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Combination of CDNF and Deep Brain Stimulation Decreases Neurological Deficits in Late-stage Model Parkinson's Disease

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Abstract—Several neurotrophic factors (NTF) are shown to be neuroprotective and neurorestorative in pre-clinical animal models for Parkinson's disease (PD), particularly in models where striatal dopamine neuron innervation partially exists. The results of clinical trials on late-stage patients have been modest. Subthalamic deep brain stimulation (STN DBS) is a proven treatment for a selected group of advanced PD patients. The cerebral dopamine neurotrophic factor (CDNF) is a promising therapeutic protein, but its effects in animal models of late-stage PD have remained under-researched. The interactions of NTF and STN DBS treatments have not been studied before. We found that a nigral CDNF protein alone had only a marginal effect on the behavioral deficits in a late-stage hemiparkinsonian rat model (6-OHDA MFB). However, CDNF improved the effect of acute STN DBS on front limb use asymmetry at 2 and 3 weeks after CDNF injection. STN lesion—modeling chronic stimulation—had an additive effect in reducing front limb use in the cylinder test and apomorphine-induced rotation. The combination of CDNF and acute STN DBS had a favorable effect on striatal tyrosine hydroxylase. This study presents a novel additive beneficial effect of NTF and STN DBS, which might be explained by the interaction of DBS-induced endogenous NTFs and exogenously injected CDNF. SNpc can be reached via similar trajectories used in clinical STN DBS, and this interaction is an important area for future studies. **© 2018 The Authors. Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).**

Key words: CDNF, MANF, GDNF, neurotrophic factor, median forebrain bundle, 6-hydroxydopamine, STN DBS.

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease. The cardinal symptoms of PD—tremor, rigidity, postural instability, and bradykinesia—are mainly caused by shortage of dopamine due to dopamine neuron death in the substantia nigra pars compacta (SNpc). The best available drugs—levodopa in combination with DDC and COMT inhibitors and dopamine agonists—act by replacing lost dopamine in the brain, leading to alleviation of the motor symptoms. However, in the late stages of the disease, these drugs lose their effectiveness or begin to cause debilitating side effects. Deep brain stimulation (DBS) of the subthalamic nucleus (STN) provides an efficient symptomatic control for some patients whose PD has progressed to a late stage (Krack et al., 2003; Castrioto et al., 2011).

Experimental DBS is often termed high-frequency stimulation (HFS), alluding to the finding that only frequencies higher than about 60 Hz alleviate the motor symptoms produced in the animal models of PD (Fogelson et al., 2005), and the standard 130-Hz frequency used produces mainly the inhibition of STN cells (Tai et al., 2003). In most animal studies, stimulation comes from an external pulse generator that limits the duration of continuous HFS to hours, or at the most, days. Long-term STN HFS and DBS can be mimicked by the lesioning of the STN (STNL), and originally DBS was realized to reproduce the effects of therapeutic stereotactic lesions (Benazzouz et al., 1993). The efficacy of STNL was originally verified by chemical ibotenic acid (IBOT) lesions in a primate MPTP model of PD (Bergman

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Abbreviations: 5-HT, serotonin, 6-OHDA, 6-hydroxydopamine; CDNF, cerebral dopamine neurotrophic factor; DA, dopamine; DBS, deep brain stimulation; DOPAC, 3,4-dihydroxyphenylacetic acid; GDNF, glial cell line-derived neurotrophic factor; HVA, homovanillic acid; IBOT, ibotenic acid; MANF, mesencephalic astrocyte-derived neurotrophic factor; MFB, medial forebrain bundle; NTF, neurotrophic factor; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; STN, subthalamic nucleus; STNL, lesion of subthalamic nucleus; TH, tyrosine hydroxylase.

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et al., 1990). Furthermore, STNL has been used in patients with PD (Jourdain et al., 2014). Additionally, STN DBS has both local excitatory effects aside from inhibition (McIntyre et al., 2004) and complex network level effects (McIntyre and Hahn, 2010) such as effects on beta coupled high-frequency activity in motor cortex (Yang et al., 2014). According to animal studies, DBS is able to cause increased striatal dopamine transmission (Meissner et al., 2001; Walker et al., 2009; Pazo et al., 2010) and affect the downstream motor nuclei (Shehab et al., 2014). There is some experimental evidence that DBS may have a neuroprotective effect (Temel et al., 2006; Harnack et al., 2008; Spieles-Engemann et al., 2010; Musacchio et al., 2017), but clinical evidence for neuroprotection is lacking (Harnack and Kupsch, 2010).

There has been an extensive search for drugs that can stop the progression of dopamine neuron degeneration and restore dopaminergic phenotype and function in dying neurons (Airavaara et al., 2012b; Bartus and Johnson, 2017a,b). Neurotrophic factors (NTFs) are able to protect and even restore dopamine neuron degeneration. NTFs are a group of proteins with variable biological actions (Airaksinen and Saarma, 2002; Lindholm et al., 2016). Importantly, both glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) have been shown to be neurorestorative in rodent and primate models of PD *in vivo* (Airavaara et al., 2012b). The efficacy has been found in partial lesion models where tyrosine hydroxylase (TH) immunoreactive fibers in the striatum still exist. Recently, it was reported that the retrograde transport of cerebral dopamine neurotrophic factor (CDNF) from the striatum to substantia nigra (Voutilainen et al., 2011) depends on striatal dopamine innervation (Mätlik et al., 2017). Additionally, BDNF (Spina et al., 1992), FGF-2 (Otto and Unsicker, 1990) and VEGF (Yasuhara et al., 2004) have been effective preclinical trials. Two NTFs—GDNF and neurturin—have been in clinical trials, where they have been administered directly into the striatum either GDNF as a recombinant protein or NRTN in an adeno-associated virus (AAV) gene therapy, but their efficacy has been variable (Kordower et al., 1999; Gill et al., 2003; Lang and Obeso, 2004; Lang et al., 2006; Penn et al., 2006; Patel and Gill, 2007; Bartus and Johnson, 2017b). Clinical studies have usually been carried out on late-stage patients, but a recent NRTN study showed positive effects in a subgroup of patients with less-progressed disease (Olanow et al., 2015). Analysis of the brains of PD patients has shown that there is minimal or no putaminal TH immunoreactive innervation after 5 years post-diagnosis (Kordower et al., 2013). Therefore, the relatively rapid development of putaminal dopaminergic axonopathy should be taken into account in future trials (Tenenbaum and Humbert-Claude, 2017), particularly when there are radiolabeled ligands with which to study the functionality of dopamine terminals. Similarly, several NTFs have been effective in partial lesion models of PD; but with complete lesions, they have not restored dopamine neurites in the striatum (Airavaara et al., 2012b; Domanskyi et al., 2015).

The CDNF/MANF protein family has a neurorestorative potential similar to GDNF and NRTN (Lindholm et al., 2007; Voutilainen et al., 2009; Lindahl et al., 2017). In a rat 6-OHDA model of PD where 6-OHDA was administered to the striatum, both CDNF and mesencephalic astrocyte-derived neurotrophic factor (MANF) have been shown to be neuroprotective and neurorestorative (Lindholm et al., 2007; Voutilainen et al., 2009, 2011; Ren et al., 2013; Bäck et al., 2013a). This was also the case for CDNF in a mouse MPTP model of PD (Airavaara et al., 2012a). However, the effects of CDNF have not been studied in medial forebrain (MFB) 6-OHDA models of PD where dopamine depletion is nearly complete. In the clinic, STN DBS is used mostly for late-stage PD, where the caudate putamen is devoid of TH+ fibers and dopamine depletion is already severe. Therefore, the likelihood of any neurorestorative therapy alone increasing endogenous dopaminergic activity is limited.

The aim of the current experiments was to study whether CDNF has a neurorestorative effect in a late-stage parkinsonian model (MFB lesion) and whether CDNF and DBS are synergistically neurorestorative in this model. A MFB 6-OHDA lesion was used instead of a striatal lesion, and CDNF in combination with or without STN DBS was employed. STNL was used to model long-term DBS.

EXPERIMENTAL PROCEDURES

Animals

A total of 242 male Wistar rats weighing 220–300 g at the time of first operation were used. Rats were housed under a light–dark cycle at an ambient temperature of 20–23 °C. Food pellets (Harlan Teklad Global diet, Holland) and tap water were available *ad libitum*. The experimental design was approved by the Committee for Animal Experiments of the University of Helsinki and the chief veterinarian of the County Administrative Board for 2008–2010 and by the National Animal Experiment Board for 2011–2015. Animal experiments were conducted according to EU regulations (EU Directive 2010/63/EU) and Finnish legislation (Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes [497/2013] and the Government Decree on the Protection of Animals Used for Scientific or Educational Purposes). The laboratory experiment protocol approval numbers were ESAVI/5459/04.10.03/2011 and ESAVI/6959/04.10.03/2012.

Surgical procedures

The stereotaxic operations were performed under isoflurane anesthesia, as described in previous studies (Lindholm et al., 2007); (Voutilainen et al., 2009). 6-Hydroxypamine (6-OHDA, 10 μg) was injected into either the left anterior (A/P −2.0, L/M +2.0, D/V −8.3; adapted from (Shi et al., 2004) for the STN HFS and STN lesioning experiments (Experiments 3 and 4) or left posterior medial forebrain bundle (A/P −4.4, L/M +1.2, D/V −8.3) as described in (Hudson et al., 1993, 1994) for the NTF only experiments (Experiments 1 and 2). NTFs (GDNF, Amgen, Thousand Oaks, CA, USA; human recombinant CDNF (produced in CHO cells by Biovian, Turku, Fin-

land)) were given post-lesion as a single, slow infusion above the left SNpc (NTFs in 10 μl PBS, 0.5 μl/min, needle left in place for additional 4 min; A/P −5.4, M/L 2.0, D/V −8.3 from skull) ipsilateral to the lesion. MS308 electrodes (Plastics1, San Diego, CA, USA) were implanted in the ipsilateral STN (A/P −3.6; M/L 2.3; D/V −8.0). All coordinates relative to the bregma were according to the stereotactic rat brain atlas (Paxinos and Watson, 1998). The electrodes were fixed to skull via dental cement and screws. After the electrode implantation, rats were housed in individual cages. Lidocaine was used as a local anesthetic during surgery. Animals received 0.025 mg/kg buprenorphine s.c. (Temgesic®; Indivior, Slough, UK) for post-operative pain relief. The left STN was lesioned via a slow infusion of IBOT (A/P −3.6; M/L 2.3; D/V −8.0; 10 μg in 1 μl PBS; #329130010, Acros Organics, Belgium). Excessive rotational behavior after STN lesioning was treated with i.p. 4% chloral hydrate 100 mg/kg (Sigma–Aldrich #C8383, St. Louis, MO, USA).

Study designs

Experiment 1, CDNF dose response given at 4 weeks after MFB 6-OHDA. The rats (n = 90) received a 6-OHDA (10 μg) injection into the left MFB. Four weeks later, the rats received CDNF 1 μg, 3.3 μg, 10 μg, 33 μg, or 100 μg (n = 12–18), GDNF 100 μg (n = 5) or PBS (n = 18) left intranigral injections. The degree of lesion and the effects of NTFs were evaluated with repeated apomorphine-induced rotation tests andTH immunoreactivity at 16 weeks after NTF administration (Fig. 1A).

Experiment 2, CDNF 10 μg given at 1 week after MFB 6-OHDA. The rats (n = 43) received a 6-OHDA (10 μg) injection into the left MFB, and 1 week later, they received 10 μg of CDNF (n = 13) or GDNF (n = 15), or PBS only (n = 15) above the left SNpc. Apomorphine-induced rotation tests were at weeks 2, 4, 6, and 8 after NTF injection (Fig. 2A). Biochemical analysis was done with TH immunohistochemistry (IHC) for the evaluation of TH+ fibers in the striatum and TH+ cell counts in the SNpc.

Experiment 3, the combination of CDNF and STN HFS in MFB 6-OHDA PD model. The rats (n = 46) received a unilateral MFB 6-OHDA injection as described above and adequate lesioning was verified with an amphetamine-induced (2.5 mg/kg, i.p., Sigma–Aldrich, Saint Louis, MO, USA) rotation test at 6 days after 6-OHDA injection. Seven days after the 6-OHDA, 26 rats received CDNF 10 μg (n = 15) or PBS injection (n = 11) above the left SNpc, and stimulation electrodes were implanted with the tips aimed at the STN. An additional nine rats were selected for the control arm without electrode. Six rats died or had to be euthanized prematurely during the experiment, leaving 22 rats surviving at the end of the experiment in the electrode arm and seven control rats without electrode. The effect of STN DBS was measured by stimulation-induced dyskinesia and by repeated cylinder tests with no stimulation (baseline) and at two stimulation amplitudes. The stimulation amplitude for cylinder tests was chosen individually for each rat based on the stimulation-induced dyskinesias; amplitude 1 was chosen to be just below any dyskinesias, and amplitude 2 was chosen as the amplitude below sustained front paw dyskinesias. The stimulation amplitudes chosen the first week after implantation were kept constant throughout the study. Biochemical analysis was done measuring striatal concentrations of dopamine (DA) and DA metabolites with high-performance liquid chromatography (HPLC) (at 4 weeks) or with TH and dopamine transporter (DAT) IHC (at 7 weeks) (Fig. 3A).

Experiment 4, the combination of CDNF and STN lesion in MFB 6-OHDA PD model. The rats (n = 63) received a unilateral MFB 6-OHDA injection as above, and after 6 days, the rats underwent amphetamine-induced rotation test to confirm adequate lesioning. After the test, 59 rats were selected to continue receiving CDNF 10 μg or PBS injection above the left SNpc and IBOT injection or PBS injection to the STN seven days after 6-OHDA injection. After CDNF and IBOT lesioning, a total of four rats were found dead in their cages or had to be euthanized prematurely, 54 rats surviving at the end of experiment (4 or 7 weeks). There were four groups in this experiment. Group one received PBS + PBS (n = 12), group two received CDNF + PBS (n = 11), group three received PBS + IBOT (n = 14), and group four received CDNF + IBOT (n = 17). The behavioral effect was measured with repeated cylinder tests and apomorphine-induced rotation tests. The biochemical effect was measured by HPLC (4 weeks) or IHC (7 weeks; Fig. 4A).

Behavioral assays

Apomorphine-induced rotations. Following a 30-min habituation period, a single injection of 0.1 mg/kg of apomorphine (Sigma Aldrich, Saint Louis, MO, USA) was administered subcutaneously, and the rotational behavior was recorded for 60 min. The results are presented as net contralateral rotations, where the ipsilateral rotations are subtracted from the contralateral rotations.

STN HFS-induced dyskinesias. On a 0–4 scale, the dyskinesia elicited by STN HFS was assessed on four different dyskinesia subgroups: orofacial, axial, front limb, and locomotive dyskinesia. The scale used was adapted from previous studies (Pavon et al., 2006) and modified: 0 = no dyskinesia, 1 = transient (< 2s) or unclear, 2 = infrequent and mild or decays strongly (< 10 s), 3 = marked, long-lasting (> 10 s), frequent (> 50%), and 4 = extreme, minimal decay (> 60 s), almost all the time. The highest score with a single or more fulfilled criteria was chosen for each data point. All the stimulation currents were scored for each dyskinesia

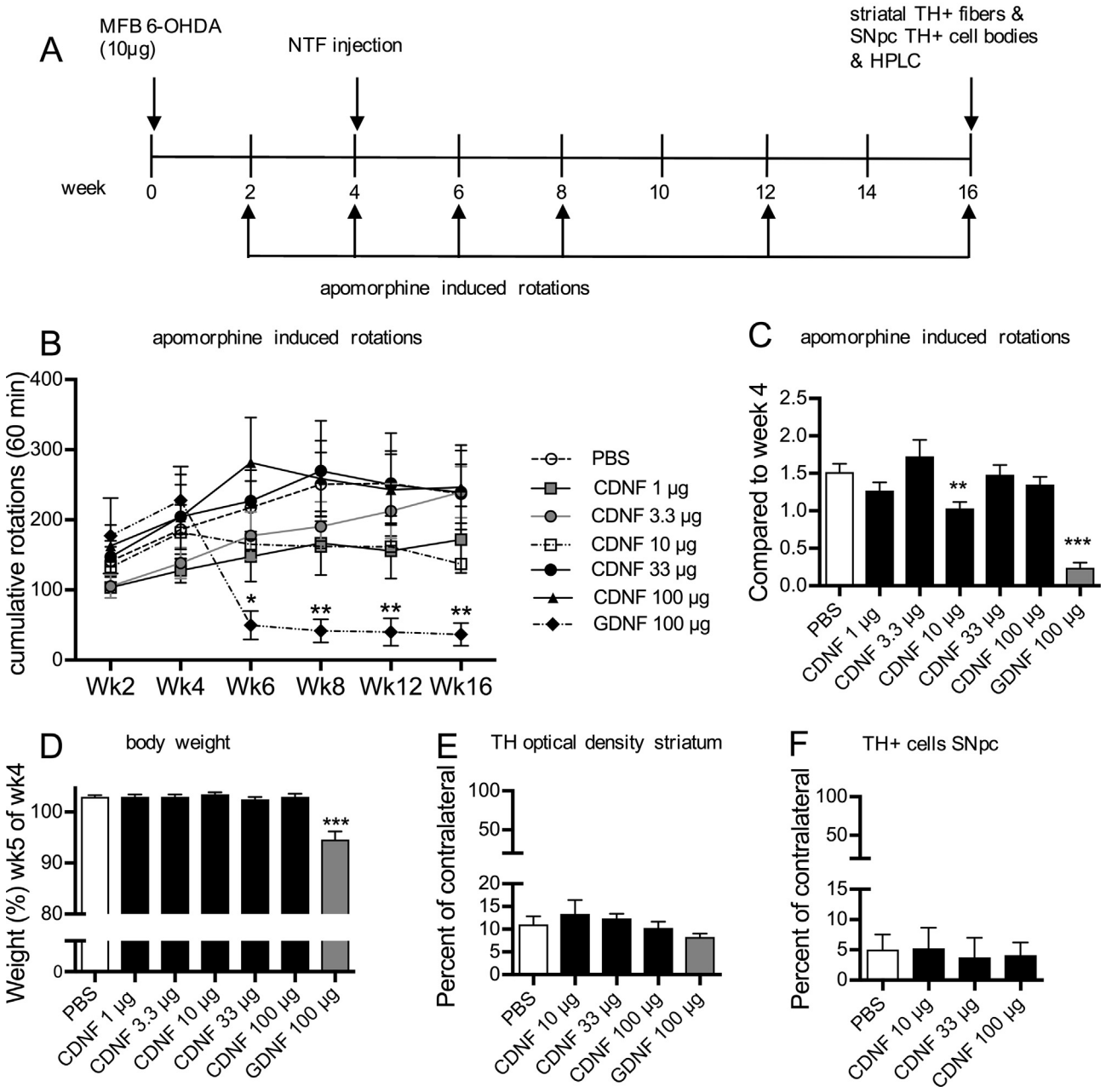

Fig. 1. Effects of various doses of intranigral CDNF injection in a 6-OHDA MFB lesion. (A) Study design of experiment 1; NTFs were administered 4 weeks after 6-OHDA. (B) Evolution of apomorphine-induced rotations over time. (C) All apomorphine-induced rotations post-NTF injection compared to rotations at 4 weeks. (D) Average body weight 1 week after growth factor injection compared to body weight before growth factor injection. (E) Tyrosine-hydroxylase-stained striatal optical density of TH+ fibers compared to contralateral side. (F) Substantia nigra TH+ cell numbers compared to the contralateral side. Data expressed as mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.0001$.

subtype. The dyskinesias were tested by increasing the current stepwise (0, 25, 50, 75, 100, 125, 150, 200, 250, 300, 350, and 400 µA) or until the development of severe dyskinesias, with each step lasting for at least 10 s (as described in our unpublished study, which Huotarinen et al. submitted). The overall amount of stimulation-induced dyskinesias was estimated by a dyskinesia score representing the sum of all dyskinesia subtypes over all amplitudes.

Cylinder tests. Rats were tested in a 25-cm diameter cylinder for the front limb contacts during the vertical rearing movement. Rats were recorded for a maximum of 10 min or until the rat reared 20 times. The counting of contacts was done from a video by a blinded observer. After the baseline cylinder test without stimulation, the rats were tested for stimulation-induced dyskinesias to determine individual stimulation amplitudes.

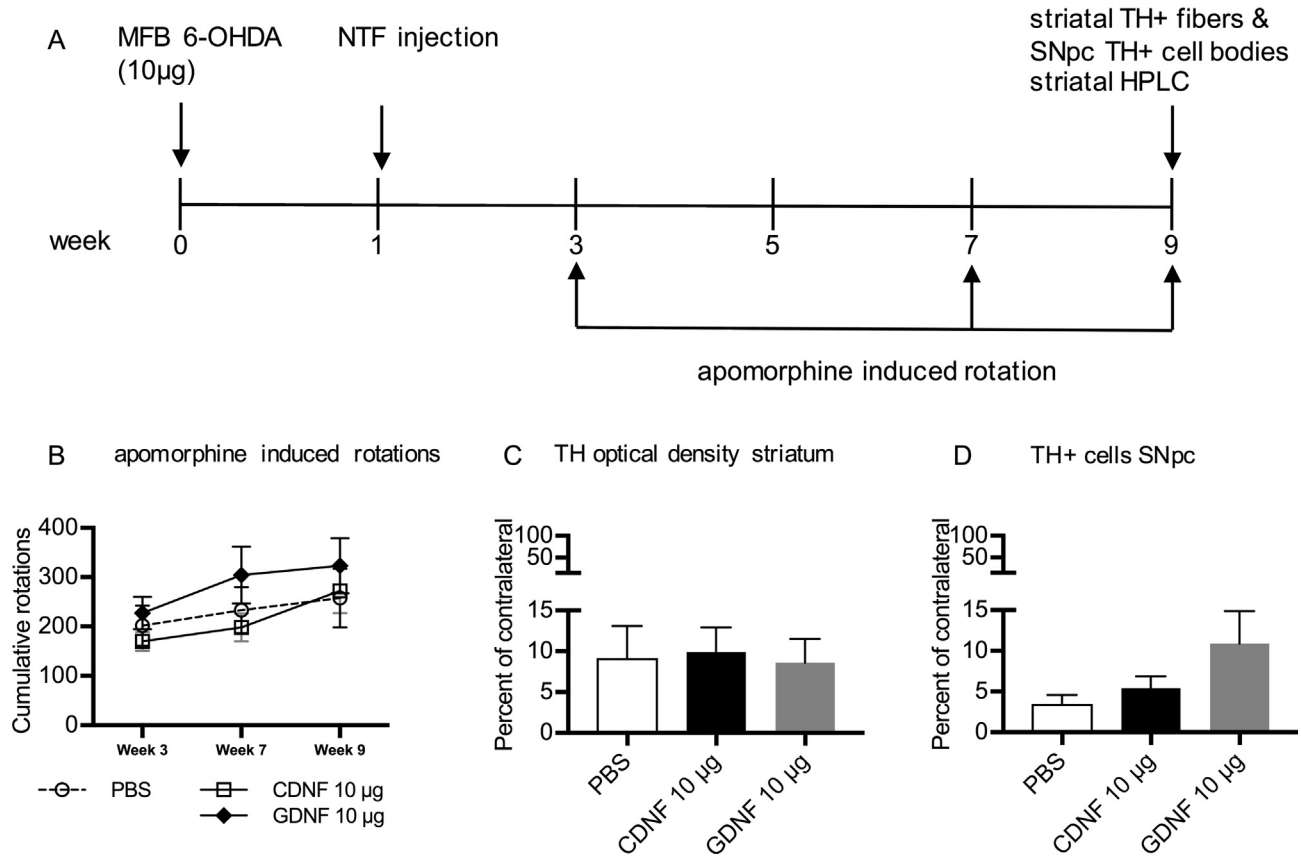

Fig. 2. (A) Study design of experiment 2 for testing intranigral CDNF 10 µg given 1 week after 6-OHDA. (B) Apomorphine-induced contralateral rotations. (C) Tyrosine-hydroxylase-stained striatal optical density of TH+ fibers compared to contralateral side. (D) TH+ cell count compared to contralateral non-lesioned site. Data expressed as mean ± SEM.

Immunohistochemistry

The animals were anesthetized with an overdose of pentobarbital (90 mg/kg, i.p., Mebunat®, Orion Oyj, Espoo, Finland) and perfused transcardially PBS followed by 4% paraformaldehyde. The removed brains were post-fixed in paraformaldehyde overnight and stored in 20% sucrose at 4 °C. Frozen brains were cut into 40-µm-thick coronal sections in a series of six with a gliding microtome (Leica Biosystems, Newcastle, UK).

In experiments 1 and 2, the free-floating sections were stained with mouse anti-TH antibody (1:2000, MAB318, Millipore, Billerica, MA, USA), as previously described (Voutilainen et al., 2009).

In experiment 3, the endogenous peroxidase activity of the free-floating sections was quenched for 30 min in 0.3% hydrogen peroxide solution. For the anti-DAT staining, the sections were incubated in 10 mM citrate buffer, pH 6.0, at 80 °C for 30 min, as previously described (Bäck et al., 2013b). To block the unspecific binding, the sections were incubated in 4% BSA and 0.1% Triton-X-100 followed by overnight incubation with mouse anti-TH antibody (1:2000, MAB318, Millipore) or rat anti-DAT antibody (1:2000, MAB369; Millipore). Following the incubation in the biotinylated secondary antibodies (1:200, anti-mouse or anti-rat, Vector Labs, Burlingame, CA, USA), the signal was enhanced using the avidin–biotin–enzyme complex (ABC-kit, Vector Labs) and visualized with 3′,3′-diaminobenzidine.

Optical density

In experiments 1 and 2, the images of the TH-stained sections were acquired with a digital camera (Nikon Corp., Tokyo, Japan) attached to the stereomicroscope (Nikon). For experiment 3, the images of the stained sections were acquired with a 3DHistech scanner (3DHistech, Budapest, Hungary) service provided by the Institute of Biotechnology (http://www.biocenter.helsinki.fi/bi/histoscanner/index.html). The optical density of the TH+ fibers in the striatum was determined from six sections from each rat by using ImagePro software (Media Cybernetics, Inc., Rockville, MD, USA). The results are given as a percentage of the intact side, which was defined as 100% as described previously (Penttinen et al., 2016).

TH+ cell counts

The number of TH-reactive cells in SNpc was estimated using StereoInvestigator software (MicroBrightfield, Williston, VT, USA) as previously described (Penttinen et al., 2016). Cells in SNpc were count bilaterally from six sections from each animal using unbiased counting

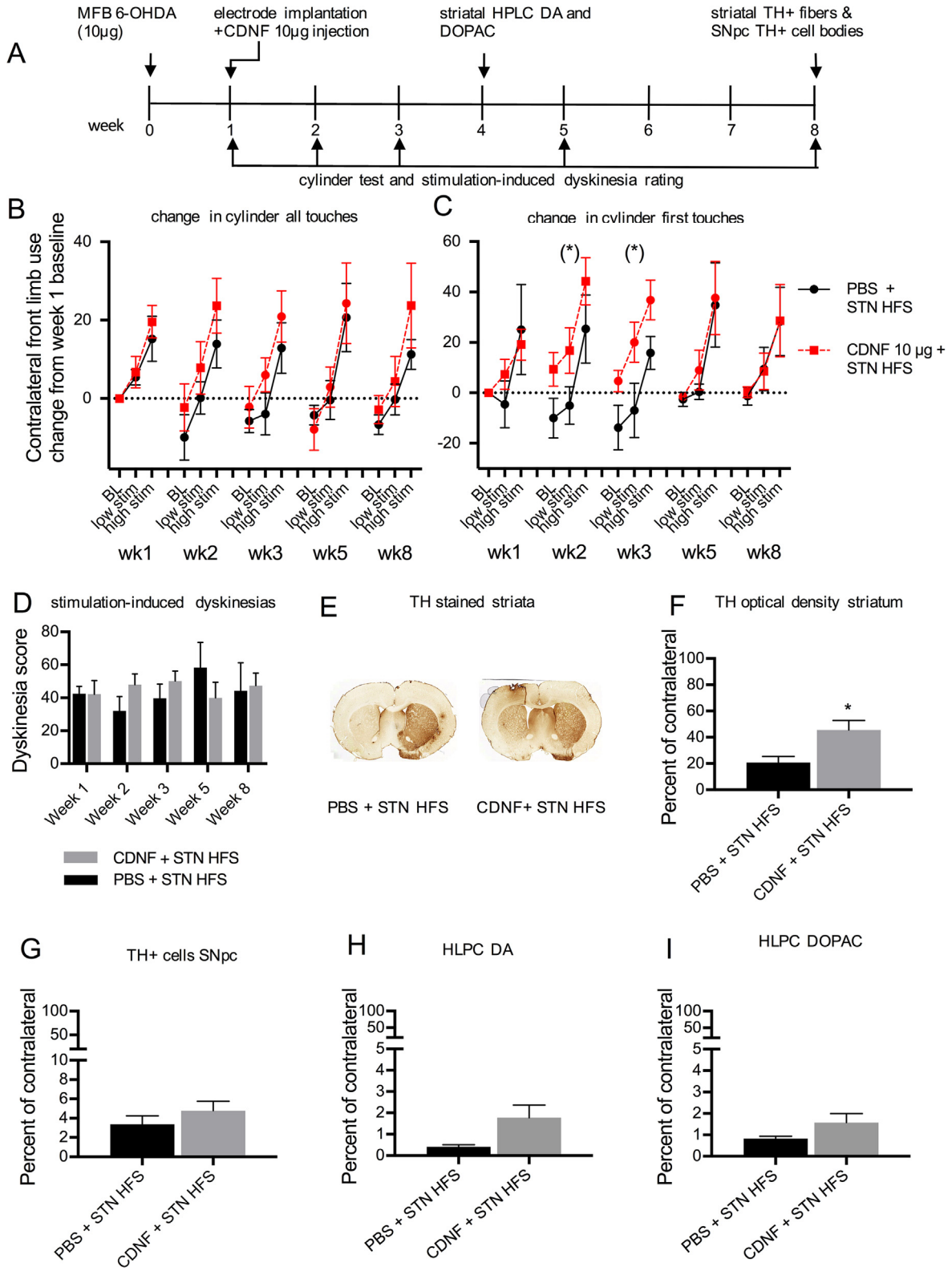

Fig. 3. The effects of intranigral (left) CDNF 10 µg combined with subthalamic stimulation (STN HFS). (A) Study design of experiment 3 for testing the effects intermittent STN HFS combined with CDNF. Cylinder tests were done on consecutive days with no stimulation, low stimulation, and high stimulation. (B) The evolution of right limb use asymmetry when all wall touches were counted in the cylinder test compared to baseline (no stimulation) of week 1. (C) The evolution of right limb use asymmetry when only first wall touches were counted in the cylinder test compared to baseline (no stimulation) of week 1. (D) Subthalamic stimulation-induced dyskinesias over time. E) Example of TH striatal optical densities at 8 weeks. (F) Tyrosine-hydroxylase-stained striatal optical density of TH + fibers compared to contralateral side at 8 weeks. (G) TH + cell counts in the substantia nigra compared to contralateral side at 8 weeks. (H) DA HPLC results at 4 weeks. (I) DOPAC HPLC results at 4 weeks. Data expressed as mean ± SEM, $^{*}p < 0.05$, (*) < 0.05 when weeks 2 and 3 were analyzed together.

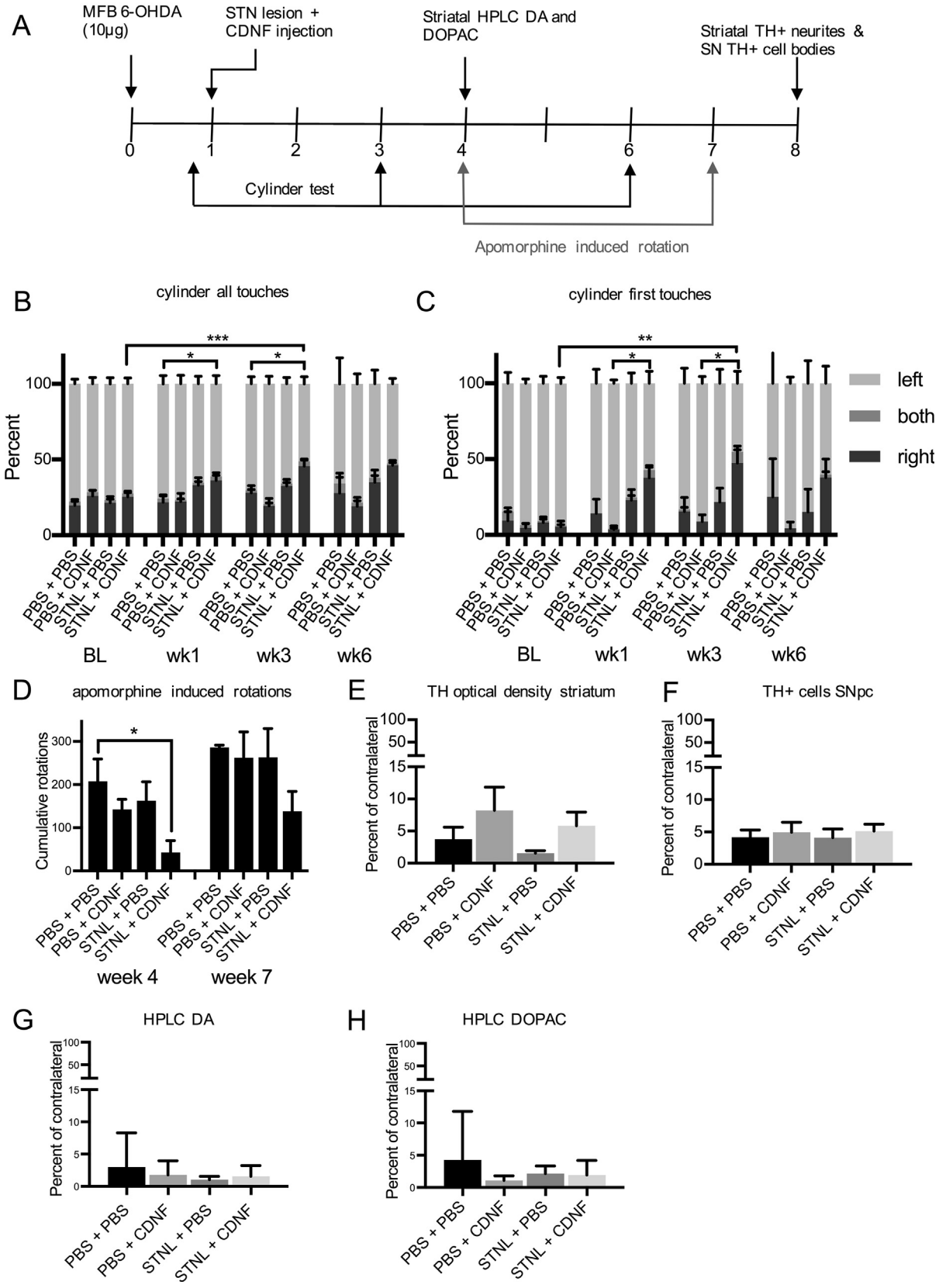

Fig. 4. (A) Study design of experiment 4 for testing the combination of intranigral (left) CDNF 10 µg and STN lesion (STNL). (B) Front limb measured by all touches with the wall during rearing movements in the cylinder test at baseline before CDNF and STNL and at 1, 3, and 6 weeks after. (C) Front limb measured by first touches with the wall during rearing movements in the cylinder test use at baseline before CDNF and STNL and at 1, 3, and 6 weeks after. (D) The number of apomorphine-induced rotations at 4 and 7 weeks after CDNF and STNL. (E) Optical density of TH immunohistochemistry of lesioned side compared to the unlesioned side. (F) The number of TH + cells in SNpc compared to the unlesioned side. (G) HPLC results for percentage of dopamine (DA) on the lesioned side compared to the unlesioned side. (H) HPLC results for percentage of DOPAC on the lesioned side compared to the unlesioned side. Data expressed as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$.

rules (Voutilainen et al., 2009). The data are presented as a percentage of the intact side.

Stimulation specifications

In experiment 3, the rats received HFS to STN through the implanted electrodes. The rats were able to move freely during all stimulations. All stimulations were conducted with a frequency of 130 Hz and pulse width of 60 μs delivered with a STG 4004 external impulse generator (Multichannel Systems, Reutlingen, Germany). The stimulations took place on weeks 1, 3, 5, and 8 after the implantation. Each weekly time point comprised three individual daily stimulation sessions. On day 1, each rat was tested for stimulation-induced dyskinesias by increasing the current stepwise from 25 μA to 400 μA or to the maximum currents tolerated by the animal. The dyskinesia was scored on a 0–4 rating scale. On the second day of stimulation, the rats were tested in a cylinder for front paw rearing activity with stimulation current just below any significant front limb dyskinesia (front limb dyskinesia rating 2, low stimulation), and on the third day, this was performed with the stimulation amplitude just below the level where stimulation produced 10 s of lasting front limb dyskinesias (front limb dyskinesia rating 3, high stimulation). As described in our currently unpublished study (Huotarinen et al., submitted). Stimulation during cylinder testing lasted for 10 min. Before euthanasia, the rats were stimulated with the higher stimulation amplitude for 60 min. The stimulation was stopped only immediately before euthanasia.

Analysis of striatal dopamine and metabolites

The rat striatum samples were homogenized in 0.5 ml of homogenization solution consisting of six parts 0.2 M $HClO_4$ and one part antioxidant solution (1.0 mM oxalic acid, 0.1 M acetic acid, and 3.0 mM L-cysteine; (Kankaanpää et al., 2002)) with a Rinco ultrasonic homogenizer (Rinco Ultrasonic AG, Romanshorn, Switzerland). The homogenates were centrifuged at 20,800*g* for 35 min at 4 °C. The supernatant was removed to 0.5-ml Vivaspin filter concentrators (10,000 MWCO PES; Sartorius, Stonehouse, UK) and centrifuged at 8600*g* at 4 °C for 35 min. Filtrates containing monoamines were analyzed using high-pressure liquid chromatography with electrochemical detection. Analyses of DA and its main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-HT were performed (Airavaara et al., 2006; Valros et al., 2015).

Statistics

All statistical analyses were done by SPSS 24.0 (IBM, Armonk, NY, USA). All variables were tested for normality with Shapiro–Wilks and equality of variance with Levene's test, and parametric or nonparametric tests were used accordingly. Comparisons between multiple groups were done with ANOVA followed by Tukey's post hoc test for a comparison between all study groups; Dunnett's test was used to compare groups that received treatments to the baseline group (vehicle only). ANOVA tests were one-way ANOVA unless otherwise stated. Nonparametric comparisons for multiple groups were done with Kruskal–Wallis test or with Friedman's test for repeated variables, followed by post hoc Bonferroni corrected Mann–Whitney test. Student's *t*-test was used when comparing two groups for parametric variables and Mann-Whitney for nonparametric variables. All statistical tests were two-sided. *P*-values < 0.05 were decided to be statistically significant.

RESULTS

Experiment 1, CDNF dose-dependent response given at 4 weeks following the 6-OHDA lesion

First, we analyzed the effects of various doses of intranigral CDNF in the rat 6-OHDA MFB model of advanced PD. There was no difference in apomorphine-induced contralateral rotations before NTF injections at 2 and 4 weeks after 6-OHDA injection (2 weeks $\chi^2(6) = 4.909$, $p = 0.555$ and 4 weeks $\chi^2(6) = 2.907$, $p = 0.820p = 0.725$)

CDNF delivered as a single intranigral injection (1, 3.3, 10, 33, or 100 μg) 4 weeks post-lesion did not produce any consistent effect on the drug-induced rotation behavior compared to the vehicle (PBS) (Fig. 1B; 6–16 weeks $\chi^2(6) = 13.4$–16.3, $p = 0.012$–0.038, all post hoc tests $p > 0.6$, across weeks 6–16 and all CDNF concentrations). GDNF at 100 μg led to a marked reduction of rotations compared to PBS (post hoc vs. PBS 0.0235, 0.0078, 0.0069 and 0.0093) and to rotations at 4 weeks at all time points after NTF injection (Friedman's $\chi^2(4) = 12.65$, $p = 0.13$, post hoc weeks 8, 12 and 16 $p = 0.050$, 0.008 and 0.020 respectively). However, when all apomorphine-induced rotations after NTF injections were analyzed together, CDNF concentrations 1 μg and 10 μg reduced the apomorphine-induced rotations compared to PBS, although not significant for 10 μg ($\chi^2(6) = 54.69$, $p < 0.0001$, post hoc 0.0307 and 0.10 respectively). When the sum of apomorphine-induced rotations was compared to weekly rotations at the week 4 baseline before NTF injection, the sum of the rotations was significantly different for CDNF 10 μg and GDNF 100 μg compared to PBS (Fig. 1C; $\chi^2(6) = 63.23$, $p < 0.0001$, post hoc $p = 0.0016$ and $p < 0.0001$ respectively).

GDNF-treated animals lost body weight at 1 week after injection ($F[6,82] = 18.623$, $p < 0.001$, post hoc GDNF vs. all other groups $p < 0.0001$, all other post hoc tests $p > 0.9$) (Fig. 1D), but CDNF had no effect on body weight.

CDNF concentrations of 10, 33, or 100 μg or GDNF at 100 μg did not have an effect on the optical density of TH + fibers in the striatum (Fig. 1E, $\chi^2(4) = 4.53$, $p = 0.34$). Different CDNF concentrations did not have an effect on the number of TH+ cells in the SNpc (Fig. 1F, $F[3,44] = 0.056$, $p = 0.982$).

Experiment 2, CDNF 10 μg given at 1 week after MFB 6-OHDA

Because there was some effect of 10 μg CDNF when given 4 weeks after the 6-OHDA lesion, we wanted to

see whether earlier injection of NTFs at 1 week after 6-OHDA would improve the behavioral recovery (Fig. 2A).

The animals were balanced into different treatment groups based on amphetamine-induced rotations before NTF injections between the treatment groups ($\chi^2(2) = 0.01$, $p = 0.99$, data not shown). Neither CDNF 10 μg nor GDNF 10 μg produced a decrease in apomorphine-induced contralateral rotations at 3, 7, or 9 weeks (Fig. 2B; $p > 0.50$ at all time points). The optical density of TH-stained striata was similar in all groups (Fig. 2C; $\chi^2(2) = 0.29$, $p = 0.86$). There was no statistically significant difference in the number of TH+ cells in SNpc (Fig. 2D; $\chi^2(2) = 2.48$, $p = 0.29$). The number of TH+ cells in the substantia nigra was 3.5% (SD 5.6), 5.4% (SD 5.6), and 10.9% (SD 15.54) compared to the contralateral side. This was not statistically significant (Fig. 2D; $\chi^2(3) = 2.48$, $p = 0.29$).

Experiment 3, the combination of CDNF and STN HFS in the MFB 6-OHDA PD model

STN DBS is a standard treatment of advanced PD, and we wanted to study the effects of combined short-term STN stimulation and intranigral CDNF in an animal model of advanced PD to see whether the effect of STN stimulation would improve in CDNF-treated animals over time (Fig. 3A).

Rats were balanced into groups based on amphetamine-induced ipsilateral rotations prior to CDNF injection and STN electrode implantations 1 week after 6-OHDA injection ($\chi^2(3) = 1.90$, $p = 0.59$).

Stimulation amplitudes used through the experiment were based on stimulation-induced dyskinesias: PBS + STN HFS group: low stimulation 106.3 μA and high stimulation 181.3 μA. For the CDNF + STN HFS group: low stimulation 146.2 μA and high stimulation 250 μA. There was a statistically significant difference between the chosen amplitudes for both low stimulation ($U = 130.5$, $p = 0.030$) and high Low- and high-stimulation amplitudes corrected the front limb use asymmetry in the cylinder test with both CDNF- and PBS-treated animals at all time points significantly ($p < 0.001$), except for the PBS group at week 1 ($\chi^2(2) = 5.56$, $p = 0.062$) and week 5 ($\chi^2(2) = 5.73$, $p = 0.057$).

There were no statistically significant differences in front limb use asymmetry between the CDNF and vehicle groups at any point (Fig. 3B and 3C; all p-values >0.1 at all time points between respective stimulations [BL, low stimulation and high stimulation]). However, contralateral front limb use increased more at 2 and 3 weeks for CDNF-treated animals compared to PBS-treated animals with both low- and high-stimulation amplitudes when compared to week 1 baselines (no stimulation); although, this was not statistically significant (Fig. 3C; $p = 0.095$ and $p = 0.21$; week 3, $p = 0.11$ and $p = 0.31$, respectively, for low and high stimulation). When first touches with the wall at 2- and 3-week time points were analyzed together, the difference was significant for no stimulation and low stimulation, but not for high-stimulation amplitudes (baseline −11.94 SD 22.82 vs. 6.98 SD 19.13, $U = 2.18$, $p = 0.029$; low stimulation −6.01 SD 25.25 vs. 15.05 SD 23.74, $U = 2.29$, $p = 0.022$ and high stimulation 20.24 SD 27.17 vs. 40.53 SD 29.45, $U = 1.93$, $p = 0.053$). There were no statistically significant differences in the absolute values of STN HFS-induced dyskinesias at any time point (Fig. 3D, $p = 0.304$–0.908).

In rats treated with the combination of CDNF and STN HFS, the optical density of TH-stained striatum was higher compared to contralateral side than in PBS and STN–HFS treated animals (Fig. 3E and F, t-test $p = 0.0347$). However, the difference in striatal optical density was not significant for DAT staining (mean 11.12 SD 3.47 in PBS vs. 15.93 SD 6.92 in CDNF co-treated rats, $U = 0.85$, $p = 0.48$). There was no statistically significant difference in the number of TH+ cells in the stimulated animals that received CDNF compared to those that received PBS (Fig. 3G; 3.37% vs. 4.79% of contralateral side, $p = 0.3335$). The average DA levels in the lesioned side were 0.40–2.34% (Fig. 3H) of the levels on the contralateral unlesioned and untreated side, and there was no difference between the study groups ($\chi^2(3) = 6.33$, $p = 0.086$). The average DOPAC levels were 1.11–4.25% of the contralateral side, and there was no difference between the study groups (Fig. 3I; $\chi^2(3) = 2.06$, $p = 0.59$). Two animals were excluded from the HPLC analysis as outliers. HVA levels were measurable only in two striata on the lesioned side. There was no difference between study groups for 5-HT concentrations and DOPAC/DA ratios. We also tested the effects of CDNF without electrode compared to PBS without electrode, and there were no statistically significant differences in HPLC or behavioral tests at 4 weeks after injections.

Experiment 4, the combination of CDNF and STN lesion in the MFB 6-OHDA PD model

Because there was a modest trend for additive or synergistic effect of short-term STN HFS and intranigrally administered CDNF, we wanted to test if STN lesion, mimicking chronic STN DBS and shown to be neuroprotective when administered early after the 6-OHDA lesion, would have a stronger synergistic effect with CDNF (Fig. 4A).

There was no difference in amphetamine-induced rotations before the CDNF injections and STN lesioning between the treatment groups ($F[3,52] = 0.007$, $p = 0.999$). In the histological analysis, 10 animals were found to have macroscopic (1 mm or over) lesions in the subthalamic area or thalamus, and they were excluded from further analysis.

The combination of STN lesion and intranigral CDNF reduced the apomorphine-induced rotations at 4 weeks, whereas STN lesion or CDNF alone did not have an effect (Fig. 4D). Only animals with a combination of CDNF and STN lesions rotated less at 4 weeks ($F[3,41] = 3.853$, $p = 0.16$, post hoc Tukey for CDNF + STNL vs PBS + PBS $p = 0.014$). At 7 weeks, there was no significant differences between the groups ($\chi^2(3) = 6.2$, $p = 0.10$).

The cylinder test results were analyzed separately for all front limb touches and first front limb touches made

with the wall during rearing movements. In the cylinder test before CDNF or STNL, there was no differences between the groups for all touches ($\chi^2(3) = 3.49$, $p = 0.32$) or first touches ($\chi^2(3) = 1.31$, $p = 0.73$).

CDNF alone had no effect on contralateral front limb use (Fig. 4B, C). At 1 week after CDNF and STNL when all touches with the cylinder wall were analyzed (animals with less than 20 touches on the wall were excluded), only the STNL + CDNF group was different from the PBS + PBS group ($\chi^2(3) = 10.16$, $p = 0.017$, post hoc $p = 0.047$). At 3 weeks, all touches with the cylinder wall were analyzed (animals with less than 20 touches were excluded), and only the rats that received both the CDNF and STN lesion used the contralateral front limb significantly more compared to the PBS + PBS group ($\chi^2(3) = 15.84$, $p = 0.001$, post hoc $p = 0.011$). At 6 weeks, when all touches with the wall were counted (animals with less than 10 touches were excluded), there was a trend that showed the CDNF + STNL animals used contralateral front limbs more compared to the CDNF + PBS group ($\chi^2(3) = 6.52$, $p = 0.089$).

When only first touches with the wall were analyzed at one week after NTF injections (animals with under 7 rearings excluded), the rats with the combination of STNL and CDNF used the contralateral front limb more compared to CDNF + PBS group ($\chi^2(3) = 11.02$, $p = 0.012$, post hoc $p = 0.012$). At week 3, the rats that received the combination of CDNF and STNL used the contralateral front limb more than those receiving only CDNF and PBS to STN (animals with under 7 rearings excluded, $\chi^2(3) = 9.28$, $p = 0.026$, post hoc $p = 0.032$). Furthermore, CDNF + STNL animals used the contralateral front limb more than the PBS + PBS group; however, this was not statistically significant (post hoc $p = 0.227$). At 6 weeks, the number of rearings was so low that no meaningful statistical analysis for first touches was possible.

When the evolution of front limb use was compared to the baseline before CDNF injection or STNL, only the treatment group with the combination of CDNF 10 μg and STNL improved their contralateral front limb use (Fig. 4B, C). This effect was seen with all touches at 3 (Friedman's $\chi^2 = 9.171$, $p = 0.027$, post hoc $p < 0.001$), all other p-values > 0.2) and for first touches at 1 and 3 weeks (Friedman's $\chi^2 = 13.91$, $p = 0.001$, post hoc $p = 0.093$ and 0.002 respectively, all other p-values > 0.40, week 6 excluded from analysis due to the low number of rearings).

The optical density of TH-stained striatum showed no statistically significant differences between the groups (Fig. 4E; $\chi^2(3) = 4.347$, $p = 0.226$). There was no difference in the number of TH+ cells in SNpc (Fig. 4F; $\chi^2(3) = 0.51$, $p = 0.92$). HPLC analysis from the striatum samples showed no statistically significant differences between the groups for dopamine content (Fig. 4G; $\chi^2(3) = 2.04$, $p = 0.57$) or DOPAC content differences between groups (Fig. 4H; $\chi^2(3) = 2.97$, $p = 0.40$). There was no difference between the study groups for 5-HT concentrations and DOPAC/DA ratios.

We also performed the previous statistical tests with the excluded rats that had a macroscopic lesion from IBOT, and this had no major influences on the results or their significance, apart from the fact that the decrease in apomorphine-induced rotations at 4 weeks for the CDNF + STNL group compared to PBS + PBS group was not significant in the other post hoc test ($F[3,50] = 2.211$, $p = 0.087$, post hoc Tukey $p = 0.085$, Dunnett's $p = 0.049$).

DISCUSSION

We studied the effects of CDNF alone or in combination with STN DBS on restoring motor function and nigrostriatal pathway phenotype in a rat MFB 6-OHDA lesion model of advanced PD. Positive synergistic behavioral effect was found without robust histochemical or neurochemical recovery. Those are in line with previous reports indicating that CDNF produces partial reversal of the behavioral 6-OHDA hemiparkinsonian defects without changes in TH or monoamine transmitter levels (Bäck et al., 2013a), and that the correlation between degree of striatal TH loss and apomorphine-induced rotations is low (Kirik et al., 1998).

CDNF alone when given after complete lesion of the nigrostriatal dopamine pathway did not have an effect in this late-stage PD model, even at higher concentrations. These results further indicate that only in partial axonopathy of SNpc dopamine neurons CDNF can restore dopamine neuron circuitry as earlier suggested (Lindholm et al., 2007; Voutilainen et al., 2011). Indeed, it has been indicated that in order for NTF-induced neurorestoration to occur there needs to be viable dopamine neurons in the SNpc (Domanskyi et al., 2015). The small putative behavioral effects did not correlate with cell numbers and fiber densities, as has been described previously (Bäck et al., 2013a). As shown in other studies, intranigral GDNF delivery of 100 μg produced a decrease in apomorphine-induced rotational behavior (Hoffer et al., 1994) without an increase in striatal TH-levels (Bowenkamp et al., 1995). However, unlike GDNF, none of the doses of CDNF produced a reduction in body weight at 1 week after NTF injection. This effect of GDNF on body weight has been described before for intranigral injections in rats (Hoffer et al., 1994; Martin et al., 1996; Lapchak et al., 1997) and monkeys (Gash et al., 1995). This implies that CDNF has a more favorable overall effect on general well-being and functioning. This overall negative finding of the efficacy of CDNF alone in the rat model of advanced PD strengthens the importance of the findings when CDNF was combined with STN HFS and STNL.

For rodent models of PD and if neuroprotection or neurorestoration by various compounds are being studied, it is customary to make moderate striatal lesions by injecting small doses of 6-OHDA into the striatum itself (Penttinen et al., 2016). The idea is that there is enough intact dopaminergic innervation left in the striatum to allow meaningful possibilities to modify it. With this technique, several NTFs have shown both neu-

roprotective and even restorative activity in rat and mouse models of PD (Airavaara et al., 2012a,b). 6-OHDA injections into the MFB used in the current study caused serious dopaminergic damage that reduced striatal dopamine levels close to zero. It is practically impossible to restore this damage by any NTF treatment, even less so if the proteins are administered close to the SN, not to the striatum. It is quite surprising that a nigral dose (100 μg administered 4 weeks post-lesion) of GDNF was able to significantly reduce the apomorphine-induced turnings without any biochemical or histochemical recovery. The mechanism of this behavioral response remains obscure although previous studies indicate that the effect is mediated by increased dopamine neurite growth in the substantia nigra pars reticulata (Bowenkamp et al., 1995). Instead, similar CDNF treatments, up to 100 μg, were much weaker, and there was no dose–response. A similar lack of efficacy of both GDNF and CDNF at 10 μg was evident, even if NTFs were given 1 week post-lesion.

Considering these mainly negative findings, it was quite rewarding to see in an equally serious nigrostriatal dopamine pathway damage model that even 10 μg of nigral CDNF was able to add the efficacy of STN HFS in the cylinder test at several time points post-lesion. The beneficial effects were accompanied by increased optical densities of TH in the striatum but not with any change in the striatal dopamine (or metabolite) levels or TH+ cells in the SNpc.

A similar finding, supporting the additive effects of CDNF and STN HFS, was seen when nigral CDNF injections were combined to STN lesion. The STN lesion is mimicking the action of prolonged STN HFS. Now, a functional recovery was seen in two behavioral tests: apomorphine-induced turning and cylinder test. No notable recoveries were seen in striatal dopamine (and metabolite) or TH immunoreactivity in the SNpc or TH optical density in the striatum.

Overall, there seems to be a putative synergistic or additive effect of intranigral CDNF and stimulation or lesioning of the STN that was seen mainly in the behavioral tests. Whether this effect is unique to CDNF is a matter for future studies. The mechanism of this putative synergistic effect requires further studies, but there are previous reports on the effects of CDNF combined with either GDNF (Voutilainen et al., 2017) or MANF (Cordero-Llana et al., 2015). Also, several NTFs are required to protect SNpc neurons in culture from MPP+ toxicity (Jaumotte et al., 2016). An interesting possibility is that some of the synergies can be explained by the NTF-like effects of STN DBS, which has been shown to increase BDNF levels in the dopaminergic system of 6-OHDA-lesioned rats (Spieles-Engemann et al., 2011), and a recent study has reported that signaling mediated by the BDNF receptor TrkB could mediate both neuroprotective and short-term behavioral effects (Fischer et al., 2017). It is well-known that neuronal activity affects the levels of many NTFs, including BDNF, GDNF, CDNF, and MANF. Moreover, neuronal and electrical activity is known to stimulate the synthesis of BDNF (Kolarow et al., 2007) and GDNF (Lonka-Nevalaita et al., 2010). Thus, the synergistic cross-talk of exogenously added and DBS-induced endogenous NTFs may at least partially explain the results obtained. It is also possible that the interaction can be mediated by a dopamine-independent mechanism, perhaps by affecting other neurotransmitter systems downstream of the nigrostriatal pathway.

Unfortunately, the observed effects seen were at least partly relatively short term, and future studies should asses the effect of longer term CDNF treatments, for example, with a minipump combined to DBS. We modeled long-term STN DBS with a STN lesion, which shares only some of the same mechanisms with STN DBS (Bacci et al., 2004), and the results might be different with continuous long-term DBS. Although STN DBS has largely replaced STN lesions, the clinical use of STN lesion has been reported also quite recently with less side-effects (Jourdain et al., 2014) whereas earliest studies with various lesioning methods had serious problems (Cooper, 1953) (Cooper, 1960). However, the use of STN lesion as a model for chronic DBS limits the translational potential of our studies. Nevertheless, our data encourage animal trials using long-term experimental DBS combined to NTF treatments to further study the translational potential of our results, although our data seem to support the hypothesis that STN DBS works at least partially by inactivating STN neural network. It is interesting that the behavioral effect of the combination was seen without changes in TH or monoamine levels, which indicates that the combination of NTF and STN DBS may be beneficial in advanced PD, where the possibilities of neurorestoration are generally quite modest (Olanow et al., 2015). This could also provide a rationale for clinical trials where NTFs could be combined with STN DBS. The NTF injection site (SNpc) used in this study can be reached through a similar trajectory as the one used for STN DBS clinically. Additionally, it has been recently suggested that DBS therapy could be combined with viral vector mediated gene therapy or stem cell implantations (Rowland et al., 2016).

We used two behavioral tests to study the behavioral effects of the combination of STN lesion and CDNF, the cylinder test and apomorphine-induced rotation. Additionally, amphetamine-induced rotations were used as a baseline measure to balance the experimental groups. Apomorphine-induced rotations develop more slowly and require the development of supersensitivity of the post-synaptic striatal neurons and thus provide indirect measurement of dopamine depletion sensitive for severe dopamine depletion relevant for the model of advanced PD used in this study (Hudson et al., 1993). Only one behavioral test was used to study the combination of STN HFS and the effect was weak and short-term. The fact that we found no robust histo- or biochemical changes stresses that these putative behavioral effects should be confirmed by other behavioral tests such as the forelimb adjusting step test and the staircase test, which could also provide for added sensitivity.

The ineffectiveness of NTFs alone in the used model of advanced PD (6-OHDA MFB lesion) indicates that NTF-treatments should be started clinically earlier in the disease. The treatment of patients with a less severe

disease status could result in better efficacy because the striatum is already lacking TH+ fibers 5 years after diagnosis (Kordower et al., 2013). Moreover, the results of clinical NRTN gene therapy trials indicate a positive effect in patients treated less than 5 years after diagnosis (Bartus and Johnson, 2017a). However, the accuracy of the initial diagnosis of PD is low (Joutsa et al., 2014), and a follow-up period of 5 years has been suggested to exclude patients with atypical PD before STN DBS (Bronstein et al., 2011); however, suggestions for earlier time points have been made (Eijkholt et al., 2017). The synergistic combination of NTFs and DBS could offer an opportunity to provide both effective symptom control and neurorestorative treatment while complying with the requirement of diagnostic follow-up.

Our study showed that there is an additive or synergistic effect of STN DBS and CDNF in an animal model of advanced PD where NTFs have not had good neurorestorative effect. This favorable interaction may be important in planning future clinical NTF trials in advanced PD patients. The better effect of the NTF and DBS combination when compared to either treatment alone provides a rationale for studying other combinations of neurorestorative and symptomatic treatments.

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RKT, MS, and MHV are inventors of a CDNF-related patent application that is owned by Herantis Pharma Plc.

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