cell group, excluding the ventral tegmental area and SN pars lateralis) was delineated using a 4X objective and the regions were then randomly sampled using a 100X objective. The thickness of each section was assessed at multiple locations within the delineated region. A three-dimensional probe consisting of a counting frame was focused through a known depth in the section and systematically distributed with known x and y steps. The counting frame area was 758 μm^2 and the disector height was 14 μm . The sampling fraction was varied to generate approximately 150 sampled neurons per animal and side. The criterion for counting a TH-positive neuron was the presence of its nucleus either within the counting frame, or touching the right or top frame lines, but not touching the left or bottom lines. Both sides (ipsilateral and contralateral to the lesion) were analysed. An unbiased estimate of the total number of TH positive neurons in SN pars compacta was then made according to the optical fractionator formula [28].

Statistical analysis

Data were analysed using two-factor ANOVA (group and time point) followed by Tukey's "honestly significant difference" (HSD) method where appropriate. The alpha level of statistical significance was set at $p < 0.05$.

Results

GDNF expression and distribution in response to LvGDNF delivery

The distribution of GDNF was visualized by immunohistochemistry at 1, 4, 10 and 35 weeks post lesion (n=3-8 per time point) (Fig 2). Immunoreactivity for GDNF was undetectable in the striatum and its output structures on either side of the brain in LvGFP-injected mice, and on the contralateral side in the LvGDNF-injected group. In the ipsilateral striatum, LvGDNF delivery caused pronounced and stable expression of the neurotrophin (Fig 2A). GDNF immunoreactivity also was observed in the GP and SN pars reticulata (Fig 2B and 2C, respectively), the terminal fields of striatofugal projections. This pattern is due to anterograde transport of the molecule, as previously reported [6,29]. In all the above areas, levels of GDNF immunoreactivity appeared maximal at 4 and 10 weeks post lesion, with some decline at 35 weeks (see right-hand column in Fig. 2A-C). The observed distribution and time course of GDNF protein expression are in line with the results reported from rat studies using the same vector construct [6].

Rotational behavior

Mice receiving either LvGFP or LvGDNF injection before the 6-OHDA lesion were evaluated in an open field test of spontaneous behaviour at 1, 2, 4 and 10 weeks post lesion. Significant overall group differences were observed at all the time points considered (Fig 3A,

$F = 50.56$, $p < 0.001$ for group effect; $F = 14.50$, $p < 0.001$ for time effect; $F = 8.35$, $p < 0.001$ for group x time interaction). In the LvGFP mice, spontaneous ipsilateral turning was prominent at 1 week post lesion (46 ± 5 turns per 10 min; Fig 3A), but progressively declined over 2 and 4 weeks, and completely ceased at 10 weeks (Fig 3A), which is in line with previous observations from this type of intrastriatal 6-OHDA lesion model [25]. In comparison with the LvGFP group, LvGDNF-treated mice showed a significantly lower rate of spontaneous ipsilateral turning at 1, 2 and 4 weeks post lesion, never exhibiting more than 6 turns per 10 min ($p < 0.05$ vs LvGFP group at each time point). Moreover, at 10 weeks post lesion animals receiving LvGDNF showed a preference to turn in the direction contralateral to the lesion, although the difference with the previous time points was not significant in post-hoc within-group comparisons (Fig 3A, $p > 0.05$).

Recordings of amphetamine-induced rotations at 2, 4, 10 and 35 weeks post lesion revealed a significant overall group difference (Fig 3B; $F = 13.74$, $p < 0.001$ for group effect; $F = 8.55$, $p < 0.001$ for time effect; $F = 1.14$, $p = 0.34$ for group x time interaction). Compared with the LvGFP group, LvGDNF mice showed a significantly lower number of amphetamine-induced rotations at 2 weeks (-33%), 4 weeks (-35%), and 10 weeks (-44 %). At the 35 weeks recording session, amphetamine-induced rotation had markedly declined also in the controls ($p < 0.05$ vs 2 week time point in LvGFP animals) and the difference between the two groups did not reach significance (Fig 3B).

Counts of nigral DA neurons

The extent of nigral DA cell loss was compared between LvGDNF- and LvGFP-injected animals at 1, 4, 10 and 35 weeks after the lesion. To this end, stereological counts of TH-positive neurons were carried out in the SN pars compacta (Fig. 4A). The analysis revealed significant overall differences between groups and time points ($F = 57.83$, $p < 0.001$ for group

effect; $F = 13.59$, $p < 0.001$ for time effect; $F = 2.36$, $p = 0.08$ for group x time interaction). At 1 week post lesion, the number of TH-positive cell bodies showed a mild reduction (approx. - 20-30% on lesioned vs. contralateral side) in both LvGFP and LvGDNF mice, and the difference between these groups did not reach significance. After 4 weeks, the number of nigral DA neurons was reduced by 74% in the LvGFP group, and by only 38% in LvGDNF-treated animals ($p < 0.05$ for LvGFP vs LvGDNF). The number of TH-positive cells remained stable between 4 and 35 weeks in each group, being significantly larger in the LvGDNF animals compared to the LvGFP controls at both 10 and 35 weeks (Fig. 4A).

Striatal TH-positive innervation

The extent of dopaminergic innervation in the striatum was examined by densitometric analysis of TH immunoreactivity (Figs. 4B and 5). Levels of TH immunostaining showed marked differences between groups and time points ($F = 107.37$, $p < 0.001$ for group effect; $F = 43.026$, $p < 0.001$ for time effect; $F = 4.67$, $p < 0.001$ for group x time interaction). In mice receiving LvGFP, TH immunoreactivity was reduced by approx. 80-90% in the caudate-putamen at 1 and 4 weeks post lesion (Figs 4B and 5A). Thereafter, the TH-positive innervation showed some long-term recovery, reaching 50% of the values on the contralateral side at post-lesion week 35 (Fig. 4B and 5A; $p < 0.05$ vs. 1 and 4 week within the same group). Animals receiving LvGDNF treatment showed a significant protection of the TH-positive innervation already at 1 week post lesion (approx 50% of control values, $p < 0.05$ vs. the LvGFP group), and a significantly enhanced long-term recovery at all later time points. In this group, TH O.D. reached 90% of intact control values at 35 weeks post lesion (Fig 5A, $p < 0.05$ vs LvGFP, $p < 0.05$ vs. 1 and 4 weeks within the same group).

TH immunoreactivity in the SN pars reticulata and GP

Densitometric analysis of TH immunoreactivity was performed also in the target of striatal projections. In the GP (Fig 5B, 6A-B), TH immunoreactivity showed overall differences between groups and time points ($F = 265.78$, $p < 0.001$ for group effect; $F = 15.44$, $p < 0.001$ for time effect; $F = 16.58$, $p < 0.001$ for group x time interaction). LvGFP mice showed a reduction of TH pallidal levels by approx. 30% on the side ipsilateral to the lesion vs. the contralateral side, which was evident already at 1 week post lesion and did not recover thereafter. By contrast, LvGDNF animals exhibited a supranormal density of TH-positive fibers at all time points (Fig 6A, $p < 0.05$ vs. LvGFP group), which increased over time and reached 320% of the contralateral values at 35 weeks (Figs 5B, 6A, $p < 0.05$ vs. 1 and 4 weeks within the same group). This increase reflected a very pronounced long-term sprouting of TH-positive fibers in the GP (Fig. 6B).

In the SN pars reticulata, TH immunoreactivity levels reflect the density of dendritic arborizations originating from DA cell bodies in the pars compacta. Densitometric analysis of TH in the reticulata showed marked overall differences between groups and time points (Fig. 5C and 6 C-D; $F = 133.07$, $p < 0.001$ for group effect; $F = 6.84$, $p < 0.001$ for time effect; $F = 20.14$, $p < 0.001$ for group x time interaction). The LvGFP group showed a reduction of TH-immunoreactivity by approx. 50%, an effect that reached significance at 4 weeks and remained stable thereafter (Fig. 6A). By contrast, LvGDNF animals did not show any loss of TH-positive fibers in the reticulata, but instead exhibited a steady increase above normal levels starting from 4 weeks post lesion (Fig. 6A-C, $p < 0.05$ vs LvGFP group at all time points), reaching 228% of the contralateral side at 35 weeks (Figs. 5C, 6 C; $p < 0.05$ vs. 1 and 4 weeks within the same group). This effect appeared to depend on hypertrophy and sprouting of TH-positive neuritic processes in this region (Fig. 6D).

Expression of phosphorylated ERK1/2

Treatment with LvGDNF caused a sustained induction of pERK1/2 immunoreactivity in the ipsilateral striatum, GP, and SN.

At 1 and 4 weeks post lesion, positive immunostaining for pERK1/2 was found in astrocytic-like cell bodies and processes (often surrounding blood vessels) in both the striatum (Fig. 7 Aa-Ff) and the GP (Fig. 7Ii-Nn) on the side ipsilateral to the lesion, with no apparent difference between the two groups. This induction was therefore attributed to an effect of the 6-OHDA lesion. In addition to this cellular staining, LvGDNF-treated mice showed pERK1/2 immunoreactivity in a fiber mesh located in the medial and lateral striatum (Fig. 7 Ee-Ff), a pattern that became much more prominent and widespread at 10 and 35 weeks (Fig. 7 Gg-Hh). Densitometric analysis of pERK1/2 immunoreactivity in the mice sacrificed at 10 and 35 weeks revealed a significant increase in striatal pERK1/2 levels following LvGDNF treatment (116.7% and 137.9 % of the contralateral side in the LvGDNF group vs. 102.7 and 107.0% in the LvGFP group at 10 and 35 weeks, respectively; unpaired t-test, $p = 0.01$ at each time point). LvGDNF-treated mice showed pERK1/2-immunoreactive fibers also in the GP (Fig. Oo-Pp).

In the substantia nigra, LvGDNF caused a marked induction of pERK1/2 in both cell bodies and neurites, which colocalized with TH immunostaining (Fig. 8C). A densitometric analysis of pERK1/2 immunoreactivity (carried out in the pars compacta region) revealed large differences between groups and time points (Fig. 8A-B; $F = 51.35$, $p < 0.001$ for group effect; $F = 5.31$, $p = 0.004$ for time effect; $F = 3.15$, $p = 0.036$ for group x time interaction). The induction of pERK1/2 by LvGDNF was apparent already at 1 week (Fig. 8A-B, $p < 0.05$ vs. LvGFP group), showing a marked increase at 10 and 35 weeks (Fig. 8A-B; $p < 0.05$ vs 1 and 4 weeks in within-group comparisons).

Expression of phosphorylated TH

TH is a cytoplasmic substrate of ERK1/2, which phosphorylates TH on Ser31 and Ser40 residues, thereby increasing its activity [30,31]. To verify that the induction of pERK1/2 by LvGDNF was paralleled by TH phosphorylation, some sections through striatum and SN were immunostained with an antibody against pSer31-TH. Immunoreactivity for pSer31-TH was measured in the striatum and the SN, showing marked overall differences between groups and time points (Fig. 9A, C, striatum; $F = 97.01$, $p < 0.001$ for group effect; $F = 15.17$, $p < 0.001$ for time effect; $F = 4.21$, $p = 0.010$ for group x time interaction. Fig. 9B, D, SN; $F = 7.57$, $p = 0.004$ for group effect; $F = 145.62$, $p < 0.001$ for time effect; $F = 8.83$, $p < 0.001$ for group x time interaction). LvGDNF caused a marked induction of pSer31-TH immunoreactivity in the ipsilateral striatum and SN, which was evident already at 1 week post lesion in both structures, and increased further at 10 and 35 weeks to reach values much above those measured on the contralateral side (Fig 9A, B; $p < 0.05$ vs LvGFP animals at all time points; $p < 0.05$ for 35 week vs 1 and 4 weeks in within-group comparisons).

Discussion

GDNF remains the most promising neurotrophin for neurorestorative treatments targeting the nigrostriatal DA pathway, and delivery methods based on viral vectors (gene therapy) represent the most efficient approach (reviewed in [18]). While the effects of viral vector-mediated GDNF delivery have been well studied in the rat, very little information is available for the mouse. Previous studies in 6-OHDA-lesioned mice had examined the effect of lentiviral GDNF delivery only at short survival times post-lesion, finding significant protection of TH-positive cell bodies when the vectors were delivered to the nigrostriatal pathway two weeks before the lesion [4]. In our study, we set out to characterize the long-term effects of LvGDNF treatment and the associated signalling responses in this mouse

model of PD. Similar to previous studies [4,6,22], lentiviral delivery of GDNF was performed two weeks before the 6-OHDA lesion. This interval was chosen based on the consideration that GDNF would require about a week to be expressed in the brain [6], and that its expression at the time of the lesion would be necessary for an optimal neuroprotective effect. Indeed, intrastriatal 6-OHDA injections are followed by a rapid phase of nigral DA cell death occurring over 2-3 days [32]. The effects of LvGDNF observed in this study should therefore be interpreted as depending both on an initial, partial neuroprotective effect and on a slower neuroregenerative effect on the nigrostriatal DA projections.

The results of this study can be summarized as follows: (i) LvGDNF achieves a robust expression of the neurotrophin in the striatum, GP and SN that is maintained over at least 35 weeks, with maximal levels of expression seen at 6 and 12 weeks post transduction (i.e. 4 and 10 weeks post lesion). This temporal pattern of GDNF expression is in line with the one reported from rat studies [6]. (ii) LvGDNF treatment protects TH-positive neurons in SN pars compacta from a neurotoxic insult, which is in line with the effects seen in rats [6] and monkeys [33]. The number of nigral TH-positive neurons remained constant from 4 weeks to 35 weeks post lesion, and no long-term down-regulation of TH expression was detected, in contrast to results obtained in the rat [6,21,34]. In this respect, the mouse model is more in line with results reported from MPTP-lesioned monkeys [33] and even PD patients [7]. (iii) LvGDNF induced progressive recovery of the striatal TH-positive innervation towards normal levels by 35 weeks, associated with a marked sprouting of TH-positive neurites in both the GP and the SN. These results are in line with data from MPTP-lesioned monkeys receiving GDNF gene therapy [33]. In contrast, studies in rats infused with LvGDNF in the striatum reported either no [6] or very modest [35] local sprouting of TH-positive fibers. One reason for this disparity might depend on a larger capacity for nigrostriatal DA fiber sprouting in the mouse compared to the rat. Indeed, LvGFP-treated mice showed significant partial recovery

of striatal TH levels between 4 and 35 weeks post-lesion (though at much lower levels than the LvGDNF treated animals). To the best of our knowledge, a similar phenomenon has never been seen in rats sustaining the same type of striatal 6-OHDA lesion and/or lentiviral injection. A large capacity for spontaneous long-term recovery of nigrostriatal dopaminergic fibers has also been reported in mice sustaining MPTP lesions [36]. Based on the results of this study, future studies aiming to address the acceleration of spontaneous recovery by LvGDNF (independent of its neuroprotective effects) in this mouse model of PD should deliver the viral vector at least two weeks post-lesion.

The partial spontaneous recovery of striatal TH fibers in the LvGFP group was not accompanied by any recovery of TH levels in either the GP or the SN reticulata, which remained significantly reduced until 35 weeks. By contrast, a time-dependent and pronounced increase in neuritic TH staining occurred in LvGDNF animals in both the GP and the SN reticulata, reaching largely supranormal levels at 35 weeks. This effect was not accompanied by any change in the number of TH cell bodies in the compacta over the time. Taken together, these data suggest that the potency of GDNF in altering the structural plasticity of DA neurons may vary between cell body and dendrites, and between proximal and distal axonal arborizations.

Controlled nigrostriatal DA fiber sprouting is certainly an important aim for any neurotrophic therapy for PD. However, the magnitude of the neuritic response induced by LvGDNF in this study, and its ectopic locations in the GP and SN reticulata, raise concerns about potential non-physiological consequences. For a future clinical application of GDNF gene therapy, it is therefore important to find means to control possible aberrant sprouting responses. This may be achieved by e.g. targeting GDNF expression to striatal astrocytes [37-39] or striatal interneurons, thereby preventing the anterograde transport of GDNF to the GP and SN.

Despite the striking TH-positive neuritic sprouting, LvGDNF-treated animals showed quite a good profile of behavioural recovery on the tests performed in this study. Indeed, LvGDNF significantly reduced amphetamine-induced rotation, as reported in rats [6,40], and suppressed the lesion-induced spontaneous ipsilateral turning bias.

Very few studies thus far have examined signalling events following *in vivo* GDNF delivery [8,29,41]. Here we show that LvGDNF infusion causes a prolonged phosphorylation of ERK1/2 in TH-positive cell bodies and dendrites in the SN. This was accompanied by the appearance of pERK1/2-immunoreactivity in fiber meshes both in the striatum and the GP, which seemed to increase gradually over time reaching very high levels at 35 weeks. This temporal pattern of pERK1/2 expression appears to match the sprouting response of TH-positive axonal projections produced by LvGDNF. Levels of pERK1/2 were more pronounced in the targets of striatal projections than in the striatum itself, corresponding to the regions where supranormal levels of neuritic sprouting occurred. These observations suggest that pERK1/2 may mediate structural plastic responses of the nigrostriatal projection following GDNF gene therapy. Our hypothesis is supported by several *in vitro* studies in cell lines and primary neuronal cultures indicating that GDNF-induced MAPK activation is causally linked with neurite outgrowth, which can be prevented using pharmacological inhibitors of MAPK pathway [42-44]. Another signalling event that was activated by LvGDNF in this study was the phosphorylation of TH at Ser31, which is known to be mediated by ERK1/2 *in vivo* [29,30]. At 10 and 35 weeks, striatal and nigral levels of pSer31-TH immunoreactivity in LvGDNF animals were increased by one-two fold above the values measured on the contralateral side. Because the striatal expression of TH at these time points did not exceed control values, these data indicate that the sprouted TH fibers in the striatum contain high levels of pSer31-TH. Since phosphorylation of TH at Ser31 is accompanied by an increased activity of the enzyme [45-47], these data are suggestive of increased DA

biosynthesis in the sprouted axon fibers. It is possible that this mechanism may help to promote behavioural recovery [48-50], and contribute to the modulation of firing rates in striatal neurons [51].

In conclusion, this study presents a model of LvGDNF gene delivery in 6-OHDA-lesioned mice which mimics several findings from non-human primate models of PD as well as observations from clinical trials of GDNF gene therapy, and in particular a long-term induction of nigrostriatal fiber sprouting in the absence of TH downregulation (reviewed in [18]). This study also provides the first report of a gradual long-term increase in ERK1/2 activation induced by LvGDNF delivery, which appears related to the intensity of the local neuritic sprouting response.

Caution should be exerted when extrapolating results from an animal model with high neuroregenerative potential to the possible effects of GDNF gene therapy in clinical trials. In PD patients, the neurotrophin would have to act on a severely compromised nigrostriatal pathway, long after the primary neurodegenerative cascade has started its course. Functional imaging studies and pathological observations from PD patients indicate, however, that DA fiber sprouting does occur at the sites of GDNF delivery (reviewed in [18]). Thus, further investigations on the signalling mechanisms that mediate nigrostriatal sprouting *in vivo* appear to be both biologically interesting and clinically relevant. Our results indicate that 6-OHDA-lesioned mice will be a very convenient experimental model for this type of studies.

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Conflict of interest

The authors have no conflict of interest to report.

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Figures

Fig. 1

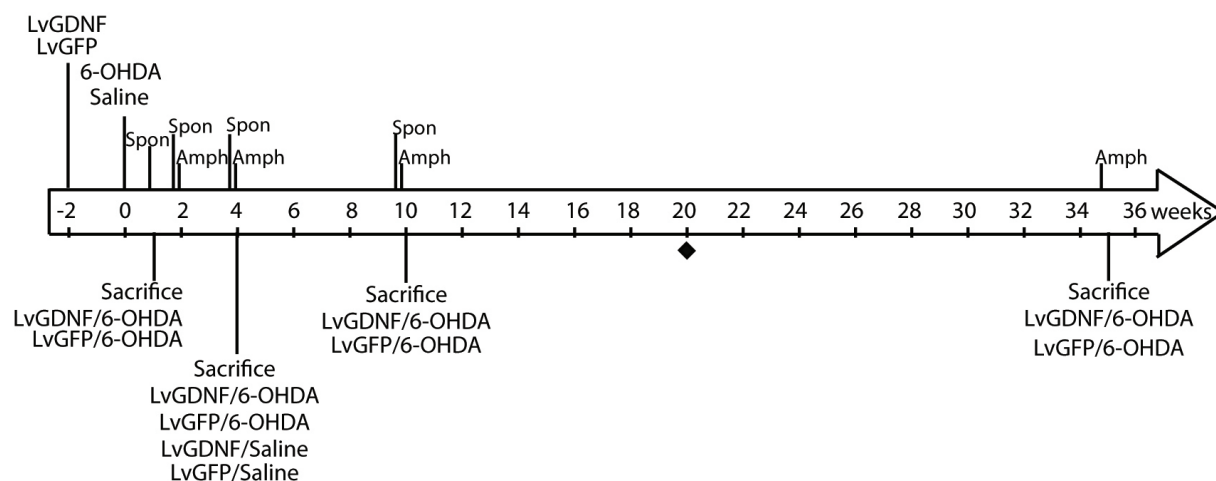


Fig 1. Time course of the study. The horizontal arrow represent the time course of the experiment. Mice were injected with LvGFP or LvGDNF two weeks before 6-OHDA lesion and tested for rotational activity at 1, 2, 4, 10 and 35 weeks post lesion. At the same time points, mice were killed for immunohistochemistry, whereas GDNF distribution and quantification were assessed at 1, 4, 10 and 20 weeks post lesion. Spon, spontaneous rotations; Amph, amphetamine-induced rotations; ♦, GDNF immunohistochemistry and ELISA.

Fig. 2

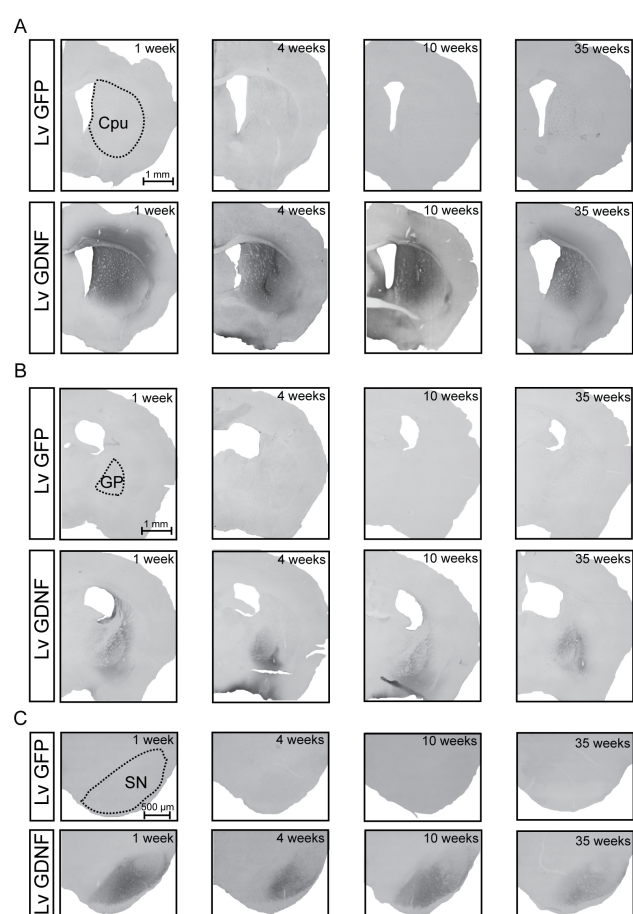


Fig 2. GDNF immunoreactivity in animals injected either with LvGFP or LvGDNF followed by 6-OHDA. Photomicrographs were obtained from the ipsilateral striatum (A), GP (B) and SN (C) at different time points after 6-OHDA lesion. GP, globus pallidus; SN, substantia nigra. Scale bar, 1 mm. (A) striatum, (B) GP and (C) SN.

Fig. 3

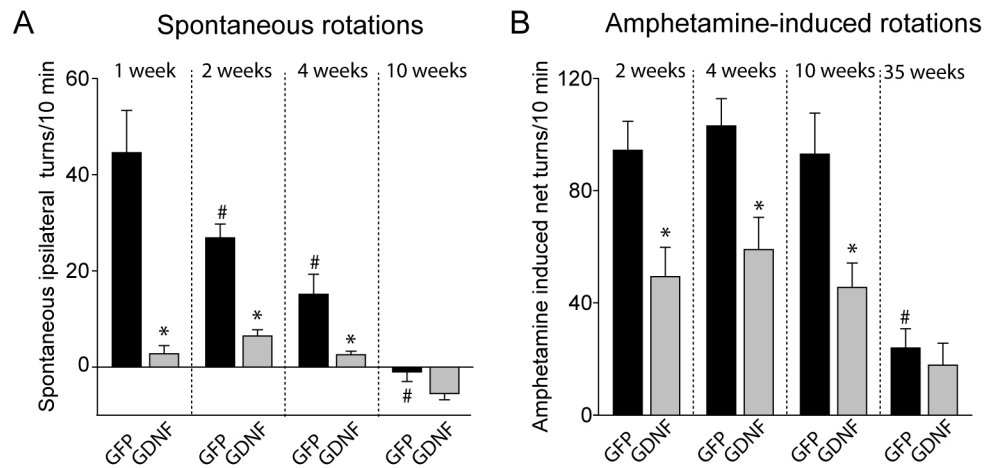


Fig 3. Behavioural recovery induced by LvGDNF. Values represent the number of ipsilateral spontaneous (A) or amphetamine-induced rotations (B) in 10 min testing session. Mice were tested for spontaneous rotations at 1, 2, 4 and 10 weeks after the 6-OHDA lesion ($n = 9 - 4$), whereas amphetamine-induced rotations test was performed 2, 4, 10 and 35 weeks post lesion ($n = 15 - 6$). Two-way ANOVA and *post hoc* Tukey's test: * $p < 0.05$ vs GFP at the same time point; # $p < 0.05$ vs GFP at 1 week.

Fig. 4

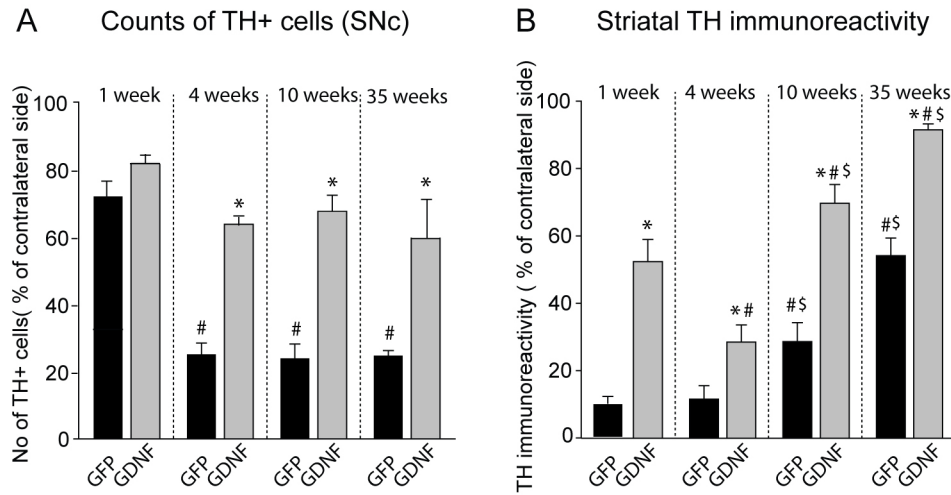


Fig 4. Neurorestorative effects induced by LvGDNF on the nigrostriatal pathway. (A) Stereological counts of TH positive cells were performed in the SNc. Values represent the number of TH positive cells in the SNc (from 7 sections per animal) on the lesion side as a percentage of the intact side. (B) Densitometric analysis of TH was performed in the striatum. Values represent the mean \pm SEM per 7-8 sections throughout striatum and are expressed as percentage of the lesioned side/intact. The time points considered are 1, 4, 10 or 35 weeks post lesion (n=9-4). Two-way ANOVA and *post hoc* Tukey's test: * $p < 0.05$ vs GFP at the same time point; # $p < 0.05$ vs 1 week in the same group; \$ $p < 0.05$ vs 4 week in the same group. SNc, Substantia nigra pars compacta.

Fig. 5

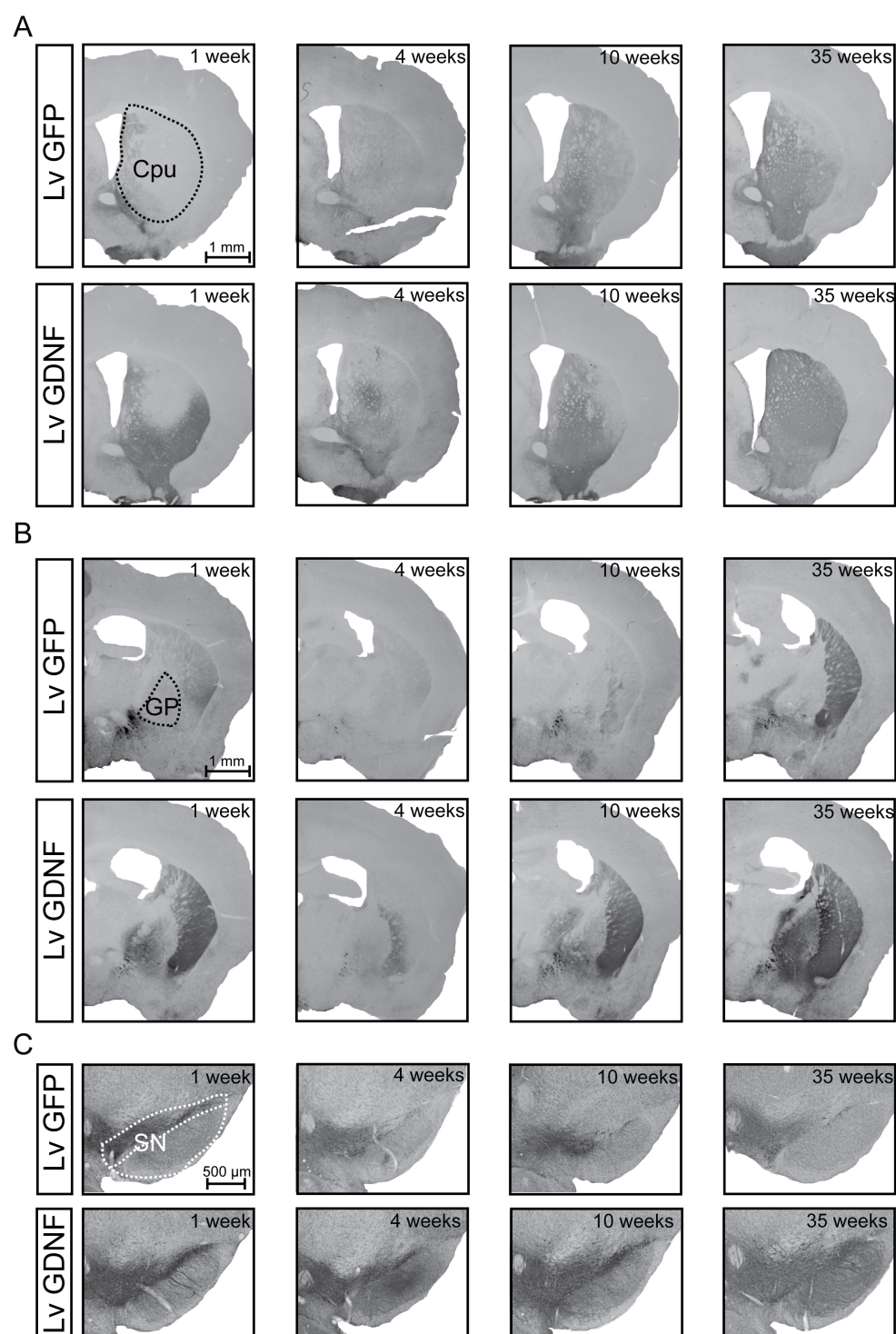


Fig 5. Overview of TH-immunostained sections show the distribution of dopaminergic fibers in the ipsilateral striatum (A), GP (B) and SN (C) of animals injected either with LvGFP or LvGDNF. TH immunohistochemistry was performed after 1, 4, 10 or 35 weeks post lesion. GP, Globus pallidus; SN, Substantia nigra. Scale, 500 μ m.

Fig. 6

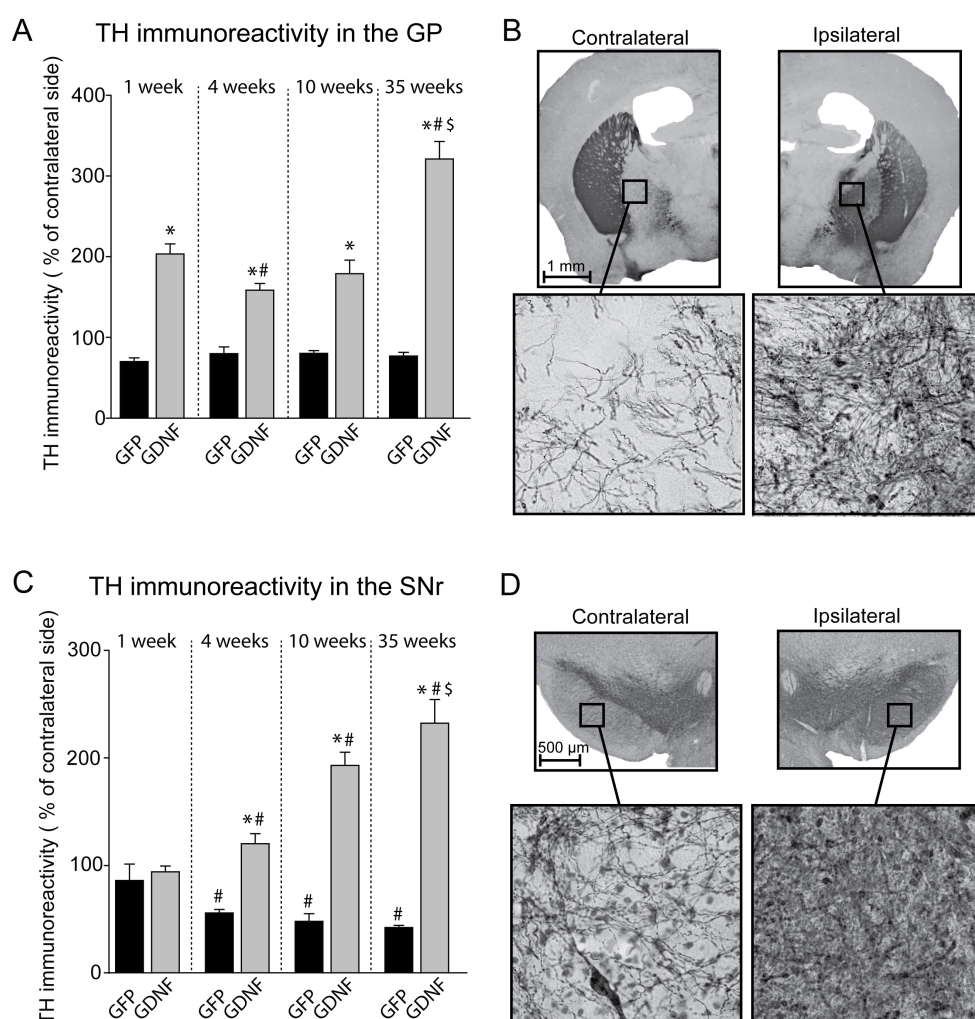


Fig 6. Densitometric analysis of TH immunoreactivity in the GP and SNr. Values represent the mean \pm SEM per 4 sections throughout GP (A) and SNr (C), and are expressed as percentage of the lesioned side/intact. Mice injected either with LvGFP or LvGDNF followed by 6-OHDA were sacrificed for TH immunohistochemistry 1, 4, 10 or 35 weeks post lesion (n=9–5). Photomicrographs show representative pictures of TH-immunostained sections at the time point 35 weeks from the GP (B) and SNr (D) with cresyl violet counterstaining. Two-way ANOVA and *post hoc* Tukey's test: * $p < 0.05$ vs GFP at the same time point; # $p < 0.05$ vs 1 week in the same group; \\$ $p < 0.05$ vs 4 week in the same group. GP, globus pallidus; SNr, Substantia nigra pars reticulata.

Fig. 7

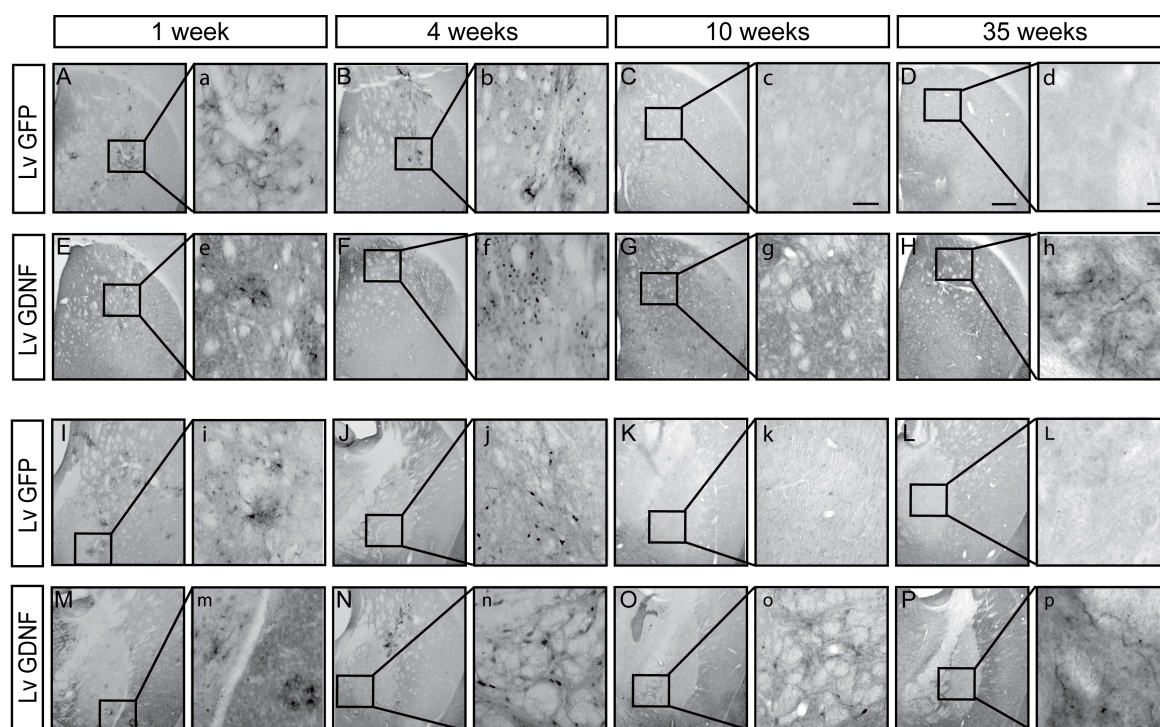


Fig 7. Pattern of pERK1/2 immunoreactivity in the striatum and the GP at different survival times. Photomicrographs show pERK1/2 immunostaining in the striatum (A-h) and in the GP (M-p) in animals that received LvGDNF or LvGFP. pERK1/2 immunohistochemistry was performed after 1, 4, 10 or 35 weeks post lesion. Pictures were taken under a 4x objective (capital letters; scale bar, 500 μ m), or 20x objective (small letters; scale bar, 100 μ m). Pictures in *d*, *h*, *l*, and *p* (35 weeks) were taken under a 100x objective (scale bar, 10 μ m). GP, Globus pallidus.

Fig. 8

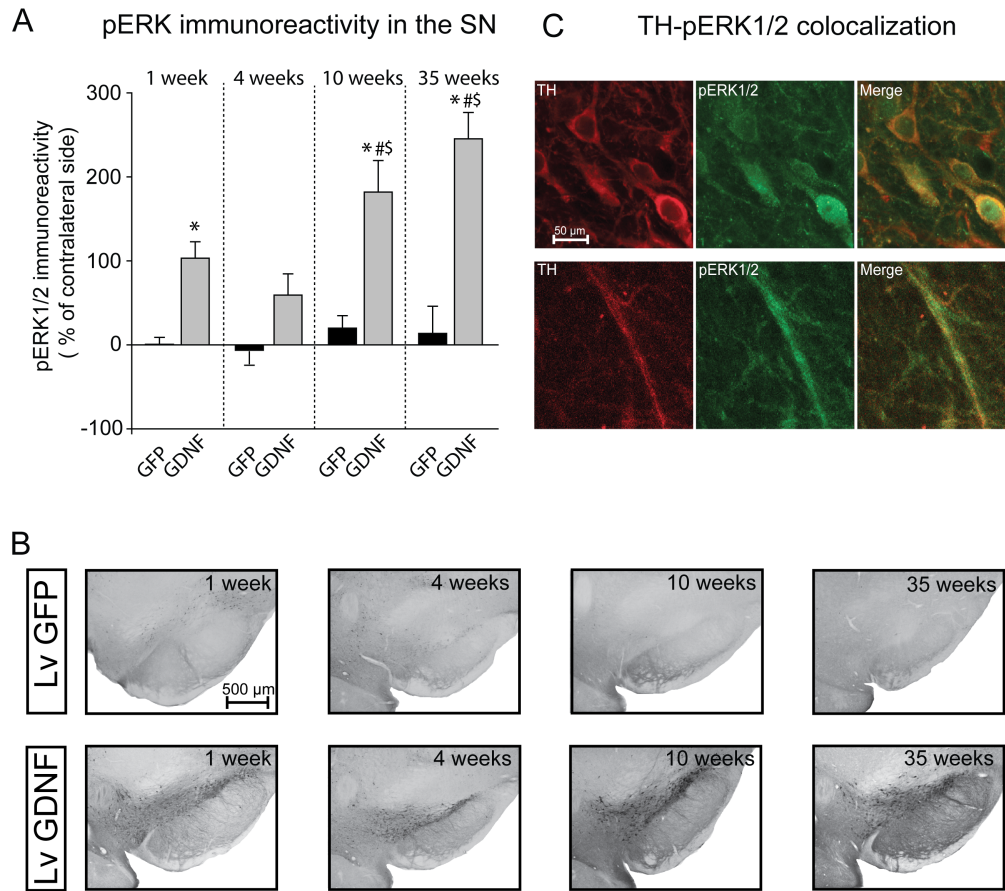


Fig 8. Sustained ERK1/2 activation in the SN was induced by LvGDNF. (A) Densitometric analysis of pERK1/2 immunoreactivity in the SN. Values on the lesion side are expressed as a percentage of the contralateral side. Two-way ANOVA and *post hoc* Tukey's test: * $p < 0.05$ vs GFP at the same time point; # $p < 0.05$ vs 1 week in the same group; \$ $p < 0.05$ vs 4 week in the same group. (B) Photomicrographs of pERK1/2 immunostaining in the SNc and SNr at 1, 4, 10 or 35 weeks post 6-OHDA lesion in animals injected either with LvGFP or LvGDNF. Scale bar, 500 μ m. (C) Dual-antigen immunohistochemistry and confocal laser scanning microscopy showed colocalization of pERK1/2 and TH in nigral cell bodies and dendrites in the LvGDNF cases. Scale bar, 50 μ m. SNc, Substantia nigra pars compacta; SNr, Substantia nigra pars reticulata.

Fig. 9

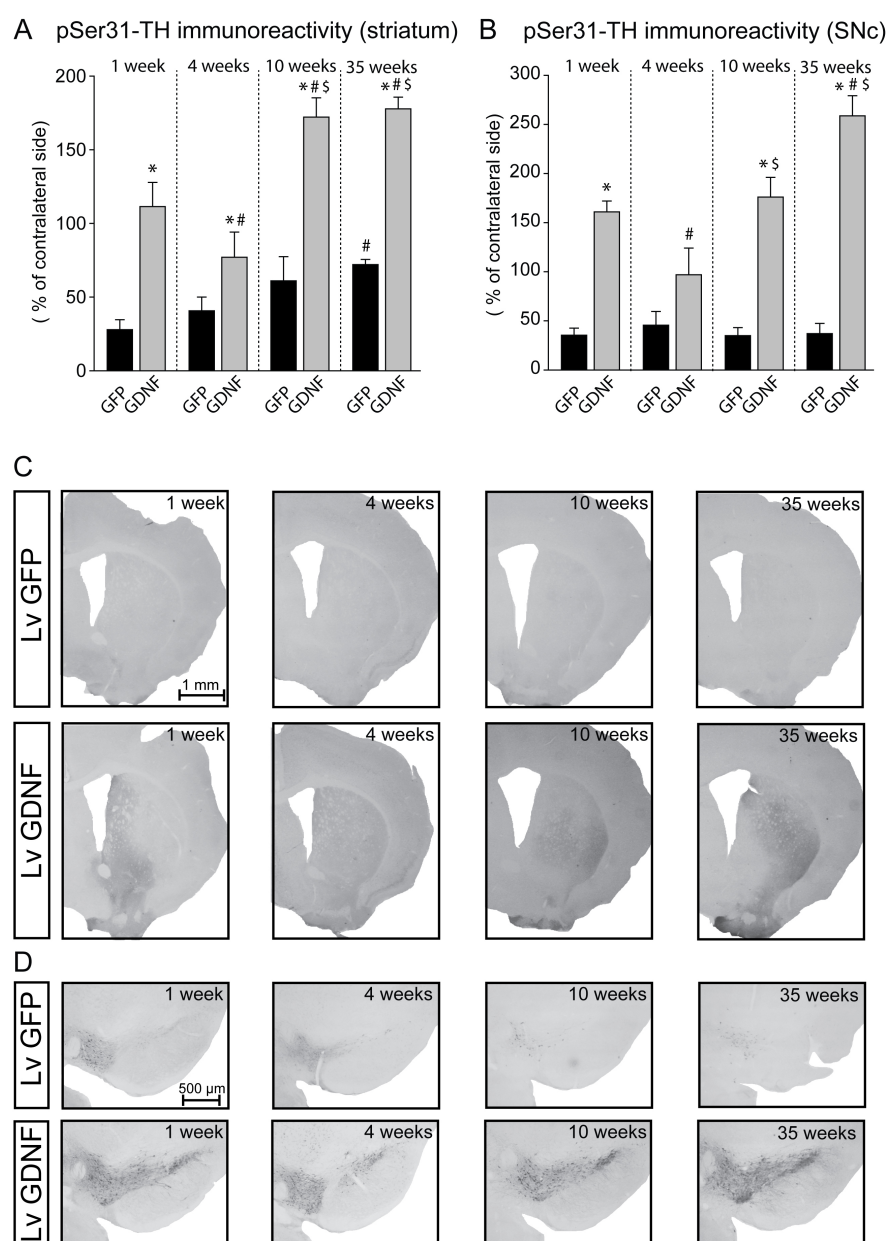


Fig 9. Densitometric analysis of pSer31-TH immunoreactivity in the striatum (A) and SNc (B) at 1, 4, 10 or 35 weeks post lesion in animals receiving LvGFP or LvGDNF (n = 7 – 4). Values from the lesion side are expressed as a percentage of the contralateral side. Two-way ANOVA and *post hoc* Tukey's test: * p < 0.05 vs GFP at the same time point; # p < 0.05 vs 1 week in the same group; § p < 0.05 vs 4 week in the same group. (C) Overview pictures of pSer31-TH immunostaining in the striatum (C) and SNc (D). Scale bar, 1 mm (striatum) or 500 μ m (SNc). SNc, Substantia nigra pars compacta.